Molecular investigation of candidate genes involved in the biosynthesis of dinoflagellate paralytic shellfish toxins


Gymnodinium catenatum GCJP10

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Abstract

Dinoflagellate species such as *Gymnodinium catenatum, Alexandrium minutum* and *Alexandrium catenella* produce potent neurotoxins, the causative agents of Paralytic Shellfish Poisoning (PSP). Molecular genetic research on these species is complicated by factors such as their symbiotic association with bacteria, unusual chromosome structure, tough cellulose cell walls (*Alexandrium*), and large amount of genomic and repetitive DNA. Little is known about how, where and when PSP toxins (PSTs) are synthesised. The basic precursors of the PST molecule(s) have been hypothesised, but no genes coding for toxin production have been definitively identified. The application of molecular methods to study armoured and unarmoured marine dinoflagellates was assessed and techniques successfully refined, including DNA and RNA isolation, flow cytometry, primer design, PCR, quantitative real time PCR, molecular cloning and sequence analysis.

Methods for detecting intra- and extra-cellular bacteria were examined, including fluorescence *in situ* hybridisation, light microscopy, agar plating and PCR. Prolonged antibiotic treatment of *G. catenatum, A. minutum* and *A. catenella* cultures reduced bacterial load but resulted in poor growth and cell death of dinoflagellates. Close bacterial associations with dinoflagellates may have an important and as yet poorly understood role in dinoflagellate health and toxicity. A dinoflagellate (eukaryotic) origin of candidate PST genes was confirmed by development of methods to isolate polyadenylated RNA not contaminated with prokaryotic (bacterial) genes. This technique was also crucial for gene expression studies.

Production of S-adenosylmethionine (SAM) is catalysed by the enzyme SAM synthetase, which is encoded by *Sam*. This enzyme is involved in many cellular metabolic processes, including the biosynthesis of PSTs. *Sam* was characterised for the first time in toxic dinoflagellates, with multiple copies of *Sam* present in individual strains. The most frequently identified copy of *Sam* was highly conserved between dinoflagellates, but dissimilar to *Sam* sequences from non-dinoflagellates. Two other candidate PST genes, S-adenosylhomocysteine hydrolase (*Sahh*) and methionine aminopeptidase

(*Map*), previously identified in the PSP dinoflagellate *Alexandrium fundyense* were cloned in *A. catenella*.

Toxin dynamics and expression of *Sam*, *Sahh* and *Map* were examined concurrently over the cell division cycle in *A. catenella*. The toxin profile was constant over the cell cycle but cellular toxin content decreased during division, suggesting that toxin was partitioned in dividing cells. Expression of *Map* and *Sahh* appeared to follow a similar pattern to rate of endocellular toxin production throughout the cell cycle. Positive toxin production occurred in the absence of light, suggesting that light was not a direct trigger for toxin production. The molecular techniques developed and sequence information and knowledge of cellular toxin dynamics gained will facilitate further characterisation of novel dinoflagellate genes.

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I would like to extend a big thankyou to my supervisors Prof. Gustaaf Hallegraeff and Dr Anthony Koutoulis. I greatly appreciate your support, guidance and enthusiasm throughout my PhD. Thank you Gustaaf for introducing me to the wonderfully diverse world of phycology and Anthony for your long-standing contribution to my knowledge of molecular biology.

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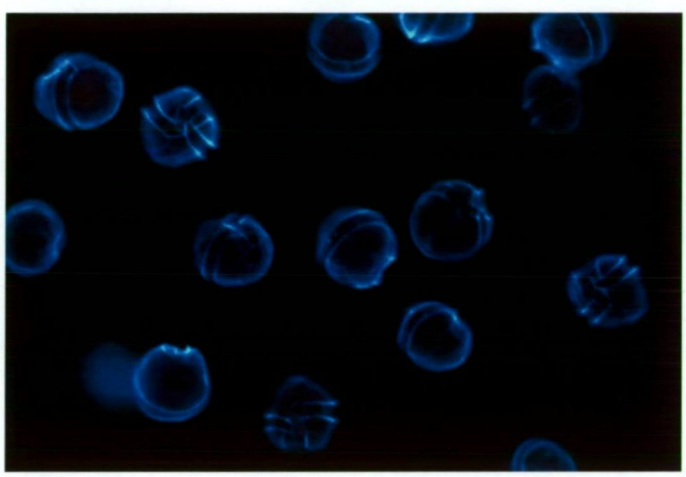
".....all the waters that were in the river turned to blood. And the fish that were in the river died; and the river stank, and the Egyptians could not drink of the water of the river....." Exodus 7:20-21



The red tide forming dinoflagellate *Alexandrium minutum* showing cellulose plates of cells stained with calcofluor.

Abbreviations

Acronym	Full name
ArgMT	arginine methyl transferase
BAMT	benzoic acid methyl transferase
BLAST	basic local alignment search tool
C	cytosine
CDC	cell division cycle
cDNA	complementary DNA
CST	cystolic sulfotransferase
CTAB	cetyl-trimethyl-ammonium bromide
DD	differential display
DEPC	diethyl pyrocarbonate
dNTPs	deoxynucleotide triphosphates
dsDNA	double stranded DNA
FISH	fluorescence *in situ* hybridisation
G	guanine
gDNA	genomic DNA
GMT	guanidine methyl transferase
GTX	gonyautoxin
H	hydrogen
H_2O_2	hydrogen peroxide
HAB	harmful algal bloom
HB	hybridisation buffer
HIS	histone like protein
HPLC	high performance liquid chromatography
LB	Luria-Bertani
LSU	large ribosomal subunit
$LiCl_2$	lithium chloride
MET	methionine aminopeptidase like protein
$MgCl_2$	magnesium chloride
mRNA	messenger RNA
MT	methyl transferase
N	nitrogen
neoSTX	neosaxitoxin
NMR	nuclear magnetic resonance (images)
NO	nitric oxide
NOS	nitrogen oxide synthase
NOHA	N-hydroxyarginine

OH	hydroxy group
O/F	oxidation-fermentation
P	phosphorus
PAPS	phosphoadenosine phosphosulfate
PAPR	phosphoadenosine phosphosulfate reductase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKS	polyketide synthase
PSP	paralytic shellfish poisoning
PST	paralytic shellfish poisoning toxin
P450s	cytochrome P450 oxidases
PVP	polyvinyl pyrrolidone
QPCR	quantitative real time PCR
Q_t	cell toxin quota
NH_2R	amine group
r	correlation coefficient
RACE	rapid amplification of complementary DNA ends
rDNA	ribosomal DNA
RNase	ribonuclease
R_{TOX}	net quantity of toxin per cell per unit time
RT-PCR	reverse transcriptase PCR
SAHH	S-adenosylhomocysteine hydrolase
SAM/AdoMet	S-adenosylmethionine
SAMS	SAM synthetase
SAMT	salicylic acid methyl transferase
SAX	saxiphilin (transferrin)
SCB	sodium channel blocking
SSU	small ribosomal subunit
ST	sulfotransferase
STX	saxitoxin
SWAV	seawater agar and vitamins
TTX	tetrodotoxin
UBI	ubiquitin
μ_{TOX}	specific rate of toxin production

Gene name	Protein/enzyme
ArgMT	arginine methyl transferase
CST	cystolic sulfotransferase
GMT	guanidine methyl transferase
Haf	histone like protein
Map	methionine aminopeptidase
NOS	nitrogen oxide sythase
PAPR	phosphoadenosine phosphosulfate reductase
P450	cytochrome P450 oxidase
Sahh	S-adenosylhomocysteine hydrolase
SAX	saxiphilin
Sam	S-adenosyl methionine synthetase gene
ST	sulfotransferase
STX	saxitoxin
Ubi	ubiquitin

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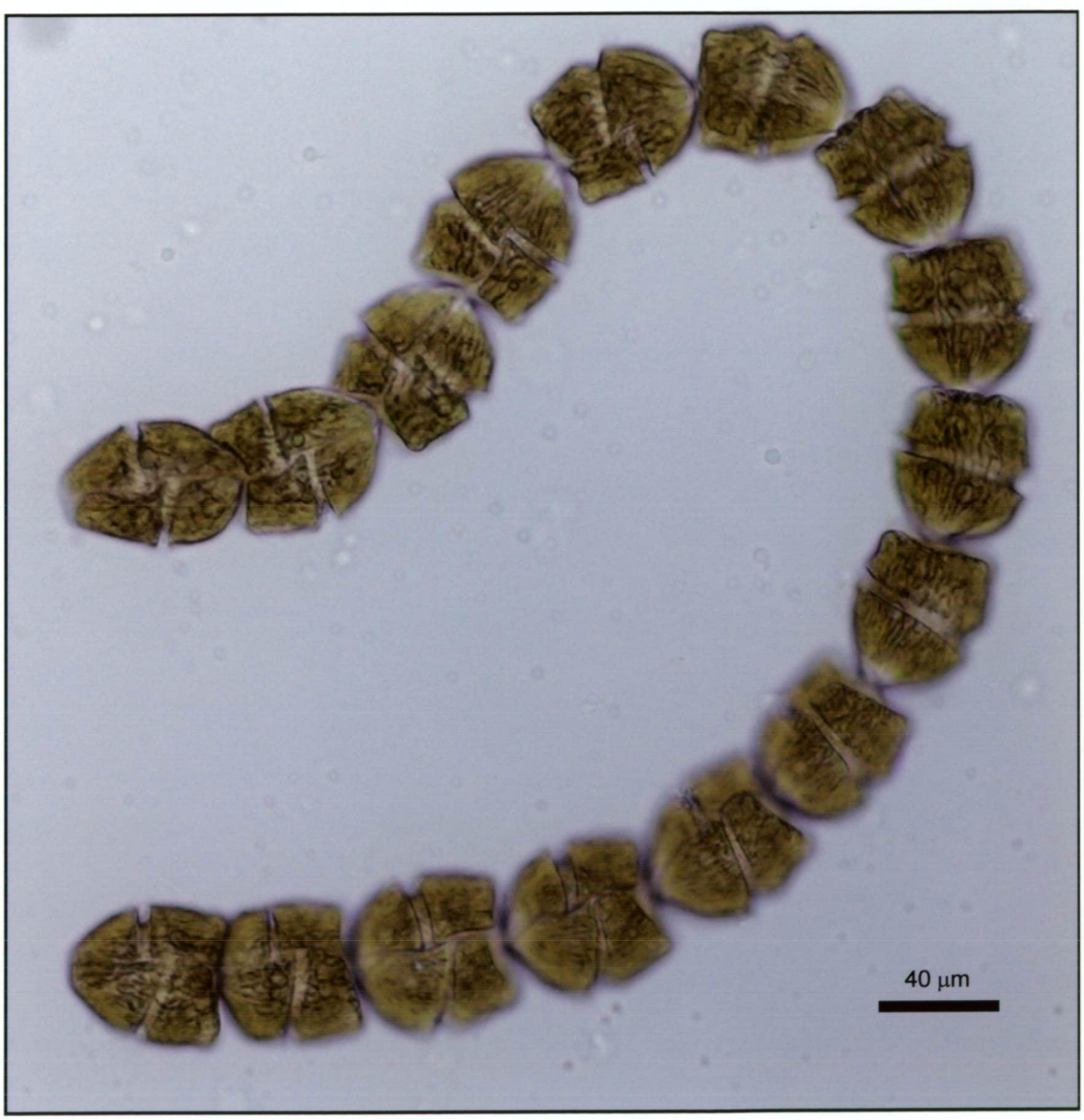
Chapter 1.

Introduction to paralytic shellfish toxin producing dinoflagellates



Molecular studies of toxic dinoflagellates are in a pioneering phase. Quite often, special techniques must be developed, since standard extraction and analysis methods appear ineffective. This thesis explores the application of genetic analysis in understanding harmful dinoflagellates responsible for Paralytic Shellfish Poisoning (PSP). The first chapter introduces PSP dinoflagellates and the significant impact that their toxins have on human health and aquaculture, highlighting the need for a genetic understanding of PSP toxin (PST) biosynthesis. In order to identify novel dinoflagellate PST genes it is important to understand the taxonomic affinities and ecophysiology of the organism. Knowledge of dinoflagellate nucleic acid extractability and DNA structure as well as the dynamics of PST production under various environmental conditions is essential. Information gleaned from these and future genetic studies will help to provide a more complete understanding of when and where PSTs are synthesised and their role in dinoflagellate ecophysiology.

1.1 DINOFLAGELLATE CELL DIVISION AND BLOOM FORMATION

On a global basis, phytoplankton provide significant quantities of food for animals in both marine and freshwater habitats (Taylor *et al.* 1987, Hader *et al.* 1998). A dense patch of algae often visible near the surface of the water is known as an algal bloom. Marine dinoflagellates generally grow by asexual reproduction, particularly when environmental conditions such as nutrient availability, water temperature and light are optimal. When a cell divides, a duplicate copy of DNA is transferred into the daughter cell, which is then able to divide itself, and so on (Fig. 1-1). If a population starts with a single cell, the number of cells can be denoted as 2^n (where n is the number of generations). Such growth is exponential and can result in a large number of cells very rapidly. Algal growth tends to follow a sigmoidal or "S-shaped" curve when plotted against time (Hwang & Lu 2000). This curve can be broken up into three phases of growth: (i) an early phase in which growth is slow (the lag phase), (ii) a middle phase in which growth is rapid (the logarithmic or exponential phase) and (iii) a final phase in which growth appears to stop and the curve plateaus (the stationary phase) (Siu *et al.*

1997). During stationary phase it is thought that the number of dividing cells equals the number of dead or dying cells.

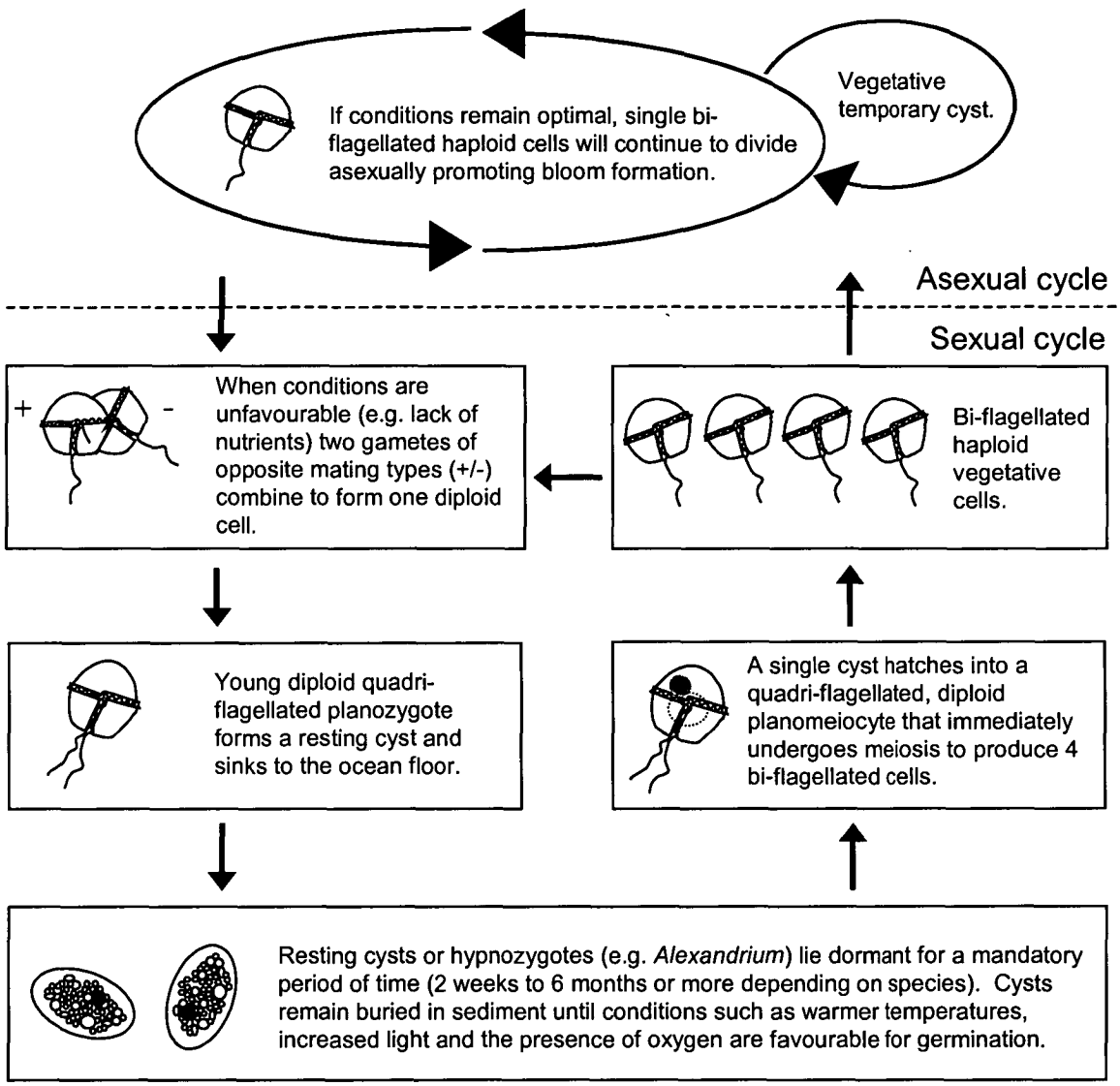


Figure 1-1. Schematic diagram of the life cycle of a dinoflagellate such as *Alexandrium* showing sexual (zygotic meiosis) and asexual reproduction. An algal bloom can occur during the asexual cycle under appropriate conditions. Similarly to the zygote, the vegetative cell can form a temporary cyst within minutes/hours of being in a stressful environment. The temporary cyst may resemble the vegetative cell in pigmentation but can be highly variable in shape and size and may or may not possess flagella. It is less tough and less well documented than the thick-walled, highly resistant sexual cyst. Sexual cysts can remain viable in sediments for up to 10 years or even longer (Anderson *et al.* 1995). Note the dark accumulation body of carotenoid pigments present in the sexual cyst and planomeiocyte.

Multiple biotic, chemical and physical factors influence the formation and maintenance of an algal bloom. These include water column stability (influenced by wind generated turbulence, ambient temperature and currents), nutrients, tides, light, sea surface temperature, salinity, grazing pressure and growth factors (Truscott 1995, Smayda 2000, Trigueros & Orive 2000). Advection and diffusion are also important physical processes that affect the fluctuation of cell densities (Yamamoto *et al.* 2002). Importantly, dinoflagellates are able to move in the water column and thus exhibit behavioural features, which contribute to a bloom. These include: phototaxis, vertical migration, pattern swimming, and aggregation, which facilitate nutrient retrieval, trace metal detoxification, antipredation, depth-keeping, and turbulence avoidance (Smayda 1997). Dinoflagellates share a low-nutrient affinity and can achieve relatively fast growth rates in nutrient-enriched environments. Upwelling systems most commonly favour bloom formation, however these are not exclusive and blooms can occur over a range of nutrient, mixing and advection combinations (Smayda 2000).

1.2 THE ENIGMA OF DINOFLAGELLATE DNA: EVOLUTION AND PHYLOGENY

Prokaryotes (Prokaryota) encompass two of the three kingdoms of life: Archaebacteria and Eubacteria; all other living organisms belong to the kingdom Eukaryota (Martin & Embley 2004). Bacteria, including the cyanobacteria, belong to the kingdom Eubacteria and are known as the Cyanophyta. It is believed that eukaryote cellular organelles like mitochondria and chloroplasts were once independent free-living prokaryotes that, during the course of evolution, became captured and internalised by eukaryote cells. The long standing theory of serial endosymbiosis was first proposed by Mereschkowsky in 1905 (van den Hoek *et al.* 1997). According to the theory, chloroplasts arose from cyanobacterial cells and mitochondria from bacteria (Falkowski *et al* 2004). These organelles are partially autonomous, they usually divide independently of the nucleus and possess their own DNA (although greatly reduced), allowing for a certain degree of genetic independence (Alberts *et al.* 1994).

Dinoflagellates are a highly diverse group of unicellular, eukaryotic, flagellated organisms that belong to the protista (phylum Dinophyta) (Taylor *et al.* 1987, Shimizu 2003, Snyder *et al.* 2003). They are nutritionally versatile and may be autotrophic (photosynthetic) or heterotrophic and/or non-photosynthetic. Dinoflagellate nuclei are structurally distinct from those of other eukaryotes (Li & Hastings 1998). Although they display eukaryotic characteristics such as the presence of a nuclear membrane, nucleoli and argyrophilic proteins (Michel *et al.* 1996), dinoflagellates are unusual in that they also display prokaryotic features such as an absence of classical centriolar structures, histones and nucleosomes (Rizzo 1991, Chan *et al.* 2002, Wong *et al.* 2003). Instead, the dinoflagellate centrosome is surrounded by a complex Golgi apparatus, localised close to the nucleus (Ausseil *et al.* 2000).

Features such as permanently condensed chromosomes (Wong *et al.* 2003) and unusual chromosome structure (Steele & Rae 1980), a very low level of basic proteins bound to the chromatin (Guillebault *et al.* 2002), and unique chloroplast genes (Zhang *et al.* 2002b) place dinoflagellates in a distinct group among eukaryotes. Even their cell division is different enough for it to be given its own name, dinomitosis (Michel *et al.* 1996). Furthermore, many species possess an unusually large amount of DNA (Wong *et al.* 2003). This may be due to repetitive sequence in the non-coding (Moreau *et al.* 1998) and coding DNA (Zhang & Lin 2003), large introns or multiple copies of genes (Zhang & Lin 2003). For instance, Zhang & Lin (2003) studied the form II Rubisco gene in the dinoflagellate *Prorocentrum minimum* and found that its coding DNA was repeated 148 +/- 16 times in the genome.

It has been known since the early 1980s that free-living dinoflagellates usually have a high number of chromosomes (20-270) (Raikov 1982), while most parasitic dinoflagellates (e.g. *Syndinium, Merodinium, Amoebophyra*) have few chromosomes (4-8) (Raikov 1995a). This wide variation in the number of chromosomes provides some indirect evidence for polyploidy in dinoflagellates (Raikov 1995a). For instance two species of the genus *Prorocentrum* have 32 and 132 chromosomes each (Costas & Goyanes

1987). Study of the ultrastructure of the nucleus of a Tasmanian strain of *Gymnodinium catenatum* (GCDE01, Microalgal Culture Collection, CSIRO Division of Fisheries, Hobart) revealed a chromosome complement of ~123, including three nucleolar organising chromosomes which associate with two nucleoli. Individual chromosomes (i) ranged in length from 2-26 μm with at least one end of each being closely associated with the nuclear membrane, (ii) were grouped (6-8) in parallel paths and (iii) were often folded through more than 90° (Rees & Hallegraeff 1991).

In dinoflagellates, the nuclear cytology of meiosis has only been studied in a few species (Parrow & Burkholder 2004) and the mechanism of DNA replication and transcription are almost completely unknown (Li & Hastings 1998). It is still unclear if there is a general pattern of dinoflagellate meiosis. Two common features of dinoflagellate sexual cycles were observed by von Stosch (1973). For the species studied, the motile zygote always retained two longitudinal flagella; and prior to zygote division, a dramatic swirling of the chromosomes (nuclear cyclosis) occurred. This cyclosis restored the asexual morphology of the cell prior to division(s) and was accompanied by chromosome pairing. Parrow & Burkholder (2004) suggest that the conspicuous appearance and duration of dinoflagellate nuclear cyclosis may be related to the condensed chromosomes and the large genome size.

Dinoflagellate generally exhibit a conventional two-divisional meiosis, although sometimes the division is uncoupled in time (Raikov 1995b, Parrow & Burkholder 2004). It has been suggested that the dinoflagellate cell cycle is regulated by universal eukaryotic mechanisms (Taroncher-Oldenburg *et al.* 1997). For instance, recent studies have found evidence for the presence of a cell division cycle 2 (*Cdc2)* like protein kinase in the dinoflagellates *Crypthecodinium cohnii* (Rodriguez *et al.* 1993) and *Gambierdiscus toxicus* (van Dolah 1995) as well as cyclins and cyclin-dependent kinases in *C. cohnii* (Leveson *et al.* 1997), Gymnodinium breve (Van Dolah & Leighfield 1999), *Karenia brevis* (Barbier *et al* 2003) and *Gonyaulax polyedra* (Bertomeu and Morse 2004). Centrosome associated proteins described from dinoflagellates include: beta-tubulin, gamma-tubulin (Ausseil *et al.*

2000), CTR 210 antigens, p72 (HSP 70), alpha-actin and p56 (cdc13), a homologue of *Schizosaccharomyces pombe* cyclin B, p80 (a nuclear and cytoplasmic protein) (Ausseil *et al.* 1999) and myosin II antigens (Ausseil *et al.* 2000).

1.3 Paralytic Shellfish Poisoning toxins

Paralytic Shellfish Poisoning (PSP) is a syndrome affecting the nervous system of animals. The causative agents are highly potent neurotoxins called PSTs most commonly produced by marine dinoflagellates. From 60 to 80 species of phytoplankton have been reported to be harmful. Of these, 90% are flagellates, notably dinoflagellates (Smayda 1997). However, some prokaryotes such as certain species of cyanobacteria (freshwater species – *Anabaena circinalis*, *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*) and true bacteria (Proteobacteria) (Sakamoto *et al.* 1992) have also been documented to produce PST-like compounds (Thain & Hickman 1995, Gallacher *et al.* 1997, Cembella 1998). For example, the symbiotic bacteria *Pseudomonas stutzeri* SF/PS and *Pseudomonas/Alteromonas* PTB-1 isolated from true PST producing *Alexandrium* spp. were shown to produce PST 'imposter' compounds (Baker *et al.* 2003).

Dinoflagellate species that produce PSTs belong predominantly to the genus *Alexandrium* (former genus: *Protogonyaulax/Gonyaulax*) (e.g. *A. fundyense/tamaranse, A. ostenfeldii* – some strains, *A. catenella* and *A. minutum*). Single species belonging to different genera that are also highly toxic are *Gymnodinium catenatum* and the tropical dinoflagellate *Pyrodinium bahamense var. compressum* (Acres & Gray 1978, Lagos *et al.* 1996, Micheli *et al.* 1996, Teegarden 1999, Hallegraeff 2002) (Table 1-1).

The first evidence that some dinoflagellates produce PSTs was in the 1930s (Taylor *et al.* 1987). PSTs are natural toxins, but their function is still unknown. It has been suggested that PSTs are produced as a deterrent to predators (such as copepods) (Teegarden 1999); as a secondary metabolite, waste product or storage product (energy reserve) (Shimizu 1996); or to reduce competition pressures (allelopathy) (Rengefors & Legrand 2001).

Whatever their function, it is almost certain that their toxic effect on higher order consumers is a secondary ramification. A number of studies have shown that phytoplankton grazers are able to detect toxins in their prey. For example, when presented with cultured *Alexandrium spp*. strains, three species of marine copepods *(Acartia tonsa, Centropages hamatus,* and *Eurytemora herdmani)* fed discriminately on the non-toxic cells by chemosensory means (Teegarden 1999). This suggests that by producing a toxin, dinoflagellates are able to decrease the risk of predation by some species. Although these toxins are harmful, understanding their mode of action may provide insight for the development of nutraceutical or pharmaceutical products. The PSTs have already been used as neurobiological tools to elucidate the sodium (Na^+) channel macromolecule and may be useful for further clarification of the Na^+ channel proteins (Kao 1986).

Table 1-1. Causative organisms of the well-documented poisoning syndrome PSP. The toxins involved are saxitoxin (STX) and other derivatives (PSTs).

Division	Causative organisms	Habitat	References
Dinophyta (dinoflagellate) Cyanobacteria* Rhodophyta?	*Gymnodinium catenatum, Alexandrium minutum, A. tamarense, A. catenella, Pyrodinium bahamense var. compressum, Anabaena circinalis, Aphanizomenon sp. (some strains)*	Marine & Freshwater	Irwin *et al.* (2003), Hallegraeff (2002), Hamasaki *et al.* (2001), Negri & Jones (1995), Lu *et al.* (2000), Pomati *et al.* (2001), Tobe *et al.* (2001), Lagos *et al.* (1996).

*Some species of bacteria have been documented to produce PST-like compounds.

PSTs are among the most potent biotoxins known. Comparison of the human lethal dose (μg per Kg body weight) of poisons places the PST saxitoxin (STX) (9 μg) in between the cobra neurotoxin (0.3 μg) and strychnine (500 μg) (Graneli *et al.* 1998). PSTs physically impair the regulatory mechanism of neurons. Neurons are the major cell type of nervous tissue, specialised for the transmission of information in the form of electrical impulses. These impulses are regulated by voltage-gated Na^+ channels in the cell membrane (Li *et al.* 1993) (Fig. 1-2). The positive charge on part of the PST molecule allows it to bind specifically to an external site on

voltage-gated Na^+ channels, blocking the passage of Na^+ ions (Fig. 1-3). Accumulation of Na^+ ions causes muscle to relax, which can result in respiratory failure (Thain & Hickman 1995).

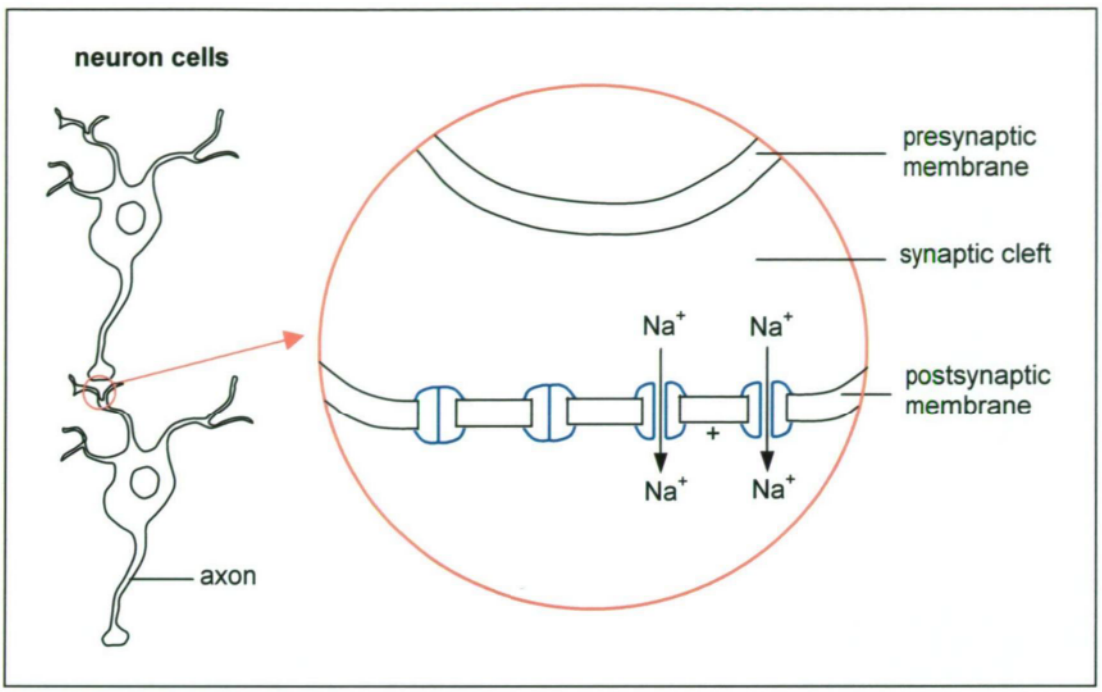


Figure 1-2. Schematic diagram of neurons showing the location of voltage-gated sodium (Na^+) channels in the postsynaptic membrane. Example of closed (left) and open (right) channels are shown. Note that Na^+ channels of a membrane are either closed or open at any one time.

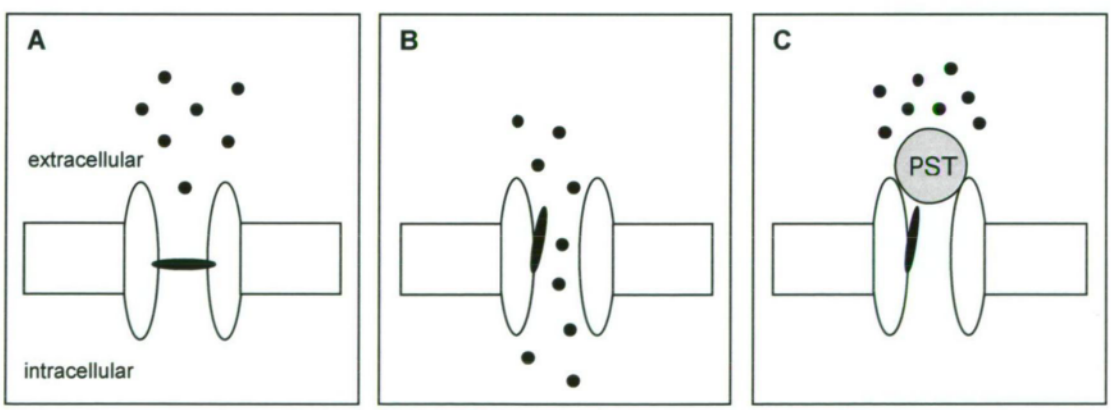


Figure 1-3. **A-C.** Schematic representation of a cross section through the membrane of a neuron, showing a voltage-gated sodium (Na^+) channel. **A.** The channel is closed and Na^+ ions (●) cannot pass through. **B.** The channel is open due to a voltage step and Na^+ ions are able to pass through the membrane. **C.** The channel is open but the passage of Na^+ ions is physically blocked by a Paralytic Shellfish Toxin (PST) molecule (e.g. STX) that is bound to an external site on the conducting pore.

To be 'gated by voltage' means that the voltage gradient across the membrane determines whether the gate obstructs the channel (i.e. whether the channel is open or closed). For example, the voltage-gated Na^+ channels of most excitable nerve cells are impermeable to Na^+ ions at potentials less than -70 mV. The gate is closed at potentials < -70 mV. However, when the membrane is depolarised to potentials > -60 mV, the probability increases that the gate will be open and that Na^+ ions will move through the channel.

PST production is endocellular (Acres & Gray 1978) and, although PSTs are water-soluble, they are not known to be excreted into the water column by dinoflagellates (see Chapter 6). However, activated charcoal has been used to recover PSTs excreted into seawater by mussels (Suzuki *et al.* 2003). Additionally, Lush *et al.* (2000) report the presence of a potent exotoxin in the culture medium of a toxic *A. minutum* strain. The PST-like activity of the cell-free medium was ~20 times higher than the endocellular PSP toxicity, as determined by toxicity to *Artemia*. Activated charcoal and Carbograph columns were used to adsorb PSTs, yet no PSTs or brevetoxin were detected by high performance liquid chromatography. Either another toxic principle(s) was active in the exocellular medium or the charcoal was not effective at trapping PSTs.

Due to their transfer in the food chain, PSTs have a widespread distribution and have been found in various marine animals (Cembella 1998). Certain consumers of these toxic dinoflagellates, namely filter feeders such as shellfish and crustaceans, accumulate and hence concentrate these toxins in their systems with no apparent adverse effects (Holmes & Teo 2002). For instance, specimens of the crab *Lophozozymus pictor* assayed by injection into mice were shown to contain more toxin than the estimated human lethal dose (Llewellyn & Endean 1989). However, there is evidence that some grazers such as copepods become physiologically incapacitated by PSTs, reducing the ability of grazers to control harmful algal blooms (Colin & Dam 2003).

1.3.1 Biodegradation

Paralytic phycotoxins are often detected in the tissues of marine filter feeding organisms such as crabs, oysters, mussels and clams (Asakawa *et al.* 1993). There is conflicting evidence as to whether shellfish are capable of biotransforming the PST they ingest. It is likely that early elimination of PST from shellfish occurs by non-selective excretion of the toxin (Suzuki *et al.* 2003). Independent studies have shown that oyster and mussel tissue possessed the same toxin composition as the *Alexandrium* cells they were fed on and toxin loss from the tissue was accounted for by excretion, suggesting that bioconversion does not occur (Lassus *et al.* 2000, Suzuki *et al.* 2003). In contrast, Yan *et al.* (2001) found that the toxin profiles of *Alexandrium* cells and mussel were different. Shellfish contamination by PSTs is a major problem for shellfish farmers and the development of methods of detoxification/depuration may become important for developing sanitary shellfish industries (Yan *et al.* 2001, Lassus *et al.* 2002). It is unlikely that mammals are able to metabolise PST molecules (Andrinolo *et al.* 1999), making the likelihood of poisoning after ingesting contaminated meat very real.

1.4 Paralytic Shellfish Poisoning: Health and Economic Issues

Blooms of PST producing dinoflagellates can have severe impacts on humans, which may be direct such as a poisoning event or indirect such as an economic loss to aquaculture. The ramifications of PST for higher order consumers can be extreme (Lehane 2001). Higher order consumers (e.g. fish, humans) may suffer death due to toxin induced respiratory failure (Acres & Gray 1978, Kan *et al.* 1986) (Fig. 1-4). Currently, the mechanism of action of PSTs at the molecular level is well known, however, there are still many unresolved questions about its pharmacokinetics and the PSP intoxication syndrome in mammals.

A study in cats found STX present in intensely irrigated organs, such as the liver and spleen, and also in the central nervous system (brain and medulla oblongata), demonstrating that STX is capable of crossing the blood-brain barrier (Andrinolo *et al.* 1999). This may explain the fast onset of poisoning

symptoms after ingestion of contaminated shellfish. In humans death may occur within 2-24 h, however less severe symptoms are commonly reported. Within 30 min of ingestion a person may feel a tingling sensation or paralysis of the lips, gradually spreading to the face, neck, hands and legs; headache, dizziness, diarrhoea, nausea and vomiting (Akaeda *et al.* 1998). Thus, PSTs have the potential to cause health and economic problems.

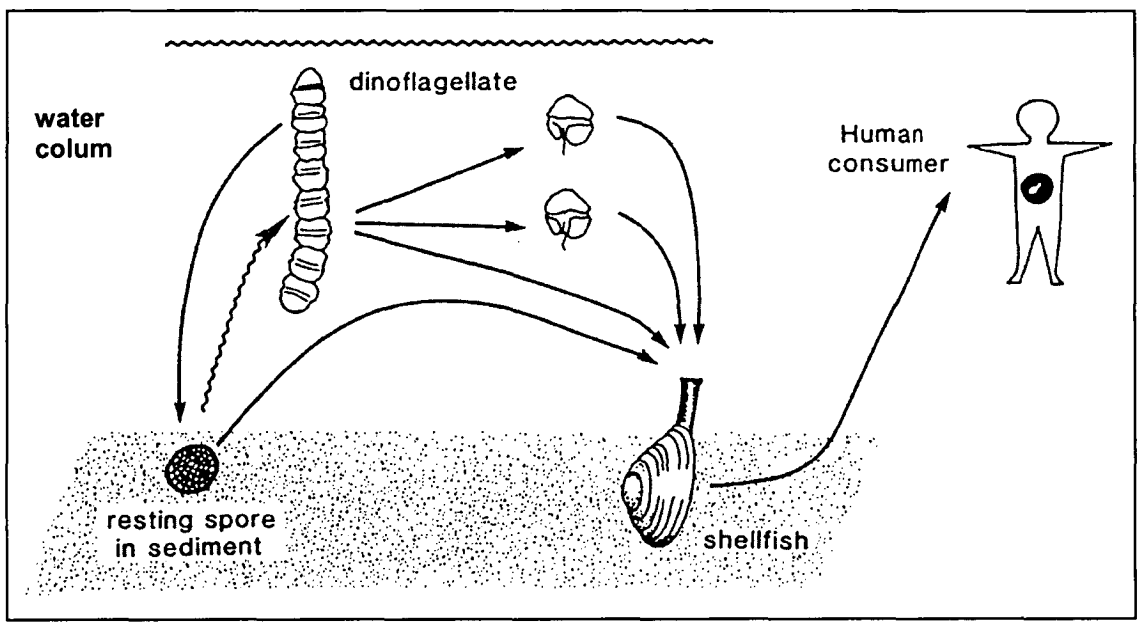


Figure 1-4. Schematic diagram showing transfer of Paralytic Shellfish poisoning Toxins (PSTs) through the food chain from dinoflagellates to human consumers (after Hallegraeff 2002).

Human food poisonings characteristic of PSP have been documented worldwide over many centuries. One of the first records of fatal human PSP was in 1793 when Captain George Vancouver and his crew landed in British Columbia in a bay now known as Poison Cove (Dale & Yentsch 1978). In the past few decades the frequency, intensity and geographic distribution of PSP appears to have increased (Fig. 1-5). It is likely that toxic algal species have spread within regions over hundreds of kilometres, moving with major water currents and storms (Anderson 1989). This apparent global increase, while partly due to an increased awareness and media attention, is also likely to be a "real" consequence of anthropogenic factors (Hallegraeff 1993, Brett 2003). It appears that human activities are having an ever-increasing impact on the occurrence of blooms. Agricultural run-off, human effluent discharge, mariculture and anthropogenic impacts on ocean margins have been linked

to increasing nitrates and/or phosphates that promote algal growth in local estuarine areas, with sometimes harmful consequences (Taylor *et al.* 1987, Graneli *et al.* 1998, Sellner *et al.* 2003). Consequently monitoring programs for the causative organisms are expanding (Hallegraeff *et al.* 1995).

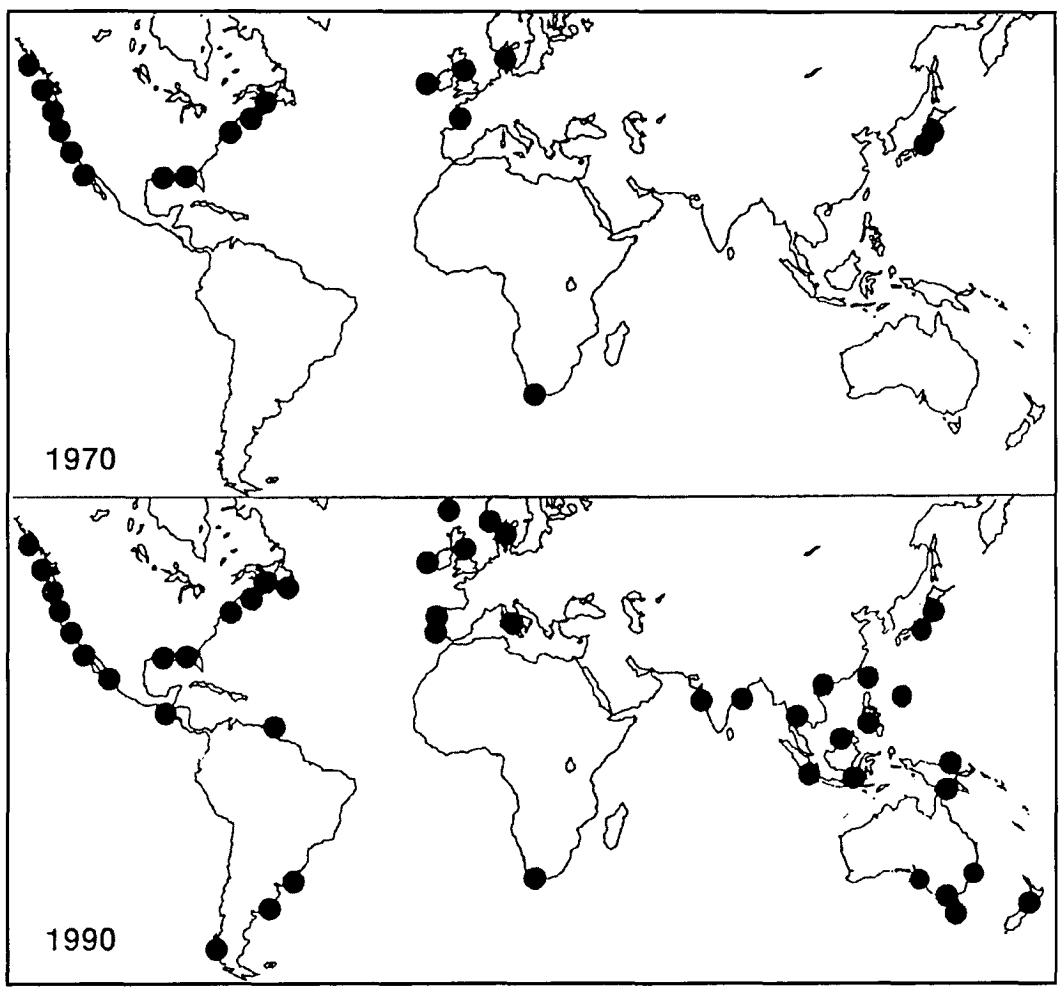


Figure 1-5. Global distribution of known PSP events in 1970 and 1990 (Hallegraeff *et al.* 1995).

The chain-forming dinoflagellate, *G. catenatum* Graham 1943, is the only known gymnodinioid species to produce PSP. This well-documented PST producer was, until recently, relatively obscure. In the period of 1940 to 1970, the presence of *G. catenatum* was reported only twice, but since then this species has been reported with increasing frequency. Its association with PSP was first reported in North West Spain in 1976, followed by the Pacific coast of Mexico in 1979 and Australia (Tasmania), Southern Japan

and Portugal in 1986 (Hallegraeff & Fraga 1998). Similarly, up until the 1970s, blooms of the PST producing dinoflagellates *Alexandrium* (*Gonyaulax*) *tamarense* and *A. catenella* were only known to occur in temperate waters of Europe, North America and Japan but have since occurred widely in the Southern Hemisphere (Hallegraeff 1993).

Modern medicine and awareness in Western society means that few deaths occur from PSP, however poisoning is still a major problem in developing, tropical countries where shellfish is an essential source of protein in the diet. The toxic dinoflagellate *Pyrodinium bahamense* var. *compressum* is distributed in mangrove areas of the tropical Indo-West Pacific region (Brunei, Indonesia, Palau, Papua New Guinea, the Phillipines, Sabah and the Solomon Islands) and in Latin America (Guatemala, Venezuela, Mexico and the tropical Caribbean). This organism caused a major PSP event in 1987 on the Pacific coast of Guatemala when 187 people were hospitalised and 26 died after consuming toxic clams (Hallegraeff 2002).

Some other PSP events include: 1) poisoning of 2 men after consumption of contaminated mussels, Nova Scotia 1978 (Acres & Gray 1978); 2) in March 1984, 8 cases of neurotoxic food poisoning occurred following the ingestion of the marine mollusc *Oliva vidua fulminans,* collected from the Bongawan seashore near Kota Kinabalu (Sabah); symptoms developed within 10-20 min and 5 children under 9 years died from respiratory failure within 3-4 h (Kan *et al.* 1986); 3) in March 1997, a large food poisoning incident due to ingestion of wild oysters, *Crassostrea gigas*, occurred at Tamano-ura, Goto Islands, Nagasaki, Japan, 26 people were poisoned, of whom 16 were hospitalised but recovered (Akaeda *et al.* 1998). Current treatment of PSP includes having the stomach pumped, respiratory support and haemodialysis.

Saxiphilin is a plasma protein identified in the bullfrog *Rana catesbeiana* (Li *et al.* 1993). Saxiphilin specifically binds STX (Llewellyn & Moczydlowski 1994) and other PST derivates. Understanding the biology of saxiphilin might allow for its pharmacological application such as in antidote therapy for PSP (Li *et al.* 1993).

The occurrence of toxic algal blooms globally often causes shellfish fisheries to close due to the threat of poisoning from contaminated shellfish, resulting in huge losses in sales. This occurs for all European coastal countries at certain times of the year (Graneli *et al.* 1998). Some shellfish fisheries have been able to combat this problem by operating in several different bays so that they can harvest from an alternate location when algal blooms are a problem. Usually PSTs are transmitted through shellfish and do not affect large fish, especially farmed fish which are primarily fed on commercial food. However fish kills have been reported, and in 1984 in the Faroe Islands, *A. tamarense* was implicated in a fish kill of 27 metric tons of farmed rainbow trout and salmon. Examination of fish gills showed acute histopathological damage (Hallegraeff 2002).

The increased awareness of PST producing dinoflagellates and their impact on public health has prompted the expansion of routine coastal monitoring programs and the development of new, sensitive detection methods (Ahmed 1991, Sellner *et al.* 2003). Detection strategies may be analytical or assay based. Analytical methods individually quantify toxins to measure total toxin content of a sample whilst assays produce a single integrated response of all toxin cogeners.

1.5 Environmental conditions: dinoflagellate growth and toxicity

Independent studies have shown a correlation between growth conditions, the cell cycle and cellular toxicity/PST composition in the dinoflagellate genus *Alexandrium.* Some environmental and nutritional factors studied include phosphorus (P) and nitrogen (N) availability, salinity, temperature and light. It is important to note that many studies are performed on batch cultures and therefore do not account for all environmental growth conditions. It has been shown that cultures grown under conditions that more closely resemble the natural environment (such as dialysis culture or temporary culture with aeration) are more toxic. It may be that the higher toxicity is attributable to these particular growth conditions which allow for elimination of low molecular weight autoinhibitory metabolites and a more efficient nutrient uptake (Marsot 1997, Wang *et al.* 2002).

Studies of *A. catenella/fundyense/minutum* have shown that growth is inhibited when P is limiting and this has consequences for toxicity (Siu *et al.* 1997/Taroncher-Oldenburg *et al.* 1999/Lippemeier *et al.* 2003, respectively). It is suggested that dividing cells repeatedly lose toxin to daughter cells such that toxin content remains stable during fast division but can increase dramatically when division is slow. The study by Lippemeier *et al.* (2003) indicates that cells continuously produce PST in the logarithmic and stationary phases. Grzebyk *et al.* (2003) found the cellular PST content of *A. minutum* to peak at the end of the logarithmic phase or early stationary phase, whilst Siu *et al.* (1997) report a maximum cellular PST content of *A. catenella* cells earlier during the logarithmic phase. More specifically, Taroncher-Oldenburg *et al.* (1999) suggest that PST production in *A. fundyense* occurs during the G_1 phase of the cell division cycle such that the longer this phase, the higher the amount of cellular toxin (refer to Chapter 6). It would therefore be expected that cells would possess the most toxin at the start of the S phase. This fits somewhat with findings by Siu *et al.* (1997) who report that cellular toxin content reached its maximum during the S phase of the cell cycle.

It may be that P availability affects the toxicity of *Alexandrium* strains differently depending on the type of PST they produce. Taroncher-Oldenburg *et al.* (1999) report that P availability influences overall cellular toxicity, not by influencing toxin production but rather the inter-conversions among STX derivatives. For instance, total concentrations of GTX 2 and 3 and C 1 and 2 were significantly higher in P-limited cultures of *A. tamarense* (formerly *fundyense*), while the levels of STX, neo and gonyautoxins 1 and 4 remained virtually unchanged. Similarly to P-limitation, Siu *et al.* (1997) found that a lack of N slowed *A. catenella* growth but toxin production was down-regulated. Bechemin *et al.* (1999) reported similar findings for *A. minutum*. The toxin content of cells was ~3 times less in N-limiting conditions and 3.5 to 7 times more in P-limiting conditions than under N:P balanced conditions (1.24 $fmol.cell^{-1}.L^{-1}$). A direct effect of N concentration on cellular toxicity in *A. tamarense* was not supported by Parkhill & Cembella (1999).

The effects of salinity on dinoflagellate growth and toxin content has been studied for *A. minutum* (Grzebyk *et al.* 2003) and *A. tamarense* (Parkhill & Cembella 1999, Wang *et al.* 2002). Parkhill & Cembella (1999) reported that although cellular toxicity varied over the growth stages, it was independent of salinity (and light) throughout the exponential growth phase. However, a positive correlation was observed between cellular toxicity and salinity-dependent growth rate, indicating that *cell toxin quota* (Q_t) may be affected by extrinsic factors, but it is not always a direct functional response to specific environmental stress.

In conclusion, there is strong evidence to suggest that the toxin content of *Alexandrium* cells is influenced by the rate of population growth, which is in turn influenced by nutrition and environmental conditions (Siu *et al.* 1997). For instance, it has been shown that growth rate is lower at low salinities (10-15 ppt) (Nguyen-Ngoc 2004) and cellular PST increases at lower salinities (15 ppt) (Grzebyk *et al.* 2003). Variations in the toxin content of *Alexandrium* cells grown at different temperature has been attributed to increasing periods of biosynthetic activity (Taroncher-Oldenburg *et al.* 1999).

1.6 DINOFLAGELLATE-BACTERIA ASSOCIATIONS

Marine dinoflagellates grown in the laboratory are generally associated with bacteria (Hold *et al.* 2001b). These may be free living, extracellular or intracellular (Seibold *et al.* 2001) to the algal cell and are sometimes symbiotic (Kodama *et al.* 1996). Bacteria associated with toxic dinoflagellates in culture have been implicated directly in the enhancement of toxin production, auto-toxigenesis (Bates *et al.* 1995, Doucette & Powell 1998, Smith *et al.* 2002) and reduction in toxicity of the dinoflagellate culture (Hold *et al.* 2001a). Presently, little is known of why dinoflagellates produce PSTs or the role, if any, of bacteria in PST production. To answer these questions, it is first necessary to understand the complex interactions between bacteria and dinoflagellates.

A number of hypotheses have been proposed regarding dinoflagellate-bacteria associations of which current research seems focused (Ishida *et al.*

1998 and references therein). There is increasing evidence that some bacteria are important for the health of dinoflagellates, while others can be detrimental (see Chapter 2). One hypothesis is that PSTs are a mechanism of reducing grazing pressure or infection by harmful bacteria (Hader *et al.* 1998). It may be that bacteria either directly or indirectly influence dinoflagellate growth and/or toxicity. Indirect influences may include the production of co-factors which inhibit or stimulate dinoflagellate toxin synthesis, as has been shown by Bates *et al.* (1995) for domoic acid production by diatoms. Additionally, bacterial secretion of signalling molecules could play a role in controlling cellular and communication processes in dinoflagellates, or bacteria could influence nutrient availability and hence dinoflagellate metabolism and toxicity (Gallacher & Smith 1999).

Smith *et al.* (2002) provide evidence that specific bacteria taxa are associated with dinoflagellates. Bacteria capable of sodium channel blocking (SCB) activity were present in PST-producing *A. tamarense* and *A. lusitanicum* cultures, while the bacterial flora isolated from a non-toxic *A. tamarense* strain showed no SCB activity (Smith *et al.* 2002). Their study found that toxigenic bacteria belonged to the alpha and gamma subclasses of the Proteobacteria. Similarly, the study by Hold *et al.* (2001b) identified bacteria in both toxic (*Alexandrium* spp.) and non-toxic (*Scrippsiella trochoidea*) dinoflagellate cultures as belonging to the same subclasses. Whether the SCB bacterial toxins are the same as dinoflagellate PSTs is unclear. Other studies have identified bacterial PST-like compounds that do not exhibit SCB activity. For instance, Baker *et al.* (2003) found five bacterial compounds with a unique fluorescence emission spectrum readily discernable from the spectrum of the PST derivative GTX4. They conclude that it cannot be ruled out that the bacteria produce PSTs, but the data clearly demonstrates that the bacteria accumulate at least five different fluorescent compounds that could be easily mistaken for PSTs.

1.7 CHEMICAL STRUCTURE OF PSTs

STX was the first described PST and since then at least 24 naturally occurring analogues have been identified from a number of organisms (Shimizu 1996). It is important to note that these compounds are only described as derivatives of STX because of the order in which they were discovered. It is not known from which compounds the different forms are derived and it is likely that many inter-convert. PSTs are alkaloids (a wide group of organic nitrogenous compounds characterised by an aromatic ring or arene) (Thain & Hickman 1995), specifically small heterocyclic guanidinium compounds (Fig. 1-6).

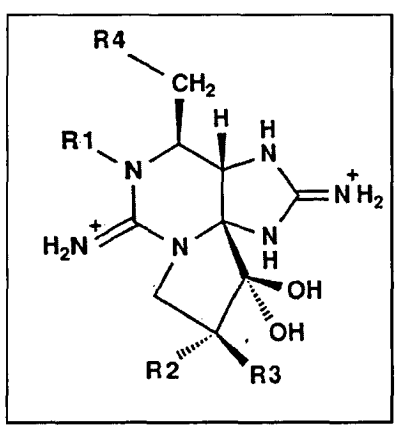


Figure 1-6. Basic structure of the Paralytic Shellfish poisoning Toxin (PST) molecule showing side groups (R1-4) which can be modified to form the different analogues.

PST derivatives differ by slight changes to their side groups, including: C-13, N-1-hydroxyl-, 11-hydroxysulfate- and 21-N-sulfo-substitutions and epimerisation at the C-11 position. These compounds are grouped according to their substitutions and hence their potency. High potency toxins include the carbamate toxins [STX and neosaxitoxin (neoSTX)] and gonyautoxins 1,2,3,4 (GTX 1-4, 8), whilst low potency toxins include the N-sulfocarbamoyl group (B1, B2, C1-4), decarbamoyl derivatives (dc-GTX1-4, dc-neo, dc-STX) and the 13-deoxy-decarbamoyl derivatives (doSTX, doGTX2,3) (Oshima *et al.* 1993, Lagos *et al.* 1996, Cembella 1998) (Table 1-2).

Table 1-2. Structural forms of Paralytic Shellfish Toxins (PSTs) and their relative potency.

Structural form	Derivatives	Potency
Carbamate	GTX1-4, GTX8, neo, STX	high
dc-carbamoyl	dc-GTX1-4, GTX8, dc-neoSTX, dc-STX	low
Sulfamate/Sulfocarbamoyl/ N-sulfocarbamoyl	B1, B2, C1-4	low
Epimers	11-β and 11-α	
Moeties	Hydroxyl (e.g. N-1), hydroxylsulfate (C-11), carbamyl	

Modifications of the basic skeleton include addition of a: hydroxyl (OH) group at N1 (R_1), O-sulfate at C-11 (R_2, R_3) or 21-N-sulfo group to produce the N-sulfocarbamoyl toxins (Fig. 1-6, Table 1-3). Groups that can be added or removed to form the different derivatives include: hydroxyl, carbamyl and hydroxysulfate moeties (Table 1-2, 1-3). Taroncher-Oldenburg *et al.* (1997) hypothesise that the C2 derivative is the first to accumulate in the biosynthetic pathway in an *A. fundyense* isolate and is subsequently transformed into the other derivatives (Fig. 1-7).

The toxicity of PST derivatives varies by over two orders of magnitude (Table 1-4) (Oshima 1995). The toxicity of STX has been calculated as 5500 Mouse Units (MU).mg^{-1} (or 5.5 MU.μg^{-1}). Therefore, if 1 µg = 5.5 MU, then 0.182 µg = 1MU (Jellett *et al.* 1995).

Table 1-3. Structure of Paralytic Shellfish Toxins (PSTs). STX, saxitoxin; GTX, gonyautoxin; neoSTX, neosaxitoxin; dc, decarbamoyl; R1-4, side groups as depicted in Figure 1-7 (after Sako *et al.* 2001).

	R1	R2	R3	R4
STX	H	H	H	$CONH_2$
neoSTX	OH	H	H	$CONH_2$
GTX1	OH	H	OSO_3^-	$CONH_2$
GTX2	H	H	OSO_3^-	$CONH_2$
GTX3	H	OSO_3^-	H	$CONH_2$
GTX4	OH	OSO_3^-	H	$CONH_2$
GTX5	H	H	H	$CONHSO_3^-$
GTX6	OH	H	H	$CONHSO_3^-$
epiGTX8(C1)	H	H	OSO_3^-	$CONHSO_3^-$
GTX8 (C2)	H	OSO_3^-	H	$CONHSO_3^-$
C3	OH	H	OSO_3^-	$CONHSO_3^-$
C4	OH	OSO_3^-	H	$CONHSO_3^-$
dcSTX	H	H	H	H
dcneoSTX	OH	H	H	H
dcGTX1	OH	H	OSO_3^-	H
dcGTX2	H	H	OSO_3^-	H
dcGTX3	H	OSO_3^-	H	H
dcGTX4	OH	OSO_3^-	H	H
11-α-hydroxySTX	H	H	OH	$CONH_2$
11-β-hydroxySTX	H	OH	H	$CONH_2$

Figure 1-7. Putative sequence of events in the biosynthesis of saxitoxin (STX) and its derivatives. Continuous lines represent most likely interconversions, as deduced from changes in total toxin composition per cell over the cell division cycle of *Alexandrium fundyense*. Dashed lines represent other possible paths (after Taroncher-Oldenburg *et al.* 1997).

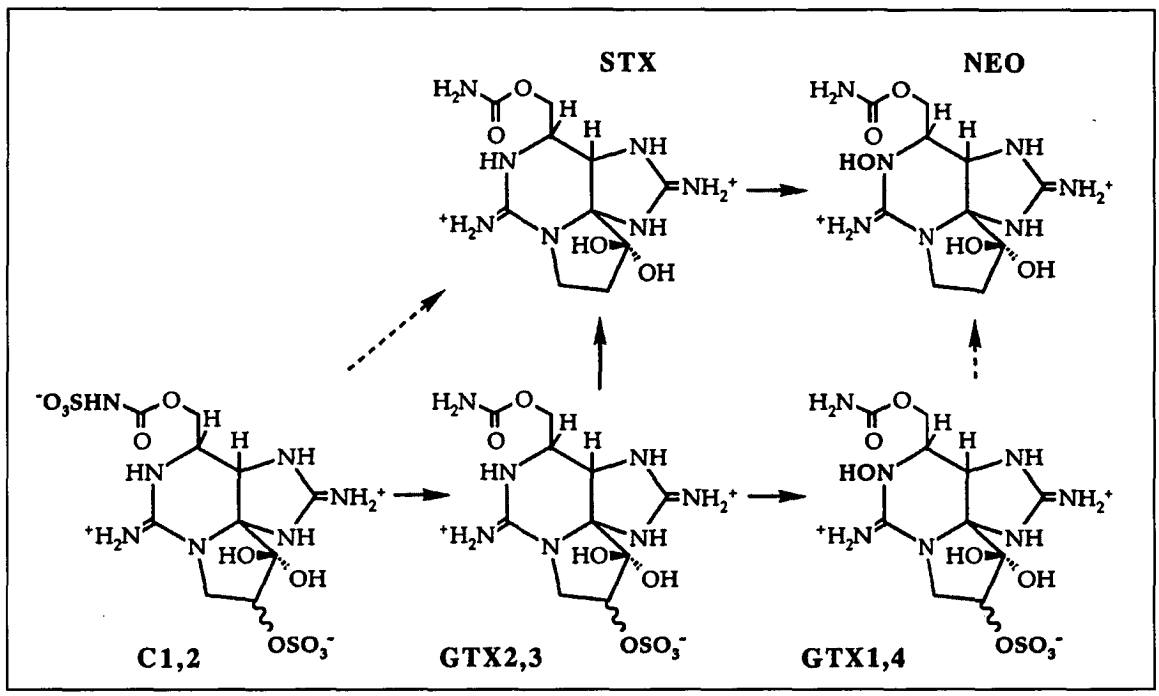


Table 1-4. Relative toxicity of Paralytic Shellfish Toxin (PST) derivatives to STX (after Oshima 1995).

PSP toxin	Toxicity factor	PSP toxin	Toxicity factor
STX	1	C2	0.10
neo	0.92	C3	0.01
GTX1	0.99	C4	0.06
GTX2	0.36	dcSTX	0.51
GTX3	0.64	dcneo	no data
GTX4	0.73	dcGTX1	no data
GTX5	0.06	dcGTX2	0.65
GTX6	no data	dcGTX3	0.75
C1	<0.01	dcGTX4	no data

1.8 PUTATIVE BIOSYNTHETIC PATHWAYS OF PST

Current research on PSTs is aimed at developing an understanding of the enzymes required to synthesise toxins, the genes that encode them, their regulation, evolution and spread through the marine community (Plumley 1997). Previous work on PSTs has focused on observing the variability in toxin production by single species or among isolates of a species, frequently as a function of environmental growth conditions. Other research can be divided into the categories of: 1) isotope feeding studies, 2) pharmacological aspects of toxins and 3) the genetics of toxin production and genetic stability (Plumley 1997, Cembella 1998). Isotopic studies have been able to identify substrates that are precursors to toxin compounds (Plumley 1997, Macpherson *et al.* 2003). At present knowledge the mechanisms of PST production in dinoflagellates, cyanobacteria and/or other bacteria are yet to be understood and knowledge of the biosynthesis of algal toxins is limited (Pomati *et al.* 2001).

Marine dinoflagellates and cyanobacteria produce an extraordinary range of chemical compounds (Shimizu 2003), some of which are toxic to animals. For instance, the cyanobacteria *Anabaena flos-aquae* and *Aphanizomenon flos-aquae* produce potent neuromuscular poisons [anatoxin and STX (PST), respectively]. Although these toxins are both alkaloids, they are chemically very different (van den Hoek *et al.* 1997). The gene clusters responsible for the production of some secondary metabolites, but not PSTs, of cyanobacteria have recently been elucidated (Shimizu 2003) but are different to those of dinoflagellates. Many dinoflagellates produce unique polycyclic ethers of polyketide origin. These are among the most complex and unique natural polyketide structures identified (Snyder *et al.* 2003). A new mechanism for the formation of the truncated polyketide backbones has recently been proposed, involving the PKS gene. This gene is responsible for the production of most dinoflagellate toxins (Shimizu 2003), yet it has not been found in *Pfiesteria* and is not likely to be involved in the production of either PSTs or *Pfiesteria* toxin.

The citric acid cycle, also known as the Krebs cycle, appears important for the production of many algal toxins. Domoic acid, a toxin produced by marine diatoms and red algae, is produced by condensation of an activated citric acid cycle derivative (e.g. α-ketoglutarate → glutamate + geranyl pyrophosphate →→→ domoic acid)*. There is evidence that dinoflagellate polyether toxins (e.g. brevetoxin, okadaic acid and ciguatoxin) are a product of a complex pathway involving ubiquitous substrates such as acetate and dicarboxylic acids from the citric acid cycle (Plumley 1997).

A study by Pomati *et al.* (2001) suggests a possible link between PST accumulation and the activation of the metabolism that leads to purine degradation in the filamentous freshwater cyanobacterium *Planktothrix sp.* FP1. The activity of allantoicase, a key inducible enzyme of purine metabolism, was used as a tool for assaying the activation of the purine degradation pathway. The enzyme and the pathway showed the best induction by: crude *E. coli* cell extracts; secondly allantoic acid, the direct substrate of allantoicase; adenine and, to a lower degree, by urea, one of the main products of purine catabolism. Differential accumulation of PSTs were observed for the different treatments, with the cyanobacterial culture induced with allantoic acid accumulating the most toxin (1.7%) in comparison with the control. From these results, Pomati *et al.* (2001) suggest a degradation pathway for the PSTs similar to purine alkaloids in higher plants. They also suggest that STX and derivatives may be converted into xanthine, urea, and further to CO_2 and NH_4^+.

In contrast, other studies provide strong evidence that PSTs, although similar in structure to purines, are not derived from the purine biosynthesis pathway. Instead, it is almost certain that in dinoflagellates, substrates from the citric acid and urea cycles are incorporated into the PST molecule (Shimizu *et al.* 1985, Shimizu *et al.* 1996). Studies have fed ^{13}C- and ^{15}N-labelled substrates to toxigenic cyanobacteria and conducted nuclear magnetic resonance analysis of the purified toxins, indicating that PSTs are not derived from purine metabolism. Rather, studies have shown that PSTs are

* multiple arrows represent multiple unknown steps

synthesised via a pathway involving arginine, S-adenosylmethionine, acetate, and other, as yet uncharacterised cellular metabolites (Shimizu *et al.* 1990a, 1990b, Shimizu & Wrensford 1993) (Fig. 1-8). Dinoflagellate toxin production has been enhanced when cultures have been grown on intermediates of the citric acid cycle (e.g. succinate, malate, fumarate). These intermediates can increase intracellular levels of acetate, arginine (via α-ketoglutarate) and/or other products that flow into the putative toxin biosynthetic pathway (Plumley 1997).

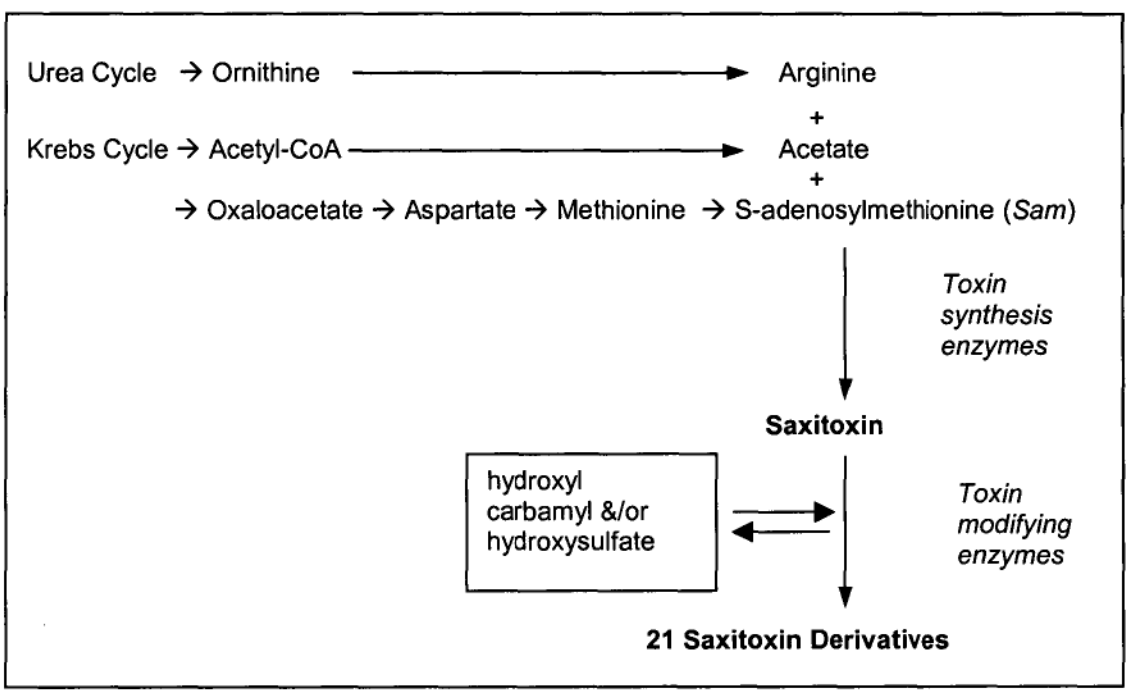


Figure 1-8. Schematic representation of the putative involvement of the citric acid (Krebs) cycle and the urea cycle in the production of saxitoxin and its derivatives (after Plumley 1997).

Although the production of PSTs by dinoflagellates and cyanobacteria has been well documented, little is known about the biosynthetic pathways of PST synthesis and degradation, their metabolic role, why some closely related dinoflagellates do not accumulate PSTs, why different strains accumulate different sets of STX analogues, and the molecular basis underlying environmental-induced changes in PST composition (Plumley 2001, Pomati *et al.* 2001). Furthermore, it has not been established whether the enzymes for PST biosynthesis are encoded by dinoflagellate DNA

(chromosomal, chloroplast or mitochondria) or other DNA sources such as bacteria, viruses or plasmids (Ishida *et al.* 1998). However, unlike most proteins, alkaloids and hence PSTs are not derived from a single gene but rather a suite of genes that act in a biochemical pathway.

Inheritance studies of the PST profile of F_1 *Alexandrium* spp. cells from parents with different toxin compositions have shown that the toxin profile can be inherited in a biparental (2:2) Mendelian fashion, or differ from the parent phenotype (Ishida *et al.* 1998). It is suggested that general recombination in toxin genomes possibly occurs, mating types (mt^+ and mt^-) are not always associated with toxin inheritance, and the genes encoding enzymes for the production of PSTs are coded in the chromosomal DNA (Sako *et al.* 1992, Ishida 1993, Ishida *et al.* 1998). The proportion of cellular PSTs is a stable property in *Alexandrium spp.* (provided cells are not exposed to physiological stress) and hence is a useful phenotypic marker (Franco *et al.* 1994, Hold *et al.* 2001a).

Two putative enzymes involved in PST biosynthesis that have been hypothesised are a: (i) methyl transferase (MT) to incorporate the methyl group of S-adenosylmethionine (SAM) into the toxin nucleus and (ii) arginine methyl transferase (ArgMT) to combine the 2-carbon precursor product of arginine and acetate into the toxin nucleus. Transfer of the methionine methyl group of SAM to the toxin nucleus probably involves an electrophilic substitution on a reactive dehydro intermediate (Shimizu *et al.* 1985). Also, two toxin modifying enzymes that are purportedly involved in the addition and/or removal of substituent groups (such as C-11 and N-21 sulfation) are N-(amino) sulfotransferases (N-ST) and O-sulfotransferases (O-ST) (see Chapter 4). The former has been shown to convert 11-α,β-hydroxySTX to the GTX 2 and 3 derivatives, while the latter could potentially modify the C-11 moiety of 11-α,β-hydroxySTX. Other enzyme groups that have been suggested include oxidoreductases (hydrolases) and carbamoylases (Fig. 1-9) (Ishida *et al.* 1998).

Molecular tools can be used to elucidate genes involved in the production of PST molecules. Two possible techniques are differential display as demonstrated by Taroncher-Oldenburg & Anderson (1998) and PCR using degenerate primers designed to known candidate genes in other organisms. Confirmation that candidate gene(s) identified from either method are linked to toxin biosynthesis, relies on knowledge of a differential system of toxin production and hence gene expression. Study of cellular toxin content during the cell cycle and growth phases may be informative.

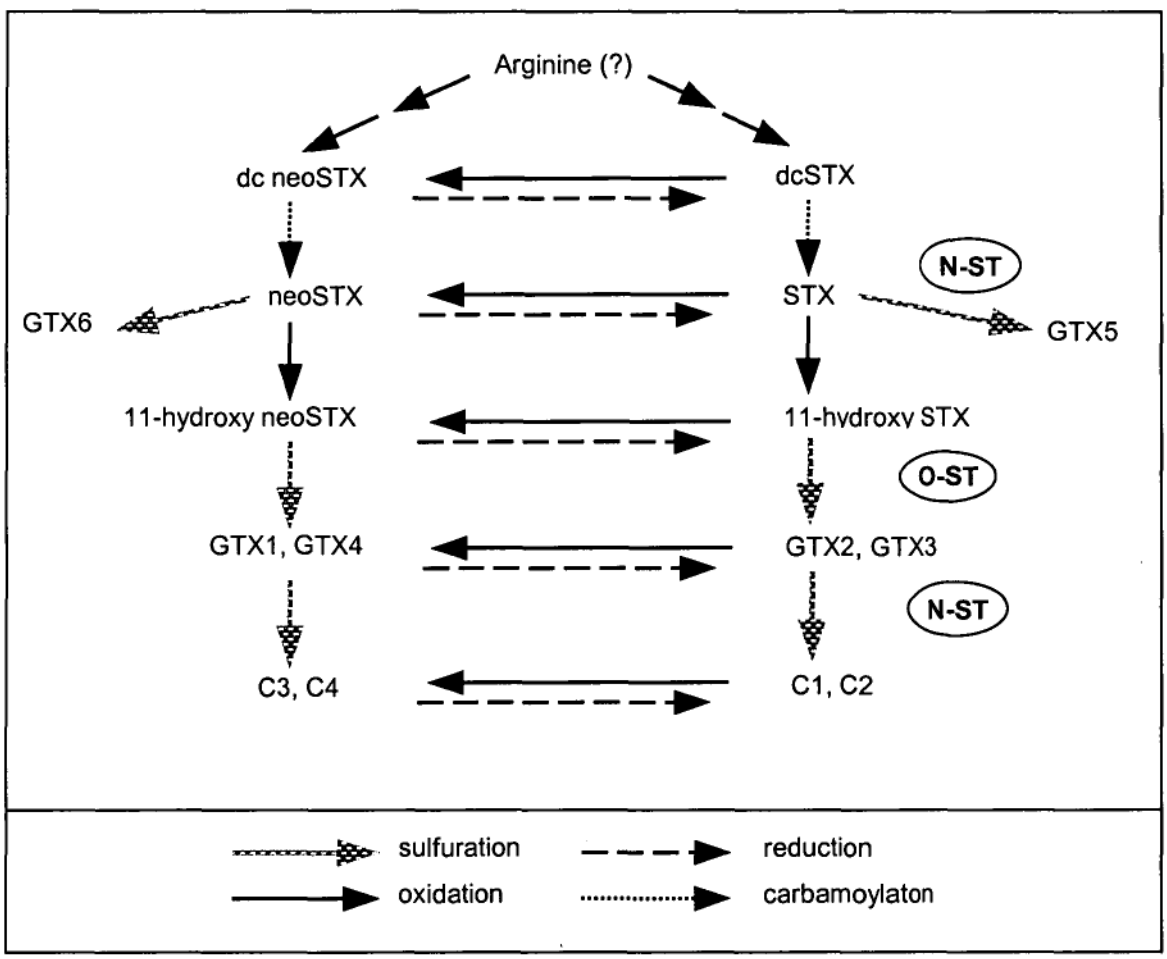


Figure 1-9. Diagram showing putative steps in the biosynthesis of PST molecules in *Gymnodinium catenatum*. Note that arrows represent enzymatic reactions in the pathway. Arginine is one of the initial substrates (after Ishida *et al.* 1998).

1.9 OVERVIEW OF DINOFLAGELLATE GENETIC STUDIES

Genetics studies of dinoflagellates, especially of chromosomal genes, is a relatively new field and extensive studies have only been performed for three species: *Gloeodinium montanum, Symbiodinium microadriaticum* and *C. cohnii.* These species can be grown on solid agar media, unlike most dinoflagellates that can only be cultured in liquid, making them easier to manipulate for genetic study (Ishida *et al.* 1998). Only relatively recently the first dinoflagellate gene encoding the luciferin-binding protein, was cloned and sequenced at the complementary DNA (cDNA) and genomic levels in *Gonyaulax polyedra* (Lee *et al.* 1993). Since then genetic studies on the biosynthesis of dinoflagellate metabolites have been slow (Rein & Borrone 1999). However, in the past 2-3 years, large-scale analysis of cDNA libraries to several species have been published: *Lingulodinium polyedr*um (1012 non-redundant sequences) and *Amphidinium carterae* (2143) (Bachvaroff *et al.* 2004), *Alexandrium tamarense* (Hackett *et al.* 2005) and *Karenia brevis* (Lidie *et al.* 2005).

The peculiarities of the dinoflagellate nucleus and nucleic acid structure (Soyer-Gobillard *et al.* 1999), along with the potentially complex bacterial associations (Alavi *et al.* 2001), armouring of some species, and lack of a dinoflagellate transformation system have hampered genetic studies (Rein & Borrone 1999). For instance, the often tough cellulose plates of armoured dinoflagellates makes these cells difficult to lyse and extract nucleic acids, symbiotic bacteria cannot be eliminated from dinoflagellate cultures (Baker *et al.* 2003) confounding genetic and toxin studies, and unusual nucleotide features may complicate primer design and gene amplification. It is already known that in several dinoflagellate species, more than 60% of thymines are replaced by a rare base, hydroxymethyluracil (Steele & Rae 1980, Herzog & Soyer 1982, Soyer-Gobillard *et al.* 1999), but whether other peculiarities exist, and whether these will effect molecular protocols such as polymerase chain reaction (PCR) is unknown.

The unique combination of morphological and genetic traits means that current molecular genetic techniques often need to be adapted to suit

dinoflagellates (see Chapter 3). When the genetics of an organism is poorly understood, as is the case with some dinoflagellates, there is a lack of nucleotide sequence information to draw upon. Polymerase chain reaction is one method of identifying novel dinoflagellate genes. This technology relies on knowledge of homologous gene(s) in other species as well as the ability to design effective primers: oligonucleotide probes that target specific genes of interest. Primer design is facilitated by genetic information about the target organism, such as codon usage, guanine (G) and cytosine (C) content, and nucleotide base modifications. When the target gene hasn't previously been identified in a related organism, degenerate (less specific) primers must be designed.

Standard methods of dinoflagellate DNA extraction and micromanipulation do not discriminate other sources of DNA. This is a problem because most dinoflagellate cultures possess microbial associations (e.g. mycoplasma, yeasts, fungi and bacteria) (Tosteson *et al.* 1989, Groben *et al.* 2000, Alavi *et al.* 2001) unless they have been treated appropriately (e.g. with antibiotics). It is likely that all laboratory grown dinoflagellate cultures will contain bacteria whether they be endosymbiotic, epiphytic or in the culture media. If this DNA is used as a template in PCR there is a chance that bacterial gene(s) will be co-amplified, with an increased risk when degenerate primers are used. A theoretical solution to this problem is to ensure that dinoflagellate DNA is pure or that the culture is axenic.

The presence or absence of bacteria does not appear to be species specific but varies between strains and species of different locations. For instance, endosymbiotic bacteria have recently been identified in the potentially toxic dinoflagellates *A. tamarense*, *A. fundyense, A.minutum* (Kodama *et al.* 1996, Lu *et al.* 2000), *A. catenella* (Cordova *et al.* 2002) and *G. catenatum* (Franca *et al.* 1996). In contrast, in a study by Biegala *et al.* (2002), intracellular bacteria were not observed in either toxic or non-toxic strains of *Alexandrium spp.* It may therefore be possible to produce axenic dinoflagellate cultures for selected strains but presumably only those that do not have an obligate relationship with bacteria. Caution must be taken when deeming a

dinoflagellate culture to be axenic. Rigorous detection methods must be utilised before strong conclusions about the presence or absence of bacteria can be made.

1.10 RESEARCH AIMS

This project is a pioneering study to investigate dinoflagellate genetics and evaluate the molecular tools available to study dinoflagellates. The first aim was to develop a method of obtaining "pure" dinoflagellate DNA, free from bacteria, as a template for PCR. Through a PCR-based approach, this study identified and investigated a novel dinoflagellate gene, *Sam*, in a range of dinoflagellates, with a focus on *A. minutum* (Fig. 1-10), *A. catenella* (Fig. 1-11) and *G. catenatum* (Fig. 1-12). *Sam* is a precursor gene that is linked to the early stages of PST biosynthesis. In order to perform gene expression studies, a simple method of preserving RNA in intact dinoflagellate cells (armoured and unarmoured) was developed. The aim was to preserve dinoflagellate cells collected at regular intervals over the cell division cycle (CDC) for extraction at a later date. A simple method of extracting and purifying total RNA, using equipment available in most laboratories, was developed.

The next aim was to investigate *Sam* and two other candidate PST genes, *Sahh* (encoding the enzyme S-adenosylhomocysteine hydrolase) and *Map* (encoding the enzyme methionine aminopeptidase), previously identified by Taroncher-Oldenburg *et al.* (1999). Taroncher-Oldenburg *et al.* (1999) report PST production to occur in *A. fundyense* cells during early G_1 phase of the CDC. The present study provided additional nucleotide sequence information for *Sahh* and *Map*. The quantitative real time PCR (QPCR) method was used to provide gene expression data for *Sahh* and *Map*, and novel information for *Sam* over the CDC of *A. catenella*. At the time of this study, to my knowledge, QPCR had not been used to study gene expression in a dinoflagellate. One of the first reports of the QPCR method applied to dinflagellates was for the detection of cells in environmental samples (Bowers *et al.* 2000).

Figure 1-10. The neurotoxic, armoured dinoflagellate *Alexandrium minutum,* a causative organism of Paralytic Shellfish Poisoning (PSP). Scale bar = 10 μm (A,B,D-G) and scale bar = 5 μm (C). A) Light micrograph of individual cell in ventral view showing girdle and longitudinal grooves. B) Light micrograph of cell showing golden brown chloroplasts. C) Scanning electron micrograph of a single cell in apical view showing apical pore (from Hallegraeff *et al.* 1991). D-G) Fluorescence light micrographs of individual cells showing cellulose thecal plates stained with calcafluor (blue) and autofluorescence of the chloroplasts (red). D) Ventral view showing distinctive girdle groove. E) Dorsal view. F) Apical view showing pore structure. G) Cell showing chloroplast shape and arrangement.

Figure 1-11. The neurotoxic, armoured dinoflagellate *Alexandrium catenella,* a causative organism of Paralytic Shellfish Poisoning (PSP). A-B) Light micrographs of individual cells in ventral view showing golden brown chloroplasts. The pinched in edges of the cell in B highlights the girdle groove. Scale bar = 15 μm (A, B, D, E, G). C) Scanning electron micrograph of a 2 cell chain showing details of the cell surface. Note the girdle and longitudinal grooves distinctive of dinoflagellates (from Hallegraeff *et al.* 1991). Scale bar = 10 μm. D-G) Fluorescence light micrographs of individual cells stained with calcafluor. D) Ventral-apical view showing thecal plates (blue) with distinctive apical pore (top of cell) and red autofluorescence of the chloroplasts below the theca. E) Ventral view showing girdle and longitudinal grooves. F) Apical view showing pore structure. Scale bar = 10 μm. G) Dorsal view showing cellulose thecal plates.

Figure 1-12. Chain forming *Gymnodinium catenatum*, a causative organism of Paralytic Shellfish Poisoning (PSP). A) Light micrograph of 3 cells in ventral view showing golden brown chloroplasts and girdle and longitudinal grooves. B-C) Fluorescence light micrographs of 4 cells in a chain showing red autofluorescence of the chloroplasts. The girdle and longitudinal grooves are visible in B whilst the dark patches in C highlight the position of the large nucleus. D) Scanning electron micrograph of a 7 cell chain showing details of the cell surface, including the coiled flagellum in the girdle groove and trailing longitudinal flagellum (from Hallegraeff *et al.* 1991). Note the cells are unarmoured. Scale bar = 40 μm.

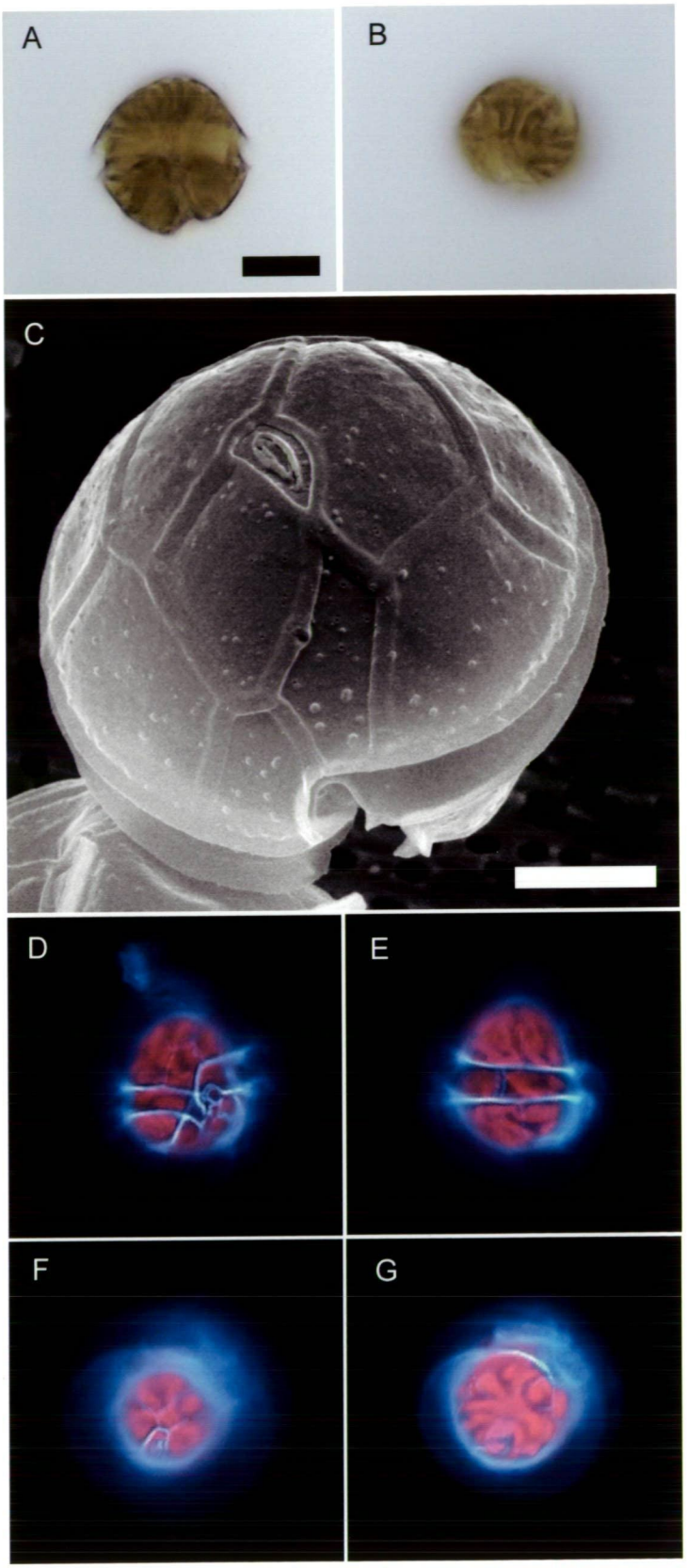


Figure 1-10.

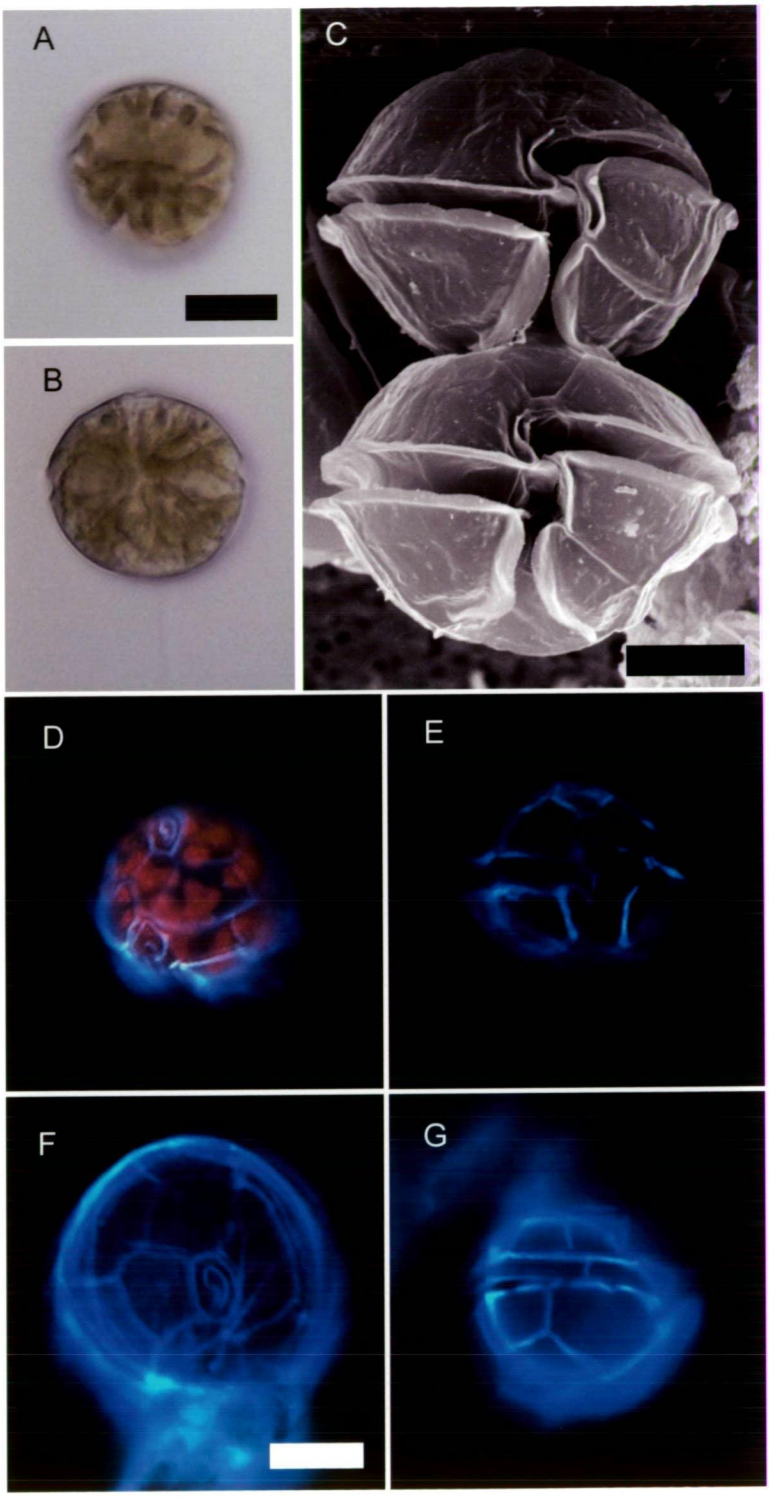


Figure 1-11.

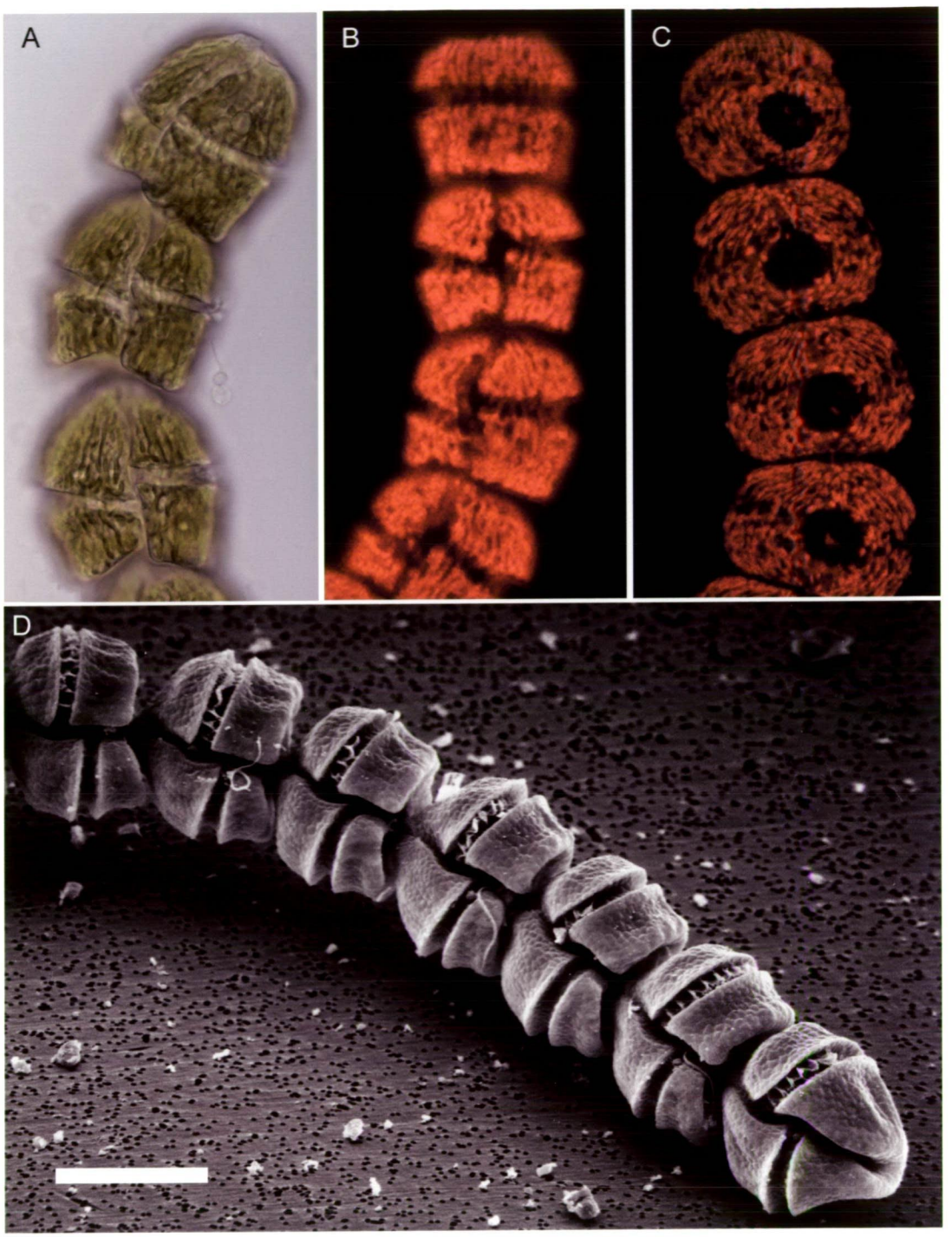


Figure 1-12.

Chapter 2: The challenge of producing axenic cultures of *Gymnodinium catenatum*, *Alexandrium catenella* and *Alexandrium minutum* for molecular biology

2.1 INTRODUCTION

The aim of producing axenic dinoflagellate cultures has usually been for application in physiology and toxicology studies rather than molecular genetics. Polymerase chain reaction (PCR) is a highly sensitive technique that is dependent on the quality and purity of the template DNA. The nature of PCR means that if there is any contaminating DNA present, even in trace quantities, it may be amplified along with the target DNA. In cases where multiple products are generated, targeting dinoflagellate genes when sequencing, is problematic. Such problems are exacerbated where primers are degenerate or the gene of interest is highly conserved between different organism kingdoms.

The most widespread application of molecular genetics in dinoflagellates has been related to species resolution and population genetics. Sequencing of ribosomal genes to study dinoflagellate evolution is extensive [*Alexandrium spp.* (Scholin *et al.* 1993, 1994, 1995, Adachi *et al.* 1994, 1996, Scholin & Anderson 1994, 1996), *Gymnodinium spp.* (Adachi *et al.* 1997, Daugjerg *et al.* 2000), *Gyrodinium* and *Amphidinium* (Daugjerg *et al.* 2000), *Prorocentrum* (Lenaers *et al.* 1989) and *Symbiodinium* (Belda-Baillie *et al.* 2002)]. Therefore there is a large bank of sequence information that can be drawn upon to study ribosomal genetics, and primers for ribosomal genes can be designed to target specific microalgal groups. The presence of contaminating DNA in PCR is not a concern in this application and template DNA can be isolated from algal cultures that contain bacteria. In contrast to ribosomal genes, other dinoflagellate nuclear genes remain virtually unstudied. There is a lack of nucleotide sequence information, and this makes primer design for nuclear genes difficult.

It has long been known that bacteria are associated with cultured dinoflagellates (Silva 1982, 1990). Studies by Cordova *et al.* (2002) found that *Alexandrium catenella* clones were simultaneously infected by different species of endocytoplasmic bacteria. Numerous studies have independently isolated and characterised bacteria associated with dinoflagellate cultures, including: *Alexandrium tamarense* (Kodama *et al.* 1988, 1990, Babinchak *et al.* 1998, Doucette & Trick 1995), *Alexandrium fundyense*, *A. catenella* and *Alexandrium minutum* (Babinchak *et al.* 1998), *A. lusitanicum* and *Gymnodinium catenatum* (Franca *et al.* 1996). However, few authors have reported methods for overcoming the problem of bacteria, particularly for molecular work (Snyder *et al.* 2003).

Antibiotic treatment is the most common method used to try and remove bacteria from dinoflagellate cultures and has had differing success. The antibiotics used must have a broad range of antimicrobial properties (Table 2-1). Some studies report that after antibiotic treatment no bacteria were detected (Hold *et al.* 2001a, Wang *et al.* 2004), while others report that bacteria-free cells could not be generated and the dinoflagellate cells were killed (Cordova *et al.* 2002). The study by ten Lohuis & Miller (1998) found antibiotic tolerance of dinoflagellates (*Symbiodinium* and *Amphidinium*) to be similar to that of fungi and diatoms, with on average 50-fold higher levels of antibiotics (kanamycin, G418 and hygromycin) required to kill 100% of dinoflagellates in comparison to higher plants. The resulting bacteria-free status of treated dinoflagellate cultures is often circumstantial because of a lack of continuous monitoring, or the limitations of the methods used to detect bacteria (Gallacher & Smith 1999).

Although it was assumed from the start of the present work that all dinoflagellate cultures contained associated bacterial flora, the extent of contamination (e.g. bacterial numbers) and whether this would affect the successful use of molecular genetic tools, was unknown. The main objective of this study was to evaluate the problems of axenic dinoflagellate culture techniques and determine whether antibiotic treatment would generate axenic cultures for use in molecular biology. Identification of bacteria to

genus and species level was not the aim; however, a basic identification (e.g. gram staining) assisted in targeting these bacteria. The species of dinoflagellates studied were *G. catenatum, A. catenella* and *A. minutum.* The ultimate goal was to obtain pure dinoflagellate DNA for use in PCR. The concept of PCR based techniques as useful tools for identifying and studying genes previously undocumented in dinoflagellates is presented.

Table 2-1. Antimicrobial properties of antibiotics used to treat dinoflagellate cultures based on literature. ×× Effectiveness against most species, × effectiveness against certain species.

Antibiotic	Gram(+) bacteria	Gram(–) bacteria	Mycobacteria	Mycoplasma	Yeasts & moulds
Gentamicin	×	××		××	
Kanamycin	××	××		××	
Penicillin G	××	×			
Streptomycin	×	××	×		
Timentin	××	××			
Nystatin					××

2.2 Materials And Methods

2.2.1 Dinoflagellate strains and laboratory culture

Dinoflagellate strains from Japan, New Zealand (Cawthron Institute, Nelson), Spain and Australia were selected as a representative sample for studying axenic culture techniques (Table 2-2). Clonal cultures of each strain were established by inoculating 50-70 ml of GSe growth medium (Blackburn *et al.* 2001) with 1 ml of mid-logarithmic phase culture. Cultures were maintained in 100 ml, 65 mm diameter cylindrical polycarbonate vials (LabServ) at 17°C (for *G. catenatum* and *A. catenella*) and 20°C (for *A. minutum*) under cool white fluorescent light (80-100 μmol photons.m^{-2}.s^{-1}) with a 12:12 h light:dark cycle. Standard sterile techniques were employed to maintain cultures. Culture vials and seawater were autoclaved at 121°C for 20 min and growth medium filtered through a 0.22 μm filter. The cultures in this investigation are

held by the University of Tasmania, School of Plant Science, Algal Culture Collection.

Table 2-2. Dinoflagellate cultures studied.

Species	Strain	Location isolated	Isolator	Date of Isolation
Gymnodinium	GCJP10	Japan	T. Ikeda	1986
catenatum	CAWD106	Manganui Bluff, New Zealand	L. McKenzie	10.08.2000
	GCPL01	Port Lincoln, SA	C. Bolch & J.M. Le Roi	17.04.1996
	GCTRA01	Triabunna, TAS	M. de Salas	24.05.2000
	GCDEO6	Derwent River, TAS	S. Blackburn	08.01.1987
	GCDE11	Derwent River, TAS	M. de Salas	01.05.2000
Alexandrium	ACSH01	Sydney Harbour, NSW	S. Norwood	1993
catenella	ACAD01	West Lakes, Adelaide, SA	M. de Salas	15.04.2000
	ACSP01	Tarragona, Spain	M. Villa-Reig	1996
	CAWD44	Tauranga, New Zealand	L. McKenzie	1996
Alexandrium	AMAD06	Adelaide, SA	S. Blackburn	27.10.1987
minutum	AMNC04	Newcastle, NSW	C. Bolch	28.08.1997

2.2.2 Solid agar media for dinoflagellate growth

A novel approach of dinoflagellate culture was tested. The ability of two *A. catenella* strains (ACAD01 and ACSP01) and two *G. catenatum* strains (CAWD106 and GCTRA01) to grow on solid agar media was examined. Between 20-40 cells were transferred onto sterile control and treatment GSe agar (0.25% and 0.5% agar) plates. Control plates lacked antibiotics while treatment plates contained four times the standard dose of penicillin G/streptomycin/gentamicin (final concentration 400/100/100 $\mu g.ml^{-1}$) (adapted from Guillard & Morton 1995). Observations of cell health were made 6 days after the plates were inoculated.

2.2.3 Axenic techniques for dinoflagellate growth

2.2.3.1 Antibiotic sensitivity trial

The antibiotic resistance of *G. catenatum*, *A. catenella* and *A. minutum* to different doses of antibiotics was initially tested on a small-scale. One ml of actively growing algal culture (Table 2-3) was added to 8-9 ml of sterile GSe media containing an antibiotic cocktail. Two treatment trials were prepared; a standard dose trial (penicillin G/gentamicin/streptamycin at a final concentration of 100/25/25 $\mu g.ml^{-1}$), and a four times standard dose as described previously. Cultures were maintained in five six well microplates (IWAKI, polystyrene 3810-006). Algal health and the presence of bacteria in the culture media were monitored under the light microscope on a weekly basis for 3 weeks. Following this trial, successive antibiotic treatments were carried out on 50-70 ml cultures to obtain enough cells for DNA isolation (Fig. 2-1).

2.2.3.2 Continuous antibiotic exposure

Continuous antibiotic exposure was also performed on dinoflagellate cultures listed in Table 2-2. Firstly, the bactericidal effectiveness of the antibiotic timentin and its effect on algal growth was tested. No report of timentin treatment of marine dinoflagellates was found in the literature. Timentin (50 $mg.ml^{-1}$) was added to GSe medium to a final concentration of 35 $\mu g.ml^{-1}$. Control (no timentin) and treatment cultures were established as described in 2.2.1. For the second treatment, 1 ml of each of the control and treatment cultures was transferred into fresh GSe medium lacking antibiotics (control) and a standard dose of penicillin G/streptomycin/gentamicin (treatment) as described previously. For the third treatment, 2-week-old cultures were transferred as previously described but the treatment dose was increased four fold. Cultures were transferred after 30 days into a fourth treatment of the same 3 antibiotics, plus 2 additional antibiotics, kanamycin 500 $\mu g.ml^{-1}$ and ampicillin 1 $mg.ml^{-1}$ (Fig. 2-1).

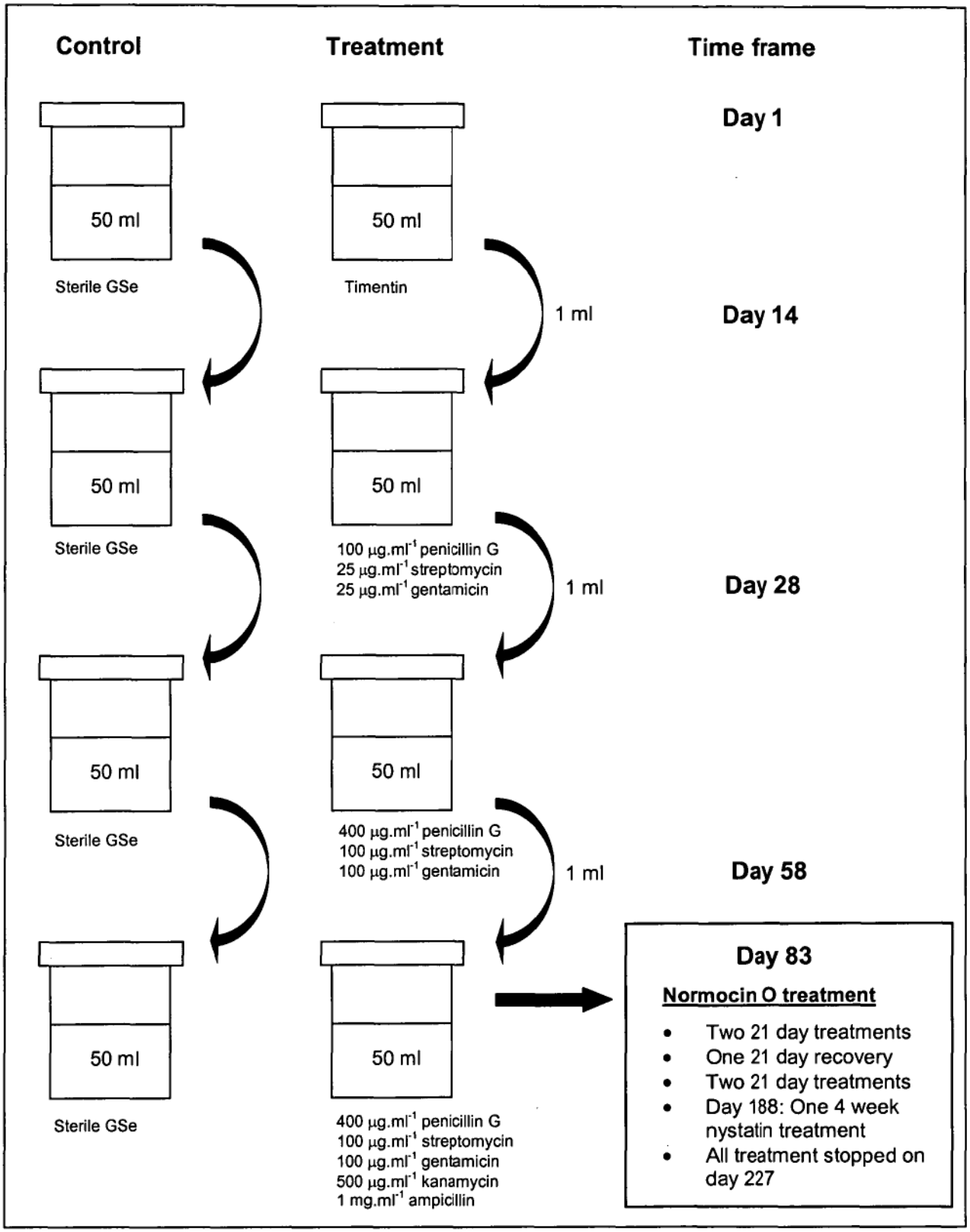


Figure 2-1. Schematic diagram showing the procedure of antibiotic treatment of dinoflagellate cultures.

Due to the slow growth of all cultures, algae were transferred 66 days later. Although fungal treatment is not standard practice for axenic culture of dinoflagellates, to be thorough, two antifungal products were used. The treatment group was inoculated into GSe containing Normocin O 100 $\mu g.ml^{-1}$ (InvivoGen, Cat. Nº. ant-nr-o), a filter sterile aqueous solution for the prevention of mycoplasma and fungal contamination in eukaryotic cell cultures. The active concentration of Normocin O should display no toxicity to the cells being treated, but this has not been shown for cultured algae. Both antibacterial components are active against Gram+ and Gram- bacteria and mycoplasma. Two successive 3-week Normocin O treatments were followed by one 3-week recovery (no treatment) and two final 3-week Normocin O treatments (Fig. 2-1).

Dinoflagellate cultures undergoing antibiotic treatment were inspected regularly (weekly-fortnightly) with the naked eye and at the light microscope level for the presence of bacteria/fungi and to examine cell health. Normocin O is not designed to eliminate fungi and bacteria that are already established in a eukaryotic cell culture. Therefore, another antifungal product, nystatin, was used. Nystatin suspension (Sigma- Aldrich Product Nº. N9767, plant cell culture tested) is an antifungal agent produced from *Streptomyces* spp. that exhibits fungicidal and fungistatic activity against yeasts and moulds (Table 2-1). Nystatin was added to freshly transferred treatment cultures, at the recommended dose of 24 ml to 1 L of growth medium. Following this, control and treated cultures were maintained in sterile GSe with no further treatment.

DNA was isolated from control and antibiotic treated algal cultures after each antibiotic exposure experiment. Fifteen to 30 ml of early to mid-logarithmic phase culture was harvested by centrifugation at 2000 rpm for 5 min. The growth medium was decanted from the algal pellet and DNA initially extracted using a phenol:chloroform:isoamyl alcohol method described by Bolch *et al.* (1998). A second method of dinoflagellate DNA isolation was developed to isolate higher quality DNA suitable for use in PCR. The CTAB method described by McKinnon *et al.* (1999) was modified slightly and combined with the phenol:chloroform:isoamyl alcohol method described

above (Appendix 1). DNA was quantified with a spectrophotometer (SmartSpec, BioRad), and gel electrophoresis (1% agarose) and aliquots diluted to 10-20 ng.μl^{-1} and stored at -20°C until use in PCR.

2.2.3.3 Mating reactions and treatment of cysts

Mating reactions to produce cysts were performed in a pairwise matrix for the 6 *G. catenatum* strains listed in Table 2-2, following the method described in Bolch (1999). Approximately 60 GCTRA01 cysts were collected using a micropipette and washed four times in sterile 28‰ salinity GSe. Twenty cysts were transferred into each of two hydrogen peroxide (H_2O_2) treatments: 500 ppm and 1000 ppm H_2O_2, left for either 1.5 h or 24 h and rinsed in sterile water. Treated and non-treated cysts were prepared for PCR. Four to 8 cysts were transferred to a 200 µl thin-walled PCR tube in 5-10 µl sterile water. Tubes were stored at -20°C until required. Samples were thawed to room temperature and cyst walls ruptured by repeated freeze-thaw and vacuum drying (Bolch 1999). Test PCR was performed using the primers SAM-FC 5'-CGACCAGGGCCACATGTTYGGNTAYGC-3' and SAM-RF 5'- GCCGGAGAAGGCGCCNCCNCCRTG-3' (Chapter 3) in a total volume of 40 µl containing a final concentration of 0.25 mM dNTPs, 3 mM $MgCl_2$ and 1 µM primers. Thirty-five amplification cycles of denaturation for 15 s at 95°C, annealing for 15 s at 55°C and extension of 2 min at 72°C were performed, with a final cycle of 10 min at 72°C. The positive control contained 10-50 ng GCTRA01 DNA extracted using the phenol-chloroform method.

2.2.4 Detection of bacteria in dinoflagellate cultures

A number of methods were used to attempt to detect the presence of bacteria in dinoflagellate cultures. These included light microscopy, inoculating dinoflagellate culture media onto seawater agar plates with vitamins (SWAV plates), GSe agar plates and Johnson's media agar plates (Johnson *et al.* 1966) to check for growth of bacterial colonies; isolation of DNA (as described in 2.2.3.2) and PCR using bacteria specific primers; and fluorescent *in situ* hybridisation (FISH) using a Cy3 labelled bacteria probe (EUB338) (Groben *et al.* 2000) (Fig. 2-2).

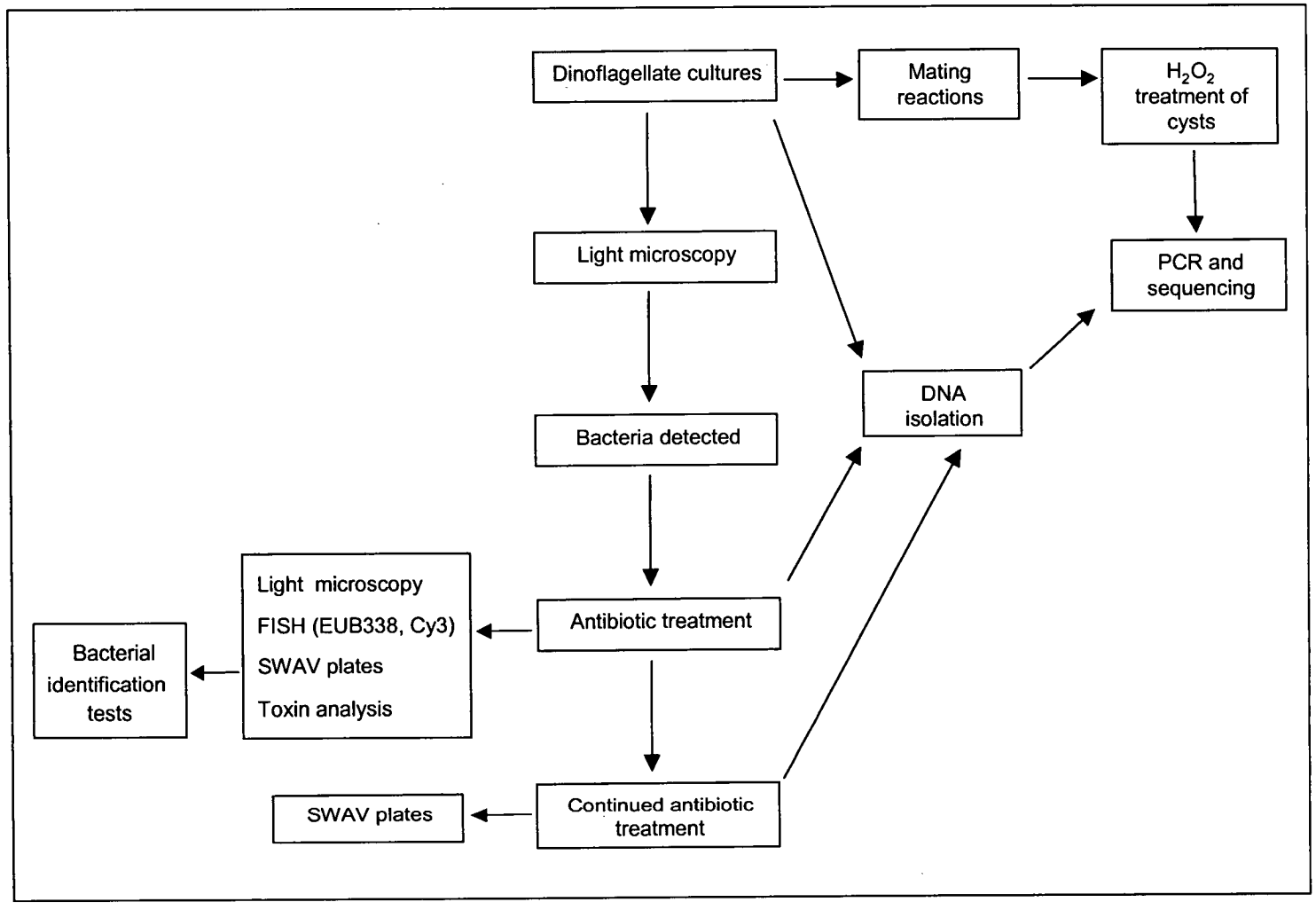


Figure 2-2. Schematic diagram of the procedure used to detect and isolate bacteria from dinoflagellate cultures.

2.2.4.1 Seawater agar plates with vitamins (SWAV)

Dinoflagellate cultures were initially examined under the light microscope for the presence of bacteria in the culture medium. Preliminary studies were conducted to identify the types of bacteria present. Firstly, SWAV plates (0.1% yeast extract, 0.1% bacteriological grade peptone, and 1.5% bacteriological grade agar) were prepared using 28‰ filtered seawater and autoclaved at 121°C for 20 min. The liquid seawater medium was cooled to 50°C and supplemented with 2.5 ml of 0.22 μm filter sterilised vitamin solution per litre (Balch & Wolfe 1976). Plates were stored at 4°C. Upon treatment of cultures for two weeks with the first antibiotic (timentin) and the third antibiotic (four times the standard dose of penicillin G/streptomycin/gentamicin) trial, SWAV plates (x2) were spread with 100 μl of each dinoflagellate culture and kept under the same conditions as for algal growth. Plain GSe medium was also spread plated as a control. Plates were monitored daily for the growth of bacterial colonies. The number, colour, shape and opacity of colonies present were recorded after 96 h.

For each of the three types of bacterial colonies detected on SWAV plates, a single colony was picked and a 16-streak plate produced. Each colony type was observed under the light microscope using oil immersion (100x magnification). Gram stain, oxidase, catalase and oxidation-fermentation (O/F) tests were preformed. The O/F test was performed under aerobic conditions to test whether the bacteria metabolised glucose by oxidation (requiring oxygen) or fermentation (independent of atmospheric oxygen). A single colony of each type was stabbed into individual tubes of O/F medium, sealed and maintaining under the laboratory conditions from which the colony was derived. Some bacteria detected in PCR were sequenced and the type of bacteria identified by the closest match produced from a GenBank search. Detailed bacterial identification was not the aim of the project and further identification was not performed.

To determine if any of the bacteria isolated above were resistant to antibiotics, SWAV plates were prepared by supplementing with 1 $\mu l.ml^{-1}$ of standard dose antibiotic stock solution (penicillin G/streptomycin/gentamicin) in conjunction with vitamins and inoculated with 100 µl dinoflagellate culture as previously described. Plain GSe medium was spread plated as a control. Plates were monitored daily for the growth of bacterial colonies. The number, colour, shape and opacity of colonies present were recorded after 96 h.

2.2.4.2 GSe and Johnson's media agar plates

Three cultures (one of each species): ACAD01, AMAD06 and GCTRA01 at the Normocin O phase of treatment were inoculated (400 µl) onto Johnson's media (complex) agar plates and monitored for bacterial growth. GSe agar (1%) plates were supplemented with 1 $\mu l.ml^{-1}$ antibiotic stock solution and inoculated with 100 µl of each of the 12 dinoflagellates cultures (timentin treated) listed in Table 2-2.

2.2.4.3 Polymerase chain reaction (PCR)

Oligonucleotide primers specific to the prokaryotic 16S ribosomal DNA (rDNA) gene (based on 1542 nucleotides) were used as a method of detecting live bacteria associated with dinoflagellate cysts or bacterial DNA potentially co-extracted with dinoflagellate DNA. Primers were chosen from the Ribosomal DNA primer database, primers for Bacterial SSU rRNA are located at the following site: http:rrna.uia.ac.be/primers/data/BS/BS_lst.html (maintained by Jan Wu yts) and published in Wilmotte *et al.* (1993). The forward primer (BSF8/20) starts at nucleotide base 8 and is 20 bp in length (corresponding to position 8-27 in *Escherischia coli*) and the reverse primer (BSR534/18) starts at nucleotide base 534 and is 18 bp in length (corresponding to position 534-517 in *E. coli*) (Table 2-3). A third primer tested was EPI277-T3R designed to a small subgroup of the bacteria class, Verrucomicrobia that may be missed by the BS primers (Petroni *et al.* 2000). PCR was conducted using H_2O_2 treated and non-treated *G. catenatum* cysts

(as described earlier) and DNA isolated from control and antibiotic treated dinoflagellate cultures as the template.

Table 2-3. Oligonucleotides for the detection of bacterial DNA in dinoflagellate DNA isolations.

Name	Sequence 5' → 3'	Length (bp)
BSF8/20	AGAGTTTGATCCTGGCTCAG	20
BSR534/18	ATTACCGCGGCTGCTGGC	18
EPI277-T3R	ATAGGTATTAACCCTCACTAAAGGGACAGATCAGCTACCCGTCTTA	46

2.2.4.4 Fluorescent in situ *hybridisation (FISH)*

Fluorescence *in situ* hybridisation (FISH) microscopy was used to detect the presence/absence of bacteria associated with dinoflagellates after successive treatment with antibiotics as described above. Fluorescence microscopy of control and Normocin O-stage treated cultures was performed for three species: *G. catenatum* (CAWD106), *A. catenella* (ACAD01) and *A. minutum* (AMAD06). Two methods were tested for the collection and preservation of cells: filtration and centrifugation.

Using the filtration method, 1 ml of each mid-logarithmic phase culture was harvested by filtration onto a black polycarbonate filter. The filter was placed in a small Petri dish, algal side up, on a piece of filter pad (Whatman) soaked in 10% formalin. The dish was sealed with parafilm and stored at 4°C for 4 h to preserve the cells. The filter was then transferred to a clean Petri dish to dry, washed with 1-2 ml of ice cold 50% PBS:50% ethanol and stored at -20°C.

Using the centrifugation method, approximately 7 ml of each mid-logarithmic phase culture was harvested by centrifugation at 2000 rpm for 5 min. The algal pellet was resuspend in 3 ml 10% buffered formalin and stored at 4°C for 4 h to preserve cells. Algal cells were re-pelleted and washed twice, firstly in 10 ml and then in 1 ml of ice cold 50% 1x PBS:50% ethanol. Cells were stored in 1 ml wash solution at -20°C.

The eubacteria probe EUB338 (Amann *et al.* 1990) labelled with the fluorescent marker Cy3 was used for detection of bacteria in preserved dinoflagellate samples. Three hybridisation techniques were trialed: (i) hybridisation on a filter, (ii) liquid hybridisation on a glass slide, and (iii) liquid hybridisation in an Eppendorf tube. Preparation of solutions is described in Appendix 2 and 3.

For hybridisation on a filter, the filters with preserved cells were cut in half or quarters and 2-4 hybridisations performed per sample, as required. In a fresh Petri dish, a piece of filter pad (Whatman) was dampened with 260 µl hybridisation buffer (HB), filters were placed algal side up and 80 µl HB and 10 µl probe (and 10 µl unmarked probe if necessary) pipetted on top (Fig. 2-3). To allow the probe to hybridise, the Petri dish was placed in a dark container lined with moist paper towel and incubated in a water bath at 46°C for 1-2.5 h. Pre-warmed (46°C) filter sterilised, autoclaved wash solution specific for the probe was prepared and DAPI (10 $\mu l.ml^{-1}$) added. Filters were transferred to clean Petri dishes containing 5 ml EUB wash solution, allowed to sink, placed back inside the moist box at 46°C and incubated for 15 min.

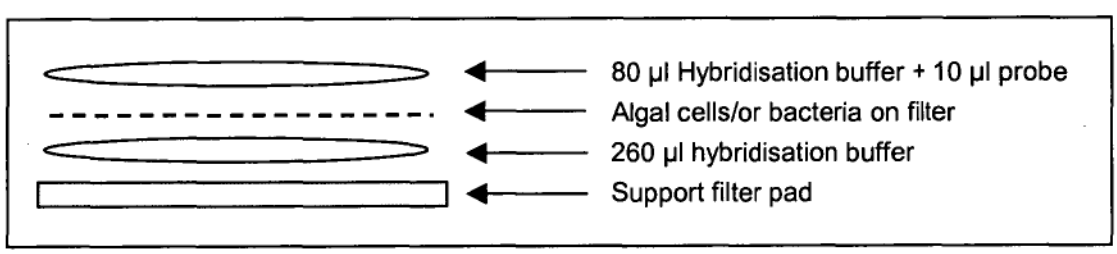


Figure 2-3. Hybridisation of formalin preserved algal/bacterial culture on a filter pad.

For liquid hybridisation on a glass slide, 5 µl of preserved algal cells were dropped onto a glass slide with wells, allowed to dry and, working in dim light, a 4 µl droplet of the probe was added followed by 16 µl HB. Slides were incubated in Petri dishes as described for the filter method. After 1.5 h, the probe was tipped off onto a fibre-free tissue and a drop of 50 µl DAPI was pipetted onto each slide followed by 400 µl EUB washing solution pre-

warmed to 46°C. Slides were placed back inside the moist box at 46°C and incubated for 15 min. The wash solution was removed and the slides left to dry in the dark.

For liquid hybridisation in an Eppendorf tube, the preserved algal sample was concentrated to 2 µl and 50 µl HB and 2 µl probe added. The tube was incubated at 46°C for 2 h. Cells were collected by centrifugation at 10,000 rpm for 1 min to form a pellet. The supernatant was removed and cells washed by adding 1 ml of 50 µl DAPI and 5 ml EUB wash solution (or 1x Tris HCl) pre-warmed to 46°C. Working in dim-light, cells were re-collected and the supernatant carefully removed. A small amount of cells (~20 µl) were smeared onto a clean slide and left to dry. All slides were observed under a Leitz DM RBE fluorescence microscope using a drop of oil underneath and on top of the coverslip.

2.3 Results

2.3.1 Solid agar media for dinoflagellate growth

The health of dinoflagellate cultures grown on GSe agar plates (0.25 and 0.5% agar) was assessed 6 days after inoculation. Health was scored as the majority of cells: (i) dead or dying (D); (ii) had formed temporary cysts (TC); or (iii) mostly alive, regular in shape and sometimes swimming (L) (Table 2-4). Cells showed a higher survival rate when inoculated onto: slushy (0.25% agar) rather than soft solid (0.5% agar) GSe plates, and control (C) rather than antibiotic treated (T) plates. All cells inoculated onto 0.5% agar plates died or formed temporary cysts whilst some strains on the 0.25% agar plates survived and showed very slow cell division. Over a 2-week period an individual chain of GCTRA01 cells increased from 6 to 12 cells, indicating an extremely slow growth rate.

2.3.2 DNA isolation

The combined CTAB and phenol:chloroform:isoamyl alcohol method for DNA isolation yielded DNA of a higher quality than the shorter phenol:chloroform

method (Bolch *et al.* 1998), as determined by spectrophotometry and its effectiveness in PCR, however, yield was reduced by up to 50%.

Table 2-4. Observations of dinoflagellate cell health 6 days after inoculation onto control (C) and treatment (T) GSe agar plates.

Strain	% Agar	Plate type	Score of cell health
ACAD01	0.25	C	D
		T	TC
	0.5	C	D
		T	TC
ACSP01	0.25	C	L
		T	TC
	0.5	C	TC
		T	TC
CAWD106	0.25	C	L
		T	L
	0.5	C	D
		T	D
GCTRA01	0.25	C	L
		T	D
	0.5	C	TC
		T	D

2.3.3 Detection of bacteria in dinoflagellate cultures

2.3.3.1 Light microscopy

At the light microscope level using oil immersion, motile bacteria were detected in the culture media of all untreated dinoflagellate strains. No noticeable reduction in bacterial numbers was observed after 3 weeks of treatment with timentin. After successive antibiotic treatment, some cultures appeared infected with fungi, however a precise diagnosis was difficult. In

certain cases a mucous appeared in cultures, forming a gelatinous matrix that trapped cell debris and confounded the visual detection of fungus.

2.3.3.2 Seawater agar plates with vitamins (SWAV) and Johnson's media agar plates

The majority of cysts washed in H_2O_2 and individually added to SWAV plates displayed bacterial growth after a period of 1-2 weeks. Three obvious bacterial colony types grew on SWAV plates inoculated with timentin treated cultures (vegetative cells) within a few days (Table 2-5). Tiny clear colonies were present in all cultures. Larger yellow round shiny colonies were present in all 3 species but only 4 strains. Larger white round shiny colonies were present in all *G. catenatum* strains and *A. minutum* strains but only 1 *A. catenella* strain (ACAD01). Two bacterial types were detected in most strains, with only 1 bacterial type detected in *A. catenella* strain ACSP01 and 3 bacterial types detected in *G. catenatum* strain GCPL01 and *A. minutum* strain AMAD06. Bacterial colonies (100s) grew on all plates with multiple species growing on top of one another. Individual colonies were successively plated to obtain individual species enabling a basic descriptive classification (Table 2-5). Identification to species level was not the aim of the project.

Inoculating timentin treated cultures on plain SWAV plates revealed high numbers of bacteria (Table 2-5). Inoculating the same cultures onto plates containing a standard dose of the antibiotic cocktail or inoculating cultures treated with 4x the standard dose of the antibiotic cocktail onto plain SWAV plates, confirmed that antibiotics had successfully reduced the number of culturable bacteria (from 100s to <<100 colonies per 100 µl culture) and possibly eliminated some bacteria types previously detected.

2.3.3.3 GSe agar plates

GSe agar plates were assessed for the growth of contaminating bacteria or fungus. No plates and therefore timentin treated cultures were free from contamination. All plates, except for cultures GCTRA01 and AMAD06,

possessed small clear bacterial colonies. GCTRA01 possessed a white branching fungal contaminant, whilst a spreading slime-like contaminant was detected in AMAD06.

Table 2-5. Observations of bacterial growth on seawater agar (1.5%) plates with vitamins (SWAV) (2 plates per strain) 96 h after inoculation with timentin treated cultures. Note, white = large milky-white, round shiny colony; yellow = large yellow, round shiny colony; clear = tiny clear colonies often overlapping other colonies; P = present; A = absent.

Strain	# Bacterial colonies/plate	Presence/absence of bacterial colonies			
		# Bacterial types	Clear	Yellow	White
GCJP10	> 500	2	P *	A	P *
CAWD106	> 500	2	P *	A	P *
GCPL01	> 500	3	P	P *	P
GCTRA01	>> 500	2	P	A	P *
GCDEO6	> 100	2	P	A	P (few)
GCDE11	> 500	2	P *	A	P *
ACSH01	300-500	2	P	P *	A
ACAD01	> 500	2	P	A	P *
ACSP01	> 200	1	P *	A	A
CAWD44	>> 500	2	P *	P *	A
AMAD06	>> 500	3	P	P (few)	P
AMNC04	>> 500	2	P	A	P

*Dominating bacteria

2.3.3.4 Polymerase chain reaction (PCR) and sequencing

Whole cyst PCR was unreliable and dinoflagellate DNA was not amplified. PCR products from SAM primers (see 2.2.3.3) were likely to have been bacterial in origin as confirmed by sequencing. A DNA fragment of ~ 500bp was produced from untreated cysts, no product was detected for cysts treated with H_2O_2, and a slightly smaller fragment of ~470bp was produced from cyst DNA extracted using the modified CTAB method (Appendix 1). The expected size based on plant *Sam* was 459 bp and from bacteria ~468 bp. Table 2-6 shows the results of PCR using the BS primers (Table 2-3) and DNA isolated from control versus treated dinoflagellate cultures. All but

1 control culture yielded a positive result, suggesting the presence of bacterial DNA in most untreated cultures. After cultures had been treated to the Normocin O-stage, only 5 out of 12 cultures gave a positive result.

Strain	Contaminated	
	Control	Treatment
GCJP10	+	+
CAWD106	+	–
GCPL01	+	–
GCTRA01	+	+
GCDEO6	+	+
GCDE11	+	+
ACSH01	+	+
ACAD01	+	–
ACSP01	+	–
CAWD44	+	–
AMAD06	+	–
AMNC04	+	–

Table 2-6. Bacteria status of control and treated (Normocin O stage) dinoflagellate cultures as determined by PCR using bacteria specific primers BSF8/20 and BSR534/18. A plus denotes a positive result in PCR, indicating the likely presence of contaminating bacterial DNA and a minus denotes that no product was detected in PCR.

The PCR product from ACAD01 was cloned and 4 clones sequenced and a BLAST search performed. The closest match for 2 clones was the 16S rRNA gene of *A. catenella* and the closest match for the other 2 clones was the 16S rRNA gene of bacteria: an uncultured Antarctic soil bacterium (Accession N°. AF419199) and a *Cytophaga*-like bacterium (Accession Nº. AF367854). Additionally, PCR of GCTRA01 and AMNC04 DNA using the BS primers yielded products of 477 bp and 450 bp. BLAST search of GenBank produced a 97.5% (418/427 bp with one gap) and 98% identity respectively to *Cytophaga* sp. KT02ds22 16S rRNA gene (Accession N°. AF235114). Additionally, a 252 bp clone matched *Cellulophaga sp.* ACEM20 16S rRNA gene (Accession Nº AY035869) with a 93% identity, and 318 bp clone matched an 'uncultured bacterium CLEAR-19 16S rRNA gene' (Accession N° AF146242) or likely *Verrucomicrobia sp.* (Dr J. Bowman pers. comm.) with

91% identities. The reverse primer EPI277-T3R did not yield a product when used alone in PCR or in conjunction with the forward primer BSF8/20.

2.3.3.5 *Fluorescence* in situ *hybridisation (FISH)*

Bacterial DNA stained with DAPI was detected in the culture media of all 3 species before and after treatment. Autofluorescence of dinoflagellate pigments in the yellow-orange spectrum (~550-620 nm) was a problem because it masked the fluorescence of the Cy3 labelled probe which emits at ~550 nm. Preliminary photobleaching tests such as exposing cells to ultraviolet light for 2 min and 35 min had minimal effect on the intensity of autofluorescence. FISH as a method for detecting bacteria was discontinued because although bacteria were detectable in the culture media, it was not possible to unambiguously screen the exterior of dinoflagellate cells for epiphytic bacteria. This was also a time consuming approach and was therefore not ideal.

2.3.4 Basic identification of bacteria

All 3 bacterial colony types (clear, yellow and white) were gram negative, rod-shaped and tested positive for the enzymes oxidase and catalase. The clear bacteria were highly motile and existed singly, not in chains. It was likely that these 3 bacteria belonged to the genus *Cellulophaga* (Dr J. Bowman pers. comm.).

2.3.5 Effect of antibiotics on dinoflagellate health

Continuous treatment of dinoflagellate cultures with antibiotics caused deterioration of dinoflagellate health. After treatment was ceased, culture health continued to deteriorate and cultures eventually died (Fig. 2-4). Regular transfer of healthier cells into fresh, non-treated GSe rescued most cultures.

When algal cells became stressed some cultures started to produce a mucous-like substance at the bottom of the culture vial and, under the light microscope, appeared to be a gelatinous matrix with trapped dead cells. The

commercial antifungal and antibacterial product, Normocin O, is designed to prevent further contamination of cultured cells rather than eliminating existing bacteria. This product did not appear to be toxic to dinoflagellate cells and its effect on the bacterial load of cultures was ambiguous. It was not known whether this product was able to prevent the colonisation of new bacteria because although bacteria were detected, they may have regrown from the reduced original population. The nystatin treatment did not seem to affect the health of *G. catenatum* cultures but appeared to slow the growth of most *Alexandrium* cultures.

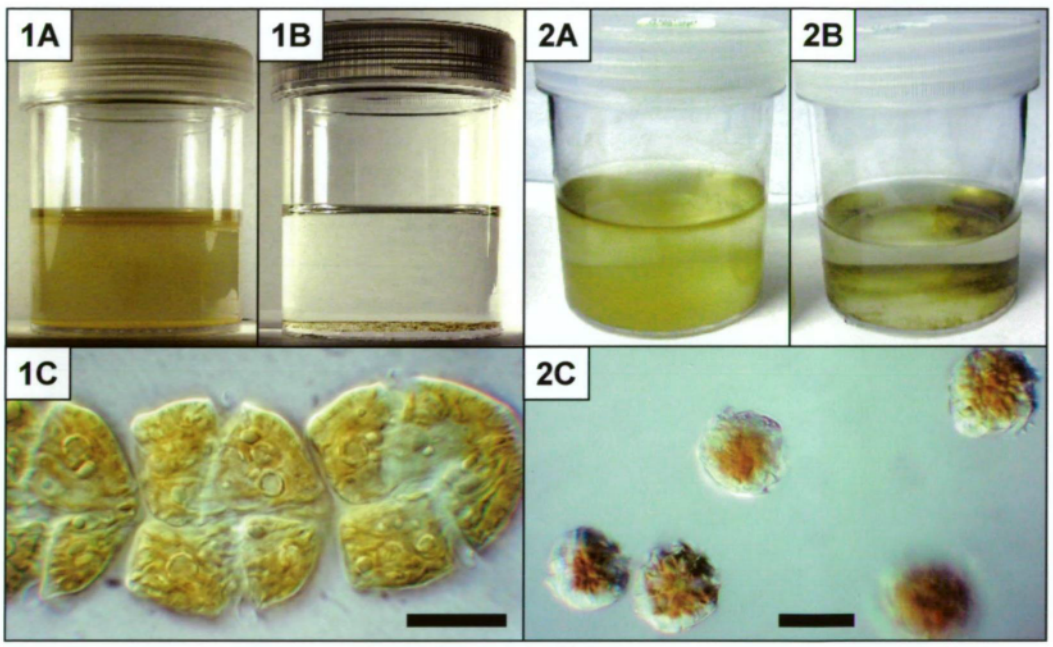


Figure 2-4. Images of two species of dinoflagellates *Gymnodinium catenatum* (1A-C) and *Alexandrium minutum* (2A-C) in culture. (A) Control culture. (B) Culture treated with antibiotics. (C) High power magnification of healthy cells under the light microscope, scale bars = 20μm. Note that cultures are the same age but the control is healthy and dense, whilst the successively treated culture has many dead or dying cells on the bottom of the vessel.

2.4 Discussion

2.4.1 Solid agar media for dinoflagellate growth

Growth of dinoflagellates on solid media has been reported for the benthic genus *Amphidinium*, the coral endosymbiont *Symbiodinium* (such as

S. microadriaticum), the heterotrophic epiphytic species *Crypthecodinium cohnii* and the freshwater species *Gloeodinium montanum* which has a non-motile benthic resting stage (Beam & Himes 1977, Taylor *et al.* 1987, Ishida *et al.* 1998). To my knowledge growth on solid media has never been reported for pelagic dinoflagellates. The study by ten Lohuis & Miller (1998) of *Amphidinium* and *Symbiodinium* is the first to genetically transform dinoflagellates, making these genera accessible to genetic manipulation. The survival of *A. catenella* and *G. catenatum* cells on soft GSe agar plates (0.25 and 0.5% agar) is the first step towards developing a method for the growth of pelagic dinoflagellates on solid media. It enabled the immobilisation of these usually highly motile cells, creating the potential to produce healthy dinoflagellate cells that could be easily isolated and manipulated. Further work is required to optimise a protocol and could include testing the method by ten Lohuis & Miller (1998) who streaked cells onto 1% agar blocks, the bottom of which were in contact with a selective algal enrichment solution.

2.4.2 Difficulties of detecting bacteria

Although some cysts inoculated onto SWAV plates did not show bacterial growth it does not rule out the presence of bacteria which may be unculturable or contained inside the cyst wall. PCR from cysts was problematic and not as effective as using purified DNA as a template. A negative result may have been due to a failed reaction because of low template or interference from cellular material as opposed to an absence of bacteria. Hydrogen peroxide as a treatment of cysts is likely to be effective in sterilising the outside of cyst walls but does not address the issue of bacteria potentially contained inside the cyst. Therefore, when the cyst is lysed to expose the dinoflagellate DNA for PCR, bacterial DNA may also be present.

Culturable bacteria may only represent a minor fraction of the bacterial flora meaning that many pelagic marine bacteria are going un-detected. It is estimated that 95% of marine bacteria are unculturable on standard laboratory media (Schut *et al.* 1993) and the exact cause of their unculturability has still not been resolved (Schut *et al.* 1993, 1997). Eilers *et*

al. (2000) predicts that with current technology, isolation of unculturable marine bacteria, particularly the gamma-proteobacterial SAR86 cluster, is limited.

Methods for detecting bacteria in dinoflagellate cultures have, in the past, been variable in accuracy and often lack precision (Biegala *et al.* 2002). Although time consuming, a combination of methods is necessary. Biegala *et al.* (2002) report the successful detection of bacteria using tyramide signal amplification-FISH combined with confocal microscopy, whilst Cordova *et al.* (2002) were able to detect intracellular bacteria by confocal and electron microscope observations. Although confocal microscopy/FISH is one of the most effective methods currently available for visually detecting bacteria inside an algal cell, it is not without complications. Detection of fluorescent probes can be compromised if they emit in the same spectrum as the autofluorescence (yellow-orange spectrum) of the chloroplast pigments (Biegala *et al.* 2002, Cordova *et al.* 2002). Using a fluorochrome that emits at a wavelength outside autofluorescence, combined with a filter to block out the autofluorescence, or enhancing the fluorescent signal by using dual labelling can overcome this problem.

To be confident that any novel genes isolated will be dinoflagellate genes, this study required all bacteria to be detectable, both intracellular and external to the dinoflagellate cell. The issue was not the location or viability of bacteria but whether any bacterial DNA was present. PCR was therefore a suitable method for assessing the bacterial status of dinoflagellate cultures. PCR is a highly sensitive technique that can detect trace quantities of DNA. Therefore, contaminating bacteria may be detected in cultures with very small bacterial loads. It is anticipated that the yield of the amplified PCR product would reflect the number of bacteria in the dinoflagellate culture; a lower yield meaning a lower level of bacterial contamination. However, the ability of the primers to detect different bacteria may also affect PCR yield.

The study by Hold *et al.* (2001a) treated cultures of the dinoflagellates *A. minutum* NEPCC 253 and *A. tamarense* NEPCC 407 with a cocktail of

streptomycin, ciprofloxacin, gentamicin and penicillin G. Residual bacteria were not detected using 17 different solid and broth bacterial growth media, by epifluorescence microscopy with the Sybr green 1 dye, or PCR amplification using universal eubacterial primers designed to target the 16S rRNA gene. However in contrast, but similar to the findings in this chapter, Cordova *et al.* (2002) report that antibiotic treatment of *A. catenella* did not generate bacteria-free cells but instead led to the killing of the host cells.

In situ hybridisation with rRNA targeted oligonucleotide probes has become a widely applied method of detecting microbial populations. It is acknowledged that the EUB338 probe routinely used to quantify members of the domain Bacteria and used in this chapter, does not detect some bacterial phyla, notably the Planctomycetales and Verrucomicrobia (Daims *et al.* 1999). Future studies should use EUB338 along with the supplementary versions, EUB338-II and EUB338-III (Vandekerckhove *et al.* 2002). Due to the difficulties highlighted with axenic culturing, innovative new methods must be developed such as the approach in Chapter 3, which solely isolates eukaryotic nucleic acids.

2.4.3 Antibiotic treatment

While antibiotics are currently the most effective method of removing most bacteria from dinoflagellate cultures, they often compromise the health of the cells (Cordova *et al.* 2002), creating a problem for molecular work. In this study, it is not known whether dinoflagellate health suffered because of a toxic effect of one or more of the antibiotics or because beneficial bacteria were lost or reduced in number. It is speculated that dinoflagellate cells may become more susceptible to infection by detrimental bacteria if the antibiotic treatment creates a "stressful" environment for cells. Additionally, the removal of one or more species of bacteria may create a window of opportunity for antibiotic resistant bacterial strains or fungus to increase in number. If dinoflagellates are being stressed RNA-based techniques may be compromised because of a change in cellular transcription rates.

It is likely that the effect of antibiotic treatment on dinoflagellate health in culture is influenced by multiple factors and will vary depending on the species and strain. Influencing factors may include the relationship between certain bacteria and dinoflagellates (e.g. symbiotic, commensal, competition), the degree of bacterial contamination, the duration of antibiotic exposure, and the tolerance of the dinoflagellate to antibiotic exposure. The ineffectiveness, re-growth or increased growth of bacteria in dinoflagellate cultures may thus be attributed to factors such as a lack of competition from the original microflora, recovery from cell injury after cessation of treatment, or, if antibiotics are used, resistant bacteria may emerge. It is possible that cryptic bacteria may be released from dinoflagellate cells into the culture medium (Gallacher & Smith 1999).

2.4.4 Conclusions

Vigilance and multiple detection methods must be applied when ascertaining the bacterial status of a culture, especially when using microscopy. For instance, previous studies have demonstrated that a dinoflagellate culture can appear bacteria-free when viewed by epifluorescence microscopy immediately after treatment but examination 5 days later has revealed low numbers of bacteria that probably re-grew from an overlooked restricted population (Gallacher & Smith 1999). This study found that treatment of dinoflagellate cultures with a high concentration dose (4x standard) of an antibiotic cocktail was effective at eliminating most of the culturable bacteria. However, it is also demonstrated that cultures exhibiting no bacterial growth on solid media can still possess bacteria detectable by fluorescence microscopy or PCR. PCR using eubacterial primers is currently the most sensitive technique for assessing the bacterial status of dinoflagellate cultures and can in part overcome the problem of detecting unculturable bacteria (Snyder *et al.* 2003). However, adaptation of this method would be required to detect Archaea and viruses and to differentiate between live and dead bacteria. From this chapter, it is notable that visual determination of PCR results can be misleading highlighting the need for sequencing. For instance, eubacterial primers where shown to detect dinoflagellate DNA in

addition to bacteria and may therefore provide a false positive result for bacteria-free cultures.

Bacteria are inherent in dinoflagellate cultures and unless treated, will always be present. The death of dinoflagellate cells after continued antibiotic treatment in the present study and others (e.g. Cordova *et al.* 2002) either suggests a toxic effect of the treatment or an essential symbiotic or commensal role of certain bacteria. It could be speculated that at least some of the dinoflagellate strains became unhealthy after treatment because of a loss of beneficial endosymbiotic bacteria. Species of *Cytophaga* have been reported to be intracellular and the *Cytophaga*-like bacterium identified by PCR in this chapter may well have been present inside dinoflagellate cells (Muller *et al.* 1999). Future work could include more thorough microscopy, including confocal and electron microscopy to detect potential endocytoplasmic bacteria before and after treatment.

Current axenic culturing methods for dinoflagellates are time consuming and bacterial detection techniques often give ambiguous results. Of the strains examined, it seems likely that truly axenic cultures are not achievable with current technology. Therefore, DNA isolated from laboratory cultures will inherently contain bacterial DNA, which co-amplifies in PCR. The results clearly showed the need for a quicker, simpler and less ambiguous approach to obtaining pure dinoflagellate DNA for molecular genetic study. It was established that cultures were contaminated and that this posed a problem for the application of molecular genetic techniques. The axenic culture approach does not provide the confidence level needed when searching for novel genes using degenerate primers and would also compromise gene expression studies. It was determined that alternative approaches were required to pursue the use of molecular genetics to study dinoflagellates in cultures contaminated with bacteria. Chapters 4 and 5 show that primers can be designed to target eukaryotic genes and the true source of novel genes isolated from xenic dinoflagellate cultures can be assessed by comparing sequences generated from total genomic DNA and eukaryotic complementary DNA.

Chapter 3: A novel, simplified technique for preservation and rapid isolation of total RNA from the toxic dinoflagellate *Alexandrium catenella*[*]

3.1 INTRODUCTION

Gene technology based on the development of molecular genetic methods for individual species is in its infancy for the group of microalgae known as the dinoflagellates. Some species such as *Alexandrium* Halim spp. and *Gymnodinium catenatum* Graham are of particular interest to molecular geneticists because they produce a unique suite of potent neurotoxins (Levin 1992, John *et al.* 2003a). Paralytic Shellfish Toxins (PSTs) accumulate in the food chain and have been known to cause severe illness and even death in humans, marine mammals and birds with huge economic losses to the aquaculture industry (Armstrong *et al.* 1978, Kan *et al.* 1986, Hernandez *et al.* 1998). Elucidation of genes in the biosynthetic pathway for PST production is the first step to understanding their ecophysiological role (Plumley 1997) and relies on the development of molecular tools specific to dinoflagellates. This knowledge may allow scientists to develop the technology to regulate, or perhaps even block, PST production.

RNA isolation is fundamental to the study of gene expression and functional genomics such as RNA silencing (Ichim *et al.* 2004, Robertson 2004, Rohr *et al.* 2004, Sarkar *et al.* 2005). Optimisation of RNA isolation from toxic microalgal is time consuming and has slowed the progress of functional studies, in particular with dinoflagellates. Available literature on dinoflagellates includes few RNA-based molecular protocols (Scholin *et al.* 1993, Mylne *et al.* 1998, Taroncher-Oldenburg & Anderson 2000, Coyne *et al.* 2004), and no simple RNA methods that do not require the sample to be immediately frozen or stored in phenol (summarised in Table 3-1). Furthermore, publications often do not provide details of the extraction process and the quality and yield of RNA.

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Table 3-1. A brief summary of RNA isolation methods in some dinoflagellates (Dinophyceae).

Species	Cell disruption method				RNA isolation method					Reference
	Grinding	N_2 bomb	Heat/ vortex	Not specified/ other	$LiCl_2$/ phenol	AGPC	Kit	Trizol	Not specified/other	
Alexandrium fundyense		✓			✓					Scholin *et al.* (1993)
Gonyaulax polyedra	✓					✓*				Bae & Hastings (1994)
Alexandrium fundyense		✓			✓ *other					Taroncher-Oldenburg & Anderson (2000)
Amphidinium carterae, Symbiodinium	✓(sand)				✓					Mylne *et al.* (1998)
Amphidinium carterae, Lingulodinium polyedrum				✓Polytron homogeniser		✓		✓		Bachvaroff *et al.* (2004)
Amphidinium operculatum				✓			✓			Barbrook *et al.* (2001)
Alexandrium tamarense	✓					*tested	✓			Martinez *et al.* (2001)
Pfiesteria shumwayae				Filtered/none				✓		Berry *et al.* (2002)
Pfiesteria shumwayae			✓		✓					Coyne *et al.* (2004)
Crypthecodinium cohnii				✓	✓					Chan *et al.* (2002)
Symbiodinium muscatinei	✓					✓*				Weis *et al.* (2002)
Pfiesteria piscicida	✓							✓		Lin & Zhang (2003)
Prorocentrum lima, P. hoffmanianum, Karenia brevis, Symbiodinium sp., Amphidinium carterae, A. operculatum and *Gymnodinium catenatum*	✓					✓*				Snyder *et al.* (2003)
Prorocentrum minimum				✓					✓	Zhang & Lin (2003)

* Denotes that the protocol was modified slightly. $LiCl_2$ denotes that lithium chloride precipitation was used; however other steps in the RNA isolation protocol were variable.

RNA isolation must often be tailored to particular algal species. The green alga *Chlamydomonas reinhardtii* Dang. (Chlorophyceae) is a model organism for flagellar biology, photosynthesis and other biological processes and is the best researched microalgal species at the molecular level (for a comprehensive review see Harris 2001). *C. reinhardtii* is a species for which RNA isolation is routinely performed. In contrast, dinoflagellates are a diverse group of microalgae and different RNA isolation methods may be needed.

There exist a number of factors that influence the ease of RNA isolation including cell size and toughness, cell density in culture and the growth habit/cellular products (e.g. mucous) associated with the culture. The first and one of the most critical steps in RNA isolation is the collection and preparation of cellular material (Chang *et al.* 1993). If amenable, cells can be processed fresh but are more commonly kept frozen or preserved in an appropriate chemical so that ribonucleases (RNases) are inactive and the sample can be processed at a later date. Another critical step is cellular disruption to release RNA. This can be achieved mechanically (Higgins & Hames 1994, Mylne *et al.* 1998), by vortexing (Lin *et al.* 2002), sonication (Liao *et al.* 1997), pressurisation or decompression (Scholin *et al.* 1993), bead milling or using a mechanical homogeniser (McGarvey *et al.* 2003, Bachvaroff *et al.* 2004). The nitrogen pump is a specialised, often expensive, piece of equipment that has successfully been used to disrupt dinoflagellate cells for RNA isolation (Scholin *et al.* 1993). A more common, less expensive technique is grinding frozen cells in liquid nitrogen with a mortar and pestle (Mylne *et al.* 1998, Martinez *et al.* 2001). In this study we evaluate the sonication as well as the Eppendorf mortar and pestle techniques (with and without sand) in terms of efficiency, reproducibility and quality of the RNA obtained from *Alexandrium catenella* (Whedon & Kofoid) Balech.

Methods for isolating microalgal RNA from cells after the disruption step using acid guanidinium thiocyanate-phenol-chloroform (AGPC) (Chomczynski & Sacchi 1987), lithium chloride ($LiCl_2$) and Trizol are lengthy and can take days. The development of commercial kits has allowed the

process to be completed much faster, usually in 1 h (e.g. Martinez *et al.* 2001). RNA isolated from *Alexandrium tamarense* (Lebour) Balech and other algae using the AGPC method contained an impurity that caused inhibition of the subsequent reverse transcriptase (RT) reaction (Martinez pers. comm.). The quality of RNA is fundamental for PCR where complementary DNA (cDNA) is the template. Polyphenolics and polysaccharides are produced in large quantities by certain microalgae and are known to hamper DNA (Galluzzi *et al.* 2004) and RNA isolation (Chang *et al.* 1993). The high molecular weight polymer polyvinylpyrrolidone (PVP) is used for the purification of nucleic acids because of its ability to bind to these contaminants (Mylne *et al.* 1998).

Efficient and complete homogenisation of cells is paramount for isolation of high quality RNA (Payton & Pinter 1999). Cells of *Alexandrium* are notoriously robust and more difficult to lyse than the more fragile groups of microalgae such as the prymnesiophytes, raphidophytes and some thin-walled dinoflagellates such as *Pfiesteria shumwayae* Glasgow & Burkholder, which do not require mechanical disruption to lyse (Coyne *et al.* 2004). The aim of the present study was two-fold. Firstly, to develop a protocol for the collection and preservation of intact *A. catenella* cells at room temperature for extraction of nucleic acids at a later date, without using toxic chemicals. This would be most useful for field samples or laboratory experiments where it is not possible to extract nucleic acids immediately and where occupational health and safety standards must be maintained. Secondly, to develop a simpler and reproducible protocol for isolation of high quality RNA from fresh and preserved *A. catenella* without the need for liquid nitrogen.

3.2 MATERIALS AND METHODS

3.2.1 Cell collection and storage

A. catenella CAWD44 cells were grown in GSe medium (salinity 28 ppt) and harvested by centrifugation for 5 min at 4,000 rpm. The volume of the pellet harvested ranged between 10-100 µl with the optimum being between 20-50 µl corresponding to ~20-50,000 cells. The culture medium was poured off

and the cells resuspended in the remaining 1 ml of liquid. Cells were re-pelleted by brief centrifugation (30 s at 8,000 rpm) in 1.5 ml Eppendorf tubes and the supernatant removed. Before the cell disruption step, cells were treated in one of four ways: i) snap frozen in liquid nitrogen; ii) resuspended in RNA*later*-ICE (Ambion); iii) resuspended in RNA*later* (Ambion) or; iv) directly resuspended in the RNA extraction buffer. To preserve cells for RNA extraction at a later date, two products were tested: (i) RNA*later*-ICE and (ii) RNA*later*.

3.2.2 Cell disruption

The effectiveness of different cell disruption treatments for RNA isolation was tested. The three main disruption treatments were: i) bead beating; ii) sonication; and iii) manual grinding with a micropestle (with and without sand). A combination of these preservation treatments and cell disruption treatments were used to determine the most suitable procedure for extracting total RNA from *A. catenella*.

To test the effectiveness of bead beating, cell pellets were first resuspended in the cell lysis solution of interest and a small quantity of sterile glass beads (0.5 mm diameter), equivalent to a volume of approximately 50 µl, were added. Glass beads were pre-sterilised by soaking in 70% nitric acid, followed by 2 rinses in DEPC treated water. Samples were kept on ice and immediately processed in a 220 V Mini Beadbeater (BioSpec Products, Inc.) at 3,800 rpm for 10-60 s depending on the species. Samples were processed three times and kept on ice between treatments.

Before sonication, cell pellets were resuspended in 500 µl of the buffer of interest and sonicated immediately on ice using an MSE 100 Watt Ultrasonic Disintegrator (Measuring & Scientific Equipment Ltd., London, England) with a probe diameter of 2.5 mm, at amplitude level 9 µm peak to peak for 10-30 s.

The effectiveness of Qiagen's RLT buffer (containing 4 µl β-mercaptoethanol/500 µl RLT) and Ambion's Plant RNA Isolation Aid (50

µl/500 µl RLT) (contains PVP), in lysing cells before and after sonication was tested and the % lysis from 3 replicates scored by light microscopic observation at 400x magnification. Results were scored as follows: (i) 0-5% lysis; (ii) 6-50% lysis and less complete lysis (i.e. cell organelles still intact and cell walls burst but not disintegrated); (iii) 51-80% lysis; (iv) 81-95% of cells lysed but some cell walls not disintegrated (i.e. sample would need more homogenisation); or (v) 96-100% lysis, lysis rapid and no further homogenisation required.

The third treatment of cell disruption was conducted using a customised glass pestle (glass rod with tip ground to fit the base of an Eppendorf tube). Algal cells were pelleted in Eppendorf tubes and either: (i) resuspended in 500 µl RNA*later* and stored at -20°C or room temperature; (ii) immediately frozen in liquid nitrogen and stored in 500 µl RNA*later*-ICE at -20°C; or (iii) immediately frozen in liquid nitrogen, thawed and stored in 500 µl RNA*later*-ICE at room temperature. Frozen cells were not allowed to thaw and 50 µl of RLT buffer added. If samples had been stored in RNA*later*-ICE or RNA*later*, they were first centrifuged at 10,000 rpm for 30 s and the supernatant removed. Frozen samples were manually ground (approximately 10 min each) with minimal thawing. If the sample started to thaw it was re-frozen in liquid nitrogen. Samples stored in RNA*later* or RNA*later*-ICE were manually ground (approximately 2 min each) with and without liquid nitrogen. Triplicate extractions without the use of liquid nitrogen were performed for ~20 µl pelleted cells (i) immersed in 500 µl RNA*later* and (ii) snap frozen and thawed in 500 µl RNA*later*-ICE and stored at room temperature for 24 h.

Sterile sand (BioSpec Products, Inc.) equivalent to a volume of 10-20 µl as used by Mylne *et al.* (1998) was tested on samples that had been frozen but not treated with a storage solution. Samples were ground as described previously.

3.2.3 RNA isolation

To isolate RNA a column based silica-membrane technology that does not use phenol:chloroform in the extraction process or ethanol for precipitation was used. The commercially available RNeasy Plant Mini Kit (Qiagen) was trialed. This kit uses cell lysis buffers that contain β-mercaptoethanol. The two lysis buffers supplied (RLT - guanidine isothiocyanate and RLC - guanidine hydrochloride) were tested with the addition of Plant RNA Isolation Aid (Ambion) according to the supplier's instructions. Qiagen produce a RNase-free DNase set, sold separately for on column treatment. RNA was eluted in 30-50 μl RNase-free water with the same eluate being placed over the column twice.

The effectiveness of two products for the removal of contaminating DNA was tested. Unlike the Qiagen DNase treatments, DNA-*free* (Ambion) and TURBO DNA-*free* (Ambion) are applied after RNA has been eluted. Both products are supplied with a DNase Inactivating Reagent that is suitable for all RNA analysis methods. The latter product contains a hyperactive TURBO DNase that is catalytically superior to the DNase supplied in the DNA-*free* kit. RNA samples were treated according to the kit protocols supplied.

The quality and yield of RNA was examined by spectrophotometry and visual inspection after formaldehyde gel electrophoresis. RNA with an absorbance at 260:280 nm of 1.8-2.0 and distinct ribosomal RNA bands was deemed to be of a high quality. High quality RNA was reverse transcribed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and the cDNA from 3-5 replicate extractions used in PCR to test for DNA contamination. RT-PCR was performed using primers to S-adenosyl-homocysteine hydrolase (*Sahh*); a gene expressed concurrently with saxitoxin biosynthesis in the toxic dinoflagellate *A. fundyense* Balech and identified by differential display (Taroncher-Oldenburg & Anderson 2000). The following primers: forward SaahF1 5'-CTATGGCGATGTCGGCAAGG-3' and reverse SahhR1 5'-ATCCACCTCCCCAACTACGG-3' were designed to *Sahh* (Accession number AF105295) and used with the cycling program: 94°C, 1 min; 35x (94 °C, 15 s; 60 °C, 45 s; 72 °C, 1 min); 72 °C, 5 min.

Reactions were prepared using PCR Master Mix (Promega) to a final volume of 20 μl, final primer concentration of 0.5 μM each and ~12-28 ng template cDNA per reaction (RT+) depending on the RNA yield. A 5 μl aliquot of each reaction (RT+ and RT-) were electrophoresed on a 2% agarose gel at 80 volts for 40-50 min.

3.3 RESULTS

3.3.1 Bead beating

Used on its own, Qiagen's RLT lysis buffer with β-mercaptoethanol and Plant RNA Isolation Aid added did not lyse cells effectively and did not prevent RNA degradation during beating. The average lysis was 6-50% from three replicates with fragments of cells still intact. Beating with glass beads was a successful treatment for disrupting cells (as determined by light microscope observations) but was not effective in preventing RNA degradation. Cells had to be beaten 3 times for 30 s at 3,800 rpm to achieve sufficient disruption (i.e. 95-100%). A single 30 s beating was generally insufficient in lysing most cells; however, by this time the friction of the beating had caused the sample to warm, which could affect the quality of RNA. Frothing of the sample was also a problem. For this reason, samples were placed on ice for 1-3 min to allow them to cool and the froth to disappear between beatings.

Despite cell lysis, when processed with Qiagen's Plant RNeasy Mini Kit, the RNeasy column generally became clogged with cell debris, suggesting that the cells had not been sufficiently homogenised. This phenomenon was not due to overloading of the column and even occurred when very small volumes (i.e. 20 μl) of cells were processed, albeit to a lesser extent. RNA isolated from samples processed by bead beating was consistently of a lower quality as determined by $A_{260:280}$ and gel electrophoresis, than the sonication and micropestle treatments. Frozen samples thawed and processed by bead beating were always degraded.

3.3.2 Sonication

After sonication for 10 s at an amplitude level of 9 μm peak to peak, lysis was complete and cells homogenised with no visible cellular debris except for some intact cells (maximum of 5% intact as determined by light microscopy). However, similar to the bead beating treatment, samples were prone to heating. An amplitude level of less than 8 μm peak to peak was ineffective to lyse cells. High quality RNA was successfully isolated from sonicated cells processed fresh, or frozen cells thawed in RNA*later*-ICE, but not from cell pellets that had been snap frozen in liquid nitrogen, thawed on ice and immediately sonicated. RNA isolated from thawed cells sonicated in RLT buffer was consistently degraded (Figs 3-1, 3-2). The timing of sonication was crucial to obtain intact RNA. If cells were sonicated for more than 10 s, RNA was degraded, even when stored in RNA*later*-ICE (Fig. 3-2). High quality RNA was obtained using the sonication treatment (Figs 3-1, 3-3).

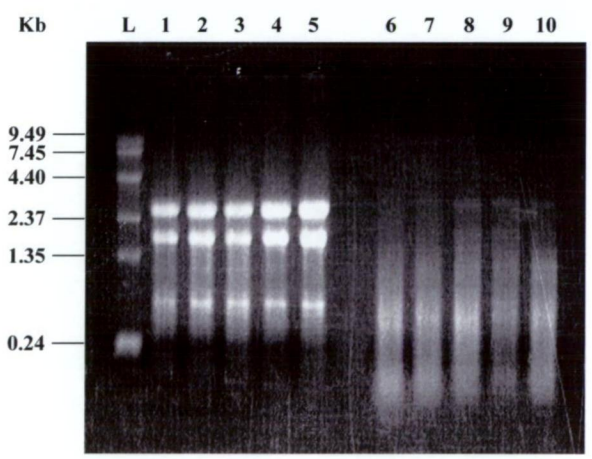

Figure 3-1. RNA isolated from fresh (1-5) vs frozen (6-10) *Alexandrium catenella* cells. Cells were processed in RLT buffer by sonication for 10 s. The RNA extracted from fresh cells (1-5) is of a high quality and does not appear to be contaminated with DNA despite there being no DNase treatment. RNA isolated from cells stored at -70°C (6-10) is highly degraded. RNA ladder (L) 0.25-9.5 Kb (GIBCOBRL).

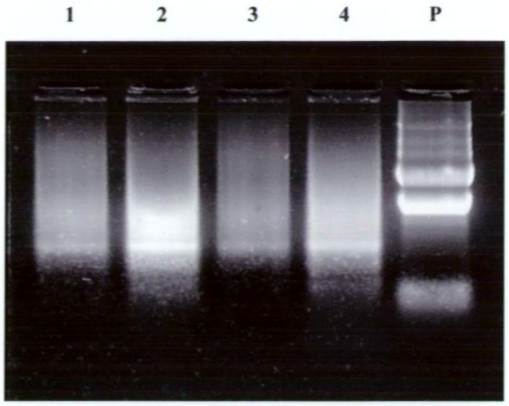

Figure 3-2. RNA isolated from *Alexandrium catenella* cells frozen at -70°C (1, 3) vs stored in RNA*later*-ICE and frozen at -20°C (2, 4) and processed in RLT buffer by sonication for 30 s. Samples were run before (1, 2) and after (3, 4) treatment with TURBO DNase. Positive control (P) - RNA isolated from the garden pea, *Pisum sativum*.

3.3.3 Micropestle

Grinding the frozen cell pellet in 50 μl of RLT buffer (with β-mercaptoethanol and Plant RNA Isolation Aid) and isolating RNA with Qiagen's Plant RNeasy Mini Kit was a reliable treatment for yielding high quality RNA. But, the nature of the sample meant that it could not be ground effectively unless it was in a semi-thawed state. Once a sample had been collected, it was crucial that the cell pellet was frozen immediately or, alternatively, RNA*later* added. Using both treatments, high quality RNA was obtained from *A. catenella* (Fig. 3-3). However, if using the freezing method the timing during grinding was crucial, making this treatment stressful. Pre-soaking cells in RNA*later* for 24 h or even 2 months at -20°C preserved the integrity of the RNA in the sample and meant that there was less urgency to keep the sample frozen during grinding.

3.3.4 DNase treatment

Regardless of the treatment used to isolate RNA, with the exception of sonication (Figs 3-1, 3-3), a large amount of DNA was co-isolated. Although on-column treatments were effective in removing most DNA, they usually left trace amounts of DNA not visible on a gel but detectable in RT-PCR. Also, they sometimes resulted in a loss of RNA (up to 20%). The most effective treatment of removing contaminating DNA was Ambion's TURBO DNA-free kit

(Fig. 3-3). RNA treated with 4-8 U TURBO DNase and used as a template for cDNA synthesis was of a high quality (Figs 3-4, 3-5).

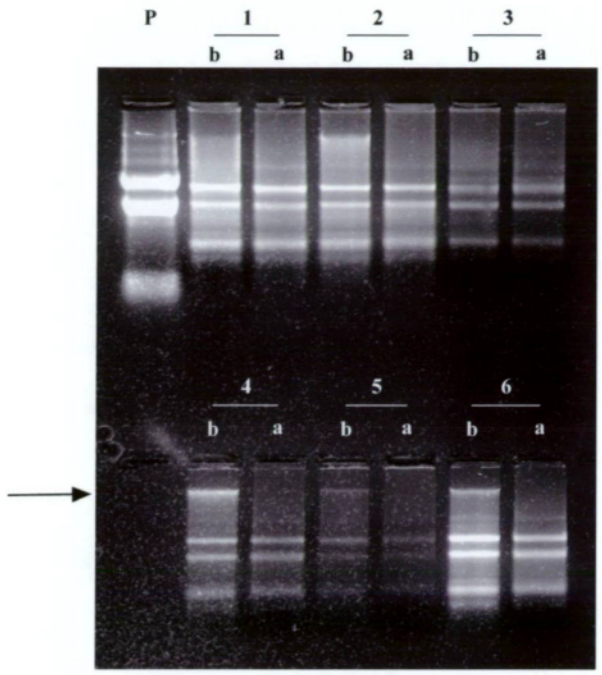

Figure 3-3. Comparison of RNA isolated using different cell treatments and disruption methods. Equal sized pellets of *Alexandrium catenella* cells were processed in RLT buffer by either sonication (10 s at 9 μm peak to peak) or grinding with a micropestle using liquid nitrogen. RNA samples were electrophoresed before (b) and after (a) treatment with TURBO DNase. (P) Positive control - the garden pea. (1-2) RNA from cells stored in RNA*later*-ICE at -20°C and sonicated (1) or ground (2). (3-4) RNA from cells stored at -70°C untreated and sonicated (3) or ground (4). (5-6) RNA from cells stored in RNA*later* at -20°C and sonicated (5) or ground (6). Note the presence of more intact high molecular weight DNA (arrow) in the samples disrupted by grinding as opposed to sonication. The contaminating DNA was successfully removed after DNase treatment in all samples except for (3), which did not appear to be contaminated.

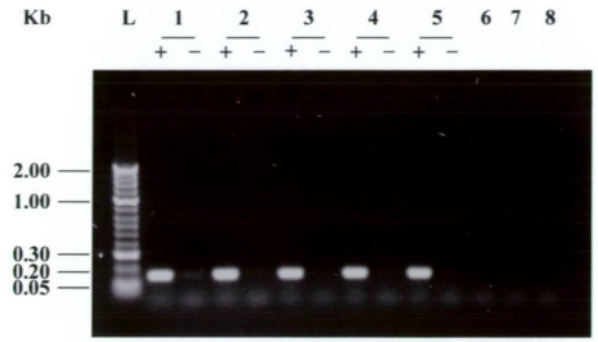

Figure 3-4. (1-5) Five replicates of reverse transcription (RT)-PCR of template cDNAs prepared with (+) and without (-) the reverse transcriptase enzyme. RNA was isolated from *Alexandrium catenella* cell pellets stored in RNA*later* at –20°C and processed in RLT buffer and liquid nitrogen using an Eppendorf micropestle. (L) HyperLadder II (Cat. Nº BIO-33039, Bioline) and (6) no template control. The 151bp amplicon was the candidate PST biosynthesis gene (*Sahh*) (Taroncher-Oldenburg & Anderson 2000).

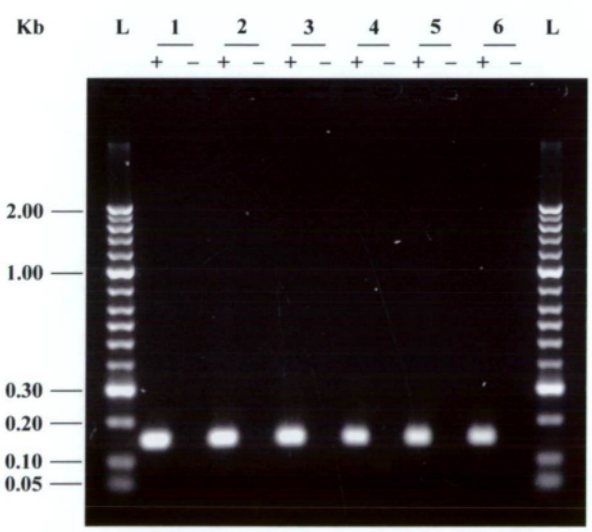

Figure 3-5. (1-6) Six replicates of reverse transcription (RT)-PCR of template cDNAs prepared with (+) and without (-) the reverse transcriptase enzyme. RNA was isolated from *Alexandrium catenella* (CAWD44) cells stored in RNA*later* (1-3) and snap frozen, thawed and stored in RNA*later*-ICE (4-6) at room temperature for 24 h and processed in RLT buffer by grinding without liquid nitrogen. (L) HyperLadder II (Cat. Nº BIO-33039, Bioline). The 151bp amplicon was the candidate PST biosynthesis gene (*Sahh*) (Taroncher-Oldenburg & Anderson 2000).

RNA isolated from five replicate samples stored in RNA*later* at -20°C and extracted using liquid nitrogen was of a high quality ($A_{260:280}$ of 1.89-1.97). The yield ranged from 166- 478 ng.μl^{-1} and was proportional to the volume of the cell pellet extracted (~15-30 μl). This cDNA was tested in RT-PCR with no detectable DNA contamination in RT minus reactions except for a faint band in replicate 1, indicating the need for thorough DNase treatment of cells with a large genome size (Fig. 3-4). Quantitative PCR for this replicate showed insignificant amplification of template in the RT minus (0.38-0.49 ng.μl^{-1}) compared to the RT plus (375-422 ng.μl^{-1}) after replication three times.

Triplicate extractions of cells stored in RNA*later* at room temperature without the use of liquid nitrogen produced RNA of an equally high quality and yield as those processed in liquid nitrogen. The $A_{260:280}$ for the three replicates was (1) 1.97, (2) 1.94 and (3) 1.92, with a yield of 252-282 ng.μl^{-1}. By comparison, the RNA extracted from thawed cells stored in RNA*later*-ICE at room temperature for 24 h was less pure and the yield ~9 fold lower (27-39 ng.μl^{-1}). The $A_{260:280}$ of the three replicates was: (4) 1.68, (5) 1.61 and (6) 1.60, but similar to replicates (1-3) tight ribosomal bands were observed on an agarose gel. Initial RT-PCR revealed some DNA contamination in the RT minus reaction for replicates (4-6) and this was removed by a second DNase treatment (Fig. 3-5).

3.4 Discussion

3.4.1 Cell storage

The most crucial step of RNA isolation from *A. catenella* is the initial cell disruption. Before cells are lysed, RNA is vulnerable to RNases, a particular problem when harvesting and disrupting the tough cells of *A. catenella* as standard lysis buffers do not protect cellular RNAs. Guanidinium is commonly used in lysis buffers like RLT for its ability to inhibit ribonucleases, but presumably in this study it could not penetrate *A. catenella* cells (Chomczynski & Sacchi 1987). In contrast, if cells were first immersed in

Ambion's RNA*later* and then transferred into RLT, the protection of RNA was improved considerably. It is likely that the alcohol component of RNA*later* allowed this solution to penetrate the algal cell walls without compromising RNA integrity, confirming the findings by Taroncher-Oldenburg & Anderson (2000).

From findings in the present study it is recommended that an RNA stabilising product such as RNA*later* be used for isolation of RNA from *A. catenella*. The advantages of RNA*later* are that it is non-toxic and stabilises and protects cellular RNA in intact, unfrozen tissue samples. Cells can be stored in RNA*later* short-term (days/weeks) at room temperature or long-term (months/years) at -20°C. Cells stored in RNA*later* can also be used for DNA isolation (Ambion). RNA*later*-ICE is advantageous for thawing frozen samples and was able to permeate cells and prevent RNA degradation during thawing. Thus sonication can be applied to frozen samples.

The only other workers to have used a commercially available RNA stabilising solution were Taroncher-Oldenburg & Anderson (2000). In their study, *A. fundyense* cells were harvested by centrifugation at 4,400 g for 5 min at 4°C, deep frozen in liquid nitrogen and resuspended in 1 ml RNA STAT-60 which includes phenol and guanidinium thiocyanate (Teltest "B", Inc., Friendswood, TX, USA). This product appears to be equivalent in performance to Ambion's RNA*later*-ICE. But unlike RNA*later*, samples were required to be frozen, making this treatment undesirable for field-based collection and laboratory experiments where speed is essential. Additionally, the inclusion of phenol and the need to extract with chloroform means that a fume cupboard is essential.

3.4.2 Cell disruption

Among the three treatments tested for disrupting *A. catenella* cells for RNA isolation, bead beating was unsuitable but grinding using a micropestle (without sand) and sonication were reliable. The addition of sand was unnecessary and dramatically reduced the yield of RNA. Compared to sonication and grinding, bead beating was less effective at breaking up cells,

rendering this treatment unsuitable for use in conjunction with column-based RNA isolation. Furthermore, it caused the sample to warm, activating RNases and resulting in RNA degradation.

Using the sonication treatment, timing was crucial to obtain intact RNA. If samples were sonicated for longer than 10-20 s RNA was always degraded, presumably due to a combination of RNA shearing and warming of the sample, which would activate RNases. High quality RNA was consistently obtained using the sonication treatment and, surprisingly, it contained minimal DNA contamination (not visible on a gel) despite no DNase treatment. This was probably due to the high molecular weight DNA becoming sheared before any RNA degradation, making it less likely to become purified by the commercial RNA isolation kit. Unlike grinding, sonication was not suitable for frozen samples. Adding RNAlater-ICE whilst thawing the sample solved this problem.

When grinding it is important not to add too much RLT buffer (50 µl is ample) to the cell pellet otherwise, upon thawing, the cells will move around rather than becoming crushed. The RLT buffer does not freeze well. Freezing causes a precipitate to form and the buffer to change consistency. Furthermore, the addition of more than 50 µl of liquid will mean that the cell pellet/suspension cannot be ground when frozen because it will settle as a lump at the bottom of the Eppendorf tube. The use of RNA*later* solved this problem because it eliminated the need for liquid nitrogen.

3.4.3 Choice of optimal protocol

This study reports on the novel application of RNA stabilising solutions from Ambion for preserving RNA in (microalgal) cells at room temperature (RNA*later*) and cells whilst thawing (RNA*later*-ICE). Additionally, two novel methods of homogenising robust (microalgal) cells for RNA isolation are discussed: (i) sonication (with and without a preservation solution) and (ii) grinding without liquid nitrogen. Sonication required the sample to be thawed and without a protective buffer was successful only for fresh samples and not

frozen samples. The ability to preserve cellular RNA without the need for freezing allowed sonication to be applied to stored *A. catenella* samples.

Confirming other studies, the present work found that the traditional method of homogenising cells by grinding in micropestle was effective and consistent for obtaining high quality RNA for *A. catenella* (not previously reported) (Table 3-1). However, a complication, especially for field sampling, is that samples must be frozen immediately and processed frozen. Importantly, this study reports a reliable treatment for collection and preservation of armoured dinoflagellate cells without the need for liquid nitrogen and which is compatible with traditional RNA isolation methods. RNA*later* was able to penetrate the cell walls of the armoured cells and preserve the nucleic acids of *A. catenella* cells stored at room temperature (Figs 3-3, 3-5). Although RNA*later*-ICE was also effective, RNA*later* was more versatile because samples did not require freezing. Immersing cells in RNA*later* allowed the micropestle technique to be used with or without liquid nitrogen and with no concern of RNA degradation. Conversely, if samples have already been frozen, RNA*later*-ICE allows greater flexibility with the extraction method chosen. Frozen cells thawed in RNA*later*-ICE, stored at room temperature for 24 h and ground using the micropestle technique without liquid nitrogen will yield RNA of a high quality for RT-PCR (Fig. 3-5).

Qiagen's Plant RNeasy Mini Kit was found to be the most reliable, quick and user-friendly method of isolating RNA. The RLT buffer in conjunction with Ambion's RNA Isolation Aid was effective for resuspending cells for homogenisation. Addition of a small amount of RLT buffer to preserved/frozen cells before grinding was beneficial because it provided some liquid to assist in the grinding, but was not essential. Immediately prior to extraction, 450 µl RLT buffer (as described earlier) was added to the ground/sonicated chilled sample. The cell extract was placed over the QIAshredder spin column and centrifuged for 2 min at 14,000 rpm. This step was essential for removing cell debris that would otherwise clog the RNeasy spin column.

3.4.4 Conclusions

Some species with thinner cell walls than dinoflagellates may require little or no physical disruption. For example, cells of the green alga *Chlamydomonas reinhardtii* Dang. can be sufficiently lysed by briefly heating at 56°C for 3 min in the lysis buffer and vortexing (unpublished observations). Likewise, the minute (2 μm) brown alga *Aureococcus anophagefferens* Hargraves & Sieburth can be lysed by incubation at 50°C for 15 min under vigorous shaking (Lin *et al.* 2000) and the small (10 μm) dinoflagellate *P. shumwayae* can be lysed by incubating in a guanidine thiocyanate lysis buffer at 60°C for 5 min (Coyne *et al.* 2004). By contrast, *A. catenella* has tough cellulose plates and the same protocols could not be applied to this species. The smaller size of cells may explain why grinding with sand worked for *Symbiodinium* Freudenthal (5-10 μm), but not for the larger *A. catenella* (30-40 μm) as in this study (Mylne *et al.* 1998).

It is concluded that extraction of cell lysates is faster (1-2 h) and the yield and purity of RNA more reliable when a column-based commercial kit is used. A recommended protocol for a mini-prep is to: (i) harvest the culture by centrifugation for 5 min at 5,000 rpm or higher if the culture is viscous; (ii) tip off the majority of the culture medium, resuspend the cells and transfer cell suspension to Eppendorf tubes; (iii) re-pellet the cells by centrifugation for 30 s at 8,000 rpm, remove the supernatant and resuspend the cell pellet (a volume of 20-50 μl is optimal) in 500 μl RNA*later* (at this stage cells can be processed immediately, stored at room temperature or 4°C short-term or at -20°C long-term); (iv) to extract RNA, re-pellet cells and remove the RNA*later*; (v) (at this stage it is unnecessary to freeze the cell pellet in liquid nitrogen) add 50 μl of RLT buffer (containing 4 μl β-mercaptoethanol and 50 μl Plant RNA Isolation Aid) (optional) and grind using a glass micropestle for ~2 min *or* sonicate 10 s at an amplitude level of 9 μm peak to peak; and (vi) add 450 μl of the same RLT buffer to the cell homogenate and proceed with kit instructions. Treatment of RNA with DNase is essential and an off-column treatment with a superior DNase such as TURBO DNase (Ambion) is recommended.

The protocol developed in this study will be used to further our understanding of functional genomics in toxic microalgae. Quantitative real time PCR has been successfully conducted to study the expression of candidate genes in the PST biosynthetic pathway of *A. catenella* (see Chapter 6). Importantly, the candidate PST gene, *Sahh*, has already successfully been amplified from cDNA produced using the optimised RNA isolation and cDNA synthesis treatments described in this chapter.

Chapter 4: The search for genes involved in paralytic shellfish toxin production

4.1 INTRODUCTION

Paralytic Shellfish poisoning Toxins (PSTs) are small heterocyclic guanidinium alkaloids. The alkaloids form the largest class of compounds produced from the secondary metabolism of amino acids (Mann 1993) (Fig. 4-1). Unlike proteins, PSTs are not encoded by a single gene but rather are the result of a series of enzymatic steps in a biochemical pathway (see 1.8). Currently, knowledge of the metabolic pathways that lead to the biosynthesis and degradation of PSTs is limited (Pomati *et al.* 2001). Section 1.8 described how PSTs are synthesised via a pathway involving arginine, S-adenosylmethionine (SAM) and acetate, which form the basic precursors of the toxin molecule. However, elucidation of toxin producing enzymes is hampered by a lack of understanding of the order in which substrates are assembled into the final molecule and because no intermediates have yet been identified (Plumley 1997). It is not known, for example, whether the bonds formed between arginine and acetate represent the first, intermediate or final step in toxin synthesis.

4.1.1 Targeting candidate paralytic shellfish toxin (PST) genes

To date no PST genes have been conclusively identified. The low degree of sequence homology of dinoflagellate genes to known sequences in GenBank is one of the many challenges for identifying toxin biosynthesis genes and determining their expression and regulatory function (Cembella *et al.* 2004). Also crucial to gene identification is an understanding of cellular features, such as DNA properties, and how these relate to the cell division cycle. The dinoflagellates are a phylum of protists whose nucleus displays unique features (see 1.2). Unusual features may necessitate the need for molecular techniques to be tailored to suit the study organism(s) as highlighted in Chapters 2 and 3. In this chapter, candidate enzymes involved in the PST

biosynthesis or degradation pathway(s) are described and the genes encoding these enzymes are studied.

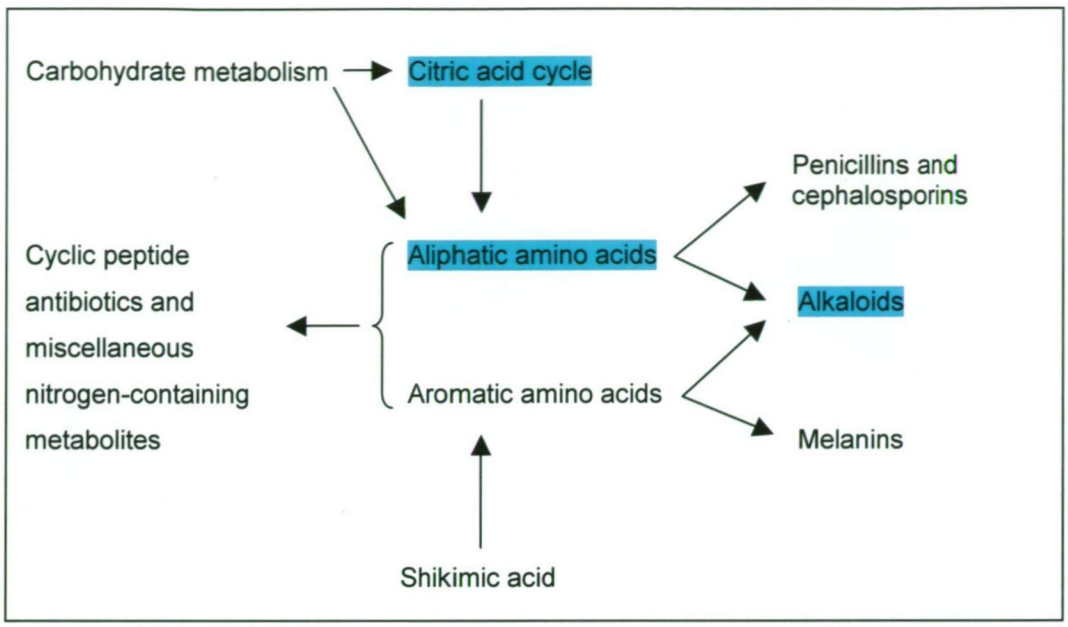

Figure 4-1. Diversity of pathways involved in the secondary metabolism of amino acids (modified from Mann 1993). The likely pathway leading to PST (alkaloid) metabolism in dinoflagellates is highlighted.

The hypothesis of candidate genes potentially involved in the biosynthesis of PST was based on knowledge gleaned from current literature on the chemical structure and properties of the toxin derivatives. Eight genes were targeted, each coding for an enzyme (protein), the function of which was to move or bind a chemical moiety. These chemical moieties are known components of the PST molecule. The genes chosen were: 1) S-adenosylmethionine synthetase (*Sam*), 2) phosphoadenosine phosphosulfate reductase (*PAPR*), 3) nitrogen oxide synthase (*NOS*), 4) cytochrome P450 oxidase (*P450*), 5) guanidine methyl transferase (*GMT*), 6) arginine methyl transferase (*ArgMT*), 7) cystolic sulfotransferase (*CST*) and 8) saxiphilin (*SAX*).

The first seven candidate genes are likely to act at different stages in the PST biosynthesis pathway, from early (*Sam*, *PAP*, *NOS, ArgMT*), to mid (*NOS*, *P450*) to late (*P450, GMT*, *CST*) acting. Saxiphilin, encoded by the eighth candidate gene *SAX*, binds STX and may therefore be involved in

regulating the activity of STX in dinoflagellates. Interestingly, it is not known whether *SAX* exists in dinoflagellates. The bioinformatics approach permitted the design of degenerate PCR primers to attempt to amplify the candidate genes.

4.1.2 Sulfotransferases (STs)

Before the dynamics and environmental control of toxin production can be understood, more information is required about the enzymes involved. Enzymatic *in vitro* assay of toxin biosynthesis relies on quantification of a product such as a toxin intermediate or end product. Oshima (1995) was able to quantify derivatives of saxitoxin (STX) by adding cell lysates from toxigenic strains as a supply of enzymes. Oshima (1995) demonstrated oxidase activity that corresponded to the conversion of two PST derivatives with 1-NH to 1-NOH side groups. Additionally, N-sulfotransferase (N-ST) activity was detected when 21-NH_2 was converted to 21-NSO_3^- in the presence of exogenously added adenosine 3'-phosphate-5'-phosphosulfate (PAPS), as also reported by Sako *et al.* (2001). Although these results are an exciting step forward, this enzyme may not have been specific to PSTs because N-ST activity was also detected in a non-toxic strain of *Gymnodinium catenatum* Graham (Oshima 1995). Since then, the first enzymes [N-ST and O-sulfotransferase (O-ST)] putatively linked to cellular dinoflagellate toxin composition have been isolated (Ishida *et al.* 1998). The properties of N-ST differed between species and the O-ST specific to C-11 and 11-α,β-hydroxySTX was different from N-ST suggesting that multiple genes encoding different STs may be involved (see 1.8, Table 4-1) (Yoshida *et al.* 1996, 1998, 2002).

Sulfated toxins dominate the toxin composition of some Paralytic Shellfish Poisoning (PSP) dinoflagellates and it is reasonable to infer that multiple sulfotransferases (STs) play a prominent role in their metabolism (Sako *et al.* 2001). Sulfation is known to be an important step in the metabolism of bioactive compounds in mammals, such as hormones and drugs (Rikke & Roy 1996, Ishida *et al.* 1998). In most cases, sulfation lessens the bioactivity, increases the water solubility, and accelerates the excretion of a

compound through the attachment of a polar sulfate group. This reaction is catalysed by a large family of STs present in the cytosol, which is why the present work focused on the cystolic STs (CSTs) as candidate enzymes in PST biosynthesis (Weinshilboum *et al.* 1997).

Table 4-1. Comparison of the properties of N-sulfotransferase (N-ST) and O-sulfotransferase (O-ST) in *Gymnodinium catenatum* (after Ishida *et al.* 1998).

	N-ST		O-ST
Substrate	STX	GTX2,3	11-α,β-hydroxySTX
	↓	↓	↓
Products	GTX5	C1,2	GTX2,3
SO_4 donor	PAPS*		PAPS*
Optimum pH	6		6
Optimum Temperature	25°C		35 °C
Metal co-factors	Mg^{2+}, Co^{2+}		no

4.1.3 Phosphoadenosine phosphosulfate reductase (PAPR)

The utilisation of sulfate by an organism requires metabolic activation to a form that can readily undergo reduction. The activated sulfate compound is phosphoadenosine phosphosulfate (PAPS) which serves as the agent for sulfate esterification in all organisms such as in the synthesis of sulfated polysaccharides (Mathews & van Holde 1996). For instance, CSTs transfer a sulfate group from PAPS to various hydroxy (OH) or amine groups (NH_2R). Since PAPS is an important sulfur donor for PSTs, the enzyme that regulates the oxido-reductive state of PAPS, PAPS reductase (PAPR), will influence the availability of substrate for CSTs and therefore the composition of PSTs in a cell. PAPR is part of the adenine nucleotide α hydrolases superfamily and uses thioredoxin as an electron donor for the reduction of PAPS to phosphoadenosine phosphate (Mathews & van Holde 1996).

4.1.4 S-adenosylmethionine (SAM) synthetase

The enzyme S-adenosylmethionine (SAM) synthetase (encoded by *Sam*) is responsible for the conversion of methionine into SAM, the main element of one-carbon metabolism in cells. Methionine is an amino acid with a sulfur-containing side chain. Radioactive labelling studies have conclusively demonstrated that the methionine methyl group of SAM is incorporated into the final PST molecule (Shimizu *et al.* 1990a, 1990b, Shimizu 1996). This transfer presumably involves multiple steps (see 1.8, Fig. 4-2) and has been suggested to involve electrophilic substitution on a reactive dehydro intermediate (Shimizu *et al.* 1985). Therefore, the enzyme SAM synthetase, which catalyses SAM production, is a key enzyme in toxin biosynthesis.

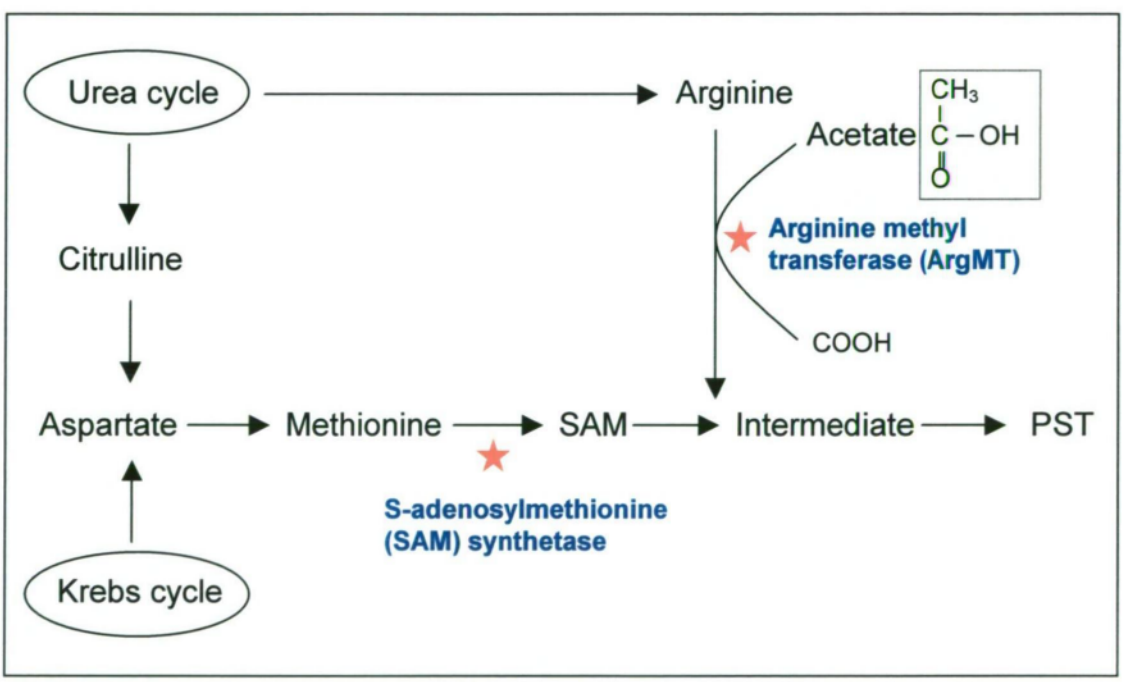

Figure 4-2. Schematic representation of the hypothetical involvement of the Krebs cycle and the urea cycle in the production of PSTs. The possible involvement of two enzymes, arginine methyl transferase (ArgMT) and S-adenosylmethionine (SAM) synthetase, in the production of the final toxin molecule is indicated (star).

4.1.5 Methyltransferases (MTs)

In Chapter 1 two putative enzymes involved in PST biosynthesis were described: (i) methyl transferase (MT) to incorporate the methyl group of SAM into the toxin nucleus and (ii) arginine methyl transferase (ArgMT) to

combine the two carbon precursor product of arginine and acetate into the toxin nucleus (Fig. 4-2). Like the STs, the MTs are a large family of enzymes. The present work selected ArgMT and guanidine MT (GMT). GMT is a logical candidate because the PST molecule contains two positively charged guanidinium groups.

4.1.6 Nitric oxide synthase (NOS)

The nitric oxide synthases (NOSs) are a class of enzymes that oxidise arginine to generate citrulline (a catalytic promotor of urea synthesis) and nitric oxide (NO). In mammals, this occurs by monooxygenation of L-arginine (L-Arg) to N-hydroxyarginine (NOHA) [i.e. the conversion of a hydrogen (H) to a hydroxyl (OH) group of a guanidinium nitrogen (N) of arginine] and the subsequent monooxygenation of this to NO and citrulline (Ost & Daff 2005, Fukuto *et al.* 1993, Moali *et al.* 2001). L-Arg and NOHA contain a guanidino group, one of the main components of the PST molecule. Since arginine is a precursor product in PST biosynthesis and that OH groups are common to many PST derivatives, it is likely that dinoflagellates possess an enzyme such as NOS that is important for PST formation (see Chapter 1, Table 1-4). Furthermore, PSTs have been hypothesised to be secondary metabolites that may be a nitrogenous waste product of cells (see Fig. 1-10). A urea cycle is a nitrogen waste cycle typically found in animals but has recently been identified in the diatom *Thalassiosira pseudonana* (Armbrust *et al.* 2004). It is therefore likely that a similar cycle exists in dinoflagellates and that NOS is involved.

4.1.7 Cytochrome P450 oxidase (P450)

A role of oxidoreductases (hydrolases) in PST biosynthesis has been previously suggested by Ishida *et al.* (1998), and hence another candidate gene(s) selected were the cytochrome P450 oxidases (P450s). P450 is a generic term for a diverse group of related oxidative enzymes, also known as oxidoreductases, which bring about oxidation by the addition of oxygen to a metabolite or by the removal of H or one or more electrons. These heme proteins bind carbon monoxide and most catalyse NADPH- and O_2-

dependent hydroxylation reactions. They have been found in bacteria, insects, fish, mammals, plants and fungi and a catalogue of cloned P450 genes can be accessed at: http://www.icegeb.trieste.it/p450/. In vertebrates, most P450s are located in the endoplasmic reticulum of liver cells where they metabolise thousands of endogenous and exogenous toxins, drugs, xenobiotics and other potentially harmful molecules. In plants, P450s participate in numerous biochemical pathways, including the synthesis of phenylpropanoids, terpenoids, lipids, cyanogenic glycosides, glucosinolates and notably alkaloids as well as plant growth regulators such as gibberellins, jasmonic acid and brassinosteroids (Chapple 1998).

Similarly to NOS, P450s have been linked to the N-hydroxylation of guanidines to N-hydroxyguanidines and the subsequent oxidative conversion to the respective urea (Clement *et al.* 1993). As discussed in Chapter 1, PSTs are tricyclic guanidinium compounds and formation of the different derivatives would require hydroxylation to occur.

4.1.8 Saxiphilin (SAX)

Saxiphilin is a ~90 kDa protein first identified in the North American bullfrog (*Rana catesbeiana*) plasma that has a novel high binding affinity (Kd, ~0.2 nM) for saxitoxin (STX) and its chemical relatives but not tetrodotoxin (TTX), also a neurotoxin and alkaloid that contains the same positively charged guanidium moiety (Morabito & Moczydlowski 1994). Saxiphilin is of particular interest because of its usefulness in the characterisation of STX congeners (e.g. other PST derivatives in dinoflagellates and cyanobacteria) and its use in bioassays for PSTs (Llewellyn *et al.* 2001). Saxiphilin is structurally related to the transferrin family: monomeric glycoproteins (~80 kDa) that bind Fe^{3+} and can exhibit anti-microbial activity. Although saxiphillin has 40-70% homology to vertebrate transferrins, it is distinct from the transferrin in bullfrog plasma in terms of its larger size, chromatographic behaviour, visible absorption spectrum and ligand-binding properties (Li *et al.* 1993). Unlike transferrins, saxiphilin does not bind Fe^{3+} and is therefore not likely to be involved in iron metabolism. A TTX binding protein from puffer-fish plasma has been discovered that also binds STX. These STX binding proteins

exhibit no amino acid sequence homology and their production and co-existence remains a biological mystery considering the only known source of STX is from dinoflagellates and cyanobacteria. The occurrence of saxiphilin in many amphibians and small freshwater fish suggests that it may directly function or be secondarily recruited as a mechanism of STX detoxification (Llewellyn *et al.* 1997).

4.2 Materials and Methods

4.2.1 Degenerate oligonucleotide primed PCR

Several techniques have been developed for isolating novel genes. These include degenerate-oligonucleotide primed PCR, differential display, and mass sequencing of genomes or complementary DNAs (cDNAs) (Lippmeier *et al.* 2002). The former bioinformatics approach was chosen in this study because it offers several advantages. Oligonucleotide design is fast, relatively low-cost and unlike the latter two methods, it allows specific genes to be targeted. Additionally, it doesn't necessitate the extraction of RNA for the production of template cDNA, a method that required tailoring to suit dinoflagellate species (see Chapter 3). However the ability to synthesise pure dinoflagellate cDNA (see Chapter 5) was an advantage when screening for novel genes because genomic DNA (gDNA) could have contained trace amounts of contaminating bacterial DNA that would potentially amplify in PCR (see Chapter 2).

4.2.2 Primer design

Degenerate primers were designed for the eight genes encoding the proteins: SAM synthetase, CST, ArgMT, GMT, NOS, P450, PAPR and SAX. Available nucleotide sequences for a range of organisms were obtained for each gene using Entrez and PubMed search engines (NCBI database). Combinations of sequences were compared in BLAST (NCBI database) to identify the degree of similarity between sequences and hence their suitability for primer design. For each gene, sequences were collated in FASTA format, aligned using ClustalX and imported into the BlockMaker multi-

sequence alignment program to generate blocks of conserved sequences. The program CODEHOPS was used to design primers for each block. Primers with the highest clamp (5' non-degenerate region) score and a low core degeneracy (3' degenerate region) score were selected. If the available nucleotide information for a gene was long (e.g. >500 bp) and there were suitable conserved regions, nested primers were designed to improve the chance of identifying the gene in dinoflagellates (Table 4-2).

Table 4-2. Primer sequences for the eight candidate PST associated genes. The only gene successfully amplified was *Sam*. The final primer pair chosen to pursue *Sam* in a range of microalgae is highlighted.

Name	Direction	Sequence 5' → 3'	Length (bp)
Sam			
SAM-FA	Forward	CGTGACCGAGGGCCAYCCNGAYAA	24
SAM-FC	Forward	CGACCAGGGCCACATGTTYGGNTAYGC	27
SAM-RFa	Reverse	GCCGGAGAAGGCGCCNCCNCCRTG	24
SAM-RD	Reverse	GTACTCGATGGTCACYTGNGTYTT	24
SAM-FG	Forward	TCGACACCTACGGCGGNTGGGGNGC	25
SAM-RJ	Reverse	GGCTTCACCACCTCCCANGTRAARTC	26
SAM-FB	Forward	CAAGACCAACATGGTGATGGTNTTYGG	27
SAM-RFb	Reverse	GGGTTCAGGTGGAAGATGGTNTTYTCRTC	29
SAM-FC2	Forward	AGATCAAGGACACATGTTYGGNTAYGC	27
SAM-FC3	Forward	TGATCAAGGTCATATGTTYGGNTAYGC	27
SAM-RF2	Reverse	ACCAGAAAAAGCACCYCCYCCTRG	24
SAM-RF3	Reverse	TCCAGAAAATGCTCCYCCYCCRTG	24
SAM/LESC-R	Reverse	AGGTCATATGTTTGGGTATGC	21
ArgMT			
ArgMT-RG	Reverse	GTCCAGGTCCCGGTTGTTYTTNKYRTT	27
ArgMT-FB	Forward	TGCATGTTCTGCGCCAARGCNGGNGC	26
ArgMT-FD	Forward	GGTGGACATCATCATCTCCGARTGGATGGGNT	32
NOS			
NOS-F	Forward	GGCACCGGCATCGCNCCNTTYMG	23
NOS-R	Reverse	CCCCTGCCGTCGCCRCANACRTANA	25
PAPR			
PAPR-F	Forward	AGTGCTGCTACCTGGGGAARKKGRWNCC	28
PAPR-R	Reverse	GGGGTACCCCTGGTCGTGNARNKSRTT	27
PAPR-BF	Forward	GATACAGGTAGACTNAAYCCNGARA	25
PAPR-FR	Reverse	TTTATGAAGTCCACAYTCYTTNGC	24

GMT			
GMT-FA	Forward	GGACTCCCCTCTGGGCAARYTNGARYT	27
GMT-FB	Forward	TTCCAGCAGGAGTCCTTCACNMGNCARGT	29
GMT-RB	Reverse	CGTTGCAGCAGATCACCCKRTGRCANGG	28
GMT-RC	Reverse	CCTCGTGGGCCAGCARCCAYTCYTT	25
P450			
P450-FA	Forward	GGCGGATGATCACCCCNRCNTTYCA	25
P450-RF	Reverse	CTCCATCATGGCGAACTTCTBNCCDATRCA	30
CST			
CST-F	Forward	CACCTACCCCAAGTCCGGNACNAMNTGG	28
CST-R	Reverse	ACGGTGAAGTGGTTCTTCCARTCNCC	26
ST1F	Forward	ACGTACCCGAAGTCNGGNAC	20
ST2F	Forward	ACTTACCCGAAGTCNGGNAC	20
ST3F	Forward	ACATACCCGAAGTCNGGNAC	20
ST4F	Forward	ACCTACCCGAAGTCNGGNAC	20
ST1R	Reverse	GTTCTTCCAGTCNCC	15
ST2R	Reverse	ATTCTTCCARTCNCC	15
SAX			
SAX-FB	Forward	CCACTACGCCGTGGCCAAGGTNAARAA	27
SAX-RD	Reverse	GGTGTTGTCGGGGCACARNARYTSRTA	27
SAX-FE	Forward	GATCCTGAAGGGGGAGGCNGAYGCNGT	27
SAX-RG	Reverse	GGAAGGCGCCCTGGTTNCCRTARTA	25

All combinations of the initially designed primers for each gene were tested in PCR on template dinoflagellate gDNA and later on cDNA (Chapter 5). Primers for *Sam* were refined to contain a lower GC content (SAM-FC2, -FC3, -RF2, -RF3), to be specific to a range of plant *Sam* only (SAM-FG, -RJ, -FB, -RFb) and to be specific to tomato (*Lycopersicon esculentum*) *Sam* Accession no. Z24741 (SAM/LESC-R). Protein sequences of plant *Sam* for *Oryza sativa* (BAA96637 and CAA81481), *Populus balsamifera subsp. Trichocarpa x Populus deltoids* (AAA20112), *Pinus banksiana Lamb.* (AAA79831) and *Arabidopsis thaliana* (P23686) were aligned with bacterial *Sam* (*Streptomyces spectabilis* - AAD22464) and primers designed to plant conserved regions that were different to bacterial *Sam.*

4.2.3 Polymerase chain reaction (PCR)

Genomic dinoflagellate DNA was extracted for use as a template in PCR using the phenol chloroform and modified CTAB methods described in Chapter 2. A positive control reaction was performed when testing newly designed primers. The primers D1R (forward) 5'-ACCCGCTGAATTTAAGCATA-3' and D2C (reverse) 5'-CCTTGGTCCGTGTTTCAAGA-3' targeted toward conserved positions of the dinoflagellate large subunit (LSU) ribosomal RNA gene (rDNA) were used to amplify a ~700 bp fragment (Scholin *et al.* 1994). The positive control and primer testing reactions usually contained 10-50 ng of dinoflagellate DNA, however more DNA (up to 200 ng) was sometimes added in later tests if no product was generated.

Initially, all primers for the eight candidate genes were tested in PCR at an annealing temperature of 55°C and subsequently reduced to 50°C and 45°C. Test PCR was performed in a total volume of 40 µl containing a final concentration of 0.25 mM dNTPs, 3 mM $MgCl_2$ and 1 µM primers. A first cycle of 2 min at 94°C was followed by 35 amplification cycles of denaturation for 1 min at 95°C, annealing for 1.5 min at 55°C and extension of 1 min at 72°C with an extension of 0.05 min per cycle, with a final cycle of 6 min at 72°C. Thermal cycling parameters were varied in subsequent test PCRs from a denaturation time of 15 s to 2 min, annealing of 15 s to 1.5 min and extension of 30 s to 4 min.

4.2.4 Cloning into a plasmid vector and sequencing

For the purpose of sequencing, PCR products were cloned into the pGEM-T-Easy Plasmid Vector System I (Cat. N°. A1360, Promega). Attempts to sequence purified PCR products directly resulted in many ambiguous bases because of the presence of multiple products, generally of the same size. Ligation reactions were prepared according to the kit instructions, except the volume was reduced to half (5 µl). Electrocompetent *Escherichia coli* cells (strain DH5α, prepared according to the manufacturer's instructions, Bio-Rad) were transformed with a Gene Pulser II electroporator (Bio-Rad) in 0.1

cm electrode gap Gene Pulser Cuvettes (Cat. Nº 165-2089, Bio-Rad) at 1.8 V, 200 Ω and 25 μF. Cells were immediately resuspended in 1 ml Luria Bertani (LB) media and incubated at 37°C for 30 min to recover before being streaked onto LB (1.5% agar) ampicillin (100 $\mu g.ml^{-1}$) X-gal/IPTG indicator plates for blue/white colour screening.

For each reaction ~10 white colonies were randomly picked using a pipette tip, dipped into individual thin-walled PCR tubes containing 6 μl of deionised water and 1 μl removed and streaked onto an LB ampicillin plate. To screen for clones with the desired insert, PCR was performed using the plasmid promoter primers SP6 5'-ATTTAGGTGACACTATAGAA-3' (Cat. No. Q5011, Promega) and T7 5'-TAATACGACTCACTATAGGG-3' (Cat. No. Q5021, Promega) which amplify the insert DNA. Tubes containing bacteria were incubated at 95°C for 5 min to lyse cells and PCR master mix added to a total volume of 20 μl (final concentration of 200 μM dNTPs, 1.5 mM $MgCl_2$ and 50 μM plasmid primers). A first cycle of 2 min at 94°C was followed by 35 amplification cycles of denaturation for 15 s at 94°C, annealing for 30 s at 60°C and extension of 1 min at 72°C, with a final cycle of 5 min at 72°C. Reactions were electrophoresed on a 1% agarose gel at 80 V for 1 h with a 0.1-12kb DNA ladder (Cat. Nº 10787-018, GIBCOBRL) and 5 or more colonies that contained the target size insert were selected for sequencing.

Target colonies were inoculated into 4-6 ml LB ampicillin media, cultured for 14-18 h and 3 ml of cells pelleted for plasmid DNA purification (Wizard Plus SV Minipreps DNA Purification System, Cat. Nº. A1460, Promega). Sequencing reactions were prepared using Beckman CEQ2000 dye terminator cycle sequencing technology. Sequencing was performed in both directions using the SP6 and T7 primers according to kit instructions (CEQ DTCS Quick Start Kit, Cat. Nº. 608120, Beckman Coulter). Half volume (10 μl) reactions were prepared in 0.2 ml thin-wall tubes. Plasmid DNA was added up to a final volume of 5 μl for a 100 fmol reaction, denatured for 3 min at 96°C, cooled to 4°C and the DTCS Quick Start Master Mix (4 μl) and 3.2 μM primer (1 μl) added. Reactions were performed in a Palm Cycler (Corbett Research) using the following thermal cycling program: 96°C 20 s, 50 °C

20 s, 60°C 4 min for 35 cycles followed by holding at 11°C. Ethanol precipitation was performed according to kit instructions. DNA pellets were resuspended in 30 μl Sample Loading Solution (formamide), loaded into a 96-well plate and sequenced in a BeckmanCEQ8000. Sequences were analysed using Sequencher software for DNA sequencing supplied by Gene Codes Corporation.

4.3 RESULTS

4.3.1 S-adenosylmethionine synthetase gene (*Sam*)

The only primer pair that successfully amplified the target gene was SAM-FA and SAM-RFa. Although primer combinations for the other genes sometimes amplified a gene fragment(s) in PCR, upon cloning and sequencing, they did not match the target gene when a BLAST search of GenBank was performed. Primers were redesigned for PAPR and CST based on plant sequences only because the closest match to dinoflagellate *Sam* in GenBank was a plant sequence. The target genes were still not amplified and for this reason *Sam* was the only gene pursued in more detail.

The primer combination SAM-FC/RFa was more suitable than SAM-FC/RD, yielding a larger single PCR product (~430 bp compared to ~220 bp) from *G. catenatum* gDNA and an optimal efficiency at a range of magnesium chloride ($MgCl_2$) concentrations (0.625 mM to 4.375 mM). The same forward primer with the reverse primer SAM-RD was less efficient and amplified multiple products at the higher $MgCl_2$ concentrations (3.125 mM and 4.375 mM) (Fig. 4-2). Although the primers SAM-FC/RFa were determined to be the most suitable for detecting dinoflagellate *Sam* their degeneracy meant that a product was amplified from a wide source of template DNA, including *Chlamydomonas reinhardtii* Dang. gDNA (~650 bp) (Fig. 4-3) and non-purified bacterial DNA (~450 bp) (Fig. 4-4). The bacteria were clear small colonies obtained from dinoflagellate cultures and streaked onto solid LB media (see Chapter 2). The strong positive result for the bacteria dipped into

the PCR reaction mixture indicated the need to refine the primers to target dinoflagellate DNA.

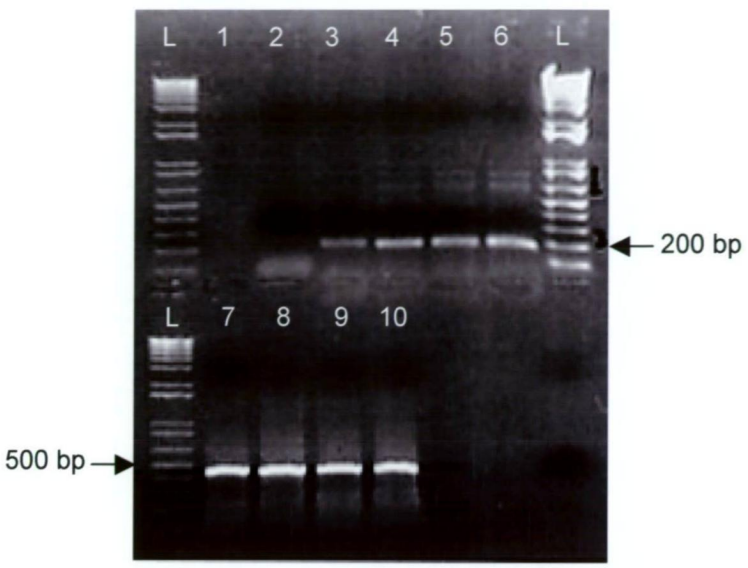

Figure 4-2. Magnesium chloride gradient PCR using primer pairs (3-6) SAM-FC/RD and (7-10) SAM-FC/RFa to optimise the amplification of dinoflagellate *Sam*. The template in all reactions except for (1), the no template control, was GCTRA01 gDNA diluted 1:50 and extracted using the phenol chloroform method. The final concentration of magnesium chloride was (3, 7) 0.625 mM, (4, 8) 1.875 mM, (1, 2, 5, 9) 3.125 mM and (6, 10) 4.375 mM. Reactions were electrophoresed on a 1% agarose gel with the DNA ladder (L) 0.1-12 kb.

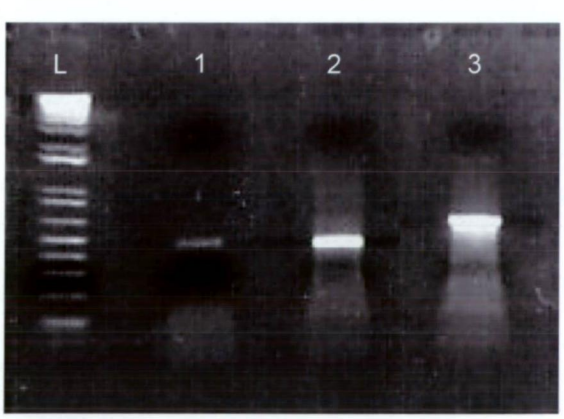

Figure 4-3. PCR using primers SAM-FC/RFa to test their sensitivity to (1) no template control (2) *Alexandrium minutum* gDNA strain AMAD06, diluted 1:50 and extracted using the phenol chloroform method and (3) *Chlamydomonas reinhardtii* gDNA. Reactions were electrophoresed on a 1% agarose gel with the DNA ladder (L) 0.1-12 kb.

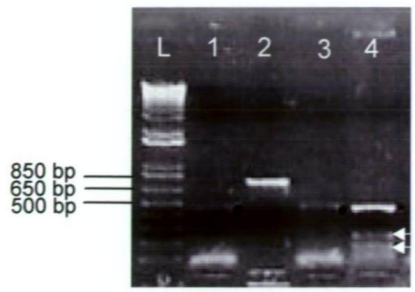

Figure 4-4. PCR using SAM-FC/RFa primers. (1) no template control, (2) positive control primers D1R-F and D2C-R, (3) 10 GCTRA01 cells and (4) clear bacteria from a contaminated dinflagellate culture. Note some smaller, lower molecular weight products were generated in (4) (white arrows). Reactions were electrophoresed on a 1% agarose gel with the DNA ladder (L) 0.1-12 kb.

The primer pair SAM-FC/RFa amplified a product when whole dinoflagellate cells or purified gDNA was used as the template. Additionally, the primers appeared to anneal to and amplify *G. catenatum* DNA more effectively than *Alexandrium catenella* (Whedon & Kofoid) Balech DNA (Fig. 4-5). Cloned gene fragments (~430 bp) obtained using the initial degenerate *Sam* primers SAM-FC/-RFa, produced a match to tomato (*L. esculentum*) *Sam* Z24743. Primers in slightly different regions to the original degenerate primers were re-designed based on sequence information for a range of plant *Sam* only to try and amplify a longer fragment of the *Sam* gene in dinoflagellates. Importantly, these new primers designated SAM-FG, -RJ, -FB and -RFb were designed to not amplify bacterial *Sam*. None of these plant primers were as effective as the original primers and sequencing with these was not pursued.

Instead, a reverse primer (SAM/LESC-R) was designed to *Sam* from tomato to test whether this was more specific to dinoflagellate *Sam* that other plant sequences. Also, a plant specific primer reduces the likelihood of amplifying potentially contaminating bacterial DNA. This primer was used with the forward primer SAM-FC and later -FC3 which was known to amplify *Sam*. This primer combination was tested in PCR using CTAB extracted gDNA from three dinoflagellate species, strains GCTRA01, ACSP01 and ACAD01. No product was generated despite reducing the annealing temperature to 40°C.

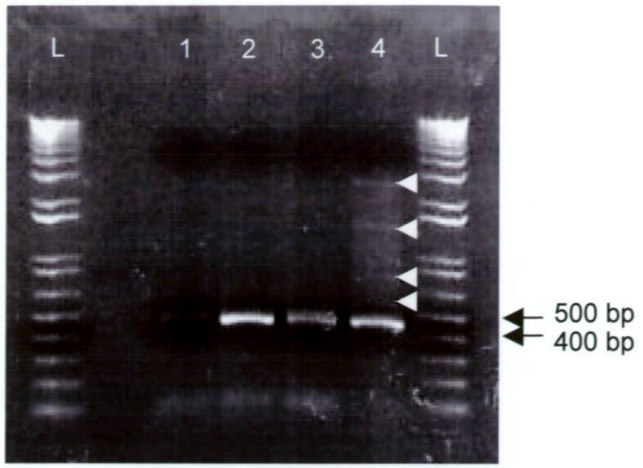

Figure 4-5. PCR using primers SAM-FC and -RFa to amplify partial *Sam* from whole dinoflagellate cells and from dinoflagellate gDNA extracted using the phenol chloroform method. (1) No template control, (2) 4 cells of GCTRA01 from a culture treated with a standard dose of antibiotics, (3) 5 cells of ACSH01 and (4) gDNA extracted from control GCTRA01 cells not treated with antibiotics. Note that higher molecular weight products less-specific to the primers were obtained in (4) (white arrowheads) indicating the need for cloning before sequencing. For details of antibiotic treatment see Chapter 2. DNA ladder (L) 0.1-12 kb.

Analysis of the frequency of nucleotide bases of the initial SAM clones (~430bp) such as CAWD106 clone1, revealed a relatively low GC content of 40.7% (Table 4-3). The original degenerate *Sam* primers were re-designed to select for regions of codon usage with a lower GC content. Two forward and two reverse primers where designed to increase the chance of producing a more dinoflagellate-specific primer. Where there was a choice, the 3rd base of the codon was selected to be an A or T over a G or C. Also, where possible, bases were chosen to be different to known bacterial *Sam*. All combinations of the primers (SAM-FC2, -FC3, -RF2 and -RF3) successfully amplified dinoflagellate *Sam* and the pair SAM-FC3 and -RF2 were chosen for further study. Later cloning of a different region of the *Sam* gene (the 3' terminus) revealed a much higher GC content (61.1%), indicating that the initial clone may not have been representative of the average GC content for the whole gene (see Chapter 5, Table 4-3). It is noted that two different species were compared, *G. catenatum* and *Alexandrium minutum* Halim.

Table 4-3. Percentage of nucleotide bases contained in partial dinoflagellate *Sam* sequences. For details of cloning AMAD06-136-7A refer to Chapter 5.

Strain	Clone	Coding bp	Base	% Base	% GC/AT
CAWD106	1	430	G	18.4	40.7
			C	22.3	
			T	34.0	59.3
			A	25.3	
AMAD06	136-7A	337	G	29.7	61.1
			C	31.5	
			T	16.6	38.9
			A	22.3	

4.4 Discussion

Identification of dinoflagellate *Sam* is an exciting step towards elucidating genes involved in the pathway of PST biosynthesis and to date is the first available sequence information for a candidate PST gene. In spite of the apparent failure to amplify the target DNA of the other seven candidate genes for PST production, this work has provided new insight into gene identification in dinoflagellates. When using a PCR-based approach to identify homologous genes, it is important to understand the phylogenetic relationship of the study organism (dinoflagellates) to other organisms. It is logical to design primers to the nucleotide sequence available for the most closely related organism. If the gene has not been identified in any closely related organism(s), such as other microalgae, then the task of finding the gene becomes harder. For this reason, it may be necessary to select a gene that shows a high level of conservation and design degenerate primers to the region of the gene that is most similar in distantly related organisms. Ribosomal DNA sequences indicate that the dinoflagellates have a common ancestor with the ciliates and Apicomplexa (Saunders *et al.* 1997). This relationship was considered when designing primers to identify candidate dinoflagellate genes.

Due to the similarity of *G. catenatum* (CAWD106) *Sam* to tomato, it was inferred that dinoflagellate *Sam* was likely to be more similar to plant *Sam* than to *Sam* available for other organisms listed in GenBank, possibly because of a more similar codon usage for this gene. Comparison of the GC content of coding nucleotide sequences available in GenBank for a range of organisms revealed some dinoflagellates, such as *Prorocentrum micans* Ehrenberg and *Karenia mikimotoi* (Miyake & Kominami ex Oda) G. Hansen & Moestrup, to have a relatively low average GC content (43%), particularly for the 3rd base of a codon (36 & 37%). This was not the case for *Alexandrium affine* (Inoue & Fukuyo) Balech/*fundyense* Balech/*tamarense* (Lebour) Balech and *Prorocentrum minimum* (Pavillard) Schiller, which were calculated to have an average GC content of around 60% (Table 4-4).

The inability to readily target dinoflagellate genes through degenerate oligonucleotide PCR has highlighted gaps in our knowledge of dinoflagellate genomes and provides direction for which future research should be targeted. It is likely that the seven unamplified genes are present in the dinoflagellate genome and that these types of proposed genes are involved in PST biosynthesis in dinoflagellates. It may be that only a few highly specialised genes are exclusive to toxin biosynthesis and the remainder of the modifications are regulated by genes coding for broad spectrum enzymes, such as SAM synthetase (Cembella 1998). It would be difficult to identify PST-exclusive genes based on similarities to the same classes of genes in other organisms. For example, the MTs and STs are a large family of enzymes with many specialised functions.

The main difficulty with a degenerate oligonucleotide PCR-based approach for identifying PST genes was in the ability to predict the codon usage of dinoflagellates. Although there is sequence information for the ribosomal genes of many dinoflagellates (used for phylogenetics), information for protein coding genes is lacking and hence we can infer little or no information on the codon usage of dinoflagellates (Table 4-4). There is increasing evidence that dinoflagellate DNA is unique in many ways and it is possible

that the frequency of codons in dinoflagellate genes is different from other organisms (Chapter 5).

Table 4-4. Codon usage for a range of species obtained from the Codon Usage Database (GenBank release 145, January 25th 2005). Note that due to a lack of sequence information available for dinoflagellate protein coding genes, the dinoflagellate codon usage (horizontal shading) was calculated from minimal sequence information compared to the other organisms represented. No *Gymnodinium* sp. coding sequences were contained in GenBank. Organisms are listed from the lowest to highest average GC content.

Species	Group	# Coding sequences	# Codons	Coding % GC	% 1st base GC	% 2nd base GC	% 3rd base GC
Cytophaga sp.	bacteria	4	1630	38	49	37	29
Anabaena circinalis	cyanobacteria	35	48025	40	52	36	31
Prorocentrum micans	dinoflagellate	1	344	43	52	42	36
Karenia mikimotoi	dinoflagellate	1	361	43	48	44	37
Pisum sativum	plant	726	287064	43	51	40	38
Arabidopsis thaliana	plant	70186	27679111	45	51	41	42
Pyrococcus abyssi	archaea	1791	544161	45	50	35	50
Homo sapiens	mammal	76893	33070915	53	56	43	59
Penicillium chrysogenum	bacteria	65	39776	55	58	44	63
Alexandrium affine	dinoflagellate	1	1238	58	59	42	71
Alexandrium tamarense	dinoflagellate	1	1238	58	60	42	72
Alexandrium fundyense	dinoflagellate	1	86	60.1	48	63	70
Prorocentrum minimum	dinoflagellate	3	665	62	54	44	89
Chlamydomonas reinhardtii	chlorophyte	697	334118	66	65	48	86
Streptomyces coelicolor	bacteria	8504	2785198	72	73	51	93

that the frequency of codons in dinoflagellate genes is different from other organisms (Chapter 5).

Table 4-4. Codon usage for a range of species obtained from the Codon Usage Database (GenBank release 145, January 25th 2005). Note that due to a lack of sequence information available for dinoflagellate protein coding genes, the dinoflagellate codon usage (horizontal shading) was calculated from minimal sequence information compared to the other organisms represented. No *Gymnodinium* sp. coding sequences were contained in GenBank. Organisms are listed from the lowest to highest average GC content.

Species	Group	# Coding sequences	# Codons	Coding % GC	% 1st base GC	% 2nd base GC	% 3rd base GC
Cytophaga sp.	bacteria	4	1630	38	49	37	29
Anabaena circinalis	cyanobacteria	35	48025	40	52	36	31
Prorocentrum micans	dinoflagellate	1	344	43	52	42	36
Karenia mikimotoi	dinoflagellate	1	361	43	48	44	37
Pisum sativum	plant	726	287064	43	51	40	38
Arabidopsis thaliana	plant	70186	27679111	45	51	41	42
Pyrococcus abyssi	archaea	1791	544161	45	50	35	50
Homo sapiens	mammal	76893	33070915	53	56	43	59
Penicillium chrysogenum	bacteria	65	39776	55	58	44	63
Alexandrium affine	dinoflagellate	1	1238	58	59	42	71
Alexandrium tamarense	dinoflagellate	1	1238	58	60	42	72
Alexandrium fundyense	dinoflagellate	1	86	60.1	48	63	70
Prorocentrum minimum	dinoflagellate	3	665	62	54	44	89
Chlamydomonas reinhardtii	chlorophyte	697	334118	66	65	48	86
Streptomyces coelicolor	bacteria	8504	2785198	72	73	51	93

Most plant P450 genes have not been cloned and it is not surprising that dinoflagellate P450 genes are also difficult to identify. It is likely that dinoflagellates possess their own unique class of P450 genes, some of which may be specific to PST biosynthesis. Therefore orthologues would not exist in other organisms and a degenerate oligonucleotide approach would not be possible. Many reports have documented the role of P450s in alkaloid biosynthesis, but in many cases, these compounds and the catalysts that function in their synthesis are found only in low levels and often in specific cell types (Chapple 1998). This has made cloning of plant P450 genes via protein purification difficult. Further, it seems likely that dinoflagellates possess unique MTs with specific functions in PST biosynthesis. Methyl transferases that act on a variety of substrates (e.g. DNA, protein, alkaloid, phenylpropanoids) all use SAM as the methyl donor (Pott *et al.* 2004). However, despite this shared role in transferring a methyl group, MTs exhibit wide amino acid sequence variation, making identification of novel MTs by degenerate oligonucleotide design difficult.

Novel MTs are continuing to be described, possibly making future identification of dinoflagellate MTs an easier task. A recent study by Pott *et al.* (2004) of plant MTs identified two novel genes, salicylic acid MT (SAMT) and benzoic acid MT (BAMT), for which the amino acid sequence shows no significant sequence identity to other MTs. Interestingly, SAMT and BAMT shared some sequence similarity with a group of N-MT involved in the biosynthesis of the alkaloid caffeine, and with jasmonic acid MT and indole-acetic acid MT. Despite these enzymes being involved in the production of compounds of different chemical type, they have been grouped into a new class of MTs designated the SABATH MT. This unique grouping was supported by the three-dimensional structure of SAMT, which was distinct from unrelated MTs found in plants (Noel *et al.* 2002). This variation may explain why the GMT and ArgMT primers in this study, although designed to conserved regions of the gene, did not amplify dinoflagellate DNA.

This study comprised a general search based on information about conserved regions of genes across different kingdoms. Although conserved

regions of proteins may also be conserved in dinoflagellates, the nucleotide bases encoding them were clearly not easy to predict. Design of primers to conserved regions based on comparison at the amino acid level often meant that thousands of primer combinations were possible. It would be helpful to have more information about the frequency of codons in dinoflagellate DNA to assist with future primer design. Clearly more nucleotide sequence information is needed for dinoflagellates before we can make accurate estimates of the average GC content and codon usage of different species. The average GC content of dinoflagellate DNA calculated in Table 4-4 was mostly based on sequence information for one gene, highlighting the limited sequence information currently available for the dinoflagellates. Preliminary sequencing of dinoflagellate *Sam* suggested that estimates of the average frequency of codon usage for one gene cannot be accurately extrapolated for the whole genome because the frequency will vary depending on the region of the gene that is considered.

The sequence information for *Sam* in this study has laid the foundation for further sequencing of dinoflagellate chromosome-encoded genes. Studies of the toxin profile of crosses between *Alexandrium* Halim sp. have shown a Mendelian inheritence of PST synthesis, with the majority of F_1 and F_2 progeny having a toxin composition identical to the parental phenotypes (Plumley 1997). It is encouraging to know that the series of genes encoding the enzymes for PST biosynthesis may be clustered or linked on the same stretch of chromosome. Therefore, well-established molecular techniques such as primer walking PCR from *Sam* and library screening could provide a means of elucidating other candidate PST genes (Plumley 2001). Chapter 5 presents sequence information for *Sam* in a range of microalgae.

Chapter 5: Sequencing the S-adenosylmethionine synthetase gene (*Sam*) in dinoflagellates

5.1 INTRODUCTION

S-adenosylmethionine (SAM) synthetase, also known as AdoMet synthetase, has been studied extensively in bacteria, yeast and animal systems but no algal SAM synthetase genes (*Sam*) have been reported. The first SAM synthetase gene to have been cloned from higher eukaryotes was the higher plant *Arabidopsis thaliana* gene *sam-1* by Peleman *et al.* (1989). Genome hybridisation indicated the presence of two *Sam* per haploid *A. thaliana* genome. Levels of *sam-1* mRNA were higher in stems and roots compared to leaves and primarily found in vascular tissue, which correlated with enzyme activity. SAM is essential to all living cells as a methyl group donor and *sam-1* can therefore be considered a housekeeping gene. However, housekeeping genes are normally expressed constitutively but *sam-1* is not (Peleman *et al.* 1989). Similar to in yeast (Thomas & Surdin-Kerjan 1987) there is evidence for a second *Sam* gene, *sam-2*, in *A. thaliana*. In the yeast *Saccharomyces cerevisia*, *sam-1* and *sam-2* are functional duplicate genes with a homology of 83% in the open reading frame (ORF) region and 92% between the polypeptide sequences (Thomas *et al.* 1988).

Peleman *et al.* (1989) described the structure of *A. thaliana sam*-1 to consist of one large ORF of 1182 bp, coding for a putative protein of 394 amino acids with a calculated molecular weight (M_r) of 43,149 D. Similarly the homologue in the bacterium *Escherichia coli*, *metK* (Accession Nº. K02129), has an ORF of 1152 bp and codes for a 384 amino acid protein (Accession Nº. P04384) with a M_r 41,941 D (Markham *et al.* 1984). However, in *S. cerevisia, sam*-1 consists of two long ORFs read in opposite directions and separated by 274 bp. ORF1 extends 1149 bp and yields 382 amino acid residues with a M_r 41,800 D (Thomas & Surdin-Kerjan 1987). The 3′ flanking regions of ORF1 and ORF2 are identical but in the opposite direction. *Saccharomyces sam-2* has one ORF of 1152 bp which translates into 384 amino acid residues and M_r 42,350 D (Thomas *et al.* 1988). The deduced amino acid sequence of *A.*

thaliana sam-1 is 49% homologous to *E. coli metK* and 57% to *S. cerevisia sam*-1 polypeptide (Peleman *et al.* 1989).

The aim of the present work was to characterise *Sam* in a range of dinoflagellates with a focus on the toxic species *Alexandrium catenella*, *Alexandrium minutum* and *Gymnodinium catenatum*. To search for additional nucleotide sequence information for *Sam*, two approaches were taken: (i) three prime rapid amplification of complementary DNA ends (3′RACE) to target the 3′ terminus and (ii) using primers designed to span the majority of the coding sequence of the *sam*-1 gene of *A. thaliana* (Peleman *et al.* 1989, Elleuch *et al.* 1998). Rapid amplification of cDNA ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA (mRNA) template between a defined internal site and unknown sequences at either the 3′- or 5′- end of the mRNA (Fig. 5-1). Three prime RACE is therefore a technique used to obtain the 3′end of a cDNA. It requires some sequence information internal to the mRNA under study. The sequence information obtained from this technique can be utilised to obtain full-length cDNA clones using the 5′RACE technique (Frohman *et al.* 1988).

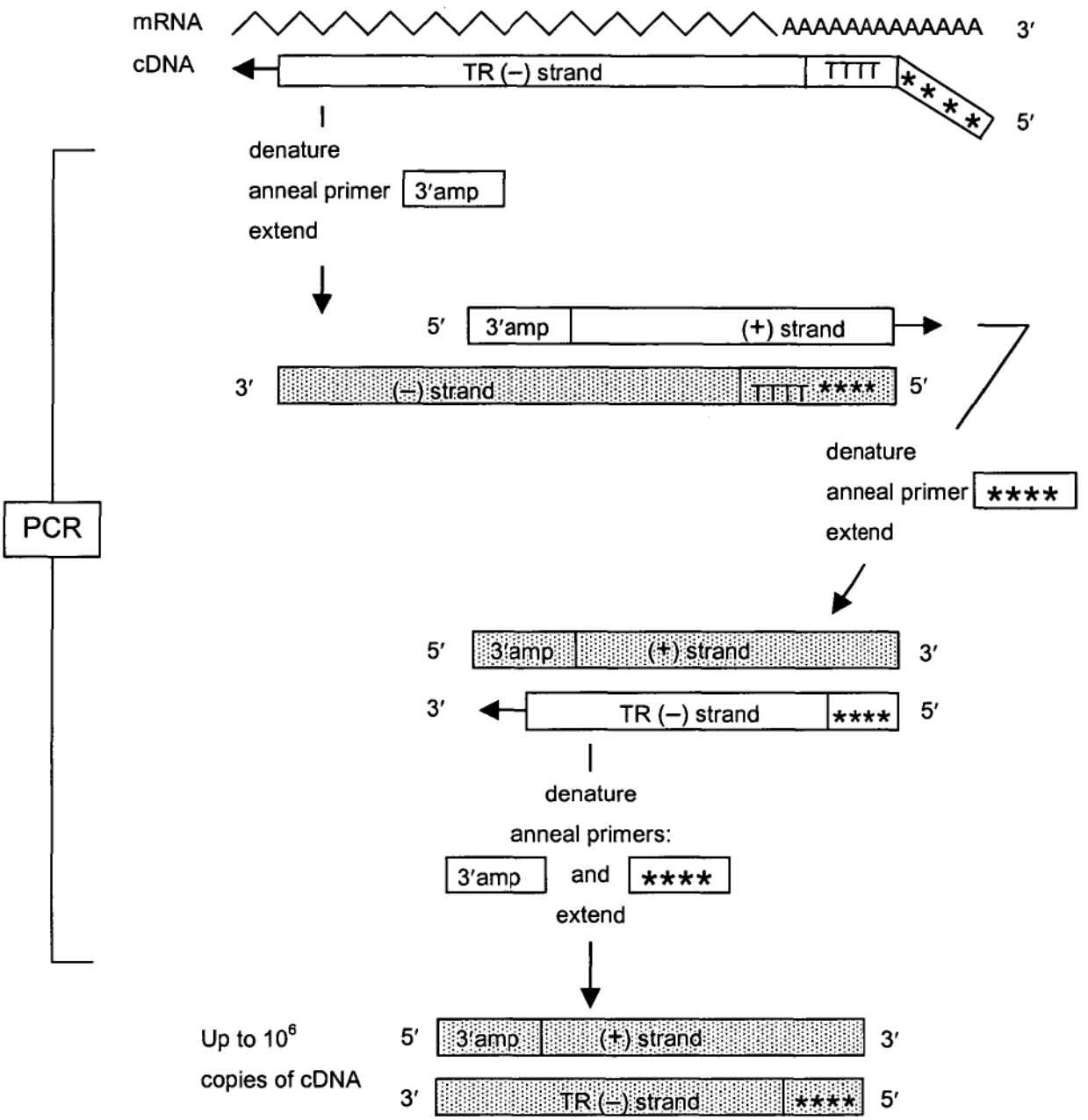

Figure 5-1. Schematic representation of the 3′RACE protocol. Primers ****TTTT $(dT)_{17}$-adaptor, 5′-GACTCGAGTCGACATCATTTTTTTTTTTTTTTT-3′. This sequence contains the *Xho* I, *Sal* I, and *Cla* I recognition sites. **** Adaptor, 5′-GACTCGAGTCGACATCG-3′. 3′amp (amp refers to amplification) specific to the gene of interest, complementary to (–) strand. Open rectangles represent DNA strands actively being synthesised; shaded rectangles represent DNA previously synthesised. At each step the diagram is simplified to illustrate only how the new product formed during the previous step is utilised. A (–) or (+) strand is designated as "truncated" (TR) when it is shorter than the original (–) or (+) strand respectively (adapted from Frohman *et al.* 1988).

5.2 Materials and Methods

5.2.1 Dinoflagellate strains and laboratory culture

Twenty one dinoflagellate strains were selected as a representative sample for sequence analysis of *Sam* (Table 5-1). Clonal cultures of each strain were established by inoculating 50-70 ml of GSe growth medium (Blackburn *et al.* 2001) with 1 ml of mid-logarithmic phase culture. Cultures were maintained in 100 ml, 65-mm diameter cylindrical polycarbonate vials (LabServ) at 17°C (for *G. catenatum* and *A. catenella*) and 20°C (for *A. minutum*) under cool white fluorescent light (80-100 μmol photons $m^{-2}.s^{-1}$) with a 12:12 h light:dark cycle. Standard sterile techniques were employed to maintain cultures. Culture vials and seawater were autoclaved at 121°C for 20 min and growth medium filtered through a 0.22 μm filter. The cultures used in this investigation are held by the University of Tasmania, School of Plant Science, Algal Culture Collection.

5.2.2 Refinement of *Sam* primers

Degenerate primers for *Sam* tested in Chapter 4 were shown to amplify the target gene from dinoflagellates. In the current chapter, dinoflagellate *Sam* was investigated using the primer pair SAM-FC3/-RF2. The optimal thermal cycling program for most dinoflagellate template DNA was a first cycle of 2 min at 94°C followed by 35 amplification cycles of denaturation for 15 s at 95°C, annealing for 30 s at 50°C and extension of 30 s at 72°C, with a final cycle of 10 min at 72°C. Sequence analysis was performed for *Sam* clones of 6 strains (CAWD106, GCJP10, ACAD01, ACSP01, AMAD06 and AMNC04) encompassing 3 species. To reduce the chance of amplifying bacterial DNA, the initial primer pair, SAM-FC3/-RF2, were substituted with primers designed to dinoflagellate *Sam* from preliminary sequence information.

Table 5-1. Dinoflagellate (Class: Dinophyceae) cultures for which partial *Sam* was sequenced.

Species	Strains	Location isolated	Isolator	Date of Isolation
Alexandrium catenella	ACAD01	West Lakes, Adelaide, SA	M. de Salas	15.04.2000
	ACSP01	Tarragona, Spain	M. Villa-Reig	1996
	ACNC50	New Castle, NSW	M. de Salas	1998
	CAWD44	Tauranga, New Zealand	L. McKenzie	1996
	ACCC01	Cowan Creek, NSW	J. Valentine	1996
	ACAM03	Monterey Bay, California, USA	C. Scholin	04.1993
Alexandrium minutum	AMAD06	Adelaide, SA	S. Blackburn	27.10.1987
	AMNC04	Newcastle, NSW	C. Bolch	28.08.1997
Alexandrium tamarense	ATBB01	Bell Bay, TAS	C. Bolch	1990
	ATHK01	Hong Kong	C. Bolch	1998
Alexandrium ostenfeldii	AOKT02	Kettering, TAS	M. de Salas	01.2003
Gymnodinium catenatum	GCJP10	Japan	T. Ikeda	1986
	CAWD106	Manganui Bluff, New Zealand	L. McKenzie	10.08.2000
	GCDEO6C	Derwent River, TAS	S. Blackburn	08.01.1987
	GCDE11	Derwent River, TAS	M. de Salas	01.05.2000
Karenia brevisulcata	KBNZ01	Wellington Harbour, New Zealand	L. McKenzie	1998
Karenia umbella	KUTN05	Taranna, TAS	M. de Salas	19.10.2001
Karlodinium micrum	KDMSR01	Swan River, Perth, WA	M. de Salas	11.03.2001
Noctiluca scintillans	NOCTILUCA	Parson's Bay, Nubeena, TAS	J. Marshall	14.03.2002 field sample
Prorocentrum micans	PMPL01	Port Lincoln, SA	M. de Salas	24.07.2001
Takayama tasmanica	TTDEO3	Derwent River, TAS	M. de Salas	03.05.2001

Two different *Sam* genes initially amplified were chosen and new primers (dinoSAM-1F/-1R, -2F/2R) specific to dinoflagellate *Sam* were designed to two representative clones: (i) SAM-1 from the *G. catenatum* strain CAWD106 and (ii) SAM-8 from the *G. catenatum* strain GCJP10 (Table 5-2, Appendix 4). A single primer pair was designed to each clone and one pair (DinoSAM-1F/-1R to clone SAM-1) selected for further sequence analysis in a range of microalgae. The position of the DinoSAM primers is indicated in Figs 5-2 and 5-3.

Table 5-2. List of primers designed to: (1) dinoflagellate *Sam* (DinoSAM) to pursue *Sam* in a range of microalgae, (2) dinoflagellate *Sam* for 3'RACE (3'RACE-SAM) to obtain the 3'terminus, and (3) the higher plant *Arabidopsis thaliana Sam* (Athal-SAM1) from Elleuch *et al.* (1998) to try and identify full length *Sam* in dinoflagellates.

Name	Direction	Sequence 5′ → 3′	Length (bp)
DinoSAM-1F	Forward	GCACCTTTACCTCCGTAGG	19
DinoSAM-2F	Forward	GTGCGGCACCACCGTAGGTG	20
DinoSAM-1R	Reverse	CCAGCGAGACCGAGAACTAC	20
DinoSAM-2R	Reverse	TCAGTGAAACCCGTGAGTTG	20
3'RACE-SAM-1F	Forward	TTAATCTTTGCCAACAT	17
3'RACE-SAM-2F	Forward	CTCATTGGCATCTGAATA	18
Athal-SAM1-F	Forward	CCAGTATGGACGATTCAAGGCTTGC	25
Athal-SAM1-R	Reverse	GGTCGTCCCTTCCGAAGTGTCC	22

5.2.3 Polymerase chain reaction (PCR)

Dinoflagellate genomic DNA (gDNA) was extracted for use as a template in PCR using the phenol chloroform and modified CTAB methods described in Chapter 2. For the algal strains: ACCC01, ACAM03, ATBB01, ATHK01, AOKT02, TTDE03, KBNZ01, KDMSR01, KUTN01, PMPL01, DNA was kindly provided by Dr. M. de Salas, isolated using the phenol chloroform method. The PCR program used for some template microalgal DNA, using the *G. catenatum* designed primers (DinoSAM), was an initial cycle of 94°C for 2 min followed by 5 cycles of 95°C for 15 s, 58°C for 15 s and 72°C for 1 min, 30 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 1 min, with a final cycle of 72°C for 5 min. Some strains required lower annealing temperatures of 48-55°C.

A

GGCACCTTTACCTCCGTAGGTATCAACAATTATTTTTCGCCCCGTTAATCCGGTATCTCC
ATGAGGTCCTCCAATAACGAATTTTCCCGTTGGATTGATATGGTACTTAATTTGATCATC
GAACAATTTTTGGGTCTCAACAGGCAATTGTGCCTTTACCCTAGGGATTAGAATTGAAAT
AATGTCCGACTTAATCTTTGCCAACATTTTATCATCATCTTCGTCAAAAGCATCATGCTGT
GTAGAAACTACTATCGTATCTATACGCTGTGGCTCATTGGCATCTGAATATTCTAAAGTC
ACCTGTGCTTTGGCGTCTGGTCTTAAATAAGAAATTTCTTTTCCTTCGCGACGCAGTTCT
GCCAAAGTATGCAAAATTTTGTGCGATATATCTAAAGCCAACGGCATGTAGTTCTCGGT
CTCGCTGGTC

B

TSETENYMPLALDISHKILHTLAELRREGKEISYLRPDAKAQVTLEYSDANEPQRIDTIVVSTQ
HDAFDEDDDKMLAKIKSDIISILIPRVKAQLPVETQKLFDDQIKYHINPTGKFVIGGPHGDTGL
TGRKIIVDTYGGKGA

Figure 5-2. **A.** Nucleotide sequence (antisense strand 3′-5′) for the *Sam* gene, clone SAM-1 (length 430 bp), obtained from *Gymnodinium catenatum* strain CAWD106. The reading frame is -2 such that the first codon of this fragment is ACC, coding for the amino acid threonine (T). The positions of the forward primer DinoSAM-1F (bases 2-20) and the reverse primer DinoSAM-1R (bases 428-409) are underlined. The length of the expected amplification product is 427 bp. **B.** Polypeptide sequence of 143 amino acid residues derived from the sense strand of the nucleotide sequence in A.

A

GGGGTGCGGCACCACCGTAGGTGTCCACGATAATCTTGCGACCAGTCAAACCACAATC
GCCTTGAGGTCCACCAATTACAAAGCGGCCAGTCGGGTTCACCAAATAACTGATGTCG
CCTTTGATCAACTCTTTGGGCAACACCGGTTTGATGACCTCTTCAATCACCGCCTCCCG
CCGCGTAGCCAACTCGATGTCCGGCGAATGTTGTGTGGAAAGTACAACGGTGTCGATG
CAATAAGGTTTTCCGTCTACATACTTGATCGTCACCTGCGATTTCGCATCGGGGCGCAG
CCAAGGCAGTCGTCCATCCTTGCGCAACAGGCTCTGGCGTTGAACCAGCTGATGAGAA
AGGCTGATCGCCAGCGGCATCAACTCACGGGTTTCACTGACA

B

VSETRELMPLAISLSHQLVQRQSLLRKDGRLPWLRPDAKSQVTIKYVDGKPYCIDTVVLSTQ
HSPDIELATRREAVIEEVIKPVLPKELIKGDISYLVNPTGRFVIGGPQGDCGLTGRKIIVDTYG
GAAP

Figure 5-3. **A.** Nucleotide sequence (antisense strand 3′-5′) for the *Sam* gene, clone SAM-8 (length 392 bp), obtained from *Gymnodinium catenatum* strain GCJP10. The reading frame is -2 such that the first codon of this fragment is GTC, coding for the amino acid valine (V). The positions of the forward primer DinoSAM-2F (bases 4-23) and the reverse primer DinoSAM-2R (bases 371-390) are underlined. The length of the expected amplification product is 387 bp. **B.** Polypeptide sequence of 130 amino acid residues derived from the sense strand of the nucleotide sequence in A.

5.2.4 Cloning into a plasmid vector and sequencing

For the purpose of sequencing, PCR products were cloned into the pGEM-T-Easy Plasmid Vector System I (Cat. Nº. A1360, Promega) as outlined in Chapter 4. For each reaction approximately 10 white colonies were randomly picked and whole cell PCR performed as described in Chapter 4 to screen for colonies with the target insert DNA. Reactions were electrophoresed on a 1% agarose gel at 80 V for 1 h with a 0.1-12kb DNA ladder (Cat. Nº. 10787-018, GIBCOBRL) and 5 or more colonies that contained the target size insert were selected for sequencing. Target colonies were inoculated into 4-6 ml LB ampicillin media, cultured for 14-18 h and 3 ml of cells pelleted for plasmid DNA purification (Wizard Plus SV Minipreps DNA Purification System, Cat. Nº. A1460, Promega). Sequencing reactions were prepared using Beckman CEQ2000 dye terminator cycle sequencing technology. Sequencing was performed in both directions using the SP6 and T7 primers (Chapter 4) according to kit instructions (CEQ DTCS Quick Start Kit, Cat. Nº. 608120, Beckman Coulter) in a BeckmanCEQ8000. Forward and reverse sequences were aligned using Sequencher software for DNA sequencing supplied by Gene Codes Corporation and the automated base calls checked visually by inspection of the electropherograms.

5.2.5 PAUP analysis

Nucleotide sequences for the clones SAM-1 to -105 obtained from 20 algal strains encompasing 11 species and 5 genera (Appendix 4) were aligned in ClustalX. Aligned sequences were refined by visual inspection using the computer software GeneDoc. Clones SAM-31 to SAM-105 produced using the nested primers dinoSAM-1F/-1R were shorter than clones SAM-1 to SAM-30 produced using the primers SAM-FC3/-RF2. To align all clones, missing bases at the start and end were scored with a dash and treated as missing data. If clones of a single strain were identical or differed by up to 5 bp (< 1.5%), a consensus sequence was created. If clones of a single strain differed by more than 5 bp, they were represented individually in the alignment (Table 5-3). Two files of the 26 aligned sequences were created: (i) nucleotide sequences where ambiguous bases in a consensus were scored as the most common base from the clones represented for that strain

and (ii) protein sequences produced by translation of the nucleotide sequences in (i) using the ExPASy Translation Tool located at: http://us.expasy.org/tools/dna.html (ExPASy proteomics server, Swiss Institute of Bioinformatics).

Table 5-3. List of the 25 sequences, derived from single or consensus SAM clones, aligned for phylogenetic analysis using PAUP. If more than one sequence was included for a single strain (shaded), the stain was listed with a period followed by a number corresponding to each sequence. A 26th sequence, mosquito *Anopheles gambiae* partial *Sam* (XM306865), was included as the outgroup (ANOPH). The maximum number of differences between each sequence included in a consensus was 5 bp, as indicated.

Number	Algal strain	Clone(s)	Maximum # bp differences
1	ACAD01	SAM-all (11-15)	1
2	ACSP01.1	SAM-20	-
3	ACSP01.2	SAM-17	-
4	ACSP01.3	SAM-16, -18	1
5	ACSP01.4	SAM-19	-
6	ACCC01	SAM-all (31-35)	3
7	ACAM03	SAM-all (36-39)	2
8	ACNC50	SAM-all (41-45)	5
9	AMNCO4	SAM-all (26-30)	3
10	AMAD06	SAM-all (21-25)	3
11	ATBB01	SAM-all (46-50)	2
12	ATHK01	SAM-all (51-55)	3
13	AOKT02.1	SAM-66,-67,-68	5
14	AOKT02.2	SAM-70	-
15	CAWD106	SAM-all (1-5)	2
16	GCDE06C	SAM-all (71-75)	5
17	GCDE11	SAM-all (76-80)	2
18	GCJP10	SAM-all (6-10)	2
19	TTDE03	SAM-all (96-100)	2
20	KDMSR01	SAM-all (86-90)	2
21	KBNZ01.1	SAM-92, -93, -95	3
22	KBNZ01.2	SAM-91	-
23	KUTN05	SAM-all (101-105)	2
24	NOCTILUCA	SAM-all (61-65)	5
25	PMPL01	SAM-all (56-60)	4
26	ANOPH	Accession N^{o}. XM306865	-

Analysis of sequence similarity at the nucleotide and protein levels was performed using the computer program PAUP (Swofford DL, 1998. Phylogenetic Analysis Using Parsimony version 4, Sinauer Associates, MA,

USA). Phylogenetic trees were derived under the assumptions that all sequence positions were unordered characters with equal weight. For consensus nucleotide and protein sequences, distance analysis was performed using the heuristic search options and the best tree produced was shown. Insect *Anopheles gambiae* (XM306865) *Sam* was included as the outgroup.

5.2.6 Restriction enzyme digests and Southern blots

Genomic DNA of *A. catenella* CAWD44 was extracted using the CTAB method (Chapter 2). Restriction enzyme digests were performed on 5 μg of high quality DNA ($A_{260:280}$ 1.6-1.8). An eight times concentration of the minimum amount of enzyme to DNA was tested. A control containing no enzyme, in which the DNA should remain undigested, was prepared alongside digest reactions. The following 11 enzymes (and buffers) were tested: *EcoR109*I (DraII) (Y+/Tango) (MBI Fermentas), *Hind*III (E) (Promega), *BamH*I (E) (Promega), *Sal*I (D) (Promega), *Xba*I (Y+BSA) (MBI Fermentas), *EcoR*I (H) (Promega), *Msp*I (B+BSA) (Promega), *Hae*II (B+BSA) (Promega), *Apa*I (A+BSA) (Promega), *Not*I (Y+BSA) (Promega) and *Sma*I (Y+BSA) (MBI Fermentas). The final reaction mixture contained BSA 0.1 μg μl^{-1} and RNase 20 μg ml^{-1}. Digested and control DNA was electrophoresed on a 0.7% agarose gel at 25 V for 4 h with a 0.1-1kb DNA ladder (Cat. Nº. DMW-100L, GIBCOBRL). Southern blots were performed using Hybond N+ membranes (Amersham, Australia).

5.2.7 Isolation of total RNA, northern blot and first strand cDNA synthesis

Total RNA was isolated from a selection of dinoflagellate cultures: *A. minutum*, AMAD06 and AMNC04; *A. catenella*, CAWD44; and *G. catenatum*, CAWD106, using both the sonication method (northern blot) and the optimised micropestle method (cDNA synthesis) described in Chapter 3. RNA was DNase treated using Ambion's DNA-free kit (Cat. Nº. 1906) and/or TURBO DNA-*free* (Cat. Nº 1907, Ambion) treatment. The quality and purity of RNA was assessed by spectophotometry and visual inspection after

denaturing formaldehyde gel electrophoresis. RNA with an $A_{260:280}$ 1.8-2.0 and distinct ribosomal RNA bands was of a high quality. RNA for northern blotting was denatured for 10 min at 65°C, elecrophoresed on a formaldehyde gel with a 0.24-9.5 kb RNA ladder (Cat. Nº. 15620-016, GIBCOBRL) and nothern blots prepared using Hybond N+ membranes (Amersham, Australia) according to the manufacturer's instructions.

To obtain cDNA 3′ ends (Fig. 5-1), 5 μg of total RNA from each culture was reverse transcribed using the Superscript III first-strand Synthesis System for RT-PCR (Cat. Nº. 18080-051, Invitrogen). An oligo $(dT)_{20}$ primer was used to target the polyA tail of eukaryotic mRNA, eliminating the possible amplification of bacterial genes in PCR. The RNA, primer and dNTPs mix was denatured at 65°C for 5 min, cooled and 10 μl cDNA synthesis mix [2 μl 10 x RT buffer, 4 μl 25 mM $MgCl_2$, 2 μl 0.1M DTT, 1 μl RNaseOUT (40 $U.\mu l^{-1}$), 1 μl Superscript III RT (200U/μl)] added. cDNA was synthesised at 50°C for 50 min and the reaction terminated by heating to 85°C for 5 min. Following reverse transcription, 1 μl RNase H was added to chilled cDNA and incubated at 37°C for 20 min. cDNA was stored at -20°C for use in PCR. A test PCR was conducted on dinoflagellate gDNA and cDNA from the following strains, *G .catenatum* CAWD106, and *A. minutum* AMAD06 and AMNC04, using primers dinoSAM-1F and -1R (Table 5-2) to confirm whether first strand cDNA synthesis had been successful and whether the dinoflagellate *Sam* primers were effective on cDNA.

5.2.8 Hybridisation

Plasmid DNA of clone SAM-1 was used for probing Southern and northern blots. The 430 bp coding gDNA insert to be used as a hybridisation probe was isolated by 2 sucessive PCRs, the first using plasmid primers SP6 and T7 and the second using primers SAM-FC3/-RF. The PCR product was purified and 100 ng labelled using random primers and [α-^{32}P]dCTP. Hybridisation was performed in a buffer containing 7% SDS, 1 mM EDTA, 0.14 M NaH_2PO_4 and 0.36 M Na_2HPO_4, pH 7.2, in a BioRad hybridisation oven at 65°C overnight and washed six times in 2x SSC + 0.1% SDS manually by shaking for 1 min each time, followed by three washes in 1x

SSC + 0.1% SDS for 30 min in the hybridisation oven at 65°C. Autoradiography was performed using intensifying screens and Kodak X-OMAT films at -70°C.

5.2.9 3′-end amplification of cDNAs (3′RACE)

The resulting cDNAs were subjected to PCR 3′-end amplification by a series of PCRs. Initially two site specific primers were tested, SAM-FC3 and dinoSAM-1F (Tables 4-2, 5-2) in conjunction with the oligo $(dT)_{17}$-adaptor primer (Fig. 5-1). Amplification conditions were as follows: 1 cycle of 94°C for 4 min followed by 35 cycles at 94°C for 15 s, 50°C for 30 s, and 72°C for 1 min. The resultant PCR reaction mixture was diluted 1:10 with sterile water and 1 μl used as a template in a second, nested PCR. Two dinoflagellate *Sam* specific primers were tested, 3′RACE-SAM-1F and -2F (Table 5-2), in conjunction with the adaptor primer minus the $(dT)_{17}$ sequence (Fig. 5-1), on cDNA of the *A. minutum* strain AMAD06. Amplification conditions were as follows: 1 cycle of 94°C for 4 min followed by 35 cycles at 94°C for 15 s, 50°C for 30 s, and 72°C for 1.5 min.

PCR products obtained from 3′RACE were cloned into the pGEM-T-Easy Plasmid Vector System I (Cat. Nº. A1360, Promega) as described previously. The nucleotide sequences of the isolated clones were determined with a capillary BeckmanCEQ8000 sequencer, using Beckman CEQ2000 dye terminator cycle sequencing technology (Beckman Coulter). The *A. minutum* strain AMAD06 was selected for further study (refer to section 5.3.3 and 6.2.1.1). The nucleotide sequence similarity of clone SAM-136-7A resulting from the forward primers SAM-FC3 (first round PCR) and 3′RACE-SAM-1F (nested PCR) was compared to *Sam* of other organisms.

5.2.10 PCR using primers specific to the *sam-1* gene of *Arabidopsis thaliana*

To try and obtain further sequence information for dinoflagellate *Sam* and to augment 3′ RACE, primers Athal-SAM1-F/-R (Elleuch *et al.* 1998) specific to the *sam-1* gene of *A. thaliana* (NM179246, GenBank) were tested (Table 5-2). The Athal primers amplify a 1801 bp fragment overlapping the major part of the *sam-1* gene coding sequence and part of the 35 S promoter. The product amplified in *A. thaliana* contains one open-reading frame of 1182 bp, coding for a putative protein of 394 amino acids.

PCR was conducted on gDNA and cDNA templates for dinoflagellate strains AMAD06, AMNC04 and CAWD106. Reactions were prepared using a commercial PCR Master Mix (Cat. Nº. M7502, Promega) to a final volume of 50 µl, final primer concentration of 0.8 µM each and ~20-25 ng template cDNA per reaction (RT+) and 100 ng of gDNA. Amplification conditions were as follows: a single cycle of 2 min at 94°C followed by 35 amplification cycles of denaturation for 1 min at 94°C, annealing for 1.5 min at 50°C and extension of 3 min at 72°C, with a final cycle of 5 min at 72°C.

PCR products amplified from cDNA of strain CAWD106 and AMAD06 were cloned into a plasmid vector. For each strain, 20 clones were screened in PCR for the target insert size using vector primers SP6 and T7 (Chapter 4). Three CAWD106 clones (SAM-170-4, SAM-171-11 and SAM-172-12) and 4 AMAD06 clones (SAM-173-14, SAM-174-15, SAM-175-16 and SAM-176-18) were sequenced (method described previously). A nucleotide search using the program Basic Local Alignment Search Tool (BLAST) (NCBI database) was performed for each edited sequence of the full length cloned insert.

5.3 Results

5.3.1 Sequencing and PAUP analysis of genomic DNA *Sam* clones

Both the degenerate primer pair, SAM-FC3/-RF2, and the dinoflagellate specific primer pair, DinoSAM-1F/-1R, successfully amplified *Sam* genes

from 11 dinoflagellate species (Table 5-3). Primers DinoSAM-2F/-2R designed to the less common clone SAM-8 (GCJP10) amplified the target size product and could be useful for later studies. The length of the cloned PCR amplicons for the degenerate primers ranged from 399-438 bp with the most common length being 430 bp, and was a consistent 388 bp for the SAM-1 specific primers (Fig. 5-4). The frequency of codons for CAWD106 clone SAM-1 (Table 5-4) and the translated sequence similarity to *Sam* from a range of organisms (Table 5-5) are presented. At the nucleotide level, no significant match of SAM-1 to sequences in GenBank was found. The closest match at the protein level was to the arthropod *Anopheles gambiae* (85% positives) and the bacterium *Cytophaga hutchinsonii* (83% positives), however no closer match to the kingdom Protista was observed. *Alexandrium* clones SAM-21 to -25 (AMAD06) and SAM-31 to -35 (ACCC01) were equally similar to *A. gambiae*. Dinoflagellate SAM equally matched some bacteria, plant, nematode, fungus, arthropod, mollusc, bird, and mammal sequences (Table 5-5).

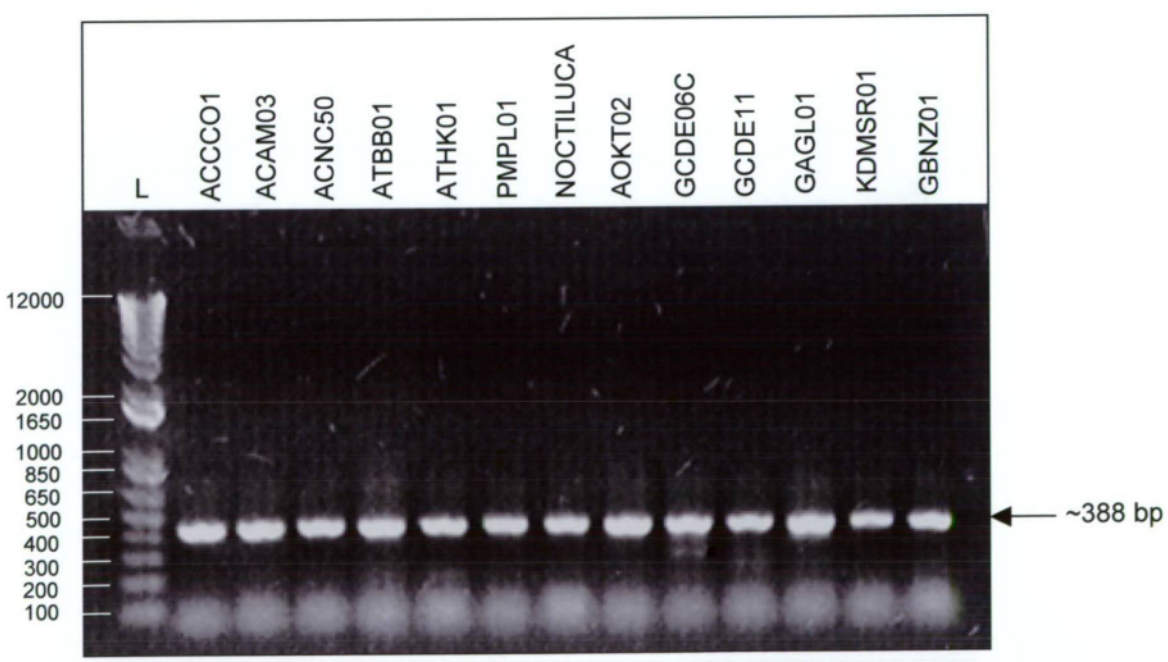

Figure 5-4. Example of the consistent sized PCR products amplified, from template gDNA from 13 dinoflagellate strains and 9 species (Table 5-1), using the *Sam* primers dinoSAM-1F/-1R (Table 5-2). The same size ampicon was produced from PCR of other dinoflagellate strains as determined by agarose gel electrophoresis and cloning into a plasmid vector (refer to Appendix 4). (L) DNA ladder 0.1-12 kb.

Multiple copies of *Sam* that differed by more than 5 bp, were amplified from 3 out of 20 dinoflagellate strains. Four different copies of *Sam* were amplified from strain ACSP01 using degenerate primers whilst SAM-1 specific primers amplified 2 copies of *Sam* from AOKT02 and KBNZ01 (Table 5-3 and Fig. 5-5). Upon translation, ACSP01.2 and ACSP01.3 were functionally similar (Fig. 5-6). Three groupings of *Sam* were evident at the protein level (Fig. 5-7). The first contained 3 sequences representing two *Alexandrium* strains, ACSP01 and AMAD06. The second and largest grouping contained 18 sequences representing the most common copy of *Sam* amplified from most dinoflagellate strains (18 out of 20). These 18 consensus sequences were almost identical at the nucleotide and protein levels. At the protein level, the only differences between sequences 4-21 were for GCDE11 (16) and KDMSR01 (17) that contained a proline (P) instead of a serine (S) at residue 15 and differed from each other in a single residue at position 86 (Fig. 5-7). At this site, KDMSR01 contained a S whilst all other sequences contained an isoleucine (I), except GCJP10 (26), which was the most different strain to all other sequences (Fig. 5-6).

The third grouping contained four different copies of *Sam* from five sequences that were different to groups 1 and 2. The same copy was amplified from two *Alexandrium* strains, ACSP01 and AOKT02. The Japanese dinoflagellate isolate of *G. catenatum*, GCJP10, and the arthropod *A. gambiae* (25) were represented here, making group 3 diverse (Fig. 5-6).

Table 5-4. Frequency of amino acids and codons for the 430 bp CAWD106 *Gymnodinium catenatum* clone SAM-1.

Amino acid	Total	Codon	Frequency/ 143 amino acids	Frequency/ thousand ratio	Amino acid	Total	Codon	Frequency/ 143 amino acids	Frequency/ thousand ratio
M	2	ATG	2	13.9	**T**	11	ACT	2	13.9
W	0	TGG	0	0			ACA	1	6.9
C	0	TGT	0	0			ACC	5	34.9
		TGC	0	0			ACG	3	20.9
D	14	GAT	11	76.9	**P**	7	CCT	3	20.9
		GAC	3	20.9			CCA	3	20.9
E	9	GAG	4	27.9			CCC	0	0
		GAA	5	34.9			CCG	1	6.9
F	3	TTT	1	6.9	**V**	7	GTT	4	27.9
		TTC	2	13.9			GTA	2	13.9
H	5	CAT	4	27.9			GTC	0	0
		CAC	1	6.9			GTG	1	6.9
K	12	AAA	8	55.9	**L**	12	CTT	0	0
		AAG	4	27.9			CTA	1	6.9
N	3	AAT	2	13.9			CTC	0	0
		AAC	1	6.9			CTG	1	6.9
Q	6	CAA	3	20.9			TTA	4	27.9
		CAG	3	20.9			TTG	6	41.9
Y	5	TAT	2	13.9	**R**	6	CGT	2	13.9
		TAC	3	20.9			CGA	1	6.9
I	15	ATT	9	62.9			CGC	1	6.9
		ATA	4	27.9			CGG	0	0
		ATC	2	13.9			AGA	1	6.9
A	9	GCT	2	13.9			AGG	1	6.9
		GCA	4	27.9	**S**	7	TCT	2	13.9
		GCC	3	20.9			TCA	2	13.9
		GCG	0	0			TCC	0	0
G	10	GGT	2	13.9			TCG	2	13.9
		GGA	7	48.9			AGT	0	0
		GGC	0	0			AGC	1	6.9
		GGG	1	6.9					

Table 5-5. Comparison of the polypeptide sequence of the dinoflagellate clone SAM-1 to *Sam* from other organisms. A translated BLAST search was performed and a selection of the organisms producing the closest match are listed in taxanomic groups. Accession # = the reference number of the sequence in GenBank, Ident = the % of identities, Pos = the % of positives, Length = the number of amino acids in the alignment and Gaps = the % of gaps inserted into the query (dinoflagellate) sequency to align it with the subject sequence. If Gaps = 0, gaps may have been inserted into the subject sequence. Mosquito, *Anopheles gambiae*, *Sam* is highlighted because of the high similarity to SAM-1 and other dinflagellate clones such as SAM-21 to -25 (AMAD06) (87% Pos) and SAM-31 to -35 (ACCC01) (84% Pos).

Accession #	Species	Organism	Ident	Pos	Length	Gaps
ZP00307689	*Cytophaga hutchinsonii*	Bacteria	71	83	145	1
BAD46810	*Bacteroides fragilis*	Bacteria	63	78	155	7
CAG49861	*Staphylococcus aureus*	Bacteria	53	71	140	0
P54419	*Bacillus subtilis*	Bacteria	48	68	139	0
AAA66932	*Saccharomyces cerevisiae*	Fungi	48	66	143	2
EAA65815	*Asperigillus nidulans*	Fungi	49	62	146	2
UM05019	*Ustilago maydis*	Fungi	48	63	146	2
AAN31489	*Phytophthora infestans*	Fungi	44	64	146	2
AAG17035	*Pinus contorta*	Higher Plant	47	70	140	0
AAK29409	*Elaegnus umbellata*	Higher Plant	45	64	146	2
CAE72642	*Caenorhabditis briggsae*	Nematoda	51	65	146	2
EAA01957	*Anopheles gambiae*	Arthropoda	73	85	143	0
CAA54567	*Drosophila melanogaster*	Arthropoda	46	58	146	2
AAT06208	*Modiolus americanus*	Mollusca	46	65	146	2
AAT06195	*Asterina miniata*	Echinodermata	45	61	146	2
AAT06214	*Monosiga brevicollis*	Choanoflagellida	45	63	146	2
AAH43970	*Xenopus laevis*	Amphibia	48	61	146	2
XP421512	*Gallus gallus*	Birds	47	62	146	2
CAA48726	*Homo sapiens*	Mammalia	47	62	146	2
BAD06937	*Mus musculus*	Mammalia	47	62	146	2

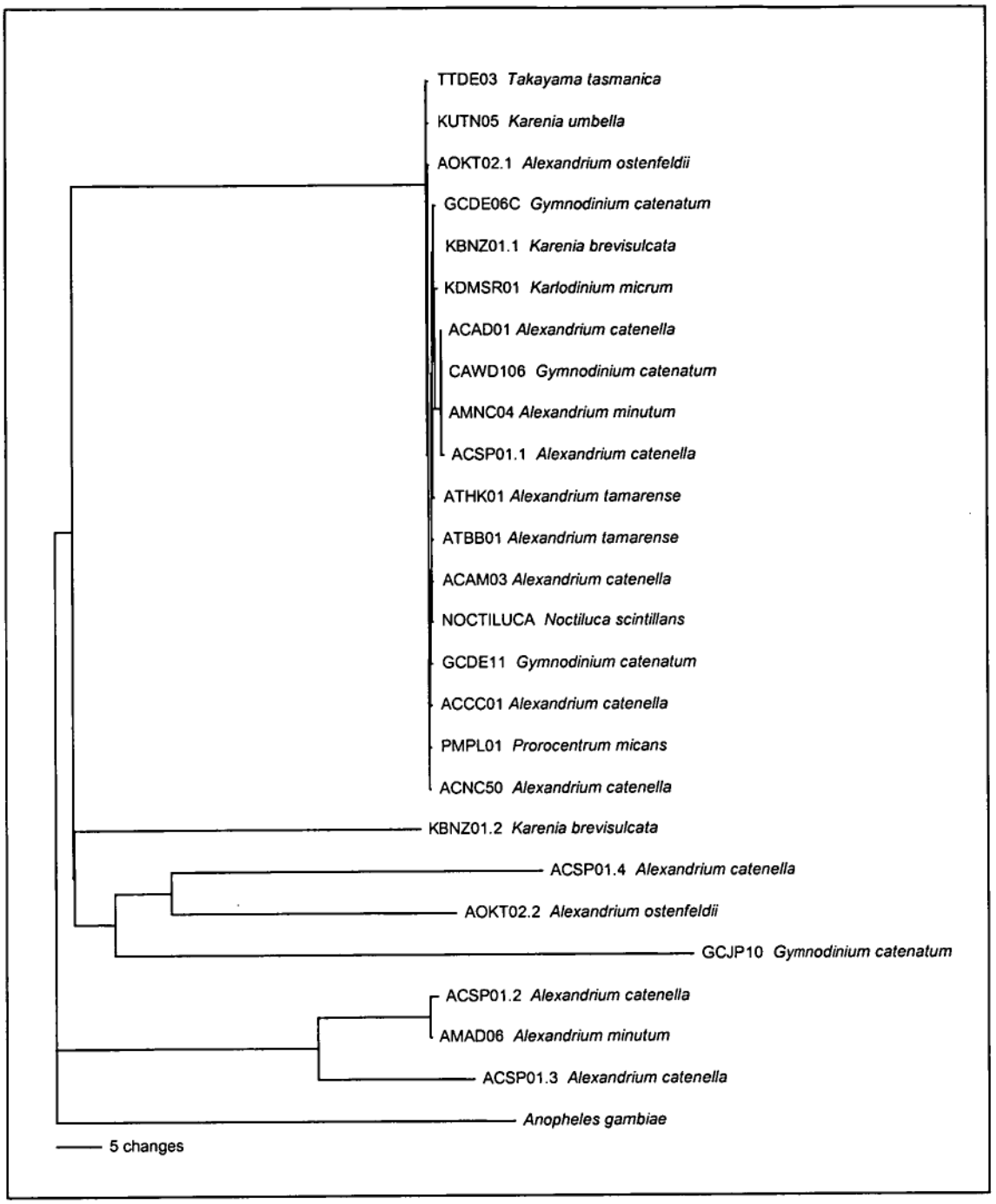

Figure 5-5. Consensus tree showing the similarity of dinoflagellate *Sam* nucleotide sequences based on the number of base differences for clones 1 to 25 listed in Table 5-3. Dinoflagellate clones are listed as the strain name followed by the species name. Strains for which multiple *Sam* genes were cloned are indicated with a period and the copy number. The outgroup is *Anopheleles gambiae Sam*.

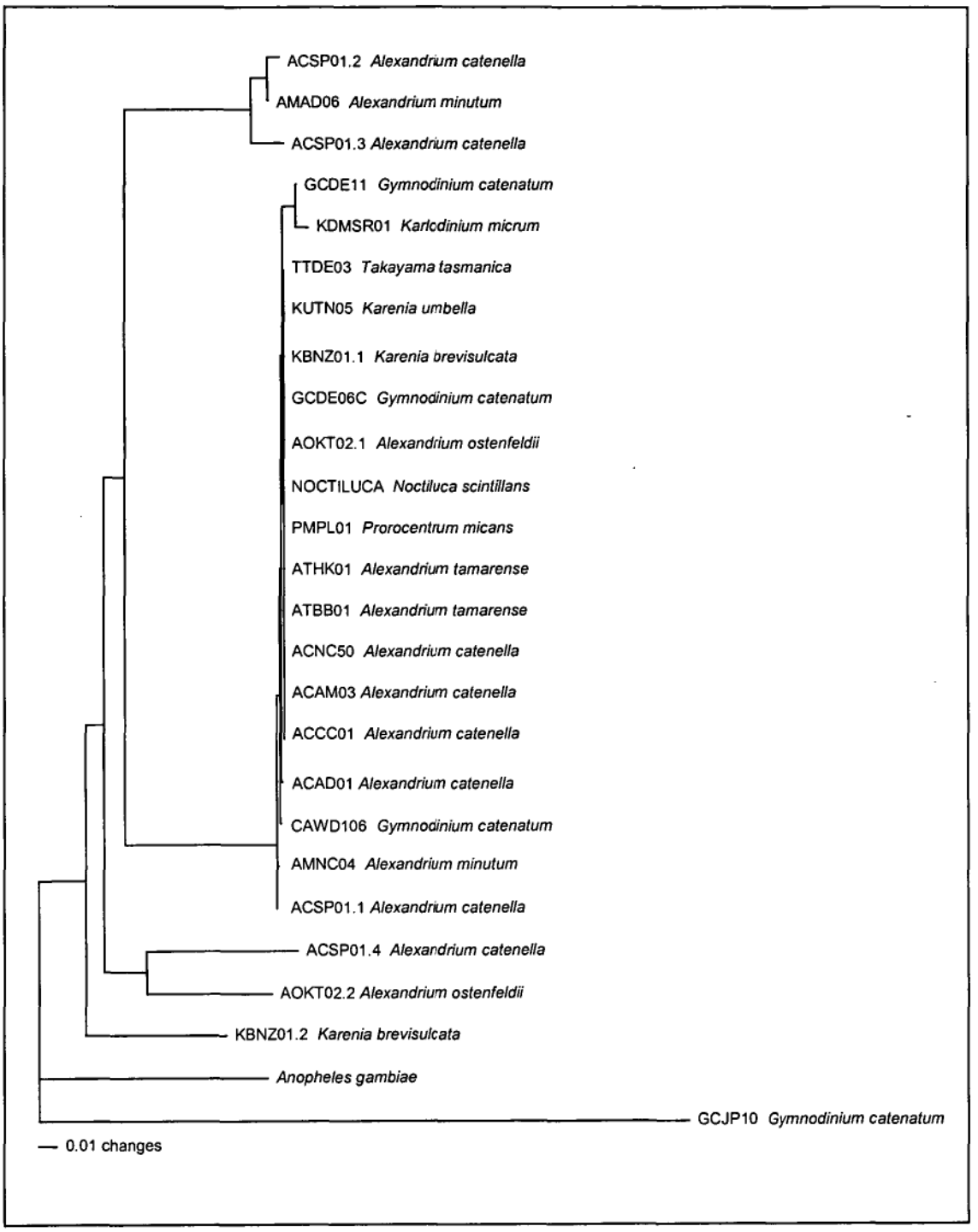

Figure 5-6. Consensus tree showing the similarity of dinoflagellate protein *Sam* sequences based on the number of amino acid residue differences for clones 1 to 25 listed in Table 5-3. Dinoflagellate clones are listed as the strain name followed by the species name. Strains for which multiple *Sam* genes were cloned are indicated with a period and the copy number. The outgroup is *Anopheleles gambiae Sam*.

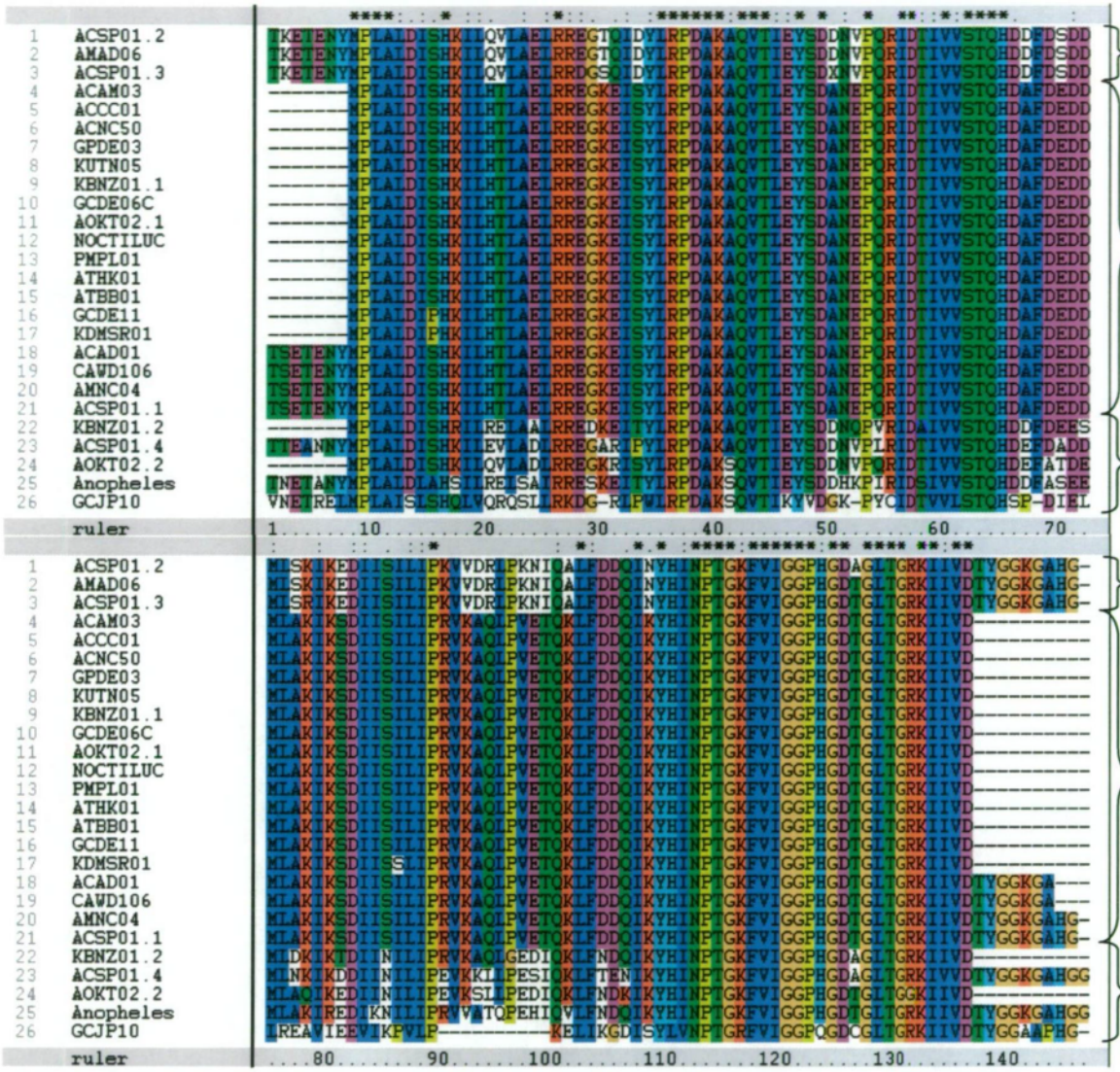

Figure 5-7. ClustalX protein sequence alignment for partial dinoflagellate *Sam* 1 to 25 listed in Table 5-3 (1 to 24 and 26 above) compared to *Anopheles gambiae Sam* (25 above). The numbering (left margin above) reflects the three groupings of sequences, by ClustalX, according to homology. The largest brackets indicate the most common copy of *Sam* identified in dinoflagellates. Asterisks denote amino acid residues that are identical in all sequences, colons denote amino acid residues that are identical except for one sequence and a period denotes amino acid residues that are identical except for two sequences. Default amino acid colours have been used.

5.3.2 Restriction enzyme digests and Southern and northern blots

Restriction enzymes did not appear to digest dinoflagellate *A. catenella* (CAWD44) gDNA effectively and subsequently the copy number of *Sam* could not be determined using this method. Instead, the presence of multiple copies of *Sam* was confirmed from sequence analysis. Visual inspection of

gDNA digest reactions by agarose gel electrophoresis revealed activity of only one enzyme on *A. catenella* DNA, *EcoR109*I (*Dra*II), which was known to have a single recognition site in clone SAM-1 at base 362. All other enzymes tested were not expected to restrict the SAM-1 sequence.

Results of the Southern blot confirmed the presence of a SAM gene(s) in CAWD44 which was not amplified by the degenerate SAM primers or dinoSAM-1F/-1R primers. The 430 bp insert of clone SAM-1 hybridised to RNA from the three species tested: *G. catenatum*, *A. catenella* and *A. minutum*, however intense labelling of the RNA ladder suggested non-specific labelling of the probe. Therefore, the results of the northern blot could not be interpreted with confidence. Southern blot analysis revealed poor restriction activity of *Dra*II as well as activity of *Sma*I that was not visible on the agarose gel. Unlike the RNA ladder, the probe did not hybridise to the DNA ladder (Fig. 5-8).

5.3.3 3′RACE and sequencing of cDNA clones

Clones derived from PCR using the primers dinoSAM-1F/-1R on cDNA produced a sequence identical to gDNA. Therefore, the fragment of *Sam* amplified did not contain an intron. Twenty clones produced from 3′RACE of AMAD06 cDNA using the forward primer 3′RACE-SAM-1F were sequenced, with 2 clones of 516 bp and 513 bp (SAM-135-6A and SAM-136-7A respectively) producing a match to known *Sam* sequences in GenBank. Twenty clones produced from 3′RACE of AMAD06 cDNA using the forward primer 3′RACE-SAM-2F were sequenced, with one 515 bp clone (SAM-158-9A) producing a match to known *Sam* sequences in GenBank. The 3′ terminal *Sam* clone AMAD06-136-7A was used for further primer design for quantitative real time PCR (QPCR) in Chapter 6 (Fig. 5-9).

Comparison of the closest matching sequences from a translated BLAST search (NCBI) of clone SAM-136-7A revealed no close match to representative organisms from any of the kingdoms (Table 5-6). The alignment retrieved was for the coding region of SAM-136-7A starting at base 14, a conserved region of the gene. The closest matches at the polypeptide

level from a BLAST search of GenBank was the bacterium *Clostridium perfringens*, the fungus *Cryptococcus neoformans* and the apicomplexan protozoan *Plasmodium falciparum*.

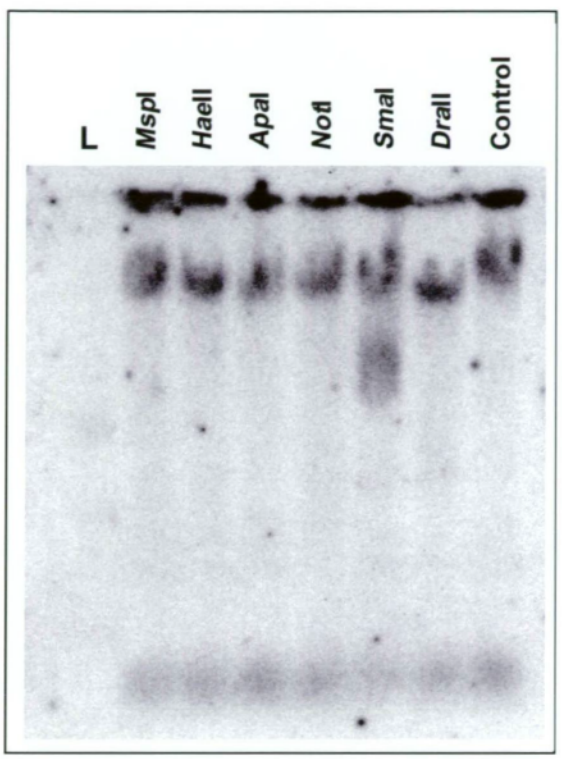

Figure 5-8. Southern blot analysis of *Alexandrium catenella* (CAWD44) probed with the [α-^{32}P]dCTP labeled *Sam* clone of *Gymnodinium catenatum* (CAWD106), SAM-1. Genomic DNA was digested with the following 6 restriction enzymes: *Msp*I, *Hae*II, *Apa*I, *Not*I, *Sma*I and *Dra*II. (L) DNA ladder and (Control) undigested gDNA.

A

GACTCGAGTCGACATCGGAGTGGCGAAGCCGCTCTCGCTCTTCGTCGAGACCTACGG
CACTGAGCAGGGCGCGCTCAGTGCGGAGGACATCACCAACGTCATCAAGATCGCGTT
CGACTGCCGTCCTGGTGCCATCGCCATGTCCTTGGCGCTCCGCGAGCCCAAATACCA
GGAGACCGCGGCGTACTGCCACTTCGGCCGCGAGGCCGTCACCAAGGACGGCAAGA
AGTTCTTCGAGTGGGAGAGTGCCAAGGACTTGTCGAAATACAAGTCCATGACCTCAGC
CCAGGTCACCGCGGAGCTCAAGGACAGCAACTACCTCACCAAGTGGGTGGATTGAGC
CTGTGCTTTGCCCGCGCCAAGGGCGGCCTGTGCAGTTCCAGAATCTCTCGTCTGGGG
CGTTGCATGTTTTTGTTTTTCCTGTTTTTCTCAGTCCGGTGTATATGCCACCTCAACTCA
GATCCCCATGTCCAGTTGGACTGGTGCGGTGAGGAGAGTGCGACTACGGACCGCAAA
AAAAAAAAAAAAAAA

B

TRVDIGVAKPLSLFVETYGTEQGALSAEDITNVIKIAFDCRPGAIAMSLALREPKYQETAAYC
HFGREAVTKDGKKFFEWESAKDLSKYKSMTSAQVTAELKDSNYLTKWVDStopACALPAP
RAACAVPESLVWGVACFCFSCFSQSGVYATSTQIPMSSWTGAVRRVRLRTAKKKKK

Figure 5-9. **A.** Nucleotide sequence (sense strand 5′-3′) for the 3′terminus of a *Sam* gene, clone SAM-136-7A (length 531 bp), obtained from *Alexandrium minutum* strain AMAD06. The reading frame is +2 such that the first codon is ACT, coding for the amino acid threonine (T). The stop codon TGA in bold is at bases 338-340. The 3′ untranslated region (3′UTR) including the stop codon is 176 bp in length, spanning bases 338-513. The structural region of the gene is shaded and the 3′UTR is unshaded. **B.** Polypeptide sequence for the SAM-136-7A clone in A, reading frame +2. The coding region (112 amino acids) is shaded and the 3′UTR is unshaded. The position of the stop codon is indicated in bold. The underlined sequence in A and B is the region of the gene that matches *Sam* in other organisms from a BLAST serach of GenBank.

Table 5-6. Comparison of the polypeptide sequence of the dinoflagellate clone SAM-136-7A to *Sam* from other organisms. A translated BLAST search was performed and the organisms producing the closest match are listed in taxanomic groupings. Accession # = the reference number of the sequence in GenBank, Ident = the % of identities, Pos = the % of positives, Length = the number of amino acids in the alignment and Gaps = the % of gaps inserted into the query (dinoflagellate) sequency to align it with the subject sequence.

Accession #	Species	Organism	Ident	Pos	Length	Gaps
BAB81883 NP_563093	*Clostridium perfringens*	Bacteria	58	73	63	0
ZP_00340788	*Rickettsia akari*	Bacteria	51	62	77	0
AAO34962 NP_781025	*Clostridium tetani*	Bacteria	44	56	88	0
ZP_00307689	*Cytophaga hutchinsonii*	Bacteria	42	54	99	13
AAO78325 NP_812131	*Bacteroides thetaiotaomicron*	Bacteria	43	55	100	13
ZP_00120745 AAN25569	*Bifidobacterium sp.*	Bacteria	42	56	92	0
AAQ66880 NP_905981	*Porophyromonas gingivalis*	Bacteria	56	64	64	0
AAW40933 XP_566752	*Cryptococcus neoformans*	Fungi	46	62	83	0
AAN31489	*Phytophthora infestans*	Fungi	45	62	82	0
EAK85879 XP_402634	*Ustilago maydis*	Fungi	45	59	83	0
AAG13449 NP_704761	*Plasmodium falciparum*	Apicomplexa	44	56	87	0
CAH88842	*Plasmodium chabaudi*	Apicomplexa	43	57	88	0
EAA18424	*Plasmodium yoelii*	Apicomplexa	42	57	88	0
CAH99282	*Plasmodium berghei*	Apicomplexa	40	57	88	0
CAA55794 Q95032	*Acanthamoeba castellanii*	Acanthamoeba	45	60	83	0
S51671	*Acanthamoeba castellanii*	Acanthamoeba	44	57	87	0
AAB38500 P93254	*Mesembryanthemum crystallinum*	Higher Plant	48	61	84	0
AAA33274 T10710	*Dianthus caryophyllus*	Higher Plant	47	64	84	2
BAC81655	*Pisum sativum*	Higher Plant	43	60	94	3
AAA81378 P50301	*Actinidia chinensis*	Higher Plant	48	64	85	3
AAR15895	*Nicotiana tabacum*	Higher Plant	48	62	85	3
CAA48726	*Homo sapiens*	Mammalia	43	57	82	0

5.3.4 Rubisco

A search of GenBank with sequences of cloned PCR products obtained using the *A. thaliana Sam* primers, revealed no match to *Sam*. All clones produced no significant similarity to sequences in GenBank except for clone AMAD06 SAM-175-16 which closely matched form II rubisco of the dinoflagellates *Heterocapsa triquetra* (94.2 % identical amino acids) (AY826897) (Patron *et al.* 2005) and *Prorocentrum minimum* (93.5 % identical amino acids) (AY169184) (Zhang & Lin 2003) at the protein level (Fig. 5-10). Comparison of the primer sequence for *A. thaliana sam-1* to the amplified rubisco sequence (1-851 bp) produced an exact match of the forward primer (site 1-25 bp) and some similarity to the reverse complement of the reverse primer (site 830-851 bp) (Fig. 5-11).

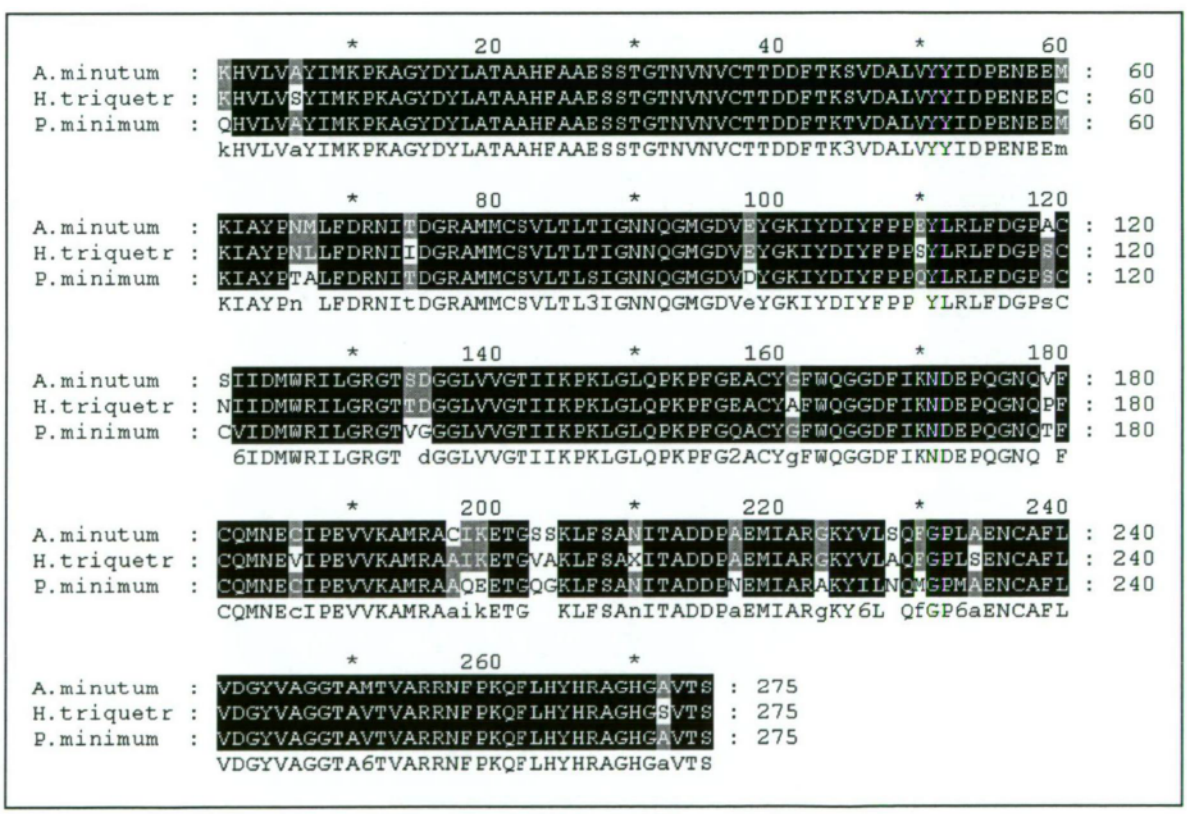

Figure 5-10. Protein sequence alignment for the complete N-terminus and partial large chain of the form II Rubisco gene produced using the program GeneDoc. Three sequences are compared, *Alexandrium minutum* (AMAD06) clone 'SAM-175-16', *Heterocapsa triquetra* (AY826897) and *Prorocentrum minimum* (AY169184) obtained from GenBank. Black shading indicates residues that are conserved in all sequences, grey shading indicates residues that are conserved in 2 sequences and no shading indicates that the residue differs in all 3 sequences.

CCAGTATGGACGATTCAAGGCTTGCAAGCACGTGCTCGTGGCCTACATCATGAAGCC
CAAGGCCGGCTACGACTACCTCGCGACGGCGGCGCACTTCGCCGCTGAGTCCTCCA
CCGGCACCAACGTGAATGTCTGCACCACCGATGACTTCACCAAGTCCGTGGACGCCT
TGGTCTACTACATCGATCCCGAGAACGAGGAGATGAAGATCGCCTACCCCAACATGCT
CTTCGACCGCAACATCACGGACGGCCGCGCTATGATGTGCTCTGTCCTCACCCTCAC
CATCGGCAACAACCAGGGCATGGGCGATGTGGAGTACGGCAAGATCTACGACATCTA
CTTCCCCCCGGAGTACTTGCGGCTCTTCGACGGCCCCGCCTGCAGCATCATCGACAT
GTGGCGCATCTTGGGCCGCGGCACATCTGACGGTGGCCTCGTCGTCGGCACCATCAT
CAAGCCCAAGCTCGGCTTGCAGCCCAAGCCCTTCGGCGAGGCTTGCTACGGCTTCTG
GCAGGGCGGTGACTTCATCAAGAACGACGAGCCCCAGGGCAACCAGGTCTTCTGCCA
GATGAACGAGTGCATCCCGGAGGTGGTCAAGGCCATGCGCGCTTGCATCAAGGAGAC
GGGCAGCTCCAAGCTCTTCTCGGCGAACATCACTGCCGACGACCCCGCCGAGATGAT
CGCTCGTGGTAAGTATGTCCTCTCGCAGTTTGGGCCTCTCGCGGAGAATTGCGCCTTC
CTCGTCGACGGCTATGTCGCCGGCGGCACTGCCATGACTGTGGCCAGGAGGAACTTC
CCCAAGCAGTTCCTCCACTACCACCGCGCG**GGTCACGGCGCCGTGACGAGCC**

Figure 5-11. Nucleotide sequence of AMAD06 clone SAM-175-16 which spans the complete N-terminus and partial large chain of the form II rubisco gene. Underlining indicates the primer binding sites for the forward primer Athal-SAM1-F (site 1-20 bp) and the reverse primer Athal-SAM1-R (site 830-851 bp) designed to *A. thaliana sam-1*. Nucleic acids in bold font indicate an exact match to the primer.

5.4 Discussion

5.4.1 Identification and sequence analysis of *Sam* in dinoflagellates

Comparison of the sequence of cloned PCR products from gDNA and cDNA revealed that they were the same gene, confirming a eukaryotic dinoflagellate origin and not derived from a contaminating prokaryote such as bacteria. Similarly, Snyder *et al.* (2003) provided evidence to support a dinoflagellate origin of the polyketide synthase (PKS) gene by showing that the same gene was amplified from polyadenylated RNA, the lack of PKS expression in light-deprived cultures, residual phylogenetic signals, resistance to methylation-sensitive restriction enzymes, and the lack of hybridisation to bacterial isolates.

The resistance of *Karenia brevis* (dinoflagellate) DNA to methylation-sensitive restriction enzymes (Snyder *et al.* 2003) lends support to the findings in the present work of poor restriction enzyme activity on *A. catenella* gDNA. It is likely that *A. catenella* gDNA, like the DNA of the dinoflagellates *Prorocentrum micans* (Herzog & Soyer 1982, Soyer-Gobillard *et al.* 1999),

Amphidinium carterae, Crypthecodinium cohnii, Peridinium triquetrum and *Symbiodinium microadriaticum* (Rae & Steele 1978, Steele & Rae 1980), has a high incidence of thymine substitution for hydroxymethyluracil. Therefore, it is possible that restriction enzymes which recognise a target sequence containing thymine, or are sensitive to methylation, would not be effective on dinoflagellate DNA. Additionally, the potentially complex secondary structure of dinoflagellate DNA; which is permanently condensed, usually present in high amounts per cell (Guillebault *et al.* 2001) and believed to be in a cholesteric liquid crystalline phase inside the chromosome core (Livolant & Bouligand 1980), may result in target restriction sites being inaccessible to some restriction enzymes.

Dinoflagellate DNA has been reported to be inhibitory to many restriction enzymes (Lee *et al.* 1993, Snyder *et al.* 2003). Snyder *et al.* (2003) tested 10 enzymes for restriction activity on dinoflagellate DNA, in particular to *Karenia brevis* and *P. lima*. Similar to this chapter, none of the enzymes, *EcoR*I, *BamH*, *Xho*I, *Kpn*I, *Pst*I or *Hind*III, all of which contain adenine or thymine in their recognition sequence, were effective. These enzymes would be expected to be inhibited by the sustitution of thymine by hydroxymethyluracil. Methylation sensitive enzymes can also be inhibited by dinoflagellate DNA. Snyder *et al.* (2003) reported that *Apa*I, *Msp*I and *Hae*II, enzymes insensitive to internal methylation, were effective on *P. lima* DNA, however in the present study, ApaI and MspI did not cut *A. catenella* DNA. The restriction site for *EcoR109*I (*Dra*II) was known to be present in clone SAM-1 (G^GACC) but hybridisation of SAM-1 to *A. catenella* gDNA revealed poor restriction activity. DraII is blocked by methylation of the internal cytosine at position C-5 (G^GA**C**C) (Schnetz & Rak 1988), which possibly occurs in dinoflagellate DNA.

Although a common copy of *Sam* was identified in the majoity of dinoflagellate strains analysed, including five *A. catenella* strains, the same copy was not detected in the *A. catenella* strain CAWD44. Potentially this copy may not be present in the genome of CAWD44. An alternative, more likely scenario, is that there is sequence variation at the primer target site.

Sequence variation for partial fragments of *Sam* amplified from individual strains (e.g. ACSP01, KBNZ01 and AOKT02), showed that multiple copies of *Sam* are present in the genomes of dinoflagellates and that genetic variation exists between species and strains.

The most commonly amplified copy of dinoflagellate *Sam* showed homology at the protein level to arthropod *Sam* from *Anopheles gambiae* (Diptera) and hence this sequence was used as an outgroup for construction of distance trees. As discussed in Chapter 4, the dinoflagellates are suggested to have a common ancestor with the Apicomplexa and ciliates (Alveolata) (Saunders *et al.* 1997). A close match to these groups was anticipated; however, information for *Sam* for these groups in GenBank was limited. A match in GenBank at the protein level to the Apicomplexa was obtained for the 3′ terminal clone SAM136-7A. However, this clone equally matched bacteria and fungi *Sam* (Fig. 5-6). The cloned copy of *Sam* from the Japanese isolate of *G. catenatum*, GCJP10, was different to all other dinoflagellate strains. Surprisingly, at the protein level, GCJP10 was more distinct from dinoflagellate *Sam* than the arthropod *A. gambiae* was from dinoflagellate *Sam*. It is possible that a different *Sam* was isolated from GCJP10 that was not detected in the other dinoflagellates. A different outgroup *Sam* would need to be identified to interpret the GCJP10 *Sam* data.

Despite the lack of a PCR product from *A. catenella* CAWD44 gDNA and cDNA using degenerate *Sam* primers, and primers specific to the *G. catenatum* CAWD106 clone SAM-1, the SAM-1 probe showed cross reactivity to CAWD44 in Southern and northern blots (Fig. 5-8). Therefore, at least one copy of *Sam* was shown to be present in *A. catenella* strain CAWD44. The amplification of a copy of *Sam* from CAWD44 using 3′RACE provided evidence to support the presence of multiple copies of *Sam*. Whether this gene was the 3′ terminus of the same *Sam* gene amplified in other dinoflagellates remains to be determined. In future studies it would be useful to attempt 5′RACE to obtain the 5′ end of dinflagellate *Sam*.

Isolation of additional SAM synthetase genes in microalgae will allow the expression of other copies to be studied (Chapter 6) and determine whether both (or more) genes are biologically equivalent or whether they have specialised functions. For example, *sam*-1 was isolated from the higher plant *A. thaliana* but genomic blot analysis indicated the presence of a second *Sam* gene, *sam*-2, a situation similar to that in yeast (Thomas *et al.* 1988, Peleman *et al.* 1989). Therefore, in *A. thaliana*, *sam*-1 is a member of a gene family consisting of two different copies per haploid *Arabidopsis* genome (Peleman *et al.* 1989). The large amount of chromosomal DNA in dinoflagellates suggests polyploidy and therefore genes will be represented multiple times. Recent characterisation of dinoflagellate genes (Okamoto *et al.* 2001, Lin *et al.* 2002, Zhang & Lin 2002, Bertomeu & Morse 2004) has provided evidence that many genes are often tandemly repeated and display a high frequency of single nucleotide polymorphisms which supports the contention that many genes are present in high copy numbers. A significant frequency of single nucleotide polymorphisms in the third codon position that do not change the translation has been observation in the cDNA library analysis of *Lingulodinium* and *Amphidinium* (Bacharoff, 2004) and *K. brevis* (Lidie *et al* 2005).

A likely scenario in this study is that the degenerate *Sam* primers were amplifying similar ORFs of the one gene and/or duplicate *Sam* in the dinoflagellate genome. Sequencing 5 clones per strain may not have been sufficient, in some cases, to detect all *Sam* copies that were amplified in PCR (Appendix 4). Future study should involve more extensive sequencing for strains such as ACSP01 where four different copies of *Sam* were detected from sequence analysis of five clones.

5.4.2 Identification of novel dinoflagellate genes

Primers for *Sam* in *A. thaliana* did not amplify *Sam* in dinoflagellates despite their successful use by Belbahri *et al.* (2000) in tobacco. Instead, the form II rubisco gene was amplified. Not surprisingly, comparison of the primer sequence to the start and end of *A. catenella* form II rubisco revealed a high degree of homology despite the primers being designed to *Sam*. The

different codon usage of dinoflagellates (kingdom Protista) compared to *A. thaliana* and organisms from other kingdoms (Chapter 4), combined with the likely repetition of the coding DNA of the form II rubisco gene (repeated 148 +/- 16 times in the genome of *P. minimum*) (Zhang & Lin 2003) may account for the accidental amplification of rubisco. Other dinoflagellate genes have also been reported to be present in high copy numbers. For example, a gene for a luciferin-binding protein in *Gonyaulax polyedra* is present as ~1000 copies per cell (Lee *et al.* 1993). It is possible that *Sam* is also highly repeated in the dinoflagellate genome and this may explain the amplification of multiple different copies of *Sam* from some dinoflagellate strains.

The distinct codon usage of dinoflagellates was evident by the lack of a match of the clones SAM-1 and SAM136-7A to *Sam* in GenBank at the nucleotide level. However, at the protein level, the clones showed homology to *Sam* from a range of different organisms (Table 5-5). Without sequence information for the target gene in a closely related organism, possibly even at the species level, identification of dinoflagellate genes is problematic (Chapter 4). For instance, the current study found that despite primers being designed to a dinoflagellate sequence, they did not consistently detect *Sam* in other dinoflagellate species and strains.

The preliminary codon usage information for *G. catenatum* clone SAM-1 provides important insight for future primer design to target dinoflagellate genes. Further sequencing of chromosomal genes, such as *Sam*, will advance current knowledge of dinoflagellate codon usage which is limited. Such knowledge will facilitate the discovery of genes in the biosynthetic pathway of PST production. This chapter demonstrates that dinoflagellate genes can be identified in non-axenic cultures through confirmation of the nucleotide sequence in eukaryotic cDNA compared to gDNA.

Chapter 6: Expression of putative toxin genes during the cell division cycle of *Alexandrium catenella*

6.1 INTRODUCTION

6.1.1 Dinoflagellate cell division cycle, growth and toxicity

The biological function of Paralytic Shellfish poisoning Toxins (PSTs) in dinoflagellates is unknown. Little is known about the regulation of PST biosynthesis, and no genes in the pathway have been definitively identified (Taroncher-Oldenburg & Anderson 2000). Biosynthetic pathways can be regulated at many different levels. For instance, expression of a specific enzyme may be controlled through transcription or translation events that in turn may be influenced by environment. It is generally accepted that in the dinoflagellate genus *Alexandrium* Halim, endocellular PST content varies with the growth phase (e.g. exponential versus stationary) (Marsot 1997, Parkhill & Cembella 1999) and the cell division cycle (CDC) phase (Kim *et al.* 1993, Taroncher-Oldenburg *et al.* 1997). However, the influence of environmental factors (light, temperature, salinity and nutrients) on *cell toxin quota* (Q_t) is less clear (Kim *et al.* 1993, Siu *et al.* 1997, Parkhill & Cembella 1999, Hwang & Lu 2000, Grzebyk *et al.* 2003) (see 1.1 and 1.5). Although it has been well documented that toxicity of cultured *Alexandrium* cells is typically less than those in natural populations (Cembella *et al.* 1988, Marsot 1997), the environmental control is unknown.

A study of *A. tamarense* (Lebour) Balech by Parkhill & Cembella (1999) indicated that Q_t was independent of exogenous environmental factors (light, salinity, inorganic nitrogen) throughout the exponential growth phase. While a role of nitrogen and phosphorus in cellular PST cycling has been suggested (John & Flynn 2000), it is likely that PST biosynthesis is not directly regulated by phosphate (Taroncher-Oldenburg *et al.* 1999). Rather, studies have suggested that a deficiency of phosphate indirectly affects Q_t through other cellular process, such as protein and amino acid metabolism (Anderson *et al.* 1990, John & Flynn 2000). However, unlike phosphorus, studies have shown that a source of nitrogen is required for PST synthesis

(Flynn *et al.* 1996) but this can be recycled within the cell, rather than solely from inorganic nitrogen taken into the cell (Anderson *et al.* 1990, John & Flynn 2002).

The Q_t may vary substantially within a strain and represents an equilibrium between the rate of anabolism, catabolism, leakage/excretion, cell growth and division (Cembella 1998, John & Flynn 2002). The relationship between growth rate and Q_t is poorly defined and has been reported to be both a non-definitive relationship (Ogata *et al.* 1987) or an inverse relationship (Proctor *et al.* 1975). Analysis of dinoflagellate toxicity during the cell cycle is complicated by the diurnal cycle. For instance, Taroncher-Oldenburg *et al.* (1999) report that toxin production by *Alexandrium fundyense* was discontinuous over the CDC and coincided with light. Either toxin production was specific to the cell cycle stage, was activated by light, or, all three events were linked.

The typical eukaryotic CDC is divided into four stages, initiated with mitotic division (M) (cytokinesis), followed by three stages of interphase, G_1, S and G_2. In the first stage, G_1, cells have one copy of the genome and are metabolically very active. When cells enter S phase they begin to actively synthesise DNA. This phase extends until chromosome replication occurs and the DNA content of the cell has doubled. Finally, cells enter G_2 which lasts until M (Fig. 6-1) (Yeung & Wong 2003).

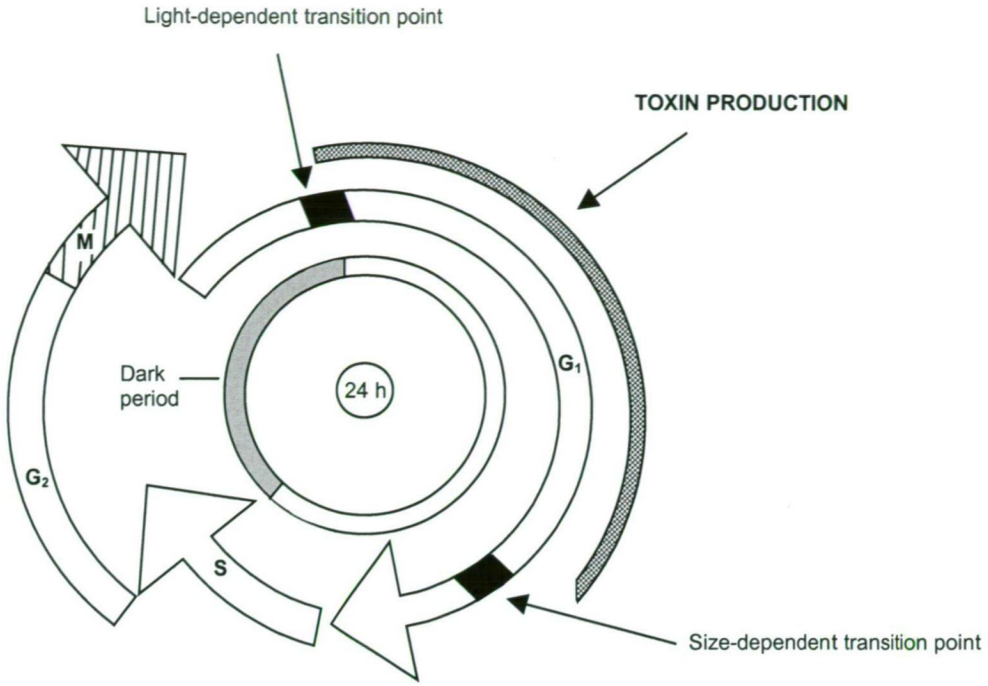

Figure 6-1. Schematic representation of cell cycle events in *Alexandrium fundyense* cells during synchronous growth on a 14:10 light:dark cycle. Open bar – light phase; G_1 – gap 1 (postmitotic); S – DNA synthesis phase; G_2 – gap 2 (premitotic); M – mitosis (after Taroncher-Oldenburg *et al.* 1997).

6.1.2 *Alexandrium catenella*, CAWD44

Alexandrium catenella Whedon & Kofoid (Balech) was the first dinoflagellate to be linked to PSP (Sommer & Meyer 1937). Members of the genus *Alexandrium* are gonyaulacoid dinoflagellates. All are marine, thecate and photosynthetic and the genus contains more neurotoxic members than any other harmful algal genus, although some are non- or weakly-toxic (Taylor & Fukuyo 1998). This study chose *A. catenella* strain CAWD44 (Cawthron Institute, Nelson, NZ) because of its relatively fast exponential growth phase in laboratory culture (0.65 divisions per day at 20°C) (de Salas 1999) and its known production of PSTs (MacKenzie *et al.* 2004). The genus *Alexandrium* was favoured over another known PST producer, *G. catenatum,* because it is more amenable to bulk culture and molecular techniques.

The CAWD44 strain was isolated from Tauranga, New Zealand by L. MacKenzie in 1997 and maintained in the Cawthron Culture Collection of

Microalgae. The strain was kindly donated to the Microalgal Culture Collection at the School of Plant Science, University of Tasmania, Australia where it is maintained as a uni-algal, clonal but non-axenic culture. CAWD44 has a Japanese subribotype (de Salas 1999) within the temperate Asian ribotype (as described by Scholin & Anderson 1996). Ribosomal sequence information from this strain and others indicate a common origin of New Zealand, Australian and Japanese strains of *A. catenella* (Scholin & Anderson 1996).

It has been well documented that dinoflagellates undergo diel vertical migration in the water column, migrating up to the surface during the day and down during the night (van den Hoek *et al.* 1997, Kamykowski *et al.* 1998). This ability of dinoflagellates to exhibit behavioural "swimming" results in an uneven distribution of cells in laboratory cultures. In order to gain an accurate reflection of the density of cells in culture, it is essential that the cells be equally distributed within the culture. Samples must therefore be mixed before a sub-sample is taken for cell counting.

Preliminary studies showed that aeration and regular gentle mixing do not affect the health of *A. catenella* and *Alexandrium minutum* Halim cultures, whilst the impact of agitation on *G. catenatum* cultures was severe. Although *A. catenella* and *G. catenatum* are both chain forming species, the cells of *G. catenatum* differ because adjacent cells are connected via the cytoplasm (Rees & Hallegraeff 1991). When cells are agitated, these cytoplasmic connections break causing damage to the cells and making this species less amenable to bulk culture. The cell division apparatus may also be damaged by turbulence (Yeung & Wong 2003).

6.1.3 Candidate cell cycle and/or toxin related genes

The PST profile of dinoflagellates is claimed to have a genetic basis (Sako *et al.* 1992, Kim *et al.* 1993, Sako *et al.* 1995). Sako *et al.* (1992) crossed *A. catenella* strains with different toxin compositions and analysed toxin composition and mating type of F1 progenies. Results showed toxicity to be inherited in a Mendelian fashion. Additionally, studies by Taroncher-

Oldenburg *et al.* (1997, 1999) demonstrated a link between the CDC and PST production in the dinoflagellate *Alexandrium fundyense* Balech. Further work identified three genes potentially linked to the CDC and/or PST biosynthesis in *A. fundyense*: *Haf* encoding a histone like protein (HIS), *Map* encoding a methionine aminopeptidase like protein (MAP) and *Sahh* encoding the protein S-adenosylhomocysteine hydrolase (SAHH) (Taroncher-Oldenburg & Anderson 2000). These genes showed differential expression during the cell cycle phases of synchronised cultures, as identified by analysing radiolabelled PCR products on denaturing polyacrylamide gels. The gene *Sahh* was down-regulated, while *Map* and *Haf* were up-regulated during the early G_1 phase and toxin production.

6.1.4 S-adenosylhomocysteine hydrolase (SAHH)

A side product of S-adenosylmethionine (SAM) mediated methylation reactions is S-adenosylhomocysteine (SAH). The enzyme S-adenosylhomocysteine hydrolase (SAHH), ecoded by *Sahh*, catalyses the reversible cleavage of SAH into adenosine and homocysteine (Caggese *et al.* 1997). Thus SAHH has a key role in the regulation of the SAM pathway and will affect PST production in dinoflagellates (see 4.1.4). However, the activity of SAHH will affect all methyl-dependent pathways in cells, not only the methylation steps in saxitoxin (STX) biosynthesis. SAHH is an essential enzyme and is highly conserved, with up to 80% sequence homology throughout all kingdoms (Tehlivets *et al.* 2004).

6.1.5 Methionine aminopeptidase (MAP)

Methionine aminopeptidase (MAP) is an enzyme that regulates the translation of many proteins and enzyme activities and is essential for the viability of an organism. Genes encoding MAP have been shown to be essential for cell growth in *Drosophila* (Cutforth & Gaul 1999) and *Escherichia coli* (Chang *et al.* 1989). The importance of MAP to the metabolism of a cell means that it is likely to be a key enzyme in regulating toxin biosynthesis via complex interactions (Taroncher-Oldenburg & Anderson 2000).

6.1.6 Histone like protein (HIS)

Although typical nucleosome structures and histone proteins are lacking in dinoflagellate nuclei, small basic histone-like proteins (HISs) have been reported in the dinoflagellates: *Lingulodinium polyedrum* Stein (Chudnovsky *et al.* 2002), *A. fundyense* (Taroncher-Oldenburg & Anderson 2000) and *Crypthecodinium cohnii* Biechler (Wong *et al.* 2003). The function(s) of these proteins are unknown. Sequence alignment of the chromosomal HISs of *C. cohnii* revealed significant similarity to the bacterial DNA-binding proteins and the eukaryotic histone H1 proteins. Phylogenetic analysis supported that these HISs may have originated from the HISs of bacteria (Wong *et al.* 2003) but no clear function has been defined (Sala-Rovira *et al.* 1991). Rather than having a structural or stabilising function they may be involved in transcriptional regulation or initiation.

The partial gene fragment of 576 bp, encoding a histone like protein (*Haf*), identified by Taroncher-Oldenburg & Anderson (2000) in *A. fundyense* showed a high similarity to histone genes. Study of HISs in *C. cohnii* (Sala-Rovira *et al.* 1991) and *A. fundyense* (Taroncher-Oldenburg & Anderson 2000) suggest a differential or cyclic de novo synthesis, similar to the cell cycle-dependent expression shown for histones in plants (Callard & Mazzolini 1997).

6.1.7 Aims

This study aimed to reproduce and extend the cell cycle experiments by Taroncher-Oldenburg & Anderson (2000) for the dinoflagellate *A. catenella* strain CAWD44. Instead of differential display, the latest technology of quantitative real time PCR (QPCR) was used to study gene expression. Three candidate toxin-related genes were selected: *Sahh* and *Map* identified by Taroncher-Oldenburg & Anderson (2000) and the novel dinoflagellate gene S-adenosylmethionine synthetase (*Sam)* (Chapters 4 and 5). A fourth gene, *Haf*, was chosen as a likely cell cycle-related gene to act as a comparison to *Sahh*, *Map* and *Sam* expression. *Haf* was not identified in *A. catenella* and therefore not pursued further (Appendix 5). Ubiquitin (*Ubi*) was

used as an internal control (Albrecht *et al.* 1998) to display a uniform expression over the CDC.

The main aim was to examine toxin production and the activity of *Sahh*, *Map* and *Sam* concurrently over the different phases of the CDC, and assess whether a correlation existed. Toxin production has frequently been misinterpreted as the Q_t (toxin.cell^{-1}), rather than as the net rate of toxin production (the biosynthetic rate minus the various loss terms) (Cembella 1998). Changes in the *net quantity of toxin per cell per unit time* (R_{TOX}) and the *specific rate of toxin production* (μ_{TOX}) (developed by Anderson *et al.* 1990) can be calculated even when the various loss terms are unknown. The introduction of these rate variables has improved the interpretation of times and conditions of toxin production and the effect of growth rate.

6.2 Materials and Methods

6.2.1 Primer design for quantitative real time PCR (QPCR)

6.2.1.1 S-adenosylmethionine synthetase gene (Sam)

To study *Sam* expression in *A. catenella* CAWD44, multiple primer pairs suitable for QPCR were designed and tested (Table 6-1). Four approaches were taken. Firstly, three sets of primers were designed to *G. catenatum* CAWD106 clone SAM-1, which was the *Sam* clone type most common in the dinoflagellate strains sequenced (refer to 5.3.1, Fig. 6-2). To predict potential intron-exon boundaries and therefore splice sites for the SAM-1 nucleotide sequence, the program Net Plant Gene Server located at: http://www.cbs.dtu.dtu.dk/services/NetPGene was used. The first primer pair, F1-/R1-QPCR-SAM (Table 6-1), was designed using the computer program Primer3 located at: http://www.broad.mit.edu/cgi_bin/primer/primer3 following the guidelines for QPCR. The expected amplicon length was 142 bp. Additionally, two sets of primers, F2-/R3-QPCR-SAM and F3-/R2-QPCR-SAM (Table 6-1) were designed visually without using a computer program, which were expected to amplify fragments of 165 bp and 177 bp respectively (Fig. 6-2). Test PCR was performed using a standard PCR

Master Mix (Cat. Nº. M7502, Promega) and Platinum SYBR Green qPCR SuperMix-UDG (Cat. Nº 11733-038, Invitrogen). Plasmid DNA for clone SAM-1, diluted to 1 ng.μl^{-1}, was used as a positive control. The amplification conditions were as follows: 1 cycle of 95 °C for 1 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, and one final cycle of 72 °C for 5 min.

Secondly, a primer pair F4/R4-QPCR-SAM2 (Table 6-1) was designed to identical regions of the *Alexandrium* clones: ACSP01 SAM-17 (Fig. 6-3) and AMAD06 SAM-21 to SAM-25 (Fig. 6-4). These clones were selected because they were different to SAM-1. The expected amplicon length was 206 bp. Test PCR was performed alongside primers F4-/R4-Cterm-SAM discussed below.

Thirdly, degenerate primers (F4-/R4-Cterm-SAM) (Table 6-1) were designed to the C-terminus of *Sam*. The C-terminus of *Sam* is highly conserved as indicated by alignment of GeneBank sequences for *Escherischia coli* (bacteria), *Saccharomyces* (bacteria), *Staphylococcus* (bacteria), *Neurospora* (fungi), *Caenorhabditis* (nematode), *Drosophila* (insect), *Rattus* (mammal) and *Pisum sativum* (higher plant) (CD: pfam02773.11 S-AdoMet_Synt_C structure PSSM-Id:23370) (Fig. 6-5). The same is true for dinoflagellate *Sam*, which is identical to a partial fragment of *Anopeheles gambiae* (insect) *Sam* C-terminus at the protein level (Chapter 5, Fig. 6-6). Primers F4-/R4-Cterm-SAM were designed to the most conserved region produced by the GeneBank multi-sequence alignment. The program CODEHOP was used to determine suitable degenerate primers. Primer sequences were subsequently modified to concur with the codon usage of *Alexandrium* clones AMAD06 SAM-21 to SAM-25 and ACSP01 SAM-17 for the corresponding residues. This meant a lower GC content. The expected product size was 195 bp. Test PCRs were performed on CTAB extracted gDNA and cDNA using a standard PCR Master Mix (Cat. N° M7502, Promega) and the following amplification conditions: one cycle of 95°C for 5 min followed by 35 cycles at 94°C for 30 s, gradient of 45-55°C for 1 min, and 72°C for 1 min, with a final cycle of 72°C for 10 min.

Table 6-1. Primer sequences for four known dinoflagellate genes: *Sam*, *Sahh*, *Haf* and *Map*. The primer pairs successfully used in QPCR for *Alexandrium catenella* CAWD44 are shaded.

Name	Sequence 5' → 3'	Length (bp)
Sam		
F1-QPCR-SAM	GGCTCATTGGCATCTGAATA	20
R1-QPCR-SAM	ACTACATGCCGTTGGCTTTA	20
F2-QPCR-SAM	CGAGAACTACATGCCGTTGG	20
R3-QPCR-SAM	ATCGTATCTATACGCTGTGG	20
F3-QPCR-SAM	ACCAGACGCCAAAGCACAGG	20
R2-QPCR-SAM	AATTGTGCCTTTACCCTAGG	20
F4-Cterm-SAM	GGTCTTAAAATCATYGTGGATCANTAYGG	29
R4-Cterm-SAM	GGCTCGGCCACTCCDATNGCRTA	23
F4-QPCR-SAM2	GGCAGAACTTAGAAGAGAGG	20
R4-QPCR-SAM2	CAACCTTTGGAATCAGAATGG	21
SAM-3'136-1F	GACCTACGGCACTGAGCAGG	20
SAM-3'136-1R	GGCCGAAGTGGCAGTACGCC	20
SAM-3'136-2F	CGCTCACCGCTGATGACATCACC	23
SAM-3'136-2R	GCCGCAGTTTCCTGGTACTTGG	22
Ubi		
UBI-F	ATGCAGATYTTTGTGAAGAC	20
UBI-R	ACCACCACGRAGACGGAG	18
UBI-Gcat-R	ACCACCACGCAGACGGAG	18
Sahh		
Sahh-F1	CTATGGCGATGTCGGCAAGG	20
Sahh-R1	ATCCACCTCCCCAACTACGG	20
Haf		
Haf-F1	AGGTTCTGCCCGAGCTCCGG	20
Haf-R1	CATGCCAGGCCACTCTCAGG	20
Map		
Map-F1	GATGTCGCTGAGCGCGAAGG	20
Map-R1	TGGCTCCAGGGTGAAGGTGG	20

GACCAGCGAGACCGAGAACTACATGCCGTTGGCTTTAGATATATCGCACAAAATTTTGC
ATACTTTGGCAGAACTGCGTCGCGAAGGAAAAGAAATTTCTTATTTAAGACCAGACGCC
AAAGCACAGGTGACTTTAGAATATTCAGATGCCAATGAGCCACAGCGTATAGATACGAT
AGTAGTTTCTACACAGCATGATGCTTTTGACGAAGATGATGATAAAATGTTGGCAAAGAT
TAAGTCGGACATTATTTCAATTCTAATCCCTAGGGTAAAGGCACAATTGCCTGTTGAGAC
CCAAAAATTGTTCGATGATCAAATTAAGTACCATATCAATCCAACGGGAAAATTCGTTAT
TGGAGGACCTCATGGAGATACCGGATTAACGGGGCGAAAAATAATTGTTGATACCTACG
GAGGTAAAGGTGCC

Figure 6-2. Nucleotide sequence for the *Sam* gene, clone SAM-1 (length 430 bp), obtained from *Gymnodinium catenatum* strain CAWD106 (refer to Chapter 5). Primer binding sites are underlined and correspond to the following positions in order: F2-QPCR-SAM = bases 13-32, F3-QPCR-SAM = bases 109-128, R3-QPCR-SAM = bases 158-177, R2-QPCR-SAM = bases 266-285. The positions of primers F1-QPCR-SAM = bases 140-159 and R1-QPCR-SAM = bases 18-37 are shaded.

A

AACAAAGGAGACTGAAAATTATATGCCTTTGGCGCTTGATATTTCCCACAAGATCCTTCA
AGTCTTGGCAGAACTTAGAAGAGAGGGCACGCAAATAGATTATTTAAGACCAGATGCAA
AAGCACAAGTAACTATTGAGTATTCCGATGATAACGTTCCTCAACGTATAGATACGATTG
TGGTTTCCACGCAGCATGATGATTTTGATTCGGATGATCAAATGCTATCAAAAATTAAAG
AAGATATTATCTCCATTCTGATTCCAAAGGTTGTGGATAGGTTACCAAAAAACATTCAAG
CCCTTTTTGATGATCAGATTAACTATCACATAAACCCAACAGGAAAATTTGTGATTGGTG
GCCCGCATGGAGAT**G**CGGGACTTACCGGTCGTAAAATAATTGTGGATACCTATGGGGG
CAAAGGAGCCCACGGAGG

B

TKETENYMPLALDISHKILQVLAELRREGTQIDYLRPDAKAQVTIEYSDDNVPQRIDTIVVSTQ
HDDFDSDDQMLSKIKEDIISILIPKVVDRLPKNIQALFDDQINYHINPTGKFVIGGPHGD**A**GLT
GRKIIVDTYGGKGAHGG

Figure 6-3. **A.** Nucleotide sequence for *Sam* clone SAM-17 (length 435 bp), obtained from *Alexandrium catenella* strain ACSP01 (refer to Chapter 5). The reading frame is +2 such that the first codon of this fragment is ACA, coding for the amino acid threonine (T). The positions of the forward primer F4-QPCR-SAM2 (bases 67-86) and the reverse primer F4-QPCR-SAM2 (bases 252-272) are underlined. The length of the expected amplification product is 206 bp. The nucleotide sequence is identical to clone SAM-21 to SAM-25 except for base 374 which is a G (bold) not an A, resulting in a codon for alanine (A) as opposed to threonine (T) in SAM-21 to SAM-25. **B.** Polypeptide sequence of 145 residues derived from A. The primer sites in A are underlined. The partial region of the conserved C-terminus aligned in Fig. 6-7 is shaded in A and B.

A

AACAAAGGAGACTGAAAATTATATGCCTTTGGCGCTTGATATTTCCCACAAGATCCTTCA
AGTCTTGGCAGAACTTAGAAGAGAGGGCACGCAAATAGATTATTTAAGACCAGATGCAA
AAGCACAAGTAACTATTGAGTATTCCGATGATAACGTTCCTCAACGTATAGATACGATTG
TGGTTTCCACGCAGCATGATGATTTTGATTCGGATGATCAAATGCTATCAAAAATTAAAG
AAGATATTATCTCCATTCTGATTCCAAAGGTTGTGGATAGGTTACCAAAAAACATTCAAG
CCCTTTTTGATGATCAGATTAACTATCACATAAACCCAACAGGAAAATTTGTGATTGGTG
GCCCGCATGGAGATACGGGACTTACCGGTCGTAAAATAATTGTGGATACCTATGGGGG
CAAAGGAGCCCACGGAGG

B

TKETENYMPLALDISHKILQVLAELRREGTQIDYLRPDAKAQVTIEYSDDNVPQRIDTIVVSTQ
HDDFDSDDQMLSKIKEDIISILIPKVVDRLPKNIQALFDDQINYHINPTGKFVIGGPHGDTGLTG
RKIIVDTYGGKGAHGG

Figure 6-4. **A.** Nucleotide sequence for identical *Sam* clones SAM-21 to SAM-25 (length 435 bp), obtained from *Alexandrium minutum* strain AMAD06 (refer to Chapter 5). The reading frame is +2 such that the first codon of this fragment is ACA, coding for the amino acid threonine (T). The positions of the forward primer F4-QPCR-SAM2 (bases 67-86) and the reverse primer F4-QPCR-SAM2 (bases 252-272) are underlined. The length of the expected amplification product is 206 bp. The nucleotide sequence is identical to clone SAM-17 except for base 374 which is an A (bold) not a G, resulting in a codon for threonine (T) as opposed to alanine (A) in SAM-17. **B.** Polypeptide sequence of 145 residues derived from A. The primer sites in A are underlined. The partial region of the conserved C-terminus aligned in Fig. 6-7 is shaded in A and B.

Fourthly, a primer pair (SAM-3'136-1F/-1R) (Table 6-1) was designed to the *Alexandrium minutum* AMAD06 SAM 3'-end clone SAM3'-136-7A obtained by 3'RACE (see 5.2.9, Fig. 6-7, Appendix 4). Primers (10 μm) were tested in PCR on *A. catenella* CAWD44 and *Gymnodinium catenatum* CAWD106 cDNA using the amplification conditions: one cycle of 95°C for 5 min followed by 35 cycles at 94°C for 15 s, 50°C for 45 s, and 72°C for 1 min, and one final cycle of 72°C for 5 min. Reactions were prepared using a commercial PCR Master Mix (Cat. Nº. M7502, Promega) to a final volume of 20 μl, final primer concentration of 1 μM each and ~10-25 ng template cDNA per reaction (RT+). PCR products were purified, cloned and sequenced as described previously. The target amplicon was a short fragment of 152 bp. A second primer pair, SAM-3'136-2F/-2R (Table 6-3), was designed by visual inspection to be specific to the resultant CAWD44 clone SAM-3'136-9-8. These final *A. catenella* CAWD44 specific primers were used in QPCR to amplify a 112 bp product (see 6.2.6, Fig. 6-7).

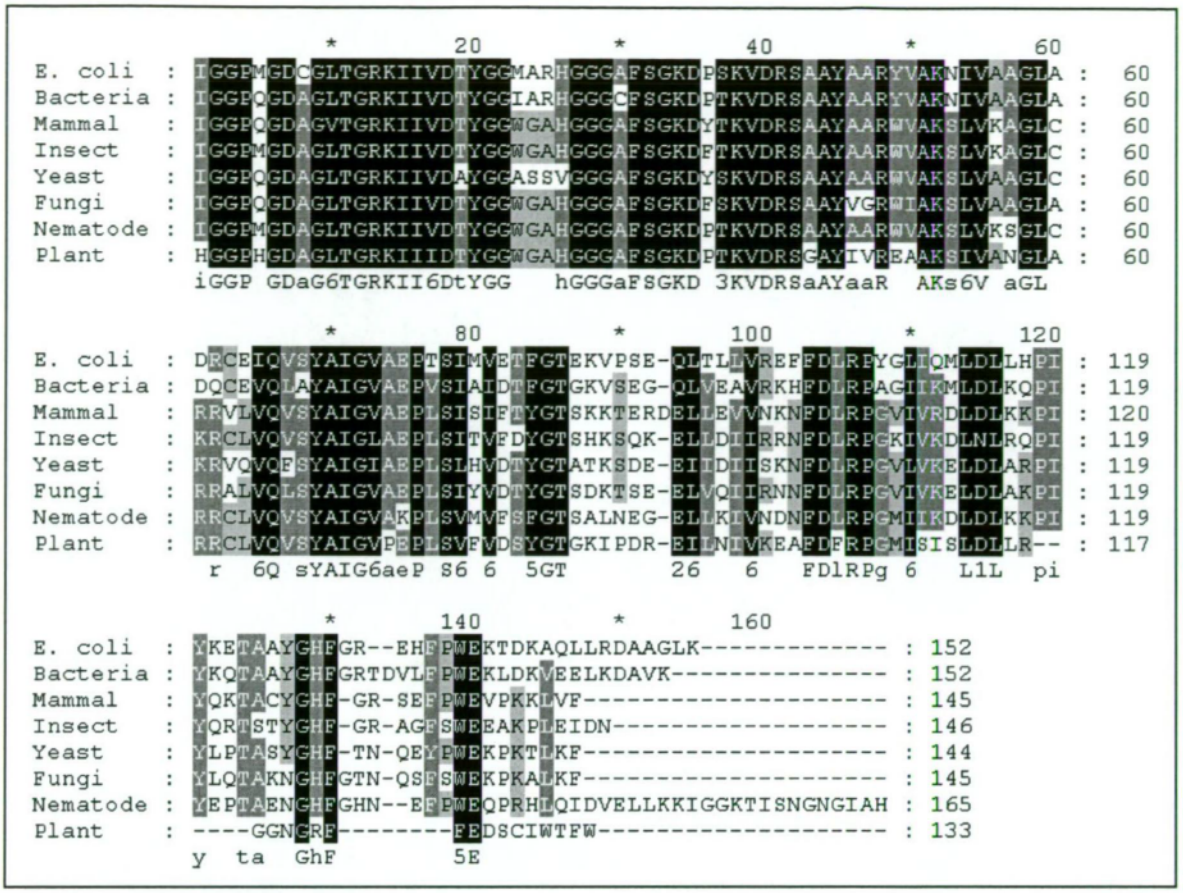

Figure 6-5. Protein sequence alignment for the C-terminus of *Sam* produced using the program GeneDoc. Eight sequences, representing 5 Kingdoms, are compared. Black shading indicates residues that are conserved in all sequences, dark grey shading indicates residues that are conserved in at least 6 sequences and light grey shading indicates residues that are conserved in 5 sequences. The forward primer F4-Cterm-SAM was designed to residues 13-21 (RKIIVDTYG) and the reverse primer F4-Cterm-SAM was designed to residues 68-75 (YAIGVAEP).

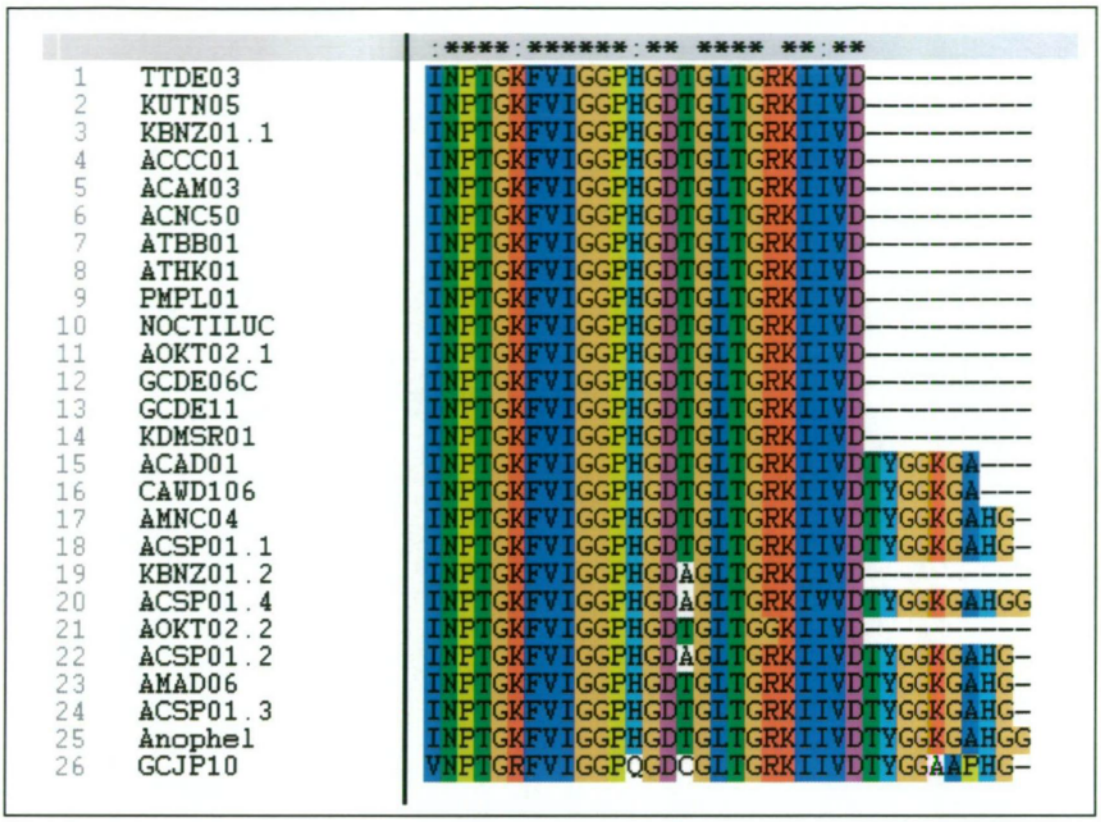

Figure 6-6. Protein sequence alignment for the partial C-terminus of the *Sam* gene from 25 dinoflagellate clones (1-24, 26) and the insect, *Anopheles gambiae* (25, Anophel) produced in ClustalX (refer to Chapter 5, Table 5-3). Default amino acid colours have been used. Asterisks denote amino acid residues that are identical in all sequences and colons denote amino acid residues that are identical in all but one sequence.

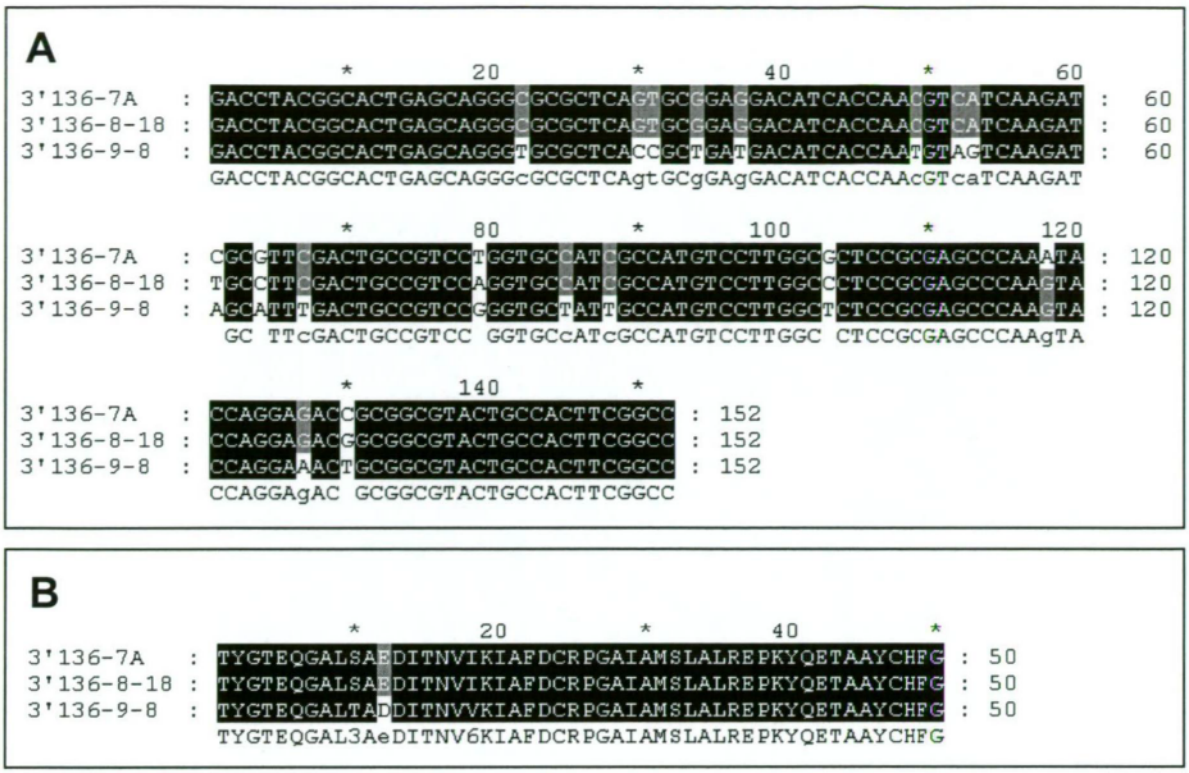

Figure 6-7. **A.** Nucleotide sequence alignment for the 152 bp clones SAM-3'136-8-18 (*Gymnodinium catenatum* CAWD106) and SAM-3'136-9-8 (*Alexandrium catenella* CAWD44) with the corresponding partial fragment of the clone SAM-3'136-7A (*Alexandrium minutum* AMAD06). Primers SAM-3'136-1F/-1R designed to the clone SAM-3'136-7A (refer to Chapter 5) and used to obtain clones SAM-3'136-8-18 and SAM-3'136-9-8 are located at positions 1- 20 bp and 133-152 bp respectively. Primers SAM-3'136-2F/-2R designed to clone SAM-3'136-9-8 (CAWD44) are located at positions 24-46 bp and 114-135 bp. The consensus sequence (unshaded) is listed below the three aligned sequences. Bases shaded black are identical in all 3 sequences whilst bases shaded grey are conserved in 2 sequences. **B.** Protein sequence alignment for the clones SAM-3'136-8-18 (CAWD106), SAM-3'136-9-8 (CAWD44) and SAM-3'136-7A (AMAD06) in A. The CAWD44 clone differs from the first two clones at three amino acid residues, 10 [threonine (T) not serine (S)], 12 [aspartic acid (D) not glutamic acid (E)] and 18 [valine (V) not isoleucine (I)].

6.2.1.2 *S-adenosylhomocysteine hydrolase gene* (Sahh), *Methionine aminopeptidase gene* (Map), *Histone like protein gene* (Haf)

A single pair of primers was designed to the coding sequence of each of three partial nucleotide sequences from *A. fundyense* encoding: (1) *Sahh* mRNA partial cds (585 bp) (AF105295), (2) *Map* mRNA complete cds (1-291) (AF105293) and (3) *Haf* mRNA complete cds (15-272) (AF105294) (Taroncher-Oldenburg & Anderson 2000) by visual inspection. Primers were tested in PCR on *A. catenella* gDNA and cDNA using the following amplification conditions: one cycle of 95°C for 1 min followed by 35 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and one final cycle of 72°C for 5 min. Primers for *Sahh* (Sahh-F1/-R1) and *Map* (Map-F1/-R1) were used in QPCR to study gene expression over the CDC of *A. catenella*. The expected amplicon length for *Sahh* 151 bp (Fig. 6-8), *Map* was 150 bp (Fig. 6-9) and *Haf* 146 bp (Fig. 6-10).

A

GTGAACGACTGCGTGACAAAGTCCAAGTTCGATAATGTGTACGGGTGCCGTCACTCGT
TGCCGGACATCATGCGTGCCACAGATGTCATGATTGGGGGCAAGCGCGCCCTGATCTG
CGGCTATGGCGATGTCGGCAAGGGCTGTGCTTTGGCCATGCGCGGAGCCGGAGCCCG
TGTCTTGATTACCGAGATTGACCCAATCTGCGCGCTTCAGGCGTGCATGGAGGGCTTC
CAGGTTGTCACTCTGGAGTCCGTAGTTGGGGAGGTGGATATTTTTACGACGACCACGG
GCAACTTCAAGATCATCACTCTGGAGCATATGAAAAAGATGAAGAACAATGCCATCGTC
GGCAACATTGGCCACTTCGACAACGAGATTGAGATGGCTGAGCTGGAAGGAATGCCGG
GCATCAAGGTGGAGAACATCAAGGCACAGGTTGACCGCTTCATATTCCCAGATGGCCA
CGGTATCATCGTTCTCGCCTCCGGCCGGCTCTTGAACTTGGGCTGTGCCACAGGCCAT
CCCTCCTTCGTCATGTCTTGCTCCTTCACCAATCAGGTGTTGGACAACTTGACTTTGCTT
CAA

B

VNDCVTKSKFDNVYGCRHSLPDIMRATDVMIGGKRALICGYGDVGKGCALAMRGAGARVLI
TEIDPICALQACMEGFQVVTLESVVGEVDIFTTTTGNFKIITLEHMKKMKNNAIVGNIGHFDNE
IEMAELEGMPGIKVENIKAQVDRFIFPDGHGIIVLASGRLLNLGCATGHPSFVMSCSFTNQVL
DNLTLLQ

Figure 6-8. **A.** *Alexandrium fundyense Sahh* mRNA, partial cds (AF105295) of 585 bp (Taroncher-Oldenburg & Anderson 2000). The sites of the QPCR primers Sahh-F1/-R1 are underlined and should amplify a 151 bp fragment corresponding to bases 120-270.
B. Translation of *A. fundyense Sahh* (AAD20318) that codes for 195 amino acid residues. The primers Sahh-F1/-R1 should amplify the region underlined from amino acid residues 41-90.

A

GTCAGCGATGTCGCTGAGCGCGAAGGGTTTGGTGTTGTCAAGACCTTGGTTGGTCATG
GCATTGGCGAGTTTTTCCATGGAGTGCCTCAGGTCTTCCACTGTCGAAACAGCGACAAT
CGAAAGATGCAGGAAGGCACCACCTTCACCCTGGAGCCAGTACTTACAGAGGGGTCAC
GGGATTGGATAACGTGGGATGACGGGTGGACAGTTGCTACAAGTGACCATGGCCGCG
CTGCCCAATTTGAGCACACGCTGCTGATCACTGCCAATGGCTGTGAGGTCATGACTTGA
TGCATCTGCGGCGGCATCTGACCCACGGTGCGGTATGCGACTCCTGCCATGCCAACC
GCTGTGGCCTGCTCCCCCGTTCAAAAAAAAAA

B

VSDVAEREGFGVVKTLVGHGIGEFFHGVPQVFHCRNSDNRKMQEGTTFTLEPVLTEGSRD
WITWDDGWTVATSDHGRAAQFEHTLLITANGCEVMT

Figure 6-9. **A.** *Alexandrium fundyense Map* mRNA (AF105293) of 380 bp and partial cds (1-291) (Taroncher-Oldenburg & Anderson 2000). The sites of the QPCR primers Map-F1/-R1 are underlined and should amplify a 150 bp fragment corresponding to bases 7-156. The grey shading indicates the structural region of the gene and the 3'UTR is unshaded. **B.** Translation of *A. fundyense Map* (AAD20316) that codes for 96 amino acid residues. The primers Map-F1/-R1 should amplify the region underlined from amino acid residues 3-52.

A

CACGAGCCCGTTCGATGAGCCCCTCGGCGACCAGATCCCGGACGCGAACAGCGGGG
GCATCTCGCAGGACAAGGCTGCGGACAAGTTCTGCCAGAGGTTCTGCCCGAGCTCCG
GTTGCCACTGCGGAGGTGAAGAAGTTGGGCGTCTTCACTGTCCCAGGCCCCTACAATA
TCAAGTTGAGGGAGAAGCCCGCCACCCAGGCCAGCAAGTGGAAGGTGTGCCTGAGAG
TGGCCTGGCATGTATTCCCTTTAAAGGCTTGCAAGCTCTGTTAGAGCATCAGGCTGAAG
AGCTTTGCTTGGAATTGCAACTGGAGCAGCAGGAGGCTCATCTACAGGCTCTTCTTCGC
GCTTCCGATCCTGGGCCCTCTAAGGAGAAGAAGGGCAAGATGAAAAGTAGGAAGTGAT
GCCGGTGATGTTGCTCAGCTCCAGTGGCCTGCTCTCAGAGTTGATTTGCTAGGTCAGT
CATTTTGAACTCTGTGGTGTGCATTCTGCACGCAGTATTTACTTTGTAAATCTCTCAGGC
GATCCTCTTCATTACATTGTGTGGAGGCGGATTAAGACGTTGTCAAAAAAAAAA

B

MSPSATRSRTRTAGASRRTRLRTSSARGSARAPVATAEVKKLGVFTVPGPYNIKLREKPAT
QASKWKVCLRVAWHVFPLKACKLC

Figure 6-10. **A.** *Alexandrium fundyense Haf* mRNA (AF105294) of 576 bp and complete cds (15-272) (Taroncher-Oldenburg & Anderson 2000). The grey shading indicates the structural region of the gene, whilst the upstream and downstream regions of the gene are unshaded. The sites of the QPCR primers Haf-F1/-R1 are underlined and should amplify a 146 bp fragment corresponding to bases 95-240. **B.** Translation of *A. fundyense Haf* mRNA (AF105294) to a protein sequence of 85 amino acid residues.

6.2.1.3 *Ubiquitin gene* (Ubi)

Degenerate ubiquitin (*Ubi*) primers (Albrecht *et al.* 1998) were used to amplify dinoflagellate ubiquitin using the PCR program: one cycle of 3 min at 98°C, 35 cycles of 30 s at 94°C, 45 s at 55°C and 1 min at 72°C, with a final cycle of 5 min for 72°C. PCR products were cloned into a plasmid vector and sequenced. The reverse primer, UBI-R was subsequently modified at the degenerate base R (A or G) to be a cytosine (C), which was present in dinoflagellate ubiquitin, and denoted UBI-Gcat-R (Table 6-1).

6.2.2 Experimental outline

The *A. catenella* strain CAWD44 was grown in culture under a 10:14 dark:light cycle. Preliminary studies replicated twice showed that (i) cells were synchronised to within approximately 45 to 50% (conservative calculation of division in a 2 h period) with or without a long dark period exposure (block release synchronisation method) used by Taroncher-Oldenburg & Anderson (2000) (Appendix 6), (ii) total toxin increased over the dark:light phase (Appendix 7) and (iii) the toxin profile did not change over the dark:light phase (Appendix 8). The time of cytokinesis for CAWD44 was shown to begin around 2.5 h before the culture room lights came on (-2.5 h), with the majority of cells having divided by 1.5 h after lights on (+1.5 h). Therefore, to capture cells over all stages of the CDC, a time series from -2.5 h to +3.5 h was chosen for the first cell cycle Experiment (1). For the second cell cycle Experiment (2), the same time series was covered but extended to include three later intervals (+4.5 h, +5.5 h and +12.0 h).

The two experiments were performed separately using the same methods. A 20 L carboy was filled with 15 L sterile 0.2 μm filtered 28 ppt. seawater containing GSe nutrients (Blackburn *et al.* 2001). The carboy was inoculated with 100 ml of exponentially growing *A. catenella* CAWD44 culture to obtain a starting density of 10 to 30 cells.ml^{-1}. Experiments commenced when the cell density reached 5-800 cells.ml^{-1}: 732 cells.ml^{-1} (Experiment 1) and 579 cells.ml^{-1} (Experiment 2), and the cultures were in the early exponential growth phase.

Samples for cell counts and flow cytometric analysis were harvested at hourly intervals. Larger sub-samples were harvested at two-hourly intervals for RNA isolation and toxin analysis (Tables 6-2, 6-3). Prior to harvesting each sample, the carboy was swirled and rocked gently to obtain an even distribution of cells. Swirling the carboy was effective in vertically mixing the culture but it concentrated cells centrally. To avoid this problem, the carboy was also rocked from side-to-side. Samples were collected via a tap at the bottom of the carboy which was flushed of trapped culture before each harvesting.

Table 6-2. Sampling procedure for the analysis of the cell cycle of *Alexandrium catenella* CAWD44 over one dark:light period. The experimental outline for two experiments, Experiment 1 and Experiment 2, is shown where the sampling time (h) refers to the time until light exposure (-) or the time of light exposure (+). Two additional samples were collected in Experiment 2, one at +5.5 h and one at +12.0 h. Abbreviations for sample types collected: RNA – RNA isolation, Toxin – Toxin analysis, Flow – Flow cytometry, Count – Cell count.

	Dark			Light							
Time (am-pm)	4.30	5.30	6.30	7.00	7.30	8.30	9.30	10.30	11.30	12.30	11.00
Time (h)	-2.5	-1.5	-0.5	0	+0.5	+1.5	+2.5	+3.5	+4.5	+5.5	+12.0
Samples	RNA Toxin Flow Count	Flow Count	RNA Toxin Flow Count	None, lights on	Flow Count	RNA Toxin Flow Count	Flow Count	RNA Toxin Flow Count	Flow Count	RNA Toxin Flow Count	RNA Toxin Flow Count

Table 6-3. Four sample types collected at regular intervals over the cell division cycle of *Alexandrium catenella* CAWD44. A single sample was collected at each time point. The time of sampling is outlined in Table 6-2.

Purpose of sample	Volume harvested (ml)	Treatment
RNA isolation	1000-1500	Pellet and resuspend in 450µl RNA*later*
Toxin analysis	250	Filter → store in 0.5 M acetic acid
Flow cytometry	200	Concentrate to 2-4 ml → store in 5% formalin
Cell count	3-5	Store in glass vials in Lugol's solution

6.2.3 Cell concentrations

For each sub-sample collected at hourly intervals, a small volume (3-5 ml) was preserved in 2-3 drops of Lugol's solution and the density of cells determined by counting at least 400 cells in a Sedgewick-Rafter counting chamber (Table 6-3). Cell concentrations are presented individually for Experiments 1 and 2, rather than as a mean as done in the three carboy experiment of Taroncher-Oldenburg *et al.* (1997).

6.2.4 Flow cytometry

For flow cytometry, approximately 200 ml of culture was transferred to four 50 ml Falcon tubes, centrifuged immediately for 4 min at 4,000 rpm and the cell-free medium poured off. Cell pellets were resuspended gently in the remaining ~1 ml of medium, transferred to a single glass vial, preserved in a final concentration of 5% formalin (Table 6-3) and stored at 4°C. To prepare samples for flow cytometry fixed cells were transferred to Eppendorf tubes, centrifuged for 1 min at 6-8,000 rpm and the supernatant removed. To extract pigments such as chlorophyll, cells were resuspended in 1.5 ml of ice cold (-20°C) methanol and incubated at 4°C for 20-30 min or until cells looked bleached. To rinse cells samples were re-pelleted, the methanol removed, cells resuspended in 1 ml cold (4°C) 1x PBS, re-pelleted and the PBS removed. Cellular DNA was stained with cold (4°C) 100 $\mu g.ml^{-1}$ propidium iodide and cells stored in the dark at 4°C for 2-3 h to allow up take of the stain.

The cells of *Alexandrium* settle on the bottom of the tube. Immediately prior to analysis, it was essential to thoroughly resuspend each sample. Analysis was performed on a Coulter Elite ESP flow cytometer equipped with a 100 W high-pressure mercury arc lamp (excitation using a 488 nm air cooled argon laser and fluorescence collected using a 600 nm dichroic LP and 575 nm BP). The percentage of cells in the cell cycle phases: G_1, S and G_2+M, for each sampling event, were calculated from histograms plotted using the software WinMDI version 2.8. Percentages were adjusted to exclude cell debris.

6.2.5 Toxicological analysis

Preliminary (Appendices 7 and 8) and final experimental (Table 6-3) cell cycle PST analysis was performed for *A. catenella* CAWD44. Cultures were gently filtered onto 47 mm glass-fibre filters (Whatman GFC or GF/F), placed in 3 ml of 0.05 N acetic acid and stored at -20°C. Samples were thawed and sonicated for 1-3 min or until filters were disintegrated, packed in ice and shipped overnight to Andrew Negri (Australian Institute of Marine Science, Townsville QLD) for PST analysis.

The specific toxin production rate per hour, μ_{Tox}, was defined as the change in toxin concentration (cell^{-1} or ml^{-1} of culture) relative to the existing concentration in the synchronised cultures (Taroncher-Oldenburg *et al.* 1997).

Where, $$\mu_{Tox} = \frac{\ln (T_1 / T_0)}{t_1 - t_0}$$

This calculation was based on the definition of the specific toxin production rate in asynchronous cultures, μ_{Tox} (d^{-1}) by Anderson *et al.* (1990). Values T_1 and T_0 are the toxin concentrations (cell^{-1} or ml^{-1} of culture) at times t_1 and t_0 and in this experiment μ_{Tox} (h^{-1}) was calculated. It is necessary to use the ln average concentration because the dinoflagellate are growing exponentially between t_1 and t_0 (refer to 1.1). To determine whether cells were losing toxin, it was essential to compare the rate of toxin production relative to the concentration of cells (μ_{Tox}.h^{-1}.ml^{-1}) to the rate of toxin production relative to the volume of culture (μ_{Tox}.h^{-1}.ml^{-1}) for each steady state. The rate of toxin production represents an instantaneous rate value for t_1 relative to t_0. For this reason rate values were plotted for time point t_1 and not half way between the two time points.

Human error in cell counts (Culverhouse *et al.* 2003) and errors incurred during quantitation of PSTs through HPLC (e.g. sample loss) (John & Flynn 2000) can significantly influence the findings and must be taken into account. The maximum error commonly associated with these methods is ±20%

(Sullivan 1993). An error of 20% was allowed for and only differences in toxicity of >20% were considered in the interpretation of the results.

6.2.6 Quantitative real time PCR (QPCR)

Total RNA was extracted, using the optimised micropestle technique outlined in Chapter 3, from *A. catenella* CAWD44 samples collected at regular intervals over the CDC (refer to Tables 6-2, 6-3). A single sample of a population of cells was collected at each time point for each experiment and RNA extracted. Four samples (1-4) for Experiment 1 and six samples (1-6) for Experiment 2 were extracted, where 1 = 2.5 h before lights on, 2 = 0.5 h before lights on, 3 = 1.5 h after lights on, 4 = 3.5 h after lights, 5 = 5.5 h after lights on and 6 = 12 h after lights. RNA was treated with TURBO DNA-*free* (Cat. Nº. 1907, Ambion) and the quality and purity of RNA assessed by spectophotometry and visual inspection after denaturing formaldehyde gel electrophoresis. RNA with an $A_{260:280}$ 1.8-2.0 and distinct ribosomal RNA bands was of a high quality.

cDNA was synthesised from 4 μg of total RNA in a volume of 60 μl using the Superscript III first-strand Synthesis System for RT-PCR (Cat. Nº. 18080-051, Invitrogen) and an oligo $(dT)_{20}$ primer to target the polyA tail of eukaryotic RNA. The RT sample was diluted 1:5 and 2 μl (for *Sahh*, *Map* and *Sam*) and 4 μl (for *Ubi*) used in a total QPCR volume of 20 μl containing 0.5 μM primers and 1x Platinum SYBR Green qPCR SuperMix-UDG (Cat. Nº. 11733-038, Invitrogen). Triplicate QPCR reactions were performed for each RT+ and RT- cDNA sample on a Rotorgene2000 (Corbett Research). Standard curves were performed for each gene in every QPCR run using serially diluted plasmid DNA containing the gene of interest. The dilution series started at 1 $ng.\mu l^{-1}$ which was diluted 10 fold 6 times (i.e. 10^{-1} to 10^{-6}). The following temperature profile was used for all primers with adjustment of the annealing temperature: one hold of 15 min at 95°C followed by 60 repeats of 15 s at 95°C, 20 s at 60°C (*Sam*, *Sahh* and *Map*) or 55°C (*Ubi*), 30 s at 72°C and 15 s at 74°C. Relative concentrations (Ct values) for the study genes *Sam*, *Sahh* and *Map* were normalised to the internal standard, *Ubi* (Albrecht

et al. 1998). Melt curve analysis was performed to confirm the presence of a single peak and therefore that a single sized transcript was being amplified.

Correlation coefficients (r) were calculated in Microsoft Excel to determine the reliability of a linear relationship between toxin production rate and gene expression and cell numbers. A value of +/-1.0 indicates a 100% correlation, which can be positive or negative.

6.3 Results

6.3.1 Primer selection for QPCR

6.3.1.1 S-adenosylmethionine synthetase gene (Sam)

The first *Sam* primer pair (F1-/R1-QPCR-SAM) did not amplify a product from cDNA or gDNA of *A. catenella* or *G. catenatum* in PCR. Neither was the positive control DNA, plasmid clone SAM-1, amplified. The two primer pairs designed visually (F2- and R3-QPCR-SAM and F3- and R2- QPCR-SAM) successfully amplified the target gene from *G. catenatum* CAWD106 cDNA but not from *A. catenella* CAWD44 cDNA using both standard PCR Master Mix (Promega) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Therefore all primer pairs designed to *G. catenatum* clone SAM-1 were not usable on *A. catenella* CAWD44 DNA (Table 6-4). No splice sites were found in the nucleotide sequence of SAM-1.

Comparison of the nucleotide and translated protein sequences of the *Sam* clones for dinoflagellate strains: ACSP01, AMAD06 and CAWD106, representing 3 species, revealed a conserved C-terminus at the protein level (Fig. 6-11). Clone SAM-1 of *G. catenatum* CAWD106 was ~18% different to clone SAM-17 of *A. catenella* ACSP01 and clones SAM-21 to SAM-25 of *A. minutum* AMAD06 at the C-terminus. However, at the protein level there was only one amino acid residue difference. The degenerate primers F4/R4-Cterm-SAM and the primer pair F4/R4-QPCR-SAM2 specific to *Alexandrium* clones ACSP01 SAM-17 and AMAD06 SAM-21 to SAM-25 did not amplify the target *Sam* from *A. catenella* CAWD44 cDNA or gDNA (Table 6-4).

Table 6-4. Summary of primer pairs designed and tested for compatibility with *Alexandrium catenella* CAWD44 cDNA to study the expression of *Sam* using QPCR. Whether the primer pair amplified the target *Sam* gene in PCR of *A. catenella* (CAWD44) or *Gymnodinium catenatum* (CAWD106) cDNA is indicated with a 'Yes', 'No' or 'N/A' (not applicable). The final primer pair used in QPCR, SAM-3'136-2F/-2R is shaded.

Primer pair	Designed to:	PCR product		Expected size bp
		CAWD44	CAWD106	
F1-/R1-QPCR-SAM	CAWD106 clone SAM-1	No	No	142
F2-/R3-QPCR-SAM	CAWD106 clone SAM-1	No	Yes	165
F3-/R2-QPCR-SAM	CAWD106 clone SAM-1	No	Yes	177
F4-/R4-QPCR-SAM2	ACSP01 clone SAM-17 and AMAD06 clone SAM-21 to -25	No	N/A	206
F4-/R4-Cterm-SAM	Multi-sequence alignment, adjusted to ACSP01 and AMAD06 above	No	No	195
SAM-3'136-1F-/-1R	AMAD06 clone SAM-3'136-7A	Yes	N/A	152
SAM-3'136-2F-/-2R	CAWD44 clone SAM-3'136-9-8	Yes	N/A	112

A

ACSP01	ATAAACCCAACAGGAAAATTTGTGATTGGTGGCCCGCATGGAGATGCG GGACTTACCGGTCGTAAAATAATTGTGGAT
AMAD06	ATAAACCCAACAGGAAAATTTGTGATTGGTGGCCCGCATGGAGATACG GGACTTACCGGTCGTAAAATAATTGTGGAT
CAWD106	ATCAATCCAACGGGAAAATTCGTTATTGGAGGACCTCATGGAGATACC GGATTAACGGGGCGAAAAATAATTGTTGAT

B

ACSP01	INPTGKFVIGGPHGDAGLTGRKIIVDT
AMAD06	INPTGKFVIGGPHGDTGLTGRKIIVDT
CAWD106	INPTGKFVIGGPHGDTGLTGRKIIVDT

Figure 6-11. **A.** Nucleotide sequences (sense strand 5'-3') of the C-terminus of *Sam* clones for 3 dinoflagellate strains: clones SAM-17 (ACSP01), SAM-21 to 25 (AMAD06) and SAM-1 (CAWD106). **B.** Polypeptide sequence of 27 amino acid residues derived by translation of the nucleotide sequences in A. Nucleotide base and amino acid residue differences between the 3 sequences are shaded.

A

GACCTACGGCACTGAGCAGGGTGCGCTCACCGCTGATGACATCACCAATGTAGTCAAG
ATAGCATTTGACTGCCGTCCGGGTGCTATTGCCATGTCCTTGGCTCTCCGCGAGCCCA
AGTACCAGGAAACTGCGGCGTACTGCCACTTCGGCC

B

TYGTEQGALTADDITNVVKIAFDCRPGAIAMSLALREPKYQETAAYCHFG

Figure 6-12. **A.** Nucleotide sequence for 152 bp CAWD44 clone SAM-3'136-9-8. The reading frame is +2 such that the first codon is ACC. **B.** Translation of A into a protein sequence of 50 amino acid residues.

Figure 6-13. Translated protein sequence (50 amino acid residues) of the *Alexandrium catenella* clone CAWD44-3'136-9-8 (152 bp) aligned with partial *Sam* from *Phytophthora infestans* (AAN31489) in GeneDoc. Common residues are indicated in a consensus line below. Note that numbers refer to the grouping of amino acids in terms of their shared chemical properties.

6.3.1.2 S-adenosylhomocysteine hydrolase gene (Sahh)

PCR of *A. catenella* CAWD44 and *G. catenatum* GCTRA01 cDNA and gDNA, using the primer pair Sahh-F1/-R1, yielded a 151 bp amplicon (Fig 6-14, Appendix 5). The nucleotide sequence of all clones was identical and the closest match (98%) in GenBank was to *A. fundyense Sahh* (148/151 identities).

6.3.1.3 Methionine aminopeptidase gene (Map)

PCR of CAWD44 gDNA and cDNA using the primers Map-F1 and Map-R1 yielded products of 150 bp as expected (Fig. 6-15). Nucleotide sequences for cDNA and gDNA clones were identical and the closest match from a Blast search in GenBank was to *A. fundyense Map* with a 98% match and 148/150 identities.

A

CTATGGCGATGTCGGCAAGGGCTGTGCCTTTGCCATGCGCGGAGCTGGAGCCCGTGT
CTTGATTACCGAGATTGACCCAATCTGCGCGCTTCAGGCGTGCATGGAGGGCTTCCAG
GTTGTCACTCTGGAGTCCGTAGTTGGGGAGGTGGAT

B

YGDVGKGCAFAMRGAGARVLITEIDPICALQACMEGFQVVTLESVVGEVD

Figure 6-14. **A.** Nucleotide sequence for the 151 bp *Sahh* clones of *Alexandrium catenella* CAWD44, Sahh-4-4 (gDNA) and Sahh-16-1 (cDNA) and *Gymnodinium catenatum* GCTRA01, Sahh-6-1 (gDNA) (Appendix 5). The reading frame is +2 such that the first codon is TAT. **B.** Translation of A into a protein sequence of 50 amino acid residues.

A

GATGTCGCTGAGCGCGAAGGGTTTGGTGTTGTCAAGACCCTGGTTGGTCATGGCATCG
GCGAGTTTTTCCATGGAGTGCCTCAGGTCTTCCACTGTCGAAACAGCGACAATCGAAAG
ATGCAGGAAGGCACCACCTTCACCCTGGAGCCA

B

DVAEREGFGVVKTLVGHGIGEFFHGVPQVFHCRNSDNRKMQEGTTFTLEP

Figure 6-15. **A.** Partial nucleotide coding sequence (sense strand 5'-3') of 150 bp for *Map* (cDNA and gDNA) of *Alexandrium catenella* CAWD44. **B.** Polypeptide sequence of 50 residues derived from A.

6.3.1.4 Histone like protein gene (Haf)

A single 332 bp clone, Haf-1-1, produced from a PCR of *Gymnodinium catenatum* strain GCTRA01 (refer to Table 2-2 in Chapter 2) gDNA and primers Haf-F1/-R1, was sequenced. A BLAST search at the nucleotide level produced no match to *A. fundyense His* except for an exact match of the first 20 bp, which corresponded to the forward primer site (Fig. 6-16). Instead a translated search produced a match of approximately 43-47% identities to bacteria and higher plant aminotransferases.

AGGTTCTGCCCGAGCTCCGGCGCAGAGATACAGGGAGCTACGGAGATGTCTGGTTACAA
CGTTAAACGCGGTCGGAGCCTGCTTAACACCCCCGGCCCAACCAACGTGCCAGAGCAG
GTTCAGCAAGCCATGGTGCGCAACCCCCTGGATCTTGGCGATCCGCGCGCACTTTCAAT
GATCGAGACCTGCTTCCGGGATCTGAAGAAAATCTTCAAGACCGAGCACGAAATTTTCAT
GTACGCCGCCAACGGCCATGGCGCCTGGGAAGCCGCCCTGGTCAACACCATGTCCCCG
GGCGACCTGCTGCTTGTGCCGGAGCTCGGGCAGAACCTA

Figure 6-16. Nucleotide sequence for the 332 bp GCTRA01 gDNA clone Haf-1-1. The first 20 bp, corresponding to the forward primer site (underlined), exactly matched the *A. fundyense Haf* sequence.

6.3.1.5 Ubiquitin gene (Ubi)

Seven *Ubi* clones (UBI-177 to UBI-182) obtained from PCR of *G. catenatum* gDNA were sequenced and five different consensus sequences obtained (Fig. 6-17). Clones UBI-177 and UBI-178 (511 bp) were identical, and clones UBI-181 and UBI-183 (228 bp) were identical except for base 147 (A or C). Other clones were the following lengths: UBI-179 (430 bp), UBI-180 (226 bp) and UBI-182 (165 bp). All clones produced a match to *Ubi* of various forms in GenBank. *A. catenella* ubiquitin was subsequently cloned using the degenerate forward primer (UBI-F) and the dinoflagellate reverse primer (UBI-Gcat-R).

6.3.2 Toxin analysis over the cell division cycle

The toxin profile of *A. catenella* CAWD44 did not change over the CDC (Fig. 6-18). The fractions neoSTX, dcSTX, STX and GTX2 were not detected and only trace amounts of C1, C3, GTX1 and GTX3 were present (Table 6-5), with C3 only occurring at 1.5 h and 3.5 h of light exposure (Fig. 6-18). A large proportion of the low specific toxicity C2 toxin was detected. In Experiment 1, the average C2 content (47.9 fmol.cell^{-1}) comprised approximately 44% of the total average toxin (108.9 fmol.cell^{-1}). While in Experiment 2, the average C2 content (29.8 fmol cell^{-1}) comprised approximately 35% of the total average toxin (86.5 fmol.cell^{-1}). As a result, the toxicity of CAWD44 cultures was low, at approximately 4.9 pgSTX equivalents.cell^{-1} (Experiment 1) and 4.3 pgSTX equivalents.cell^{-1} (Experiment 2) (Table 6-5).

Table 6-5. Concentration (mol%) of PST derivatives and total toxicity (pg STX equivalents.cell^{-1}) of *A. catenella* CAWD44 cultures (Experiment 1 and 2) averaged over one light:dark phase.

Exp.	C1	C2	C3	C4	GTX1	GTX3	GTX4	GTX5	GTX6	Toxicity
1	1.83	44.01	0.10	4.76	1.36	0.60	8.33	16.02	22.99	4.86
2	0.86	34.45	0.06	4.61	0.85	0.40	11.90	16.53	30.43	4.25

In both experiments, the concentration of cells gradually increased over the study period. An approximate doubling was observed, with the majority of division occurring over the dark:light transition (-0.5 h to +1.5 h) (Fig. 6-19). Cells of *A. catenella* CAWD44 were suitable for flow cytometry and, unlike *G. catenatum* strains GCNZ01 and GCTRA01, they did not form chains that clogged the capillary. Cells of CAWD44 were generally single unless undergoing division, in which case pairs of cells where observed. Flow cytometry indicated that *A. catenella* cells were already entering G_2+M phase 2.5 h before activation of the lights (-), with the majority of cells in Experiment 1 (~86%) re-entering G_1 phase by +1.5 h of light exposure (for cell numbers refer to Fig. 6-19 and for cell % refer to Fig. 6-20). In Experiment 2, cell division was less synchronous and two waves of S phase were evident, the first occurring between -2.5 h and –1.5 h (dark phase) and the second approximately 4 h later at +2.5 h to +3.5 h (light phase) (Fig. 6-20). The first burst of division over the dark:light transition was larger than the second small burst of division as indicated by the large proportion of cells (56%) in G_2+M phase at -0.5 h.

The total toxin content calculated per cell and per culture volume (Appendix 9) changed over the duration of both experiments (Fig. 6-19). Changes in toxin content.cell^{-1} were similar to toxin.ml^{-1} for Experiment 2 but not for Experiment 1 (Fig. 6-20, Table 6-6). In Experiment 1, total toxin.cell^{-1} showed a steadily increasing loss of toxin (the amount of toxin lost increasing by 3.6-3.7% over each 2 h period), with a total loss of 48% over the 8 h period (Table 6-6, Fig. 6-19, Appendix 9). In Experiment 2, total toxin cell^{-1} was variable over the 8 h period and unlike Experiment 1, toxin increased in the initial 4 h (52.4% and 10.5%) (Table 6-6, Fig. 6-19, Appendix 9). Toxin remained relatively stable over the next 4 h (-8.3/+6.4% cell^{-1} and -6.5/+7.2% ml^{-1}).

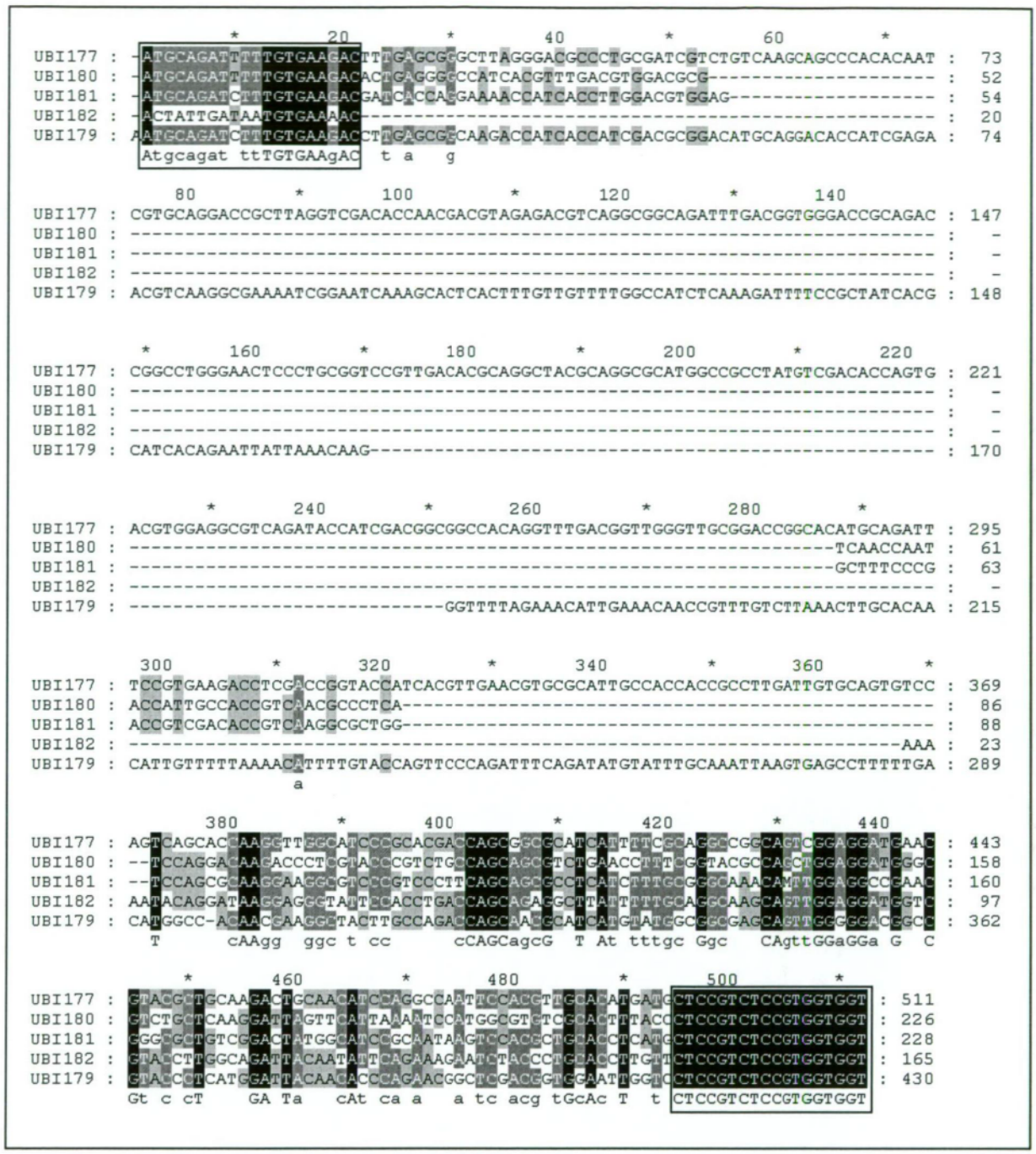

Figure 6-17. Nucleotide alignment of five different *Gymnodinium catenatum* ubiquitin clones obtained using primers UBI-F/-R (Albrecht *et al.* 1998). Note the clones UBI-177/-188 and UBI-181/-183 are the same. The 20 bp corresponding to the forward primer ATGCAGAT**Y**TTTGTGAAGAC (top box) is an exact match except for clone UBI-182. Both bases for the degenerate base Y (cytosine, C or thymine, T) are represented in the different clones. The 18 bp corresponding to the binding site of the reverse primer 5'-ACCACCACG**R**AGACGGAG-3' (lower box) is an exact match to all clones. No degeneracy was observed and the degenerate base R (i.e. Y if reverse complement) was unnecessary. Instead, the dinoflagellate specific primer UBI-Gcat-R 5'-ACCACCACGCAGACGGAG-3' was used in further studies.

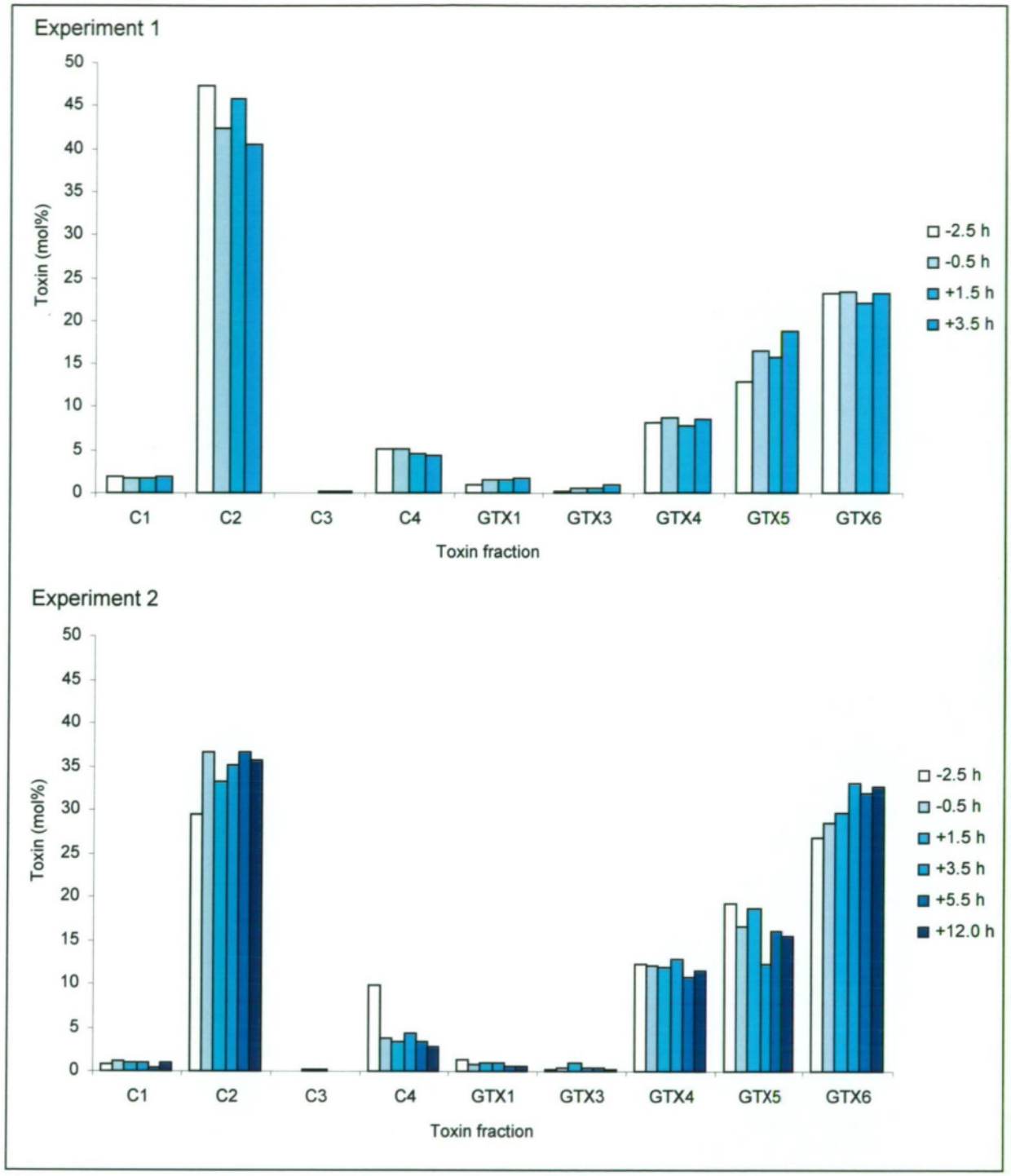

Figure 6-18. Toxin profile of *Alexandrium catenella* CAWD44 at 2-h intervals (from -2.5 h to +3.5 h) over the cell division cycle for Experiment 1 (upper graph) and Experiment 2 (lower graph). Two additional samples were taken for Experiment 2, one at +5.5 h and one at +12 h of light exposure. The time until lights on (cells in the dark phase) is indicated with (-) and the time since the lights came on (cells in the light phase) is indicated with (+).

In Experiment 1, toxin production rates compared $cell^{-1}$ and ml^{-1} were different, whereas in Experiment 2, toxin production rates compared $cell^{-1}$ and ml^{-1} followed the same trend (Fig. 6-21). When calculated on a cellular basis, the rate of toxin production in Experiment 1 was always negative and correlated inversely with cell numbers (r = -0.85), which increased. When calculated on a volume basis, which does not take into account cell density, a positive rate of toxin was evident. Toxin production in Experiment 2, although positive at a number of time points (-2.5 h to +1.5 h and at +5.5 h), also showed a strong negative correlation with cell numbers (r = -0.90). Although positive, the rate of toxin production declined as cell numbers increased (Figs 6-19, 6-22).

Immediately prior to light exposure (time point -0.5 h), the rate of toxin production ml^{-1} in Experiment 1 was negative but was positive after light exposure (time point +1.5). Toxin production may have been triggered by light, but the positive rate of toxin production at -0.5 h (ml^{-1} and $cell^{-1}$) in Experiment 2 does not support this (Fig. 6-20). Instead, toxin content of cells appeared to be related to the cell cycle phase and the rate of cell division (Figs 6-19, 6-20).

In both experiments, the rate of toxin production.ml^{-1} was positive between time points +0.5 h to +2.5 h, a period when a high proportion of cells were in early G_1 phase. The fastest rate of cell division observed in both experiments was between the dark:light transition (-0.5 h and +1.5 h), with an increase of 36.7% of cells in Experiment 1 and a lower increase of 27.5% of cells in Experiment 2 (Fig. 6-19). A decrease in toxin.ml^{-1} occurred in both experiments but this difference was <20% except for Experiment 1 between time point 1.5 and 3.5 h after lights on (23.6% decrease) (Table 6-6). Flow cytometry indicated that the proportion of cells in G_2+M phase (with double the complement of DNA) peaked at -0.5 h in both experiments (1=46% and 2=56% cells in G_2+M) and dropped after the lights came on, indicating that cytokinesis was occurring (Fig. 6-20).

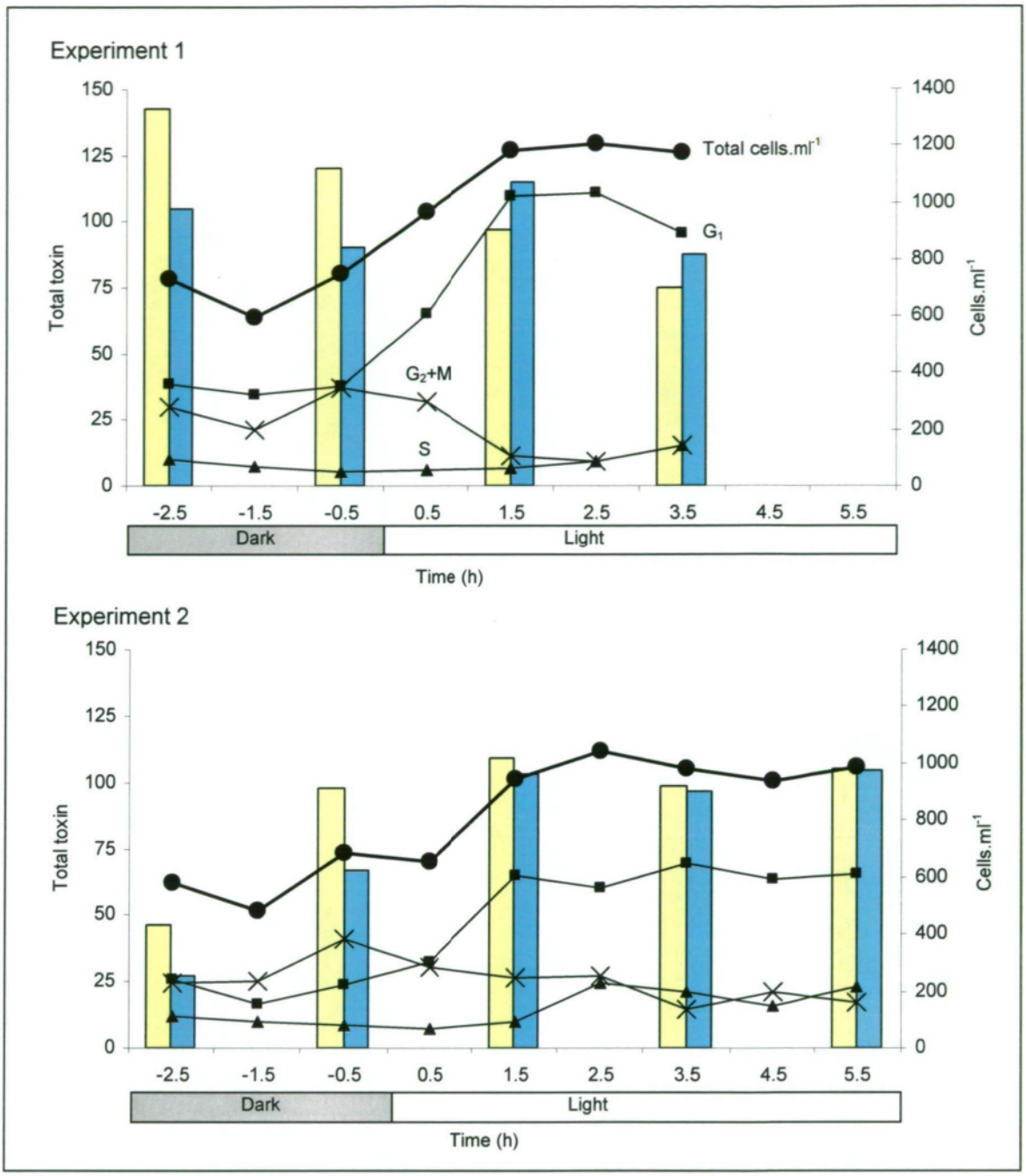

Figure 6-19. Total toxin content per cell ☐ and per ml ▬ of *Alexandrium catenella* CAWD44 cells plotted with the total cells per ml of culture (●) and the number of cells in the different cell cycle phases: G_1 (■), S (▲), G_2+M (X). The same time points (hours) in the dark phase and light phase for these two independent experiments are shown, with two extra time points recorded for Experiment 2 (4.5 h and 5.5 h).

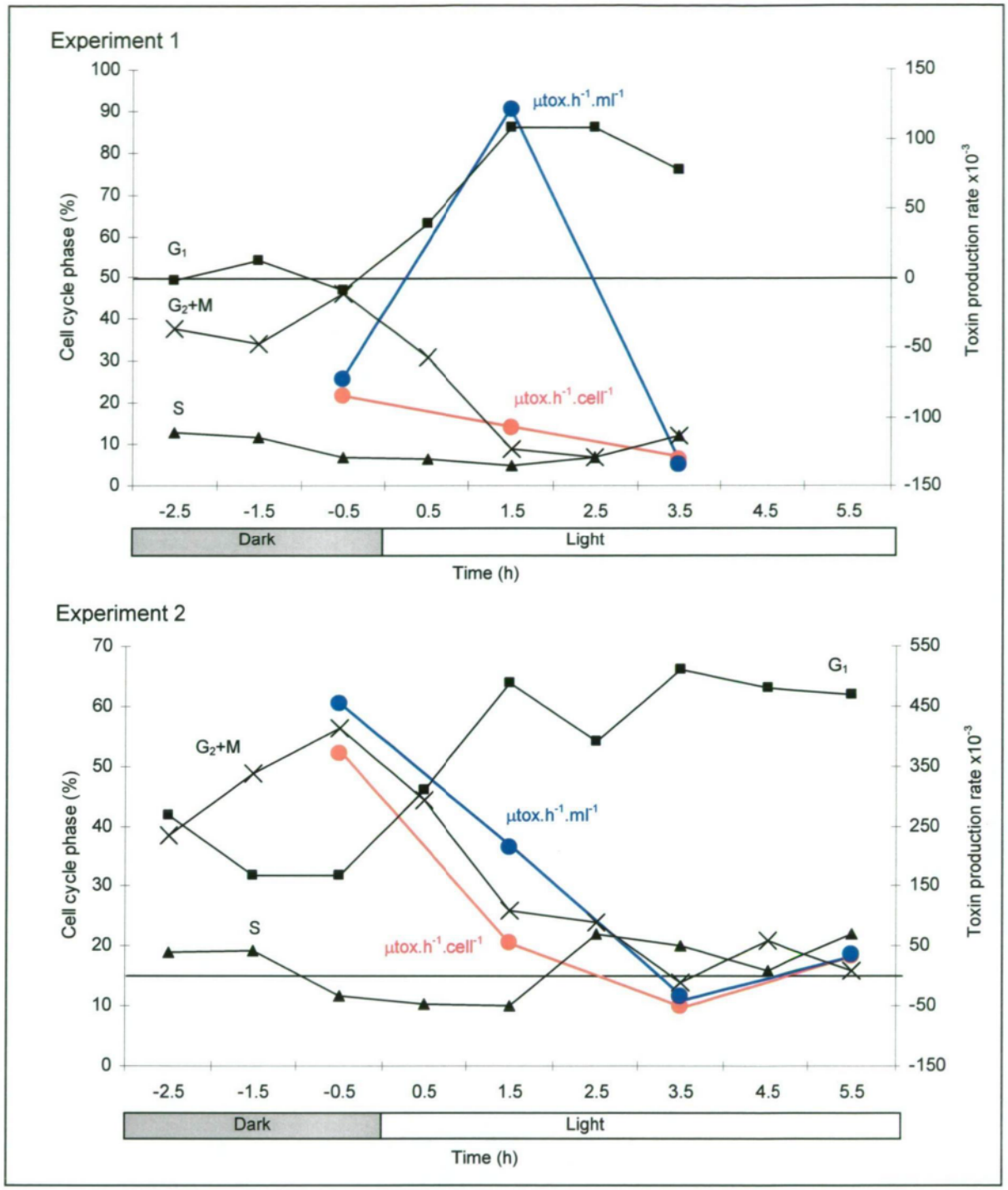

Figure 6-20. Proportion of *Alexandrium catenella* CAWD44 cells (%) in the different cell cycle phases: G_1 (■), S (▲), G_2+M (X), compared to the toxin (all STX derivatives) production rates (●) µtox.h^{-1}.$cell^{-1}$ (red) and µtox.h^{-1}.ml^{-1} (blue). The same time points (hours) in the dark phase and light phase for these two independent experiments are shown, with two extra time points recorded for Experiment 2 (4.5 h and 5.5 h). A line to indicate zero toxin production rate is shown. It is important to note that the scale of the toxin production axis is ~2.3 times smaller in Experiment 1 compared to Experiment 2.

Table 6-6. Change in toxin content (cell^{-1} or ml^{-1}) of *Alexandrium catenella* CAWD44 cultures at 2-h intervals over a single dark (-): light (+) phase. Change refers to the % difference in total toxin relative to the previous value. In Experiment 2, an additional time point at +5.5 h was analysed. A decrease in toxin from the previous time point is indicated with a ↓ and an increase with a ↑. The column '% difference' refers to the difference between the '% change' columns (cell^{-1} and ml^{-1}) for that experiment. Shaded values indicate a substantial (>20%) difference in the change in toxin.cell^{-1} compared to the change in toxin.ml^{-1} not accountable by error.

	Experiment 1			Experiment 2		
Time (h)	% change in toxin (cell^{-1})	% change in toxin (ml^{-1})	% difference	% change in toxin (cell^{-1})	% change in toxin (ml^{-1})	% difference
-2.5						
-0.5	↓ 15.7	↓ 13.7	2.0	↑ 52.4	↑ 59.9	7.5
+1.5	↓ 19.4	↑ 27.4	46.8	↑ 10.5	↑ 35.1	24.6
+3.5	↓ 23.0	↓ 23.6	0.6	↓ 8.3	↓ 6.5	1.8
+5.5				↑ 6.4	↑ 7.2	0.8

In both experiments, a substantial (>20%) difference in the toxin content cell^{-1} compared to the toxin.ml^{-1} (not accountable for by error) was recorded (Table 6-6). Both differences were recorded as an increase in the toxin content of the culture volume relative to the toxin content of cells at time point +1.5 h (the first time point sampled after exposure to light). At this time point cell density was increasing (Fig. 6-19). The largest difference, an increase of 46.8%, was observed in Experiment 1, with an increase of 24.6% in Experiment 2 (Table 6-6).

6.3.3 Gene expression and toxin production rate

The RNA isolated from *A. catenella* cells collected at intervals over the CDC was of a high quality as determined by spectrophotometry and contained no visible contaminating DNA (Fig. 6-21). Fine scale (2 h) changes in gene expression and toxin production were observed (Fig. 6-22).

The relative expression pattern of *Sam*, *Sahh* and *Map* were different in the two experiments and thus gene expression did not correspond solely to the dark:light cycle. In Experiment 1, the expression of *Sam* did not change between time points -2.5 h and -0.5 h but showed an increase of 24% after lights on (Fig. 6-22). *Sahh* and *Map* showed a similar pattern of expression

in Experiment 1 with the highest expression level (100%) at -2.5 h followed by a drop of 44% (*Sahh*) and 61% (*Map*) at -0.5 h. In Experiment 2, the pattern of expression of *Sam*, *Sahh* and *Map* was similar, particularly over the dark:light transition. The highest expression (100%) was observed at time point -0.5 h, an increase of 52% (*Sam*), 46% (*Sahh*) and 41% (*Map*) from time point -2.5 h. Expression of all genes dropped after lights on (time point +1.5), with *Sam* and *Sahh* dropping by 46% and 55% respectively and *Map* less dramatically by 21%. The same trend was not observed in Experiment 1, with expression levels increasing after light exposure, particularly for *Sahh* and *Map*.

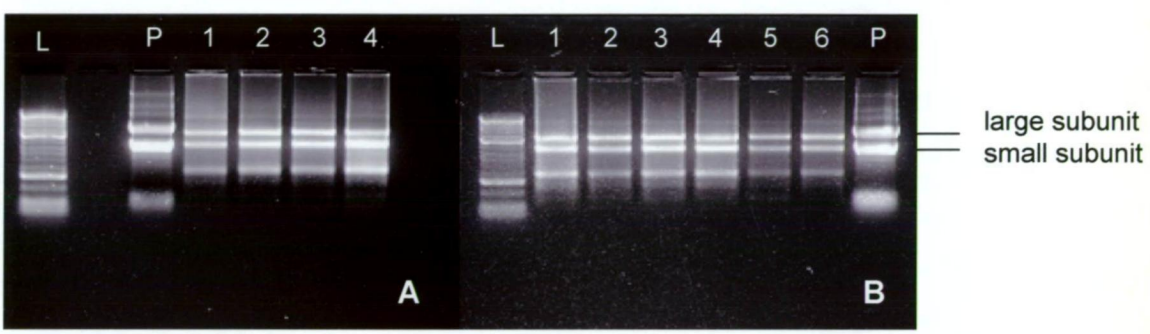

Figure 6-21. RNA isolated from *A. catenella* (CAWD44) cells from cell cycle Experiment 1 (**A**) and 2 (**B**). Prior to RNA extraction, cells were stored in Ambion's RNA*later*. Frozen cells were ground using an Eppendorf micropestle and processed in RLT buffer using Qiagen's Plant RNeasy Mini Kit. RNA was subsequently treated with Ambion's TURBO *DNase*. RNA was extracted from cells at a series of intervals over the cell division cycle where (–) indicates that the sample was collected in the dark and (+) indicates the sample was collected when the culture room lights were on. (1-6) 1 = –2.5 h, 2 = –0.5 h, 3 = +1.5 h, 4 = +3.5 h, 5 = +5.5 h and 6 = +12. (P) Positive control: RNA of the garden pea, *Pisum sativum* treated with Qiagen's on column *DNase*. RNA ladder (L) 0.25-9.5 Kb (Cat. Nº 15620-016, GIBCOBRL). Note that the RNA yield varied depending on the amount of starting material and grinding.

The rate of toxin production.ml^{-1} was not correlated to cell density (Table 6-7). Despite the strong negative correlation between toxin production.ml^{-1} and cell density (r = -0.98) in Experiment 2, this correlation was inconsistent with Experiment 1 (r = 0.31). Of the three genes, *Map* showed the strongest correlation to the rate of toxin production.ml^{-1} and this was consistent in both experiments (r = 0.97 and 0.98) (Table 6-7). *Sahh* also showed a consistent positive correlation to the rate of toxin

production.ml^{-1} (r = 0.68 and 0.73), although lower than the correlation for *Map*. In relation to the rate of toxin production, the pattern of *Sam* expression was inconsistent between the two experiments (Fig. 6-22). Although a strong positive correlation was observed for *Sam* and the rate of toxin production.ml^{-1} (r = 0.89) in Experiment 2, a poor negative correlation (r = -0.52) was observed in Experiment 1 (Table 6-7).

Table 6-7. Correlation coefficients (r) calculated in Microsoft Excel for the rate of toxin production.ml^{-1} of culture compared to gene expression and cell density (cells.ml^{-1}). For both experiments, coefficients were calculated for data collected at 2-h intervals over one dark:light phase from -2.5 h to +3.3 h, with one additional sample point at +5.5 h for Experiment 2.

	Toxin production rate.ml^{-1}	
	Experiment 1	Experiment 2
Sam	-0.52	0.89
Sahh	0.68	0.73
Map	0.97	0.98
Cells.ml^{-1}	0.31	-0.98

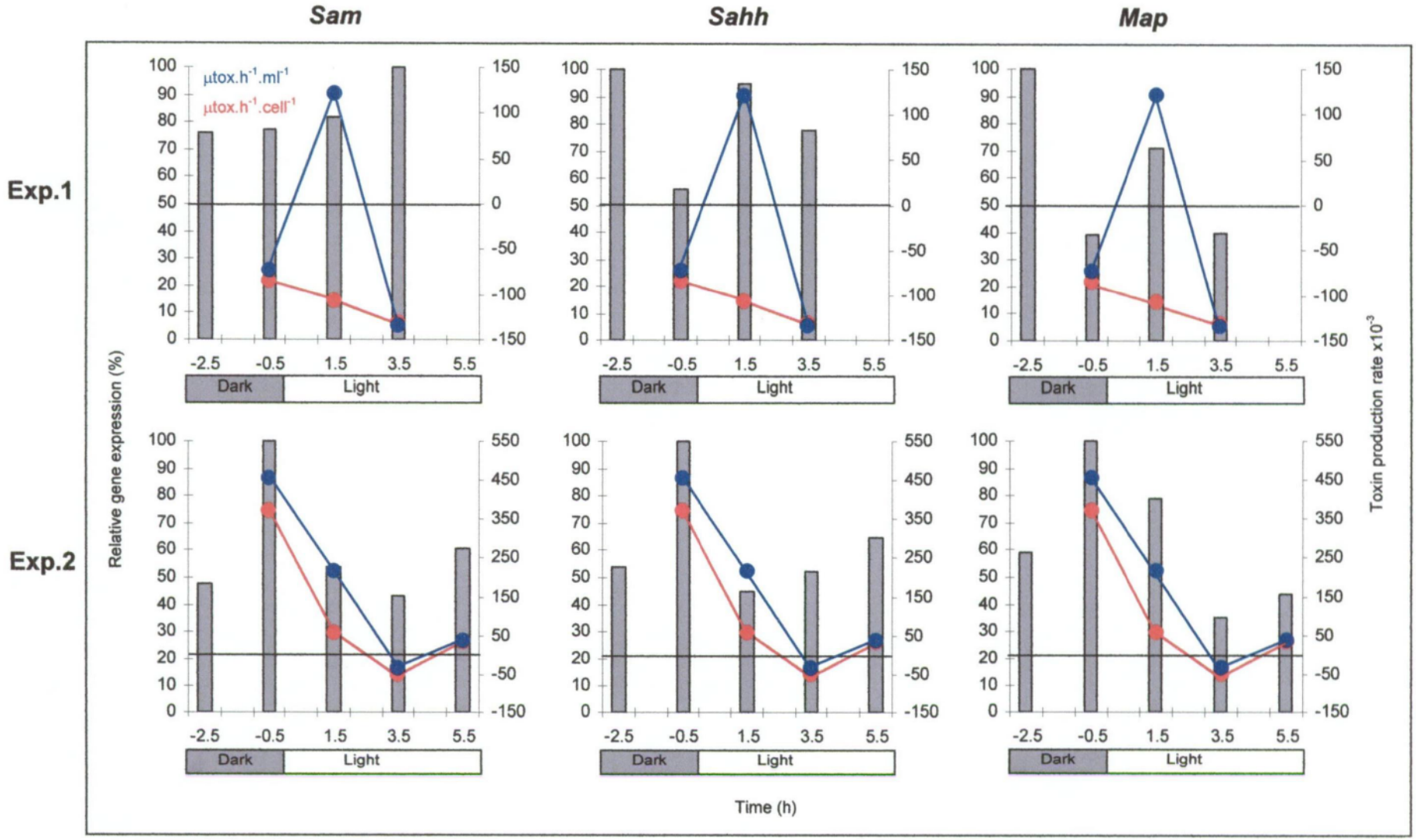
Sam
Sahh
Map
Exp.1
Exp.2
µtox.h-1.ml-1
µtox.h-1.cell-1
Relative gene expression (%)
Toxin production rate x10-3
Time (h)
Dark
Light
-2.5
-0.5
1.5
3.5
5.5

Figure 6-22. Relative expression levels (bars) of three putative toxin genes: S-adenosylmethionine (*Sam*), S-adenosylhomocysteine hydrolase gene (*Sahh*) and methionine aminopeptidase (*Map*) over the cell division cycle of *Alexandrium catenella* CAWD44. The rate of total toxin (all STX derivatives) production (●) calculated per cell (red) and per ml (blue) is indicated. In Experiment 1, all toxin production rates were negative and the scale is ~6.9 times smaller than in Experiment 2. Samples were collected at 2-h intervals before (dark) and after (light) the culture room lights came on.

6.4 Discussion

This work provides the first characterisation of cellular toxin dynamics and gene expression concurrently over the CDC of *A. catenella* CAWD44. Light and the cell cycle phase were examined as factors that could influence toxin biosynthesis. The expression of one novel dinoflagellate gene, *Sam*, and two *A. catenella* genes, *Sahh* and *Map* previously identified in the PSP dinoflagellate *A. fundyense*, were investigated. The gene ubiquitin (*Ubi*) was identified for the first time in a dinoflagellate and used as an internal control for QPCR. Gene expression studies were made possible by the development of a simple RNA extraction protocol (Chapter 3).

Two approaches for comparative studies of toxin production in *Alexandrium* include: (i) to compare toxin production of non-toxic and toxic strains or (ii) to study a single toxic strain at times of differential toxin production. The second approach was taken for a number of reasons. The University of Tasmania holds several non-toxic *A. tamarense* strains from the unique non-toxic Australian ribotype, which is genetically distinct from the toxic north American and Asian ribotypes (John *et al.* 2003b). In comparison, *A. catenella* strains from all ribotypes are consistently toxic. Consequently, no two toxic and non-toxic strains of the same species and of the same ribotype could be compared. To avoid confounding factors when comparing gene expression it would be essential that the cultures differed solely in their ability to produce PSTs.

In order to identify times in the cell cycle when toxin was produced, the method of Taroncher-Oldenburg & Anderson (2000) was followed. However, the authors identified many differentially expressed genes during toxin production, including CDC related genes. Therefore, this study selected three candidate toxin related genes and used QPCR technology, rather than differential display. QPCR offers advantages over the differential display method for studying gene expression. Errors in quantitation of RNA and cDNA were accounted for by normalising gene expression to *Ubi*. Additionally, the assessment of gene expression in QPCR was based on gene copy number rather than on visual interpretation of radiolabelled PCR

products, and should have been able to detect finer scale variation. This was considered useful when observing small-scale fluctuations in cellular toxin production and gene expression, which occurred between 2-h sampling events.

Flow cytometry was successfully applied to *A. catenella* CAWD44 to determine the relative proportions of cells in the different cell cycle phases. Determination of absolute genome size would require the development of size standards suitable for the often-large genome size of dinoflagellates. A high chromosome count has been reported for the genus *Alexandrium*, with *A. tamarense* possessing 143 chromosomes (Rees & Hallegraeff 1991). Importantly, standard concentrations of propidium iodide appeared insufficient to saturate the DNA of *A. catenella* cells and therefore a higher concentration (100 $\mu g.ml^{-1}$) was used.

In accordance with the literature for *Alexandrium* (such as Cembella *et al.* 1990), the toxin profile of *A. catenella* CAWD44 did not change over the CDC. Sako *et al.* (1992) reported that in all *A. catenella* strains examined, the mole percentage of toxin composition did not significantly change in any growth phase. Similarly to Cawthron's findings, CAWD44 did not produce neoSTX, dcSTX and STX and only trace amounts of GTX1 and GTX3 were detected. However, unlike Cawthron, this study did not detect any GTX2 and the cellular concentrations of GTX6, GTX5 and GTX4 were slightly higher. Large quantities of the low specific toxicity C2 derivative were detected, with Cawthron reporting 85.4 $fmol.cell^{-1}$ (Veronica Beuzenberg pers. comm.) and this study finding a lower average of 47.9 $fmol.cell^{-1}$ (Experiment 1) and 29.8 $fmol.cell^{-1}$ (Experiment 2), which explains the relatively low total toxicity (9.0 $pgSTX\ equivalents.cell^{-1}$, Veronica Beuzenberg pers. comm.) and (4.2 to 4.9 $pgSTX\ equivalents.cell^{-1}$, this study) of CAWD44 cells. Variation in toxicity measured as Q_t may have been influenced by the cell density, growth phase and/or age of cells (Sako *et al.* 1992, Kim *et al.* 1993), intensity of light exposure (Ogata *et al.* 1987) as well as bacterial load of the culture (Hold *et al.* 2001a). Future work should study gene expression under conditions

known to enhance PST synthesis such as low light (Ogata *et al.* 1987) and compare this to times of lower (e.g. high light) or no toxin production.

6.4.1 Cell division cycle timing

A study of *A. catenella* by Siu *et al.* (1997) reported that the G_1 phase ended at approximately 3 h after onset of darkness, and the G_2+M phase had begun at 4 h. Similarly, Taroncher-Oldenburg *et al.* (1997) showed that *A. fundyense* entered S phase 3-6 h after the onset of darkness with cell proliferation lasting for approximately 9 h. In the present study, *A. catenella* CAWD44 cells had already entered S phase and G_2+M phase at the commencement of the two experiments at 2.5 h before light exposure. Therefore, an extended sampling time is required in future experiments to determine when DNA synthesis commences for this isolate. The intensive sampling requirements, low density of cells and subsequent large volumes required to be harvested, meant that sampling events were restricted to the time of predicted toxin production and experiments had to be conducted individually. Future work could involve continuous sampling over multiple light:dark phases at more regular intervals (e.g. 1-h instead of 2-h), but would require larger culture volumes. Due to the complex behaviour of *Alexandrium* which is influenced by turbulence (MacIntyre *et al.* 1997, Karp-Boss *et al.* 2000, Yamamoto *et al.* 2002, Sullivan *et al.* 2003) and therefore the difficulty of mixing cultures (John & Flynn 2002), it is likely that cell counts from large cultures (such as bag cultures) would not accurately reflect cell densities. Cell counts from sub-samples would not be reliable for estimating cell growth rates and an alternative method, such as flow cytometry (used in this study), would be essential for the determination of cell division timing and the relative proportion of dividing cells.

6.4.2 Gene expression and cellular toxin dynamics

A sudden decrease in endocellular toxin has been reported to coincide with cell division in *A. tamarense* and *A. catenella* (Kim *et al.* 1993). It is suggested that dividing cells repeatedly lose toxin to daughter cells such that Q_t remains stable (Lippemeier *et al.* 2003) or decreases (John & Flynn 2002)

during fast division. The latter was true in Experiment 1, while in Experiment 2, a slower rate of division was observed and Q_t increased and then stabilised over the 8-h period. It may have been that while some cells were dividing, others were synthesising toxin, resulting in a counteracting effect on the average Q_t, with Q_t low in newly divided cells and high in older cells.

Endocellular toxin was analysed and reported as a total relative to the culture volume (toxin.ml^{-1}) or as a total average relating to cell density (toxin.cell^{-1}). Comparison of both values allowed the interpretation of whether toxin had been partitioned in dividing cells or if there had been a net loss of toxin. If toxin had been lost from cells due to partitioning in daughter cells, then this would appear as a decrease in toxin.cell^{-1} and stable or increase in toxin.ml^{-1} (remembering that toxin.ml^{-1} is a measure of endocellular toxin and does not include any toxin potentially contained in the medium). A decrease in toxin.ml^{-1} would indicate a net loss of endocellular toxin, which could have been lost to the surrounding medium either by excretion, leakage or cell lysis (John & Flynn 2002). A loss of cellular toxin could also be accounted for by turnover/degradation of toxin within the cell. During fast cell division and nitrogen stress, competition for nitrogen precursors required for PST synthesis may occur (Siu *et al.* 1997, John & Flynn 2002).

Exocellular PSTs dissolved in seawater can be detected using an activated charcoal column and HPLC analysis (Suzuki *et al.* 2003), but the technique is yet to be refined. Also, it is unclear whether dinoflagellates actively excrete PSTs (see 1.3). Future work measuring both the endocellular and exocellular PST content of a culture would be valuable in the interpretation of a loss of cellular toxin.

Synchronised cultures are difficult to achieve with dinoflagellates (Taroncher-Oldenburg *et al.* 1997) and this was only partially achieved in the present study. RNA extracted from cells sampled at discrete time points represented an average of the physiological status of cells in several different metabolic states. In both experiments, the most synchronous division occurred over the dark:light transition with light potentially acting as a trigger for cytokinesis.

Although the presence of two populations of dividing cells was likely in Experiment 2, the first division which occurred over the dark:light transition was the largest.

Toxin production appeared to be discontinuous over the CDC and was related to the CDC phase. Toxin production coincided with a higher proportion of cells in the early G_1 phase, confirming findings by Taroncher-Oldenburg & Anderson (2000) but in contrast to the study of *A. catenella* by Siu *et al.* (1997) which report PST synthesis to occur in the S phase. Taroncher-Oldenburg & Anderson (2000) report the time of toxin production to coincide with light exposure but it was not known whether the two were coupled. In the present study, the expression of *Sam*, *Sahh* and *Map* was different between the two experiments and did not show the same trend over the dark:light transition. Therefore, the changes observed in the expression of these genes were not solely induced by light exposure. Furthermore, a positive rate of cellular toxin production was observed in the dark, contradicting current thinking that toxin production occurs only in the light (Ogata *et al.* 1987, John & Flynn 2002).

The most interesting gene was *Map*, which showed a strong and consistent positive correlation (r) with the rate of toxin production in the culture. These findings support those of Taroncher-Oldenburg & Anderson (2000) who found that *Map* was upregulated during toxin production. Therefore *Map* is a good candidate in the biosynthetic pathway for PST production. Similarly to *Map*, the expression of *Sahh* was also positively correlated with the rate of toxin production in both experiments, an observation contradictory to those by Taroncher-Oldenburg & Anderson (2000). The latter authors found that *Sahh* was down regulated during toxin production and the early G_1 phase of the CDC. These results are inconclusive as to whether *Sahh* is involved in PST production.

The methionine methyl group of SAM is incorporated into the PST molecule in dinoflagellates (Shimizu *et al.* 1990a, 1990b, Shimizu 1996), implicating an as yet undefined role of *Sam* in toxin production (refer to 4.1.4). It is likely

that *Sam* is involved early in the PST biosynthesis pathway and may not regulate toxin production directly. The expression of *Sam* was inconsistent with the rate of cellular toxin production between the two experiments. Therefore *Sam* activity did not solely relate to toxin production. The two experiments conducted were independent and showed different rates of cell division suggesting that different cellular processes were occurring, possibly explaining different patterns of *Sam* expression. The CDC therefore complicated interpretation of toxin production and gene expression.

Isolation of a single metabolic pathway for toxin production is problematic. The three genes examined, *Sam*, *Sahh* and *Map* are involved in methyl metabolism and are therefore essential to normal functioning of dinoflagellate cells. It is logical to infer that these genes must be involved in many metabolic pathways, including PST biosynthesis. It is probable that *Sam* and *Sahh* are not solely toxin-related genes and are involved in other metabolic pathways (Thomas & Surdin-Kerjan 1987, Chiang 1998 respectively). Repeated experiments and longer sampling may provide key information about gene expression and toxin production at discrete time points during synchronous growth.

6.4.3 Conclusions

This study has demonstrated the potential of QPCR as a means of studying transcriptionally regulated genes in dinoflagellates. Identification of the genes required for toxin biosynthesis and the mechanism(s) by which they are regulated should provide insight into the functional significance of PSTs. Firstly, like Plumley (1997) and Cembella (1998), this study recognises the need for a better understanding of the cellular toxin dynamics of dinoflagellates. While net changes in toxin content are known to occur, knowledge of the gross rates of change due to synthesis and loss, and their regulatory basis, are weak (John & Flynn 2002).

The present study has highlighted gaps in our knowledge of dinoflagellate genomes, particularly of toxigenic strains. Further understanding of the codon usage, base modifications and secondary structure of dinoflagellate DNA will facilitate the identification of toxin related genes as well as other

novel dinoflagellate genes. Future identification of genes specific to PST biosynthesis may provide genetic markers to identify the presence of toxigenic species. Such markers would have applications for aquaculture and ballast water monitoring. Although it is unlikely that *Map*, *Sahh* and *Sam* are involved exclusively in PST production, the identification of the novel dinoflagellate gene *Sam*, and the study of the expression of candidate toxin related genes using QPCR is significant. Importantly, the strong positive correlation of *Map* expression and the rate of toxin production in *Alexandrium sp.* in two independent experiments and in a study by separate authors (Taroncher-Oldenburg & Anderson 2000), strongly supports the involvement of this gene in PST production in dinoflagellates. *Sahh* is also of interest as a candidate PST related gene and more extensive research of the expression of *Sahh* and *Map* during toxin production would be valuable.

Chapter 7: General discussion

7.1 APPROACH AND MAIN FINDINGS

In order to identify novel dinoflagellate PST gene(s) it is important to understand the taxonomic affinities and ecophysiology of the organism. Understanding the phylogenetic relationship between dinoflagellates and other organisms is necessary where a PCR-based approach is used to identify homologous genes (Chapter 4). It is practical to select the most closely related organism for which the gene of interest is known and design primers to this gene. If the gene has not been identified in any closely related microalgae (as was the case with the candidate PST gene *Sam* encoding the enzyme S-adenosylmethionine synthetase), then the task of primer design becomes more difficult. It may be necessary to select a gene that shows a high level of conservation and design degenerate primers to the region of the gene that is most similar in distantly related organisms. An understanding of cellular features such as the properties of the DNA and, in this case, their relationship to the cell division cycle was fundamental to gene identification. Throughout this thesis standard molecular techniques were tailored to suit the unusual features of dinoflagellates.

Marine dinoflagellates exist in association with a range of microbiota, predominantly bacteria (Chapter 2). Laboratory grown dinoflagellate cultures commonly contain numerous bacterial contaminants that may be present in the culture medium (Gallacher *et al.* 1997, Groben *et al.* 2000), epiphytic on dinoflagellate cells (Biegala *et al.* 2002, Simon *et al.* 2002), or intracellular in dinoflagellate cells or cysts (Silva 1990, Cordova *et al.* 2002). Very few molecular protocols have been developed for dinoflagellates. Dinoflagellates possess numerous unusual DNA properties and cell division features (reviewed in de la Espina *et al.* 2005), including permanently condensed chromosomes (Costas & Goyanes 2005), unusually large amounts of nuclear DNA (Wong *et al.* 2003), unusual chromosome structure (Steele & Rae 1980), a very low level of basic proteins bound to the chromatin (Guillebault *et al.* 2002), unique cell division (Michel *et al.* 1996), unique chloroplast genes (Zhang *et al.* 2002a), often repetitive sequence in the non-coding

(Moreau *et al.* 1998) or coding DNA (Zhang & Lin 2003), large introns or multiple copies of genes (Zhang & Lin 2003) and, in some species, a high frequency of the rare base hydroxymethyluracil (Steele & Rae 1980, Soyer-Gobillard *et al.* 1999). The complexities and lack of technical knowledge on dinoflagellate DNA, and the close association of dinoflagellates and bacteria make the study of dinoflagellate genetics a challenging task. These factors have posed a major stumbling block in understanding the regulation of dinoflagellate PST production at the cellular and genetic levels.

The association of bacteria and dinoflagellates in culture presents difficulties for molecular genetic research (Chapter 2). For example, the dinoflagellate strains in this study could not be cultured without associated bacteria, and genomic DNA extractions were contaminated with bacterial DNA. Therefore, PCR-based identification of novel dinoflagellate genes from genomic DNA was compromised. A practical approach for isolating dinoflagellate genes was developed. To confirm that *Sam* amplified from genomic DNA (Chapters 4 and 5) was truly dinoflagellate (eukaryotic) in origin and not from a contaminating bacterium (prokaryote), the same gene was amplified from complementary DNA (cDNA) synthesised using eukaryotic specific primers [Oligo (dT) primers] (Chapter 6). The ability to isolate intact total RNA was fundamental to cDNA synthesis and gene expression studies (Chapter 3).

Different RNA preservation and isolation methods were evaluated. A nitrogen decompression chamber has previously been used for disruption of dinoflagellate cells (Scholin *et al.* 1993, Taroncher-Oldenburg & Anderson 2000), and while effective, this method is expensive. A simpler, more amenable method of isolating dinoflagellate RNA, applicable to armoured and unarmoured dinoflagellates, using equipment that is more widely available in molecular laboratories was developed (Chapter 3). This required taking into account the physical properties of various dinoflagellate species. A method of preserving RNA in intact dinoflagellate cells, suitable for samples collected in the field or when samples could not be processed immediately, was developed. Extraction of high quality RNA from

dinoflagellate cells was confirmed using quantitative real time PCR in the study of candidate PST genes (Chapter 6).

The candidate PST gene *Sam* was identified for the first time in dinoflagellates (Chapter 4). Sequence information for *Sam* in a range of dinoflagellates was presented, with multiple copies of *Sam* being identified in individual isolates (Chapter 5). At the nucleotide level, dinoflagellate *Sam* showed no significant similarity to *Sam* from sequences of other species in GenBank, highlighting the different codon usage of dinoflagellates. At the protein level dinoflagellate *Sam* was conserved but was not more similar to any particular group of organisms. The closest match of the most frequently isolated copy of *Sam* was to the mosquito, *Anopheles gambia*, possibly reflecting the lack of available sequence information for the Protista. The improved knowledge of dinoflagellate codon usage through sequencing of *Sam* should facilitate the discovery of novel dinoflagellate genes.

The only other study known to have sequenced any candidate PST genes was by Taroncher-Oldenburg & Anderson (2000). Two highly expressed genes were identified in the PSP dinoflagellate *Alexandrium fundyense*, *Sahh* encoding the enzyme S-adenosylhomocysteine hydrolase and *Map* encoding the enzyme methionine aminopeptidase. Their study showed that expression of *Sahh* (downregulated) and *Map* (upregulated) coincided with a positive rate of toxin production, which occurred during early G_1 phase of the cell division cycle.

The correspondence between the expression of *Sam*, *Sahh* and *Map* and PST biosynthesis and/or degradation was investigated (Chapter 6). The marine dinoflagellate *Alexandrium catenella* was used as a model as it is amenable to bulk culture. Other known PST producing dinoflagellates such as *Gymnodinium catenatum* are more difficult to culture in bulk. PST production in *A. catenella* cells was measured concurrently with gene expression over the cell division cycle (Chapter 6). The times and conditions when PST synthesis was active versus inactive were assessed (Chapter 6). A comprehensive knowledge of the toxicity, toxin profile, and how these

parameters change under different environmental growth conditions was required for such work (reviewed in Chapter 1).

As found by Taroncher-Oldenburg & Anderson (2000), *Map* was upregulated during times of positive toxin production per cell. Upregulation of *Sahh* (Chapter 6) was inconsistent with data from Taroncher-Oldenburg & Anderson (2000). Gene expression and toxin production did not consistently coincide with the dark:light transition, suggesting that light was not a direct trigger for toxin production. The findings of a positive rate of toxin production half an hour before light exposure was contradictory to the results observed in Taroncher-Oldenburg & Anderson (2000) and the model developed by John & Flynn (2002), which report that the cellular toxin quota only increased in the light phase.

7.2 FUTURE DIRECTIONS

It is probable that some of the genes in the PST biochemical pathway are unique and, although these genes may belong to common gene families, homologous genes would not exist in non-PST producing organisms. Therefore, degenerate primer design cannot be used to identify all PST genes. A lack of knowledge of the intermediate molecules in the PST biosynthesis pathway means that we are currently limited to searching for the few enzymes (and genes that encode them) at the terminal steps of the pathway, or those indirectly associated with PST synthesis such as *Sam*, *Sahh* or *Map*. Therefore, with current knowledge, this method is most successfully used to identify precursor and toxin modifying genes only. A system biology approach will be required to fully understand PST biosynthesis at both the genetic and functional levels: to elucidate the biochemical pathway(s) involved, the physiological and environmental parameters that influence cellular toxicity, and the role of PSTs in dinoflagellate metabolism. Isolation of a single PST gene could facilitate the identification of other PST genes by applying standard molecular techniques such as primer walking PCR (Plumley 2001).

Methods for identifying toxin genes are in the early stages of development (Plumley 1997). Methods used in the past to understand the biochemical pathway of PST synthesis have been: (i) stable isotope studies (Shimizu *et al.* 1985, Shimizu 1996), (ii) purifying and characterising enzymes (Ishida *et al.* 1998, Plumley 2001), (iii) differential display to identify candiate genes (Taroncher-Oldenburg & Anderson 2000) and (iv) the present work of degenerate primer design and PCR on cDNA. These methods provided an understanding of the toxigenicity of dinoflagellate strains, however they are not without their problems and it is expected that other approaches will be needed to identify the genetic basis of dinoflagellate PST synthesis.

The successful application of differential display for identification of candidate PST genes indicates that other global gene expression methods, such as microarray and subtractive hybridisation technologies, could be applicable in the study of PST genetics. Another approach, albeit time consuming, could be to clone the entire genome of a toxigenic dinoflagellate in *Escherichia coli* and screen transformed colonies for toxin production (Plumley 1997). Ideally, toxic and non-toxic strains of the same ribotype within species should be included in studies to provide a valid genetic comparison. However, at present, such cultures are not available. Mutagenesis has been proposed as a method for obtaining non-toxic isolates from toxic isolates (Plumley 1997). However, this method would be time consuming and is problematic for PST producing dinoflagellates (Plumley 1997). The ability to grow *Gymnodinium catenatum* cells on soft agar (see Chapter 2) is a positive step towards being able to develop a method of mutagenesis in PST producing dinoflagellates.

The focus of this thesis has been on PST producing dinoflagellates, however some species of cyanobacteria (Negri & Jones 1995, Velzeboer *et al.* 2001) and other bacteria (Silva 1990, Cordova *et al.* 2002) produce neurotoxins identical to those of dinoflagellate PSTs (Llewellyn *et al.* 2001). It has not been conclusively established that bacteria are involved in dinoflagellate PST biosynthesis. Strategies to investigate the importance of bacterial genes in the biosynthesis of toxic compounds were investigated by Plumley *et al.* (1999). PST genes may have evolved once (Plumley 1997), probably in a

prokaryote and these may have been transferred to other aquatic microorganisms through endosymbiosis. Cembella (1998) discusses a polyphyletic origin of the capacity for PST production. Some authors suggest that the neurotoxic cyanobacterium *Anabaena circinalis* may provide a parallel yet simpler system in which to study PST biosynthesis (Pomati & Neilan 2004). For example, it has been suggested that cyanobacteria may be more amenable to molecular genetic protocols such as mutational analysis of transformants, however this has not been demonstrated (Plumley 2001).

7.3 SIGNIFICANCE AND CONCLUSIONS

Dinoflagellates are a diverse group of microalgae with numerous peculiar genetic features (Costas & Goyanes 2005) that render dinoflagellates useful models to provide novel insight into cellular processes (Guillebault *et al.* 2001). Elucidation of genes involved in the biosynthesis and degradation pathways in PSP dinoflagellates has the potential to provide scientists with useful tools for: (i) detection of harmful species before they reach high enough densities associated with toxicity, to cause problems to humans and other mammals, (ii) estimation of the toxicity of species based on the activity of a gene or genes, (iii) the ability to control toxin production of microalgae *in vitro*, with the potential to up-regulate or turn off toxin production all together (this would have spin-offs for identifying other toxin related genes and perhaps also pharmacological benefits), (iv) performing inheritance studies of toxicity at genetic and physiological levels and (v) investigating the production of PSTs by cyanobacteria and other bacteria. Information gleaned from this study will facilitate future genetic studies which will potentailly answer the questions: what genes control the biochemical pathway of PST synthesis and what role do PSTs play in dinoflagellate ecophysiology?

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APPENDIX 1

Modified CTAB DNA extraction protocol for dinoflagellates. Volumes for a mini prep are described which uses a maximum of a 100 mg tissue, equivalent to ~100 µl of packed cells. The procedure can be scaled up accordingly and was modified from step 7 onwards.

1. Prepare CTAB buffer in fumehood (500 µl/sample):
    i. CTAB buffer (pre-filtered) 12.5 ml
    ii. PVP (polyvinyl pyrrolidone) 0.5 g
    iii. β- Mercaptoethanol 1M (toxic) 25 µl (i.e. 0.02 ml/10 ml buffer)
2. Extract 15-50 ml mid-log phase dinoflagellate culture by centrifugation at 2,000 rpm for 5 min.
3. Transfer concentrated cells to 1.5 ml eppendorf tubes and centrifuge at 10,000 rpm for 30 s to form a pellet. Pellet should be ~30-100 µl in size.
4. Resuspend pellet in 500 µl CTAB buffer (in fumehood). Add 5 µl Proteinase K (20mg/ml) to each sample. Mix well.
5. Incubate at 65°C for 60 min, mixing occasionally.
6. Extract with 600 µl chloroform:isoamyl (24:1). Mix slowly by inversion, then faster. Centrifuge for 10 min at 4°C. Place supernatant in a fresh tube.
7. Add 1/10th volume 3 M NaAcetate pH5.2 (sterile filtered) = 50 µl. Add 2 volumes (900 µl) ice cold 95-100% ethanol.
8. Do not mix. Place tube on ice 2 min. Mix by inversion. A cloud of precipitate DNA should form at interphase.
9. Place in ice bath for 10 min or freezer (up to an hour) to allow DNA to precipitate.
10. Centrifuge at 14,000 rpm for 5 min and discard ethanol.
11. Carefully wash pellet with 400 µl 76% ethanol/0.01M NaAcetate for 10 s. Centrifuge to remove wash solution.
12. Air dry upside-down or vacuum dry (no heat). Do not let the DNA pellet dry too much.
13. Dissolve pellet in appropriate amount of distilled water.

APPENDIX 2

Preparation of hybridisation solutions for fluorescence *in situ* hybridisation.

EUB

1.04 g NaCl
10 ml Tris HCl (0.04M) pH 7.4
200 µl SDS (1%)
9.8 ml MilliQ (→ 20ml)

β and γ

1.04 g NaCl
10 ml Tris HCl (0.04M) pH 7.4
200 µl SDS (1%)
7 ml deionised formamide
2.8 ml MilliQ (→ 20 ml)

α

1.04 g NaCl
10 ml Tris HCl (0.04M) pH 7.4
200 µl SDS (1%)
4 ml deionised formamide
5.8 ml MilliQ (→ 20 ml)

Cytophaga/Flavo

1.04 g NaCl
10 ml Tris HCl (0.04M) pH 7.4
200 µl SDS (1%)
*3 ml deionised formamide
6.8 ml MilliQ (→ 20 ml)
*can use between 4-7 ml (i.e. β, γ, α)

Make up solution lastly adding formamide. Filter sterilise (before or after adding formamide) and dispense into aliquots of approx. 3-5 ml. If stored frozen, solutions will keep for at least one year.

APPENDIX 3

Preparation of wash solutions for fluorescence *in situ* hybridisation (FISH). For the fixation and probe hybridisation of cells on filters, Dr Jenny Skerrat (CSIRO Marine Research, Hobart, Australia) has successfully used EUBwash for all subclasses of bacteria i.e. α, β, γ, and *Cytophaga* species. In this study, the EUBwash was successfully used for three species of dinoflagellate: *Gymnodinium catenatum, Alexandrium catenella* and *Alexandrium minutum*.

EUB (100 ml)

2 ml Tris HCl (1M) pH 7.4
1 ml EDTA (0.5M) pH 8.0
1 ml SDS 1%
5.62 g NaCl
96 ml H_2O

β, γ and cytophaga/flavo (100 ml)

2 ml Tris HCl (1M) pH 7.4
1 ml EDTA (0.5M) pH 8.0
1 ml SDS 1%
0.46 g NaCl
96 ml H_2O

α (100 ml)

2 ml Tris HCl (1M) pH 7.4
1 ml EDTA (0.5M) pH 8.0
1 ml SDS 1%
1.32 g NaCl
96 ml H_2O
Filter sterilise (optional)

Stock solutions required:

- 0.04M Tris HCl pH 7.4
  (1.211 g in 200 ml milliQ water). Autoclave and store in fridge.
- 0.10 Tris HCl pH 7.4
  (3.141 g in 200 ml milliQ water). Autoclave and store in fridge.
- 0.5M EDTA pH 8.0
  (37.2 g in 200 ml milliQ water). Autoclave and store in fridge.
- 1% SDS. Do not filter, autoclave (may froth) and store at room temperature. Alternatively store in fridge.
- 1x Tris HCl. Filter sterilise, autoclave, store in fridge.

Appendix 4

Dinoflagellate DNA clones obtained by PCR using different primer combinations to the gene S-adenosylmethionine synthetase (*Sam*). Clones that contained different sized inserts, including the expected size, were deliberately picked for sequencing. Clones listed were sequenced and a BLAST search of GenBank performed to determine which clones matched *Sam*. Clones 1 to 115 produced the closest match to *Sam* genes in GenBank whilst only some clones from 130 onwards matched *Sam* (as indicated by highlighting). Clone 175 matched the form II rubisco gene from dinoflagellates. (No insert) indicates that the cloning was unsuccessful, (?) indicates that the full length of the insert was not determined and the insert was not likely to be *Sam*, (No match) indicates that the sequence did not produce a match to any sequences in GenBank, and (Poor seq) indicates that the sequencing results were of poor quality and a search of GenBank could not be performed. Note that sequencing of clones 81 to 85 for *Gymnodinium aureolum* GAGL01 isolated from Gipsland Lakes, VIC, Australia, revealed no insert.

Clone #	Primers	Strain	DNA	Clone Name	Insert bp
Clones produced using degenerate primers with a reduced GC content to plant Sam					
1	SAMFC3/RF2	CAWD106	gDNA	SAM-1	430
2	SAMFC3/RF2	CAWD106	gDNA	SAM-2	430
3	SAMFC3/RF2	CAWD106	gDNA	SAM-3	429
4	SAMFC3/RF2	CAWD106	gDNA	SAM-4	431
5	SAMFC3/RF2	CAWD106	gDNA	SAM-5	430
6	SAMFC3/RF2	GCJP10	gDNA	SAM-6	399
7	SAMFC3/RF2	GCJP10	gDNA	SAM-7	399
8	SAMFC3/RF2	GCJP10	gDNA	SAM-8	392
9	SAMFC3/RF2	GCJP10	gDNA	SAM-9	No insert
10	SAMFC3/RF2	GCJP10	gDNA	SAM-10	399
11	SAMFC3/RF2	ACAD01	gDNA	SAM-11	430
12	SAMFC3/RF2	ACAD01	gDNA	SAM-12	430
13	SAMFC3/RF2	ACAD01	gDNA	SAM-13	No insert
14	SAMFC3/RF2	ACAD01	gDNA	SAM-14	430
15	SAMFC3/RF2	ACAD01	gDNA	SAM-15	430
16	SAMFC3/RF2	ACSP01	gDNA	SAM-16	435
17	SAMFC3/RF2	ACSP01	gDNA	SAM-17	435
18	SAMFC3/RF2	ACSP01	gDNA	SAM-18	435
19	SAMFC3/RF2	ACSP01	gDNA	SAM-19	436
20	SAMFC3/RF2	ACSP01	gDNA	SAM-20	438
21	SAMFC3/RF2	AMAD06	gDNA	SAM-21	435
22	SAMFC3/RF2	AMAD06	gDNA	SAM-22	435
23	SAMFC3/RF2	AMAD06	gDNA	SAM-23	435
24	SAMFC3/RF2	AMAD06	gDNA	SAM-24	435
25	SAMFC3/RF2	AMAD06	gDNA	SAM-25	435
26	SAMFC3/RF2	AMNC04	gDNA	SAM-26	438
27	SAMFC3/RF2	AMNC04	gDNA	SAM-27	438
28	SAMFC3/RF2	AMNC04	gDNA	SAM-28	438
29	SAMFC3/RF2	AMNC04	gDNA	SAM-29	438
30	SAMFC3/RF2	AMNC04	gDNA	SAM-30	438

Clones produced using primers specific to dinoflagellate Sam clone SAM-1					
31	DinoSAM1F/1R	ACCC01	gDNA	SAM-31	388
32	DinoSAM1F/1R	ACCC01	gDNA	SAM-32	388
33	DinoSAM1F/1R	ACCC01	gDNA	SAM-33	388
34	DinoSAM1F/1R	ACCC01	gDNA	SAM-34	388
35	DinoSAM1F/1R	ACCC01	gDNA	SAM-35	388
36	DinoSAM1F/1R	ACAM03	gDNA	SAM-36	388
37	DinoSAM1F/1R	ACAM03	gDNA	SAM-37	No insert
38	DinoSAM1F/1R	ACAM03	gDNA	SAM-38	388
39	DinoSAM1F/1R	ACAM03	gDNA	SAM-39	388
40	DinoSAM1F/1R	ACAM13	gDNA	SAM-40	388
41	DinoSAM1F/1R	ACNC50	gDNA	SAM-41	388
42	DinoSAM1F/1R	ACNC50	gDNA	SAM-42	388
43	DinoSAM1F/1R	ACNC50	gDNA	SAM-43	388
44	DinoSAM1F/1R	ACNC50	gDNA	SAM-44	388 (1 gap)
45	DinoSAM1F/1R	ACNC50	gDNA	SAM-45	388
46	DinoSAM1F/1R	ATBB01	gDNA	SAM-46	388
47	DinoSAM1F/1R	ATBB01	gDNA	SAM-47	388
48	DinoSAM1F/1R	ATBB01	gDNA	SAM-48	388
49	DinoSAM1F/1R	ATBB01	gDNA	SAM-49	388
50	DinoSAM1F/1R	ATBB01	gDNA	SAM-50	No insert
51	DinoSAM1F/1R	ATHK01	gDNA	SAM-51	388
52	DinoSAM1F/1R	ATHK01	gDNA	SAM-52	388
53	DinoSAM1F/1R	ATHK01	gDNA	SAM-53	388
54	DinoSAM1F/1R	ATHK01	gDNA	SAM-54	388
55	DinoSAM1F/1R	ATHK01	gDNA	SAM-55	388
56	DinoSAM1F/1R	PMPL01	gDNA	SAM-56	388
57	DinoSAM1F/1R	PMPL01	gDNA	SAM-57	388
58	DinoSAM1F/1R	PMPL01	gDNA	SAM-58	388
59	DinoSAM1F/1R	PMPL01	gDNA	SAM-59	388
60	DinoSAM1F/1R	PMPL01	gDNA	SAM-60	388
61	DinoSAM1F/1R	NOCTILUCA	gDNA	SAM-61	388
62	DinoSAM1F/1R	NOCTILUCA	gDNA	SAM-62	388
63	DinoSAM1F/1R	NOCTILUCA	gDNA	SAM-63	388
64	DinoSAM1F/1R	NOCTILUCA	gDNA	SAM-64	388
65	DinoSAM1F/1R	NOCTILUCA	gDNA	SAM-65	388
66	DinoSAM1F/1R	AOKT02	gDNA	SAM-66	388
67	DinoSAM1F/1R	AOKT02	gDNA	SAM-67	388
68	DinoSAM1F/1R	AOKT02	gDNA	SAM-68	388
69	DinoSAM1F/1R	AOKT02	gDNA	SAM-69	No insert
70	DinoSAM1F/1R	AOKT02	gDNA	SAM-70	388 (3 gaps)
71	DinoSAM1F/1R	GCDE06C	gDNA	SAM-71	388
72	DinoSAM1F/1R	GCDE06C	gDNA	SAM-72	388
73	DinoSAM1F/1R	GCDE06C	gDNA	SAM-73	388
74	DinoSAM1F/1R	GCDE06C	gDNA	SAM-74	387 (1 gap)
75	DinoSAM1F/1R	GCDE06C	gDNA	SAM-75	388
76	DinoSAM1F/1R	GCDE11	gDNA	SAM-76	388
77	DinoSAM1F/1R	GCDE11	gDNA	SAM-77	No insert
78	DinoSAM1F/1R	GCDE11	gDNA	SAM-78	No insert
79	DinoSAM1F/1R	GCDE11	gDNA	SAM-79	388
80	DinoSAM1F/1R	GCDE11	gDNA	SAM-80	388
86	DinoSAM1F/1R	KDMSR01	gDNA	SAM-86	388
87	DinoSAM1F/1R	KDMSR01	gDNA	SAM-87	No insert

88	DinoSAM1F/1R	KDMSR01	gDNA	SAM-88	388
89	DinoSAM1F/1R	KDMSR01	gDNA	SAM-89	388
90	DinoSAM1F/1R	KDMSR01	gDNA	SAM-90	388
91	DinoSAM1F/1R	KBNZ01	gDNA	SAM-91	388 (3 gaps)
92	DinoSAM1F/1R	KBNZ01	gDNA	SAM-92	388
93	DinoSAM1F/1R	KBNZ01	gDNA	SAM-93	388
94	DinoSAM1F/1R	KBNZ01	gDNA	SAM-94	No insert
95	DinoSAM1F/1R	KBNZ01	gDNA	SAM-95	388
96	DinoSAM1F/1R	TTDE03	gDNA	SAM-96	388
97	DinoSAM1F/1R	TTDE03	gDNA	SAM-97	No insert
98	DinoSAM1F/1R	TTDE03	gDNA	SAM-98	388
99	DinoSAM1F/1R	TTDE03	gDNA	SAM-99	388
100	DinoSAM1F/1R	TTDE03	gDNA	SAM-100	No insert
101	DinoSAM1F/1R	KUTN05	gDNA	SAM-101	No insert
102	DinoSAM1F/1R	KUTN05	gDNA	SAM-102	388
103	DinoSAM1F/1R	KUTN05	gDNA	SAM-103	388
104	DinoSAM1F/1R	KUTN05	gDNA	SAM-104	388
105	DinoSAM1F/1R	KUTN05	gDNA	SAM-105	388
106	DinoSAM1F/1R	AMAD06	gDNA	SAM-106	?
107	DinoSAM1F/1R	AMAD06	gDNA	SAM-107	?
108	DinoSAM1F/1R	AMAD06	gDNA	SAM-108	?
109	DinoSAM1F/1R	AMAD06	gDNA	SAM-109	?
110	DinoSAM1F/1R	AMAD06	gDNA	SAM-110	?
111	DinoSAM1F/1R	AMNC04	gDNA	SAM-111	?
112	DinoSAM1F/1R	AMNC04	gDNA	SAM-112	?
113	DinoSAM1F/1R	AMNC04	gDNA	SAM-113	?
114	DinoSAM1F/1R	AMNC04	gDNA	SAM-114	?
115	DinoSAM1F/1R	AMNC04	gDNA	SAM-115	?
Clones produced by 3' RACE					
130	3'RACE-SAM1F	AMAD06	cDNA	SAM-130-1A	?
131	3'RACE-SAM1F	AMAD06	cDNA	SAM-131-2A	Poor seq
132	3'RACE-SAM1F	AMAD06	cDNA	SAM-132-3A	?
133	3'RACE-SAM1F	AMAD06	cDNA	SAM-133-4A7	?
134	3'RACE-SAM1F	AMAD06	cDNA	SAM-134-5A	?
135	3'RACE-SAM1F	AMAD06	cDNA	SAM-135-6A	516
136	3'RACE-SAM1F	AMAD06	cDNA	SAM-136-7A	513
137	3'RACE-SAM1F	AMAD06	cDNA	SAM-137-8A	595
138	3'RACE-SAM1F	AMAD06	cDNA	SAM-138-9A	?
139	3'RACE-SAM1F	AMAD06	cDNA	SAM-139-10A	?
140	3'RACE-SAM1F	AMAD06	cDNA	SAM-140-11A	334
141	3'RACE-SAM1F	AMAD06	cDNA	SAM-141-12A	?
142	3'RACE-SAM1F	AMAD06	cDNA	SAM-142-13A	410
143	3'RACE-SAM1F	AMAD06	cDNA	SAM-143-14A	?
144	3'RACE-SAM1F	AMAD06	cDNA	SAM-144-15A	?
145	3'RACE-SAM1F	AMAD06	cDNA	SAM-145-16A	?
146	3'RACE-SAM1F	AMAD06	cDNA	SAM-146-17A	350
147	3'RACE-SAM1F	AMAD06	cDNA	SAM-147-18A	?
148	3'RACE-SAM1F	AMAD06	cDNA	SAM-148-19A	?
149	3'RACE-SAM1F	AMAD06	cDNA	SAM-149-20A	350
150	3'RACE-SAM2F	AMAD06	cDNA	SAM-150-1B	?
151	3'RACE-SAM2F	AMAD06	cDNA	SAM-151-2B	687
152	3'RACE-SAM2F	AMAD06	cDNA	SAM-152-3B	Poor seq
153	3'RACE-SAM2F	AMAD06	cDNA	SAM-153-4B	768
154	3'RACE-SAM2F	AMAD06	cDNA	SAM-154-5B	Poor seq

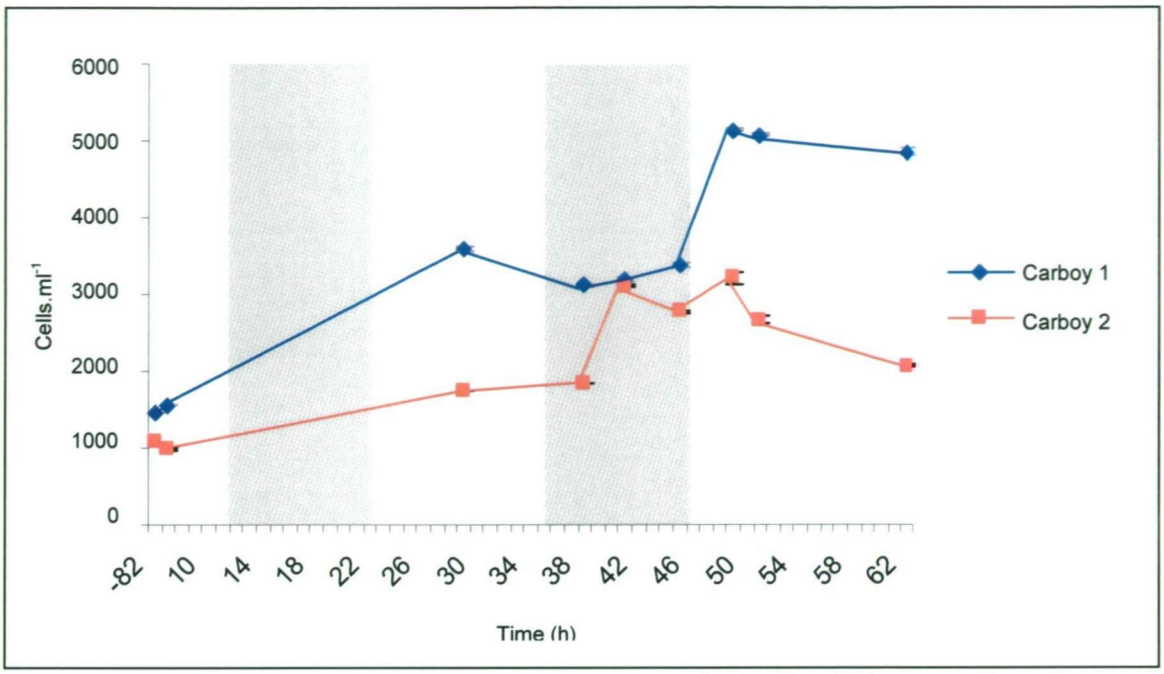
155	3'RACE-SAM2F	AMAD06	cDNA	SAM-155-6B	Poor seq
156	3'RACE-SAM2F	AMAD06	cDNA	SAM-156-7B	Poor seq
157	3'RACE-SAM2F	AMAD06	cDNA	SAM-157-8B	?
158	3'RACE-SAM2F	AMAD06	cDNA	SAM-158-9B	515
159	3'RACE-SAM2F	AMAD06	cDNA	SAM-169-10B	?
160	3'RACE-SAM2F	AMAD06	cDNA	SAM-160-11B	Poor seq
161	3'RACE-SAM2F	AMAD06	cDNA	SAM-161-12B	?
162	3'RACE-SAM2F	AMAD06	cDNA	SAM-162-13B	?
163	3'RACE-SAM2F	AMAD06	cDNA	SAM-163-14B	Poor seq
164	3'RACE-SAM2F	AMAD06	cDNA	SAM-164-15B	?
165	3'RACE-SAM2F	AMAD06	cDNA	SAM-165-16B	?
166	3'RACE-SAM2F	AMAD06	cDNA	SAM-166-17B	?
167	3'RACE-SAM2F	AMAD06	cDNA	SAM-167-18B	146
168	3'RACE-SAM2F	AMAD06	cDNA	SAM-168-19B	?
169	3'RACE-SAM2F	AMAD06	cDNA	SAM-169-20B	?
Clones produced from primers specific to *Arabidopsis thaliana* Sam					
170	Athal SAM1F/1R	CAWD106	cDNA	SAM-170-4	No match
171	Athal SAM1F/1R	CAWD106	cDNA	SAM-171-11	No match
172	Athal SAM1F/1R	CAWD106	cDNA	SAM-172-12	No match
173	Athal SAM1F/1R	AMAD06	cDNA	SAM-173-14	No match
174	Athal SAM1F/1R	AMAD06	cDNA	SAM-174-15	No match
175	Athal SAM1F/1R	AMAD06	cDNA	SAM-175-16	Rubisco
176	Athal SAM1F/1R	AMAD06	cDNA	SAM-176-18	No match
Clones produced from primers designed to clone SAM-136-7A (see Chapter 6)					
197	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-197	202
198	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-198	156
199	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-199	202
200	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-200	388
201	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-201	800
202	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-202	805
203	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-203	292
204	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-204	152
205	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-205	152
206	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-206	138
207	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-207	112
208	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-208	84
209	SAM-3'136-1F/1R	CAWD44	cDNA	SAM-209	111
210	SAM-3'136-1F/1R	CAWD44	gDNA	SAM-210	113

APPENDIX 5

Dinoflagellate DNA clones obtained by PCR using primers to the genes ubiquitin (*Ubi*), S-adenosyl-homocysteine hydrolase (*Sahh*), methionine aminopeptidase (*Map*) and histone like protein (*Haf*). Clones that contained different sized inserts, including the expected size, were deliberately picked for sequencing. Clones listed were sequenced and a BLAST search of GenBank performed to determine which clones matched the target gene (as indicated by highlighting).

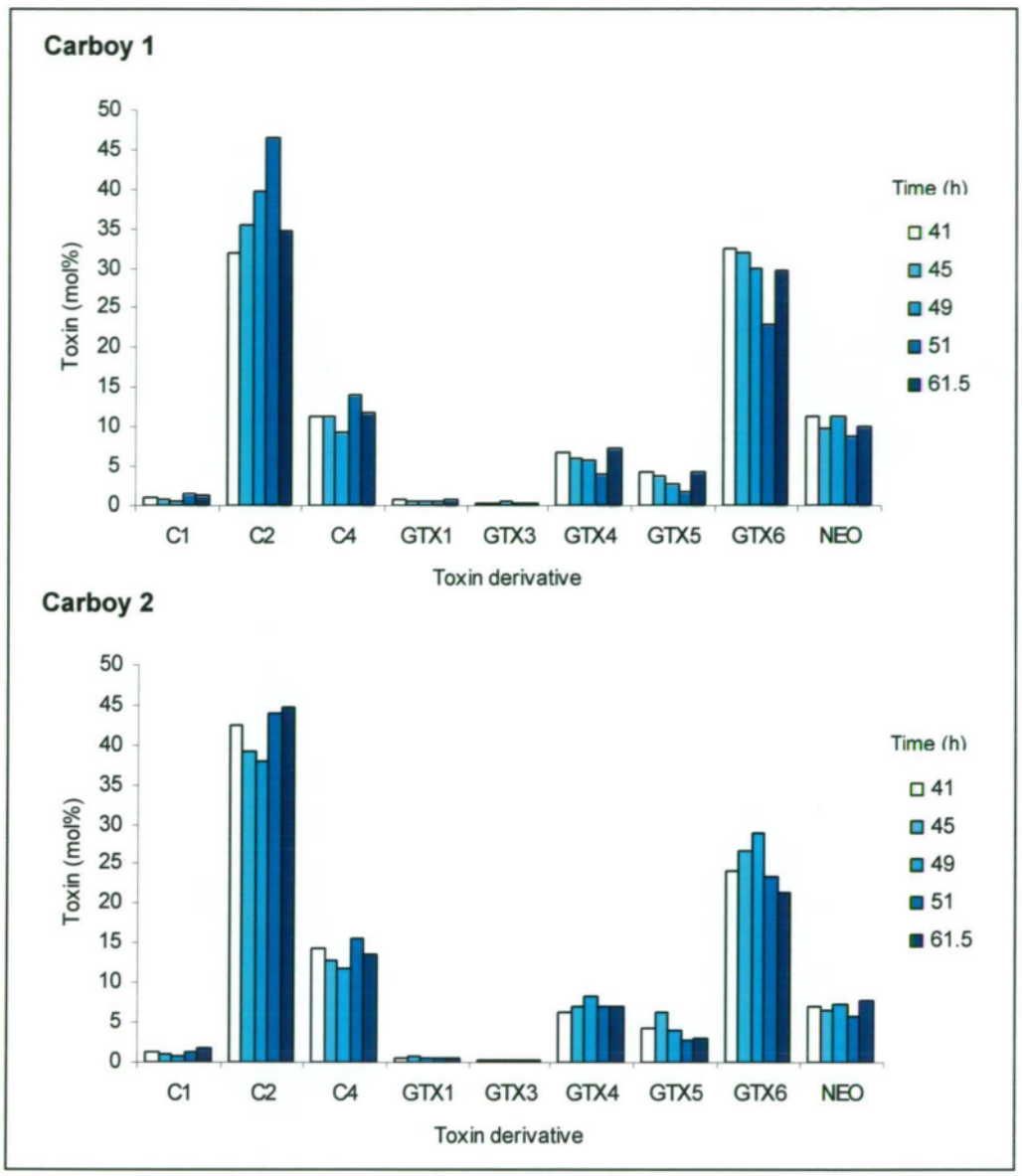
Clone number	Primers	Strain	DNA	Clone Name	Insert bp
177	UBI F/ R	CAWD106	gDNA	UBI-1	511
178	UBI F/ R	CAWD106	gDNA	UBI-3	511
179	UBI F/ R	CAWD106	gDNA	UBI-5	430
180	UBI F/ R	CAWD106	gDNA	UBI-6	226
181	UBI F/ R	CAWD106	gDNA	UBI-7	228
182	UBI F/ R	CAWD106	gDNA	UBI-8	165
183	UBI F/ R	CAWD106	gDNA	UBI-12	228
184	Sahh F1/ R1	CAWD44	gDNA	Sahh-4-4	151
185	Sahh F1/ R1	CAWD44	gDNA	Sahh-5-5	347
186	Sahh F1/ R1	GCTRA01	gDNA	Sahh-6-1	151
187	Sahh F1/ R1	GCTRA01	gDNA	Sahh-7-2	220
188	Sahh F1/ R1	GCTRA01	gDNA	Sahh-8-3	257
189	Sahh F1/ R1	GCTRA01	gDNA	Sahh-9-4	410
190	Sahh F1/ R1	AMNC04	gDNA	Sahh-11-1	871
191	Sahh F1/ R1	AMNC04	gDNA	Sahh-14-4	308
192	Sahh F1/ R1	CAWD44	cDNA	Sahh-16-1	151
193	Map F1/R1	CAWD44	gDNA	Map-1-1	562
194	Map F1/R1	CAWD44	gDNA	Map-3-3	436
195	Map F1/R1	CAWD44	gDNA	Map-5-5	150
196	Map F1/R1	CAWD44	cDNA	Map-6-1	150
197	Haf F1/R1	GCTRA01	gDNA	Haf-1-1	332

APPENDIX 6



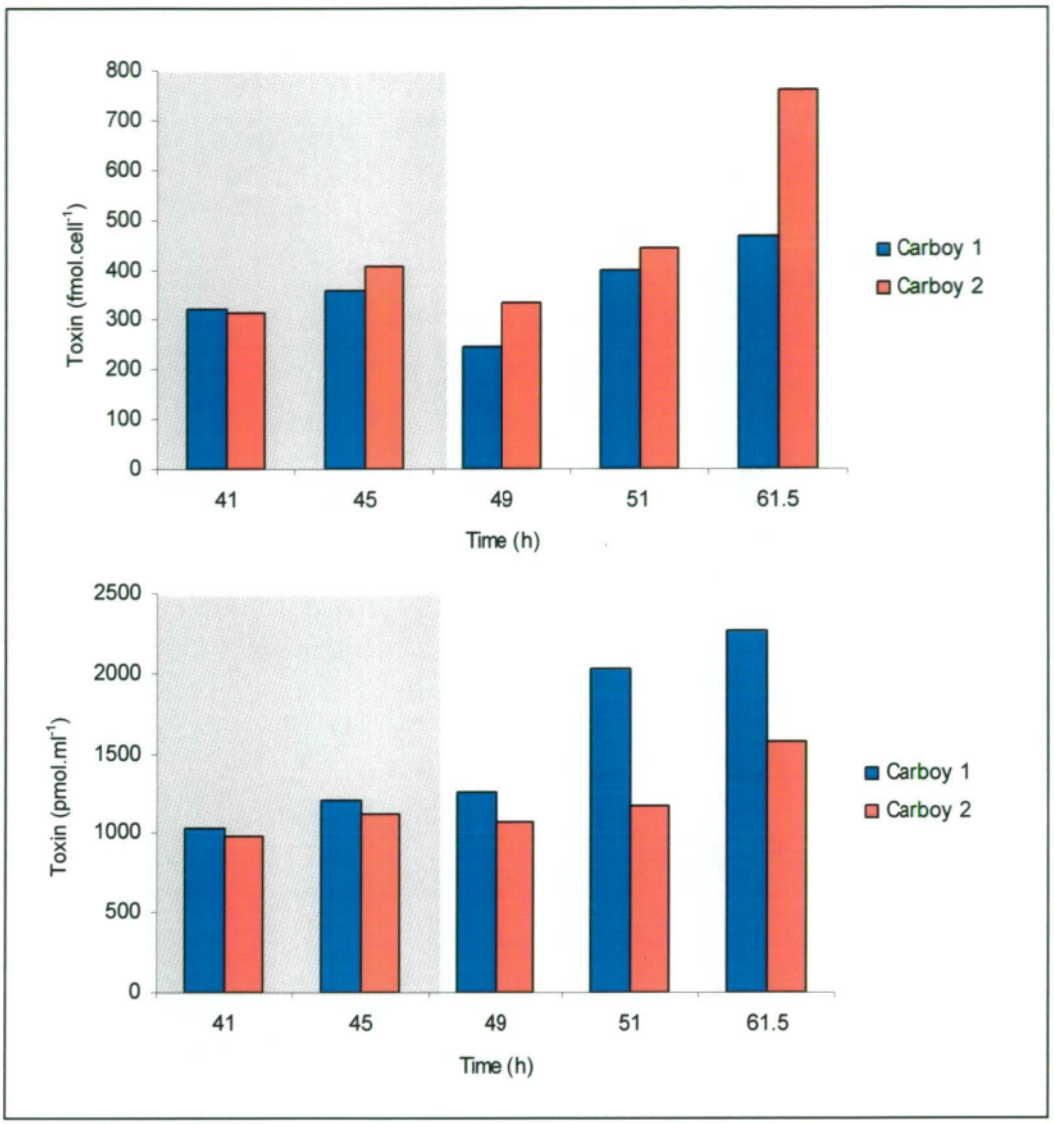
Concentration of *Alexandrium catenella* CAWD44 cells in replicate carboys (1 and 2) before entering 82 hours of dark (-82) and after release (hour zero) into a regular 10:14 dark:light cycle. The dark phases are indicated with shading. Toxin analysis was performed for hours 41 to 61.5 (see Appendices 7 and 8).

APPENDIX 7



Proportion of paralytic shellfish toxin derivatives over one dark:light phase of *Alexandrium catenella* CAWD44. Results for two replicate carboys (1 and 2) are presented at hours 41 to 61.5 after release (hour zero) from a long dark period (see Appendix 6) into a regular 10:14 light:dark cycle. Time points (h) 41 and 45 were taken in the dark and 49, 51 and 61.5 in the light.

APPENDIX 8



Total paralytic shellfish toxin content of *Alexandrium catenella* CAWD44 represented as toxin per cell (upper graph) or toxin per ml of culture (lower graph). Both graphs show an increase in toxin over a single light and dark (shaded) period with this trend most evident for toxin.cell^{-1} for carboy 2 (red bars, upper graph) and toxin.ml^{-1} for carboy 1 (blue bars, lower graph). The time refers to the number of hours after release from a long dark exposure (see Appendix 6).

Appendix 9

Experiment 1. Toxin analysis of *Alexandrium catenella* CAWD44 at 2-h intervals over the cell division cycle from 2.5 h before the culture room lights come on (-) to 3.5 hours after lights on (+).

Time (h)	Mol%									Total toxin (fmol.cell^{-1})	Total toxin (pmol.ml^{-1})
	C1	C2	C3	C4	GTX1	GTX3	GTX4	GTX5	GTX6		
-2.5	1.99	47.27	0.00	5.07	0.92	0.24	8.27	12.99	23.26	142.84	104.6
-0.5	1.71	42.44	0.00	5.10	1.44	0.63	8.66	16.57	23.45	120.40	90.3
+1.5	1.65	45.91	0.16	4.53	1.45	0.65	7.87	15.78	22.00	97.07	115.0
+3.5	1.98	40.42	0.25	4.36	1.64	0.88	8.51	18.73	23.23	74.73	87.9

Experiment 2. Toxin analysis of *Alexandrium catenella* CAWD44 at 2-h intervals over the cell division cycle from 2.5 h before the culture room lights come on (-) to 5.5 hours after lights on (+). One extra time point at 12.0 h after lights on was recorded.

Time (h)	Mol%									Total toxin (fmol.cell^{-1})	Total toxin (pmol.ml^{-1})
	C1	C2	C3	C4	GTX1	GTX3	GTX4	GTX5	GTX6		
-2.5	0.84	29.52	0.00	9.75	1.25	0.28	12.26	19.24	26.87	46.5	26.9
-0.5	1.15	36.69	0.00	3.77	0.71	0.43	12.15	16.67	28.43	97.7	67.0
+1.5	1.03	33.24	0.17	3.45	0.93	0.88	11.87	18.73	29.70	109.2	103.3
+3.5	0.97	35.01	0.21	4.33	0.99	0.30	12.84	12.34	32.99	98.6	96.6
+5.5	0.32	36.56	0.00	3.43	0.63	0.30	10.80	16.03	31.93	105.3	104.1
+12.0	0.88	35.67	0.00	2.91	0.62	0.23	11.46	15.54	32.69	61.8	71.5