UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING, SCIENCE AND MATHEMATICS
SCHOOL OF OCEAN AND EARTH SCIENCE

MOLECULAR TECHNIQUES FOR INVESTIGATING TOXIC DINOFLAGELLATE SPECIES IN THE WESTERN ENGLISH CHANNEL, UK AND IN BAHRAIN COASTAL WATERS OF THE ARABIAN GULF.

By

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ABSTRACT

Some species of marine microalgae produce toxins that have major impacts on aquaculture and fisheries and can cause human illnesses through their accumulation in shellfish. Dinoflagellates account for 75% of all toxic microalgal species and are considered primarily responsible for the current expansion and regional spreading of harmful algal bloom outbreaks in the sea. The aim of this investigation was to develop a protocol using a combination of molecular methods for specifically detecting, identifying and enumerating harmful algal species in natural water samples from the English Channel and Bahrain coastal waters.

Initially, a fluorescent *in situ* hybridization (FISH) protocol with a monolabelled probe was assessed for detecting *Alexandrium tamarense* cultured cells, but was shown to produce a weak hybridization signal and only a small fraction of the target species were detected. Following this, two new species specific molecular primers were developed for use with polymerase chain reaction (PCR) to specifically differentiate between two closely related species (*Karenia mikimotoi* and *Karenia brevis*) of the harmful dinoflagellate genus *Karenia. K. brevis* produces brevetoxins and is responsible for neurotoxic shellfish poisoning in coastal regions of the Gulf of Mexico. *Karenia mikimotoi* is less toxic but has been linked with fish kills and is usually considered the species causing blooms in some Atlantic coastal regions of Europe. The 28S large subunit ribosomal RNA (28S LSU rDNA) of *Karenia mikimotoi* and its morphologically and genetically similar relative *Karenia brevis* were sequenced and the two sets of primers shown to be specific for detecting the two individual target species. Nested PCR allowed an unbiased identification of the bloom forming *Karenia* species occurring in Lugol's preserved water samples previously collected from the western English Channel, identifying only *Karenia mikimotoi* to be present. In some preserved water samples collected from Bahrain coastal waters both *K. brevis* and *K. mikimotoi* were shown to be present using these primers. To allow specific enumeration of *Karenia mikimotoi* cells in preserved water samples, a protocol based on catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) was developed. The technique was optimized for quantification of the target species by epifluorescence microscopy and flow cytometry. Epifluorescence microscopy was shown to be superior for the quantification of the target species, although flow cytometry was demonstrated to be a promising technique for developing an automated detection system. Absolute numbers of *K. mikimotoi* cells estimated using the CARD-FISH probe did not correlate with inverted light microscope counts of Lugol's preserved water samples from the western English Channel. However, positive hybridized relative cell counts compared to total DAPI stained phytoplankton cells did show a strong correlation indicating some loss of cells during processing of samples. These molecular methods were then sequentially applied to the potentially harmful species *Bysmatrum granulosum* isolated from Bahrain coastal waters using a newly designed 18S SSU rRNA probe. The CARD-FISH protocol was used to monitor changes in absolute cell concentrations of *Bysmatrum granulosum* in preserved water samples collected from Arad Bay, Bahrain from June to November 2006. CARD-FISH derived absolute cell counts were strongly correlated to those derived from inverted light microscope counts of Lugol's preserved water samples, proving the useful application of this method.

This investigation was one of the first to successfully use the CARD-FISH molecular technique for the detection and enumeration of harmful algal species in natural water samples. It could therefore be applied for monitoring a range of harmful dinoflagellate species providing a sensitive early warning method for their detection in coastal waters.

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Declaration of authorship

I, Layla J. Hazeem, declare that the thesis entitled "Molecular techniques for investigating toxic dinoflagellate species in the western English Channel, UK and in Bahrain coastal waters of the Arabian Gulf" and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- This work was done wholly or mainly in candidature for a research degree at this University;
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotation, the thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- None of this work has been published before submission.

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CHAPTER 1

Introduction

1.1 General introduction

Phytoplankton are major primary producers in oceanic and neritic waters and have long been considered to be highly dispersed taxa with large population sizes (Medlin et al. 2000). The main eukaryotic groups of marine phytoplankton include diatoms, dinoflagellates and coccolithophores with diatoms estimated to contribute up to 45% of the total oceanic primary production (Field et al. 1998). In temperate and tropical coastal waters dinoflagellate species are important primary producers and some species produce extensive and dense populations often referred to as blooms. Dinoflagellates exhibit a great diversity in their nutritional types, including autotrophs, mixotrophs and parasites. It is difficult therefore to generalize about the role of dinoflagellates in ecosystems because of their large ecological diversity. Photosynthetic dinoflagellates are significant primary producers whereas the non-photosynthetic forms acquire their nutrition through the uptake of dissolved organics, extracellular digestion or by phagotrophy. Most dinoflagellates are free-living although some species exist as parasites (Steidinger and Tangen 1996). A few photosynthetic species exist as beneficial symbionts or “zooxanthella”, in a great variety of hosts. These have considerable ecological importance because of their abundance and widespread occurrence, particularly in tropical coral communities (Taylor 1987). Additionally, dinoflagellates are particularly important in the global cycling of the elements N, O, S, P and C and are becoming even more significant as the growth in human population results in the alteration of marine biogeochemical cycling of these and other elements. For example, human-induced changes in the global nitrogen cycle have already had multiple consequences, including dramatic increases in the frequency, extent and duration of blooms of some marine dinoflagellate species during the last two decades (Hallegraeff (2004). About 60 dinoflagellate species are known to produce water or lipid

soluble toxins. The majority of toxin-producing dinoflagellates are photosynthetic, estuarine or coastal shallow waters forms and many are capable of producing benthic resting cysts, which tend to produce monospecific populations (Graham and Wilcox 2000). Some Diatoms (e.g. *Pseudo-nitzschia*), Prymnesiophytes (e.g. *Prymnesium*), and prokaryotic cyanobacteria (e.g. *Oscillatoria*) also produce toxins and when conditions are optimal for their growth they can form dense aggregations often referred to as Harmful Algal Blooms (HABs) (Hallegraeff 2004).

1.2 Harmful algal blooms

There are estimated to be about 5000 living phytoplankton species (Sournia et al. 1991), of which some 300 species are known to cause harmful effects on the marine environment. These effects include water discoloration and oxygen depletion problems and species that are known to produce toxins that can cause human shellfish poisoning (Hallegraeff 2004). The three different types of harmful algal species can be grouped as follows:

1. Species which produce harmless water discolorations, but under exceptional conditions in sheltered bays, blooms can grow so dense that they cause indiscriminate kills of fish and invertebrates due to oxygen depletion.
2. Species which produce potent toxins that can find their way through the food chain to humans, causing a variety of gastrointestinal and neurological illness, such as paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), ciguatera fish poisoning, neurotoxic shellfish poisoning (NSP) and cyanobacteria toxin poisoning.
3. Species, which are non-toxic to humans, but harmful to fish and invertebrates (especially in intensive aquaculture systems) by damaging or clogging their gills.

Harmful algal blooms (HABs) have increased in frequency, intensity and geographical distribution in the past two decades (Hallegraeff 2004). For example, *Alexandrium minutum* which was first described from waters off Egypt and is now known to occur world-wide in coastal waters of Australia, Ireland, England, France, Spain, Portugal, Italy, Turkey, the

east coast of North America, Thailand, New Zealand, Taiwan and Japan (Yuki 1994, Lilly et al. 2005, Nascimento et al. 2005, Touzet et al. 2007). There are several possible explanations for the increase in algal bloom events: the scientific awareness of the toxic species has increased; the high utilization of coastal waters for aquaculture; plankton blooms are stimulated by cultural eutrophication and/or unusual climatological conditions; and when dinoflagellate resting cysts are transported in ships' ballast water or shellfish stocks translocated from one area to another (Hallegraeff 2004).

Nowadays, monitoring harmful algae is the only way to protect humans and their food resources of marine origin from the poisonous effect of HABs. Basic knowledge about harmful algal species distribution, succession and population dynamics is essential to predict and mitigate their effects. The crucial step to predict HAB phenomena is to both identify and quantify HAB species and their adaptations that might initiate their development in particular hydrodynamic and ecological conditions (Zingone et al. 2000).
While some organisms such as the dinoflagellates *Karenia brevis* (=*Gymnodinium breve)*, *Alexandrium* and *Pyrodinium* appear to be affected by natural of coastal nutrient enrichment events, other algal bloom species appear to be stimulated by "cultural eutrophication" from domestic, industrial and agricultural wastes. There is no doubt that increasing human interest in utilizing coastal waters for aquaculture is leading to an increased awareness of toxic algal species. Most importantly, those responsible for management decisions on nutrient loading of coastal waters (including decisions on agricultural and deforestation activities in catchments areas) should be more aware that one probable outcome of increased nutrient loading will be an increase in harmful algal blooms (Hallegraeff 2004).

1.3 Harmful Dinoflagellates

Some dinoflagellates produce toxins that can affect many types of organisms. Table 1.1 includes types of dinoflagellate toxins involved in shellfish and fish poisoning when consumed by humans. The chemical structure of some dinoflagellate toxins is shown in Fig. 1.1. Some dinoflagellate for example the genus *Alexandrium* contain species that

produce a range of toxic substances and the species *Alexandrium tamarense* includes both toxic and non toxic strains (Cembella et al. 1987) that occur in different regions of the world.

Table 1.1: Some harmful effects on humans associated with dinoflagellate toxic species

Toxin family	Effect	Causative organisms
Saxitoxin	Paralytic shellfish poisoning (PSP)	*Alexandrium catenella* , *Alexandrium minutum* , *Alexandrium tamarense* , *Gymnodinium catenatum* , *Pyrodinium bahamense var.compressum.*
Brevetoxin	Neurotoxic shellfish poisoning (NSP)	*Karenia brevis,* brevetoxin produced by other speceis of *Karenia* is not yet confirmed.
Okadaic acid	Diarrhetic shellfish poisoning (DSP)	*Dinophysis acuminata* , *Dinophysis acuta* , *Dinophysis fortii* , *Dinophysis norvegica* , *Prorocentrum lima.*
Ciguatoxin	Ciguatera fish poisoning (CFP)	*Gambierdiscus toxicus, (Ostreopsis siamensis and Coolia monotis* not confirmed*).*
Azaspiracid	Azaspiracid shellfish poisoning (AZP)	*Protoperidinium crassipes.*

(Modified from Anderson 2001, Hallegraeff 2004)

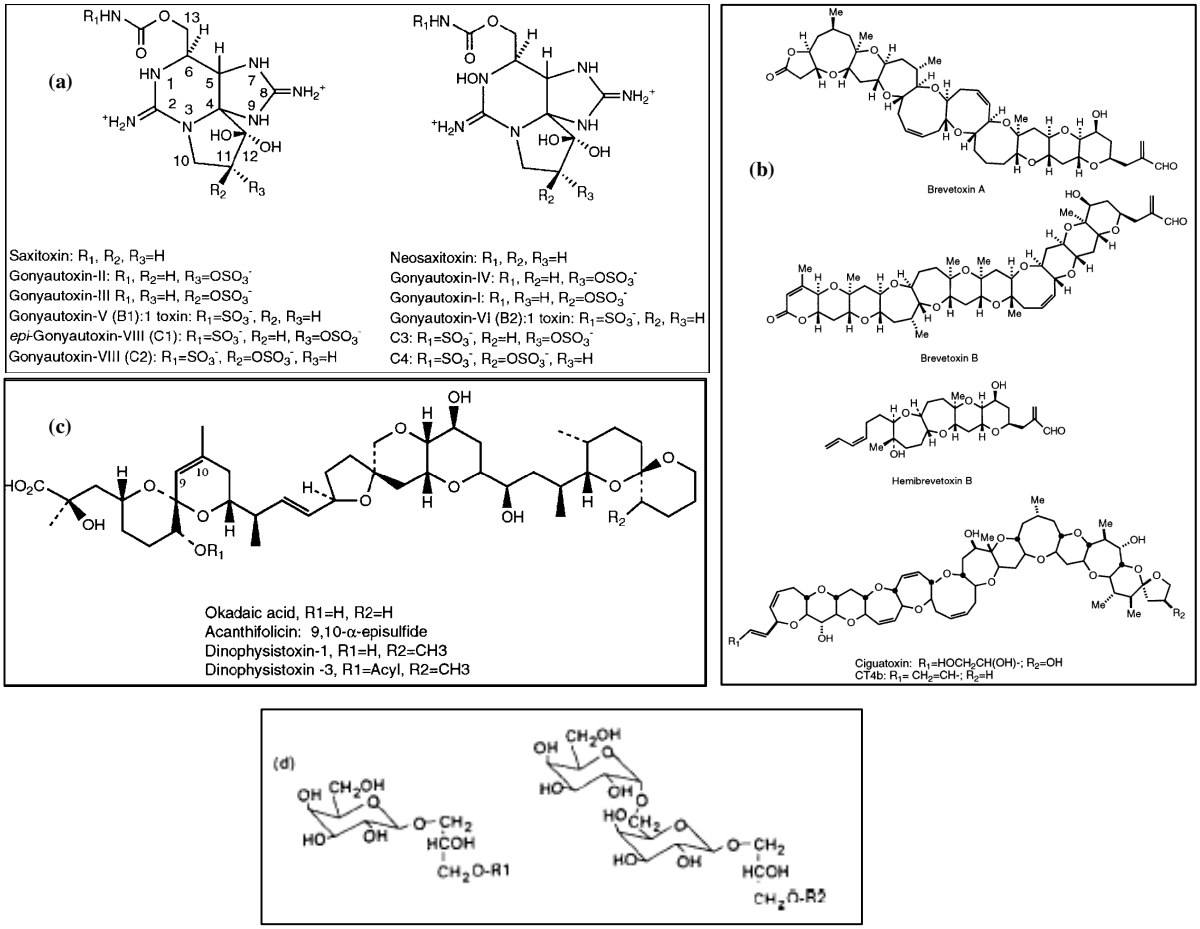


Figure 1-1: Examples of chemical structures of dinoflagellate toxins. a: Representative structures of PSP toxins produced by *Alexandrium* species., divided into saxitoxin and neosaxitoxin series (further structure variation comes from the presence or absence of O- and N-sulfate moieties and stereochemical differences); b: Brevetoxins produced by the Florida red tide species *Karenia brevis* (*Gymnodinium breve*) and ciguatoxin congeners by *Gambierdiscus toxicus*, a tropical dinoflagellate; c: Structure of okadaic acid and its derivatives, potent protein phosphatase 1 and 2A inhibitors produced by some *Prorocentrum* and *Dinophysis* species. (a-c: source: Shimizu 1996); d: Hemolysins from *Amphidinium* carterae and *Karenia mikimotoi* (source: Anderson 1996)

More than 80% of the eukaryotic algal taxa that produce defined phycotoxins belong to Dinophyceae. The bioactive secondary metabolites produced by dinoflagellates may play diverse roles (currently unknown) in intracellular regulation of cell growth and metabolism as well as in extracellular regulation of population growth via allelochemical interactions (Cembella and John 2006). It is still not known why some phytoplankton species produce toxins while most do not, or why some strains of the same species are toxic while others are

not (Shimizu 1996). Some microalgae do not produce toxins themselves, but toxin production is caused by intracellular bacteria (Gallacher et al. 1997). In some cases, toxin production is also stimulated by the presence of grazers (Granèli and Flynn 2006). Amongst the suggestions made about the biological role of toxin production are that toxins may act as a deterrent against predation or as nutrient-storage compounds synthesized when the nutrient supply is imbalanced (John and Flynn 2002). Toxin synthesis requires the availability of a source of nitrogen and of light, but it is fastest in low rather than high light (i.e. it is promoted by high availability of nitrogenous metabolites with relatively low availability of carbon). Additionally, phosphorus stress could promote toxicity. Oshima (1995) reported the role of ATP-dependent enzymes for the interconversion of some PSP toxins; hence the phosphorus status may be expected to affect the toxin profile. Toxin accumulation occurs primarily under unbalanced nutrient conditions, and when cells are not growing optimally. This suggests that production is only sometimes advantageous, even though production costs appear minor (John and Flynn 2002). The respiratory cost of toxin synthesis is assumed to be similar to that for all other nitrogenous components in the cell (costed at 1.5 g C g N^{-1}) (Flynn 2001). The toxin production is essentially being selection-neutral and this explains why toxin production ability varies so greatly among and within species and strains. However, it is quite likely that in both harmful algae and other phytoplankton groups, many other secondary metabolites are also produced that are indigestible to grazers thus affecting predator-prey interactions.

Biosynthetic evidence from stable isotope-labelling studies indicates that most if not all polyether phycotoxins are produced via polyketide pathways, in which acetate units are added sequentially from acetyl-CoA within a pathway regulated by polyketide synthases (PKS) (Cembella and John 2006, Shimizu 1996). The relationship between growth and toxin production has been extensively reviewed for the dinoflagellates that produce saxitoxin and analogues, polyether toxins, and diatoms that synthesize domoic acid by Cembella and John (2006).

1.4 Dinoflagellates life cycles

Phytoplankton reproduce commonly vegetatively or asexually. Many phytoplankton are able to reproduce sexually however, enabling genetic exchange and maintaining genetic variation within a population. Resting stages can be formed from vegetative cells or as part of the sexual life cycle (Blackburn and Parker 2004). Dinoflagellates form two basic types of resting stages or cysts: (1) hypnozygotic cysts, a product of sexual reproduction; and (2) temporary (pellicular) cysts, also referred to as thin walled cysts. Stressful environmental conditions, such as turbulence or nutrient limitation appear to induce sexuality and formation of resting cysts in some dinoflagellate species. Temporary cysts are also formed under adverse conditions. However, temporary cysts, as opposed to resting cysts, can quickly re-establish a vegetative, motile existence, when conditions become favorable again (Fistarol et al. 2004). Hypnozygotic cysts can remain dormant in the sediment until conditions become favorable and then germinate to produce new vegetative cells (Steidinger and Garccès 2006). The ability to produce cysts is important with respect to dispersal of harmful dinoflagellates, providing survival through adverse conditions and genetic recombination when sexuality is involved in their formation (Anderson et al. 2004). Fig. 1.2 is a diagrammatic summary showing the different stages of a dinoflagellate life cycle, using *Alexandrium tamarense* as a model.

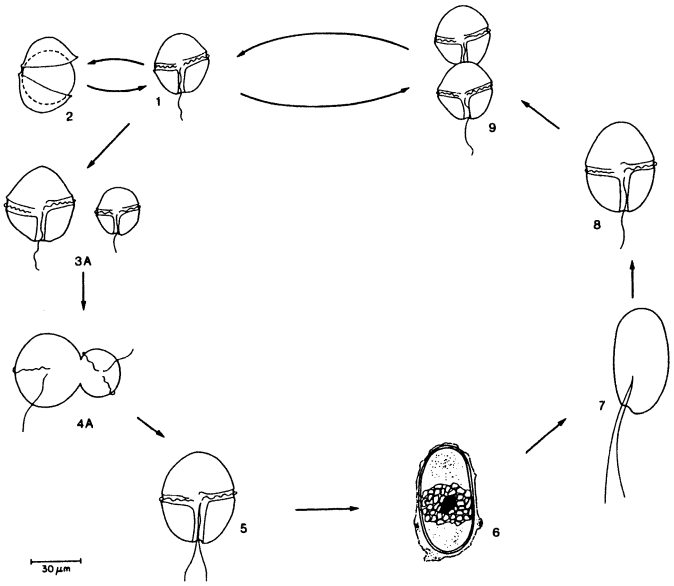


Figure 1-2: Life-cycle diagram of a dinoflagellate, using *Alexandrium tamarense* as a model. Stages are identified as follows: 1, vegetative, motile cell; 2, temporary or pellicle cyst; 3, anisogamous "female" and "male" gametes; 4, fusing gametes; 5, swimming zygote or planozygote; 6, resting cyst or hypnozygote; 7, 8, motile, germinated cell or planomeiocyte; 9, pair of vegetative cells following division (source: Anderson 1998).

1.5 The genus *Karenia*

The genus of *Karenia* G. Hansen & Moestrup gen. nov. is a member of the family Gymnodiniaceae and also includes both toxic and non-toxic species. In addition to the genus *Karenia*, the Gymnodiniaceae Lankester, 1885 (Steidinger and Tangen 1996) includes the genera *Amphidinium* Clapare`de & Lachmann, *Cochlodinium* Schütt, *Gymnodinium* Stein, *Gyrodinium* Kofoid & Swezy, , *Karlodinium* J. Larsen gen. nov., *Woloszynskia* Thompson (Shao et al. 2004) and *Akashiwo* G. Hansen & Moestrup gen. nov., the latter according to Daugbjerg et al. (2000).

Rapid identification and clarification of the phylogenetic affiliations of toxic algal species are key research topics as there are significant taxonomic uncertainties with respect to

identifying causative organisms. The criteria traditionally used for the identification of these species were based on cellular morphological aspects and biochemical data (Taylor 1980, Wood and Leathan 1992). In athecate dinoflagellates, there are additional difficulties in morphological identification (Shao et al. 2004) because they tend to be more delicate and frequently burst or collapse during chemical fixation (Graham and Wilcox 2000). Species belonging to Gymnodiniaceae are athecate and they lack many distinct features. Morphological characteristics of Gymnodiniaceae species have been shown to be insufficient for their classification by traditional approaches (Zardoya et al. 1995) which thus presents a significant problem in harmful bloom monitoring programs. Therefore, the determination of the unique genotypes of these species to avoid problems associated with their phenotypic plasticity has become necessary.

The marine dinoflagellate genus *Karenia* currently contains 13 species: *K. asterichroma*, *K. bicuneiformis*, *K. brevis*, *K. brevisulcata*, *K. concordia*, *K. cristata*, *K. digitata*, *K. longicanalis*, *K. mikimotoi* have been listed in "Biodiversity occurrence data provided by: Field Museum of Natural History, Museum of Vertebrate Zoology, University of Washington Burke Museum, and University of Turku (Accessed through GBIF Data Portal, www.gbif.net, 2009-03-23)": *K. umbella* has been reported in de Salas et al. (2004a), *K. bidigitata*, *K. papilionacea* and *K. selliformis* in Haywood et al. (2004). Table 1.2 shows the distribution and toxicity of the different species of *Karenia* in the world ocean.

Under the light microscope, the different species of *Karenia* are difficult to distinguish from each other (Steidinger et al. 1989), even though the light microscope is still primarily used to identify phytoplankton. The consequences of the difficulty in microscopic identification are that described species of *Karenia* can be misidentified. The environmental threat posed by these species becomes more complicated when morphologically similar species of *Karenia* tend to co-occur with described species, and there may be synergistic toxic effects caused by the presence of multiple species (Haywood et al. 2004). Internationally, the cosmopolitan nature of some species like *Karenia* and other dinoflagellates known or considered to be toxic or harmful to humans and wildlife has increased the need for species specific cell detection methods. For example, *Karenia selliformis* is known to produce the toxin gymnodimine (Seki et al. 1996) and has been

isolated and described from New Zealand (Haywood et al. 2004), but it was also found to be present in Kuwait where it was associated with a large fish kill (Glibert et al. 2001, 2002, Heil et al. 2001). Additionally, at least three other species of *Karenia* (*K. mikimotoi*, *K. selliformis* and *K. papilionacea*) have been recorded to co-occur with *K. brevis* in the Gulf of Mexico (Haywood et al. 2007). The morphological similarities between these species may have resulted in their misidentification as *K. brevis* in the past (Haywood et al. 2004). In New Zealand, several algal species, including *Karenia mikimotoi* and *K. brevisulcata* and *K. concordia* were found to bloom simultaneously in 2002 (Chang and Ryan 2004).

The use of molecular approaches to discriminate between different species at all taxonomic levels have been widely accepted (Avise 1994). Ribosomal RNA (rRNA) gene sequences are commonly used in taxonomic and phylogenetic studies of different taxa (Shoa et al. 2004). Additionally molecular techniques based on using DNA probes have been developed for making rapid, accurate identification of dinoflagellates, particularly toxic forms. In the following section, the different molecular techniques that have been previously developed for the detection and enumeration of harmful algal species are summarized.

Table 1.2: Known distribution and toxicity of the different species of *Karenia*

Speceis	Distribution	Toxicity	Reference
K. brevis	Gulf of Mexico	cause NSP, fish kills, asthma-like symptoms in humans through aerosolization of toxins	Daugbjerg et al. 2000
K. mikimotoi	Widespread : Australia, Denmark, Irland, Japan, Korea, Norway, Scotland, UK	hemolytic and ichthyotoxins	Taylor et al. 2004
K. brevisulcata	Wellington Harbour, New Zealand	causes fish kills	Taylor et al. 2004
K. longicanalis	Hong Kong Harbour and the southwestern coastal waters of Hong Kong	Not associated with harmful effects	Yang et al. 2001
K. digitata	Hong Kong and Japan	Ichthyotoxic, causes fish kills	Yang et al. 2000
K. umbella	Tasmanian coastal waters, Australia	Ichthyotoxic	De Salas et al. 2004a
K. asterichroma	Tasmanian coastal waters, Australia	caused salmonid mass mortality, but its ichthyotoxicity not yet confirmed	De Salas et al 2004b
K. bicuneiformis	Gordan's Bay, South Africa	Not associated with harmful events	Botes et al. 2003
K. bidigitata	New Zealand	Toxicity not confirmed	Haywood et al 2004
K. concordia	Hauraki Gulf, New Zealand	Believed to cause NSP and respiration distress in humans. Cell extracts of *K. concordia* shown to be haemolytic and cytotoxic (Chang et al. 2008)	Chang & Ryan 2004, Chang et al. 2008
K. cristata	South Africa	Eye, nose. throat and skin irritations in humans as well as extensive mortality of sub-and intertidal fauna. Found to produce brevetoxin in culture	Botes et al. 2003
K. papilionecea	Reported from New Zealand only	Toxicity not confirmed	Haywood et al. 2004
K. selliformis	New Zealand and reported in Kuwait	May produce gymnodimine toxins	Haywood et al. 2004

1.6 A review of molecular methodologies for the detection and enumeration of harmful algal species

The detection and enumeration of specific algal species in water samples is essential for understanding the HAB phenomena. The traditional microscopic identification methods require a high level of expertise to distinguish fundamental morphological characteristics indicative of HAB dinoflagellate species. In many cases, distinguishing between the different organisms is difficult using morphological features only. In addition some species *Alexandrium tamarense* for example, includes both toxic and non-toxic forms that are morphologically very similar but can be separated genetically (John et al. 2003b). Separation between the toxic and the non-toxic strains can only be achieved using molecular criteria (Scholin et al. 2004). "Molecular probes" refer to biological molecules that include lectin, antibodies, DNA and RNA. Each set of probes shares the ability to selectively adhere to molecules specifically associated with a particular species or group of species. Therefore, these probes can be used for detecting specific organisms even when they occur in complex natural communities (Scholin and Anderson 1998). The use of these molecular probes has increased and now can even be used for monitoring as well as conducting HAB risk assessments (Scholin et al. 2004).

1.7 Application strategies of nucleic acid probes to whole cells and cell homogenates

Molecular probes applied to HAB species can be used either with whole cells or cell homogenates. Fig.1.3 shows a flow diagram of the different molecular methods that can be used for the detection of harmful algal species. Detection protocols that depend on whole cells require that the target species remain intact throughout the protocol; therefore preservation of water samples is an essential step before applying the probe. Probes which are fluorescently labeled bind to specific indicator molecules which are present on the cell surface or within the margins of the cell wall and these can be examined by epifluorescence microscopy or flow cytometry. To date, whole cell protocols for HAB species have been applied using lectins, antibodies and ribosomal RNA (rRNA) targeted DNA probes. In

contrast, molecular techniques based on using cell homogenates require cell lysis. The probes in this case are applied to crude homogenates that contain the indicator molecules. The detection and quantification of these indicator molecules then reveals the presence of the target species, and its abundance in the original sample can be estimated. Both rRNA and rDNA probes can be used with cell homogenates (Scholin et al. 2004).

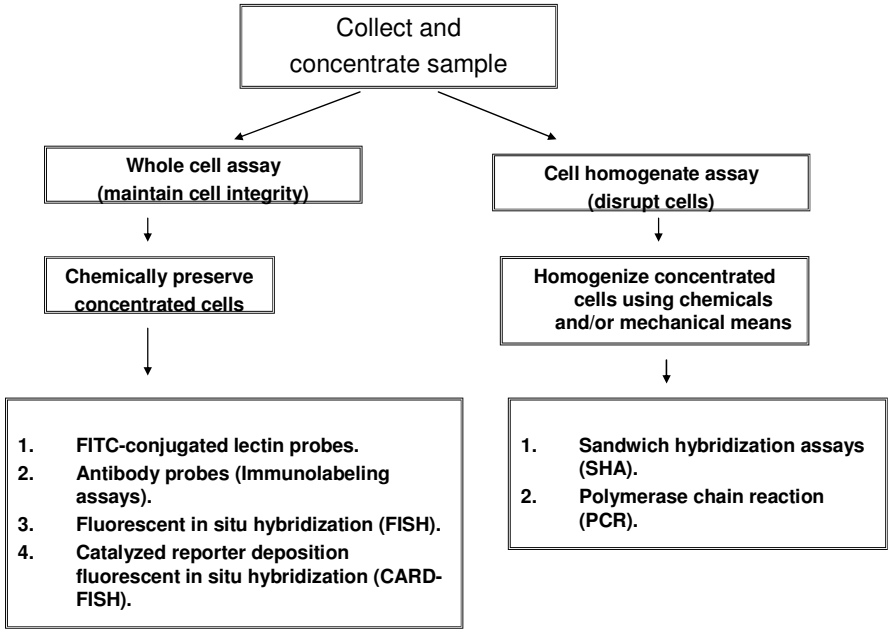


Figure 1-3: A flow diagram showing the different molecular methods that could be used for the detection of harmful algal species.

Molecular methods using cell homogenates:

1.7.1 *Sandwich hybridization assays (SHA)*

Sandwich hybridization assays (SHA) can be applied to cells where their nucleic acids are liberated into solution (crude cell homogenate), and the presence of the rRNA of particular interest is detected indirectly by colorimetric. A positive reaction in the sandwich hybridization system reveals the presence and relative abundance of molecules that complement both capture and signal probe sequences (Scholin et al. 1997). The protocol of sandwich hybridization starts by using living cells and then homogenizing the concentrated

sample in a chaotropic solution that both liberates nucleic acids and protects them from degradation. Subsequently, two separate hybridization reactions are performed: capturing the target nucleic acid sequences (DNA or RNA) from the crude lysate using an oligonucleotide tethered to a solid support, and binding of an enzyme-tagged signal probe to a sequence near that of the capture site. Visualization of capture probe/rRNA/signal probe sandwiches is accomplished enzymatically, yielding colorimetric or chemiluminescent products. The extent of color development or chemiluminescence can provide a measure of the relative abundance of target species in the original sample (Scholin et al. 1997, 1999, 2000, 2004). The SHA method has been widely used on species of *Pseudo-nitzschia* (Scholin et al. 1999, 2000) and *Alexandrium spp.* (e.g. Anderson et al. 2005).

1.7.2 *Polymerase chain reaction (PCR)*

Polymerase chain reaction (PCR) is an enzymatic process, based on the binding of complementary strands of nucleic acids, whereby minute numbers of target sequences present in a sample are amplified specifically, hence leading to their detection and manipulation (Scholin 1998a). PCR is considered one of the most sensitive techniques for looking for small amounts of target nuclear sequences in more complex samples. The protocol has concentrated almost completely on nuclear encoded rRNA and rDNA sequences with the 18S small subunit gene (SSU rDNA) having been the most common targets as they include highly conserved regions common to most organisms (Scholin et al. 2004). This is considered as an advantage because primers designed to amplify 18S genes are available, allowing sequencing to determine unique sequences within variable regions which can then be targeted as unique molecular "signatures". Once a potential signature sequence is identified, a pair of oligonucleotide primers (forward and reverse) is designed to bind to unique sequences within or flanking that target. The first step in developing a PCR-based protocol is the determination of a proper nucleic acid or gene target sequence that has the preferred specificity, at species, genus or other taxonomic level. Successful amplification depends on well-designed primers that have been checked against databases to assure that they are unique and will not cross-react. Further, they must bind effectively to

the target, should have similar annealing temperatures and should not bind to each other (Scholin et al. 2004). Fig. 1.4 shows a diagram of the different reactions of PCR thermal cycle. PCR primers, extraction of nucleic acids from sample material, and application of one or more amplification protocols are required. The following is a brief summary of the different amplification protocols.

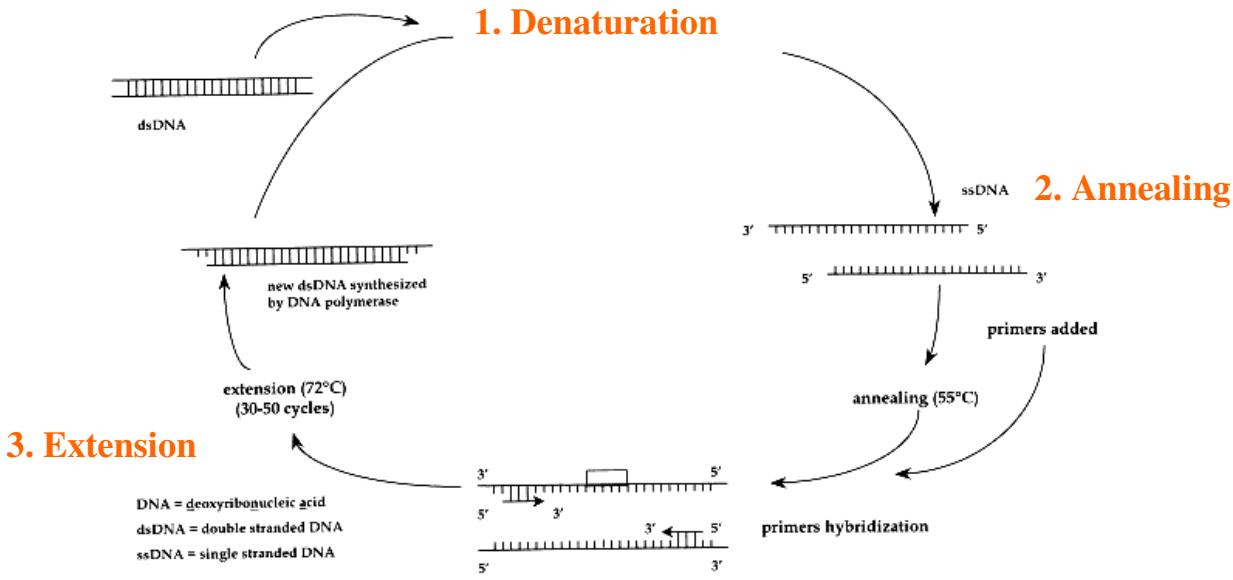


Figure 1-4: Scheme of the Polymerase Chain Reaction (PCR) (from Gachet et al. 1998).

A. *Direct non-quantitative PCR detection of target DNA:*

The simplest and the most common type of PCR reaction is intended to amplify a target sequence that can be visualized on an agarose gel after electrophoresis and staining of the DNA.

B. *Direct quantitative PCR with fluorescent probe (QPCR):*

In this protocol, an oligonucleotide probe with both a fluorophore and a quencher molecule (Taqman^{TM}) are used in addition to oligonucleotide primers, and an instrument capable of excitation and detection of fluorescent signal is essential. In the PCR reaction, the quencher prevents fluorescence when the probe molecule is intact. But, when the probe binds to its complementary region in amplicons at each cycle, the Taq polymerase releases the

fluorescent and quencher molecules into solution as a result of its exonulease activity. When excited by light of the appropriate wavelength, the fluorescent molecule can emit light. Therefore, the relative fluorescence is directly related to the number of amplicons (free fluorescent molecules in solution), and the cycle at which this fluorescence can be detected is directly related to the number of target molecules in the initial reaction mixture (Scholin et al. 2004).

C. *Heteroduplex mobility assays (HMA):*

In this method two PCR reactions are initially conducted using taxon-specific primers (genus or higher taxonomic rather than species-specific). One PCR reaction is performed using a defined template or 'driver', while the other uses a DNA template from an unknown culture or environmental sample. The two reaction products are then mixed in a 1:1 ratio, denatured at high temperature and hybridized when the mixture cools. The resultant hybridized amplicons are then detected by electrophoresis on a polyacrylamide gel. When the driver and unknown are identical, all hybrids produced will be homoduplex DNA amplicons (perfectly complementary strands from the same organism) and a single band will become visible on the gel. If the unknown is distinct from the driver, then both homoduplex and heteroduplex DNA amplicons (nearly complementary strands from two organisms, but containing mismatches) will produce bands on the gel, as heteroduplexes migrate slower than homoduplexes. The pattern of the DNA bands will be characteristic of the species or strain once the same driver and reaction conditions are maintained. HMA assays are useful for identifying unknown cultures, determining if a single or more species in the taxon of interest is present, and they can facilitate the direct sequence discovery (Scholin et al. 2004).

Molecular methods using whole cells:

1.7.3 *Fluorescent (FITC) - conjugated lectin probes*

Lectins are carbohydrate-binding proteins (Scholin and Anderson 1998) of non-immune source that precipitate glycoconjugates or agglutinate cells and they are present in many

organisms, from bacteria to higher vertebrates (Goldstein et al. 1980). They are made up of non-enzymatic secretory proteins or glycoproteins that have vital physiological functions as recognition molecules inside a cell, between cells, or between organisms (Hori et al. 1996). It has been suggested by Hori et al. (1996) that lectins and carbohydrate containing lectin receptors may generally be found on the cell surfaces of several species of microalgae. Fluorescein isothiocynate (FITC)-conjugated lectins are fluorochrome probes with the capability of differentiation of morphologically similar microalgae species (Rhodes et al. 1995). There are many applications of FITC-lectin probes for discriminating harmful algal bloom species (e.g., Rhodes et al. 1995, Cho et al. 1998, Hou et al. 2008).

1.7.4 *Antibody probes (immunolabeling assays)*

One of the most common types of probes used in HAB studies are antibodies probes (Vrieling and Anderson 1996). These antibody probes bind to molecules known as "antigens". The term "antigens" includes peptides, glycoproteins, carbohydrates, toxins and other components which are molecular constituents of cell walls, membranes, components of the cytoplasm and cellular exudates. The specific antibodies offer a sensitive means of identifying microorganisms without the need for culturing (Veal et al. 2000). Mostly antibodies have been used in laboratory experiments and very few applied to field studies.
There are two types of antibodies: monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) and they are used for immunological labeling. The polyclonal antibodies are a suite of antibodies that will bind to many different antigens or epitopes and those antibodies are normally obtained from an animal serum when inoculated with an antigen (Scholin et al. 2004). The heterogeneity associated with the polyclonal antibodies gives a greater likelihood of binding to non-targets organisms (Vesey et al. 1997). In contrast, monoclonal antibodies react only with a single epitope and are produced from cultured cells rather than live animals. The protocol of producing the mAbs is achieved by fusing antibody-producing cells from the host animal with a culturable cell line. Pure cultures of these hybrid cell lines then can be maintained indefinitely (in theory) and large batches of specific mAbs with specific reactivity can be produced consistently. The advantages of

mAbs over pAbs are significant when issues of epitope specificity and long-term supply are of concern (Scholin et al. 2004).

Antibodies could be either directly conjugated to a suitable label such as an enzyme or fluorochrome or a second antibody conjugated to a label is used for the detection of antibodies. Directly-conjugated antibodies require only a single incubation step. Direct coupling generally results in lower non-target binding as the use of a secondary antibody increases the degree of cross reactivity and non-specific binding. Therefore, for specific detection mAbs are preferred to pAbs and direct conjugation is preferable to indirect immunofluorescence (Vesey at al. 1994). Another version of the secondary labeling protocol is known as "enhanced". Enhanced assays are used to increase the number of reporter molecules per antibody, thus increasing the overall signal output (Vrieling et al. 1993).

1.7.5 *Fluorescently labelled, ribosomal targeted DNA (rRNA) probes for identifying whole cells*

Fluorescent *in situ* hybridization (FISH) involves the preparation of short sequences of single stranded DNA (probes), which are complementary to the DNA signature sequences of the target organisms. These oligonucleotide probes are synthetically produced DNA molecules which are generally 18-35 bases long. They can be labelled with radioactive, fluorescent, chemiluminescent, or colorimetric moieties (molecules) and these are usually attached to the 5' end of the probe (Tyrrell et al. 1997). These probes hybridize, or bind, to the complementary DNA and because they are labelled, the location of these sequences of DNA can be detected. FISH assays have been widely used by microbiologists in the detection of marine prokaryotes (Amann et al. 1995) and have been adapted for the study of eukaryotic phytoplankton, and in particular, for research and monitoring of HAB species (e.g. Scholin et al. 1996). Oligonucleotide (DNA) probes for identifying HAB species applied in the whole cell format have been used to target signature sequences of the small subunit (18S SSU) large subunit (28S LSU) and the internal transcribed spacer regions (ITS1 and 2) to identify phytoplankton at various taxonomic levels from classes down to

species or strains (Groben and Medlin 2005). Applications of these methods have focused on species of *Alexandrium* (John et al. 2003a, b, 2005, Sako et al. 2004), *Pseudo-nitzschia (*Miller and Scholin 1996, 2000) and *Karenia brevis* (Mikulski et al. 2005). There are a number of commercially available oligonucleotide probes that are routinely used for the detection of different HAB species (Scholin et al. 2004). Problems encountered using the FISH technique however include: lack of cell, or signal, low signal intensity caused by small numbers or insufficient accessibility of the target molecules (i.e. rRNA) and different concentrations of the target molecules at different growth stages of cells (Amann et al. 1995).

1.7.6 *Catalyzed reporter deposition fluorescent in situ hybridization (CARD-FISH)*

Detectable FISH-signal with monolabelled fluorescent oligonucleotide probes requires a few thousand rRNA target molecules per cell (Amann et al. 1995). As mentioned above, the standard procedure of FISH is limited to the identification of microorganisms with high ribosome content, but those with low ribosome content cannot be detected due to dormancy or low physiological activity (Wagner et al. 2003). A good potential way to deal with this deficiency is the use of oligonucleotide probes labelled with the enzyme horseradish peroxidase (HRP). As outlined in Fig. 1.5, *in situ* hybridization is followed by catalysis and accumulation of fluorescent tyramide at the HRP-molecules during tyramide signal amplification (TSA). In the first application of this method Schönhuber et al. (1997) detected even less labelled cells than with monolabelled oligonucleotide probes. Pernthaler et al. (2002a) improved the method however, which is referred to as CARD-FISH (catalyzed reporter deposition), by increasing the permeabilization of the target cells leading to an increase of detection rates from 46% to 86%. CARD-FISH has been applied to marine prokaryotes (e.g. Biegala et al. 2002) and nano and picoeukaryotes (e.g. Not et al. 2002, 2004, Pernthaler et al. 2003, Biegala et al. 2003) but rarely applied to detect harmful algal species, e.g. Töbe et al. (2006) has used this method to detect the toxic Haptophyte *Prymnesium parvum* and the dinoflagellate *Alexandrium*.

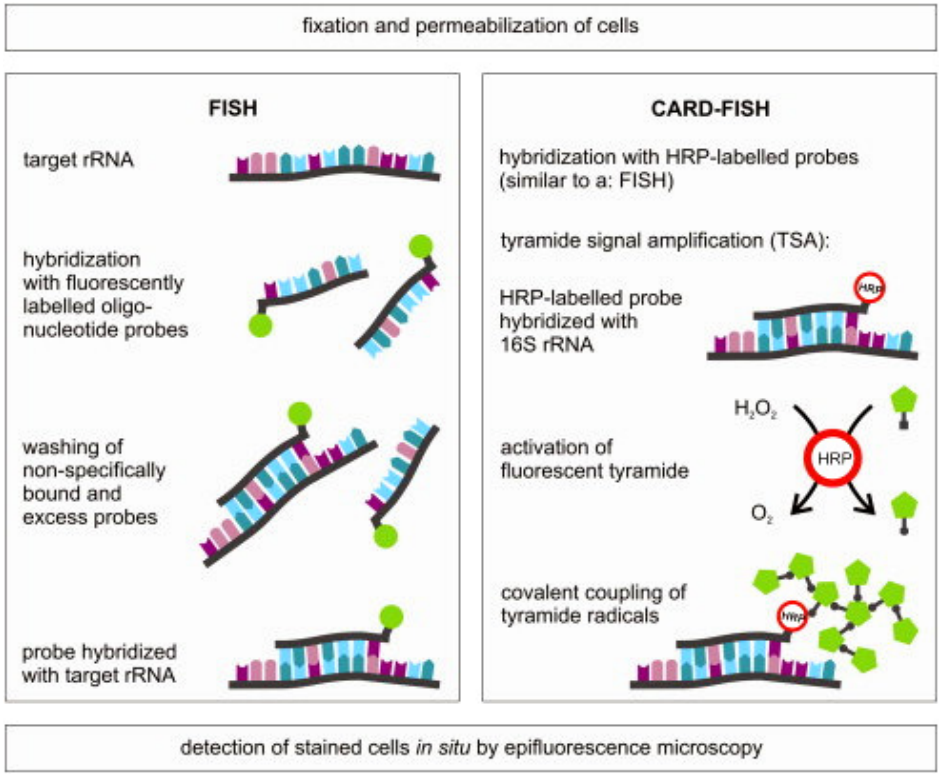


Figure 1-5: Principle steps of (a) FISH and (b) CARD-FISH (from Eickhorst and Tippkötter 2008).

As outlined above, molecular assays using whole cells provide information on the size and shape of the labelled cells. Additionally, they are useful for examining population dynamics and structure. In contrast, when examining field samples, cell homogenate assays (SHA and PCR) only provide information on the target species and no information is provided on the specificity of the probes. Consequently, to determine the specificity of probes, extensive inspection of non-target species must be undertaken and it is critical to maintain a subsample of each field sample to visually check if the target species is present when a positive result is recorded from the assays (Tyrrell et al. 1997).

There are several molecular-based techniques which are now widely used for the detection/ identification and enumeration of harmful algal species around the world. However, to date, it appears that there is no single type of molecular technique or probe sufficient or optimal to fulfill these objectives. Therefore, for the detection of some harmful algae, different probes should be applied. Individual's personal preference, practical background and available laboratory equipment are key factors for applying specific probes and techniques

for a given species in a given region. Molecular probes must be adapted to be appropriate with the geographic region of interest despite the type of the probe or the application format. Several harmful algal species show differences on a molecular scale that is not always obvious morphologically; some probes will identify a particular organism in one geographic region but may not identify the same (morphologically similar) organism in another (Scholin et al. 2004). Accordingly, the reactivity of molecular probes towards target and non-target species must be established empirically, and molecular description of those species should be conducted in combination with analyses based on morphology (Scholin et al. 2004). For example, *Alexandrium tamarense* which is a cosmopolitan species does not have a single species specific probe because the species exists as a series of different strains identifiable through application of lectin, antibody and rRNA-targeted probes. Consequently, description of the probe reactivity should include both species and origin of strain whenever possible.

Genes used for phylogenetic and taxonomic studies

Ribosomal RNA (rRNA) gene sequences are now widely used in taxonomic and phylogenetic studies of different taxa. Generally, assays are targeted at rRNA because there are large numbers of copies in cells, thus offering a large signal per cell (Amann 1995). However, the different subunits and regions of the rRNA gene have different degrees of sequence variability; therefore these sequences may have varying suitability for comparison analyses at the inter-generic level or inter-species level (Adachi et al. 1996b). To date, the most widely used genes are the 18S small and 28S large subunit rRNA genes (18S SSU and 28S LSU rDNA) because they have a mosaic of conserved and highly variable domains which allow the design of oligonucleotide probes which can be targets from Kingdom through to strain level (Amann 1995) and there is a large database available for the 18S SSU and 28S LSU rDNA for sequence comparisons. However, the rDNA internal transcribed spacer (ITS) region, consisting of the ITS-1, 5.8S and ITS-2 sequences, separates three ribosomal genes in the rDNA cistron of eukaryotes (Apples and Honeycutt 1986), and is known to evolve faster than the 18S and 28S rDNA sequences. Additionally, the ITS regions have high base substitution rates and well-documented length variation within various organisms (Shao et al. 2004). The use of the ITS sequences for phylogenetic

and biogeographic studies of marine algae is growing, for example, for green algae (Bakker et al. 1992, 1995), diatoms (Zechman et al. 1994) and dinoflagellates (Adachi et al. 1997, Baillie et al. 2000).

1.8 Aims and objectives

The aim of this investigation was to develop a protocol using a combination of molecular methods for specifically detecting, identifying and enumerating harmful algal species in natural water samples from the English Channel and Bahrain coastal waters. This type of investigation may significantly contribute to the harmful algal species monitoring programs.

Fluorescent *in situ* hybridization (FISH) with monolabelled probes and polymerase chain reaction (PCR) were chosen because they were well established protocols at the time of starting this investigation and widely used for the detection and enumeration of several harmful algal species. Additionally, the 18S SSU and the 28S LSU rDNA genes were targeted in this study because they are used in taxonomic and phylogenetic studies of various taxa, they have different degrees of sequence variability that are suitable for comparing inter-generic or inter-specific differences (Adachi et al. 1996b) and several genus- and species- specific oligonucleotide probes for these genes are commercially available.

Several harmful or toxic dinoflagellate species were studied during the different stages of the research. *Alexandrium tamarense* was used in the first part of the research as a model organism that is responsible for paralytic shellfish poisoning (PSP) events in coastal areas globally. *Karenia spp.* was targeted because it is known to cause extensive harmful algal blooms in the western English Channel but there existed some ambiguity in the literature with respect to the dominant species of *Karenia* present in these waters. Contrary to most other reports, Llewellyn et al. (2005) suggested from HPLC pigment data that *K. brevis* might be the bloom forming species in the western Channel region whereas most other

reports have stated that it is *K. mikimotoi*. Therefore, one objective was to solve this ambiguity in the identification of the *Karenia* species present in that particular area.

The objectives of the research reported in this thesis were as follows:

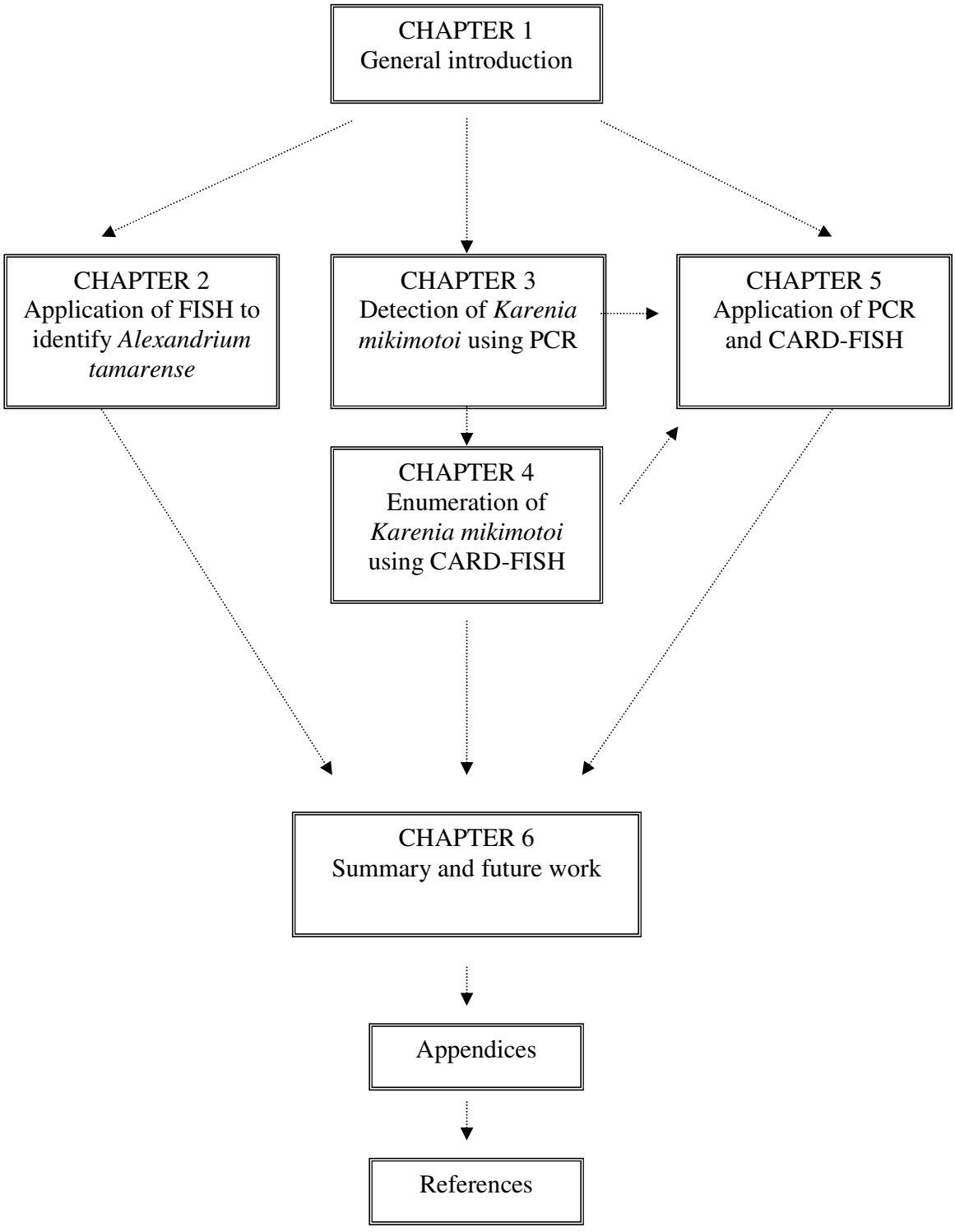
1. To assess a method based on fluorescent *in situ* hybridization with a monolabelled probe (FISH) to specifically detect the toxic microalgal species *Alexandrium tamarense* (Chapter 2).

2. To develop new species-specific molecular primers for use with polymerase chain reaction (PCR) to specifically differentiate between two closely related species of the harmful dinoflagellate genus *Karenia,* allowing an unbiased identification of the bloom forming *Karenia* species occurring in samples collected from the western English Channel (Chapter 3).

3. To use the primers developed above in combination with catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) to enumerate cells of the *Karenia* species present in water samples collected from the western English Channel (Chapter 4).

4. To apply the previously developed molecular PCR and CARD-FISH methods to identify and enumerate a harmful dinoflagellate species, *Bysmatrum granulosum* in samples collected from Bahrain coastal waters (Chapter 5).

1.9 Thesis structure



CHAPTER 2

Molecular detection of *Alexandrium tamarense* (Dinophyceae) using fluorescent *in situ* hybridization with monolabelled oligonucleotide.

2.1 Introduction

Alexandrium tamarense is an armoured, marine planktonic dinoflagellate that has been associated with toxic paralytic shellfish poisoning (PSP) in many coastal regions (John et al. 2005). It was first described as *Gonyaulax tamarensis* from the Tamar estuary near Plymouth, UK (Lebour 1925) cited in John et al. (2003b) and has been reported to occur from temperate to sub-Arctic regions and in tropical latitudes (Taylor 1984). The dinoflagellate genus *Alexandrium* Halim (Balech 1995) consists of more than 20 species (Balech 1985) and many are known to produce PSP toxins (Shumway 1990, Shumway and Cemeblla 1993, John et al. 2003a, Touzet and Raine 2006, Cho et al. 2008). The toxins cause severe symptoms in humans that ingest contaminated shellfish (Cho et al. 2008). The three species *Alexandrium tamarense* (the selected species in this study), *Alexandrium catenella*, and *Alexandrium fundyense* (Cembella 1998, Scholin 1998b) are collectively known as "*A. tamarense* species complex" (John et al. 2003b, 2005). According to nucleotide sequence analyses of the 18S small subunit of the ribosomal gene (SSU rDNA), the 28S large subunit of the ribosomal gene (LSU rDNA) and internal transcribed spacer (ITS) regions of the ribosomal gene (rDNA), the three species do not separate into three morphotypes, but instead the analyses have separated them into phylogenetic clades (ribotypes) based on their geographic origin (Guillou et al. 2002, John et al. 2005, Penna et al. 2008). These are the temperate Asian (TA), Tropical Asian (TROP), Tasmanian (TASM), West European (WE), and North American (NA) and finally the Mediterranean Sea (ME) clades (John et al. 2005). The *A. tamarense* morphotype is the only species compared to the other two in the *A. tamarense* species complex that occurs globally and is present in all the clades listed above (John et al. 2005).

Cembella et al. (1987) found that the '*A. tamarense* species complex' contains toxic and non-toxic strains that occur in different regions of the world. However to date, all strains from the Temperate Asian (TA), Tropical Asian (TROP) and North American (NA) clades have been shown to be potentially toxic (John et al. 2005).
Scholin (1998b) stated that the toxic and the non-toxic *A. tamarense* strains are indistinguishable according to both morphological and sub cellular criteria (e.g. ribosomal RNA (rRNA) gene). A good possible solution for the ambiguity of a confident identification of morphologically indistinguishable taxa is the application of molecular probes that specifically bind to a specific species or strain and therefore could be used to provide definitive identification (John et al. 2005).

Different molecular techniques that employ species-specific gene markers were previously investigated for the detection of *A. tamarense* and or/ *A. tamarense* species complex. Sandwich hybridization assays (SHA) (e.g. Anderson et al. 2005), fluorescent *in situ* hybridization (FISH) (e.g. John et al. 2003b, 2005) and various methods based on polymerase chain reactions (PCR) (e.g. Guillou et al. 2002, Hosoi-Tanabe and Sako 2005b) can identify *Alexandrium spp*. quite rapidly and simply. Table 2.1 is a list of the different probes and methods used to identify *A. tamarense* and/ or the *A. tamarens*e species complex.

Fluorescent *in situ* hybridization (FISH) with monolabelled probes is a fast, cheap and easy way to detect organisms in cultures and environmental water samples and does not require any further detection steps after hybridization (Motor and Göbel 2000). The FISH protocol often targets ribosomal RNA because large numbers of rRNA copies are found in cells, thus offering a large signal per cell (Amann 1995). In addition, whole cell hybridization provides information on the size and shape of the labelled cells which confirms the specificity of probes (Tyrrell et al. 1997).

The main objective of this initial part of the study was to detect and identify *A. tamarense* morphotype using a previously published monolabelled probe (ATAM01) designed by John et al. (2003b) employing a FISH technique with monolabelled probes and to evaluate

the specificity of the probe using other non-target dinoflagellate cultured cells. Then to employ similar techniques for the identification of HABs caused by *A. tamarense* and other toxic/harmful dinoflagellate species that occur either in temperate or tropical coastal waters.

Table 2.1: Probes and molecular methods used previously for the detection of *A. tamarense* or *A. tamarense* species complex.

Method	Probe used to detect *A. tamarense*	Specific for	Reference
FISH	cTAM-F1	*A. tamarense*	Adachi et al. (1996a)
Monoclonal antibodies and FISH	LSU rRNA (NA1)	North American *Alexandrium* species cluster	Anderson et al. (1999)
Nested PCR	LSU rDNA (Alex1)	*Alexandrium spp.tamarensis* complex	Guillou et al. (2002)
DNA dot blot and FISH	SSU rDNA (ATAM01)	*A. tamarense* species complex	John et al. (2003b, 2005)
FISH	LSU rRNA (Atm1)	North American *Alexandrium* species cluster	Sako et al. (2004), Hosio-Tanabe and Sako (2005a)
Monoclonal antibodies, FISH and sandwich hybridization assay (SHA)	LSU rRNA (NA1)	North American *Alexandrium* species cluster	Anderson et al. (2005)
Real- time PCR	LSU rDNA	North American *Alexandrium* species cluster	Hosio-Tanabe and Sako (2005b)
FISH	LSU rDNA (TAMAD2)	*A. tamarense*	Kim et al. (2005)
Loop medaited isothermal amplification (LAMP)	5.8S rRNA (FIP, BIP, F3, B3)	*A. tamarense*	Wang et al. (2008)

2.2. Materials and methods:

2.2.1 *Cultures*

A number of unialgal cultures were obtained from the Plymouth Culture Collection of Marine Algae (PLY; Plymouth, UK) as indicated in Table 2.2 and grown in 100 mL Erlenmeyer flasks in L2 medium (Guillard and Morton 2004), minus silicate (Guillard and Ryther 1962). All cultures were maintained at 18-20 ºC in a temperature controlled incubator with a 12:12 hour light: dark (L: D) photocycle, at a photon flux density of 80 µmol photons m^{-2} s^{-1}**.** The cultures were occasionally gently mixed and subcultured every three weeks.

Table 2.2: Algal species used.

Algal species	Origin	Strain number
Alexandrium tamarense (Lebour) Balech	SW England, (estuarine)	PLY: 173
Alexandrium minutum Halim	Fleet lagoon, Dorset UK	PLY: 669
Karlodinium veneficum Braarud	Norway, inshore	PLY: 517
Glenodinium foliaceum Stein	SW England (brackish)	PLY: 499

2.2.2 *Fluorescent* in situ *hybridization (FISH) with monolabelled probes*

Monolabelled probes with fluorescent stain (6-FAM) attached at the 5' end, were used in the first stage of this project (Table 2.3). The probes were purchased from Thermo Fisher Scientific (Ulm, Germany) and were received in lyophilized form. The material was resuspended in filtered-sterile distilled water to a final concentration of 200 ng μL^{-1}, subdivided in small aliquots of 50 µL volumes, and stored frozen at -20 ºC. The protocol used in this study was a modification of John et al. (2003b, 2005).

Algal cells were fixed prior to filtration and probe hybridization in order to retain cell integrity and morphology. 10 mL of cultured cells were fixed with 20% paraformaldehyde (PFA, Sigma- Aldrich) to produce a solution of 1% final concentration for one hour at room temperature or for 24 hours at 4 °C prior to filtration. Cells were then collected onto a 25 mm diameter, 5.0 µm (pore-size) cyclopore membrane filter (Fisher Scientific, UK), fitted into a 25 mm diameter standard filtration unit, filtered under < 100 mmHg vacuum to prevent cell damage, and dehydrated in an ethanol series (50%, 70% and 96% [v/v], 5 min each). Subsequently, the filters were dried and labelled with black pen and cut by a sterile razor blade into 8 small segments. The filter sections were placed on glass slides and each filter section was covered with probe-buffer mix. The monolabelled probe (final concentration 50 ng μL^{-1}) was added to formamide hybridization buffer (900 mM NaCl, 20 mM Tris-HCl [pH 8.0], formamide [concentration depends on the probe used, Table 2.3], 0.01% [w/v] sodium dodecyl sulfate [SDS]) in 1:10 ratio; 40 µL of the mixture was needed for every filter segment.

The hybridization step was continued for 2-4 hours at 46 ºC in a hybridization oven (HB-1000 Hybridizer) in a humid chamber. To terminate the labeling reactions, the filters were rinsed with a pre-warmed washing buffer (5 M NaCl stock reagent [concentration depends on concentration (%) of formamide in hybridization buffer, Table 2.3], 20 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.01% [w/v] SDS) to remove excess probes for 15 min at 48 ºC in a water bath (Grant). Filters were incubated in the solution without shaking. Subsequently, filters were dried and mounted on glass microscope slides with a mixture of CitiFluor (CitiFluor Ltd, London, UK) as an anti-fade agent, with 4', 6'- diaminidino- 2-phenylindoline solution (DAPI, $1 \mu g \mu L^{-1}$, Sigma) as a counterstain. The filter sections were inspected under a Zeiss Axiovert 200M inverted epifluorescence microscope (Carl Zeiss) with motorized stage, equipped with a ×40 UV Plan Apochromat objective and excitation/ emission filters 360/ 420 for DAPI and 490/ 515 for FITC and an automated image analysis system KS300 (Image Associates). Detection efficiency was calculated as FISH detectable cells (%) = 100 × (cells detected with rRNA FISH visualized by FITC)/ (cells visualized by DAPI).

To test and evaluate the specificity of the previously designed and tested ATAM01 probe to target *A. tamarense*, four different cultured cells (*Alexandrium tamarense*, *Alexandrium minutum*, *Karlodinium veneficum,* and *Glenodinium foliaceum)* were mixed in equal proportions (10 mL each) and hybridized with four different probes; the probe sequences, hybridization conditions and references are given in Table 2.3. EUK 1209R was used as a positive control probe, which is an eukaryote-specific probe; the ATAM01 probe was used as a specific probe that should react only with *Alexandrium tamarense;* negative control probes (EUB338R and NON338) were used to account for nonspecific labeling and the inherent autofluorescence of cells, which might contribute to the positive ATAM01 signal.

Table 2.3: Probes used for Fluorescent *in situ* Hybridization.

Probe name	Specific for	Probe Sequence [5′- 3′]	Probe location (gene/region)	FA (%)[b]	NaCl in washing buffer (mM)	Source
EUK1209R	Eukaryotes	GGGCATCACAGACCTG	18S rDNA (1195-1211)[a]	40	56	Giovannoni et al. (1988)
EUB338R	Eubacteria	GCTGCCTCCCGTAGGAGT	16S (338- 355)[a]	35	80	Amann et al. (1990a)
NON338	Non target organism	ACTCCTACGGGAGGCAGC	16S(338- 355)[a]	35	80	Wallner et al. (1993)
ATAM01	*Alexandrium tamarense species complex*	TTCAAGGCCAAACACCTG	18S rDNA	20	225	John et al. (2003b, 2005)

a *Escherichia coli* numbering of the small subunit rDNA.
b Formamide concentration for *in situ* buffer

2.3 Results

FISH with monolabelled probes

Attempts to use the previously developed and tested fluorescent *in situ* hybridization method with monolabelled oligonucleotide probe to target cultured *A. tamarense* were unsuccessful for many reasons. Firstly, signal intensity was too low to distinguish labelled cells from the autofluorescence of non-target cells and from background fluorescence. Furthermore, the species-specific ATAM01 probe gave a positive hybridization signal with both the target species and other non-target species used in this study and the percentage of positive hybridization was low.

When the four different algal cultures (*Alexandrium tamarense*, *Alexandrium minutum*, *Karlodinium veneficum* and *Glenodinium foliaceum)* were mixed and hybridized with the four different probes (Table 2.3), EUK1209R probe was found to be capable of labeling all four species (Fig. 2.1A) (95% of the hybridized cells showed positive hybridization). Both EUB338R and NON338 probes did not stain the cells used (Fig. 2.1B, C) (no positive hybridization). ATAM01 was used to aid in detecting *A. tamarense* cells in mixed populations of different cultures, but the percentage of positive hybridization was low (2% positive hybridization) (Fig. 2.1D). The non-detection of cells may have been caused by the loss of cells by the FISH processes (e.g. during washing). Additionally it was difficult to discriminate between the target species and the non-target species (photograph not shown), i.e. the hybridization signal intensity was not strong enough to discriminate between stained and non stained cells.

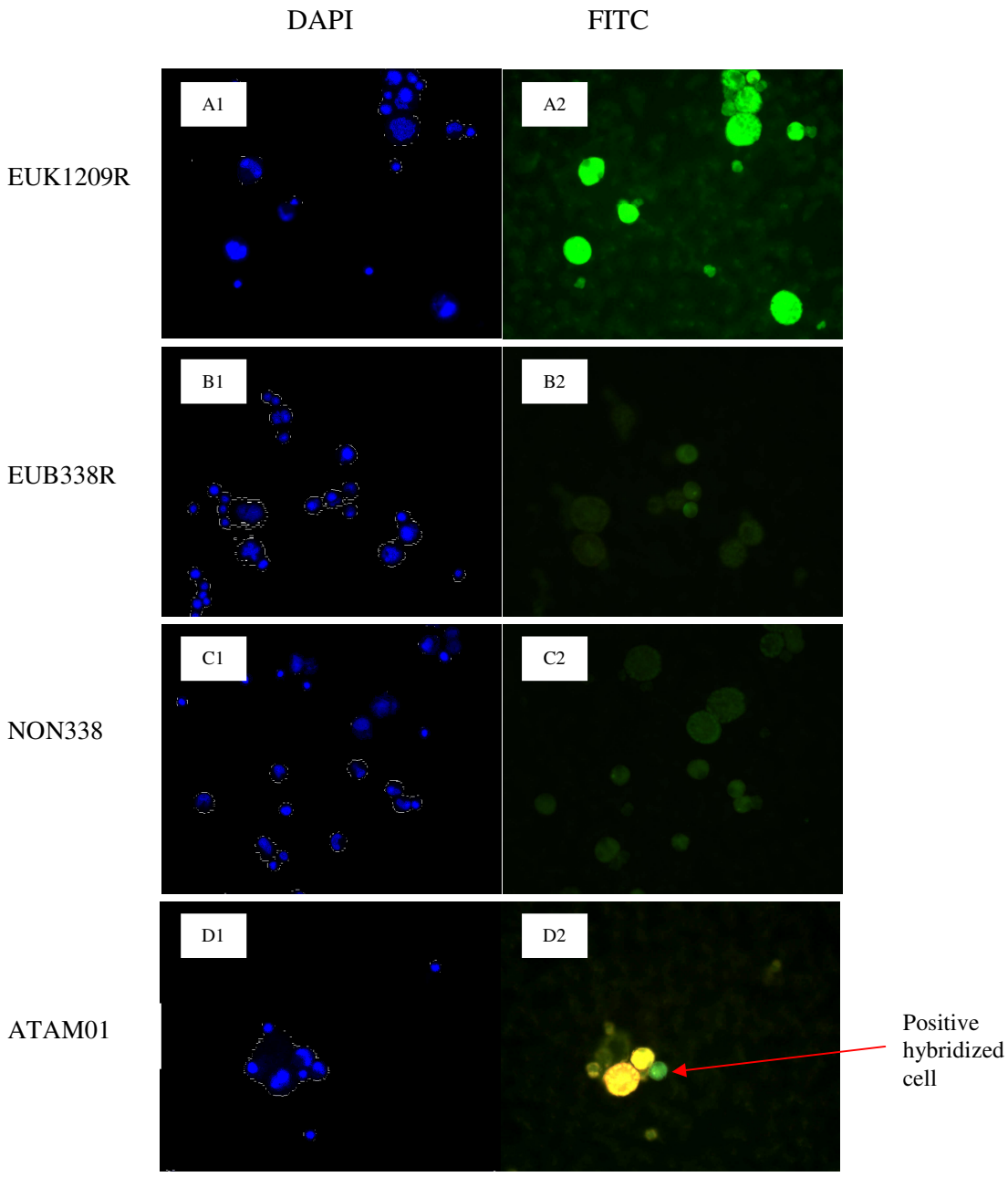


Figure 2-1: Micrographs of the mixed four cultures hybridized with different probes. (A1, A2): EUK1209R; (B1, B2): EUB338R; (C1, C2): NON338; (D1, D2): ATAM01 (left: DAPI- stained cells; right: FITC- stained cells).

2.4 Discussion

The molecular probe that was selected in this study was based on the 18S small subunit ribosomal gene (SSU rDNA) designed for the entire '*A. tamarense* species complex' (John et al. 2003b, 2005). This probe has been previously found to be 100% specific for the entire group of target organisms (i.e. *A. tamarense*/ *A. catenella*/ *A. fundyense*), and was at least one nucleotide mismatch to all known non-target organisms (John et al. 2003b, 2005). John et al. (2005) demonstrated the problem of the strong autofluorescence of cells that occur within FISH methods which can mask the hybridization signals, but recommended that laboratory cultures should be harvested during exponential growth phase because they contain more ribosomes than cells in stationary phase. In field samples, the fixation time and washing with ethanol could be increased to allow ethanol to extract more chlorophyll (Chl.) and therefore the autofluorescence of cells will be minimized. In this study, the probe showed non-specificity in the detection of *A. tamarense* species as it produced positive hybridization signals with the target species as well as other non-target species used for the evaluation of the probe. The percentage of positive hybridization was difficult to assess because of the great loss of cells that may have been due to the filtration and washing steps. Additionally, 1% paraformaldehyde may not be sufficient to fix the large *Alexandrium tamarense* cells used in this part of the study.

Previously developed and tested monoclonal antibodies, fluorescent *in situ* hybridization (FISH) using SSU rDNA and LSU rDNA probes to target different *Alexandrium* species (Table 2.1) have encountered many problems. Anderson et al. (1999) demonstrated that both cell surface proteins and ribosomal RNA concentration would vary under different environmental conditions. Therefore, different signal intensities could be expected using either monoclonal antibodies targeting cell surface proteins or oligonucleotide targeting rRNA. The cell permeability and the accessibility of the target protein and nucleic acid could vary greatly with the physiological condition of the cells (Anderson et al. 1999). Similar findings were achieved by Adachi et al. (1993, 1996a).

As was mentioned in the introduction polymerase chain reaction (PCR) was found previously to be successful in the detection and identification of many harmful algal bloom species (e.g. Godhe et al., 2001, Guillou et al.2002, Hosoi-Tanabe and Sako 2005b, Anderson et al. 2005) but it would be better to couple this molecular method with other molecular methods that involve the intact cells (e.g. FISH with amplification step) to minimize non-specific detection due to cross reactivity with non-target species.

2.5 Conclusions

The preliminary experiments conducted during the first part of the current study involved the evaluation of a previously designed and tested fluorescent *in situ* hybridization method using a monolabelled oligonucleotide probe (ATAM01), and has shown non-specific detection of the target species. The percentage of positive hybridization was low and the hybridization signal intensity was weak and difficult to discriminate between the positively hybridized cells and the autofluorescence of cells or even the background fluorescence. Therefore a molecular method based on polymerase chain reaction (PCR) and a FISH method involving amplification steps (catalyzed reporter deposition-fluorescent *in situ* hybridization, CARD-FISH) were chosen for later work.

CHAPTER 3

Molecular detection and identification of *Karenia mikimotoi* (Dinophyceae) and *Karenia brevis* (Dinophyceae) in cultures and seawater samples.

3.1 Introduction

The dinoflagellate genus *Karenia* includes several identified species including *K. mikimotoi* (Miyake et Kominami *ex* Oda) Hansen & Moestrup, previously described as *Gyrodinium aureolum, Gymnodinium* cf. *nagasakiense* and *Gymnodinium mikimotoi* (Hansen et al. 2000, Vanhoutte-Brunier et al. 2008) and *K. brevis* (Davis) Hansen & Moestrup formally known as *Gymnodinium breve* (Daugbjerg et al. 2000, Haywood et al. 2004).

K. brevis produces potent toxins (brevetoxin) that can find their way through the food chain killing fish, invertebrates, birds, and marine mammals (Landsberg and Steidinger 1998, Tester et al. 2000). In humans, brevetoxin cause illness termed neurotoxin shellfish poisoning (NSP) (Taylor et al. 2004). Until recently, NSP produced by *K. brevis*, was considered to be endemic to the coastal waters of the Gulf of Mexico, including the south-west coast of Florida and New Zealand coastal waters (McFarren et al. 1965, Taylor et al. 2004) but *K. brevis*- like species have also been reported from the Western Atlantic, Spain, Greece, Japan, and Australia (Taylor et al. 2004, Walsh et al. 2006). The ichthyotoxic dinoflagellate *K. mikimotoi* produces hemolytic glycolipids that cause gill damage in fish (Yasumoto et al. 1990, Parrish et al. 1998), and has been linked to fish kills in Japan and Norway (Takayama and Adachi 1984). Assessing the environmental threat posed by these two toxic species is complicated by the fact that in some regions, e.g. the Gulf of Mexico *K. mikimotoi* has been reported to co-occur with *K. brevis* (Steidinger et al. 1998, Haywood et al. 2004, 2007).

Historically, microscopic observations of live and preserved water samples have been required to monitor phytoplankton species known to produce toxins but considerable taxonomic expertise is required to distinguish between closely related species like *K. brevis* and *K. mikimotoi* (Haywood et al. 2004). Pigment analysis of phytoplankton samples without any knowledge of the phytoplankton population at the species level, can result in serious errors in the pigment-derived taxonomic composition of phytoplankton assemblages (Irigoien et al. 2004). More recently however, several investigators have explored the use of molecular genetic probes as tools to enhance our capability for rapidly detecting low concentrations of specific groups or species of microalgae to complement traditional microscopic cell-detection methods (Scholin et al. 2004).

Molecular methods based on genomic information and including fluorescence *in situ* hybridization (FISH) (Simon et al. 1997), lectin probes (Anderson et al. 1999) and polymerase chain reaction (PCR) (Scholin et al. 1999) have been introduced for the detection and identification of some harmful algal species. These assays can be rapid, accurate, simple and effective for the investigation of large numbers of samples (Kamikawa et al. 2006). Several molecular probes and primers have been previously designed to detect *K. brevis* in the Gulf of Mexico (e.g. Gray et al. 2003, Mikulski et al. 2005, Haywood et al. 2007). *K. mikimotoi* has also been targeted using molecular techniques in Southwest India (Godhe et al. 2001), off French Coasts (Guillou et al. 2002) and in Japanese waters (Kamikawa et al. 2006).

An attempt was made early in this study to test previously designed 18S small subunit ribosomal gene (SSU rDNA) primers designed by Godhe et al. (2001). The primers were used to detect and discriminate between *K. mikimotoi* and *K. brevis,* and apply them to measure cell concentration semi-quantitatively in cultures.

Two species-specific molecular probes were later developed, one for *K. mikimotoi* and one for *K. brevis* based on their 28S large subunit ribosomal gene (LSU rDNA) sequences. This region contains three domains (D1, D2 and D3) with the conserved core region upstream domain D3 and the hypervariable regions D1 and D2 which constitutes one of the fastest

evolving segments in rRNA-encoded eukaryotic DNA (Lenaers et al. 1989, 1991, Hansen et al. 2000, Guillou et al. 2002) and have the potential to be used for taxonomic and phylogenetic analyses of closely related species (Lenaers et al. 1991). Furthermore, a large set of sequences of the 28S LSU rRNA from many dinoflagellate species are available in GenBank (Guillou et al. 2002) for sequence comparisons. Because the aim was to detect *Karenia sp.* cells in pre-bloom conditions, i.e. when dinoflagellate cells in general and *Karenia sp.* in particular are rare in environmental samples, a nested PCR method was chosen. A first round of PCR using general dinoflagellate primers ($D1R_f$ and $D3B_r$) was used to amplify the target gene (the D1 and D3 parts of the LSU rDNA). This first PCR produced enough DNA for testing the specific primers designed in this study. The second amplification used PCR products obtained from the first amplification to specifically detect the presence or absence of the target species. This method is proposed for the detection of target species found in low cell densities for routine surveys, due to its high sensitivity, specificity and simplicity. Fig. 3.1 shows an illustration of nested PCR process. The target DNA (D1-D3 region of the 28S LSU rDNA gene) undergoes the first run of PCR with the first set of primers (D1R and D3B, shown in green). The product from the first reaction undergoes a second run with the second set of primers (*K. mikimotoi* or *K. brevis* primers, shown in red). The second primer set is intended to amplify a secondary target within the first run product.

The designed 28S LSU rDNA primers were used to confirm if *K. mikimotoi* is the main species present in water samples collected from the western English Channel during summer 2003 (bloom period, Fig. 3.2 shows SeaWiFS images for the time series of *K. mikimotoi* bloom in 2003), 2006, 2007 and 2008 and determine if *K. brevis* cells are also present. The designed 28S LSU rDNA probes were then applied on environmental water samples collected from Arad Bay, Bahrain of the Arabian Gulf during summer 2006, 2007 and 2008.

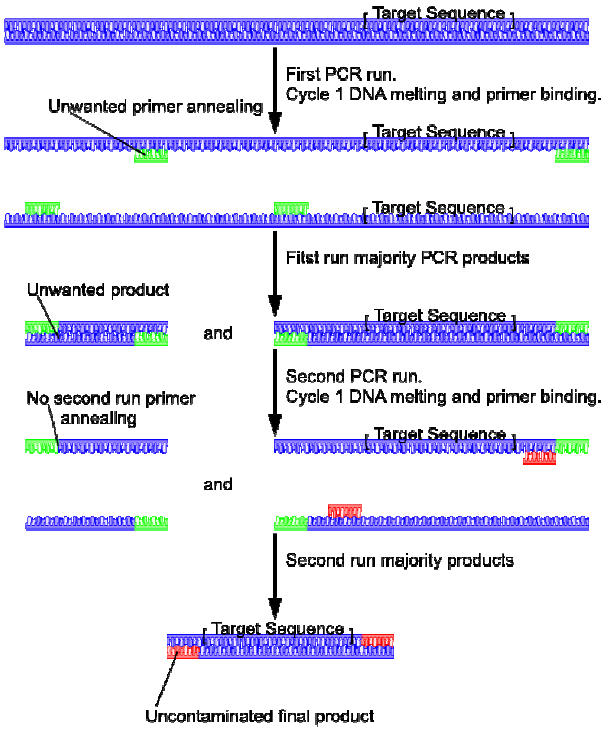


Figure 3-1: A diagram showing the principle of nested PCR (from www.Wikipedia.org)

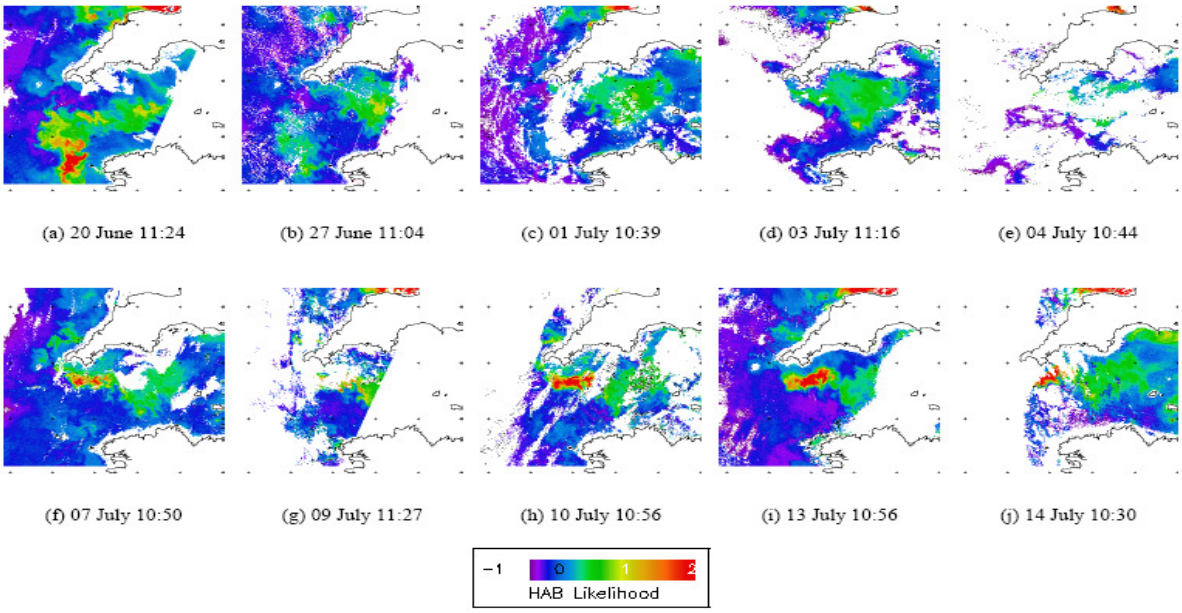


Figure 3-2: Time series HAB likelihood maps for the *Karenia mikimotoi* classifier showing the U.K. South Western Approaches and the development of a bloom between 20 June 2003 and 14 July 2003 (source: Shutler et al. 2005).

3.2 Materials and methods

3.2.1 *Dinoflagellate strain cultures*

A number of marine dinoflagellate strains were obtained from Plymouth Culture Collection of Marine Algae (PLY; Plymouth, UK) or Provasoli-Guillard National Centre for Culture of Marine Phytoplankton (CCMP; West Boothbay Harbor, Maine, USA) culture collections as listed in Table 3.1. Strains were grown in batch cultures in Guillard's L2 medium (Guillard and Morton 2004), minus silicate (Guillard and Ryther 1962), in 100 mL conical flasks in a temperature controlled incubator at 18-20 ºC, with 12:12 h light: dark (L: D) cycle and irradiance of 80 µmol photons m^{-2} s^{-1} with gentle mixing of the cultures and subculturing every three weeks.

Table 3.1: List of algal strains used in the present study, origin of isolates and strain number.

Algal strain	origin	Strain number
Karenia mikimotoi (Miyake et Kominami ex Oda) G. Hansen & Moestrup	English Channel, Carbis Bay, Devon	PLY: 705
Gymnodinium nagasakiense (=Karenia mikimotoi) Takayama & Adachi	Pacific: NW	PLY: 561
Karenia brevis (Davis) G Hansen et Moestrup	Sarasota, Florida USA	CCMP: 2228
Gymnodinium simplex (Lohmann) Kofoid & Swezy	English Channel	PLY: 368
Karlodinium veneficum Braarud	Norway, inshore	PLY: 517
Glenodinium foliaceum Stein	SW England (brackish)	PLY: 499
Alexandrium tamarense (Lebour) Balech	SW England, (estuarine)	PLY: 173
Alexandrium minutum Halim	Fleet lagoon, Dorset UK	PLY: 669

3.2.2 *18S small subunit rDNA primers for the detection of* K. mikimotoi *in cultures*

As a first step towards the development of a simple, quick and consistent method for the detection of *K. mikimotoi* in both cultures and seawater samples, species-specific primers designed by Godhe et al (2001) and based on the 18S small subunit ribosomal RNA gene (SSU rDNA) were initially tested. The primer sequences are listed in Table 3.2.

Table 3.2: Sequences of primers used in this study.

Species	Target gene	Annealing position	Primer sequence		Reference
			Forward primer sequence 5'-3'	Reverse primer sequene 5'-3'	
Dinoflagellate-Group	28S rDNA	(D1R: 24-31; D3B :1011-992)[a]	ACCCGCTGAATTTAAGCATA [$D1R_f$]	TCGGAGGGAACCAGCTACTA [$D3B_r$]	D1R (Scholin et al. 1994a) D3B (Nunn et al. 1996)
K. mikimotoi	28S rDNA	(Forward: 575-595; Reverse: 790-770)[b]	GCT**CT**GCATGAAGGTTG**TTG**	CACAATTGATTGGTCGGTTG	current study
K. brevis	28S rDNA	(Forward: 613-633; Reverse:828-808[b]	GCT**TG**GCATGAAGGTTG**C**T**A**	CACAATTGATTGGTCGGTTG	
K. mikimotoi	18S rDNA	(Forward: 276-297; Reverse: 1730-1709)[b]	TGCATCAGCTGGCGAATGATC	CAGGAA**C**TGAACACTGCGGCA	Godhe et al. (2001)
K. brevis	18S rDNA	(Forward: 275-296; Reverse: 1729-1708)[b]	TGCATCAGCTGGCGAATGATC	CAGGA**T**CTGAACACTGCGGCA	

a. Annealing position refer to the LSU rDNA of *Prorocentrum micans* (Lenaers et al. 1989).

b. Annealing position refer to the LSU of *K. mikimotoi* and *K. brevis* sequences obtained in this study (Appendix 1).

Dinoflagellate DNA extraction

Approximately 10 mL of exponentially growing cells from cultures listed in Table 3.1 was harvested by centrifugation (bench-top Eppendorf, MIKRO 22R, Hettich Zentrifuge) at

14000 rpm (19060*g*) for 10 min at room temperature and transferred to 1.5 mL Eppendorf tubes. Pelleted cultures were frozen at -20 ºC prior to DNA extraction.

Total genomic DNA was extracted from pelleted cells by adding 200 µL of a solution of 2% [w/v] of CTAB (Cetyl trimethylammonium bromide) buffer [1.4 M NaCl, 20 mM EDTA, 100 mM Tris- Cl, pH 8] containing 2% [w/v] polyvinylpyrrolidone (PVP) and 0.5% [v/v] ß-mercaptoethanol prewarmed to 60 °C according to Doyle and Doyle (1987). Subsequently, cells were vortexed for 1 min, until the mixture was homogenous and a further 800 µL of CTAB buffer was added and again vortexed. Samples were then kept at 60 °C for 30 min with regular mixing. Proteins were removed by extraction in an equal volume of chloroform:isoamylalcohol (24:1), then DNA was precipitated in 2× volume of cold isopropanol and 0.1× volume 7.5 M ammonium acetate at -20 ºC for at least one hour. DNA was recovered by centrifugation at 14000 rpm (19060*g*) for 15 min in a microcentrifuge. The supernatant was discarded, and the pellet was washed in 1000 µL of cold wash buffer (76% (v/v) ethanol containing 10 mM ammonium acetate). The supernatant was discarded, and the DNA pellet air-dried and resuspended in sterile 20 µL TE buffer (10 mM Tris-Cl; 1 mM EDTA) [pH 8].

Polymerase Chain Reaction method (PCR)

Amplification with 18S SSU rDNA species-specific primers (Table 3.2) was carried out in a thermal cycler (BIO-RAD DNA Engine) as described by Godhe et al. (2001). PCR reactions were run in 200 µL reaction tubes in volumes of 25 µL reaction mixture consisting of 1×Taq polymerase buffer (New England BioLabs, UK) with 0.5 µM of each primer (Thermo, Ulm, Germany), 200 µM of each deoxynucleotide triphosphate (dNTPs; New England BioLabs, UK) and approximately 10 ng of extracted DNA used as a template, 1 U of Taq polymerase (New England BioLabs, UK) and sterile Milli-Q water. All PCR reactions were commenced with an initial denaturation at 94 ºC for 5 min, followed by 30 cycles of 1 min at 94 ºC, annealing at 57 ºC for 1 min, and extension at 72 ºC for 1 min. After completion of the cycles, extension was completed at 72 ºC for 5 min. 10 µL of the PCR reaction mixture was loaded together with 2.5 µL of dye buffer onto 1.5% (w/v) agarose gel in 1×TAE buffer (0.04 M Tris-Cl, 0.04 M acetic acid, 0.001 M EDTA)

containing 1 mg mL^{-1} ethidium bromide. PCR bands on gels stained with ethidium bromide were later visualized under UV transillumination (Bio-Rad, Hercules, CA, USA).

3.2.3 *Semi-quantitative PCR measurements using 18S SSU rDNA* K. mikimotoi *species-specific primers*

The PCR based method was tested to determine the concentration of cultured *K. mikimotoi* cells semi-quantitatively, using the 18S SSU rDNA *K. mikimotoi* primers designed by Godhe et al. (2001). To construct a concentration-based curve, DNA was extracted from a range of different number of cells. A small volume of culture containing a known number of *K. mikimotoi* cells was pipetted onto a microscope slide and observed under the microscope. These were washed carefully to avoid loss of cells twice with sterile water and thereafter directly filtered onto a small piece of 5.0 µm (pore-size) cyclopore membrane filter (Fisher Scientific, UK). The number of cells ranged between 10 and 10^5 cells. Filters containing cells were immediately immersed in sterile Milli-Q water in a 200 µL PCR tube and kept at -20 ºC prior to analysis. To prevent the loss of cells using CTAB DNA extraction protocol, cells were used directly for DNA amplification (i.e. crude DNA from disrupted cells was amplified). Disruption of cells was induced by a freeze-thaw process. Due to false negative PCR results with high numbers of cells (more than 500 cells), cultured samples containing more than 500 cells were centrifuged, concentrated and finally sterile Milli-Q water was added to the PCR tubes and stored at -20 ºC. On the day of analysis, PCR tubes were thawed on ice and the PCR method (PCR thermal cycle and PCR conditions) used as listed in section 3.2.2. Quantification of positive DNA bands was performed using Bio-Rad QuantityOne software (version 4.1.1). The positive DNA bands were identified by comparison against a 1 Kbp low DNA mass ladder (Low DNA Mass Ladder, Invitrogen, UK).

3.2.4 *Development of 28S LSU rDNA primers (DNA extraction, PCR amplification, determination of 28S LSU rDNA gene sequences, and primer design)*

Due to the poor specificity of 18S SSU rDNA *K. brevis* primers, a decision was taken to design new primers based on the 28S LSU rDNA partial sequences (≈1000 bp) of *K. mikimotoi* and *K. brevis*. Approximately 10 mL of culture media of exponentially growing *K. mikimotoi* and *K. brevis* was harvested by centrifugation at 19060*g* for 10 min at room temperature and transferred to 1.5 mL Eppendorf tubes. Pelleted cultures were frozen at -20 ºC prior to DNA extraction. Total genomic DNA was extracted following the protocol listed in section 3.2.2 and according to Doyle and Doyle (1987).

Total cellular DNA was used as templates to amplify about 1000 bp of the large subunit (LSU) ribosome gene (rDNA) using terminal primers $D1R_f$ (Scholin et al. 1994) and $D3B_r$ (Nunn et al. 1996). Primer sequences are shown in Table 3.2. PCR amplification was performed on volumes of 25 µL, each consisting of 1×Taq polymerase buffer including 2 mM $MgCl_2$, 200 µM dNTPs (Roche Diagnostic Ltd, UK), 0.2 µM of each primer (Thermo, Ulm, Germany), 0.1 mg mL^{-1} Bovine Serum Albumin (BSA; Roche Diagnostic Ltd, UK), approximately 10 ng of DNA template, and 2.5 U of Taq DNA polymerase (Qiagen, UK). Amplification with the 28S LSU rDNA primers was carried out in a thermal cycler (BIO-RAD DNA Engine) using similar conditions to Hansen et al. (2000) as follows: one initial cycle of denaturation at 95 ºC for 5 min, followed by 32 cycles of denaturation at 94 ºC for 1 min, annealing at 52 ºC for 1 min, and extension at 72 ºC for 3 min. The temperature profile was completed by a final extension cycle at 72 ºC for 6 min. DNA PCR products were loaded on 2.0% agarose gels stained with ethidium bromide in 1×TAE buffer, followed by examination under UV transillumination (Bio-Rad, Hercules, CA, USA). PCR products were excised from the gel using a sterile scalpel and were then purified from the agarose using QIAquick PCR purification kit (Qiagen, MO BIO Laboratories, INC, USA) and were commercially sequenced (Geneservice Ltd, Cambridge, UK) using the same primers ($D1R_f$ and $D3B_r$).

The 28S LSU rDNA partial sequences of cultured cells of the two strains of *K. mikimotoi* (PLY: 705 and PLY: 561) and *K. brevis* were imported into CAP3 software (Huang and Madan, 1999) to obtain consensus sequences. These were then aligned against each other to check for base pair differences using ClustalW2 (Thompson et al. 1994, Larkin et al. 2007). Species-specific primers were designed using Primer3 (Rozen and Skaletsky 2000). Table 3.2 shows the designed primer sequences; one of the primers (Forward) has been designed to include four base pair differences between the two species (note that both *K. mikimotoi* and *K. brevis* have the same reverse primer sequence). The specificity of the primers for their target species was checked against GenBank database using BLAST (Basic Local Alignment Search Tool) within the NCBI (National Centre for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1990). The selected primer sequences were 20 nucleotides in length with GC content of 50% to get a more uniform annealing temperature during PCR.

3.2.5 *28S LSU rDNA species-specific primers*

To evaluate the species-specific 28S LSU rDNA primers designed in this study, PCR was performed on extracted DNA from several dinoflagellate algal cultures (Table 3.1) used as negative controls as well as the target species *K. mikimotoi* and *K. brevis*. DNA was extracted from 10 mL of cultured cells after harvesting them by centrifugation in a microcentrifuge according to Doyle and Doyle (1987) and as outlined in section 3.2.2. Direct PCR reactions using 28S LSU species-specific primers were run in total volumes of 25 µL, each consisting of 1× Taq polymerase buffer including 2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, 0.1 mg mL^{-1} BSA, 10 ng of DNA template and 2.5 U of Taq polymerase. Amplification with species-specific primers was carried out in a thermal cycler as follows: initially 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 1 min. A further extension was then completed at 72 °C for 2 min. The expected PCR fragment was approximately 200 base pairs. PCR products were cooled and loaded on 1 mg mL^{-1} ethidium bromide stained 2.0% (w/v) agarose gels in 1×TAE buffer followed by examination under UV transillumination. Annealing temperatures were optimized along a

temperature gradient from 55 °C to 60 °C using 10 ng of DNA templates. The optimal annealing temperature was determined from tests performed on the cultures listed in Table 3.1.

3.2.6 *Environmental water samples*

Western English Channel environmental samples

Seawater samples were collected from the western English Channel during summer 2003 (during a *Karenia* bloom period), 2006, 2007 and 2008 (Fig. 3.3, and Table 3.3). 100 mL of sea water was preserved with Lugol's iodine solution (50 g KI and 25 g I_2 in 500 mL Milli-Q H_2O used at a final concentration of 1%) for later microscopic examination. 10 mL of each Lugol's fixed sample was placed in a 10 mL sedimentation chamber (Hydrobios) and left to settle for 24 hours. *Karenia* cells as well as other phytoplankton were identified following Tomas (1997) and counted along two diagonal transects of the sedimentation chamber base at ×400 using a Leica DM IRB inverted microscope (the estimated counting error was < 10%). The concentration of cells mL^{-1} (*C*) was determined using the following equation (Anderson and Throndsen 2004):

$C = N \times (Ba/Bc)/V$

Where N = *number of cells in the two diagonal transects*

Ba= *area of chamber base* (mm^2) (=572 mm^2)

Bc= *area of counted transects* (mm^2) (=54 mm^2)

V= *10 mL*

Initially water samples collected in 2006 were preserved with formalin (5% final concentration) and processed according to the formalin-methanol method described by Godhe et al. (2002). PCR products however could not be produced from DNA extracted from the formalin preserved samples. A method was therefore developed based on extracted DNA from Lugol's preserved samples and nested PCR. 50 mL of each Lugol's

preserved water sample was filtered through a 25 mm GF/F filter (Whatman, Maidstone, UK) and kept at -20 ºC prior to DNA extraction.

DNA was extracted from the environmental filters according to Doyle and Doyle (1987) protocol as previously listed. Extracted DNA was first amplified using the 28S LSU rDNA dinoflagellate group-specific primers $D1R_f$ and $D3B_r$ (Table 3.2) and the obtained products used as template DNA for the next reaction with species-specific primers (28S LSU rDNA *K. mikimotoi* and *K. brevis* primers) listed in Table 3.2, i.e. nested PCR as described above. PCR products on gels were visualized under UV transillumination and a few positive DNA products were excised from the gel using a sterile scalpel and were subsequently purified from the agarose using QIAquick PCR purification kit (Qiagen, UK). These were commercially sequenced (Geneservice Ltd, Cambridge, UK) using the 28S LSU rDNA species-specific *K. mikimotoi* primers (positive PCR products produced using *K. mikimotoi* primers). Then the 28S LSU rDNA sequences were imported into CAP3 software (Huang and Madan 1999) to obtain consensus sequences. These sequences were then aligned against 28S LSU rDNA partial sequences of both *K. mikimotoi* and *K. brevis* obtained previously in this study to find base pair differences using ClustalW2 (Thompson et al. 1994, Larkin et al. 2007).

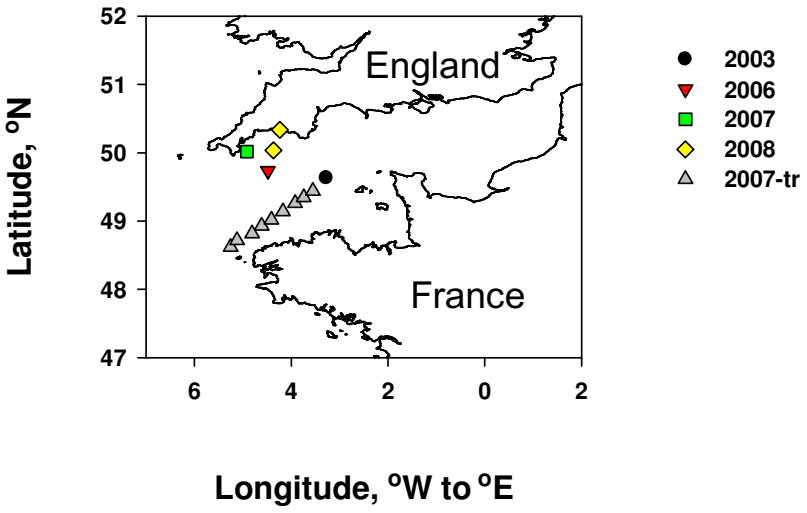


Figure 3-3: A map of the western English Channel showing the locations where water samples were collected (tr = surface water samples collected during a Ferry Box transect).

Environmental water samples from Arad Bay, Bahrain

Several seawater samples were collected from Arad Bay (Bahrain) during summer 2006 (April-November), summer 2007 (3.05.07, 23.06.07 and 13.09.07) and finally one sample during summer 2008 collected on 30.04.08. 100 mL of each seawater sample was fixed with Lugol's iodine solution (final concentration 1%) and stored in a cold dark place for later microscopic identification of phytoplankton and for molecular analyses using protocols developed during other parts of the study. More details of seawater samples collected from Bahrain will be given in Chapter 5.
Karenia sp. cells were found in several samples during microscopic examination. Therefore, the designed 28S LSU rDNA *K. mikimotoi* and *K. brevis* primers were applied to DNA extracts to confirm the presence of *Karenia sp.* following the protocol listed above.

3.3 Results

3.3.1 *18S SSU rDNA* K. mikimotoi *species-specific primers*

The 18S SSU rDNA primers designed by Godhe et al. (2001) and tested during the first part of the study amplified DNA fragments of the expected size (1433bp) from cultured cells of *K. mikimotoi*, but did not produce DNA products with most of the other unialgal cultures used as non-target species. However, *K. mikimotoi* primers were found to produce positive PCR products with *K. brevis* (Fig. 3.4).

Several experiments were conducted to increase the specificity of the 18S SSU rDNA *K. mikimotoi* species-specific primers. Only *K. mikimotoi* and *K. brevis* were used as *K. brevis* was the only non-target species that showed positive PCR products using the 18S SSU rDNA species-specific *K. mikimotoi* primers. These included decreasing the number of PCR cycles from 30 to 20 cycles and gradually increasing the annealing temperature above 57 °C to determine the optimum annealing temperature that would discriminate between the target species *K. mikimotoi* and the non-target species *K. brevis* (i.e. positive PCR products with *K. mikimotoi* only).

Decreasing the number of PCR thermal cycles from 30 to 20 did not increase the specificity of the 18S SSU rDNA *K. mikimotoi* primers (Fig. 3.5). However, raising annealing temperature above 57 °C did enhance the specificity of the primers. The optimum annealing temperature that would discriminate between *K. mikimotoi* and its closest relative *K. brevis* was shown to be 72 °C (Fig. 3.6).

Once the PCR method had been optimized for the detection of *K. mikimotoi* in cultures using 18S SSU rDNA primers, the same conditions were used for the detection of *K. brevis* in cultured cells using *K. brevis* primers (only the reverse primer is different from the one used for *K. mikimotoi*, see Table 3.2). The same thermal cycle and PCR conditions listed in section 3.2.2 were applied using both *K. brevis* culture (as target species) and *K. mikimotoi* culture (as non-target species). As shown in Fig. 3.7, the 18S SSU rDNA *K. brevis* species-specific primers yielded positive PCR products (1433bp) with both the target species *K. brevis* and the non-target species *K. mikimotoi* even using the optimized PCR thermal conditions (i.e. annealing temperature 72 ºC).

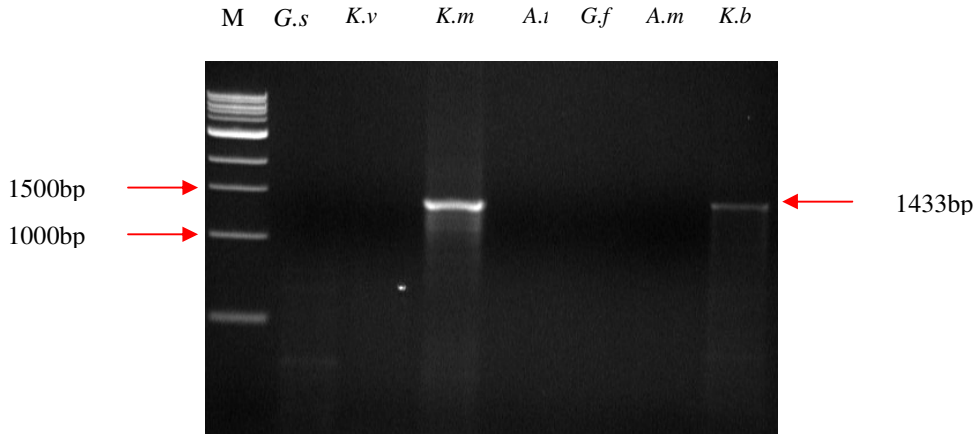


Figure 3-4: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using *K. mikimotoi* 18S SSU rDNA species-specific primers and DNA template from *K. mikimotoi* and other non-target species. The expected length of fragment is 1433bp. Lane 1: indicate molecular marker (1Kbp plus DNA ladder) (M). Lane 2: *Gymnodinium simplex (G.s)*; Lane 3: *Karlodinium veneficum (K.v)*; Lane 4: *Karenia mikimotoi (K.m)*; Lane 5: *Alexandrium tamarense (A.t);* Lane 6*: Glenodinium foliaceum (G.f);* Lane 7*: Alexandrium minutum (A.m);* Lane 8*: Karenia brevis (K.b).*

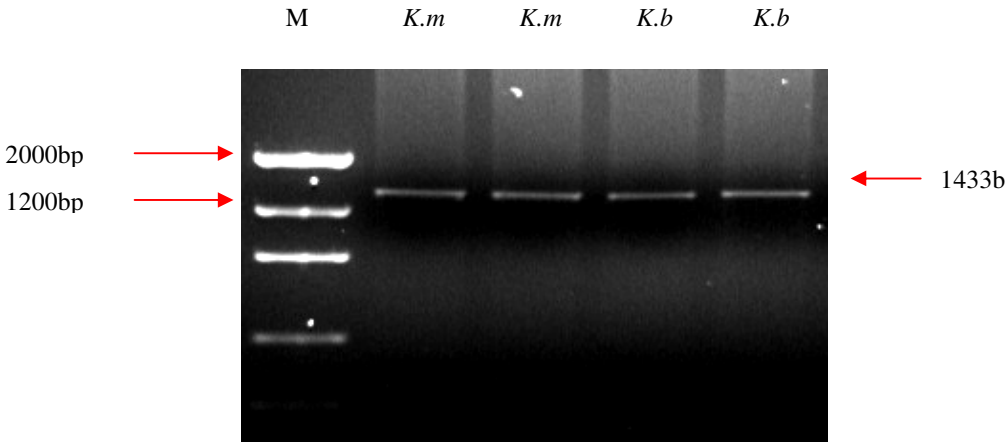


Figure 3-5: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using *K. mikimotoi* 18S SSU rDNA species-specific primers and DNA template from *K. mikimotoi* and *K. brevis*. Reducing the number of PCR thermal cycles from 30 to 20. Lane 1: indicate molecular marker (1 Kbp low DNA mass ladder) (M); Lanes 2 and 3: *K. mikimotoi (K.m)*; Lanes 4 and 5: *K. brevis (K.b)*.

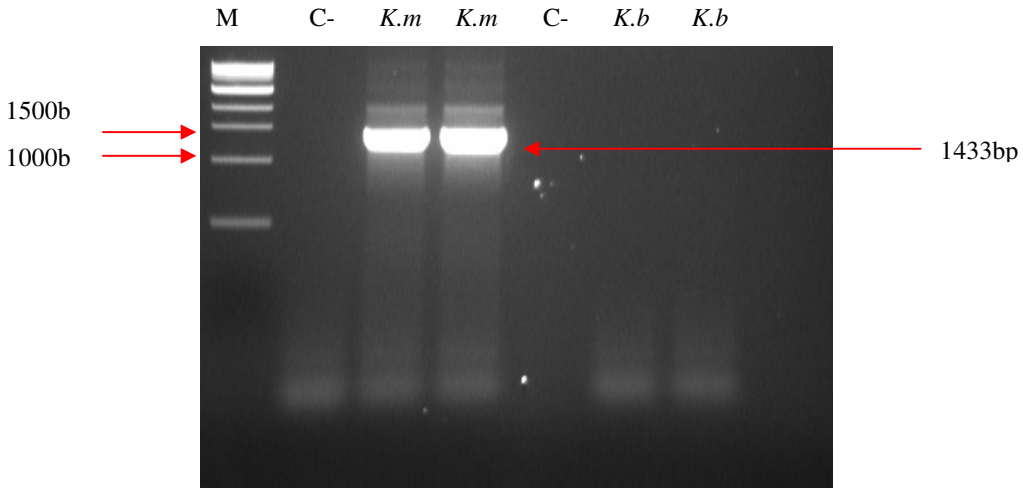


Figure 3-6: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using *K. mikimotoi* 18S SSU rDNA species-specific primers and DNA template from *K. mikimotoi* and *K. brevis*. Annealing temperature 72 ºC. Lane 1: indicate molecular marker (1Kbp plus DNA ladder) (M). Lanes 2 and 5: negative control (C-); Lanes 3 and 4: *K. mikimotoi (K.m)*; Lanes 6 and 7: *K. brevis (K.b).*

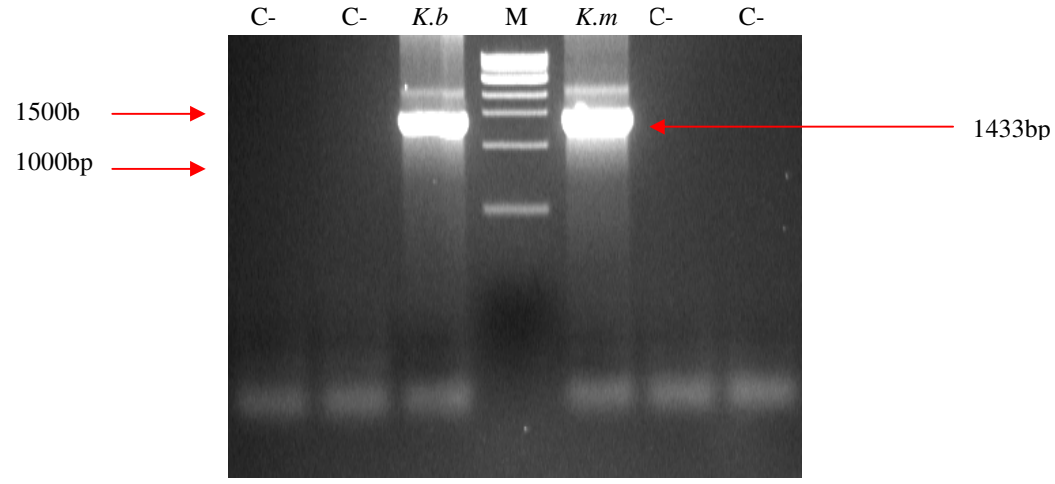


Figure 3-7: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using *K. brevis* 18S SSU rDNA species-specific primers and DNA template from *K. mikimotoi* and *K. brevis*. Lane 4: indicate molecular marker (1Kbp plus DNA ladder) (M). Lanes 1, 2, 6 and 7: negative controls (C-); Lane 3: *K. brevis (K.b)*; Lane 5: *K. mikimotoi (K.m)*.

3.3.2 *Semi-quantitative PCR measurements using 18S SSU rDNA* K. mikimotoi *species-specific primers*

The preliminary tests conducted to develop a semi-quantitative method to estimate *K. mikimotoi* cell concentrations in cultures were inconclusive. There was no clear relationship between the number of vegetative cells and the concentration of amplified DNA measured relative to a 1 Kbp low DNA mass ladder using QuantityOne software (Fig. 3.8, 3.9) although there was some indication of a positive trend at cell concentrations below 400.

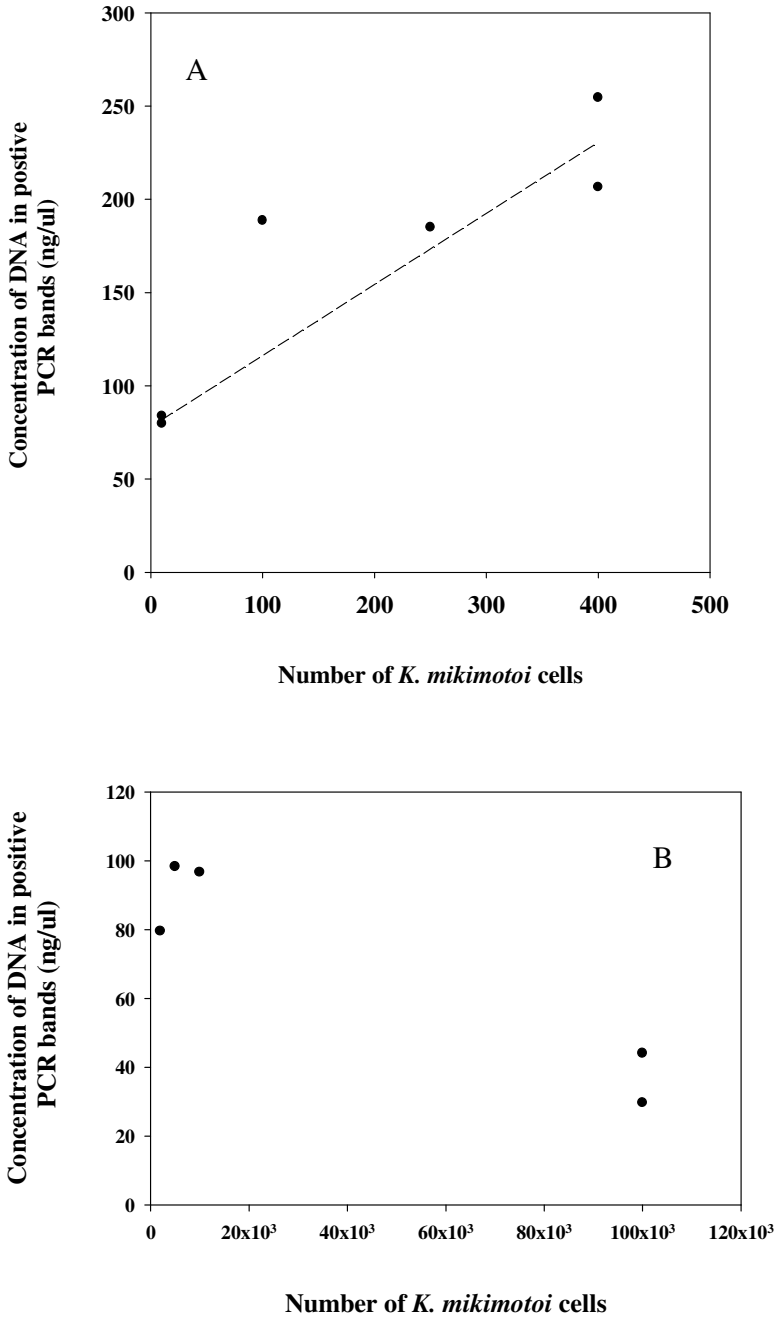


Figure 3-8: Plots showing relationship between numbers of *K. mikimotoi* cultured cells and concentrations of amplified DNA in positive PCR products. (A) Number of *K. mikimotoi* between 10 and 400 cells (dashed line indicates positive trend line); (B) Number of *K. mikimotoi* between 2000 and 100000 cells.

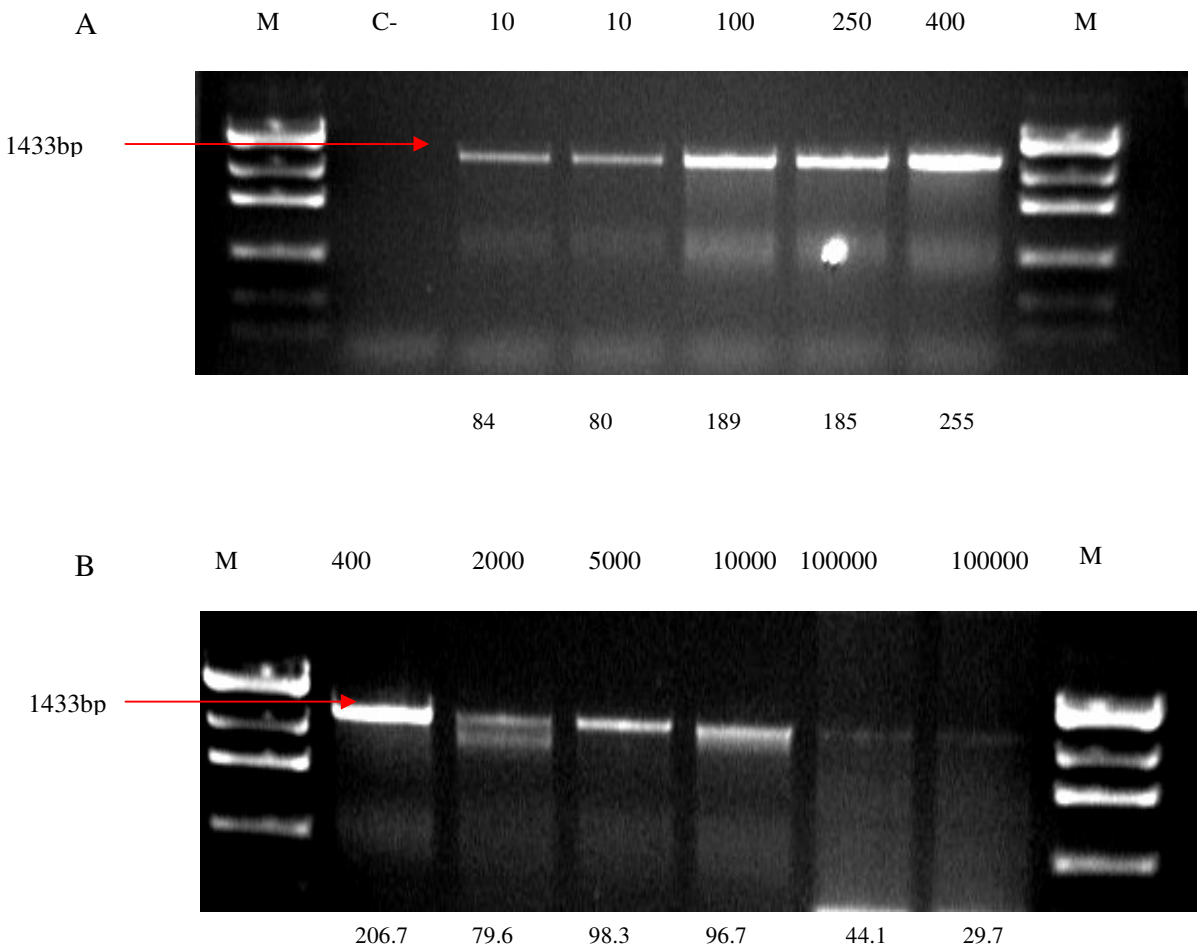


Figure 3-9: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using 18S SSU rDNA *K. mikimotoi* species-specific primers and DNA template from *K. mikimotoi* cultured cells. Numbers shown on the top of the gels represent the number of *K. mikimotoi* cells used for PCR method and the ones beneath the gels represent the concentration of DNA in the positive PCR products estimated using QuantityOne software in comparison to the 1 Kbp low DNA mass ladder. M: Molecular marker; C-: negative control.

3.3.3 *Comparison of 28S LSU rDNA partial sequences and design of species-specific primers of* K. mikimotoi *and* K. brevis

The D1-D3 region of the 28S LSU rDNA partial sequences (*ca.* 1000 bp) of the two strains of *K. mikimotoi* (PLY: 561 and PLY: 705) were 100% identical. There were however 25 base pair differences between *K. mikimotoi* and *K. brevis* in this region of the 28S LSU

rDNA. The D1-D3 region partial nucleotide sequences of *K. mikimotoi* and *K. brevis* cultured cells are listed in Appendix 1. *K. mikimotoi* species-specific forward primer sequence was aligned to different 28S LSU rDNA *K. mikimotoi* sequences using ClustalW2. The primer sequence had no mismatches when compared with *K. mikimotoi* strains isolated from Australia (AF200679), New Zealand (U92249), Japan (U92247, AF200681), Norway (AF200682) and from France (AF318223, AF318224). It does however have at least six base pair mismatches with the *K. brevis* culture used in the present study (CCMP: 2228) and other published sequences of different *K. brevis* strains from GenBank (e.g. AF200677, U92248). When the *K. brevis* species-specific forward primer sequence (Table 3.2) was compared to different *K. brevis* and *K. mikimotoi* LSU rDNA sequences, the forward primer had no mismatches with the *K. brevis* (CCMP: 2228) culture sequence and two other strains from GenBank (AF200677, U92248), but had at least four mismatches with *K. mikimotoi* sequences (PLY: 561, PLY: 705, present study cultures) and all other *K. mikimotoi* strain sequences from GenBank (AF200679, U92249, U92247, AF200681, AF318223 and AF318224).

3.3.4 *Designed 28S LSU rDNA species-specific primers*

The 28S LSU rDNA primer set designed in this study were successful in yielding PCR products of approximately 200 bp from their target species. They did not produce PCR products with other non-target species used to test the specificity of both primer sets. The primers designed in this study are characterized not only by having at least 4 base pair mismatches between the two closely related species, but they were chosen to be 20 nucleotides in length and have GC% content of 50% to achieve a more uniform annealing temperature during PCR thermal cycle. Different annealing temperatures were tested to minimize non-specific binding and reduce the cross reactivity with the non-target species. The optimum annealing temperature to discriminate between the two closely related species was found to be 60 ºC. Under these conditions the primers were shown to have very high specificity and sensitivity and there was a consistency in the results when tested on target and non target laboratory cultured species and field samples. Fig. 3.10A shows positive PCR products using *K. mikimotoi* primers with DNA extracted from *K. mikimotoi* (lane 2),

but no detectable products with DNA extracted from *K. brevis* cells (lane 3) or five other dinoflagellates cultured species tested. Similarly Fig. 3.10B shows positive PCR products using *K. brevis* primers but no detectable products with *K. mikimotoi* or the other five dinoflagellates. These results clearly demonstrate the specificity of the two 28S LSU rDNA primers.

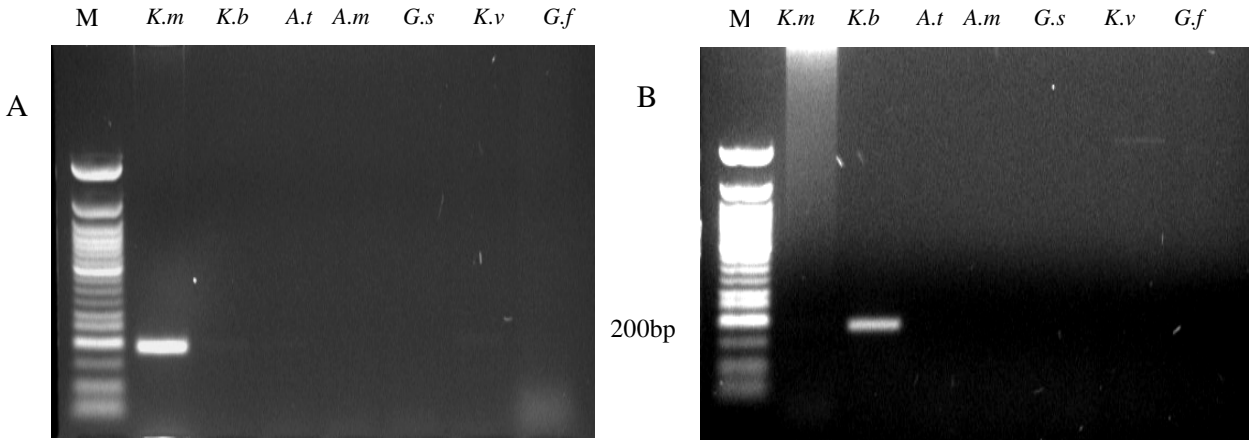


Figure 3-10: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using 28S LSU rDNA *K. mikimotoi* species-specific primers (A) and 28S LSU rDNA *K. brevis* species-specific primers (B) and template DNA from different dinoflagellate cultures. Lane 1: molecular marker (50bp plus DNA ladder) (M); Lane 2: *Karenia mikimotoi (K.m)*; lane 3: *Karenia brevis (K.b)*; Lane 4: *Alexandrium tamarense (A.t)*; Lane 5: *Alexandrium minutum (A.m)*; Lane 6: *Gymnodinium simplex (G.s)*; Lane 7: *Karlodinium veneficum (K.v)*; Lane 8: *Glenodinium foliaceum (G.f).*

3.3.5 *Environmental water samples*

Western English Channel water samples

Direct PCR using the 28S LSU rDNA species-specific primers designed above for *K. mikimotoi* and *K. brevis* often produced very faint bands or no products at all (data not shown). Therefore, nested PCR was performed on DNA extracted from all Lugol's preserved seawater samples. When a nested-PCR method was applied to DNA extracted from 50 mL of Lugol's preserved water samples, bright bands were seen on the gel and PCR using *K. mikimotoi* primers was positive in some samples even when cells were not

detected by microscopy (i.e. < 1 cell mL^{-1}, Table 3.3). A few positive PCR products amplified using *K. mikimotoi* primers were sequenced to confirm that nucleotide sequences of positive DNA products were identical to those obtained from *K. mikimotoi* cultured cells previously sequenced using 28S LSU rDNA dinoflagellate group primers.

Fig. 3.11A shows an example of positive PCR products produced using 28S LSU *K. mikimotoi* species specific primers on DNA extracted from a *Karenia* bloom water sample collected from the western English Channel during 2003 at 5 m. However, no PCR products were produced using 28S LSU *K. brevis* species-specific primers on DNA extracted from the same water sample (Fig. 3.11B). Fig. 3.12A-C shows PCR products produced using 28S LSU *K. mikimotoi* species specific primers on DNA extracted from different water samples collected from off Falmouth, from samples collected on FerryBox cruises in the western English Channel and off Plymouth (see Table 3.3). No PCR products were produced using *K. brevis* primers when applied on the same water samples (gels not shown).

Microscopic identification and quantification of cell densities of the different phytoplankton genera (Table 3.4) revealed that small flagellates (< 5 µm in size) were the dominant group in most seawater samples. The number of diatom species identified in the Lugol's fixed samples exceeded the number of dinoflagellate species. In the 2003 bloom sample, it was found that *Karenia sp.* cells formed about 99% of the phytoplankton present in the sample, and no diatoms were observed. Similarly, no diatoms were detected in 2006 Falmouth samples and *Karenia sp.* was present in all but two samples in a relatively high concentration (6 contained cell concentrations of *Karenia sp.* between 24 and 320 cells mL^{-1} by microscopy) and also gave positive PCR products with 28S LSU rDNA primers; *Karenia sp.* was not detected by microscopy (< 1 cells mL^{-1}) or the nested PCR method in two of the 8 samples. *Karenia sp.* was present at lower cell concentrations in samples collected off Falmouth during summer 2007; 1 cell mL^{-1} in a water sample collected at 37 m depth and 10 cells mL^{-1} in the thermocline at 12 m; however nested PCR was positive for all samples despite the low concentration of *Karenia sp.* cells. Water samples collected on a FerryBox sampling transect during summer 2007 showed positive PCR products and

detectable *Karenia sp.* cells by microscopy in 8 samples and no detectable cells by microscopy or PCR products in one sample. Water samples collected off Plymouth in April 2008 (inshore and at the offshore E1 station) were positive by the nested-PCR method even when cells were not detected by microscopy. Diatoms were present in high concentrations in samples collected during 2007 from Falmouth and from FerryBox cruises.

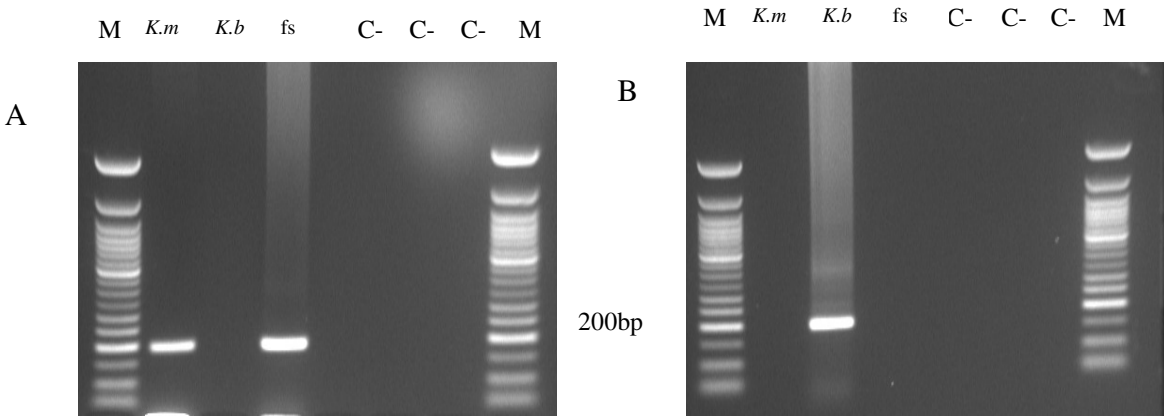


Figure 3-11: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using DNA extracted from a surface water sample collected from FerryBox survey during a bloom period in 2003. Nested PCR was performed using first the 28S LSU dinoflagellate primers D1R and D3B, the second PCR using the 28S LSU rDNA species-specific primers of *K. mikimotoi* and *K. brevis* (A) PCR products using *K. mikimotoi* species-specific primers and (B) PCR products using *K. brevis* species-specific primers. Lanes 1 and 8: molecular marker (50bp plus DNA ladder) (M); Lane 2: *K. mikimotoi (K.m)*; Lane 3: *K. brevis (K.b)*; Lane 4: surface water sample collected on FerryBox cruise (fs); Lanes 5, 6 and 7: negative controls (C-).

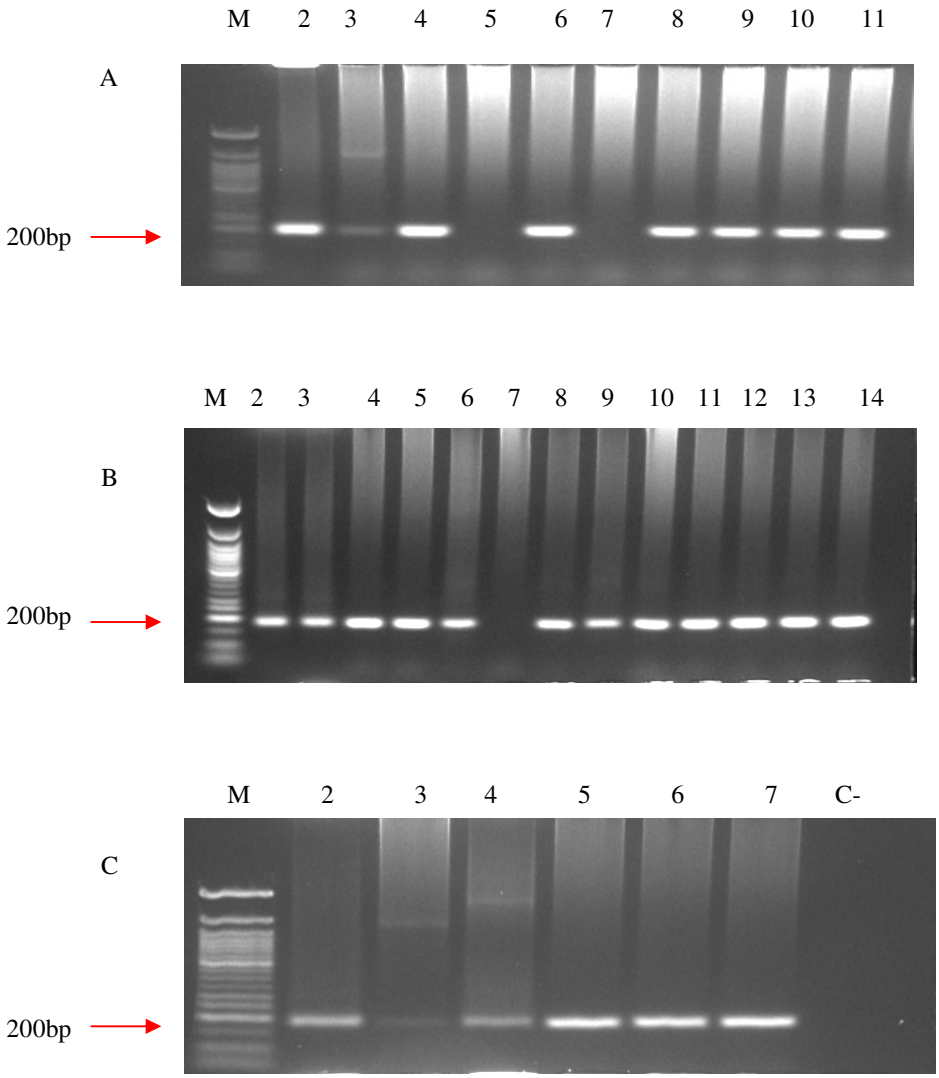


Figure 3-12: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using nested PCR which was performed using first the 28S LSU dinoflagellate primers D1R and D3B, the second PCR using the 28S LSU rDNA species-specific primers of *K. mikimotoi* and template DNA from environmental water samples collected from:
(A) Off Falmouth (summer 2006): Lane 1: molecular marker (50bp plus DNA ladder); Lane 2: *K. mikimotoi*; Lane 3: *K. brevis*; Lanes 4-11: water samples collected offshore from Falmouth.
(B) Off Falmouth and FerryBox transect water samples (summer 2007): Lane 1: molecular marker; Lane 2: *K. mikimotoi*; lanes 3-5: Different Falmouth water samples; Lanes 6-14: water samples collected on a FerryBox transect.
(C) Plymouth water samples (April 2008): Lane 1: molecular marker; Lane 2: *K. mikimotoi;* Lane 3: *K. brevis*; Lanes 4-7: Plymouth water samples; Lane 8: negative control. M: molecular marker; C-: negative control.

Table 3.3: Detection of *Karenia sp.* in western English Channel water samples using PCR analysis and microscopy counts.

Date of collection	Location	PCR detection		Microscopic detection range (cells mL^{-1})[e]
		+ve	-ve	
10.07.2003	FerryBox [a]	1		6004
6.07.2006	Falmouth [b]	6	2	<1-320
11.07.2007	Falmouth [c]	3		1-10
13.07.2007	FerryBox [b]	8	1	<1-30
2.04.2008	Plymouth [b]	2		<1
3.04.2008	Plymouth [d]	2		<1

[a] a seawater sample collected from a bloom area at 5m
[b] samples collected from surface water at different locations
[c] samples from 0, 12 & 37m
[d] samples from 0 & 10m
[e] microscopy detection limit of 1 cell mL^{-1}

Table 3.4: Quantitative record of phytoplankton taxa present in water samples collected from western English Channel (no. cells/ mL)

species	FerryBox 2003 [1]	Falmouth 2006 [2]								Falmouth 2007 [3]			FerryBox 2007 [4]								Plymouth 02.04.08 [5]		Plymouth 03.04.08 [6]	
		1	2	3	4	5	6	7	8	0m	12m	37m	1	2	3	4	5	6	7	8	9	1	2	3	4
DINOFLAGELLATES																									
Karenia sp.	6004	24		30		91	321	86	180	2	10	1	6		7	10	22	30	20	11	13				
Prorocentrum gracile	4		17	6	7	3	6	2	4																
Prorocentrum sp.																			11						
Scrippsiella sp.		1		5	5	1	3		8		2		6	1	13	1	15	5		3	3				
Oxytoxum sp.					1	2	3	3											8	7					
Gonyaulax sp.																			9						
Ceratium sp.			1		3	1		4	7																
FLAGELLATES																									
small flagellates		297	1754	2606	1523	81	12	523	386	289	475	652	106	211	591	872	3982	4259	4013	2078	340				
Euglenoids		11	7	53	39	1	2		2		1		25	7	4		85	122	83			7	4	8	6
DIATOMS																									
Guinardia sp.										25	288														
Pleurosigma sp.										42	23	10				1		1		5	1	2	2	2	2
Dactyliosolen sp.										4	3	2							98	14					
Nitzschia sp.										12	99	22	207	23	147	78	37	30	206	1					
Rhizosolena sp.										19	16	4						1	28	5	11				
Thalassiosira sp.										1	16														
Ceratulina sp.											1														
Chaetoceros sp.											4														
Grammatophora sp.											4	4										3			
Coscinodiscus sp.												16													
ciliate		10	15	14	13	1	8	2	18	2	5	1	2					6		2	5	2		6	3

1. Water sample collected during a FerryBox transect at 5m.
2: Surface water samples collecetd from different locations off Falmouth
3: Water samples collected off Falmouth at different depths (0, 12 and 37m)
4: Surface water samples collected during a FerryBox transect
5: Two different surface water samples.
6: Water samples collected from two different depths (0 and 10m)

Seawater samples from Arad Bay, Bahrain

Initially Lugol's preserved water samples collected from Arad Bay (Bahrain) were examined using light microscopy. Microscopic identification revealed that *Karenia sp.* was present in a few water samples at low concentrations (more details of cell densities will be given in Chapter 5). Therefore nested PCR was performed on DNA extracted from all Lugol's preserved seawater samples collected from Bahrain coastal areas (Arad Bay). Bright bands were seen on the gel, and PCR using 28S LSU rDNA *K. mikimotoi* primers was positive in 4 samples and 28S LSU rDNA *K. brevis* primer was positive in 2 samples.

Table 3.5 shows a list of water samples analyzed by microcopy and by PCR using 28S LSU rDNA *K. mikimotoi* and *K. brevis* primers. Positive PCR products were produced using *K. mikimotoi* species specific primers on DNA extracted from 2 surface water samples collected during summer 2006 (15.08.06 and 3.09.06) and 2 surface water samples collected during summer 2007 (3.05.07 and 23.06.07). Additionally, positive PCR products were produced using *K. brevis* species-specific primers on DNA extracted from 1 surface seawater sample collected on 1.11.06 and 1 surface water sample collected on 23.06.07 (Table 3.5, Fig. 3.13). *Karenia sp.* was also detected in these Lugol's fixed water samples using a light microscope.

No positive PCR products were produced from 9 seawater samples collected during summer 2006, 1 in 2007 and 1 in 2008 using 28S LSU rDNA *K. mikimotoi* primers. Similarly, PCR was negative with 10 seawater samples collected in 2006, 2 in 2007 and 1 in 2008 using 28S LSU rDNA *K. brevis* primers. There was a good agreement between the PCR method and the microscopic detection of *Karenia* species in these samples. Positive PCR products from gels were in this case not sequenced.

Table 3.5: Detection of *Karenia sp.* in Arad Bay, Bahrain water samples using PCR analysis and microscopic detection.

Year of collection	No. of collected samples	Positive Microscopic detection of *Karenia sp.*	*K. brevis* PCR detection		*K. mikimotoi* PCR detection	
			+ve	-ve	+ve	-ve
Summer 2006	11	3	1	10	2	9
Summer 2007	3	2	1	2	2	1
Summer 2008	1	0	0	1	0	1

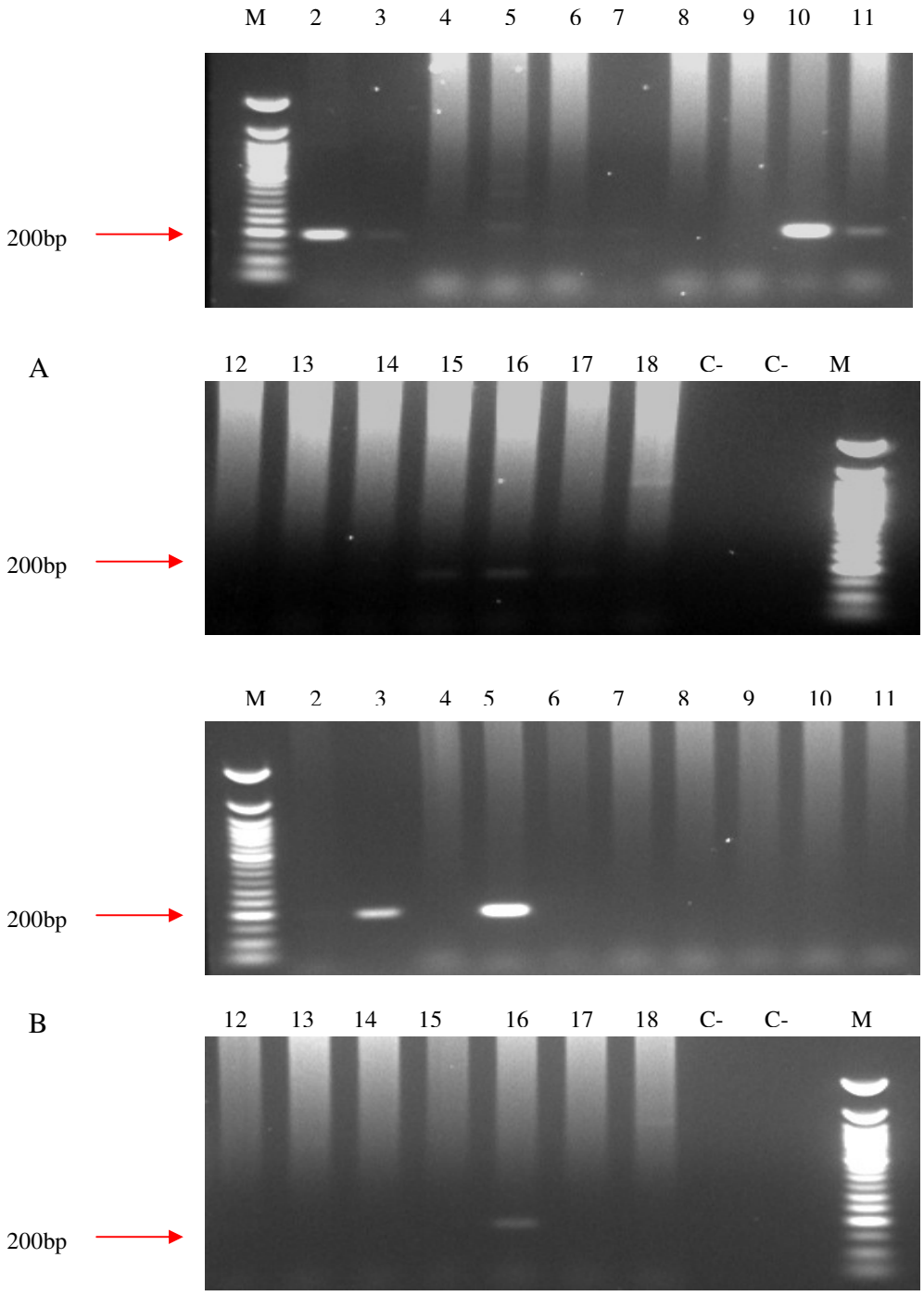


Figure 3-13: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using nested PCR which was performed using first the 28S LSU dinoflagellate primers D1R and D3B, the second PCR using the 28S LSU rDNA species-specific primers of *K. mikimotoi* (A), using the 28S LSU rDNA *K. brevis* primers (B) and template DNA from environmental water samples collected from Arad Bay (Bahrain) (2006-2008, see Table 3.5). Lanes 1 and 21: molecular marker (50bp plus DNA ladder) (M); Lane 2: *K. mikimotoi;* Lane 3: *K. brevis*; Lanes 4-18: seawater samples collected from Bahrain; Lanes 19 and 20: negative controls (C-).

3.4 Discussion

3.4.1 *Detection of two closely related* Karenia *species with 28S LSU rDNA primers*

Godhe et al. (2001) previously designed species specific 18S SSU rDNA PCR primers for the detection of *K. mikimotoi* and showed that the primers amplified a product of expected size from cultured cells of *K. mikimotoi* but did not yield products from a range of closely related non target species including *Pentaphardinium tyrrhenicum*, *Heterocapsa triquetra*, *Cachonina hallii*, *Prorocentrum micans*, *Gymnodinium catenatum* and *Gyrodinium impudicum.* However, they did not use *K. brevis* as a non target species in their study, which is known to be the closest relative to *K. mikimotoi*. Initially Godhe et al. (2001) 18S SSU rDNA species-specific primers (sequences shown in Table 3.2) were tested in this study and positive PCR products (1433bp) were achieved with DNA extracted from *K. mikimotoi* cells (after slight modification of the original PCR thermal cycle) but no products were obtained with *K. brevis* cultured cells. The 18S SSU rDNA forward and reverse primer sequences were checked in GenBank using BLAST and it was found that there is only one base pair difference between *K. mikimotoi* species-specific reverse primer and *K. brevis* 18S SSU rDNA nucleotide base sequence (Table 3.3). However, when the 18S SSU rDNA *K. brevis* species-specific primer was tested on *K. mikimotoi* and *K. brevis* cultured cells, positive PCR products were obtained with both species. Sako et al. (1998) reported that the 18S SSU rDNA gene was difficult to use for discriminating *K. mikimotoi* from other closely related species because the homologies of sequences in this gene among dinoflagellates is very high. Additionally, the semi-quantitative PCR test applied to calculate cell concentrations of cultured *K. mikimotoi* using the 18S SSU rDNA primers was not encouraging. It was found that there was no clear relationship between the concentration of DNA produced after PCR amplification and the number of *K. mikimotoi* vegetative cells. This could have been due to the high concentration of the target DNA (dinoflagellate contain multiple copies of the 18S SSU rDNA gene). Furthermore, the PCR chemicals used for conducting this part of the experiments were found to be not optimal (New England BioLabs DNA Polymerase) for semi-quantitative PCR.

Following these preliminary results with the 18S SSU rDNA species-specific primers, new primers were designed targeting the 28S LSU rDNA gene. The LSU rDNA gene encompasses highly variable regions intermixed with very conservative areas (Daugbjerg et al. 2000) and has shown previously to be species-and sometimes strain-specific (e.g. Michot et al. 1984, Lenaers et al. 1989). In addition the 28S LSU rDNA gene of many dinoflagellate species has been sequenced and these are available in GenBank (Guillou et al. 2002).

3.4.2 *28S LSU rDNA Primers characteristics and sensitivity*

The designed 28S LSU rDNA primers used in this study have at least 4 base pair mismatches between *K. mikimotoi* and *K. brevis* compared to the previously tested 18S SSU rDNA primers that have only single base pair mismatches between the two species. The 28S LSU rDNA sequences (≈1000 bp) of the two *K. mikimotoi* strains used in this study and from 7 strains from different geographic origins available in GenBank, all showed identical sequences when compared to the designed species-specific primers. This comparison agrees with previous results of Hansen et al. (2000) and Guillou et al. (2002). The 28S LSU rDNA gene sequences of all *K. brevis* strains deposited in GenBank also had similar sequences despite their geographic origin. Mikulski et al. (2005) compared five *K. brevis* cultures isolated from the Texas Gulf coast, the Florida Gulf coast, and the Atlantic coast of Florida, and detected no differences in the 28S LSU rDNA gene sequence among those isolates. Positive PCR products were obtained when the 28S LSU rDNA primers were applied to Lugol's fixed samples collected from the western English Channel and from Bahrain coastal waters. The 28S LSU rDNA species-specific primers designed in this study could be globally used therefore for the detection and identification of *K. mikimotoi* and/or *K. brevis* due to their high specificity and sensitivity.

3.4.3 *DNA extraction and nested PCR methods*

The CTAB method used for the extraction of DNA from both cultures and Lugol's preserved environmental water samples proved to efficiently extract DNA from low cell concentrations. The sensitivity of this DNA extraction method is thus suitable for monitoring harmful species of algae at low cell concentrations prior to the occurrence of algal blooms (Kamikawa et al. 2006). In addition to the effective CTAB DNA extraction method, nested-PCR using first dinoflagellate group 28S LSU rDNA primers followed by species specific primers for the target species allows the detection of toxic species at very low concentration in natural water samples. Direct PCR products were obtained on DNA extracted from *Karenia sp.* cultures (both live and Lugol's fixed) using the 28S LSU rDNA species specific primers for both species. The sensitivity of this method was demonstrated as positive PCR products could be produced from single cultured cells of *Karenia*. PCR products using the same method however could not be obtained from DNA extracted from natural samples even when cell concentrations were high. With natural water samples, direct PCR resulted in faint bands or no bands on gels when examined under UV transillumination as previously reported by Godhe et al. (2001). This could have been due to the presence of large amount of DNA from other dinoflagellate species present in the field samples that can mask the target DNA, or the target template DNA is present in very few copies. Therefore in order to achieve positive PCR products with natural water samples a nested PCR method had to be used involving initial amplification with dinoflagellate 28S LSU rDNA primers. The lowest concentration of *Karenia sp.* detected by microscopy in Lugol's preserved samples collected from the western English Channel was 1 cell mL^{-1} (July 2007) and the maximum concentration was 6000 cells mL^{-1} in surface waters during a bloom in July 2003 (Table 3.3). Using the nested PCR method, positive PCR products were obtained from both these samples (Table 3.3). According to this study results, 0.02 cells mL^{-1} is the PCR detection limit (assuming one cell present in 50 mL filtered seawater sample). Godhe et al. (2001) using a nested PCR approach with 18S SSU rDNA *K. mikimotoi* primers obtained positive PCR products with a field sample containing 0.7 cells mL^{-1} (lowest detection limit by microscopy). A water sample collected from the French

coast containing 0.01 cells mL^{-1} of *K. mikimotoi* was successful in giving positive PCR products using 28S LSU rDNA Kmik1 primers (Guillou et al. 2002).

It is not possible to use the nested PCR method to estimate cell abundance in environmental water samples. In addition, real-time PCR (QPCR) cannot be used for the same objective as positive PCR products were not achieved using DNA template extracted directly from field samples (i.e. direct PCR) and because of the nature of the dinoflagellate genome (multiple copy numbers of the 28S LSU rDNA gene). The 28S LSU rDNA copy number in both *K. mikimotoi* and *K. brevis* need to be established before a real time PCR method can be used to determine the cell concentrations of species in both cultures and more significantly in environmental water samples. The rDNA copy number in eukaryotes is generally correlated with genome size and cell volume (Prokopowich et al. 2003). *Alexandrium minutum* for example contains around 1000 rDNA copies (Galluzzi et al. 2004) and large dinoflagellates such as *Akashiwo sanguinea* have rDNA gene copy numbers that reach the highest values observed for plants (≈25000 in plants). Therefore for quantification of cell number in natural samples, other methods should be investigated such as fluorescent *in situ* hybridization (FISH). However, nested PCR is very useful for the initial detection and identification of harmful algal species and can be used as an early warning system for the presence of harmful algal species in low concentrations (Godhe et al. 2001).

PCR-based methods are very specific and sensitive (positive PCR signals are seen even if the number of cells is very low (< 1 cell per mL) in field samples) and do not require the use of cloning and incubating steps (Guillou et al. 2002). Furthermore, it is important to note that in most water samples analyzed during this study, there was a heterogeneous population of phytoplankton present (Table 3.4) but this did not affect the sensitivity and specificity of detecting and confirming the presence of *K. mikimotoi* in the western English Channel.

Godhe et al. (2002) found that Lugol's solution and various ethanol concentrations produced negative effects on PCR results. However, Lugol's solution used in this study as a fixative, showed very encouraging results. Sequences obtained from positive PCR products

(amplified from Lugol's fixed samples), were compared to the 28S LSU rDNA *K. mikimotoi* nucleotide sequence obtained from dinoflagellate group primers ($D1R_f$ and $D3B_r$) as well as other published sequences from GenBank from the same strain. The results showed no negative effect from this fixation and long term storage especially in the case of the 2003 bloom sample, on PCR results and no alteration of base pairs had taken place. Similar findings were achieved by Bowers et al. (2000), Tengs et al. (2001) and Guillou et al. (2002).

A study by Llewellyn et al. (2005) reported HPLC-pigment data and microscopy cell counts from water samples collected from the time-series sampling station L4 off Plymouth in the western English Channel. They suggested the presence of the pigment gyroxanthin was indicative of the dinoflagellate *K. brevis* in water samples collected during late summer and autumn at this station between 1999 and 2002. However gyroxanthin is known to also be present in *K. mikimotoi* as well as *K. brevis* (Örnólfsdóttir et al. 2003) and the reported presence of *K. brevis* at L4 by Llewellyn et al. (2005) has now been accepted as an error (Carol Llewellyn personal comm.). There was no indication from the current study that *K. brevis* is present in the western English Channel waters.

3.4.4 *Significance of microscopic examination and algal detection*

Karenia sp. is difficult to identify to species level using a light microscope due to difficulties in preserving the structure of naked dinoflagellates with different fixatives tending to distort characteristic details of cells. Additionally, many taxonomic problems are associated with *Karenia sp.* because morphological features are more likely to vary in response to changes in environmental conditions and growth stages (Godhe et al. 2001, Haywood et al. 2004). Despite the uncertainties and difficulties in identifying phytoplankton using a light microscope, it is still primarily used in research but more recently together with other practical approaches (e.g. HPLC detection of pigments (Irigoien et al. 2004)).

The PCR based method developed in this study should allow simultaneous detection of both *K. mikimotoi* and *K. brevis* in a single sample with minimal sample handling. Additionally, this method is more sensitive in the detection of *Karenia sp.* cells in comparison to traditional light microscopy i.e. PCR detection limit is 0.02 cell mL^{-1} compared to a detection limit of 1 cell mL^{-1} using a light microscope. However, the community structure of seawater samples could be studied in more detail using a light microscope. For example, in the 2003 *Karenia sp.* bloom sample, light microscopic examination of the water sample revealed the dominance of *Karenia sp.* with no diatoms detected in the sample. This suggests that the high density of *Karenia sp.* may inhibit the growth of diatoms due to possible toxic effects and similar findings were reported previously in the Ushant frontal system of the English Channel (Arzul et al. 1993). In the case of water samples collected from Arad Bay, Bahrain, the Lugol's fixed samples were studied using a light microscope, and *Karenia sp.* cells were detected in a few samples. Therefore, a test using the newly designed 28S LSU rDNA *K. mikimotoi* and *K. brevis* primers was performed on all samples. The few positive PCR products detected in some samples from Bahrain coastal water in 2006 and 2007 highlight the need for a monitoring and research program to investigate the possible occurrence of *K. mikimotoi* and/or *K. brevis* blooms in these waters.

3.5 Conclusions

It has been demonstrated in this study that the two designed 28S LSU rDNA species-specific probes can unequivocally differentiate *K. mikimotoi* and *K. brevis* cells from a range of non-target dinoflagellate cultured cells. *K. mikimotoi* has been shown to be the bloom forming species present in the western English Channel, and *K. brevis* is not present in that area. The nested PCR method proved very efficient in detecting these toxic species even if they are present at low concentration in the water column and it therefore could be used in HABs monitoring programs.

CHAPTER 4

Molecular enumeration of *Karenia mikimotoi* (Dinophyceae) in cultures and water samples from the western English Channel.

4.1 Introduction

An effective HAB monitoring programme requires the application of methods that allow the detection and enumeration of harmful species at low concentration (e.g. during bloom initiation). In Chapter 3, the 18S SSU rDNA *K. mikimotoi* primers designed by Godhe et al. (2001) were shown to be capable of discriminating between the target species *K. mikimotoi* and the non-target species *K. brevis* after slight modification of the PCR thermal conditions. However, preliminary attempts to develop a semi-quantitative PCR method to estimate cell abundance were not fully successful.

Subsequently, species-specific primers to target either *K. mikimotoi* or *K. brevis* were designed in this study based on the 28S LSU rDNA gene and were shown to be capable of discriminating between *K. mikimotoi* and *K. brevis* in culture, and detecting *K. mikimotoi* in environmental water samples collected from the western English Channel. One approach to quantifying the number of target cells in a water sample would therefore be to use a quantitative PCR (QPCR) method. However, as the copy number of the 28S LSU gene is not known for *K. mikimotoi* and *K. brevis*, it was realized that the QPCR method was not applicable. Therefore an alternative molecular method for the quantification of *Karenia* cells in natural water samples based on fluorescent *in situ* hybridization (FISH) was developed.

FISH is a widely used molecular approach that has been successfully employed to detect several harmful algal species (see Chapter 2 for details). Increasing the speed and reliability of FISH analysis has been developed by coupling FISH with flow cytometry (Amann et al. 1990b, Wallner et al. 1993) and has been applied to marine nano- and picoeukaryotes (Lim et al. 1993, Simon et al. 1995). However, the signal intensity was often too low to allow the

detection of small cells by flow cytometry. Therefore, several protocols of fluorescent amplification have been suggested e.g. the use of multiple probes or multiple fluorescent labels on one probe (DeLong et al. 1989, Amann et al. 1990a,b, Trebesius et al. 1994, Ouverney and Fuhrman 1997, Hoshino et al. 2001). The application of these amplification techniques however was limited: when non-specific binding was not a limitation to cell detection, only rapidly growing or large cells (>3 µm) could be detected (Lim et al. 1993, Wallner et al. 1993). Tyramide signal amplification of FISH (TSA-FISH) was first used with immunoassays (Bobrow et al. 1989, 1991, Adams 1992) then introduced to marine microbiology (Lebaron et al. 1997, Schönhuber et al. 1997, 1999) and found to be a powerful amplification technique that improved the fluorescent signal of hybridized cells 20 to 40 times (Schönhuber et al. 1997) over that of the background and even to 100 fold (Speel et al. 1999).

The TSA-FISH method is now widely used as catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) in microbial ecology (Schönhuber et al. 1997, Wagner et al. 1998, Pernthaler et al. 2002a, b). TSA-FISH has been successfully applied to free-living heterotrophic marine bacteria (Lebaron et al. 1997, Pernthaler et al. 2002a) and on environmental samples with a high fluorescent background (Schönhuber et al. 1997, Biegala et al. 2002), on photosynthetic microorganisms such as cyanobacteria (Schönhuber et al. 1999, West et al. 2001) and picoeukaryotes (Not et al. 2002, 2004). Several probes have been designed to detect the newly discovered algal groups Pelagophyceae (Anderson et al. 1993), Bolidophyceae (Guillou et al. 1999) and Picobiliphytes (Not et al. 2007). The coupling of TSA-FISH with flow cytometry has proved to be applicable for estimating picoeukaryotic abundance and diversity in natural environmental samples (Biegala et al. 2003, Not et al. 2004).

Quantification of harmful algal species

Monitoring harmful algal blooms (HABs) normally involves traditional methods of microscopy to discriminate harmful species. However, fluorochrome-labelled probes used in whole cell hybridization was shown by Groben et al. (2004) to be a fast alternative to the

traditional methods in the identification and detection of harmful phytoplankton. Toxic diatoms and dinoflagellate species of the genera *Pseudo-nitzschia* and *Alexandrium* that are responsible for amnesic and paralytic shellfish poisoning respectively have been identified in cultures and natural samples employing the FISH method (Miller and Scholin 1996, Adachi et al. 1996a, Scholin et al. 1996, Anderson et al. 1999, 2005, Sako et al. 2004, John et al. 2005, Hosio-Tanabe and Sako 2006).

Oligonucleotide probes targeting unique signature sequences in the 28S LSU rDNA gene have been designed previously to target *K. brevis* to include two base pair differences, as compared to *K. mikimotoi* (Mikulski et al. 2005). It was demonstrated in their study that the oligonucleotide probe designed to target *K. brevis* was capable of specifically labelling *K. brevis* in cultures and bloom samples to the exclusion of a range of other algal species, including *K. mikimotoi*, using fluorescent *in situ* hybridization (FISH) with monolabelled probes (Mikulski et al. 2005). In the current study several unsuccessful attempts to use FISH with monolabelled probes were initially undertaken to test published oligonucleotide probe sequences that target *Alexandrium tamarense* (see Chapter 2). The hybridization signals conferred by the monolabelled fluorescent probe was however not sufficient. In natural water samples, it has been demonstrated that FISH with mono-labelled probes can miss non-growing or starving cells because of low cellular rRNA content (Eilers et al. 2000, Oda et al. 2000) and the true abundances of particular target organisms might not be detected using FISH, especially in oligotrophic environments (Pernthaler et al. 2002b). Therefore, the main objective of this part of the study was to develop a method to detect and enumerate *K. mikimotoi* cells using the FISH approach that involves a signal amplification step (CARD-FISH). To date there has only been one other reported study of using CARD-FISH for detecting harmful algae in seawater (Töbe et al. 2006).

Experimental plan

To achieve the above objective, a method similar to that known as the full cycle rRNA approach (Amann et al. 1995) was used. Initially DNA from cultured cells of *K. mikimotoi* and its closest relative *K. brevis* was isolated, followed by PCR amplification of the 28S

large subunit ribosomal gene (LSU rDNA) and gene sequencing. Then the sequences were subjected to a comparative sequence analysis, in which a unique signature sequence was determined and used to design species-specific primer sets for both *K. mikimotoi* and *K. brevis*. After successful application of the designed primers on cultures and environmental water samples collected from the western English Channel (Chapter 3), the forward primer nucleotide sequence of *K. mikimotoi* was used to commercially synthesize an HRP-oligonucleotide probe to target *K. mikimotoi*. The use of the CARD-FISH approach was then evaluated to detect and enumerate *K. mikimotoi* in the laboratory. Non-labelled competitor probe was used to increase the probe specificity and to address non-specific binding of probes. Flow cytometry was also used to rapidly distinguish labelled *K. mikimotoi* and unlabeled *K. brevis* as well as other non-target dinoflagellate species in cultures. The finalized protocol allowed the abundance of *K. mikimotoi* to be estimated in seawater samples collected from the western English Channel during summer 2003, 2006, 2007 and 2008. Both epifluorescence microscopy and flow cytometry were used to quantify *K. mikimotoi* after CARD-FISH and the two counting methods compared.

4.2 Materials and methods

4.2.1 *Cultures*

Algal cultures used in this part of the study are listed in Table 3.1. Details of algal strains and culture conditions were previously listed in Chapter 3.

4.2.2 *Catalyzed Reporter Deposition Fluorescent in situ Hybridization (CARD-FISH)*

The protocol used in this study was a slightly modified version of a protocol reported previously (e.g. Biegala et al. 2002, 2003). The hybridization experiments were conducted

using cultured cells in exponential growth phase when cells contain the highest number of ribosomes.

The cell surfactant pluronic (10% [v/v] stock solution, Sigma Aldrich) was added to the cells at a final concentration of 0.1% [v/v], to minimize cell loss and clumping of cells. For cultured cells, 10 mL of each species was fixed with 20% [w/v] paraformaldehyde (PFA, Sigma- Aldrich) final concentration 1%, for one hour at room temperature or for 24 hours at 4 ºC. The fixed samples were then filtered onto a 25 mm diameter, 5 µm (pore-size) cyclopore membrane filter (Fisher Scientific, UK) fitted onto a 25 mm Millipore glass filtration system under < 100 mmHg vacuum to prevent cell damage. The cells were then dehydrated in an ethanol series (50%, 70% and 96% [v/v], 5 min each). Subsequently, to avoid cell loss, filters were embedded in low–gelling point agarose (concentration 0.2% [w/v]) and dried face-down in a Petri-dish covered with parafilm at 20-40 ºC for 10-30 min. The filters were then removed from the agarose by pipetting 96% [v/v] ethanol onto the filters and peeling them off very carefully and placed on a paper tissue to dry. Filters were then cut using a sterile scalpel into 8 equal segments. To ensure full permeabilization, cells were incubated in 50 mL of 0.01 M HCl for 10 min at room temperature then washed in 50 mL 1× phosphate buffered saline pH 7.4 (PBS, Sigma), sterile water and 96% (v/v) ethanol for 1 min each; the preparations were then air-dried and either processed directly or stored at –20 ºC until further processing.

FISH analysis on these pre-treated filter sections were performed using 5' horseradish peroxidase (HRP) - labelled probes. The probes that were used in this part of the study were received in a lyophilized form, and a portion of this material was resuspended in filter-sterile distilled water to a final concentration of 200 ng.μL^{-1}, subdivided into aliquots of 50 µL, and stored frozen at -20 ºC. Probe sequences, hybridization conditions and references are given in Table 4.1.

Filter sections were placed on a glass slide covered with parafilm then each covered with buffer-probe mix and incubated at 46 ºC in a hybridization oven (HB- 1000 Hybridizer) in a humid chamber for a minimum of 2 hours. The hybridization buffer and probe working solution (final concentration 50 ng μL^{-1}) were mixed in a 10:1 ratio. 1 µL of *K. brevis* probe used as a competitor (50 ng μL^{-1}, not labelled with HRP) was added in hybridization

experiments which involved the use of the *K. mikimotoi* probe. 40 µL of the mixture was required for each filter section. The hybridization buffer contained 900 mM NaCl, 20 mM Tris-HCl [pH 8.0], 0.01% [w/v] sodium dodecyl sulfate (SDS), 10% [w/v] dextran sulfate (Sigma), 2% [w/v] blocking reagent (Roche) and varying amounts of formamide, depending on the probe used (Table 4.1). The blocking reagent was prepared in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5). Washing was done in 50 mL of pre-warmed washing buffer with varying amounts of NaCl, depending on the probe used (Table 4.1), 5 mM EDTA, 20 mM Tris-HCl [pH 8.0] and 0.01% [w/v] SDS in a water bath (Grant) at 48℃. To equilibrate the probe-delivered HRP, filters were transferred into 15 mL of 1× phosphate buffered saline (PBS) for 15 min at room temperature. To remove excess buffer, the filter sections were dabbed onto blotting paper. Tyramide signal amplification was performed using custom fluorescent isothiocynate (FITC)-labelled tyramide (Pernthaler and Amann 2004). One part of tyramide solution was added to 300 parts of amplification buffer (1× PBS, 2 M NaCl, 0.1% [w/v] blocking reagent, 10% [w/v] dextran sulfate, 0.0015% [v/v] H_2O_2) for 15 min at 46 ℃ in the dark. Filter sections were then placed onto blotting paper to remove excess tyramide and washed with 1×PBS for 15 min at 46 ℃ followed by subsequent washes in sterile MQ water and 96% ethanol for 1 min. Subsequently, filters were air dried and either processed directly or stored at –20 °C prior to processing. Counter-staining of CARD-FISH preparations with 4', 6'- diaminidino- 2-phenylindoline (DAPI, 1 $\mu g.\mu L^{-1}$, Sigma) and microscopic evaluations were performed as described previously by Glöckner et al. (1996). Cells were counted under a Zeiss Axiovert 200M inverted epifluorescence microscope (Carl Zeiss) with motorized stage, equipped with ×40 and ×63 UV Plan Apochromat objectives and excitation/emission filters 360/420 for DAPI and 490/515 for FITC and an automated image analysis system KS300 (Image Associates). At least 300 cells were counted per filter section (three filter sections from three different experiments were counted); the mean values and the standard deviation were determined. Probe positive cells were presented as a fraction of DAPI-stained cells. Table 4.2 lists the CARD-FISH protocol used in the present study.

Table 4.1: Probes used for CARD-FISH analysis, (FA: formamide [v/v]).

Probe	Specificity	Sequence (5'- 3') of probe	Target site (rRNA positions)	FA (%)	NaCl in washing buffer (mM)	Source
EUK1209R[a]	**Eukaryotes**	GGGCATCACAGACCTG	**18S(1209- 1223)**	**40**	**37**	**Giovannoni et al. (1988)**
EUB II[a]	**Eubacteria**	GCAGCCACCCGTAGGTGT	**16S**	**50**	**19**	**Daims et al. (1999)**
EUB III[a]	**Eubacteria**	GCTGCCACCCGTAGGTGT	**16S**	**50**	**19**	**Daims et al. (1999)**
EUB338/I[a]	**Eubacteria**	GCTGCCTCCCGTAGGAGT	**16S**	**50**	**19**	**Amann et al. (1990a)**
NON338[a]	**No target organisms**	ACTCCTACGGGAGGCAGC	**16S**	**50**	**19**	**Wallner et al. (1993)**
GMIKI02[a,1]	***K. mikimotoi Species complex***	TTCCGGGCAAGGTCGAAA	**28S**	**40**	**37**	**Sako et al. (1998)**
K. mikimotoi[b,1]	***K. mikimotoi***	CAGGAACTGAACACTGCGGCA	**18S (1730-1709)**	**50**	**19**	**Godhe et al. (2001)**
K. mikimotoi[b,2]	***K. mikimotoi***	GCTCTGCATGAAGGTTGTTG	**28S (575-595)**	**60**	**9**	**The present study**
Kbprobe -7[a] **(competitor)**	***K. brevis***	GCTGGTGCAGATATCCCAG	**28S (877-896)**	_	_	**Mikulski et al. (2005)**

1. Reverse primer sequence

2. Forward primer sequence.

a. Purchased from Thermo Fisher Scientific, Ulm Germany.

b. Purchased from Biomers, Ulm Germany.

Table 4.2: CARD-FISH protocol for dinoflagellate species (Modified from Sekar et al., 2003).

Stage	Step no.	Description
Fixation	1	Fix cells with 20% PFA (1% final concentration) for 1 hr at room temp.(RT) or for 24 hrs at 4 °C.
	2	Filter fixed cells onto 5 µm cylopore membrane filter.
	3	dehydrate cells in an ethanol series (50%, 70%, 96%, 5 min each).
Embedding	4	Dip filters in 0.2% low-gelling point agarose and place filters face-down in a Petri-dish covered with parafilm and air dry at 20-40 °C (10-30 min).
	5	dehydrate in 96% ethanol at RT for 1 min.
	6	Air dry filters.
Permeabilization	7	If necessary, incubate in 0.01M HCl for 10 min at RT.
	8	Wash with 1×PBS (1 min at RT)
	9	Wash thoroughly with Milli-Q water (1 min at RT)
	9	Wash with 96% ethanol (1 min at RT).
	10	Air dry filters and cut in sections.
Hybridization	11	Place filter sections on a microscopic slide.
	12	Mix probe, competitor (if required) and formamide hybridization buffer in 1:1:10 ratio, 40µl of the mixture is needed for each filter segment.
	13	Incubate filters in hybridization oven at 46 °C for at least 2 hrs.
	14	Wash filter sections in prewarmed washing buffer in water bath (15 min at 48 °C), do not air dry filters after washing.
	15	Remove excess liquid with blotting paper, but do not let filter section dry out.
Tyramide signal amplification	16	Incubate filters in 1×PBS (15 min at RT)
	17	Dab filter sections on blotting paper, but do not let filter sections dry out
	18	Incubate filters in tyramide-amplification buffer mixture at 46 °C for 15 min in the dark.
	19	Dab filter section on blotting paper
	20	Wash in 1×PBS for 15 min at 46 °C.
	21	Wash in milli-Q water for 1 min at RT
	22	Wash in 96% ethanol for 1 min at RT
	23	Air dry filter sections
	24	Counter stain with DAPI

4.2.3 *Evaluation of CARD-FISH by Flow cytometry*

The CARD-FISH protocol for analysis of cells by flow cytometry was as described in the previous section (4.2.2) with slight modification. Exponentially-growing laboratory cultures were labelled with *K. mikimotoi* probe, CARD-FISH was employed without embedding filters in low-gelling point agarose. The protocol was carried out in a micropipette tip to minimize loss of cells during the different stages of CARD-FISH. Two 1000 µL micropipette tips were cut using a sterile scalpel so that one could be inserted inside the second and a small segment of 5 µm cyclopore membrane filters held in between. 500 µL of Paraformaldehyde fixed cultured cells were filtered and dehydrated in an ethanol series (50%, 70% and 96%, 5 min each). Subsequently, cells were permeabilized with 0.01 M HCl for 10 min and washed in 1×PBS, sterile Milli-Q water then 96% (v/v) ethanol for one min each. FISH and tyramide signal amplification steps were carried out as outlined in section 4.2.2. During the hybridization and amplification steps, micropipette tips holding the filter were sealed with parafilm to prevent loss of liquid and to avoid dehydration of filters. Finally, cells were back washed into plastic centrifuge tubes using sterile filtered seawater. Evaluation of oligonucleotide probe binding was performed using CellQuest software (Becton Dickinson, Oxford, UK) used to operate the flow cytometer (FACSCalibur Becton Dickinson equipped with a 15 mW 488 nm laser). For analysis of CARD-FISH samples, green fluorescence (fluorescein) was collected through the FL1 detector using 500-560 nm band pass filter. Data were acquired by using density plots of side scatter (SSC) versus log green fluorescence. Yellow-green beads of 0.5 µm diameter (Fluoresbrite Microparticles; Polysceinces) were used as a flow cytometric internal standard. WinMDI software 2.9 (http://flowcyt.salk.edu/software.html) was used to generate data plot illustrations.

4.2.3 *Application of the 28S LSU rRNA* K. mikimotoi *probe to enumerate* Karenia sp. *in Lugol's fixed environmental samples*

Seawater samples were collected from a number of locations in the western English Channel (Fig. 3.2, Chapter 3) and fixed with 1% Lugol's iodine solution and kept in a dark

cold place prior to CARD-FISH analysis. The CARD-FISH method was then applied on 20 mL of 8 fixed seawater samples to evaluate the sensitivity of the 28S LSU rRNA probe designed in this study to detect *Karenia sp.* in natural seawater samples. The seawater samples were washed with 10% sodium thiosulphate (Fisons Scientific, England) to equilibrate with the iodine in the Lugol's solution. The CARD-FISH protocol was then followed as listed above (4.2.2) using the designed 28S LSU rRNA probe. Epifluorescence microscopy was used to calculate the percentage of positive hybridized cells. This was then compared to the number of *Karenia sp.* cells counted as a percentage of total algal cells in 10 mL of Lugol's fixed sample using light microscopy. Additionally, the absolute number of positive hybridized cells counted using epifluorescence microscope was compared to *Karenia sp.* cells counted using light microscopy.

A seawater sample collected during summer 2003 (*Karenia sp.* bloom) was analyzed using CARD-FISH by flow cytometry. 20 mL of Lugol's fixed sample was washed with 10% sodium thiosulplate, and then labelled using the 28S LSU rRNA probe. The cells were then back washed into sterile seawater and analyzed using flow cytometry.

4.2.4 *Alignment of the different* K. mikimotoi *species-specific probes using BLAST*

The nucleotide sequences of GMIKI02, 18S SSU rRNA, and the designed 28S LSU rRNA probes were checked using GenBank to search for other eukaryotes that have exact matches using BLAST (Basic Local Alignment Search Tool) within the NCBI (National Centre for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1990).

4.2.5 *Phylogenetic analysis of* K. mikimotoi

Due to positive hybridization signals obtained using the 28S LSU GMIKI02 probe with two non-target species (*Gymnodinium simplex* and *Karlodinium veneficum*), an attempt was

made to reveal the phylogenetic relationship between *K. mikimotoi* and these two species, as well as with its closest relative *K. brevis*.

10 mL of each culture (*Karenia mikimotoi*, *Karenia brevis*, *Gymnodinium simplex* and *Karlodinium veneficum*) were concentrated by centrifugation at 14000 rpm (19060g). Genomic DNA was extracted using the CTAB method previously described in Chapter 3 according to Doyle and Doyle (1987). Total cellular DNA was used as templates to amplify about 1000 base pairs of the large subunit (LSU) ribosome gene (rDNA) using terminal primers $D1R_f$ (Scholin et al. 1994a) and $D3B_r$ (Nunn et al. 1996). Primer sequences (Table 3.2, Chapter 3) and PCR conditions were similar to conditions described by Hansen et al. (2000) as outlined in detail in Chapter 3. DNA fragments were checked in 1 $mg.mL^{-1}$ ethidium bromide stained 2.0% (w/v) agarose gels in 1×TAE buffer, followed by examination under UV transillumination. PCR products were excised from the gel using a sterile scalpel and were then purified from the agarose using QIAquick PCR purification kit (Qiagen, MO BIO Laboratories, INC, USA) and were commercially sequenced (Geneservice Ltd, Cambridge, UK) using the same primers ($D1R_f$ and $D3B_r$).

The 28S LSU rDNA sequences of cultured species were imported into CAP3 software (Huang and Madan 1999) to obtain consensus sequences. Consensus sequences were then aligned against each other and against published 28S LSU rDNA sequences of different dinoflagellate species from GenBank to check for base pair differences using ClustalW (Thompson et al. 1994, Larkin et al. 2007).

Phylogenetic analysis of the alignments was obtained using Phylip version 3.66 (Felsenstein 1993). Trees were constructed based on the distances calculated using the neighbor-joining (NJ) analysis. The reliability of the trees was tested and evaluated by bootstrapping (1000 replicates) using neighbor-joining (NJ). Trees were viewed using Tree View version 1.6.6. The different sequences selected from GenBank were chosen to make the association of the target species (*K. mikimotoi* and *K. brevis*) clearer. The 28S LSU rDNA sequence of *Alexandrium catenella* was used to polarize the in-group. Strain information and GenBank accession numbers of the different species used to generate the phylogenetic tree are listed in Table 4.3.

Nucleotide similarity which is used to quantify the similarity between the different species sequences was calculated for four dinoflagellate species of the present study. These species as mentioned above were *Karenia mikimotoi*, *Karenia brevis*, *Gymnodinium simplex* and *Karlodinium veneficum*. The selection was based on results obtained from CARD-FISH experiments to reveal the similarity between these species.

Table 4.3: Culture collection number or accession numbers for 28S LSU rDNA sequences and strain information of the dinoflagellate used in constructing the phylogenetic tree.

Species	Culture collection number /GenBank Accession number	Location and region/ country of isolation
Karenia mikimotoi *	PLY: 561	Pacific : NW
Karenia brevis *	CCMP: 2228	Florida, Sarasota
Gymnodinium simplex *	PLY: 368	English Channel
Karlodinium veneficum *	PLY: 517	Norway
Gymnodinium mikimotoi	AF200681	Japan
Karenia mikimotoi strain NOAA-2	AY355460	USA, Florida, Sarasota
Karenia brevisulcata	AY243032	New Zealand, Cawthron Institute
Karenia selliformis	U92250	New Zealand
Karenia brevis strain NOAA-1	AY355458	USA, Florida, Charlotte Harbor
Karenia papilionacea	U92252	Hawke's Bay, North Island, New Zealand
Karenia umbella	AY263963	Australia:Taranna, Tasmania
Karlodinium veneficum	DQ114466	Strain “Plymouth 103”
Gymnodinium galatheanum	AF200675	Strain “K-0522”
Scrippsiella trochoidea	AY628427	Unknown
Prorocentrum triestinum	AY863010	Unknown
Alexandrium catenella	AB265207	Strain “ACY12”

*Species used in the present study.

4.3 Results

4.3.1 *CARD-FISH analyses using epifluorescence microscopy*

28S LSU rRNA GMIKI02 probe

As part of the development of the molecular protocol to enumerate *K. mikimotoi* in cultures, a published species-specific probe GMIKI02 (Sako et al. 1998, Table 4.1) was tested to target *K. mikimotoi* against a wide range of other dinoflagellate algal species. Different concentrations of formamide hybridization buffer were used to get the highest percentage of positive hybridization with *K. mikimotoi*. 10 mL of *K. mikimotoi* culture was filtered and cells hybridized with the GMIKI02 at different formamide concentrations (40%, 45%, 50% and 55%), and the optimum formamide concentration that would produce the highest percentage of positive hybridization was determined. 40% formamide hybridization buffer was found to yield the highest percentage of positive hybridization 95.9% [± 2.1] (Fig. 4.1) and the optimum hybridization signal intensity.

To evaluate the specificity of the probe, 10 mL of different algal cultures (*Gymnodinium simplex*, *Karlodinium veneficum*, *Alexandrium minutum* and *Glenodinium foliaceum*) was fixed and hybridized with GMIKI02 probe using the optimum formamide hybridization buffer concentration (*K. brevis* was not included in this part of the experiment because it was not then in the culture collection). When the GMIKI02 species-specific probe was tested on other non-target species using 40% formamide hybridization buffer, the HRP-probe was found to give positive hybridization signals with the target species as well as other non-target species (Fig. 4.2, 4.3). The results showed that there was no significant differences in the percentage of positive hybridization (*Karenia mikimotoi* 95.9% [± 2.1]; *Gymnodinium simplex* 71.1% [± 4.6]; *Karlodinium veneficum* 73.0% [± 15]; *Alexandrium minutum* 90.3% [± 7.6], Fig. 4.2). No data is included in Fig. 4.2 for *Glenodinium foliaceum* as very few cells remained on the filter sections after CARD-FISH analysis. The

few *Glenodinium foliaceum* remaining cells did show a positive signal however. The hybridization signal intensity was almost the same between the different cultures (Fig. 4.3).

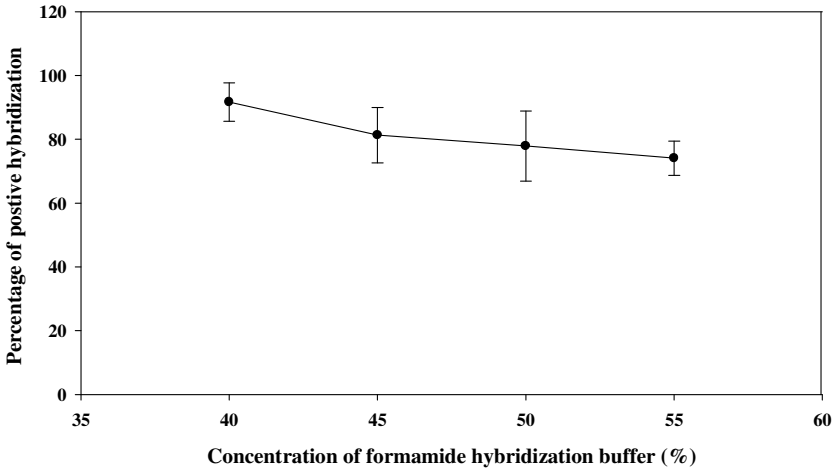


Figure 4-1: A plot showing percentage of positive hybridization after applying CARD-FISH using *K. mikimotoi* cultured cells at different concentrations of formamide and GMIKI02 probe, (error bars corresponds to standard deviation, n= 3).

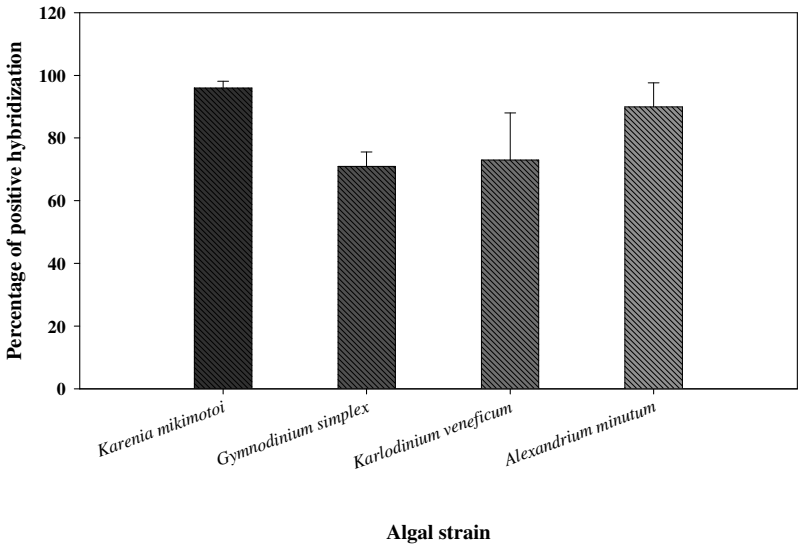


Figure 4-2: Percentage of positive hybridization using GMIKI02 probe at 40% formamide hybridization buffer, (error bars corresponds to standard deviation, n= 3).

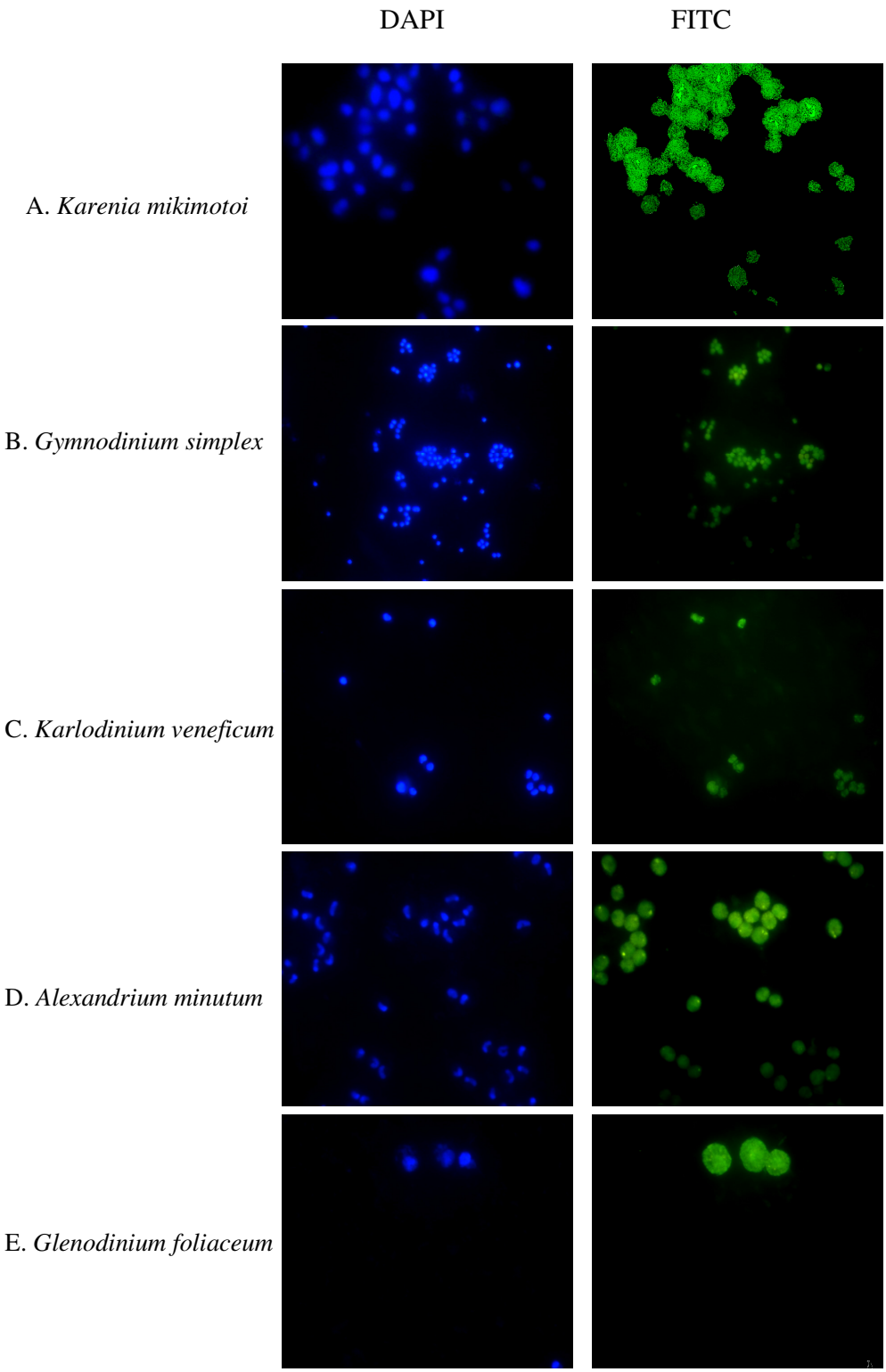


Figure 4-3: Micrographs of different algal strains using epifluorescence microscope with DAPI stained cells (left), GMIKI02 FITC probe stained cells (right). A: *Karenia mikimotoi*; B: *Gymnodinium simplex*; C: *Karlodinium veneficum*; D: *Alexandrium minutum* and E: *Glenodinium foliaceum* (total magnification of ×400).

The sensitivity of the species-specific probe GMIKI02 to detect *K. mikimotoi* in a mixed population of different dinoflagellate cells was tested by mixing 10 mL of *K. mikimotoi* with 10 mL of *Gymnodinium simplex, Karlodinium veneficum, Alexandrium minutum* and *Glenodinium foliaceum.* Subsequently, the CARD-FISH protocol was applied using several probes: GMIKI02 (*K. mikimotoi* species-specific probe); EUK1209R as the general eukaryotic probe and the two negative controls: EUBI-III (general probes used to target bacterial cells) and NON338 (which has no target species).

The EUK1209R probe was found to produce a high percentage of positive hybridization 88.6% [±8.2] and high hybridization signal intensity (Fig.4.4A). Percentage of positive hybridization with GMIKI02 probe was also high of 91.8% [±3.2] (Fig. 4.4B). The negative controls that were used to assess the autofluorescence of the different mixed dinoflagellate cells (EUBI-III and NON338) did not yield stained cells (Fig. 4.4C & D).

CARD-FISH with high formamide concentrations

Due to non-specific hybridization results obtained from the above experiment using the GMIKI02 probe, formamide hybridization buffer above 50% was used to increase the specificity of the species-specific probe GMIKI02 (increasing formamide concentration increases the stringency of binding). 10 mL of the dinoflagellate cultures used in the previous experiment (*Karenia mikimotoi, Gymnodinium simplex, Karlodinium veneficum, Alexandrium minutum* and *Glenodinium foliaceum*) was fixed and hybridized with GMIKI02 at 55%, 65%, 75% and 85% formamide hybridization buffer. Positive hybridization signals were obtained with two non-target species *Gymnodinium simplex* and *Karlodinium veneficum* as well as the target species *K. mikimotoi*. The positive signal greatly decreased at formamide concentration > 55% with the other non-target species (*Alexandrium minutum* and *Glenodinium foliaceum* (Fig. 4.5, 4.6)**.**

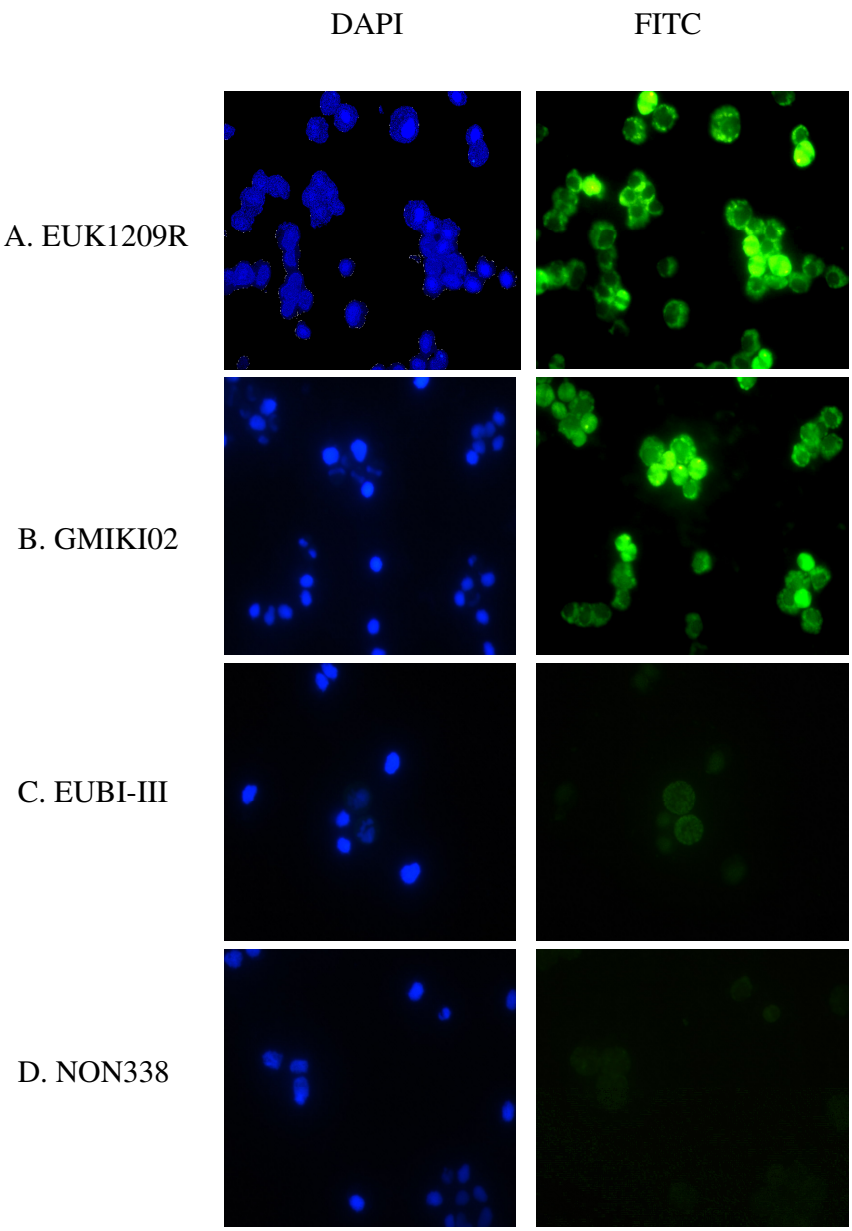


Figure 4-4: Micrograph of *K. mikimotoi* mixed with other algal strains. DAPI stained cells (left), FITC stained cells (right). A: EUK1209R; B: GMIKI02; C: EUBI-III; D: NON338.

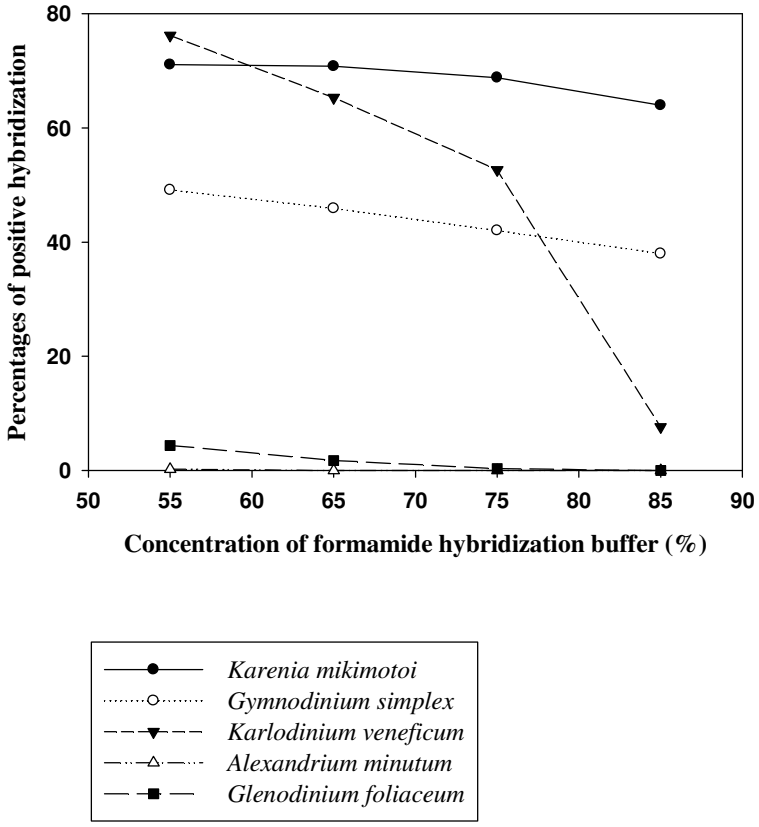


Figure 4-5: Percentages of positive hybridization with different algal strains using GMIKI02 probe and high formamide concentrations.

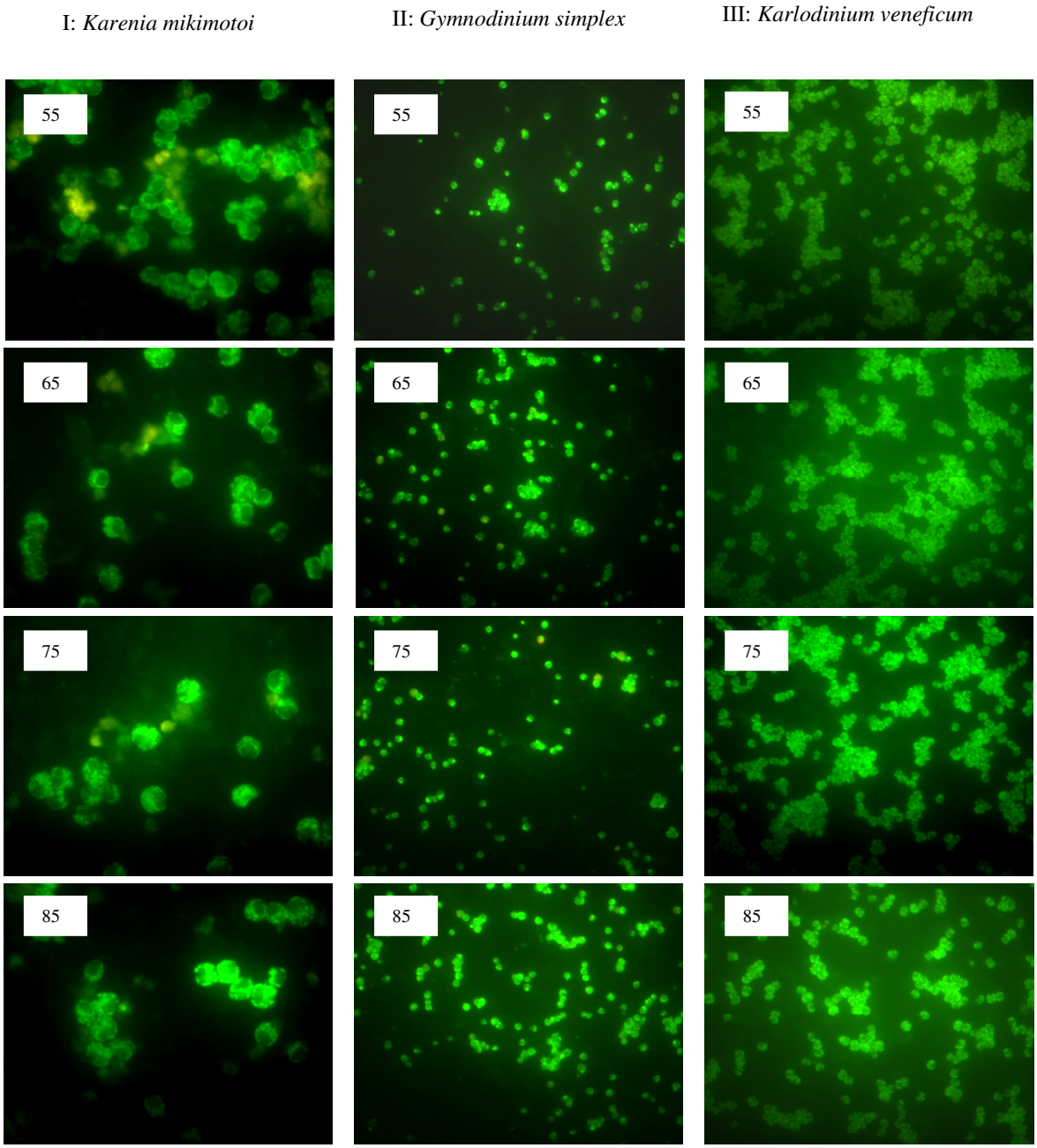


Figure 4-6: Micrographs of the different algal strains hybridized with GMIKI02 probe using high formamide concentrations (total magnification ×400). I: *Karenia mikimotoi*; II: *Gymnodinium simplex*; III: *Karlodinium veneficum*; IV: *Alexandrium minutum*; V: *Glenodinium foliaceum*. Numbers shown are the concentration (%) of formamide hybridization buffer.

IV: *Alexandrium minutum* V: *Glenodinium foliaceum*

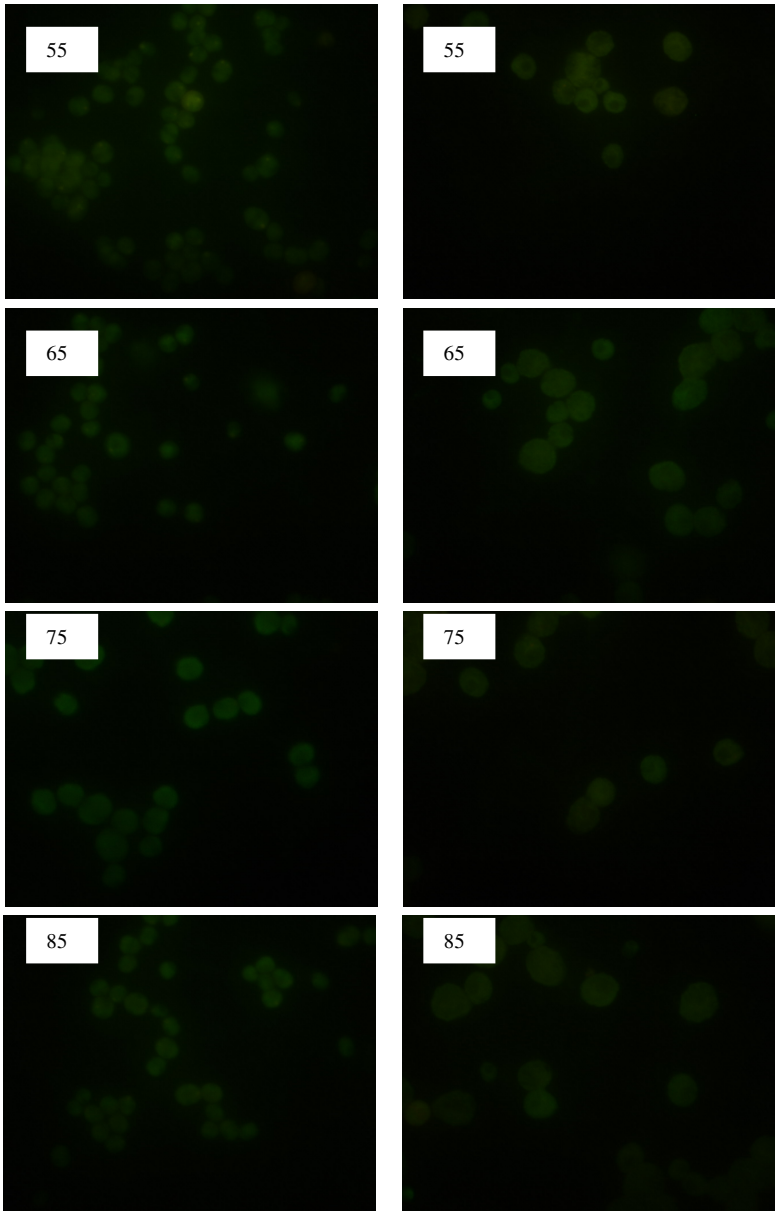


Figure 4-6: Continued

After the addition of *K. brevis* to the culture collection, CARD-FISH was applied to 10 mL of *K. brevis* cultured cells to examine the specificity of GMIKI02 probe at 55% formamide hybridization buffer. *K. brevis* was found to produce high percentage of positive hybridized cells [88%, ±5.4] (micrograph not shown).

18S SSU rRNA probe

The *K. mikimotoi* species-specific probe based on the nucleotide sequence of the 18S SSU rDNA reverse primer designed by Godhe et al. (2001) was tested to target *K. mikimotoi* against its closest relative *K. brevis* (which was used as the main non-target species). 10 mL of *K. mikimotoi* and 10 mL of *K. brevis* cultures were hybridized at different concentrations of formamide hybridization buffer (20%, 40%, 50%, 60% and 80%). Non-labelled species-specific probe of *K. brevis* (Mikulski et al. 2005) was used as a competitor to increase the HRP-labelled oligonucleotide probe specificity and to address non-specific binding problems and was mixed with the labelled species-specific *K. mikimotoi* probe and formamide hybridization buffer in 1:1:10 ratio in the hybridization step. 50% formamide hybridization buffer was found to be the optimal concentration that would produce the highest percentage of positive hybridization and to discriminate between the target *K. mikimotoi* and the non-target *K. brevis* species (Fig. 4.7). *K. mikimotoi* showed 94.6% [±1.7] positive hybridization compared to only 14.3% [±4] with *K. brevis*. Fig. 4.8 shows FITC epifluorescence images taken for *K. mikimotoi* and *K. brevis* after applying CARD-FISH.

The 18S small subunit rRNA probe was tested on other non-target species (*Gymnodinium simplex, Karlodinium veneficum, Alexandrium minutum, Alexandrium tamarense* and *Glenodinium foliaceum*) using the optimum concentration of formamide hybridization buffer (50%). Table 4.4 shows that *K. mikimotoi* was the only species that produced strongly positive hybridization after labelling with the species-specific probe. Negative hybridization was obtained with other non-target species. A few positive hybridized cells were produced with *K. brevis* however. Subsequently, to test the sensitivity of the probe to target *K. mikimotoi* in mixed populations of other algal species, a known concentration of

K. mikimotoi cultured cells was artificially mixed with a known concentration of other non-target dinoflagellate cultured cells and CARD-FISH was applied using the 18S SSU rRNA probe. The percentage of positive hybridization after CARD-FISH was compared to the initial percentage of *K. mikimotoi* cells (from microscopic cell counts) mixed with other algal species listed above. It was found that the percentage of cells showing positive hybridization (17.3%, ±5.9) was in a good agreement with the original percentage of *K. mikimotoi* cultured cells (21.6%) mixed with other non-target species before applying the CARD-FISH technique. Fig. 4.9 shows DAPI and FITC epifluorescence images of positive hybridized *K. mikimotoi* cells mixed with other non- target species after CARD-FISH.

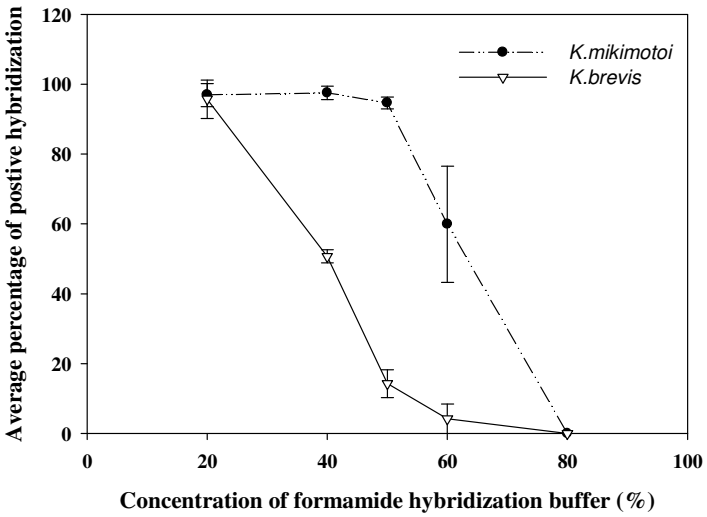


Figure 4-7: A plot showing percentage of positive hybridization using 18S SSU rRNA probe at different concentrations (%) of formamide hybridization buffer. (error bars corresponds to standard deviation, n= 3).

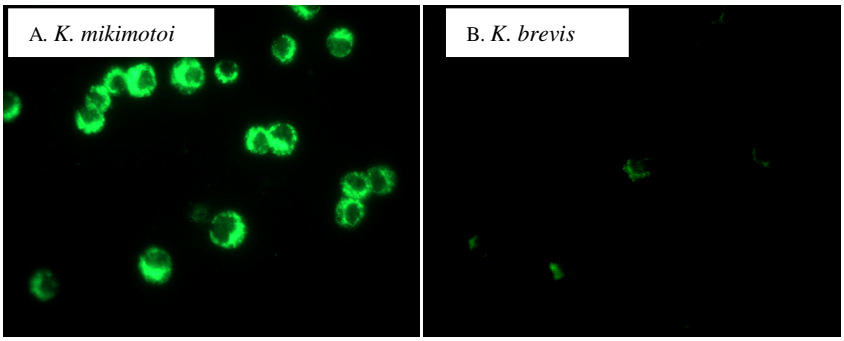


Figure 4-8: FITC epifluorescence images of *K. mikimotoi* and *K. brevis* after CARD- FISH using 18S SSU rRNA probe at 50% formamide hybridization buffer. (A) *K. mikimotoi*; (B) *K. brevis*.

Table 4.4: A list of algal species used to test cross-reactivity and specificity of 18S SSU rRNA probe targeting *K. mikimotoi*. The table shows the concentration of different cultured cells used for CARD-FISH, and the calculated % of *K. mikimotoi* cells in mixed cultures (light microscopy cell counts) and % of positive hybridization after applying CARD-FISH using epifluorescence microscope.

Species	Positive hybridization	Concentration of cells
Karenia mikimotoi	+	52000
Karenia brevis	+/-	13200
Glenodinium foliaceum	-	27600
Gymnodinium simplex	-	20600
Karlodinium veneficum	-	87600
Alexandrium minutum	-	32600
Alexandrium tamarense	-	7600
% of *K. mikimotoi* in the mixed cultures		21.6 [1]
Average % positive hybridization		17.3 [±5.9] [2]

[1] estimated from microscopic cell counts.
[2] estimated from positive hybridized stained cells.

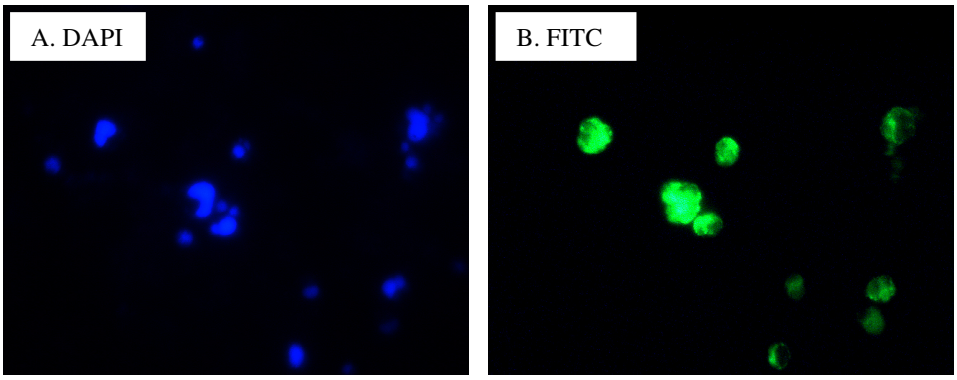


Figure 4-9: DAPI (A) and FITC (B) epifluorescence images of *K. mikimotoi* cultured cells mixed with other non-target species after applying CARD-FISH using 18S SSU rRNA probe.

28S LSU rRNA probe

Firstly, *K. mikimotoi* species-specific probe (based on the 28S LSU rDNA forward primer sequence of *K. mikimotoi* designed in this study, Chapter 3) was tested to target *K. mikimotoi* against the closest relative *K. brevis*. The optimal hybridization conditions were determined by hybridizing the 28S LSU rRNA probe to 10 mL of *K. mikimotoi* and 10 mL *K. brevis* cultured cells at different formamide concentrations (20%, 40%, 50%, 60% and 80%). The non-labelled species-specific probe of *K. brevis* was mixed with the labelled species-specific *K. mikimotoi* probe and hybridization buffer in 1: 1: 10 ratio and used in the hybridization step.

60% hybridization buffer was found to be the optimal concentration that would discriminate between the target *K. mikimotoi* and the non-target *K. brevis* species (Fig. 4.10). The positive hybridization was 95.4% [±0.4] with *K. mikimotoi* and was only 3.4% [±1.1] with *K. brevis*. Fig. 4.11 shows FITC epifluorescence images taken of *K. mikimotoi* and *K. brevis* cells after applying CARD-FISH.

Fig. 4.12 shows the *in situ* accessibility data of the newly designed 28S species-specific probe using 26S rRNA *Saccharomyes cerevisiae in situ* accessibility map (Inácio et al. 2003). According to the accessibility map, the designed probe belongs to brightness class III (classes I to VI, with class I the brightest).

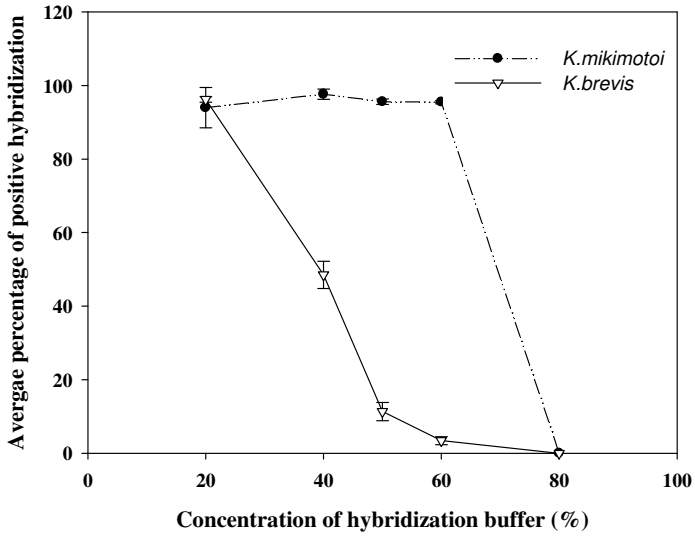


Figure 4-10: A plot showing percentage of positive hybridization using the 28S LSU rRNA probe at different concentrations of formamide hybridization buffer (%). (error bars corresponds to standard deviation, n= 3).

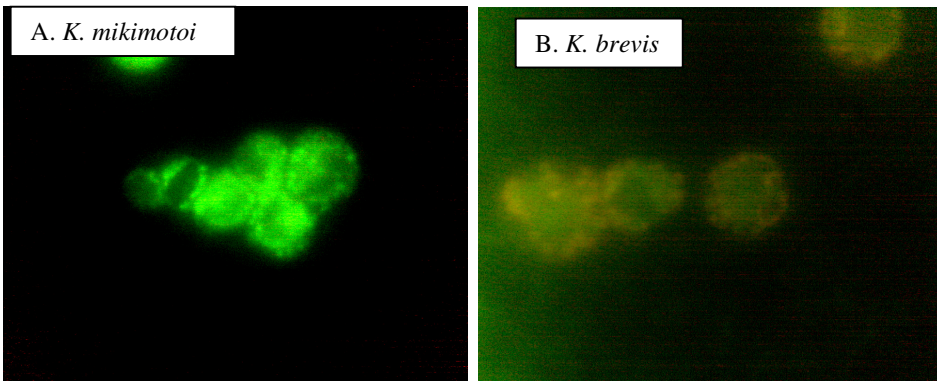


Figure 4-11: FITC epifluorescence images of *K. mikimotoi* and *K. brevis*, after CARD-FISH using 28S LSU rRNA probe, 60% formamide hybridization buffer and using *K. brevis* probe as competitor. (A) *K. mikimotoi*; (B) *K. brevis* (total magnification of ×630).

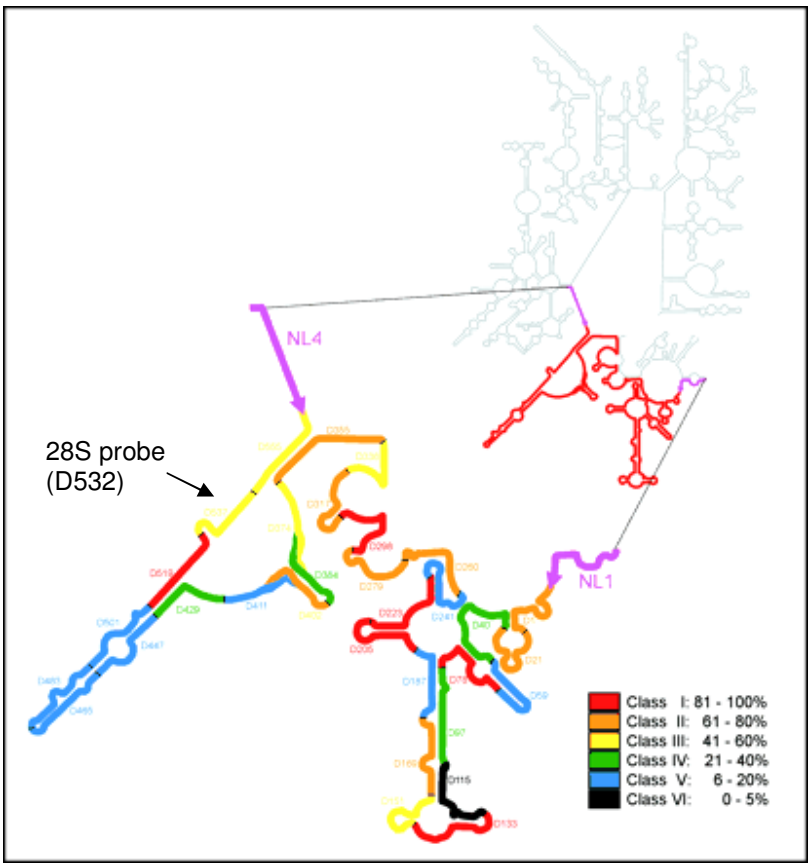


Figure 4-12: A model of the *S. cerevisiae* 26S rRNA secondary structure in which the D1 and D2 domains (delimited by the NL1 and NL4 primer target sites) are enlarged. The position of the newly designed 28S probe is indicated.

4.3.2 *Evaluation of flow cytometry to detect* Karenia sp. *after CARD-FISH*

18S SSU rRNA probe

Flow cytometry analysis of the 18S SSU rDNA species-specific probe showed that it could distinguish *K. mikimotoi* from other non-target species (Fig.4.13A-F). The density plots of FITC versus side scatter revealed two distinct cell clusters, indicating successful discrimination between labelled *K. mikimotoi* cells (gated region R3) and the unlabelled *K. brevis* cells (gated region R2 in Fig. 4.13E) or other non-target dinoflagellate cells (gated region R2) in Fig. 4.13F. Although both taxa (*K. mikimotoi* and *K. brevis*) had similar side scatter properties due to their similar sizes (gated region R2 in Fig. 4.13A-C); they were separated based on the difference in FITC fluorescence attributable to positive reaction of *K. mikimotoi* with the HRP-labelled probe. Gated region R2 in Fig. 4.13D shows different cell sizes of the different dinoflagellate cultures used in the experiment. Gated region R1 represents yellow-green beads of 0.5µm diameter (Fluoresbrite Microparticles; Polysceinces) that were used as a flow cytometric internal standard.

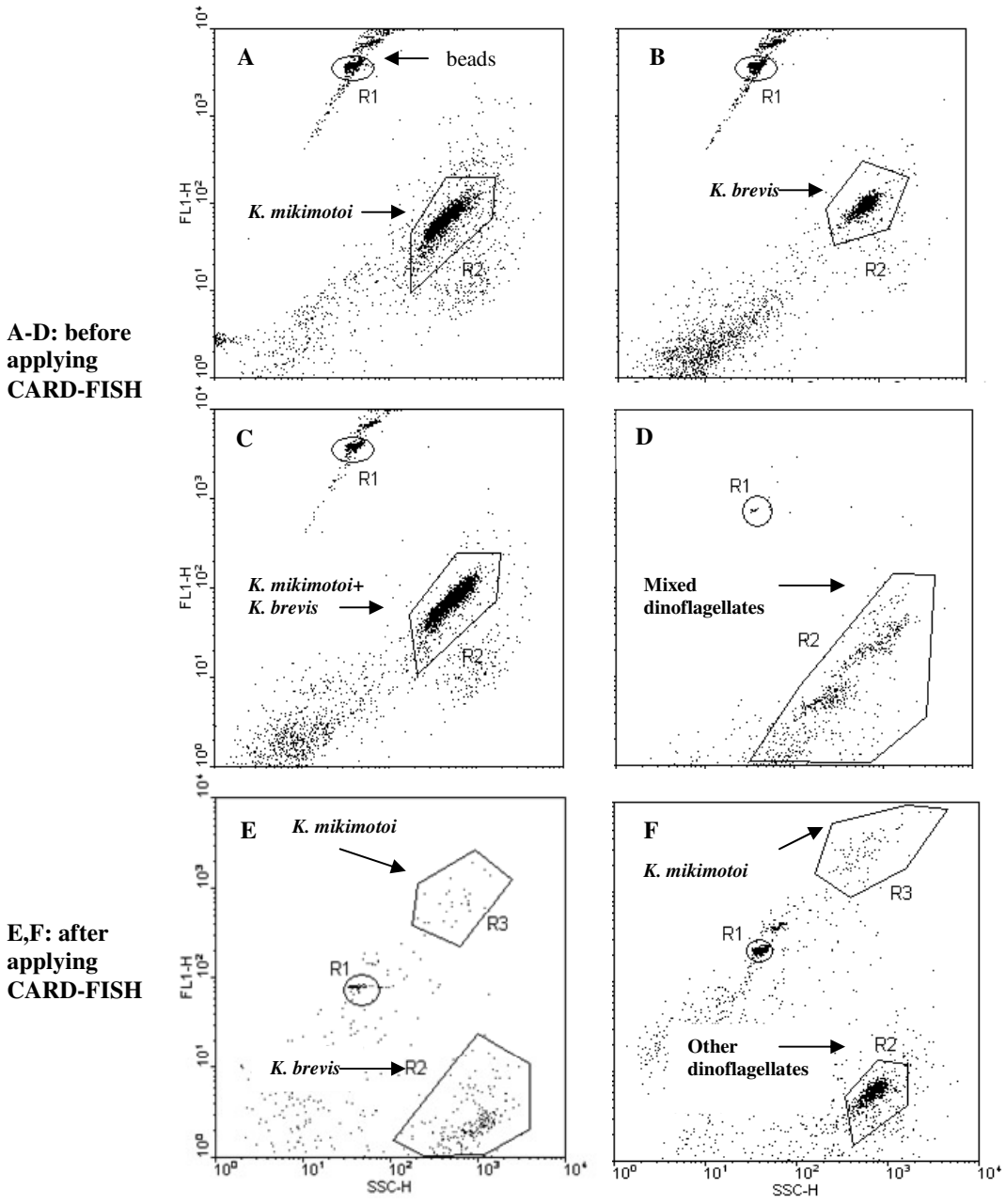


Figure 4-13: Scatter plots of flow cytometry data showing the difference between 18S SSU rRNA positive-labelled *K. mikimotoi* and non-positive *K. brevis* and other non-target cells. A: *K. mikimotoi* (R2); B: *K. brevis* (R2); C: mixed cultures of *K. mikimotoi* and *K. brevis* (R2); D: *K. mikimotoi* mixed with other non-target dinoflagellate cultures (R2). Plots A- D: without applying CARD-FISH. E: *K. mikimotoi* (R3) mixed with *K. brevis* (R2); F: *K. mikimotoi* (R3) mixed with other dinoflagellates (R2), Plots E and F: after applying CARD-FISH (the probe-labelled *K. mikimotoi* cells (gated region R3) can be distinguished from the non-labelled dinoflagellate cells (gated region R2; cells did not react with the probe). R1 represent the yellow-green beads. Note all values are in arbitrary units.

28S LSU rRNA probe

The 28S LSU rDNA probe targeting *K. mikimotoi* was shown to be capable of discriminating between *K. mikimotoi* and its closest relative *K. brevis* (Fig. 4.14A-D)**.** As mentioned above**,** both *K. mikimotoi* and *K. brevis* have the same side scatter (Fig. 4.14A-C), but could be separated based on the difference in FITC fluorescence attributable to positive reaction of *K. mikimotoi* with the HRP-labelled probe (Fig. 4.14D). In Fig. 4.14D gated region R3 represents the labelled *K. mikimotoi* cells; gated region R2 shows the non-labelled *K. brevis* cells and gated region R1 is the yellow-green beads of 0.5µm diameter that were used as a flow cytometric internal standard.

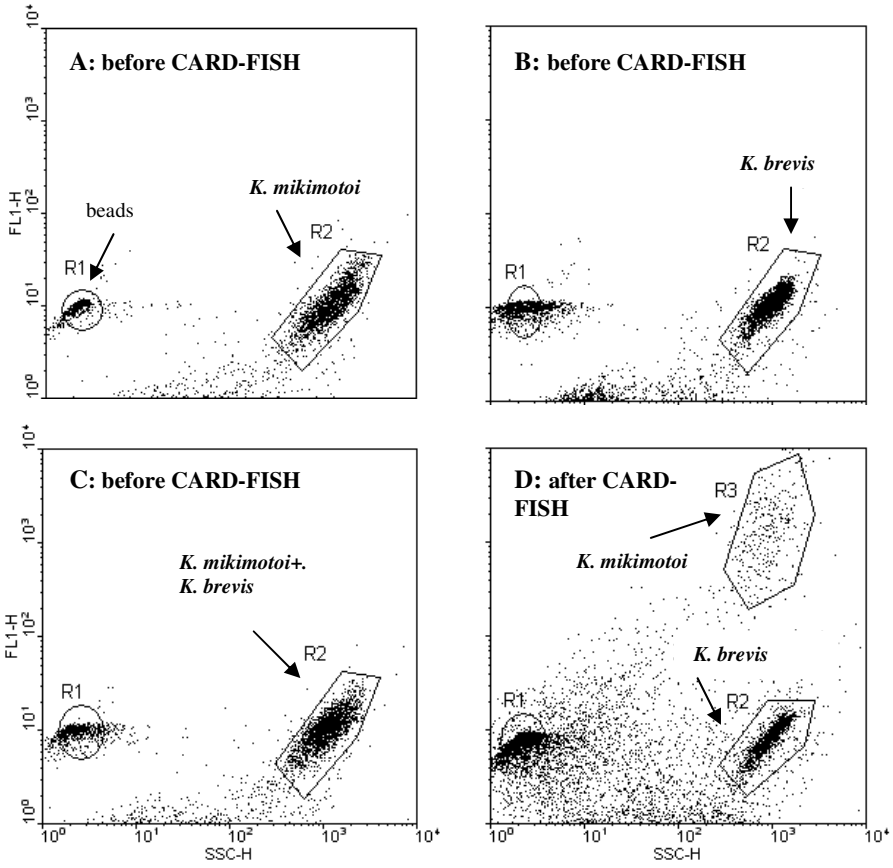


Figure 4-14: Scatter plots of flow cytometry data relating FITC-fluorescence (FL1-H) with side size scatter (SSC-H) using the 28S LSU *K. mikimotoi* probe. A: *K. mikimotoi* (R2); B: *K. brevis* (R2); C: mixed *K. mikimotoi* and *K. brevis* (R2); (A- C): cells before applying CARD-FISH. D: probe-labelled *K. mikimotoi* cells (R3) and non-labelled *K. brevis* cells (R2) (cells after applying CARD-FISH with 28S LSU rDNA probe). R1 represent the yellow green beads.

Comparison between 18S SSU and 28S LSU rDNA species-specific probes used for the quantification of K. mikimotoi *in mixed populations of the two closely related species (*K. mikimotoi *and* K. brevis*) in cultures*

Cells of *K. mikimotoi* and *K. brevis* were mixed in a known proportion (approximately 1:1 ratio) then the CARD-FISH method applied to the mixture using the 18S SSU and the 28S LSU rRNA probes. The percentage of positive hybridization was calculated using both epifluorescence microscopy and flow cytometry. Table 4.5 shows that the percentage of positive hybridization produced using the 28S LSU rRNA probe by epifluorescence microscope was in a good agreement with the original percentage (i.e. 50%) of *K. mikimotoi* cells before CARD-FISH. The percentage of positive hybridization was 45.6% [±4] using the 28S LSU rRNA. The percentage of positive hybridization was 27.6 [±4] with the 18S SSU rRNA probe. In comparison, the percentage of positive hybridization was lower with the two probes when analyzed using flow cytometry. Percentage of positive hybridization was 36% with the 28S LSU rRNA probe and only 13% with the 18S SSU rRNA.

Table 4.5: Comparison of positive hybridization after applying CARD-FISH on mixed population of *K. mikimotoi* and *K. brevis* cultured cells using epifluorescence microscopy and flow cytometry.

Probe	Method used for counting	Postive hybridization (%) [± SD]
28S LSU rRNA	Epifluorescence microscope	45.6 [±4]
28S LSU rRNA	Flow cytometry	36
18S SSU rRNA	Epifluorescence microscope	27.6 [±4]
18S SSU rRNA	Flow cytometry	13

Fig. 4.15 shows that both the 18S SSU and 28S LSU rRNA probes were capable of distinguishing between the target species (*K. mikimotoi*) and the non-target species (*K. brevis*) when analyses were performed using flow cytometry. However, when the

fluorescence values were compared, it was found that the fluorescence signal intensity was higher in the case of the 18S SSU rRNA probe. Measurements of fluorescence intensity were made using WinMDI 2.9 software and the probe-conferred fluorescence was determined as the FL1 values of single cells lying in a gate that was defined in an SSC-versus-FL1 dot plot. The standardized cell-probe conferred fluorescence was obtained by dividing the probe values by the fluorescence values of the reference beads (0.5 µm yellow-green beads). The estimated fluorescence intensity was 190.3 relative units for the labelled *K. mikimotoi* cells and 1.2 relative units for non-labelled *K. brevis* using the 18S SSU rRNA probe. In contrast, the fluorescence intensity was 135.2 relative units for the labelled *K. mikimotoi* cells and 1 relative unit for the non-labelled *K. brevis* cells using the 28S LSU rRNA probe.

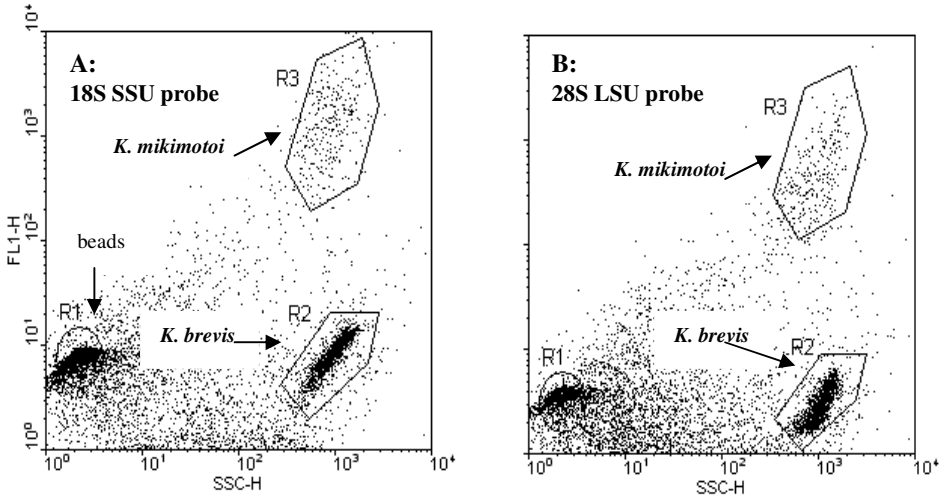


Figure 4-15: Scatter plots of flow cytometry data relating FITC-fluorescence (FL1-H) with side size scatter (SSC-H) using the 18S SSU and 28S LSU probes. A: *K. mikimotoi* (R3) and *K. brevis* (R2) after applying CARD-FISH using the 18S SSU rRNA probe; B: *K. mikimotoi* (R3) and *K. brevis* (R2) after applying CARD-FISH using the 28S LSU rRNA probe. R1 represents the yellow-green beads.

4.3.3 *Application of the 28S LSU rRNA probe to enumerate* Karenia sp. *in Lugol's fixed field samples*

According to the above experimental results, the 28S LSU rRNA probe was used to calculate *Karenia sp.* cell concentrations in water samples collected from different locations in the western English Channel. *Karenia sp*. was previously counted in Lugol's preserved water samples using light microscopy (Chapter 3). Table 4.6 shows that the absolute/relative abundance of positive hybridized cells after CARD-FISH was lower than the absolute/relative abundance of *Karenia sp.* cells counted in all Lugol's fixed samples using a light microscope. The comparison between the absolute number of *Karenia sp.* counted using a light microscope and the absolute number of positive hybridized cells after CARD-FISH showed no correlation (Pearson Product Moment correlation, $p > 0.05$, $R = 0.65$, $n = 7$; note that the 2003 bloom data was excluded) as shown in Fig. 4.16A. In contrast, there was a significant correlation between the percentage of *Karenia sp.* counted using a light microscope and the percentage of positive hybridized cells (Spearman Rank Order correlation, $R = 0.99$, $p < 0.05$, $n = 8$) as shown in Fig.4.16B. Fig. 4.17 shows an example of epifluorescence microscope images taken for a Lugol's fixed seawater sample after applying CARD-FISH, *K. mikimotoi* cells showed good positive hybridization signal intensity.

Table 4.6: Comparison between total numbers of positive hybridized cells counted after CARD-FISH technique and total *Karenia sp*. cell counts using light microscopy, and percentage of positive hybridized cells in seawater after applying CARD-FISH with percentage of *Karenia sp*. in Lugol's fixed samples.

Seawater sample	Absolute no. of positive hybridized cell counts using CARD-FISH (no.cells in 10 mL)	Absolute no. of *Karenia sp* . cell counts in Lugols fixed samples (no.cells in 10 mL)	% of positive hybridized cell counts using CARD-FISH [e]	% of *Karenia sp.* in Lugols fixed samples
Falmouth 2007 [a]	64	95	1	1
FerryBox 2007/sample 5 [b]	100	223	0.5	0.5
FerryBox 2007/ sample 6[b]	90	297	0.7	0.7
FerryBox 2007/ sample 7 [b]	14	201	0.4	0.5
Falmouth 2006/ sample 5 [c]	556	912	48.5	50.3
Falmouth 2006/ sample 6 [c]	396	3213	83.6	90.2
Falmouth 2006/ sample 8 [c]	304	1803	29	29.7
FerryBox 2003 [d]	2900	60038	88.2	99.9

a: A water sample collected off Falmouth at 12 m.
b: Surface water samples collected from a FerryBox transect.
c: Surface water samples collected from different locations off Falmouth.
d: A water sample collected from a FerryBox transect at 5 m.
e. Counted as a fraction of total DAPI stained cells

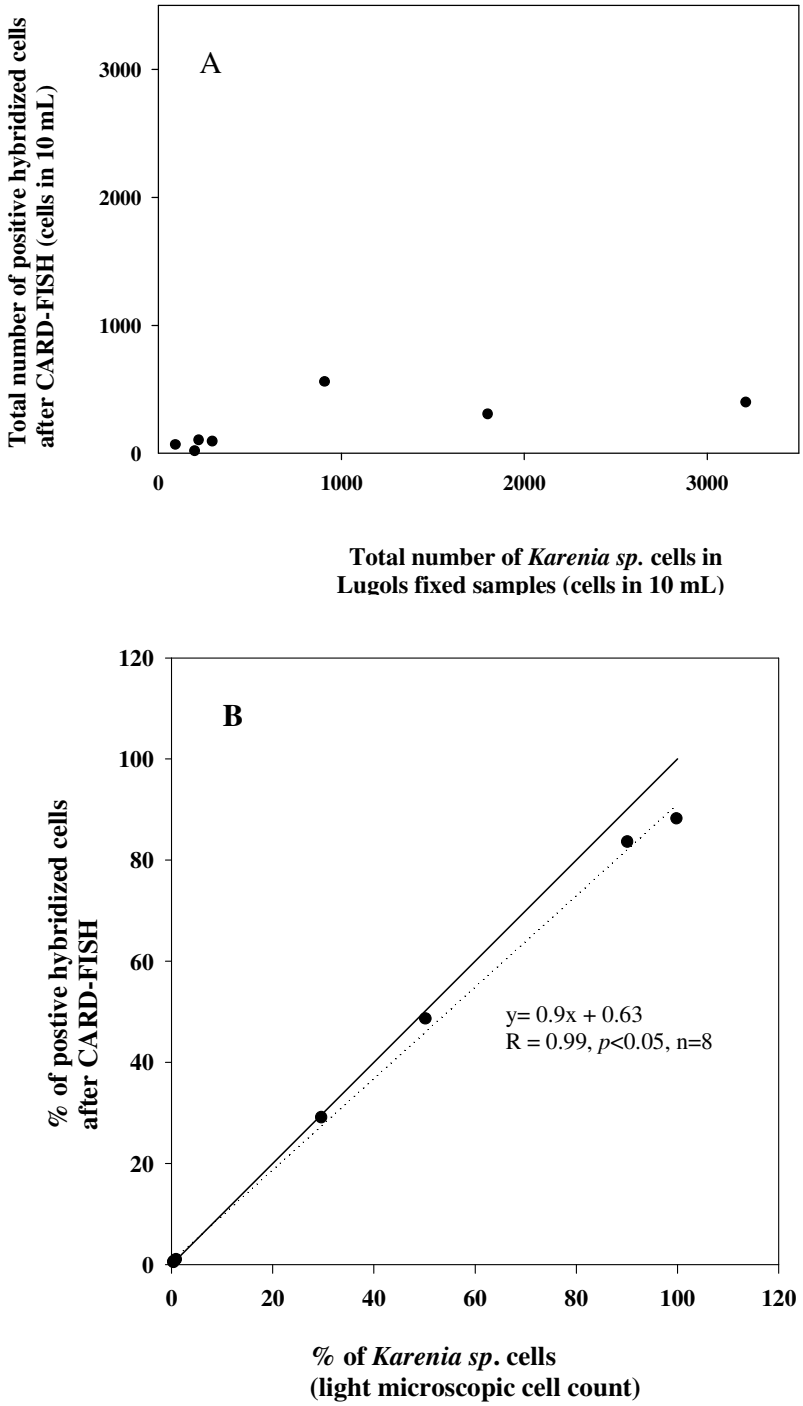


Figure 4-16: (A) Comparison between total number of *Karenia sp.* cells in Lugol's fixed samples and total number of positive hybridized cells after applying CARD-FISH; (B) comparison between % of *Karenia sp*. cells in Lugol's samples and % of positive hybridized cells after CARD-FISH. The solid line in B corresponds to a 1:1 relationship and the dotted line is a linear regression fit.

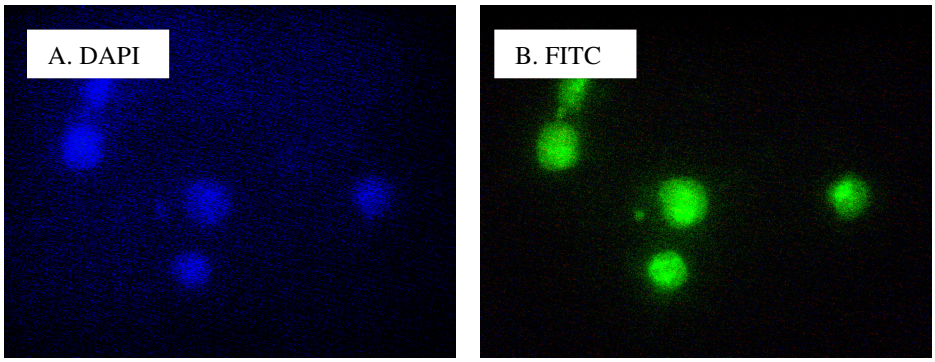


Figure 4-17: (A) DAPI and (B) FITC epifluorescence images of seawater samples collected from a FerryBox cruise during summer 2003 after applying CARD-FISH technique using 28S LSU rDNA *K. mikimotoi* species-specific probe.

Flow cytometric analysis of the 28S LSU rDNA CARD-FISH probed cells of FerryBox 2003 seawater sample

The CARD-FISH technique in conjunction with flow cytometry was applied to one of the seawater samples found to contain a high concentration of *Karenia sp.* cells (a water sample collected during a FerryBox transect in July 2003, concentration of *Karenia sp.* cells was 6004 cells mL^{-1}). Fig. 4.18A shows the water sample before applying CARD-FISH (gated region R2 represents non-labelled *Karenia sp.* cells) and gated region R3 in Fig. 4.18B represents labelled *Karenia sp.* cells after applying CARD-FISH and could be distinguished from non-labelled *Karenia sp.* cells without applying CARD-FISH (gated region R2), i.e. stained and non-stained *Karenia sp.* mixed together. Region R1 represents yellow-green beads of 0.5µm diameter that were used as a flow cytometric internal standard.

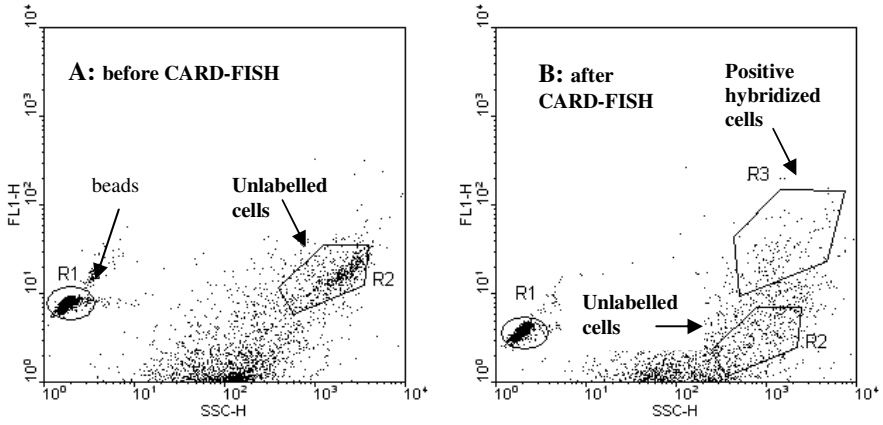


Figure 4-18: Scatter plots of flow cytometry data relating FITC-fluorescence (FL1-H) with size side scatter (SSC-H). (A): Seawater sample collected in a FerryBox 2003 cruise without applying CARD-FISH, R2 represents unlabelled cells; (B): The same water sample after applying CARD-FISH with 28S LSU rRNA probe of *K. mikimotoi*, R2 represents unlabelled cells and R3 positive hybridized cells. R1 is yellow green beads.

4.3.4 *Alignments of the different* K. mikimotoi *probes using BLAST*

The nucleotide sequences of the different species specific *K. mikimotoi* probes were checked using GenBank (http://www.ncbi.nlm.nih.gov) to search for dinoflagellate strains that have 100% exact matches using BLAST. Table 4.7 shows that the probe designed in the present study was optimal compared to the previously published probes as it has a 100% exact match only with *K. mikimotoi*. The 28S LSU rRNA GMIKI02 probe designed by Sako et al. (1998) showed 100% exact match with both *K. mikimotoi* and *K. brevis*. Both *Gymnodinium simplex* and *Karlodinium veneficum* which showed positive hybridization after applying CARD-FISH (4.3.1) using the GMIKI02 probe are not available in the BLAST results (Table 4.7). The 18S SSU rRNA probe designed by Godhe et al. (2001) showed a 100% exact match with *K. mikimotoi, Gyrodinium aureolum* and *Gyrodinium fusiforme*.

The nucleotide sequence of the 28S LSU probe designed in the present study was then compared to 28S LSU gene sequences of different *Karenia* species obtained from GenBank. ClustalW software was used to determine the number of base pair differences.

Table 4.8 shows that there are 6 base pair differences between the probe nucleotide sequence and the other species of *Karenia* in that region. It was only the target species *K. mikimotoi* (28S LSU rDNA partial sequence obtained in this study and a second 28S LSU rDNA sequence obtained from GenBank) that showed 100% homology to the probe sequence.

Table 4.7: The different species specific *K. mikimotoi* probe sequences and the dinoflagellate species that showed 100% match with the probe sequences according to BLAST.

Species	GenBank accession number
GMIKI02 probe 5'-TTCCGGGCAAGGTCGAAA-3'*	
Karenia mikimotoi	EF492505
Karenia brevis	EF492504, EF492503, EF492502, EF492501, DQ847434, AF352822, AF352821, AF352820, AF352819, AF352818, AF274259
Gymnodinium breve	AF172714, AJ415518
Gyrodinium aureolum	AF172713, AJ415517
Gymnodinium cf. mikimotoi	AF009216
Gymnodinium mikimotoi	AF009131, AF022195
18S SSU rRNA probe 5'-CAGGAACTGAACACTGCGGCA-3'*	
Karenia mikimotoi	EF492505
Gyrodinium fusiforme	AB120002
Gyrodinium aureolum	AF172713, AJ415517
Gymnodinium cf. mikimotoi	AF009216
Gymnodinium mikimotoi	AF009131
28S LSU rRNA probe 5'-GCTCTGCATGAAGGTTGTTG-3'*	
Karenia mikimotoi	EU165311, EF469238, U92249, U92247, AY355460
Gymnodinium mikimotoi	AF200682, AF200681, AF200680, AF200679, AF200678, AF318224, AF318223

***Probe sequence**

Table 4.8: Base pair differences between 28S LSU *K. mikimotoi* probe and the different *Karenia* species 28S LSU sequences (from GenBank) using ClustalW. The different base pairs are underlined.

Probe/speceis	Aligments	GenBank Accession numer
***K.mikimotoi* probe**	5'-GCTCTGCATGAAGGTTGTTG-3'	
***K. mikimotoi* - present**	GCTCTGCATGAAGGTTGTTG	**This study**
***K. mikimotoi* -GenBank**	GCTCTGCATGAAGGTTGTTG	**EF469238**
K. bidigitata	GCTCAGCATGGAAATTGGGG	**AY947663**
K. umbella	GCTCAGCATGGAAATTGGGG	**EF469239**
K. asterichroma	GCTCAGCATGGAAATTGGGG	**AY590123**
***K. brevis* -present**	GCTCAGCATGGAAATTGGGG	**This study**
***K. brevis* -Genbank**	GCTCAGCATGGAAATTGGGG	**EU165308**
K. selliformis	GCTCAGCATGGAAATTGGGG	**U92250**
K. papilionacea	GCTCAGCATGGAAATTGGGG	**AY590124**
K. brevisulcata	GCTCAGCATGGAAATTGGGG	**AY243032**
K. cristata	GCTCAGCATGGAAATTGGGG	**AY243963**

4.3.5 *Phylogenetic analysis of* K. mikimotoi *and distance tree*

Fig. 4.19 shows that *K. mikimotoi* forms a sister group to *K. brevis*. The different *K. mikimotoi* strains (present study sequence and published ones AY355460, AF200681) show an unresolved topology, as their sequences are identical. *Gymnodinium simplex* (present study) clusters strongly (77.2 bootstrap) with the thecate dinoflagellate *Prorocentrum triestinum. Karlodinium veneficum* show unresolved topology with other *Karlodinium veneficum* strains sequences from GenBank (*Karlodinium veneficum* DQ114466 and *Gymnodinium galatheanum* AF200675 (now known as *Karlodinium veneficum*). Table 4.9 shows that *K. mikimotoi* and *K. brevis* have the highest percent sequence similarity (98.4%).

Table 4.9: Percent similarity between different algal cultures species used in the present study based on the 28S LSU gene (partial sequences).

	Percentage similarity			
	Karenia brevis	*Karenia mikimotoi*	*Karlodinium veneficum*	*Gymnodinium simplex*
Karenia brevis	100			
Karenia mikimotoi	98.4	100		
Karlodinium veneficum	87.4	87.1	100	
Gymnodinium simplex	68.7	68.7	71.5	100

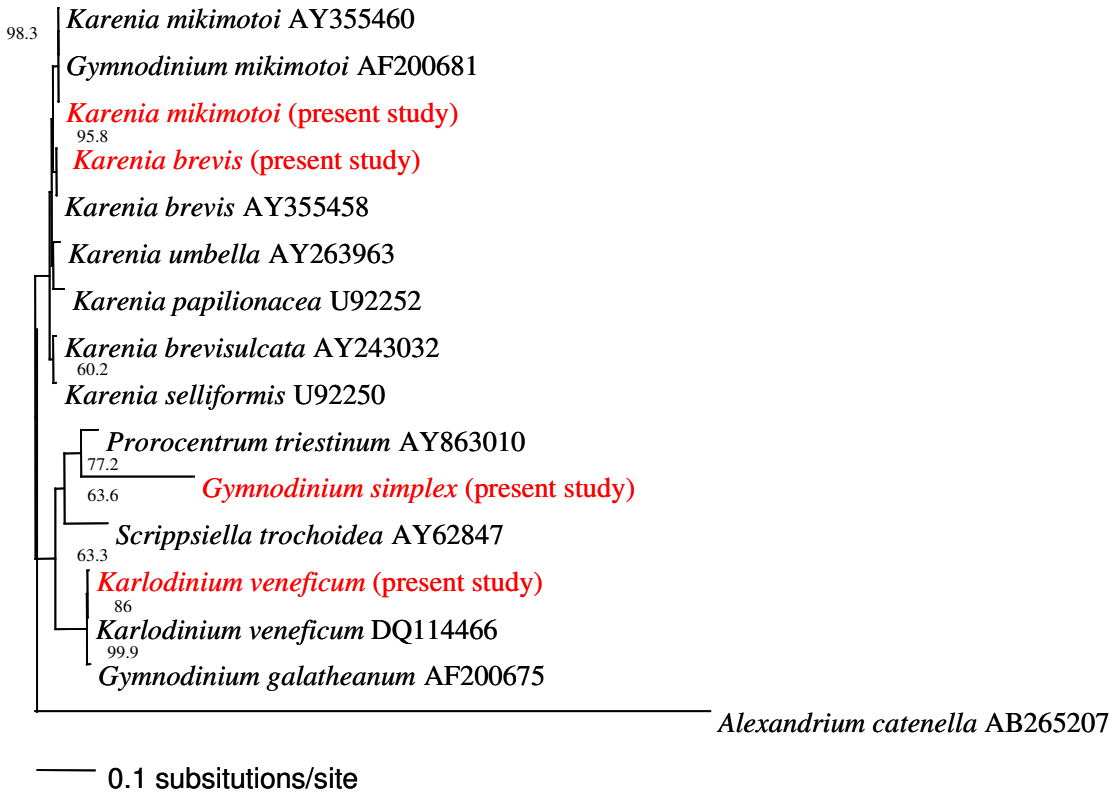


Figure 4-19: Phylogeny of 16 species of dinoflagellates inferred from about 1000 base pairs of nuclear-encoded 28 LSU rDNA (domains D1-D3). Numbers on branches denote bootstrap values (1000 replicates) calculated using neighbor-joining (NJ). The reconstruction was based on neighbor-joining (NJ) method. *Alexandrium catenella* AF265207 was used to root the trees.

4.4 Discussion

4.4.1 *Probes targeting* K. mikimotoi

It was demonstrated that both the 18S SSU rRNA designed by Godhe et al. (2001) and the 28S LSU probe designed in this study were both successful in discriminating between the target species *K. mikimotoi* from its closest relative *K. brevis* using both epifluorescence microscopy and flow cytometry applying CARD-FISH protocol. BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) showed that the 18S SSU rRNA probe has 100% homology with the 18S SSU nucleotide sequences of *K. mikimotoi* and *Gyrodinium fusiforme* (which was not used as non-target species in this study) whereas the 28S LSU rRNA probe showed 100% homology with *K. mikimotoi* only. Competitor probes (helper oligonucleotides) are normally designed to perfectly match at the homologous site with the non-target sequence and they are synthesized without a fluorescent label (Pernthaler et al. 2001) or designed to bind to regions adjacent to that of the target probe (Fuchs et al. 2000). In this study un-labelled probe targeting *K. brevis* (the probe was designed by Mikulski et al. (2005) based on the 28S LSU rRNA gene) was found to greatly decrease the cross-reactivity and has addressed the binding properties of the probes when used.

The 28S LSU rRNA GMIKI02 probe designed by Sako et al. (1998) and used in the first part of the experiment showed no mismatch (100% homology) with *K. mikimotoi* and *K. brevis.* This explains the non-specificity of the probe when tested on these two cultured cells. Additionally, unexplained cross-reactivity was not eliminated using the 28S LSU rRNA GMIKI02 probe with both *Gymnodinium simplex* and *Karlodinium veneficum* (which were not in the BLAST results, Table 4.6) regardless of the increase in the stringency binding properties of the probe (i.e. increasing formamide concentration in the hybridization buffer).

When the 18S SSU rRNA probe and the 28S LSU rRNA probes were tested on pure cultures of *K. mikimotoi* and *K. brevis*, the latter probe showed a higher percentage of positive hybridization (Fig.4.7, 4.10). This was confirmed when a higher percentage of positive hybridization was achieved using the 28S LSU probe compared to the 18S SSU

rRNA probe when applied to the mixed cultures of *K. mikimotoi* and *K. brevis* cells in a 1:1 ratio. In this case, the percentage of positive hybridization after applying CARD-FISH was estimated using both epifluorescence microscope and flow cytometry (Table 4.4). This difference could have been due to the fact that the 28S LSU rRNA probe has a higher binding affinity to its target site than the 18S SSU rRNA probe and its binding properties are more stable than the longer 18S SSU rRNA probe (20bp in the case of 28S rRNA probe and 21bp in the 18S rRNA probe). Furthermore, the quantification performed using epifluorescence microscopy was superior to quantification performed using flow cytometry. This variation in cell counts between epifluorescence microscopy and flow cytometry could be due to incomplete removal of cells into the sterile seawater after completing CARD-FISH protocol for analysis using flow cytometry. Biegala et al. (2003) used Pluronic solution and sonication to prevent cell loss for analysis of CARD-FISH by flow cytometry. The combination of a cell surfactant and sonication allowed excellent quantification of picoeukaryotes in their study. Sekar et al. (2004) tested different protocols to increase cell removal from membrane filters into suspension for analysis using flow cytometry. NaCl-Tween at 37 °C for 30 min, followed by vortexing was found the best strategy. In the present study, cell loss was minimized by performing the protocols in sealed micropipette tips and carefully sealing them with parafilm during the hybridization step to prevent loss of hybridization buffer-probe solution and dehydration of filters. It was found that vortexing the tube containing cells attached to a membrane filter was sufficient for removal of a high percentage of cells into the seawater. Further modification of the protocol is needed however to reduce the loss of cells during the different steps of CARD-FISH analysis and increase detachment of cells from membrane filters prior to flow cytometry analysis.

The 28S LSU rRNA probe was used to enumerate *Karenia sp.* cells in environmental water samples collected from different locations of the western English Channel. The quantification of positive hybridized cells was performed using epifluorescence microscopy and compared to the number of *Karenia sp*. cells counted in the Lugol's fixed samples using light microscopy (Table 4.5). The percentage of positive hybridized cells was in a good agreement with the *Karenia sp.* cells counted as a percentage of the total algal cells in

the samples. In contrast, it was found that the absolute number of positive hybridized cells counted using epifluorescence microscopy after CARD-FISH was much lower than the total *Karenia sp.* cells counted in Lugol's fixed samples using light microscopy. This difference in the two counting approaches could have been due to the low number of cells and their uneven distribution on filters. Pernthaler et al. (2001) has suggested that in FISH and TSA-FISH methods, absolute number of cells should not be determined from filters after hybridization, but only the percentage of positive hybridized cells. Cells are never distributed evenly on membrane filters, resulting in a higher error of the total DAPI counts (Pernthaler et al. 2001). It was demonstrated earlier that the 28S LSU *K. mikimotoi* probe produced 95.4% [±0.4] positive hybridized cells using the *K. mikimotoi* cultured cells due to the fact that even in laboratory cultures there are few dead cells and others with low ribosome number that would not show positive hybridization. Furthermore, the environmental water samples were fixed with Lugol's iodine solution and cells fixed with Lugol's iodine might become less permeable to the oligonucleotide probes or this fixative may mask the target sites of the oligonucleotide probes or may even cause some structural modification of ribosomes. Furthermore, long fixation time and CARD-FISH processes might cause damage to the naked dinoflagellates.

West et al. (2001) found that the fluorescent signal from *Prochlorococcus marinus* and *Prochlorococcus sp* cultured cells were significantly brighter than those obtained in natural water samples particularly in deep waters. In this study, it was demonstrated that there was a slight decrease in the fluorescence signal intensity (labelling efficiency) in Lugol's fixed environmental water samples compared to the Paraformaldehyde fixed cultured cells when analyses were performed using flow cytometry (Fig. 4.17). There are many factors that might cause the drop in fluorescence intensity in Lugol's fixed natural samples. Firstly, as mentioned above, long fixation in Lugol's iodine solution may mask the probe target sites. Furthermore, *Karenia sp.* cells from natural samples may have different growth stages and therefore have different concentrations of rRNA per cell. Finally, there might be some variations in structural and physiological properties between natural populations and cultured cells that reduce the accessibility of the target sites in the former (West et al. 2001).

Evaluation of CARD-FISH using the 18S SSU rRNA and the 28S LSU rRNA probes with cultured *K. mikimotoi* and *K. brevis* using flow cytometry revealed that the 18S SSU rRNA has higher fluorescence signal intensity than the 28S SSU rRNA probe (Fig.4.14). Brightness of FISH signals are affected by many factors. These include cell contents of rRNA (this should not be the case however in exponential growth phase cultured cells used for this part of the experiment), probe affinity and the accessibility of the probe binding site on the rRNA (Wagner et al. 1998, 2003, Yilmaz and Noguera 2004, Yilmaz et al. 2006). Possible reasons that explain the lower fluorescence signal in the case of the 28S rRNA probe are that the target sites were less accessible to the oligonucleotide probe and the probe may belong to a lower brightness class (Fuchs et al. 1998, 2001, Behrens et al. 2003, Inácio et al. 2003). Fuchs et al. (1998) and Fuchs et al. (2001) have demonstrated that the accessibility of the probe target sites for *Escherichia coli* on the 16S and 23S rRNA molecules are different because of the higher-order structure of the ribosome. This leads to marked differences in FISH signal intensity (Wagner et al. 2003). Constructing accessibility data for the 18S SSU and 28S LSU rRNA probes used in this part of the study was not possible because the target sites of the two probes were not included in the 18S and 28S consensus accessibility maps constructed previously by Behrens et al. (2003) and Inácio et al. (2003) for *Saccharomyces cerevisiae* (Eucarya). To increase the fluorescence signal using the 28S LSU rRNA probe, longer hybridization times become essential as suggested by Yilmaz et al. (2006). Nevertheless, when the 28S LSU rRNA probe was used to detect *Karenia sp.* in a seawater sample known to contain a high concentration of *Karenia sp.*, it was capable of detecting the positive hybridized cells and discriminating them from non-labelled cells (Fig. 4.17).

The signal intensity of microbial cells with low rRNA content has been increased previously by using multiple species-specific oligonucleotide probes (Amann et al. 1990b). Accordingly, an attempt could be made to use the two *Karenia* 18S SSU and 28S LSU rRNA probes together to increase both the fluorescence signal intensity and the detection efficiency of *Karenia sp.* in environmental samples with possible low rRNA content.

4.4.2 *CARD-FISH protocol*

One main objective of the application of molecular methods in ecological studies has been the detection and enumeration of particular species of microorganisms in their natural environment. The evaluation of the CARD-FISH technique modified and applied in this study using epifluorescence microscopy is an important stage toward this objective. Direct enumeration of both cultured and naturally occurring *Karenia sp.* was achieved using this method by *in situ* hybridization with HRP-labelled oligonucleotide probes. In addition, the method was applicable to Lugol's fixed seawater samples. CARD-FISH analysis performed in this study with known concentrations of *K. mikimotoi* and *K. brevis* cultured cells clearly demonstrated that the method could detect a high proportion of cells using epifluorescence microscopy. In natural samples a number of factors (e.g. a range of cell types, sizes, physiological states, and aggregations) will reduce the proportion of cells detected by CARD FISH analysis.

Cell fixation is a crucial step in any successful FISH experiment. Paraformaldehyde was used in this study as a fixative and was effective in preserving naked dinoflagellates. Aldehyde fixation tends to be superior at preserving cell morphology and keeping cells intact during FISH steps (Tyrrell et al. 1997). However, for the natural water samples, Lugol's iodine solution was used as a fixative and was found to have some effect on both the detection efficiency (positive hybridized cell counts after CARD-FISH were lower than the light microscopic cell counts) and the fluorescence signal intensity (fluorescence signal was lower than the fluorescence signal intensity achieved with cultured cells fixed with Paraformaldehyde).

Some problems were encountered while developing the CARD-FISH protocol with getting the probe to penetrate the cell wall due to the clumping of cells during filtration. To solve this problem, a Pluronic solution was used before cell fixation with Paraformaldehyde. Washing cells with different concentrations of ethanol greatly decreased the autofluorescence of the different cultured cells used in the experiments. Additionally, the fluorescent signals obtained with TSA using the HRP-probes were sufficient to fully mask

the autofluorescence of the cells. Similar observations were made by Schönhuber et al. (1999) and West et al. (2001) for the detection of cyanobacteria that possess strong background autofluorescence and of picoeukaryotes (Biegala et al. 2002, 2003).

The penetration of the large HRP molecule (molecular weight [MW] 40 000) did not require an enzymatic permeabilization step probably because the naked dinoflagellates used in this study lack a cell wall. Biegala et al. (2002) did not encounter problems of HRP molecule penetration even with thecate dinoflagellates.

The percentage of positive hybridization was calculated as a fraction of DAPI stained cells. DAPI is a highly specific and sensitive fluorescent DNA stain and can be used to enumerate eukaryotic cells in natural water samples (Porter and Feig 1980). The bright blue fluorescence of DAPI stain improves the visualization of cells and therefore the accuracy of cell counting. This counting protocol was acceptable because both cultured cells and environmental water samples were filtered onto 5 µm pore size cyclopore membrane filter, thus only cells greater than 5 µm were retained on the filters. The principle of this counting method was achieved previously by Hicks et al. (1992) and Biegala et al. (2002). However, in the case of small cells and water samples containing detrital aggregates, this counting method was not applicable (Lim et al. 1996).

Both the HRP probes used in this study and the modified CARD-FISH protocol were sufficient to detect *Karenia sp.* cells in seawater samples even when present at low cell densities (lowest *Karenia* cell density counted using a light microscope was 95 cells in a 10 mL of water sample collected off Falmouth at 12 m during summer 2007, Table 4.6).

4.5 Conclusions

In this part of the study, two species-specific *K. mikimotoi* probes based on the 18S SSU rRNA gene and the 28S LSU rRNA gene were used coupled with the CARD-FISH method

to enumerate *K. mikimotoi* cells from cultures and natural water samples using both epifluorescence microscopy and flow cytometry. The 28S LSU probe was optimal for the quantification of *Karenia sp.* in seawater samples using the epifluorescence microscope. In contrast, for quick and rapid detection of *Karenia sp.* in seawater samples, the 18S SSU rRNA probe is considered to be superior using flow cytometry. Future efforts should therefore concentrate on combining the two probes (18S SSU and 28S LSU probe) for both the detection and quantification of *K. mikimotoi* in natural seawater samples. Quantification of cells using epifluorescence microscopy was more accurate and the details of hybridized cells could be easily seen. Further modifications of the CARD-FISH protocol are required to minimise cell loss when using flow cytometry to quantify stained cells. Nevertheless, the separation of *K. mikimotoi* from its closest relative *K. brevis* by flow cytometry is very useful, since it demonstrates the potential for using an automated detection system in conjunction with the species-specific rRNA probe without using the more labour intensive epifluorescence microscope.

CHAPTER 5

Molecular identification and enumeration of the potentially harmful dinoflagellate *Bysmatrum granulosum* (Dinophyceae) in Bahraini coastal waters of the Arabian Gulf.

5.1 Introduction

5.1.1 *Background information about the study area*

The second sampling area in this study was located in coastal waters of Bahrain, which is one of the Arabian Gulf countries. A brief description of the main marine characteristics of the Arabian Gulf are first outlined then a more detailed description of the Bahrain marine environment, a description of the sampling area and the harmful algal bloom events and species recorded in Bahrain and the Arabian Gulf countries are listed.

The marine environment of the Arabian Gulf region

The Arabian Gulf lies in the sub-tropics, north of the tropics of Cancer in west Asia between 24º- 30º N and 48º - 57º E. Eight countries are situated around the coast of the Gulf: Bahrain, Iran, Iraq, Kuwait, Oman, Qatar, Saudi Arabia and the United Arab Emirates (Al-Zayani 2003) (Fig. 5.1).

Very high salinity is a distinguishing characteristic of the Arabian Gulf. This is due to limited water circulation, weak currents, and limited freshwater flowing into the sea coupled with very high evaporation rates due to the extremely hot climate. These factors have a negative impact on the biota and limit the diversity of marine and coastal species (IUCN 1987). Unlike temperate marine regions, the salinity distribution of the Gulf is more consistent throughout the year. Salinity generally rises from 36.5-37 near the Strait of Hormuz (Fig. 5.1) to about 41-42 off the Saudi Arabian shores. The high surface salinity is evident on the shallow side of the Gulf, i.e. along the southwestern and southern coast (Chao et al. 1992).

Typical surface water temperature in the Gulf is about 33 °C in summer with little spatial variation. The winter surface temperature is about 22°C near the Strait of Hormuz, decreasing northwestwards to about 16 °C near the head of the Gulf (Chao et al. 1992). Researchers have found that the water temperature of the Gulf has the greater influence on seawater level, whereas the salinity has the greater influence near the Strait of Hormuz (Fig.5.1, due to the entrance of the water from the Gulf of Oman, which is less saline than the Arabian Gulf (Al-Madani and Al-Sayed 2001).

The marine environment of Bahrain

Location of Bahrain

The Kingdom of Bahrain consists of an archipelago of 36 islands located on the southern shores of the Arabian Gulf, sandwiched between its large neighbour Saudi Arabia to the west, and Qatar to the east (Fig. 5.2). Bahrain is located in the Arabian Gulf at longitudinal 50° 22′ 45"- 50° 49′ 45" N and latitudes 25° 32′ 20"- 26° 17′ 10" E (Directorate of Statistics 1996). It is approximately 15 miles (22 Km) off the east coast of the Arabian Peninsula, 150 miles west of the coast of Iran and 18 miles north-west of the coast of the Qatar Peninsula (Al-Zayani 2003). The area is located in an arid and semiarid zone, with a mainly dry and hot climate, the average rainfall being approximately 74 mm per annum, mostly occurring in winter. The humidity in the area is very high for most of the year, and especially when south-easterly winds blow (Al- Zayani 2003).

The physical marine environment of Bahrain

Tides and Currents: Bahrain is located close to the amphidromic point (a point within a tidal system where the tidal range is almost zero, and there is almost no vertical movement) of the diurnal tides so that, unlike the northern and southern end of the Gulf, Bahrain experiences semi-diurnal tides with a tidal range of up to two meters. The net circulation around Bahrain is clockwise. This is a result of shallower water depths in the strait between Bahrain and Qatar as well as phase differences within the tides in the Gulf. This mechanism

is of great importance to the generation of salinity fronts around the island. The circulation pattern in conjunction with the reduced water exchange caused by the sill and natural evaporation due to high (summer) temperatures creates a considerably higher salinity to the south of Bahrain and into Dawhat Salwa (D in Fig.5.2) (Vousden 1988, Al-Madani and Al-Sayed 2001). This also has a pronounced effect on the geographical distribution of the biota. Many species that are less salinity-tolerant will disappear moving south as salinity levels increase thereby reducing diversity (Vousden 1988).

Temperature: High air temperatures and insulation levels are characteristic features of the Gulf. Although thermal ranges are less in the island climate of Bahrain than on the mainland of Arabia, summer temperatures regularly exceed 40 °C. Coastal sea temperatures are also maximal during this period typically averaging 38 °C and intertidal pool temperatures often exceed 40 °C (Vousden 1988, Uwate and Shams 1999).
In winter months, coastal sea temperatures normally drop to between 14 to 18 °C (Price et al. 1984). These extremes in temperature obviously must present tolerance limitations for many of the intertidal organisms but must be seen in context with regard to the damping effects of the tidal regimes and its effect on the distribution of the intertidal biota. The limited depth of the Gulf results in a relatively small thermal capacity, allowing water temperatures to track air temperatures, and this undoubtedly limits the number of species that can survive in the area (Vousden 1988). Sea temperatures and salinities are found to be closely related as a direct result of the climate and the shallow and partially land-locked nature of this region (Vousden and Price 1985). Sea temperatures parallel air temperatures closely, due to the small thermal capacity of the shallow waters.

Salinity: Within the Gulf, salinities are generally high due to the effect of high temperatures and consequent evaporation. This is particularly evident during the hotter months of July and August when the highest salinity gradients can be seen around the coastal waters of Bahrain. On the west coast of the Island, salinities fluctuate between 52 and 58 with 60 being the normal recorded level for the south-western coastline. On the east coast where currents velocities are generally higher, salinities are in the 42- 45 range (Vousden 1988).

In areas of restricted water flow such as tidal pools, bays or lagoons, higher salinity values are often recorded. For example a salinity of 80 has been measured at high tide over Sabkha flats in the Hawar Archipelago and 70 within the lagoon at Ras Al Mumatallah (H and R in Fig. 5.2) (Vousden 1988, Al-Madani and Al-Sayed 2001).

Water quality: Water quality is as expected for a shallow sub-tropical coastal area with such extremes of temperature and high salinities. Nutrient levels are low favoring the development of corals whereas higher levels would undoubtedly favor the growth of macro algae. Chlorophyll levels in the water column are also low especially offshore and primary production is mostly benthic rather than phytoplanktonic (Basson et al. 1977, Price et al. 1984, Vousden and Price 1985, Vousden 1985) with very high levels of chlorophyll recorded from the surface of intertidal mud (Price et al. 1984) due to the presence of cyanobacteria and diatoms.

Human impacts on the marine environment are obvious in Bahrain and can be divided into two components: pollution, and physical alteration and destruction of habitats. Marine pollution in Bahrain can also be divided into industrial pollution and non-industrial pollution. The main non-industrial pollution discharge to the marine environment is untreated domestic sewage. In Bahrain, most untreated sewage is discharged directly into the sea, causing physical, chemical and biological contamination, especially if the discharge area has low currents and shallow depths (Al-Zayani 2003).

Sampling Area:

Water samples were collected from Arad Bay (Fig.5.2) which is located to the North of the main Island of Bahrain (26° 14′ 23"N (26.240°), 50° 37′ 48"E (50.530°)). It was declared as a nature reserve in 2003. It is a safe area for migrant birds, shrimps and rare marine life. This nature reserve safeguards a number of marine life that were facing extinction due to coastal reclamation works being carried out in most areas of the country. It is registered as one of the Marine protected areas in UNEP (United Nations Environment Programme) and the site code is 313506. The Government of Bahrain in late 2006 initiated a new

conservation project in the sampling area called "The Arad Bay project" aimed at protecting the natural habitat of migratory birds. The Government simultaneously wants to create awareness among the public and the project includes the creation of a 3.5km long walkway along the boundaries of the back waters of Arad in order to allow the public access to the area.

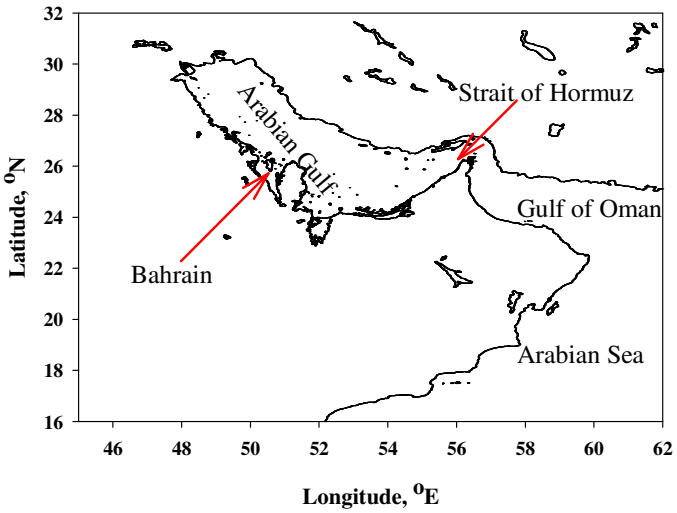
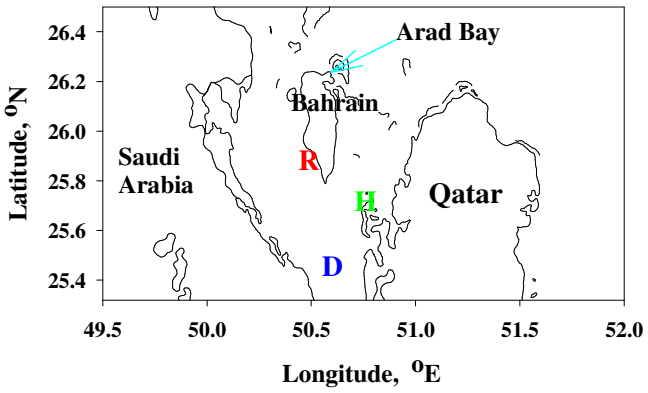


Figure 5-1: A map showing the Arabian Gulf region.



R: Ras Al mumtalah
H: Hawar Islands
D: Dawhat Salwa

Figure 5-2: A map showing the sampling area (Arad Bay). (R, H and D refer to regions mentioned in text).

5.1.2 *Harmful algal blooms in the Arabian Gulf*

Phytoplankton species lists from the Arabian Sea (Subba Rao and Al-Yamani 1998) suggest that some potentially harmful species are present within the region. In September of 1999, a major fish kill occurred in Iranian coastal waters and was immediately followed by wild and aquaculture-related fish kills in Kuwaiti coastal waters (September-October, 1999). Fish mortality (at least in Kuwaiti waters) was related to the presence of a red tide outbreak of the dinoflagellate *Gymnodinium sp***.** identified by Kuwaiti EPA (Environmental Public Authority) personnel on October 2, 1999 based upon high cell counts of *Gymnodinium sp* (> 6×10^6 cells L^{-1}). Following the peak of the *Gymnodinium sp.* bloom, red water caused by *Myrionecta rubra* (=*Mesodinium rubrum*) was observed (Heil et al. 2001). Subba Rao et al. (1999) described a bloom of the dinoflagellate *Gyrodinium instriatum* in Kuwait Bay in 1997 that was characterized by high biomass (>200µg chlorophyll *a* L^{-1}) and primary production rates (>500 µg C L^{-1} h^{-1}) but was not apparently toxic to fish or shellfish. Blooms of the photosynthetic ciliate *Myrionecta rubra* were also reported in Kuwaiti Bay in October of 1995, with chlorophyll *a* concentrations up to 160 µg L^{-1} and cell concentrations of 1.08×10^6 cells L^{-1} (Al-Yamani et al. 1997).
In August and September 2001, Kuwait Bay, experienced a massive fish kill involving over 2500 metric tons of wild mullet, due to the bacterium *Streptococcus agalactiae*. In the Bay, this event was preceded by a small fish kill of gilthead sea bream in aquaculture net pens associated with a bloom of the dinoflagellate *Ceratium furca*. This event was nearly 100-fold larger than the previous major fish kill in this region, which was recorded in 1999 and associated with a red tide (Heil et al. 2001).

The only mass fish mortality recorded in Bahrain waters was caused by the Bacteria *Streptococcus iniae* (Yuasa et al. 1999). Red tide events from Bahrain coastal waters have not been recorded in the literature, but *Gymnodinium mikimotoi (=Karenia mikimotoi)* has been identified in some areas of the Gulf (Khamdan 2000 unpublished report). Table 5.1 lists a summary of the recorded bloom events in the Arabian Gulf.

Table 5.1: Recorded bloom events in Bahrain and the Arabian Gulf.

Toxic algae/ organism	Country, time of event	Reference
Nitzschia and *Gymnodinium sp.*	Kuwait, 1997	Subba-Rao et al. (1999)
Gymnodinium splendens	Bahrain, September 1998 and April 1999	Khamdan (2000) (unpublished report)
Gymnodinium nagaskiense(=Gymnodinium mikimotoi =Karenia mikimotoi).	Kingdom of Suadi Arabia and Bahrain, January 1987; Kuwait, September- October 1999	Khamdan (2000) (unpublished report)
Karenia selliformis	Kuwait, October- December, 1999	Ismail W, Kuwait Insitute for Scientific Research*
Gymnodinium sp .	Kuwait, 1999	Heil et al. (2001)
Phaeocystis sp	Kuwait, May 2004	Ismail W, Kuwait Insitute for Scientific Research*
Streptococcus bloom followed by *Ceratium furca* , *Gymnodinium catenatum* , *Gyrodinium impudicum* and *Pyrodinium bahamense* var. *compressum.*	Kuwait, 2001	Glibert et al. (2002)
Heterosigma akashiwo	Kuwait, April, 2006	Ismail W, Kuwait Insitute for Scientific Research*
Streptococcus iniae	Bahrain, 1999	Yuasa et al. (1999)
A species belonging to *Peridinaceae*	Bahrain, June- October 1988	Khamdan (2000) (unpublished report)
Protoperidium quinquecorne and *Gonialux orientalis*	Bahrain, March 1989	Khamdan (2000) (unpublished report)
Scrippsiella trochoidea	Bahrain, April 1999	Khamdan (2000) (unpublished report)
Prorocentrum arabianum sp. nov .	Gulf of Oman, 2002	Morton et al. (2002)

* Personnel communication

5.1.3 *Work plan*

Karenia sp. (previously known as *Gymnodinium sp.*) has been recorded to cause harmful algal events in Bahrain coastal waters (Table 5.1); however, the species identification was not confirmed. Therefore, initially the objective in this part of the study was to isolate *Karenia sp.* strains from Bahrain coastal waters and perform genetic comparison with those that occur in the western English Channel. Attempts to isolate *Karenia sp.* from water samples collected following several sampling trips to Arad Bay 2006 and 2007 were not successful. However, another dinoflagellate species was found to occur in high cell densities and was subsequently isolated and grown in culture. The isolated species was identified as *Bysmatrum granulosum* by Gert Hansen (Department of Biology, University of Copenhagen). Toxicity tests performed on the isolated species using brine shrimp demonstrated that this dinoflagellate species is potentially harmful. A review of the literature of the different species of *Bysmatrum* revealed that they share many morphological characteristics and it is difficult to differentiate between them. Additionally, it was found in the present study that the identification of the species was quite difficult in the presence of other dinoflagellate species. Being a potentially harmful dinoflagellate species in Bahrain coastal water and difficult to identify, make it essential to apply the molecular approaches developed earlier in this study to this particular species. Consequently, an 18S rDNA gene sequence was obtained to perform a phylogenetic analysis and reveal the species position with respect to other dinoflagellate species. Two molecular methods (PCR and CARD-FISH) that were applied earlier in this study were then used for the detection/identification and enumeration of the isolated species *B. granulosum* in culture and in seawater samples. Species-specific primers and probes were designed according to the 18S SSU rDNA gene sequences of *B. granulosum*, and a polymerase chain reaction (PCR) method was used for detection of that species. Additionally, the CARD-FISH protocol was optimized to enumerate the isolated species using epifluorescence microscopy and flow cytometry.

5.2 Materials and methods

5.2.1 *Isolation of* B. granulosum *species and culture conditions*

B. granulosum (Ten-Hage et al) was isolated from seawater samples collected from Arad Bay, Bahrain at 26° 14′ 23"N (26.240°), 50° 37′ 48"E (50.530°) (Fig. 5.2) in June 2006.
Single cells of *B. granulosum* were directly isolated by micropipette and transferred into a 96-well plate. The single cells were grown in Guillard's L2 medium (Guillard and Morton 2004) and maintained in a temperature controlled incubator at 18-23 ºC, with 12:12 h light: dark cycle and irradiance of 80 µmol photons m^{-2} s^{-1}. After three weeks, 100-300 clonal cells (genetically identical cells generated from a single isolated cell) were transferred into a 24-well plate and more L2 medium was added to the plate to nourish cultured cells. Subsequently, cells were transferred into 12, 6 well plates and finally into small culture tubes. The cells were then sub-cultured every 4 weeks.
Cell dimensions were determined by measuring the length and width of 30 cells using an ocular micrometer on a light microscope. Microscopic photographic images were taken using an Olympus BX41 light microscope, using total magnification of ×400.
The isolated species was identified by Gert Hansen (Department of Biology, University of Copenhagen) and deposited into the Scandinavian Culture Collection of Algae & Protozoa (SCCAP) in 2008. A few fluorescent images of *B. granulosum* cells stained with Calcofluor were taken by Gert Hansen. The Fluorchrome Calcofluor is useful in defining the thecal structure plate of dinoflagellates observed under a fluorescent microscope (Fritz and Triemer 1985).
To test the toxicity of the isolated species *Bysmatrum granulosum* cells were incubated with brine shrimp for a few days in well plates. In the control experiment, brine shrimp were incubated with a non-toxic dinoflagellate *Gymnodinium simplex*. Daily observations of the behaviors of brine shrimp were recorded.

5.2.2 *DNA extraction, amplification, determination of 18S SSU and 28S LSU rDNA gene sequences and species-specific primers and probe design*

DNA extraction was performed on single cells isolated directly by micropipette. The single cells were then transferred into 200 µL PCR reaction tubes to which 20 µL of 1× PCR (Qiagen) buffer have been added. Isolated cells were kept cool during the cell-isolation procedure and were either processed immediately or stored at -80 ºC prior to PCR analysis. The isolated single cells were then directly used for DNA amplification (i.e. crude DNA from disrupted cells was amplified). Disruption of cells was induced by a freeze-thaw process. The total cellular DNA was used as a template to amplify about 1750 base pairs of the 18S small subunit ribosomal gene (18S SSU rDNA) using terminal primers $EUK328_f$ and $EUK329_r$ (Moon et al. 2000). Cellular DNA from single isolated cells was also used to amplify about 1000 base pairs of the 28S large subunit ribosomal gene (28S LSU rDNA) using terminal primers $D1R_f$ (Scholin et al. 1994a) and $D3B_r$ (Nunn et al. 1996). Primer sequences and references are listed in Table 5.2. Amplification with either the 18S SSU or 28S LSU rDNA primers was carried out in a thermal cycler (BIO-RAD DNA Engine). The 50 µL of reaction mixture contained a single cell (total cellular DNA template), 0.2 µM of each of the amplification primers, 200 µM total dNTP, 2 mM $MgCl_2$, 0.1 $mgmL^{-1}$ Bovine Serum Albumin (BSA) and 5 units of Taq polymerase (Qiagen, USA). Thermal cycle parameters to amplify the 18S SSU rDNA gene were according to Moon et al. (2000) as follows: initial denaturation at 95 ºC for 5 min, 30 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 2 min, extension at 72 ºC for 3 min. The temperature profile was completed by a final extension cycle at 72 ºC for 6 min. Amplification with the 28S LSU rDNA primers was carried out using similar conditions to Hansen et al.(2000) outlined previously in section 3.2.4.

DNA fragments were checked on 2.0% (w/v) agarose gels stained with ethidium bromide in 1×TAE buffer followed by examination under UV transillumination. PCR products were purified using QIAquick PCR purification Kit (Qiagen) and were commercially sequenced (Geneservice, Cambridge, UK) using primers listed in Table 5.2.

The 18S SSU and 28S LSU rDNA sequences of *B. granulosum* were imported to CAP3 software (Huang and Madan 1999) to obtain consensus sequences of that species. 18S SSU rRNA species-specific probes were designed using ARB software (http://www.arb-home.de) (Ludwig et al. 2004) and 18S SSU rDNA species-specific primers were designed using Primer3 (Rozen and Skaletsky 2000). The specificity of the designed primers and probes was checked against the GenBank database using BLAST (Basic Local Alignment Search Tool) within the NCBI (National Centre for Biotechnology Information) database (http: //www. ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1990) and using ARB software (Ludwig et al. 2004). The theoretical specificities of the new probe and primers were checked using the probe match function of the ARB software. The selected primer sequences were 20 nucleotides in length with GC content of more than 50% to get a more uniform annealing temperature during PCR. The probe sequence that was used in this part of the study to apply CARD-FISH was 18 nucleotides in length with GC content of 50% and was checked for its *in situ* accessibility using the 18S rRNA of *Saccharomyces cerevisiae (Eucarya)* as a reference (Behrens et al. 2003).

Table 5.2: Primers and probes used in this study.

Target organisms	Primer/ probe sequence		Target site position	Reference
	Forward primer sequence [5'- 3']	Reverse primer sequence [5'- 3']		
Eukaryotes	ACCTGGTTGATCCTGCCAG[1]	TGATCCTTCYGCAGGTTCAC[2]	Forward primer: (2); Reverese primer: (1528)[5]	Moon et al. (2000)
	GCGGTAATTCCAGCTCCAA[3]		524 [5]	Elwood et al. (1985)
Dinfolagellate-Group	ACCCGCTGAATTTAAGCATA [$D1R_f$]	TCGGAGGGAACCAGCTACTA [$D3B_r$]	D1R: (24-31); D3B: (1011-992) [6]	[$D1R_f$] Scholin et al. (1994a); [$D3B_r$] Nunn et al. (1996)
B. granulosum	GTAGGTCTGGCTTGCCTCAG	TCCTATTCACCCTCCCCTCT	Forward primer: (621-641); Reverese primer: (820-800) [7]	This study
B. granulosum	GATACTCATCAGCGGATC [4]		463 [5]	This study

1. $EUK328_f$; 2. $EUK329_r$; 3. $EUK528_f$ (used for sequencing only); 4. Probe sequence; 5. Primer/probe position correspond to *E. coli* numbering; 6. annealing positions refer to the LSU rDNA of *Prorocentrum micans* (Lenaers et al. 1989); 7. annealing position refer to the 18S rDNA of *B. granulosum* of the present study.

5.2.3 *Phylogenetic analysis of* B. granulosum *species based on the 18S SSU rDNA sequences*

All phylogenetic analyses were performed with the alignment and treeing tools implemented in the ARB program package (Ludwig et al. 2004, www.arb-home.de). The new sequence of *B. granulosum* and other dinoflagellate species sequences from GenBank were added to an existing ARB database that includes 20325 species sequences of prokaryotes and eukaryotes by using the alignment tool ARB-EDIT. Alignments were refined manually by visual inspection. Phylogenetic analyses were performed by applying maximum-parsimony (a sub-tree of the huge original tree, bootstrap analysis with 1000 replications in parsimony used to determine the robustness of topologies) and maximum-likelihood (calculated as a proper tree based on 163 species sequences) methods using a 95% similarity filter for the Alveolata group and the ARB treeing tools for nucleotide sequences. Variability of the individual alignment positions was determined by using the ARB-SAI tools. ARB was used to calculate the distance matrix between *B. granulosum* and its closest relatives according to the phylogenetic analyses based on the 18S SSU ribosomal gene sequences. Out-group: The SSU rDNA sequences from two species of *Paramecium* (*Paramecium primaurelia and Paramecium caudatum)* were used to polarize the in-group.

Attempts to obtain and use a large database of different 28S LSU rDNA sequences to construct a phylogenetic tree based on the LSU sequence of *B. granulosum* during the course of this investigation were unsuccessful. Dr. Niels Daugbjerg (Department of Biology, University of Copenhagen) was contacted to check the 28S LSU rDNA sequence of *B. granulosum* obtained in this study against his LSU rDNA database with the aim of constructing another phylogenetic tree based on that gene. However, the obtained sequence showed some unexpected base pair differences (i.e. missing 2 base pairs common to all other taxa and have one extra base pair not found in other taxa) when compared to other dinoflagellates sequences. These differences could be due to sequencing or PCR amplification bias. Due to time limitation, a decision was therefore taken to use only the 18S SSU rDNA sequences for the following steps.

5.2.4 *Testing and evaluation of* B. granulosum *species-specific 18S SSU rDNA primers*

To evaluate the designed species-specific 18S SSU rDNA primers, PCR was performed on DNA from 1 mL of *B. granulosum* cultured cells (crude DNA from disrupted cells). PCR reactions using species-specific primers were run in total volumes of 25 µL each, consisting of 1×Taq polymerase buffer including 2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, 0.1 $mgmL^{-1}$ BSA, total cellular DNA template from disrupted cells, and 2.5 units of Taq polymerase. Amplification with species-specific primers was carried out in a thermal cycler as follows: initially 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 1 min. After the cycles, extension was completed at 72 °C for 2 min. The expected PCR fragment was 200 base pairs. PCR products were cooled and visualized by electrophoresis using 2.0% (w/v) agarose gels stained with ethidium bromide in 1×TAE buffer followed by examination under UV transillumination. Annealing temperatures were optimized along a temperature gradient from 55 °C to 60 °C using cellular DNA templates of *B. granulosum*.

To evaluate the species-specific 18S SSU rDNA primers, PCR was performed on DNA extracted from different algal cultures (Table 3.1, Chapter 3) used as negative controls as well as the target species *B. granulosum*. DNA from different cultures was extracted using 1 mL of each culture following the protocol described above (i.e. disruption of cells using freeze-thaw process). PCR reactions using 18S SSU *B. granulosum* species-specific primers were run and PCR products were examined as described above.

5.2.5 *Applying the 18S SSU rDNA primers to environmental water samples from Arad Bay, Bahrain*

Surface seawater samples were collected from Arad Bay, Bahrain during summer 2006, 2007 and 2008 (Fig. 5.2). On the day of collection, 100 mL of seawater was collected and fixed with Lugol's iodine solution (final concentration 1%) and kept in a dark cool place until further analysis by PCR method. 10 mL of each Lugol's fixed sample was placed in a 10 mL sedimentation chamber and left to settle for 24 hours. *B. granulosum* and other

phytoplankton cells were identified and counted as described previously in Chapter 3, section 3.2.6.

Extraction of DNA from the Lugol's fixed water samples was as outlined in 3.2.2. DNA amplification was performed by applying nested PCR using the 18S SSU rDNA eukaryote general primers $EUK328_f$ and $EUK329_r$ (Table 5.2). Subsequently, 1 μL of the PCR obtained products was used as template DNA for the next reaction with *B. granulosum* species-specific primers listed in Table 5.2. PCR thermal conditions using the 18S SSU Eukaryote general primers and the 18S *B. granulosum* species specific primers were as mentioned above in 5.2.2 and 5.2.4, respectively.

5.2.6 *CARD-FISH protocol to enumerate* B. granulosum *using species-specific 18S SSU rRNA probe*

Epifluorescence microscopy for the detection and enumeration of B. granulosum

The protocol used in this part of the study was as described previously in Chapter 4. The hybridization experiments were conducted under the same conditions using *B. granulosum* cultured cells. FISH analysis was performed using 5' horseradish peroxidase (HRP) – *B. granulosum* labelled probe (Table 5.2).

The optimum hybridization conditions for the newly designed probe were determined by applying the designed species-specific *B. granulosum* probe at different concentrations of formamide hybridization buffer (20%, 40%, 60% and 80%) to find the highest percentage of positive hybridization with the target species (*B. granulosum*) and to discriminate it from other non-target dinoflagellate species. Table 5.3 shows the concentration of NaCl in 50 mL of washing buffer with the corresponding concentration of formamide in the hybridization buffer.

Table 5.3: Concentration of NaCl in 50 mL of washing buffer with corresponding formamide concentration in the hybridization buffer.

Concentration of formamide hybridization buffer (%)	NaCl in washing buffer (mM)
20	145
40	37
60	9
80	-

Evaluation of the 18S SSU rRNA probe to detect B. granulosum *using CARD-FISH analysis with Flow cytometry*

The protocol of CARD-FISH for analysis with flow cytometry was as described in chapter 4 with slight modification. Seawater samples containing cells of *B. granulosum* were labelled with *B. granulosum* probe employing CARD-FISH, but without embedding filters in low-gelling point agarose. The cells after hybridization were backwashed into plastic centrifuge tubes using sterile filtered seawater. Initially, the CARD-FISH protocol was performed on membrane filters as described previously in section 4.2.2 (Chapter 4). However, due to the major loss of large cells during steps of CARD-FISH on membrane filters, FISH were carried out in solution. 1000 µL of *B. granulosum* cultured cells were fixed with a solution of 20% (w/v) paraformaldehyde (final concentration 1%) for 1 hour at room temperature or for 24 hours at 4 ºC. The fixed cultured cells were then concentrated by centrifugation in a bench top microcentrifuge. Cells were then dehydrated in an ethanol series (50%, 70% and 96% [v/v], 5 min each). To ensure full permeabilization of the probe, cells were incubated with 0.01 M HCl for 10 min at room temperature. After washing in 1×PBS, sterile water and 96% [v/v] ethanol for one min each, the filters were air-dried carefully before proceeding to the next step.

FISH and tyramide signal amplification steps were carried out as outlined in section 4.2.2. Evaluation of oligonucleotide probe binding was performed using the CellQuest software

(Becton Dickinson, Oxford, UK) used to operate the flow cytometer (FACSCalibur Becton Dickinson) equipped with 15 mW 488 nm laser and used for data analysis. For analysis of CARD-FISH data, green fluorescence (fluorescein) was collected using the FL1 detector using a 500-560 nm band pass filter. Density plots of side scatter (SSC) versus log green fluorescence were generated. Yellow-green beads of 0.5µm diameter (Fluoesbrite Microparticles; Polysceinces) were used as a flow cytometric internal standard. WinMDI software 2.9 (http://flowcyt.salk.edu/software.html) was used for figures.

5.2.7 *Applying CARD-FISH protocol to Lugol's fixed samples collected from Arad Bay, Bahrain*

The CARD-FISH protocol was applied to Lugol's fixed samples using the 18S SSU rRNA probe. Initially, 2 mL of Lugol's fixed samples was washed with 100 µL of 10% sodium thiosulphate (Fisons Scientific, England) to equilibrate the iodine in the Lugol's solution. Then the CARD-FISH protocol was followed as outlined in 4.2.2 by concentrating cells by centrifugation (i.e. CARD-FISH in solution). and applied to all but one of the preserved seawater samples (13.09.07 due to insufficient volume of sample) from Bahrain coastal waters. Epifluorescence microscopy was used to calculate the absolute number of positively hybridized cells. The mean absolute number of positive hybridized cells counted using epifluorescence microscopy after CARD-FISH (number of positive cells in 1 mL) was then compared to the absolute number of *Bysmatrum sp.* cells counted in Lugol's fixed samples using a light microscope (number of cells in 1 mL).

5.3 Results

5.3.1 *Biological and ecological characteristics of* B. granulosum *species*

In 2006, the water temperature ranged from 35-38 °C and salinity from 35-45 in the study area during the sampling period between April and November. *B. granulosum* cultured cells were 40-45 µm long and 35-40 µm wide and cell growth was slow in cultures at 23 °C. It

took approximately a month for cultured cells to reach concentrations of >10 cells mL^{-1}. One isolated strain was deposited in the Scandinavian Culture Collection of Algae & Protozoa (SCCAP), and given the identification number: SCCAP K-0964. Fig.5.3 shows images of Calcofluor stained *B. granulosum* cells showing the different thecal plates (photographs A-G taken by Gert Hansen, H-I taken in NOC under an Olympus BX41 light microscope).

Brine shrimp feeding activity and mortality were investigated. During the first two days, when fed *Bysmatrum granulosum*, the brine shrimp appendage movements were hindered and complete death of brine shrimp was observed on the fourth or fifth day of incubation. In contrast, brine shrimps incubated with *Gymnodinium simplex* were in a good condition beyond five days of the experiment.

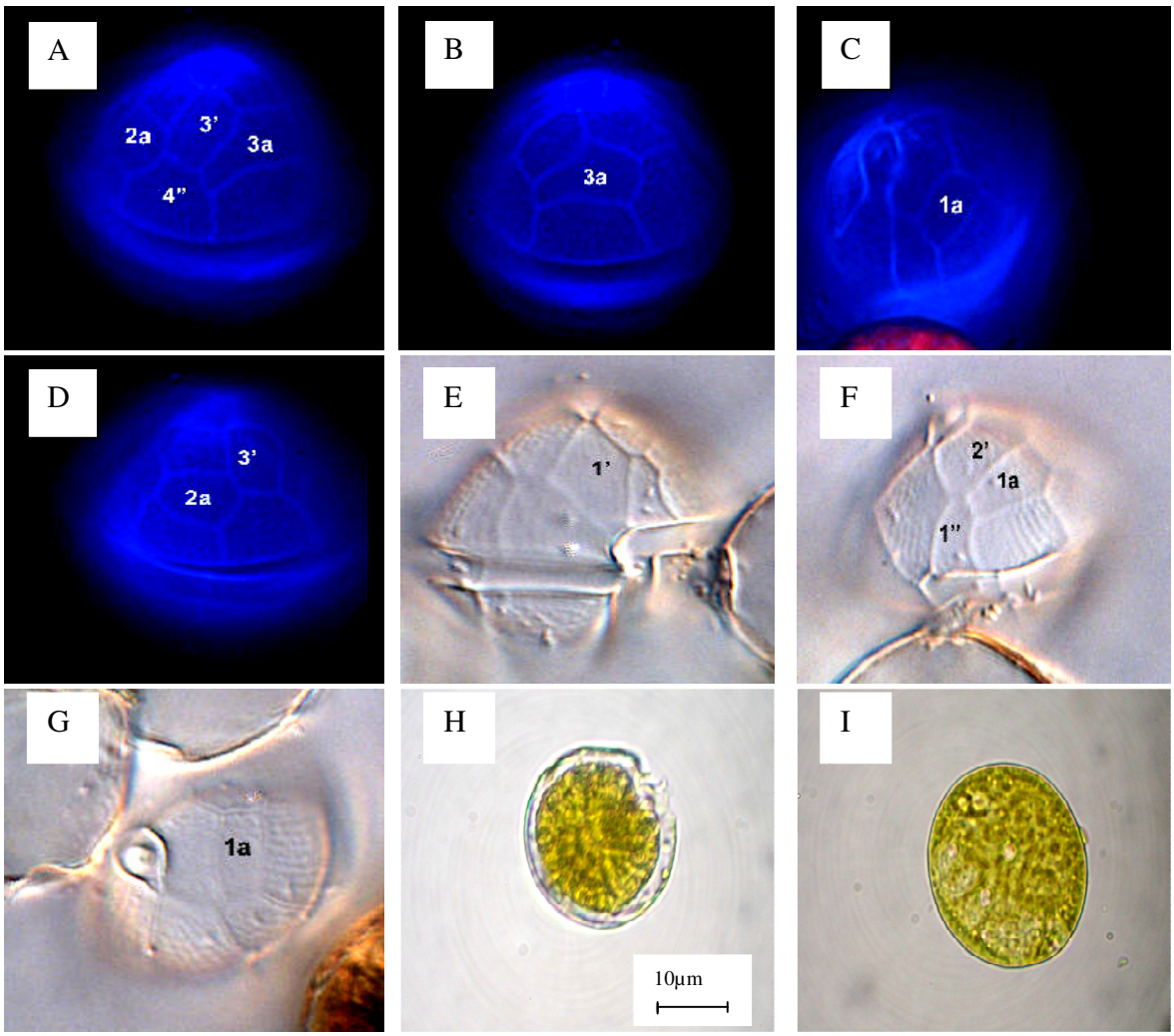


Figure 5-3: Images of *B. granulosum* (Ten-Hage et al). Photographs (A-G) taken by Gert Hansen, (A-D) cells stained with Calcofluor. Photographs (H and I) taken in this study.

5.3.2 *Determination of the 18S SSU and the 28S LSU rDNA gene sequences, and primers/ probes design*

The 18S SSU and the 28S LSU rDNA region (partial sequences) of *B. granulosum* were commercially sequenced in this study (Appendix 2). Different probes and primer sequences based on the 18S SSU rDNA sequence were designed and aligned against different

sequences in GenBank and 18S SSU rDNA database using ARB software. The 18S SSU rDNA primers and the selected rRNA probe designed in this study are listed in Table 5.2.

Specificities of the probe and primers designed in this study are illustrated by an alignment of the probe/ primers using ClustalW against several dinoflagellate species 18S SSU rDNA partial sequences obtained from BLAST results (Table 5.4). Different *Prorocentrum species, Hetecapsa sp.* And *Gymnodinium sp.* were in the BLAST results. Figure 5.4 shows the *in situ* accessibility data of the newly designed 18S species-specific probe using 16S rRNA *Saccharomyes cerevisiae in situ* accessibility map (Behrens et al. 2003). According to the accessibility map, the designed probe belongs to brightness class II (classes I to VI, with class I the brightest).

Table 5.4: Alignment of the designed primers with the closest hits according to BLAST

species	Aligment	GenBank accession number
probe [5'-3']	GATCCGCTGATGAGTATC	
Target sequence [5'-3']	GATCCGCTGATGAGTATC	
B. granulosum	GATCCGCTGATGAGTATC	This study
Prorocentrum triestinum	AATCCCTTTACGAGTACC	EF492512
Prorocentrum gracile	AATCCCTTTACGAGTACC	AY443019
Heterocapsa pygmaea	AATCCCTTTACGAGTACC	EF492500
Prorocentrum mexicanum	AATCCCTTTACGAGTACC	EU287485
Prorocentrum micans	AATCCCTTTACGAGTACC	EF492511
Gymnodinium sp.	AATCCCTTTATGAGTATC	AF022196
Forward primer [5'-3']	GTAGGTCTGGCTTGCCTCAG	
Target sequence [5'-3']	GTAGGTCTGGCTTGCCTCAG	
B. granulosum	GTAGGTCTGGCTTGCCTCAG	This study
Prorocentrum triestinum	GAGTATCTGGCTCGGCCTGG	EF492512
Prorocentrum gracile	GAGTATCTGGCTCGGCCTGG	AY443019
Heterocapsa pygmaea	GAGTATCTGGTTCGGCCTGG	EF492500
Prorocentrum mexicanum	GAGTATCTGGCTCGGCCTGG	EU287485
Prorocentrum micans	GAGTATCTGGCTCGGCCTGG	EF492511
Gymnodinium sp.	GAGCATCTGGCTCGGCCTTG	AF022196
Reverse primer [5-3']	TCCTATTCACCCTCCCCTCT	
Target sequence [3'-5']	AGAGGGGAGGGTGAATAGGA	
B. granulosum	AGAGGGGAGGGTGAATAGGA	This study
Prorocentrum triestinum	TGAGGTAATGATTAATAGGG	EF492512
Prorocentrum gracile	TGAGGTAATGATTAATAGGG	AY443019
Heterocapsa pygmaea	TGAGGTAATGATTAATAGGG	EF492500
Prorocentrum mexicanum	TGAGGTAATGATTAATAGGG	EU287485
Prorocentrum micans	TGAGGTAATGATTAATAGGG	EF492511
Gymnodinium sp.	TGAGGTAATGATTAATAGGG	AF022196

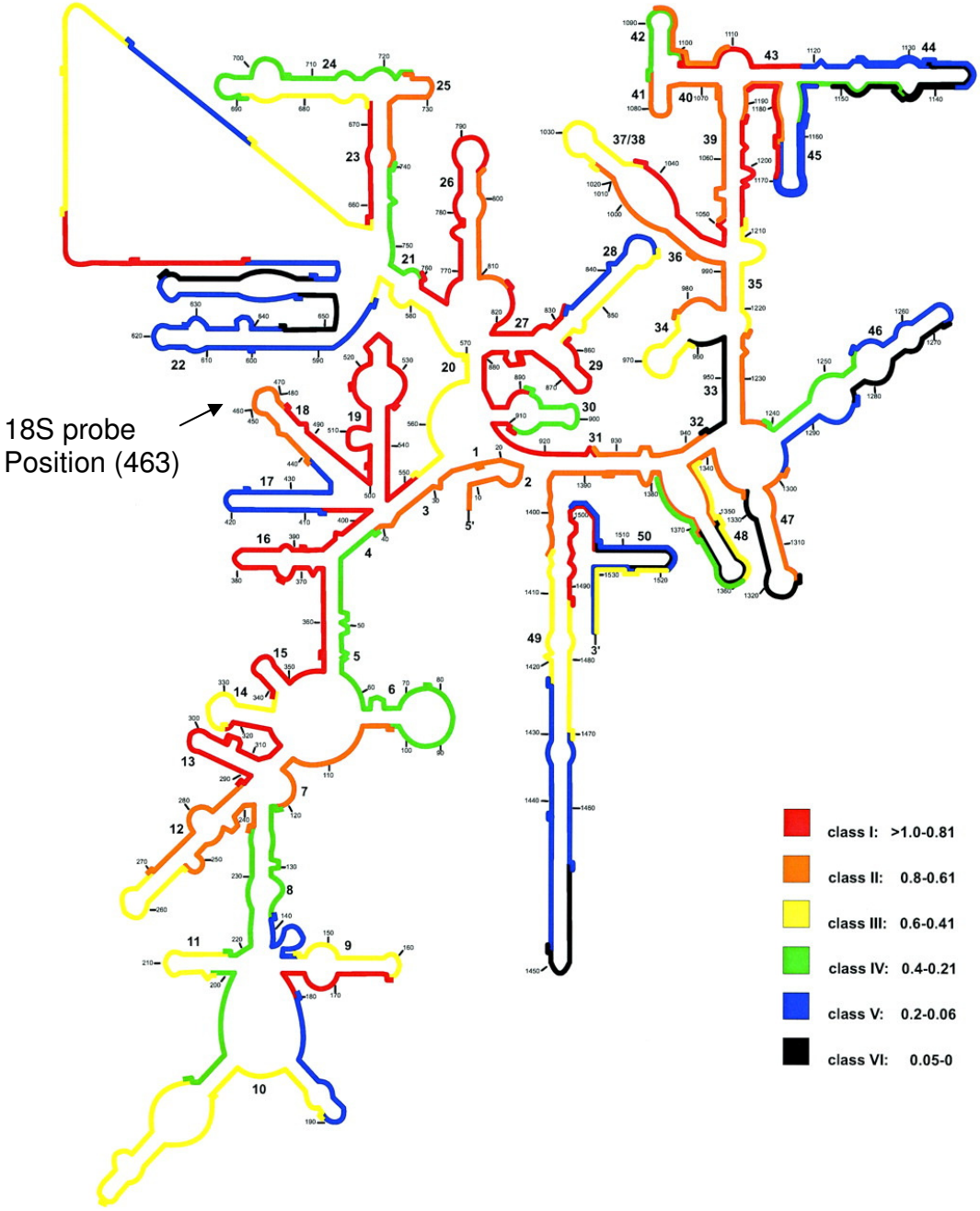


Figure 5-4: A model of the *S. cerevisiae* 16S rRNA showing the position of the newly designed 18S probe.

5.3.3 *Phylogenetic analyses of* B. granulosum

The 18S SSU ribosomal gene sequences were used to construct phylogenetic trees of the organism using ARB software. Fig. 5.5A shows a phylogenetic tree based on the maximum parsimony method and Fig. 5.5B a phylogenetic tree based on maximum likelihood (ML) method. Both maximum parsimony and maximum likelihood (ML) trees show the same tree topologies (outgroup species are shown in the maximum likelihood tree). *B. granulosum* forms a sister group relationship with a clade containing different species of *Amphidinium* (*A. klebsii*, *A. belauense*, *A. sp.* A. S1−CMSTAC025, *A. massartii*, and *A. carterae*). This relationship is strongly supported by a high bootstrap value of 93% calculated in maximum parsimony. Table 5.5 summarize percentage nucleotide sequence similarities between *B. granulosum* and its closest relatives based on neighbor joining (NJ) model. The maximum percent sequence similarity was between *B. granulosum* and *Amphidinium massaratii and Amphidinium carterae* of 81.2% nucleotides only.

Table 5.5: Percent similarity between *B. granulosum* 18S SSU rDNA nucleotide sequence (partial sequence, 1750 bp) and five different species of *Amphidinium* based on the 18S SSU rDNA nucleotide sequences, calculated using neighbor joining (NJ) model and ARB software.

	Amphidinium belauense	*Bysmatrum granulosum*	*Amphidinium massartii*	*Amphidinium sp. S1-CMSTAC025*	*Amphidinium carterae*	*Amphidinium klebsii*
Amphidinium belauense	100					
Bysmatrum granulosum	80.7	100				
Amphidinium massartii	96.9	81.2	100			
Amphidinium sp. S1-CMSTAC025	96.8	81	98.6	100		
Amphidinium carterae	96.9	81.2	100	98.6	100	
Amphidinium klebsii	99.3	80.1	96.3	96.1	96.3	100

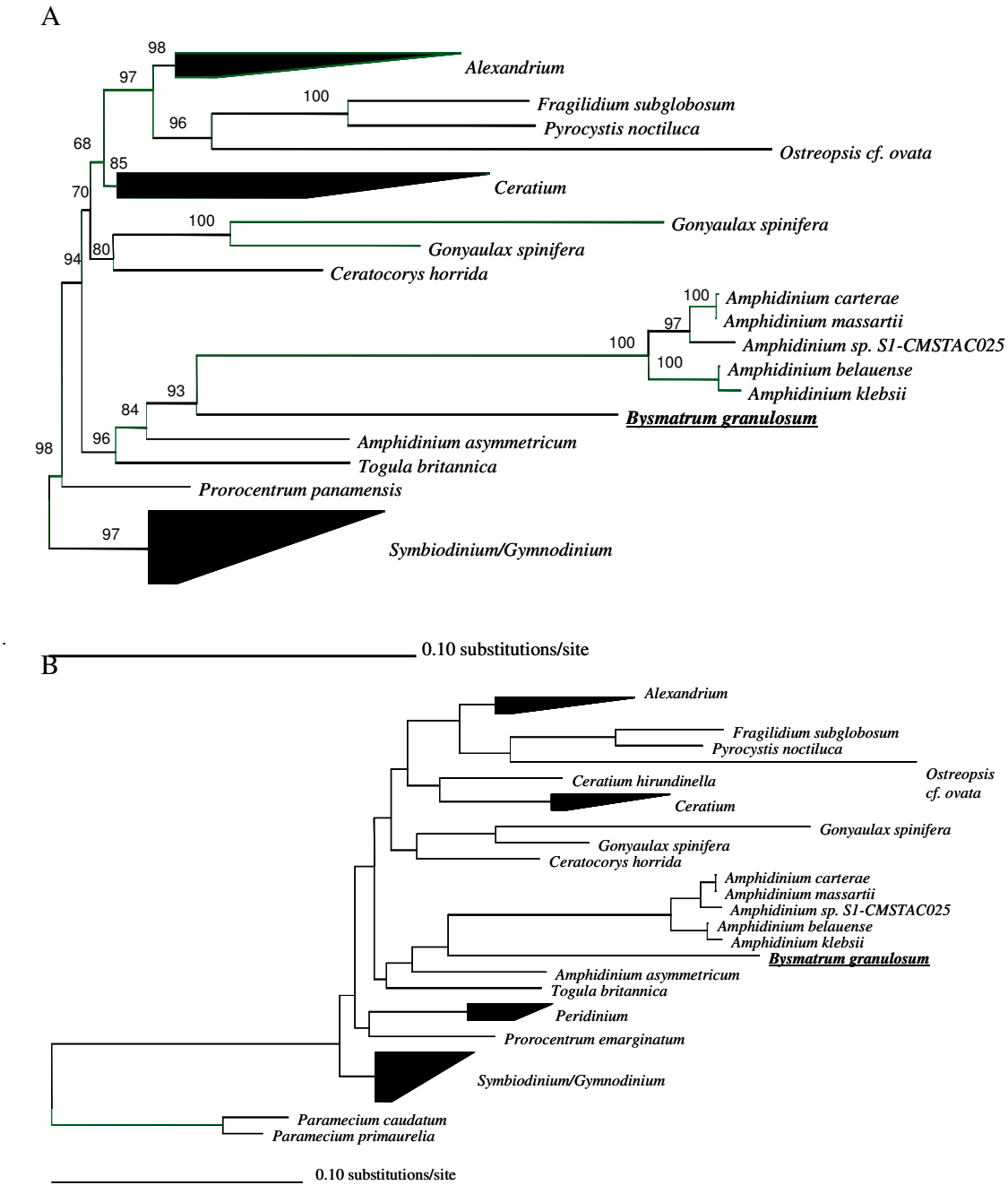


Figure 5-5: Phylogeny of different species including *B. granulosum* to show the phylogenetic position of *B. granulosum* in an evolutionary tree and its closest relatives inferred from about 1700 base pairs of nuclear-encoded 18S SSU rDNA. (A) The construction based on maximum parsimony analysis, (B) The construction based on maximum likelihood analysis (ML) method. Bootstrap values calculated for maximum parsimony tree with 1000 replications used to determine the robustness of topologies. Two *Paramecium* species were used to root the tree.

5.3.4 *Testing and evaluation of* B. granulosum *species-specific 18S SSU rDNA primers*

The 18S SSU rDNA species-specific primers were found to amplify very specifically, i.e. the target species produced DNA fragments of the expected size (200 base pairs) but no PCR products were formed with other algal species. A 60 ºC annealing temperature was found to differentiate between the target species from non-target species. *B. granulosum* primers did not yield any PCR products with a wide range of algal cultures used as negative controls (Fig. 5.6).

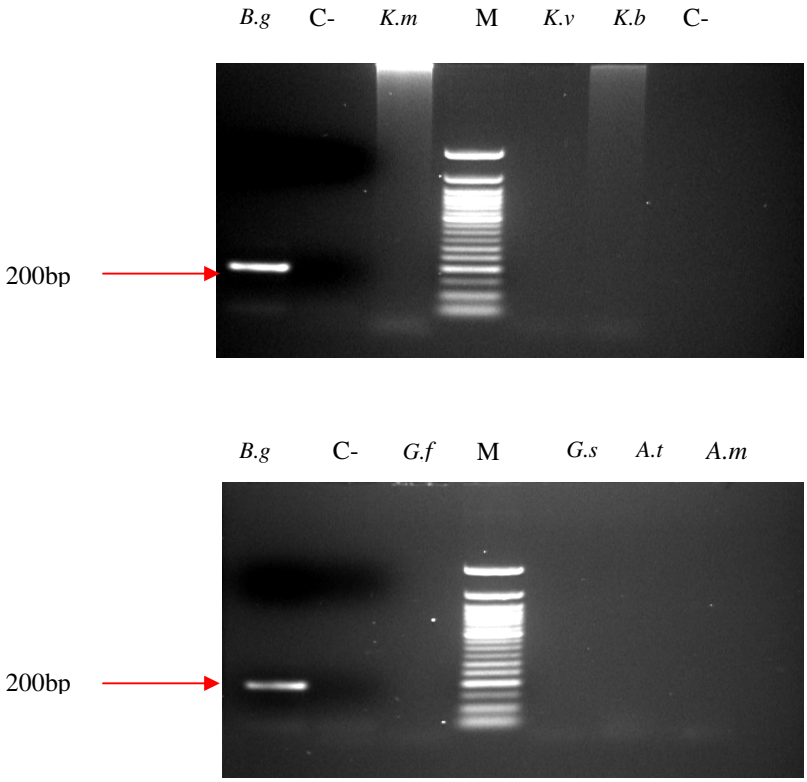


Figure 5-6: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using *B. granulosum* species-specific primers and template DNA from a *B. granulosum* culture and other non-target dinoflagellate cultures. The expected length of fragment is 200bp. Lanes 4 and 11: indicate molecular marker (50bp plus DNA ladder) (M). Lanes 2, 7 and 9: negative controls (C-); Lanes 1 and 8: *Bysmatrum granulosum (B.g)* cultured cells; Lane 3: *Karenia mikimotoi (K.m)*; Lane 5: *Karlodinium veneficum (K.v)*; Lane 6: *Karenia brevis (K.b);* Lane 10: *Glenodinium foliaceum (G.f)*; Lane 12: *Gymnodinium simplex (G.s)*; Lane 13: *Alexandrium tamarense (A.t)*; Lane 14: *Alexandrium minutum (A.m)*.

5.3.5 *Applications of* B. granulosum *primers to Lugol's fixed samples from Arad Bay, Bahrain*

Direct PCR on DNA extracted from seawater samples collected from Arad Bay using the 18S SSU species-specific primers often produced very faint bands or no products at all (data not shown). Therefore, nested PCR was performed on these samples (Fig. 5.7). Positive PCR products were produced from all samples except one (3.05.07) and *Bysmatrum* cells were detected by light microscopy in all Lugol's fixed samples except that from 3.05.07 (Table 5.5). Table 5.6 lists all the identified phytoplankton species in Lugol's fixed water samples collected from Arad Bay (estimated counting error <10%). Very high cell densities of *Bysmatrum sp.* were recorded in samples collected on 16.04.06 (1606 cells mL^{-1}) and 3.05.06 (1037 cells mL^{-1}). *Prorocentrum micans* was abundant in a water sample collected on 23.06.07 (2268 cells mL^{-1}). *Nitzschia sp.* reached a maximum concentration of 1261 cells mL^{-1} during summer 2006 (1.11.06). In general, the number of different species present in the field samples was low during the sampling periods and the number of dinoflagellate species exceeded the number of diatoms species.

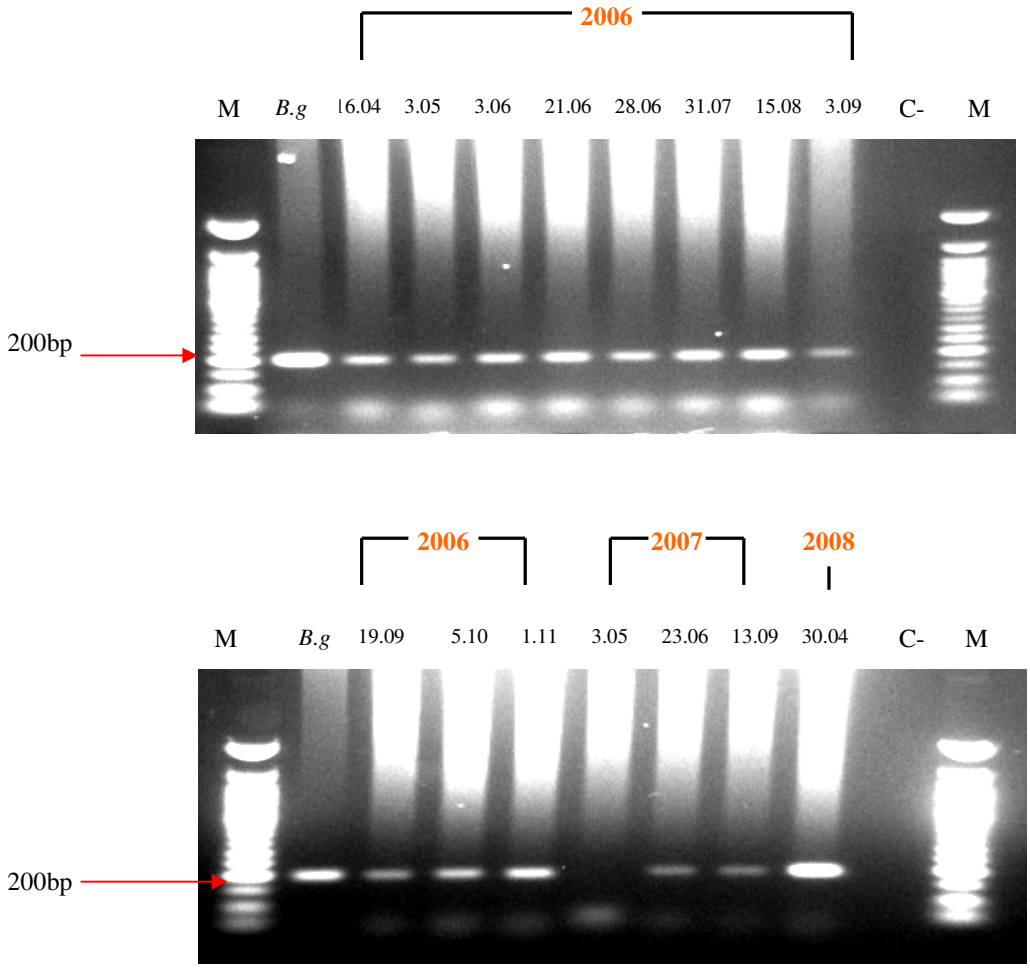


Figure 5-7: Agarose gel electrophoresis (2.0% [w/v]) showing PCR products produced using nested PCR (first PCR using the 18S SSU rDNA EUK328 and EUK329, second PCR using the 18S SSU rDNA *B. granulosum* species-specific primers and template DNA from *B. granulosum* cultured cells and environmental water samples collected from Bahrain during summer 2006, 2007 and 2008. The expected length of fragment is 200bp. Lanes 1, 12, 13 and 23: indicate molecular marker (50bp plus DNA ladder) (M). Lanes 2 and 14: *B. granulosum (B.g)* cultured cells; Lane 3: Field sample 1; Lane 4: Field sample 2; Lane 5: Field sample3; Lane 6: Field sample 4; Lane 7: Field sample 5; Lane 8: Field sample 6; Lane 9: Field sample 7; Lane 10: Field sample 8; Lane 15: Field sample 9; Lane 16: Field sample 10; Lane 17: Field sample 11; Lane 18: Field sample 12; Lane 19: Field sample 13; Lane 20: Field sample 14; Lane 21: Field sample 15; Lanes 11 and 22: negative controls (C-).(see Table 5.5 for details).

Table 5.4: A list of the surface seawater samples collected from Arad Bay, Bahrain during summer 2006, 2007 and 2008, PCR detection of *Bysmatrum sp.* using *B. granulosum* species-specific primers and *Bysmatrum sp.* cell counts in Lugol's fixed samples using a light microscope.

Field sample	Date of collection	PCR detection	Detection of *Bysmatrum sp.* in Lugols fixed samples using a light microscope [cells/mL]
1	16.04.06	+	1606
2	3.05.06	+	1037
3	3.06.06	+	125
4	21.06.06	+	60
5	28.06.06	+	19
6	31.07.06	+	28
7	15.08.06	+	18
8	3.09.06	+	5
9	19.09.06	+	3
10	5.10.06	+	5
11	1.11.06	+	264
12	3.05.07	-	<1[a]
13	23.06.07	+	739
14	13.09.07	+	82
15	30.04.08	+	88

a: not detected by microscopy.

Table 5.5: Quantitative record of phytoplankton taxa present in water samples collected from Arad Bay, Bahrain (no. cells/mL).

	2006											2007			2008
Species	sample 1 (16.04)	sample 2 (3.05)	sample 3 (3.06)	sample 4 (21.06)	sample 5 (28.06)	sample 6 (31.07)	sample 7 (15.08)	sample 8 (3.09)	sample 9 (19.09)	sample 10 (5.10)	sample 11 (1.11)	sample 12 (3.05)	sample 13 (23.06)	sample 14 (13.09)	sample15 (30.04)
DINOFLAGELLATES															
Bysmatrum sp.	**1606**	**1037**	**125**	**60**	**19**	**28**	**18**	**5**	**3**	**5**	**264**	**<1**	**739**	**82**	**88**
Prorocentrum micans	**1**			**1**	**1**	**1**							**2268**		**789**
Prorocentrum donghaiense											**190**				
Prorocentrum punctulatum													**455**		
Gonyaulax sp.						**12**	**16**	**2**			**1**				
Ceratium sp.							**5**								
Karenia sp.							**14**	**6**			**10**	**30**	**46**		
DIATOMS															
Pleurosigma sp.	**5**			**3**	**5**	**5**				**1**					
Cylindrotheca sp.	**1**						**1**								
Nitzschia sp.											**1261**				
ciliate											**155**	**28**			**5**

5.3.6 *CARD-FISH analysis using the 18S SSU rRNA B. granulosum species-specific probe*

Enumeration of B. granulosum *by epifluorescence microscopy*

The optimal hybridization conditions were determined for the newly designed *B. granulosum* 18S SSU rDNA probe. *B. granulosum* cultured cells were hybridized at different concentrations of formamide hybridization buffer (20%, 40%, 60% and 80%). 20% formamide hybridization buffer was found to produce the highest percentage of positive hybridization (94.6%, ±0.81) (Fig. 5.8). Non-target species were not used to assess the specificity of the probe due to the lack of availability. The probe was applied to seawater samples containing a high concentration of *B. granulosum* cells and 60% formamide hybridization buffer and was found to produce a high percentage of positive hybridization (93.6%, ±1.1) and yet could discriminate the target species *B. granulosum* from the non-target species present in the seawater samples. Fig. 5.9 shows DAPI and FITC images (taken using an epifluorescence microscope) of *B. granulosum* after applying CARD-FISH using different concentrations of formamide hybridization buffer.

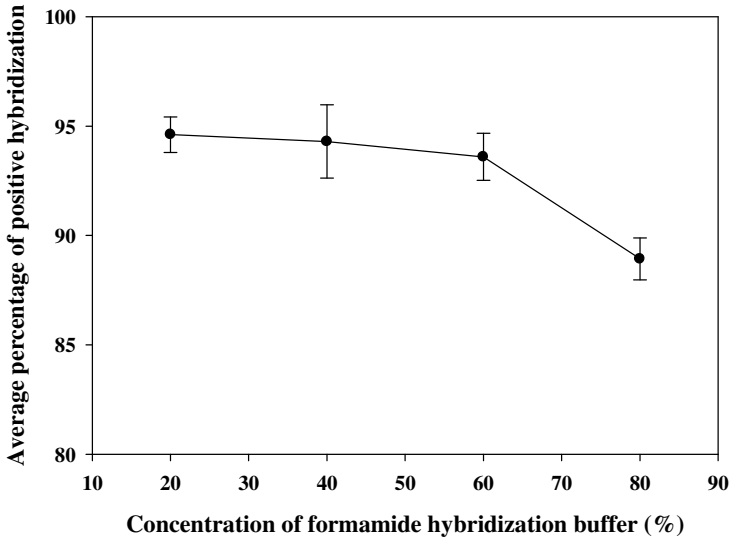


Figure 5-8: Average percentage of positive hybridization after applying CARD-FISH using the 18S SSU rRNA probe at different concentrations of formamide hybridization buffer (%) (error bars corresponds to standard deviation, n=3)

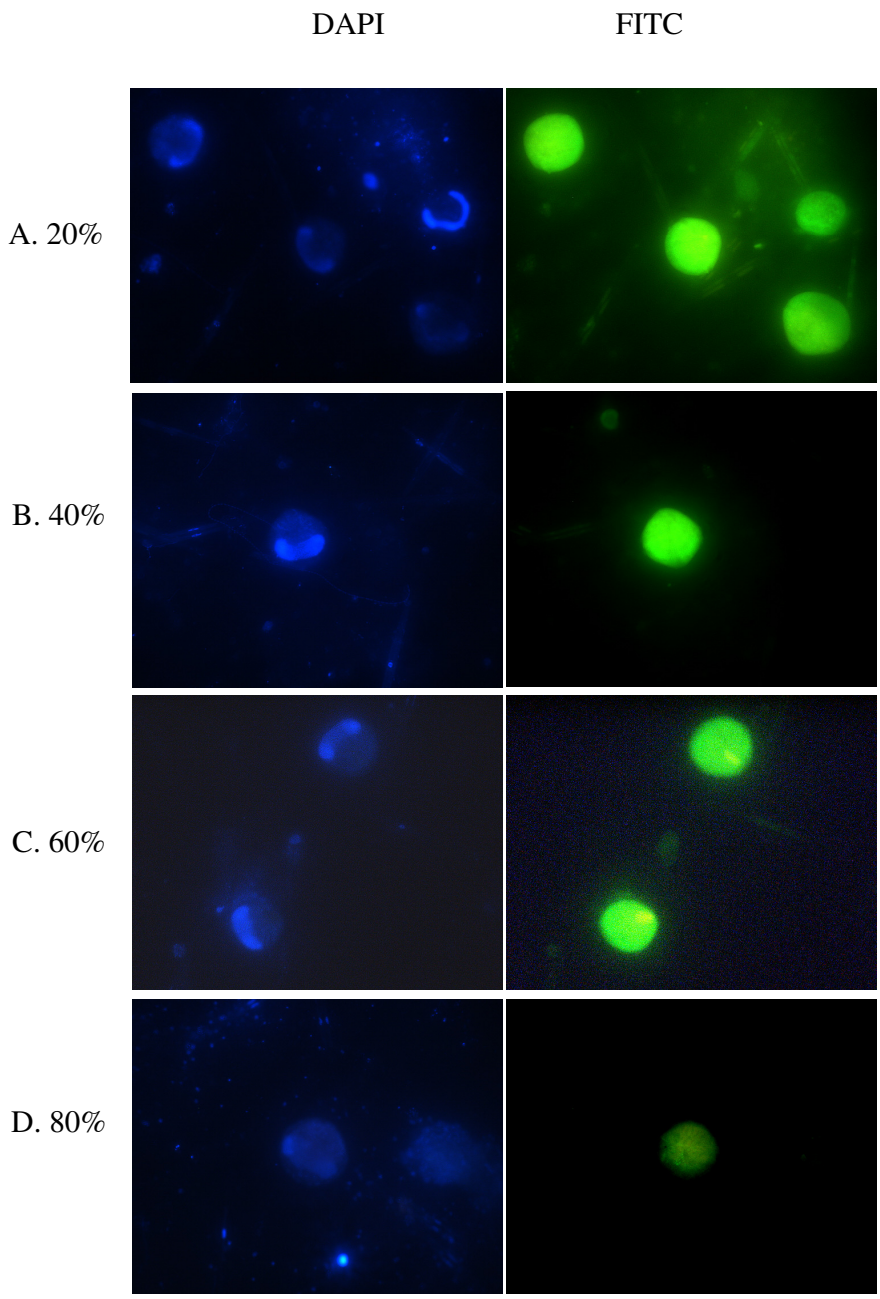


Figure 5-9: DAPI (left) and FITC (right) images of *B. granulosum* stained with the 18S SSU rRNA probe using different concentrations (%) of formamide hybridization buffer. A: 20%; B: 40%; C: 60%; D: 80%. (Total magnification of ×630).

Evaluation of CARD-FISH to detect B. granulosum *using flow cytometry*

Bysmatrum cells were obtained from a nutrient enriched seawater sample and CARD FISH applied after concentrating cells on filters, or in solution by centrifugation. Labelled cells were then analyzed using flow cytometry. The resulting two-parameter dot-plot of side scatter and fluorescence showed a well defined difference between the labelled cells that formed a clear cluster with higher fluorescence signal intensity (R3 region in Fig.5.10B) compared to the non-labelled cells (R2 in Fig.5.10A). The concentration of cells after applying CARD-FISH followed by centrifugation was higher than the concentration of cells after CARD-FISH on filters (data not shown). Gated region R1 in Fig. 5.10A and B represents the yellow-green beads of 0.5 µm diameter that were used as a flow cytometric internal standard.

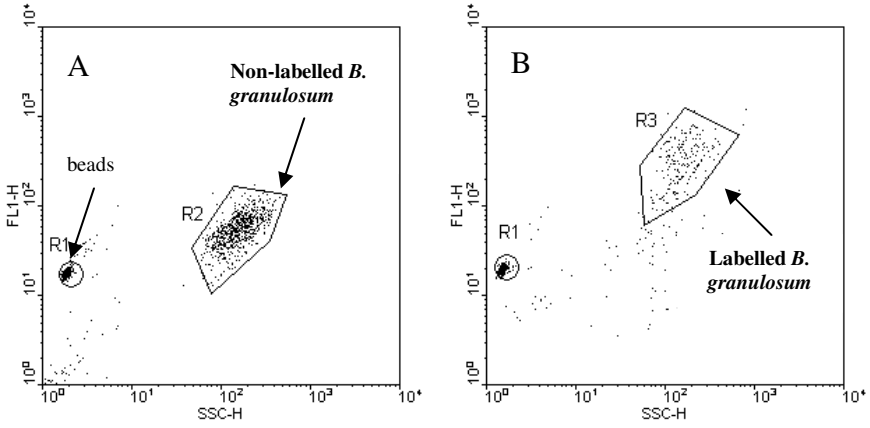


Figure 5-10: Scatter plots of flow cytometry data relating FITC-fluorescence (FL1-H) with side scatter (SSC-H). A: *B. granulosum* before applying CARD-FISH; B: *B. granulosum* after applying CARD-FISH using the 18S SSU rRNA probe.

5.3.7 *Applying CARD-FISH protocol to Lugol's fixed samples collected from Arad Bay, Bahrain*

Bysmatrum sp. cell abundance counted in Lugol's fixed samples using a light microscope and absolute number of positive hybridized cells after applying CARD-FISH using *B. granulosum* species-specific probe are shown in Table 5.5 and 5.7 respectively. A comparison of cell counts using the two methods (microscopic cell count and epifluorescence microscope positive hybridized cell count after applying CARD-FISH) is presented in Fig. 5.11 and 5.12. Time series changes in the abundance of *Bysmatrum sp*. during summer 2006 (water samples collected from April to November) are demonstrated in Fig. 5.11. The maximum concentration of *Bysmatrum sp.* was in early summer in April and May 2006 (1606 and 1037 cells mL^{-1}), following which cell numbers decreased during the hottest months of the year (June-October) with minimum concentration recorded in September (3 cells mL^{-1}) followed by an increase in cell densities (264 cell mL^{-1}) in November. Estimates of positive hybridized cells counted using an epifluorescence microscope after applying CARD-FISH was in good agreement with *Bysmatrum sp.* cell concentrations in 1 mL of Lugol's fixed field sample (Spearman Rank Order correlation, R= 0.99, $p< 0.05$, n= 14) as shown in Fig. 5.12. Fig. 5.13 shows DAPI and FITC epifluorescence images of *Bysmatrum sp.* in a Lugol's fixed seawater sample after applying CARD-FISH using the 18S SSU rRNA *B. granulosum* probe. Fluorescence signal intensity of positive hybridized cells was not negatively affected by long term fixation with Lugol's iodine solution.

Table 5.6: Average number of positive hybridized cells using the 18S SSU rDNA *B. granulosum* probe counted after applying CARD-FISH using an epifluorescence microscope.

Field sample	Date of collection	Average no. of postive hybridized cells in 1 mL (CARD-FISH) [±SD]
1	16.04.06	1395 [±5]
2	3.05.06	805 [±10]
3	3.06.06	75 [±11]
4	21.06.06	29 [±6]
5	28.06.06	8 [±2]
6	31.07.06	17[±2]
7	15.08.06	6 [±1]
8	3.09.06	1
9	19.09.06	1
10	5.10.06	1
11	1.11.06	150 [±5]
12	3.05.07	0
13	23.06.07	509 [±10]
15	30.04.08	42 [±3]

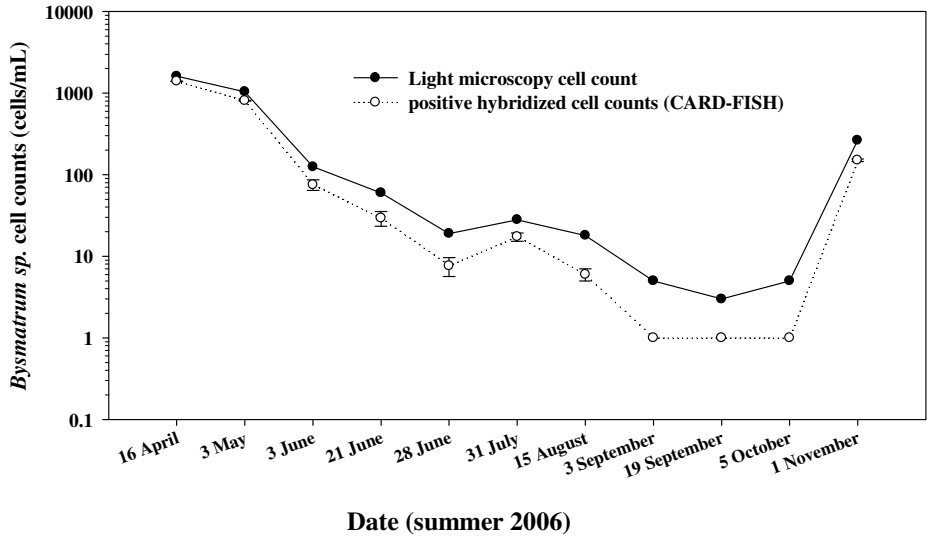


Figure 5-11: A plot showing time series changes in *Bysmatrum sp.* abundance revealed by light microscopy and CARD-FISH analysis on summer 2006 water samples. The x-axis shows the dates of sampling as indicated in Table 5.7; y-axis log scale.

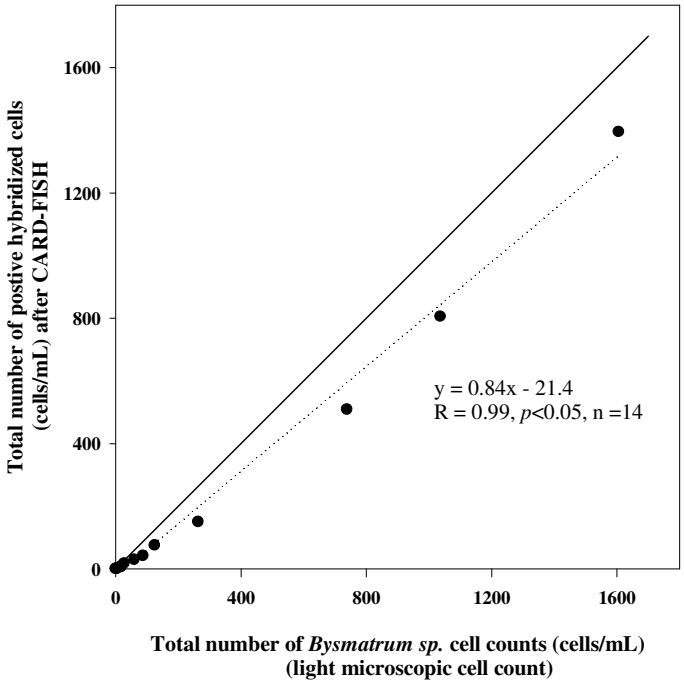


Figure 5-12: Correlation between absolute numbers of *Bysmatrum sp.* counted in Lugol's fixed samples and positive hybridized cells after applying CARD-FISH. (Straight line corresponds to a 1:1 relationship, dotted line corresponds to a fitted linear regression).

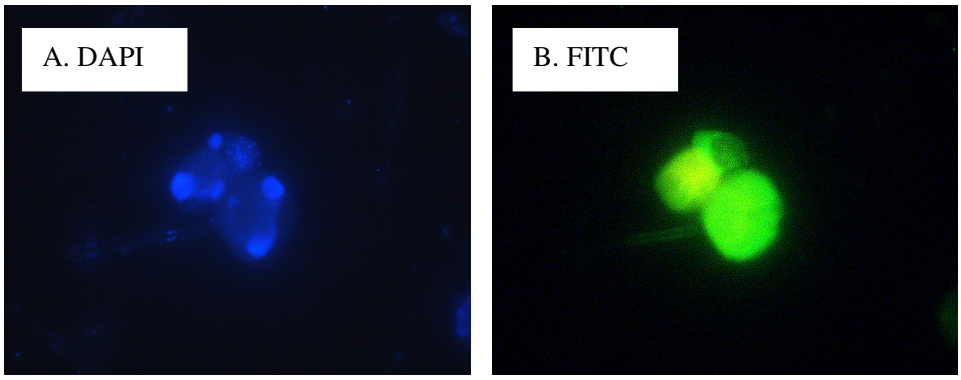


Figure 5-13: (A) DAPI and (B) FITC epifluorescence images of *Bysmatrum sp.* in Lugol's fixed seawater samples at 60% formamide hybridization buffer and labelled with HRP-*B. granulosum* probe. (Total magnification of ×630).

5.4 Discussion

5.4.1 *Biological and ecological characteristics of* Bysmatrum granulosum

The genus *Bysmatrum* is a tropical benthic scrippsielloid dinoflagellate and to date the following species have been reported: *B. arenicola* Horiguchi & Pienaar (Horiguchi and Pienaar 2000), *B. caponii* (Horiguchi & Pienaar) Faust & Steidinger and *B. subsalsum* (Ostenfeld) Faust & Steidinger (Faust and Steidinger 1998) cited in Ten-Hage et al. (2001). *B. granulosum* was first recorded by Ten-Hage et al. (2001) and isolated from sediment and coral samples from sites of La Rèunion Island (SW Indian Ocean) where water temperature ranged from 23.3 to 29 °C and salinity from 34.7 to 35.2. In this study, the isolated species was identified by G. Hansen to be *B. granulosum* and was isolated from a water sample collected from the surface in an enclosed muddy Bay in Bahrain. This isolated species was noticed to attach to the bottom of culture flasks in the Laboratory. Therefore, further examination was made of benthos (e.g. macroalgae) and sediments collected from the same area and revealed that this species is better described as bentho-planktonic due to its presence in the water column and attached to sediments or macrophytes. The water temperature ranged between 30 to 45 °C and salinity was 35 to 45 during sampling in Arad Bay.

Ten-Hage et al. (2001) described *B. granulosum* as an armoured dinoflagellate species of 40-50 µm in length and 40-46 µm in width. The cell dimensions of *B. granulosum* isolates measured in this study was within the same range (40-45 µm long and 35-40 µm wide). This species is a photosynthetic species containing many green-brown chloroplasts and the abundant plastids mask the inner cytoplasmic regions of the cells (Ten-Hage et al. 2001) (Fig. 5.3). Ten-Hage et al. (2001) recorded *B. granulosum* during a study of the benthic dinoflagellate diversity from the SW Indian Ocean, where many species are known to be toxic. The preliminary toxicity test performed in this study using brine shrimps incubated with *B. granulosum* suggested that this species is a potential harmful dinoflagellate and more toxicity tests should be conducted to determine the toxicity of the species and the toxins produced if present.

5.4.2 *Phylogenetic analysis of* B. granulosum

Phylogenetic analyses of several genes has been argued to be superior to single-gene analyses because more phylogenetic information can be produced, and because some taxa may have a fast evolutionary rate in one gene but not in another (Gontcharov et al. 2004). In this study, phylogenetic analyses of *Bysmatrum granulosum* were performed using the 18S rDNA sequences only due to the poorly resolved 28S LSU rDNA nucleotide sequence obtained. No phylogenetic analyses have been performed before for any of the three other species of *Bysmatrum* and no genetic information (i.e. 18S and 28S rDNA sequences) is available for those species. The present study is the first to perform a phylogenetic analysis of *B. granulosum* by using ARB software to show the evolutionary relationship of this species compared to other dinoflagellate species. The different reconstructions of phylogenies (maximum parsimony and maximum likelihood) obtained in this study were nearly identical, with bootstrap values calculated for parsimony tree. In the construction of the trees, *Paramecium spp*. were used as outgroup because molecular data indicate that the Ciliates and Dinoflagellates are sister taxa (Fast et al. 2002). Symbiodinium and Gymnodinium groups are the most distant species to *B. granulosum.* The two trees (Fig.5.4) show two main clusters: the first one includes the gonyaulacoid dinoflagellates *Gonyaulax*, *Ceratium*, *Pyrocystis* and *Alexandrium*. The second cluster is a large, mixed assemblage including gymnodinioid, peridinioid and prorocentroid species (named the GPP complex by Saunders et al. (1997)). Similar clustering patterns were achieved by Grzebyk et al. (1998).

Different species of *Amphidinium* were found to group together in this investigation and cluster strongly with *B. granulosum*. The long branch of *B. granulosum* and different species of *Amphidinium* reflect the large divergence of these two genera from other dinoflagellates. The intraspecific sequence variability was estimated by examining five different species of *Amphidinium*. There were only 0.7- 3.2 bp differences in the 18S SSU domains of these species. However, there was a minimum of 18.8 bp differences between *B. granulosum* and two species of *Amphidinium* (*A. massartii* and *A. carterae*) which indicates that *B. granulosum* is a different species.

The ancestral position of *Amphidinium* in Fig. 5.4 which lack armoured thecal plates, constitute evidence that the dinoflagellate ancestor may be athecate (Saldarriaga et al. 2004). *Amphidinium* branches early in a molecular tree based on the 18S SSU sequences (Saunders et al. 1997) and has received strong support as an early divergence (Zhang et al. 2007). Additionally, Grzebyk et al. (1998) constructed a phylogenetic tree with 18S rDNA sequences from unarmoured dinoflagellates, and *Amphidinium* was found to have an early diverging position. Similar findings were later obtained by Daugbjerg et al. (2000) and Jørgensen et al. (2004) using the 28S LSU rDNA sequences. It appears from all the collected data and the present study phylogeny that *Amphidinium* could be more primitive than *Bysmatrum*. It was always thought that the genus *Amphidinium* contains a polyphyletic assemblage of morphologically diverse groups (e.g. Daugbjerg et al. 2000, Saldarriaga et al. 2001). Nevertheless, in this study as well as others (e.g. Jørgensen et al. 2004) it was found that the different species of *Amphidinium* used to construct the sub-trees formed a monophyletic clade.

It was hypothesized previously that armoured dinoflagellates should be considered an evolutionary lineage distinct from unarmoured dinoflagellates (e.g. Taylor 1980, Grzebyk et al. 1998). Saldarriaga et al. (2004) and Zhang et al. (2007) demonstrated that the ancestral position of *Oxyrrhis* and *Amphidinium*, both lacking armoured thecal plates, constitutes evidence that the ancestor of dinoflagellates may be athecate. However, it was demonstrated in the two constructed trees (Fig. 5.4) that the thecate dinoflagellates cluster with the non-thecate species. Therefore, the phylogenetic analyses performed in this study agree with previous analysis that the presence of a theca could have been gained and lost many times in dinoflagellate evolution (e.g. Saldarriaga et al. 2001).

5.4.3 *18S SSU rDNA primers and HRP-oligonucleotide probe to identify and enumerate* B. granulosum

The 28S LSU rDNA region is the main target for designing species-specific probes because it contains a highly variable region (D1-D2 region) (Lenaers et al. 1989, 1991, Hansen et al.

2000). However, errors in sequencing and/or amplification produced poor nucleotide sequence of the 28S LSU rDNA gene (partial sequence) in *Bysmatrum* isolates in this study. The 18S SSU rDNA region was therefore used to design species specific primers and probes. Additionally, a wider range of 18S SSU rRNA sequences of different groups are available for sequence comparisons.

Different probes have been designed using ARB software and the primers designed using Primer 3 software according to the available database. The designed 18S SSU rDNA primers used in this study to detect *B. granulosum* were then checked in GenBank and using ARB software based on databases of 18S SSU rDNA sequences. Additionally, the 18S SSU rRNA probe selected for CARD-FISH experiments was checked for its *in situ* accessibility and found to belong to class II (there are 6 brightness classes I with the highest brightness and VI the lowest) (Behrens et al. 2003). To date, rDNA sequences of the different *Bysmatrum* species are not available for sequence comparisons.

Applying the designed 18S SSU rDNA *B. granulosum* primers to different algal cultures showed that the designed primers are specific and found to produce positive PCR products with the target species only. 60 ºC was found to be the optimum annealing temperature to discriminate between the target species and other non-target species. Under these conditions the primers were shown to have very high specificity and sensitivity and there was a consistency in the results when tested on target and non-target laboratory cultured species and field samples. The positive PCR products obtained by applying the designed primers to Lugol's fixed samples collected from Arad Bay, Bahrain revealed that the designed primers are good potential primers for a rapid and reliable detection of the target species in environmental water samples. As mentioned previously in Chapter 3, direct PCR did not produce positive PCR products with the Lugol's preserved environmental water samples even if the target species was present in relatively high concentration. This could have been due to the presence of DNA from other algal cells that may mask the target DNA. Therefore, nested PCR was used with natural water samples as direct PCR often resulted in faint bands or no bands on gels which give false negatives (i.e. to get positive

PCR products with the natural water samples, nested PCR had to be used which requires initial amplification with eukaryote 18S SSU rDNA primers).

Moreover, the CTAB DNA extraction method was found to be efficient in extracting DNA from low cell concentrations (minimum *Bysmatrum sp.* cell concentration was 3 cells per mL in a field sample 9 collected in September 2006). The sensitivity of this extraction method is suitable for monitoring *B. granulosum* at low cell densities prior to the occurrence of algal blooms. *Bysmatrum sp.* was not detected by light microscopy in one field sample collected during 2007 (3.05.07) and PCR was negative using the same water sample. It was found that there was a close agreement between microscopic detection of *Bysmatrum sp.* and PCR derived results.

The fluorescent *in situ* hybridization (FISH) method coupled with an amplification step (tyramide signal amplification, TSA) was shown in this part of the study to be successful in enumerating the potentially harmful algae *B. granulosum* both in cultures and in Lugol's fixed environmental water samples. The 18S SSU rRNA probe was used to enumerate *Bysmatrum sp.* in environmental water samples collected from Arad Bay, Bahrain during 2006, 2007 and 2008. It was found that the number of positive hybridized cells counted using the epifluorescence microscope after applying CARD-FISH corresponds well (R=0.99) with the absolute number of *Bysmatrum sp.* counted in Lugol's fixed samples using a light microscope. This reveals the importance of CARD-FISH analysis for the rapid enumeration of the potentially harmful species *B. granulosum* in seawater samples. However, the absolute number of positive hybridized cells was slightly lower compared to *Bysmatrum sp.* counted in Lugol's preserved water samples using a light microscope. This is due to the fact that when CARD-FISH was performed on cultured *B. granulosum* cells, 93.6% of the cells showed positive hybridization. 100% positive hybridization is hardly achievable because a small fraction of cultured cells might have a small number of ribosomes or they may be dead cells. Therefore, a slightly lower percentage of positive hybridization in a natural water sample containing a mixture of cells of different growth stages is expected. The 18S SSU rRNA probe designed in this study provided a good estimation of the abundance of *Bysmatrum sp.* in natural, mixed assemblages even with the

slight loss of cells. Additionally, it was demonstrated that CARD-FISH is able to detect changes in the target organism, *B. granulosum* abundance, when analysis is performed on field water samples collected over a period of time (April-November, 2006) (Fig. 5.10).

Mikulski et al. (2005) found that field samples fixed in modified saline ethanol with formalin following storage for 7 months at 4 °C showed no negative effects on the labeling intensity. In this study, it was demonstrated that field samples fixed with Lugol's iodine for several months showed good labeling intensity with no negative effect of the long fixation time. This will be useful if samples are to be stored for extended periods prior to CARD-FISH analysis.

Flow cytometry was capable of detecting *B. granulosum* stained cells with the 18S SSU rRNA HRP-labelled species specific probe after applying the CARD-FISH protocol. Positively hybridized cells were discriminated from non-labelled cells (non-target species) when the protocol was applied to a mixed population of microalgae in a seawater sample containing *B. granulosum*. Concentrating cells using centrifugation is optimal to retain high cell densities of the relatively large cells compared to filtration. Due to time limitation, flow cytometry analysis was not used to estimate the number of *B. granulosum* cells in environmental water samples collected from Arad Bay, Bahrain. However, quantifying *B. granulosum* using flow cytometry will be a challenging task because the relatively large size of the species would make it difficult to keep cells in solution during flow cytometry analysis (i.e. high sedimentation rate, therefore large cells tend to settle at the bottom of the tube during processing). Results obtained from experiments conducted using flow cytometry revealed a rapid method to detect *B. granulosum* in cultured isolates showing good fundamental sensitivity and resolution, evidently making it a potential choice for future work with field samples.

5.5 Conclusions

A potential harmful bentho-planktonic species identified as *B. granulosum* was isolated from an important ecological muddy coastal area of Bahrain in the Arabian Gulf. A unialgal culture of the isolated species was maintained and the 18S SSU and 28S LSU sequences were obtained. Phylogenetic analysis inferred from the 18S SSU rDNA sequences revealed the phylogenetic position of the species. rDNA primers and rRNA probe were designed based on the 18S SSU sequence to target *B. granulosum* and were shown to be capable of specifically labeling this species to the exclusion of a range of other algal species in laboratory cultures and in field samples of mixed populations of phytoplankton species. Future attempts should be made to design species-specific probes based on the 28S LSU rRNA region (assuming a large database of LSU rDNA sequences was available) because it contains both conserved and variable regions and has proved to be more useful for the discrimination between even different strains of the same species. The two molecular methods used in this study (PCR and CARD-FISH) are reliable and rapid techniques not only for the enumeration of *B. granulosum* in field samples but also monitoring the seasonal changes in the abundance of the target species.

CHAPTER 6

Conclusions and future work

6.1 Summary and main findings

In Chapter 2, results were presented of preliminary experiments conducted during the first stage of the present study which involved the evaluation of a fluorescent *in situ* hybridization (FISH) protocol that had been previously found successful for distinguishing between toxic and non-toxic species of the diatom genus *Pseudo-nitzschia* and different species of *Alexandrium* (Scholin et al. 1994b, 1996, Miller and Scholin 1996, Anderson et al. 1999). In this investigation, *Alexandrium tamarense* was chosen as a model organism and a FISH protocol was applied. However, the hybridization signal intensity was low and only a small fraction of the hybridized cultured cells showed positive hybridization. Cells with different growth stages would have different concentrations of ribosomes and this would eventually affect the hybridization results. As a consequence, other molecular methods were investigated.

The ambiguity in the differentiation between the two morphologically and genetically similar dinoflagellate species of *Karenia mikimotoi* and *Karenia brevis* was solved by using a polymerase chain reaction (PCR) identification method. This involved the design of two sets of primers specific to the two species based on nucleotide sequences obtained from amplifying DNA extracted from cultured cells. The primers were designed based on the 28S LSU rDNA gene sequence as it has been shown to be more useful for the discrimination between genetically similar species (Lenaers et al. 1989, 1991, Scholin et al. 1994a). Pingree et al. (1977) was one of the first to report the extent of the summer bloom of *Karenia mikimotoi* (then called *Gyrodinium aureolum*) in the western English Channel and a number of studies have since reported extensive summer blooms of *Karenia* in these waters (Garcia and Purdie 1994, Kelly-Gerreyn et al. 2004, Vanhoutte-Brunier et al. 2008). The identification of *Karenia* species in these studies were based on traditional light microscopy. One study by Llewellyn et al. (2005) however had suggested *Karenia brevis*

was present in the western English Channel. Therefore, one of the main aims of this study was to use definitive molecular techniques to determine the species of *Karenia* in this region using preserved samples that had been collected in the western English Channel over a number of years.

In Chapter 4 a successful molecular method that allows the quantification of whole cells of *Karenia mikimotoi* was optimized and applied to estimate the concentration of that species in culture and in water samples collected from the western English Channel. Two species-specific *K. mikimotoi* probes based on the 18S SSU rRNA gene and the 28S LSU rRNA gene were used and coupled with catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) method. An estimation of positively hybridized cells was performed using epifluorescence microscopy and flow cytometry. The 28S LSU probe was shown to be optimal for the quantification of *Karenia sp.* in seawater samples using epifluorescence microscopy. In contrast, for quick and rapid detection of *Karenia sp.* in seawater samples, the 18S SSU rRNA probe was shown to be superior using flow cytometry. Future efforts therefore should concentrate on combining the two probes (18S SSU and 28S LSU probe) for both the detection and quantification of *K. mikimotoi* in seawater samples. Quantification of cells using epifluorescence microscopy was shown to be more accurate and the details of hybridized cells could be easily seen. The separation of *K. mikimotoi* from its closest relative *K. brevis* by flow cytometry is very useful however, since it demonstrates the potential for using an automated detection system in conjunction with the species-specific rRNA probe without using the more labour intensive epifluorescence microscope.

In Chapter 5, an application of the earlier developed molecular methods was applied to detect and enumerate the potentially harmful bentho-planktonic dinoflagellate, *Bysmatrum granulosum,* isolated from an important ecological muddy coastal area of Bahrain in the Arabian Gulf. The 18S SSU and 28S LSU partial sequences of the isolated species were obtained and through phylogenetic analysis (from the 18S SSU rDNA sequences) the phylogenetic position of the species was inferred. The following step was to design rDNA primers and probe based on the 18S SSU rDNA gene sequence to target *B. granulosum* and

they were found to specifically label this species to the exclusion of a range of other algal species in laboratory cultures and in field samples of mixed population of phytoplankton species. At the time of conducting this part of the work, a large database of the 28S LSU rDNA sequences of different eukaryotes was not available. Additionally, the 28S LSU rDNA sequences obtained from *B. granulosum* was shown to have inconsistencies due to errors probably in the amplification processes. In the future, attempts should be made to produce a reliable 28S LSU rDNA nucleotide sequence from which a species-specific probe based on the 28S LSU rRNA region could be produced.

6.2 Application of molecular approaches to investigate harmful algal species

In coastal waters where harmful algae are known to occur any monitoring program for early warning detection of toxic algae requires a sensitive specific method for identification of the target species. Currently water samples that are routinely collected from coastal waters where shellfish are harvested or grown in aquaculture farms are investigated for the presence of harmful species by light microscopy (Fernández et al. 2004). This is a time consuming process that requires a significant level of expertise to identify accurately individual toxic species. The molecular methods employed in this investigation have shown that a specific and highly sensitive molecular probe can be developed and simply applied to seawater samples for the detection of specific toxic species of microalgae. To develop these probes, initially species causing harmful blooms should be isolated and maintained in unialgal cultures. Genetic information about the target species can be determined by extracting DNA from single isolated cells then amplifying either the 18S SSU or 28S LSU rDNA nucleotide sequences. A region of the nucleotide sequence that is specific for those organisms can be compared to a wide range of published sequences in GenBank or other available databases of different organisms to check for its specificity. Consequently, species-specific probes and primers can be designed, checked for specificity against a large number of nucleotide sequences of different algae using special software (e.g. ARB or GenBank) and then tested first with cultured cells to optimize the molecular technique for detection of a particular species. Once optimized the molecular protocols (e.g. PCR and CARD-FISH) could be applied to natural seawater samples and the target species

monitored both temporally and spatially. Figure 6.1 is a schematic diagram showing the different steps that could be followed for establishing a reliable and comprehensive technique to monitor a harmful algal species in conjunction with others like microscopic and optical methods that would give initial indication of the possible development of a harmful bloom event.

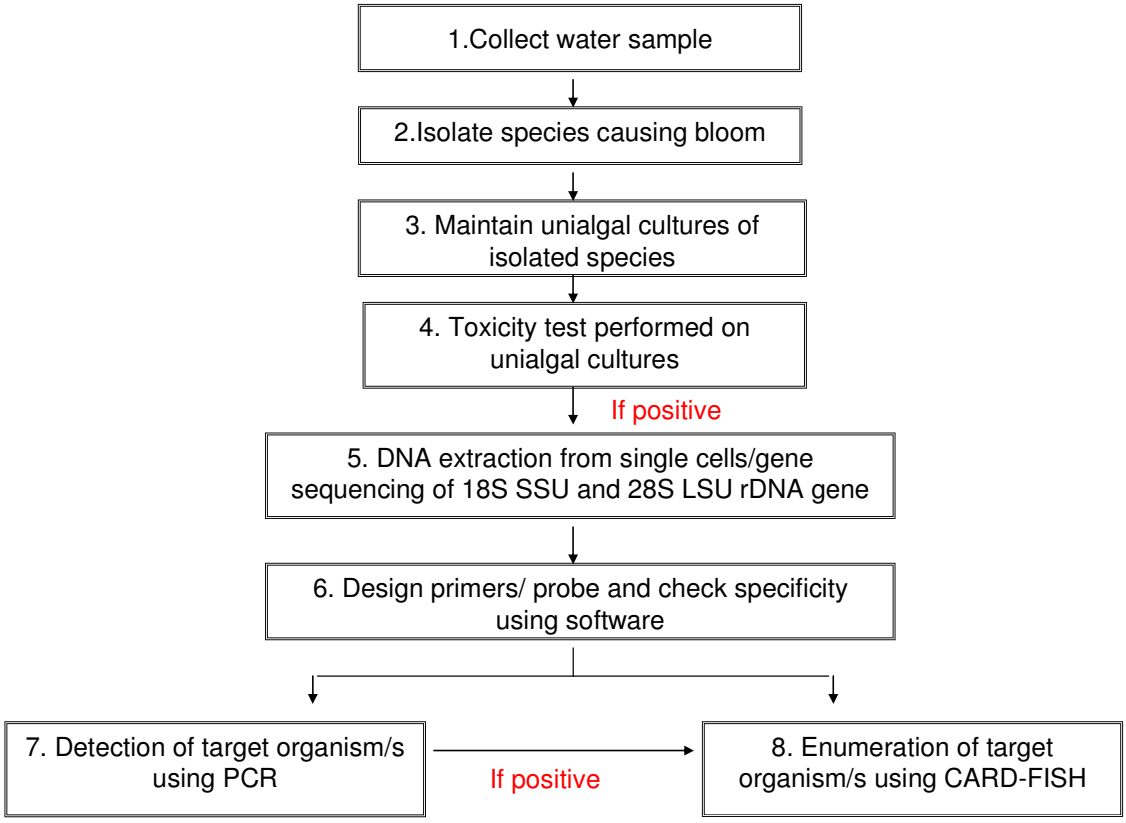


Figure 6-1: A schematic diagram illustrating the different steps to establish molecular protocols to monitor a harmful algal species.

In Chapter 5, catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) was used to monitor the changes in cell concentration of *Bysmatrum granulosum* in water samples collected from June to November 2006 in Bahrain coastal waters proving the useful application of this method.

The strategy mentioned above will be very efficient in detecting a single harmful species or when several harmful species are present concurrently in the same region. In the present study the two sets of primers designed based on the 28S LSU rDNA gene to target *Karenia mikimotoi* and *Karenia brevis* confirmed the presence of *Karenia mikimotoi* in waters of the western English Channel and the absence of any detectable *Karenia brevis* cells in these waters. In water samples collected from Bahrain coastal waters, the two sets of primers successfully detected both species using the PCR technique. The next step would be to apply the designed primers to water samples from the Gulf of Mexico where it has been suggested that both *Karenia mikimotoi* and *Karenia brevis* can be present at the same time (Haywood et al. 2007).

This investigation was one of the first to use the molecular technique catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) for both the detection and enumeration of a harmful algal species. The only other research that noted the use of this protocol to detect and enumerate harmful species was by Töbe et al. (2006) where they coupled this protocol with solid phase cytometry (SPC) to detect and enumerate *Prymnesium* and *Alexandrium* cells in cultures and environmental samples. The detection and enumeration of the labelled harmful species by the solid-phase cytometer (SPC) has the advantage of a direct combination of automated counting and epifluorescence microscopy, allowing the microscopic confirmation of each single cell detected. However, overestimation of cells might happen if false-positive particles are counted by the SPC, especially when a microscopic confirmation is not performed. Additionally, field samples might also contain a high quantity of fluorescing non-positive non-target particles. The high price of that machine and the additional costs of an epifluorescence microscope and other required equipment is a limiting factor. The manual validation of the ChemScan results is required to determine whether the system counted only positive target cells and this forms an additional time limiting factor.

To date, the factors that lead to bloom initiation are not well known for most harmful dinoflagellates, because most blooms are sporadic and detailed information on pre-bloom and initial conditions in most cases are not available (Walsh et al. 2001). Therefore, if the

above strategy is established in a specific geographic region, one can focus on studying the population dynamics of a particular harmful species that occurs in a defined geographic region and understand the different environmental factors that trigger the harmful species and lead to bloom initiation. This improved understanding will not necessarily prevent the formation of harmful bloom events; however it can significantly help in preventing human illnesses and other harmful/toxic effects caused by organisms that tend to bloom when the surrounding environmental conditions are right for bloom initiation.

6.3 Future work

There is no doubt that there is a world-wide increase in the occurrence of harmful dinoflagellate populations due to increased human activities (Hallegraeff 2004). Kim (2006) has summarized the different biological, chemical and physical strategies that have been used to control and mitigate several harmful algal species. However, the environmental effects of these mitigation and control methods should be minimized. More research should concentrate on investigating genes responsible for toxin synthesis. To date, the environmental factors that lead to toxin production, by harmful dinoflagellate species are generally poorly known, and the subject of considerable debate (Burkholder et al. 2005). As a consequence, as mentioned above investigating genes responsible for toxin production will be very efficient in controlling and or preventing the production of toxins or harmful substances produced by these toxic/ harmful organisms. Therefore, total genome sequencing is desirable for gene-expression studies. To date, there is no complete sequence available for a toxic eukaryotic algal species (Cembella and John 2006).

To establish a good harmful algal bloom monitoring program, it is more important to find a protocol that would prevent toxin production or the harmful substances produced by these harmful species when they reach high cell concentrations in the water column especially when they are triggered by the right environmental conditions that lead to toxin production. Because the toxin biosynthetic pathways and structures are unknown in most cases, bioassays may be used to target genes responsible for either toxicity expression or the toxin production.

6.4 Final remarks

The present research is considered a good contribution to the molecular methods developed and used to study and monitor harmful algal species. The molecular approach that has been developed in this study is characterized by its potential global application to study other harmful algal species. Catalyzed reporter deposition fluorescent *in situ* hybridization is a significant addition to the molecular techniques used to monitor harmful algal species because of its high sensitivity in detecting target cells at different growth stages and in different environmental conditions and would facilitate the understanding of the seasonal changes in cell concentrations of the harmful species.

APPENDIX 1

> *Karenia mikimotoi* 28S LSU partial sequence (strain PLY:561; 979 bp)

TTAAGCATATAAGTAAGCGGAGGATAAGAAACTAAATAGGATTCCCTCAGTAATGGCGAATGAACAGGGATAAGC
TCAGCATGGAAATTGGGGCCCTCGGCCTTGAATTGTAGTCTTGAGATGTGTTACCAACGGAGGCGCAGATGTAAGCC
TCTTGGAAAAGAGCGTCAGGGAGGGTGAGAGTCCCGTATGTCATCTGCAGTTCTCTGTGCACGGTGCATGTTCTAAGA
GTCACGTTCCTCGGGATTGGAGCGCAAATTGGGTGGTAAATTTCATCTAAAGCTAAATATTGGTTCGAGACCGATAGC
AAACAAGTACCATGAGGGAAAGGTGAAAAGGACTTTGAAAAGAGAGTTAAAAGTGCCTGAAATTGCTGAAAGGGAAG
CGAATGGAACCAGTTGTTCTTGGTGAGTATTGGTGTTGTCTAAAGTGATGGCTTGCCACTTCAACGCAAGTGTGGTGGCAG
GTTTTGATCTGGATGCGATACTGCTTCTCGCCTTGCATGTCAACGTCAGTTCATAATTGAGGAAAACTCTAAGGACATGGT
AATTCGCTTCCGAGTGACTGAATGTCCTCAGTTGAACTCATTTTTGAACTGCTCTCTGTGTGTCTGGTAGCACTGCTTCATG
TGCTTGCCTGCGATCTTCTGCTCTGCATGAAGGTTGTTGGTGCCAGGAGCATGTCCTTGACATTAGAACGATGACGAAATG
GTTTTATTCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATATGTGCAAGTTCACGGGCGGGAAAACCTGCTTGCG
CAATGAAAGTGACTGCTGGGGATTTTTGCACCAGCAACCGACCAATCAATTGTGAGAGGTTTTGAGTATGAGCATATCTGT
TACGACCCGAAAGATGGTGAACTATGCTTGAGAAGGGTGAAGTCAGGGGAAACTCTGATGGAGGCTCGTAGCGATACTG
ACGTGCAAATCGTTCGTCATACTTGGGTA

> *Karenia mikimotoi* 28S LSU partial sequence (strain PLY:507; 861 bp)

CTCAGCATGGAAATTGGGGCCCTCGGCCTTGAATTGTAGTCTTGAGATGTGTTACCAACGGAGGCGCAGATGTAAGCCTCT
TGGAAAAGAGCGTCAGGGAGGGTGAGAGTCCCGTATGTCATCTGCAGTTCTCTGTGCACGGTGCATGTTCTAAGAGTCAC
GTTCCTCGGGATTGGAGCGCAAATTGGGTGGTAAATTTCATCTAAAGCTAAATATTGGTTCGAGACCGATAGCAAACAA
GTACCATGAGGGAAAGGTGAAAAGGACTTTGAAAAGAGAGTTAAAAGTGCCTGAAATTGCTGAAAGGGAAGCGAATGGA
ACCAGTTGTTCTTGGTGAGTATTGGTGTTGTCTAAAGTGATGGCTTGCCACTTCAACGCAAGTGTGGTGGCAGGTTTTGAT
CTGGATGCGATACTGCTTCTCGCCTTGCATGTCAACGTCAGTTCATAATTGAGGAAAACTCTAAGGACATGGTAATTCGC
TTCCGAGTGACTGAATGTCCTCAGTTGAACTCATTTTTGAACTGCTCTCTGTGTGTCTGGTAGCACTGCTTCATGTGCTTGC
CTGCGATCTTCTGCTCTGCATGAAGGTTGTTGGTGCCAGGAGCATGTCCTTGACATTAGAACGATGACGAAATGGTTTTAT
TCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATATGTGCAAGTTCACGGGCGGGAAAACCTGCTTGCGCAAT
GAAAGTGACTGCTGGGATTTTTGCACCAGCAACCGACCAATCAATTGTGAGAGGTTTGAGTATGAGCATATCTGTTAGGA
CCCGAAAGATGGTGAACTATGCTTGAGAAGGGTGAAGTCAGGGGAAACTCTGATGGAGGCT

> *Karenia brevis* 28S LSU partial sequence (strain CCMP:2228; 752 bp)

TAGGATTCCCTCAGTAATGGCGAATGAACAGGGATAAGCTCAGCATGGAAATTGGGGCCTCCGGCCTTGAATTGTAGTCT
TGAGATGTGTTACCAACGGAGGCGCAGATGTAAGCCTCTTGGAAAAGAGCGTCAGGGAGGGTGAGAGTCCCGTATGTCAT
CTGCAGTTCTCTGTGCACGGTACATTTTCTAAGAGTCACGTTCCTCGGGATTGGAGCGCAAATTGGGTGGTAAATTTCATC
TAAAGCTAAATATTGGTTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGGTGAAAAGGACTTTGAAAAGAGAGTT
AAAAGTGCCTGAAATTGCTGAAAGGGAAGCGAATGGAACCAGTTGTTCTTGGCGAGTATTGGTGTTGTCTAAGGTGATAG
CTTGCCACTTCAACGCAAGTGTGGTGGCAGGTTTTGATCTGGATGCGATACTGCTTCTCGCCTTGCGTGTCAACGTCAGTT
CATAATTGAGGAAAACTCTAGGGACATGGTAATTTGCTTCCGGGCGACTGAATGTCCCCAGTTGAACTCATTTTTGAACTG
CTCACTGTGTGTCTGGTCGCACTGCTCCGTGTGCTTGCTTGCAATCTTCTGCTTGGCATGAAGGTTGCTAGTGCCAGGAGCA
TGTCCTTGACATAAGAACGATGACGAAATGGTTTTATTCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATATGTGC
AAGTTCACGGGCGGGAAAACCTGCTTG

> *Gymnodinium simplex* 28S LSU partial sequence (strain PLY:368; 871 bp)

CCCTTAAATTGTAATCTCAAGACGGTTTGCTAATGGGGGCGCAGGTGTAACTTGCTTGGAAAAGCGGATCATTGAGGGTG
AGAGTCCCCGTTTGTCATCTGGCAGTCCCCCGTGTACGGCATCCGCTCTACAAGTCGCGTTCCTCGGGATTGGAGCGTAAA
CTGGGTGGTAAATTTCATCTCAAGCTAAATATAGGTTCGAAACCGATAGCAAACAAGTCCCATGAGGGAAAGATGAAAAG
GACTTTGAAAAGAGAGTTAAAAGTGCCTGAACTTGCTGAAAGGAAAGCGGATGGAACCAGTCTTGCTTGGTAGAGATTGC
TTTGTACTAGGCTGATTCTGCGTGTCGTCAGCGCAAGCGTCGATGCGTTGTTTGATGCTGCCGTGCTGAGTGTTTCTTTGCC
TTGTTTGTCATCGACGGTGTGGCGATGAGGACCCCTCCTTGGGGATGGTAACTCCGATGCGTTGGTGTGAATACCCTTGGT
AGTACTCATCGCTATACTGCTACTCATTTGTGAGGCTGCGCTGCTGCGCGCCTCTGGCTGTGGCTTCACCGCTCGGTCATTG
GCTACGTGTGGCACCTTCCTTACATTCTTTTGGTGACTAAATGGTTTCATTCGACCCGTCTTGAAACACGGACCAAGGAGT
CTAACATATGTGCGAGTTCTTGGGTGGAGAAACCTACGTGCGCAATGAAAGTGATTTAGTGAGATTCTTGCATCACCAG
CCGACCGATCAATTGGGAGAGGTTTGAGCATGAGCATATCTGTTAGGACCCGAAAGATGGTGAACTATGCCTGGGAAGGG
TGAAGTCTGGGGAAACTCAGATGGAGGCTCGTAGCGATACTGACGTGCAAATCGTTCGTCATACC

> *Karlodinium veneficum* 28S LSU partial sequence (strain PLY:517, 734 bp)

GTAGTCTTTCGACGGCATTGCCAGCGGAGGCGCAGATGTAAGCCTCTTGGAAAAGAGCATCAATGAGGGTGAGAGTCCCG
TTTGTCATCTGCAGTCCTCTGTTTACGGTGTGCTTTCTAAGAGTCACGTTCCTTGGGATTGGAGCGCAAATTGGGTGGTAAA
TTTCATCTAAAGCTAAATATTGGTTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGGTGAAAAGGACTTTGAAAAG
AGAGTTAAAAGTGCCTGAAATTGCTGAAAGGGAAGCGAATGGAACCAGTCTTCTTGGTGAGATTGTTGTGCGCTATTGTG
ATTGCTTTCTGCTTCAACGCAAGTGTTGCAGTGGGTTTTGAGCATGACGCGCACTTTGTTTCTCACCTCGTGTGTCAACGTC
GGTTCAGATTTGAGGAAAACTCTAGGGACATGGTTAATTGCTCGCGAGTGATTGAATGTGCCTGGTAGAACTCATGTCTAA
ACTGATTTCCGCATGTCTGGTCGCAGTGTTCTCATTACCTGCGTCTGGGTTCGTGGCTTGTAGCTTCTGTTACTCGTCGCGG
GCTTCTGGGCCTGGTCGTGGAGCATCTCCCTGACATTAGTACGATGACAAAATGGTTTCATTCGACCCGTCTTGAAACACG
GACCAAGGAGTCTAACATATGTGCAAGTTCACGGGTGTTAAACCTGATTGCGCAATGAAAGTGACTGCTGGGATATTTGC
ACCAGC

APPENDIX 2

Bysmatrum granulosum 18S SSU and 28S LSU rDNA sequences.

>18S small subunit sequence (1699 bp)

CNTGCACATCTCAGTATAAGTCTCGGAGAAGATGAAGCTGTGAATGGCTCATTACAACAGTTATAACCTACGTGACAACC
TCTGGAAAAGGATAACTGTGGTAATTCTAGGGCTAATACCTGCACTTGCACTCAACTTCGGGGAAGAGTTGTGCTCATCGC
TCTCAGAACTATCTCAGGTACTACCTGGCTCCTCGGTGAGGCACGATGATGTTTCAAACTGCAAGATGCAAATTGGCAGTA
CGTCACCGAAGTCTCTGACCTATCAGCTTCCGAAGGTAGGGTATGGGCCTACTTTGGCAATGACGGGGTAACGGAGGATTA
GGGTTTGACTCCGGAGAGGGAGCCTGAGAAATGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCT
GACACAGGGAGGTAGTGACAAGAAATAACAATACAGGGCATTTCTGTCTTGTAATTGGAATGATTCTATTTTAGATCCGCT
GATGAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCATATGTTAAAGTTGTTG
CGGTTGAAAAGCTCGTAGTTGGACTTCTGCTGAGGGTTGCCAACCCGCCCTCTGGGCGTAGGTCTGGCTTGCCTCAGCACC
TTCTCGGAGAATGTAGATGCGCTTGACTGCGTGGTGCGCTATCTGAGGCTGTTACTTTGAGGAAATTAGAGTGCTTCAAGC
AGGTCATTGCCCTGAATACATTAGCATGGCATGACAAGTCAGAACCTCTTGTTCTTCTTTGTTGGTTTAAGAGCAGAGGGG
AGGGTGAATAGGAACAGTTGGGGGTATTCATATTTGACTGTCAGAGGTGAAATTCTTGGATTTGTGAAAGATGGACTACT
GCGAAAGCATCTGCCAGAGATGTCTTCATTGATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCCTAGT
CTTAGCCATAAACCATGCCAACTAGAGATTGGAGGTTGTCGCTCATGACCCCTTCAGCATCTTCCGAGAAATCAAAGTCTT
TGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGC
GGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACATAGTAAGGATTGACAGACTGACAGCTCTTTCTTGATT
CCATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGGTTTGTCTGGTTAATTCCGTGAACGAACGAGACCTCAACC
TGTTTAATAGTGACGCCTAACTCCGGTTAGACGGGCCACTTCTTAGAGGGACTTTGCGTGTCTAGCGCAAGGAAGTTTGAG
GCAATAACAGGTCTGTGATGCCCTTAGATGTCCTGGGCTGCACGCGCGCTACACTGATGCATTCAGCAAGTGCTGACCTTG
CCTGACAAGGCCGGGTAATCCTGTCAAGGTGCATCGTGATGGGGTCAGTCTATTGCAATTATTACTCTTTAACGAGGAATT
CCTAGTACGTGTGAGTCATCAGCTTGCGCTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAGT
GATCCGGTGAATAATTTGGACTGCTGATTTTTCGAGAGTCAAACCAGCACGAAGTTTGGTGAACCTTATCANTAGNGNAG
AGAANT

>28S large subunit partialsequence (871 bp)

GGCTGAAATTTAAGCATATAAGTAAGCGGAGGACCTGAAACTAAAGAGGAAGACCTCAGTAATGGCGAATGAACTGGGA
CACGCTCAGTGCTGGAATGGGGGTGATGAGCCTCTAACTGAGCTGCTGTGTCTTACAGCTTGCAAGAGCCTACTTTTGAGC
TTCTTGGAAAAGAGCGCCATCGAGGGTGAGAGTCCCTCCACATACCTAGAGTTCTTCTCCAATGGTGTACGTTCCTAGAGT
CGCGTTCTTTGGGACTGGAGCGCAATTGGGTGGTAAGCTGCATCTAGAGCTAATCAAACACTTGAGGCCAATAGCTGACA
AGTACTGTGAGGGAAAGATGAAAAGGACTTTGGAGAGAGAGCTAAAAGTGCCTGAAATCACTGGGGGTGAAGTGAATTA
AGCTGCTACTTTGGTGAGATTGCCCTGCAGAGTGTGGACTGGCTTCGACTCTATCTATGAGGTTGGAGCTGAGGTGCGCAG
CCTGCAGGGTGTGTCTCGCCAGAACGGTCACCAGTAGTTGCCTAGCGGGTACCTTGGTAGGTTGTGCGGTTGGCTGCATAG
CGAATTGTGCGCCTGATTTGACCTATTGGTGAACTGCTTGGAGACTCTATAAGTGGCTTGATTCGTCCCGTCTTGAAACAC
GGACCAAGGAGTCTAGCATGCGTGCAAGTGCTCTGGTGTGGAGAAACCTGACTGCAAAGTCAAGGGCGACTGCCAGACAT
TTTGCACTGGCAACCAACCAAGCAACGGGTCAAGGTTTGAGTGTGAGCACAGGTGCTAGGACCCGAAAGATGGTGAACTA
TGCCTGTGCATGGCAAACTCAGGGGAAACCCTGATGGAGGCTTCCAGCGCTACTGACGTGCTAAATCC

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