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MOLECULAR STUDIES OF KARENIA MIKIMOTOI (DINOPHYCEAE) FROM THE CELTIC SEA REGION

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By

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Abstract

K. mikimotoi has been classified under many names and has been mis-assigned to different species and genera in the North Atlantic and Pacific because of its morphological similarities to other Gymnodinoid species. It is now known to be widely distributed, but there remain unresolved questions about whether *K. mikimotoi* was introduced into the North Sea from Japanese waters, or whether it has always inhabited this region and been erroneously classified as *Gymnodinium* spp. or has been a part of the hidden flora prior to be recognised in a bloom off the Norwegian coast in 1966. To address questions about geographical genetic variation within *K. mikimotoi* and broader issues about its biogeography it was deemed important to develop a suitable diagnostic molecular marker that could then be used to monitor the presence/absence of different *K. mikimotoi* ecotypes over long time scales in European waters. This study showed that the partial rDNA LSU (D1-D2) was too conserved to separate the different strains of *K. mikimotoi,* while, the ITS region was better able to discriminate between the different strains. However, the *rbcL* gene was the most informative gene and contained sufficient substitutions to separate the different strains of *K. mikimotoi*. Specific PCR-primers were designed to amplify a variable region of the *rbc*L gene able to distinguish differences between *K. mikimotoi* isolates from the different regions. The innovative high resolution melting temperature (HRM) technique based on specific primer set allowed rapid discrimination of *K. mikimotoi* from distinct geographic localities (= sequence variants) that differed by only a single nucleotide. Moreover, this study used archival environmental samples collected from the Celtic Sea shelf-break region. The high resolution melting temperature assay successfully detected the European *K. mikimotoi* isolate within the south-western English Channel in a 1963 sample, which is prior to thefirst report of a *K. mikimotoi* bloom in Norwegian waters in 1966 and in the south-western English Channel in 1975 and in western Japan in 1965. HRM observations were further validated using clone libraries and sequencing. In summary, this data provided more information about the genotypes present over the analysed timescales, revealing that *K. mikimotoi* sub-species 2 (European and New Zealand strains) was present in south-western English Channel and south-west Ireland for over 47 years, with sub-species 1 (the Japanese isolate) being absent from all examined samples. This finding supports the hypothesis that *K. mikimotoi* isolates within Europe are not of Japanese origin and suggests that they are native species to the region.

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List of symbols and abbreviations

BLAST: Basic Local Alignment Search Tool.

***chl*-a:** Chlorophyll a**.**

CTD: Conductivity temperature depth.

CPR: Continuous Plankton Recorder.

CZCS : Coastal Zone Color Scanner.

DNA: Deoxyribonucleic Acid.

DP: Parsimony (dnapars) phylogenetic analysis.

GIS: Geographic Information Systems.

HAB: Harmful Algal bloom.

HRM: High resolution melting temperature.

ITS-region: Internal-transcribed Spacer.1, 5.8S gene and Internal-transcribed Spacer 2.

LSU rDNA: 28S Large subunit of the rRNA gene.

MB: Bayesian Analysis phylogenetic analysis using MrBayes program.

MODIS: Moderate Resolution Imaging Spectroradiometer.

NCBI: National Center for Biotechnology Information.

NJ: Distance matrix (Neighbor) phylogenetic analysis.

OTUs: Operational Taxonomic Units.

PCR: Polymerase Chain Reaction. Method to amplify a specific DNA.

***rbcL*:** Large subunit of RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase).

SeaWiFS: Sea-viewing Wide Field-of-view.

SST: Sea Surface Temperature.

TEM: Transmission Electron Microscopy.

Tm: Melting temperature of the target DNA region.

yr: year.

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Chapter 1: Introduction

1.1. Tree of Life

1.1.1. Eukaryotes

Eukaryota, Bacteria and Archaea make up the three domains of life - a classification system underpinned by DNA sequencing that was first proposed by Carl Woese in 1977 (reviewed in Woese et al. (1990)). Three decades later, Cavalier-Smith proposed the division of the Eukaryota domain into four highly diverse kingdoms of Animalia, Plantae, Protista and Fungi (Cavalier-Smith, 2004). Since then many attempts have been made to further resolve this domain (Keeling et al., 2005, Falkowski et al., 2004, Burki et al., 2007, Lane and Archibald, 2008, Minge et al., 2009, Parfrey et al., 2010, Adl et al., 2005, Keeling, 2004), with the more generally accepted being the separation of the eukaryotic lineage into six distinct monophyletic super-groups of related taxa or Kingdoms. These include the Excavata, Archaeplastida, Opisthokonta, Amoebozoa, Rhizaria and Chromaleveolata (Table 1.1, reviewed (Simpson and Roger, 2004)).

Some researchers have subsequently proposed the broad placement of those super groups into two extra mega clades, namely the Unikonts (Opisthokonta and Amoebozoa) and Bikonts (Excavata, Archaeplastida, Rhizaria and Chromaleveolata) (Stechmann and Cavalier-Smith, 2003, Richards and Cavalier-Smith, 2005), while others have argued for numerous rearrangements within these super groups (Burki et al., 2009, Burki et al., 2007, Burki et al., 2008, Cavalier-Smith, 2010). For the purposes of this study observations are set in the context the six super-group taxa as defined in Table 1.1.

Table 1. 1. A model of eukaryotic classification (Simpson and Roger, 2004).

Eukaryota super-groups	General composition
Excavata	Various flagellate protozoa
Archaeplastida (or plantae)	Land plants, green algae, red algae (Rhodophytes), and glaucophytes
Opisthokonta	Animals, fungi, choanoflagellates, etc.
Amoebozoa	Amoebae with broad pseudopods and slime moulds
Rhizaria	Foraminifera, Radiolaria, and various other amoeboid protozoa
Chromalveolata	Stramenopiles (or Heterokonta), Alveolata (Dinoflagellates, Apicomplexa and Ciliates) and Haptophyta, Cryptophyta (or cryptomonads)

1.1.2. Origins of eukaryotes

Numerous lines of evidence indicate that eukaryotic organelles (mitochondria and plastids) are of bacterial origin (Lynn, 1967, Delwiche and Palmer, 1997, Gray, 1989, Palmer, 2003, Keeling, 2004). There are many similarities between these organelles and prokaryotic cells: non-histone associated DNA; gene structure and sequence; ribosomal structure; method of division; the presence of the double membrane that surrounds them. Mitochondria are found in most eukaryotes, whereas the plastid exists mostly in photosynthentic organisms (plants and protists). Both types of organelle are hypothesized to have been derived following the endosymbiosis of bacteria, i.e. they are the ancestors of free-living bacteria that have been enslaved inside another cell. Mitochondria developed from the engulfment of a proteobacterium (Eubacteria) through a primary endosymbiotic event (Yang et al.,

1985, Gray et al., 2001, Gray and Doolittle, 1982, Gray, 1989, Gray, 1983, Andersson et al., 2003, Lynn, 1967), whereas the plastid originated from the primary endosymbiosis of a photosynthetic cyanobacterium followed, in some cases, by secondary or even tertiary endosymbiosic events (Gray, 1989, Gray, 1983, Keeling, 2004).

An endosymbiotic origin was also proposed for the nucleus, suggested to be derived from an ancient archaean that was engulfed by a bacterial-like cell (Martin, 2005), however, this is not generally accepted due to lack of cytological evidence. A viral eukaryogenesis hypothesis has also been proposed (Bell and John, 2001, Takemura, 2001) that postulates that the nucleus originated from a large double stranded viruses that infected a prokaryotic-like cell. This hypothesis found to resolve better many of the nuclear features and has been reviewed in Forterre (2006)**.**

1.1.3. Photosynthetic eukaryotes

Photosynthetic organisms can be found within the eukaryotic, prokaryotic and Archea lineages (Lake et al., 1985, Xiong et al., 2000). The blue-green algae, or cyanobacteria, are one of the Earth's more ancient life forms (Oberholster et al., 2004, Kasting and Siefert, 2002) and may have evolved 2.8 Gy ago (Cavalier-Smith, 2006): they were the first organisms to use water as an electron donor for photosynthesis and it was from within this group that the plastids of phototrophic euakaryotes were derived. The eukaryotic phototrophic organisms vary in size, colour and habitat. They fall within four of the six super-groups, namely the Excavata, Archaeplastida, Rhizaria and Chromaleveolata (Table 1.1). In the marine environment, algae (within Archaeplastida and Chromaleveolata) vary

in size. This can range from the microscopic unicellular planktonic organisms (microalgae), such as the picoplankton (< 3 µm) (Paerl, 1977, Waterbury et al., 1979, Sieburth et al., 1978) to large multicellular macroalgae, such as the giant kelps (reaching 60 m in length) (McHugh, 2003). The microalgae are ubiquitous and although most of them are marine, they also live in different freshwater habitats, such as lakes, ponds and streams, as well as on and in soil, rocks, ice, snow, plants and animals (as parasites) (Andersen, 1992). The large kelps can form forests underwater, with high biomass densities that provide the structural basis of these ecosystems (Mann, 1973, Graham et al., 2007, Santelices, 2007). Approximately 340,000 algal species have been identified, although it has been estimated that there might be in excess of 350,000 species worldwide (Brodie et al., 2007).

1.1.4. Dinoflagellates

The Dinophyceae (dinoflagellates) is a monophyletic group within Alveolata (super phylum or subgroup) that with the Chromista (Cyptophytes, Haptophytes and Stramenopiles) make up the super-group Chromalveolata (Table 1.1). The relationship between the members of the Chromalveolata is supported by morphological and molecular studies, including different molecular markers such as the ribosomal genes e.g., the commonly used SSU rDNA (Van de Peer and De Wachter, 1997, Parfrey et al., 2010, Saldarriaga et al., 2001) and LSU rDNA (Ali et al., 2001) and the plastid genes e.g., RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase) and photosystem genes (Yoon et al., 2004, Yoon et al., 2002b, Delwiche and Palmer, 1996, Valentin and Zetsche, 1990). On the other hand, the interrelationships among dinoflagellates in molecular phylogenies is still largely unresolved, especially in the deepest branches (e.g. (Daugbjerg et al., 2000, Murray et al., 2005, Hoppenrath and Leander, 2010).

The dinoflagellates are one of the larger flagellate groups, comprising around 2000 extant species and a similar number of extinct species (Taylor et al., 2008). Approximately 80% of these are found in the marine environment as free unicells, as colonies or in chains (Moestrup and Daugbjerg, 2007). About half of the described species are photosynthetic (Gaines and Elbrächter, 1987); the other half are heterotrophic (via osmotrophy and phagotrophy) (Moestrup and Daugbjerg, 2007). Stoecker (1999) reported that many dinoflagellate species are mixotrophic (combining both phototrophy and phagotrophy). The dinoflagellate nucleus (the dinokaryon) is unique (Spector, 1984, Bhaud et al., 2000, Rizzo, 1991, Dodge, 1965) in that it lacks nucleosomal histones (Rizzo, 1991), their chromosomes are always condensed and divide by a closed mitosis with an external spindle (Spector et al., 1981) and it contains a large amount of DNA per cell (3,000-215,000 Mb), i.e. more than most other eukaryotic cells (Spector, 1984): e.g., human cells which contain 2,900 Mb DNA. Some dinoflagellates, such as the noctilucoids and the parasitic blastodineans, alternate in their cycles between a dinokaryon and a more conventional, histone containing nucleus (Rizzo, 1991, Taylor et al., 2008).

Motile Dinoflagellate cells typically have two dissimilar flagella, which are either dinokont or desmokont. The dinokont cell, which is characteristic of most of the Dinoflagellates, have two flagella that arise from the ventral side with the first flagellum, the transverse flagellum, ribbon-like and encircling the cell in a semi-helical fashion, with the second whip-like flagellum the longitudinal flagellum, trailing behind the cell. In desmokont cells, such as the Prorocentrales, both flagella are inserted apically (Taylor, 1980, Taylor, 1987).
The Dinoflagellate cell wall, named the amphiesma, can be placed into one of two categories based on the presence or absence of cellulose plates in the amphiesmal vesicles (alveoli): cells with empty vesicles are designated as athecate or naked dinoflagellates,

while those cells with alveoli that contain close fitting cellulosic plates, one per vesicle, are known as thecate or armoured dinoflagellates. The patterns created by thecal plates (tabulation) play an important role in classical dinoflagellate taxonomy (Moestrup and Daugbjerg, 2007).

1.2. Harmful Algal Blooms (HABs)

Algal blooms are defined as the rapid growth or accumulation of one or more species leading to an increase in biomass for those species. These blooms can be benign or harmful, with the harmful form commonly referred to as Harmful Algal Blooms (HABs). The timing and location of HABs are tightly linked to climatic conditions. They are truly a global phenomena of increasing significance resulting in serious economic and public health impacts that have increased in frequency, intensity and geographical distribution over the last few decades (Smayda, 1990, Anderson and Garrison, 1997, Hallegraeff, 2003, Brand et al., 2012). This increase in significance has been attributed to different factors in different countries, but they are associated with increased nutrients in some regions of the world (Sellner et al., 2003). While some blooms were linked to this eutrophication (Anderson et al., 2002, Carstensen et al., 2007), others were due to anthropogenic influences (Smayda, 1990, Hallegraeff, 1993) including climatic change (Sellner et al., 2003). Increased awareness of HABs has encouraged extensive research programs that have subsequently lead to the development of many novel morphological and molecular monitoring techniques to detect any sudden HAB development and to assist in the identification of new HAB species. It is difficult, however, to quantify and classify HAB

outbreaks in order to document trends, since there are so many different types of blooms with so many different effects (Anderson, 1989).

Hallegraeff separated species that cause HABs into three groups. The first group contain non-toxic species that generally produce harmless water discolorations, but under exceptional conditions, their blooms can be so concentrated that they can cause indiscriminate kills of fish and invertebrates through oxygen depletion in the water column. Oxygen depletion can be due to high respiration by the algae (at night or at light intensities below the compensation point during the day), but more commonly is caused by bacterial respiration during the decay of the bloom. The second group are toxic species producing potent toxins that can find their way through the food chain to humans. This second type of the bloom affects the marine and fresh water biota directly, but can also have negative effects on human health either through fish or shellfish consumption, causing a variety of gastrointenstinal and neurological illnesses, or, in some cases, through water or respiratory exposure to aerolized toxins, e.g., aerolized brevitoxins. Presently, six different human syndromes are recognized as being caused by algal toxins accumulated in fin or shellfish: paralytic shellfish poisoning (PSP); diarrhoetic shellfish poisoning (DSP); amnesic shellfish poisoning (ASP); ciguateric fish poisoning (CFP); neurotoxic shellfish poisoning (NSP); Azaspirazid poisoning (AZP). Finally, the third group of species are non-toxic to humans but harmful to fish and invertebrates by damaging or clogging their gills. While the third type of HAB includes some algae that can seriously damage fish gills, either mechanically or through production of haemolytic substances, other algae kill fish through the production of extracellular neurotoxins. All three types of HAB can have a negative effect causing severe economic losses to aquaculture, fisheries and tourism operations, and can have major environmental and human health impacts (Hallegraeff, 2003).

Among 5,000 species of marine phytoplankton (Sournia et al., 1991), about 300 species are reported at times to form blooms with cell concentrations of several million per litre. They can all discolour the surface of the sea, while around 100 species have the capacity to produce potent toxins. Shellfish toxicity can occur at very low cell concentrations, i.e. <1000 cells L^{-1} for *Dinophysis* spp. The causative harmful and toxic microalgae are found within the Chromalveolates (dinoflagellates, diatoms, prymnesiophytes and raphidophytes) and Bacteria (mainly cyanobacteria). The Dinoflagellates have the highest recorded number of HAB species in the marine environment, with about 75-80 % of toxic marine microalgae belonging to the dinoflagellate group (Cembella, 2003).

1.3. *Karenia mikimotoi*

1.3.1. Classification of the genus Karenia

The genus *Karenia* G. Hansen and Moestrup 2000 is part of the Kingdom Chromalveolata, Phylum Dinoflagellata, class Dinophyceae and the family Kareniaceae. This genus includes unarmoured or naked dinoflagellates that lack cellulosic amphiesmal plates, and it belongs to the order Gymnodiniales Lankester 1885. It comprises the well-known toxic species *K. brevis* (Davis) Hansen and Moestrup 2000 and *K. mikimotoi* (Miyake and Kominami ex Oda) G. Hansen and Moestrup 2000. Species of *Karenia* were previously assigned to the genus *Gymnodinium* Stein 1878, but Daugbjerg et al. (2000) split the genus into four genera: *Gymnodinium* sensu stricto, *Akashiwo* G. Hansen and Moestrup, *Karlodinium* J. Larsen and *Karenia* G. Hansen and Moestrup, based on light and electron microscopy, nuclear-encoded LSU rDNA sequences, and pigment analyses. Members of the 'true' genus *Gymnodinium* are characterized by a certain ultrastructural feature, i.e. a horseshoe-shaped apical groove running in anticlockwise direction (Figure 1.1 A), which can only observed by transmission electron microscopy (TEM). The genus *Karenia* was created to accommodate gymnodinoid species possessing a straight apical groove (Figure 1.1 B-C) together with fucoxanthin derivatives as accessory photosynthetic pigments (Hansen et al., 2000): Biecheler (1934) and Takayama (1985) had observed the apical groove morphology previously. The genus *Karenia* is named after Karen Steidinger in recognition of her many contributions to dinoflagellate research (Daugbjerg et al., 2000). Twelve species of this genus were recently identified (Table 1.2), 10 of them have been recorded in the IOC-UNESCO taxonomic reference list of harmful micro-algae (Hansen and Moestrup, 2011) as toxic species.

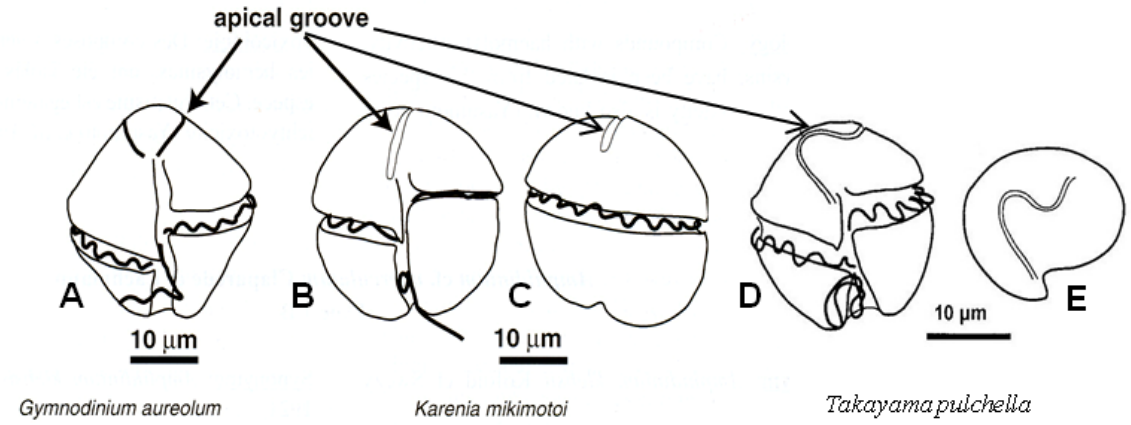

Figure 1. 1. Naked dinoflagellates classified according to type of apical groove. Counter-clockwise hourse-shoe shaped apical groove in the ventral view of *Gymnodinium aureolum* (A). Straight apical groove in the ventral view of *Karenia mikimotoi* (B). Extention of the straight apical groove in the dorsal view of *K. mikimotoi* (C). Sigmoid apical groove in the ventral view of *Takayama pulchella* (D) Sigmoid apical groove in the apical view of *T. pulchella* (E). Taken from Hansen et al. (2000) and Daugbjerg et al. (2000).

Table 1. 2. The twelve recognised species of *Karenia*.

***Karenia* species**	**Type Locality**	**Toxin**	**Harmful effects**
Karenia asterichroma de Salas, Bolch and Hallegraeff 2004	Tasmania, Australia	_	Associated with salmonid mass mortality although its ichthyotoxicity is not yet confirmed (de Salas et al., 2004a)
K. bicuneiformis Botes, Sym and Pitcher 2003*	Gordon's Bay, South Africa	_	Not associated with harmful events (Botes et al., 2003), synonym *K. bidigitata* Haywood and Steidinger 2004 from New Zealand, found to be toxic in culture (Haywood et al., 2004)
K. brevis (Davis) G. Hansen and Moestrup 2000*	Florida, Gulf of Mexico, USA	Brevetoxins	Marine animal mortalities, fish kills, NSP, respiratory irritation and asthma-like symptoms in humans (Landsberg, 2002)
K. brevisulcata (Chang) G. Hansen and Moestrup 2000*	Wellington Harbour, New Zealand	_	Associated with extensive fish kills (Chang, 1999), animal and plant mortalities, human respiratory distress, eye and skin irritations
K. concordia Chang and Ryan 2004*	North-east coast of North Island, New Zealand (Hauraki Gulf)	Brevetoxins	Associated with toxic outbreaks causing abalone and fish kills, Believed to cause NSP and respiration distress in humans (Chang, 2011, Chang and Ryan, 2004)
K. cristata Botes, Sym and Pitcher 2003*	Walker Bay, South Africa	_	Eye, nose, throat and skin irritations in humans as well as extensive mortality of sub-and intertidal fauna. Found to produce toxin in culture (Botes et al., 2003)
K. digitata Yang, Takayama, Matsuoka and Hodgkiss 2000*	Hong Kong and Japan	_	Associated with extensive fish fills (Yang et al., 2000)
K. longicanalis Z.B. Yang, I.J. Hodgkiss & G. Hansen	Hong Kong	_	Not associated with harmful effects (Yang et al., 2001)
K. mikimotoi (Miyake and Kominami ex Oda) G. Hansen and Moestrup 2000*	Gokasho Bay, Japan	Gymnocin-A, B^{+}	Associated with toxic outbreaks causing fish and invertebrate mortality (Taylor et al., 2003, Tangen, 1977, Gentien and Arzul, 1990, Landsberg, 2002, Takayama and Adachi, 1984), Found to produce toxins in Japanese cultures (Satake, 2006, Satake et al., 2002, Tanaka et al., 2005).
K. papilionacea Haywood and Steidinger 2004*	Hawke's Bay, New Zealand	Brevetoxins	Not associated with harmful events. Culture material found to produce toxin. Described and so far reported only from New Zealand (Haywood et al., 2004)
K. selliformis Haywood, Steidinger and Mackenzie 2004* (NZ and KW)	Foveaux Strait, New Zealand	Gymnodimine	Associated with toxic outbreaks causing fish kills in New Zealand (Haywood et al., 2004) and in Kuwait (Miles et al., 2003).
K. umbella de Salas, Bolch and Hallegraeff 2004*	Taranna,Tasmania, Australia	_	Fish kills only in Tasmania, killing rainbow trout and salmon (de Salas et al., 2004b)

* = Reported as a toxic micro-alga by the IOC (Moestrup, 2011); _ = unknown.

1.3.2. Historical Records

Karenia mikimotoi (Miyake and Kominami ex Oda) G. Hansen and Moestrup 2000 was previously known under different names in Pacific and European waters. The Japanese strain was first described by Oda (1935) as *Gymnodinium mikimotoi* from the samples of the red tide that occurred in the winter of 1933 at Ago Bay and the adjacent Gokasho Bays, Mie Prefecture, central Japan. In 1965, an exceptional red tide of a gymnodinioid dinoflagellate caused serious damage to fisheries in Omuru Bay, Nagasaki Prefecture. This was followed by mass mortality among fish in several areas of western Japan. The causative species was tentatively named by various investigators in different areas of Japan as *Gymnodinium* sp., *G*. sp. 1 and *G*. type-'65 (Fukuyo et al., 2003). In 1984, Takayama and Adachi (1984) described the species that caused a red tide in Nagasaki as *G. nagasakiense,* referring to the locality that suffered from this event. They differentiated between *G. mikimotoi* and *G. nagasakiense* Takayama and Adachi 1984 based on cell shape; *G. nagasakiense* was much rounder than *Gymnodinium mikimotoi* (Takayama and Adachi, 1984).

The Pacific *Gymnodinium mikimotoi* Miyake et Kominami ex Oda 1935 and the European *Gyrodinium* cf. *aureolum* Hulbert 1957 are morphologically similar resulting in a state of taxonomic turmoil for over 20 years (Takayama et al., 1998). They are generally regarded as conspecific, although genetic differences between the two populations do exist (Partensky et al., 1988). There were two distinct morphotypes described using the same species name *Gyrodinium aureolum* in USA (Hulbert, 1957), Europe and New Zealand. Neither red tides nor mass mortalities have been associated with this North American morphotype (Figure 1.1A). Conversely, an exceptional red tide occurred in coastal waters of southern Norway in 1966, accompanied by the death of sea trout (Braarud and Heimdal,

1970). The causative organism was first identified as *Gyrodinium aureolum*, a species that since then has become one of the most commonly reported to bloom in North East Atlantic waters.

A comparative study by Hansen et al. (2000) based on light and electron microscopy, nuclear-encoded LSU rDNA sequences, and pigment analysis, has shown that the European isolates formerly identified as *Gyrodinium aureolum*, *Gyrodinium* cf. *aureolum*, or *Gymnodinium* cf. *nagasakiense*, are conspecific with the Japanese *Gymnodinium mikimotoi*, *G*. sp., *G*. sp. 1, *G*. type-'65 and *G. nagasakiense*. At the same time, they were all transferred to the new genus name *Karenia* (section 1.3.1) (Daugbjerg et al., 2000) and species *mikimotoi* due to the first Japanese description of this morphology in 1935. Similarly, a New Zealand strain which was also misidentified as *Gyrodinium* cf. *aureolum* in 1981, was found to be conspecific with the Japanese and European strains based on morphological and molecular studies (Haywood et al., 2004). The original American *Gyrodinium aureolum* (reclassified as *Gymnodinium aureolum* (Hulbert) G. Hansen and Moestrup 2000), however, was found to be a different morphotype than those species that bloom in Europe and New Zealand.

Despite this reclassification, until very recently *K. mikimotoi* was still being confused with the non-toxic species *Gymnodinium aureolum* (E. M. Hulburt) G. Hansen 2000 (Hansen and Moestrup, 2011), e.g. in New Zealand (Haywood et al., 2007). In addition, morphological similarities between *K. mikimotoi* and other dinoflagellates have been reported. For example, *Takayama pulchella* (Larsen) de Salas, Bolch et Hallegraeff 2003 (formerly *Gymnodinium pulchellum* Larsen 1994, Figure 1.1 D and E) has a sigmoid apical groove (Larsen and Nguyen-Ngoc, 2004), while *Karenia brevis*, has been misidentified as *K. mikimotoi*, *K. papilionacea*, *K. brevisulcata*, *Gymnodinium aureolum* and other related

species (Steidinger et al., 2008): all are of a similar size and shape, and they are found to co-occur within single blooms.

Ultimately, extreme care should be exercised when attempting to identify these species. For example, two strains of *Karenia* sp. from Chile and Tunisia were identified as *K.* cf. *mikimotoi* based on morphological features by light microscopy only, later, however, they were confirmed to be *K. selliformis* on the basis of molecular characters and a more in depth study of their morphology (Haywood et al., 2007, Guillou et al., 2002).

1.3.3. Morphological Characteristics

K. mikimotoi cells swim using a pair of flagella; one of which is ribbon-like and wavy and called the transverse flagellum, while the other is whip-like and extends towards the posterior and is called the longitudinal flagellum (Figure 1.2 A, C and D). The cell outline is variable, ovate to almost round, flattened dorso-ventrally. The average cell length is 23.6 – 35.1 µm, with a range of 18.9 – 41.2 µm, and an average cell width of 19.4 – 30.6, µm, with a range of 15.1 – 44.3 µm (Hansen et al., 2000). The epicone is conical or hemispherical, whereas the hypocone is hemispherical, with a distinct antapical notch caused by the sulcus. The sulcus extends from the antapex to immediately above the cingulum. The cingulum encircles the equatorial part of the cell with the displacement of the proximal and distal ends smaller than 20% of the total cell length. A straight apical groove runs from near the sulcus across the apex (apical part) and a short distance down on the dorsal side of the cell. The nucleus is mainly located in the left side of the hypocone. There are several more or less oval chloroplasts, each with a conspicuous pyrenoid (Taylor et al., 2003, Hansen et al., 2000, Takayama and Adachi, 1984).

A more detailed morphological study was carried out by Hansen et al. (2000) on *K. mikimotoi* strains isolated from five different geographical areas (Japan, Denmark, Norway, Australia and England) and the American *Gymnodinium aureolum* in an attempt to resolve historical taxonomical difficulties (section 1.3.2). This investigation agreed with the general description above of *K. mikimotoi*, except that the location and the shape of the nucleus varied between strains: the nucleus in the Japanese and Norwegian strains is elongated and in the left lobe of the hypocone; the nucleus of the Australian strain is rounded and elongated and also in the left lobe of hypocone except in a few cells where it is located in the epicone; the Danish isolate has its nucleus extended to the right-side, but a 'normal' nucleus was also present in some cells; for the English isolate, in a few cells the nucleus is situated in either the left-side of the hypocone or the epicone or it occupies most of the dorsal part of the cell (Figure 1.2 E-H).

The number, shape, and colour of the plastids were also variable, both within and between *K. mikimotoi* strains. The similarity in pyrenoid structure was confirmed by TEM of Japanese and Norwegian strains. The colour of plastids was somewhat variable between strains. It appeared yellow with a greenish tinge in the Japanese strain and yellow-brown or almost orange in the Norwegian strain. The colour in the other strains was intermediate between the Japanese and Norwegian strains (Hansen et al., 2000). In addition, the position and shape of the nucleus in the American *G. aureolum* was different, i.e. found in the epicone, and the apical groove (counterclockwise horseshoe-shaped) than in isolates of *K. mikimotoi*: on this basis *Gyrodinium aureolum* was reclassified within the genus *Gymnodinium*.

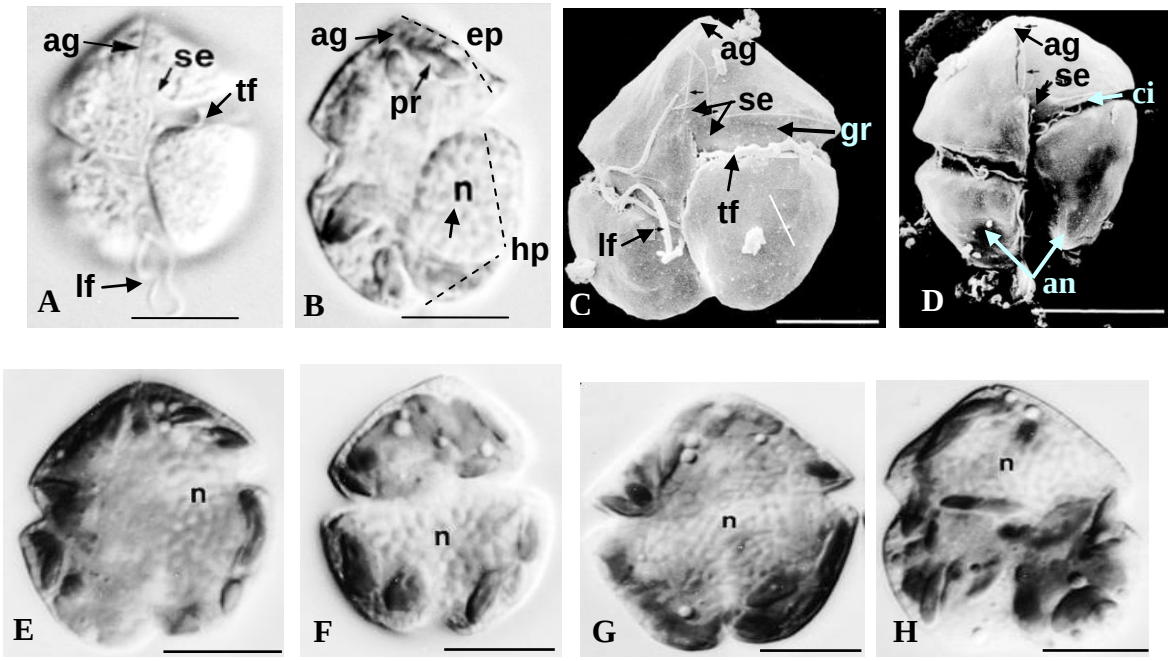

Figure 1. 2. Light micrographs (A-B, E-H) and Scanning electron micrographs (C-D) of *Karenia mikimotoi*. Notice the epicone (ep), hypocone (hp), apical part (ag), apical groove (ag), antapical part (an), sulcus (se), girdle (gr), transverse flagella (tf), longitudinal flagella (lf), pyrenoid (pr), cingulum (ci) and nucleus (n). High and low focus of the ventral view of an Australian isolate showing the rounded nucleus (n) in left lobe of hypocone (A-B). C-D shows the Japanese and Norwegian isolates, respectively. English isolate (E-H): notice the position of the nucleus in the left side of the cell (E), in the hypocone (F), in the epicone (G), occupying most of the dorsal part of the cell (H). Scale bar =10 µm. Taken from Hansen et al. (2000).

1.3.4. Life Cycle

Dinoflagellates generally multiply asexually by binary fission, but the manner of individual cell growth and cell division varies between Orders. In the Order Gymnodiniales, cell division is termed "oblique binary fission" because it occurs along a line starting at the middle of the left shoulder of the epicone (Figure 1.3), passing the flagellar pore, and reaching the middle of the right shoulder of the hypocone (Fukuyo et

al., 2003). No change in morphological characteristics, other than size, occurs during individual cell growth.

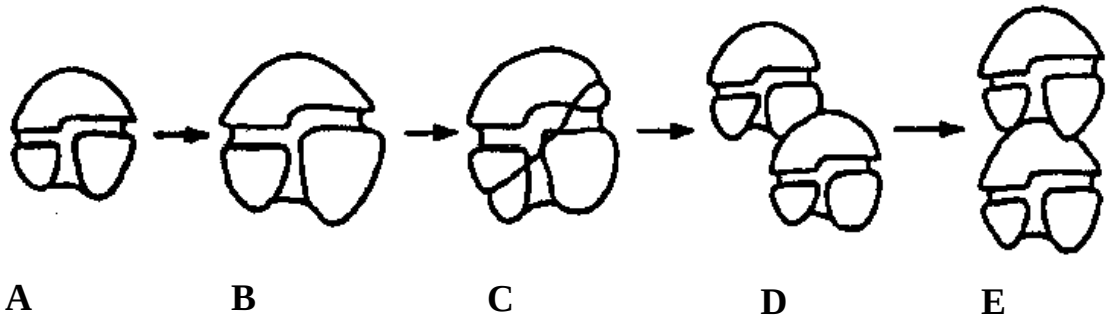

Figure 1. 3. Schematic process of asexual cell division in the Gymnodiniales. (A) vegetative cell, (B) enlargement (growing), (C) dividing cell, (D) two newly divided cells, (E) forming chain in most of the group species (Fukuyo et al., 2003).

K. mikimotoi has been described both in the field and *in vitro t*o vary in size while in the vegetative phase (Gentien, 1998). Both small and large cells (body length = 16-26.5 µm and 25.5-37 µm, respectively) have been described for this species (Partensky and Vaulot, 1989). Comparison of cultured strains of *K. mikimotoi* from the European and Japanese waters found the large cells (standard size) were typical in both strains. The Japanese strains, however, did not show any subpopulation of reduced size while the European strains showed both the small and large cells: the production of two different sized subpopulations was also recorded for the New Zealand strain (Chang, 1996).

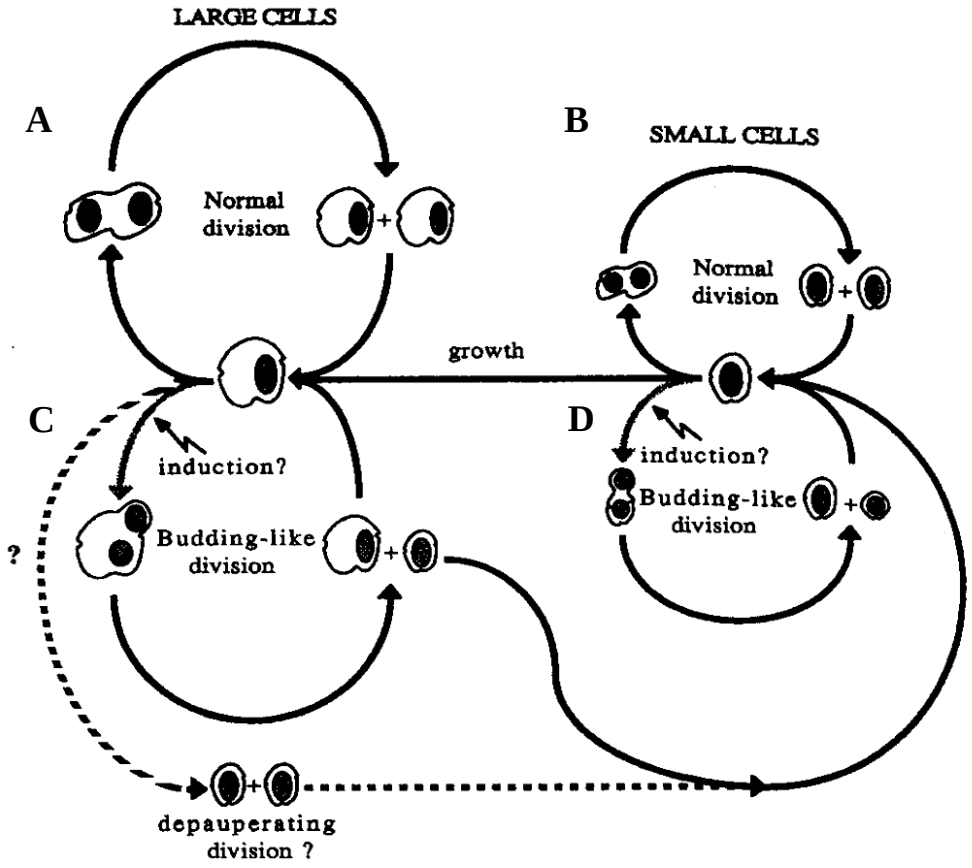

Figure 1. 4. Typical and atypical asexual division modes in large and small cell types of *Karenia mikimotoi*. Typical (normal) asexual forms of division, which give rise to two cells of similar sizes, are shown in A & B. Atypical (budding or depauperating) asexual forms of division, which give rise to two cells of unequal sizes are shown in C & D. Large and small cells (from atypical division) can either as the result of asymmetric division giving one cell of similar size to the mother size and one smaller cell, or two smaller cells can arise from the mother cell via depauperating division (the dashed arrow represents a possible but not observed division process). Atypical division modes are likely to require induction (Budding-like divisions/bended arrows). Taken from Partensky and Vaulot (1989).

The range of cell size in the European species (16-37 µm long, 13-36 µm wide) was greater than in the Japanese strains (21-34 µm long, 16-29 µm wide). Each cell type was able to divide asexually and had its own growth dynamics. They mostly represent the typical forms of cell division (Figure 1.4 A and B), which resulted in two cells of similar size, but a few examples of unequal divisions have been observed. The large cell generated a small cell by a typical budding-like division, whereas small cells generated large cells by simple enlargement of the cell body when they ceased to divide (Partensky and Vaulot, 1989).

The full life cycle of *K. mikimotoi* has not yet been fully elucidated, as the cyst stages (if any) have not been observed (Hansen et al., 2000). In addition, the conditions inducing cell size differentiation have yet to be defined (Raine, 2002). Sexual reproduction has been documented in many dinoflagellates species, but in only few species has the complete life cycle been described in detail (Fukuyo et al., 2003).

Gymnodinium nolleri Ellegaard and Moestrup 1999 is described here as an example of the 'typical' sexual life cycle for the gymnodinoid dinoflagellates (Figueroa and Bravo, 2005). The cycle starts with the fusion of gametes (Figure 1.5 A) which are either equal-sized fusing pairs (isogamy) or unequal-sized (anisogamy), though isogamy is more common. Fusing gametes combine either equatorially or with the girdles more or less perpendicular to each other. In both cases, the point of initial contact is the sulcal region. These paired cells can persist for up to eight days. Approximately five days after fusing, large, motile, heavily pigmented cells with dual trailing flagella (planozygotes) can be observed (Figure 1.5 B). Transformation of the planozygote into a hypnozygote (resting cyst) (Figure 1.5 C) involves loss of motility, shrinkage of cell contents, and the development of a thick wall within the outer membrane of the planozygote. The hypnozygotes have a dormancy period

of 33 ± 7 days. Cyst germination (Figure 1.5 D) begins by the drawing away of the protoplast from the cyst wall. The cyst wall ruptures and a single germling cell emerges inside a clear membrane formed by the inner cyst wall. The germling rotates inside this membrane for 5-20 min until the rupture of the bag finally releases it. The resulting cell is characterised by a large size (41-63 µm long, 31-40 µm wide) ovoid shape with a double longitudinal flagellum. Within 24 h to four days the planomeiocyte divides. This division is preceded by the striation and widening of the cell base that lasts for up to three days. Oblique binary fission (Figure 1.5 E) results in a two-cell chain with the apparent morphology of vegetative cells, though they can usually be formed by larger cells that subsequently separated in two individual cells (Figure 1.5 F). After the completion of gamete fusion, the planozygote can either follow the general route as described above, or divide within 4-7 days by binary fission giving rise to two individual cells (Figure 1.5).

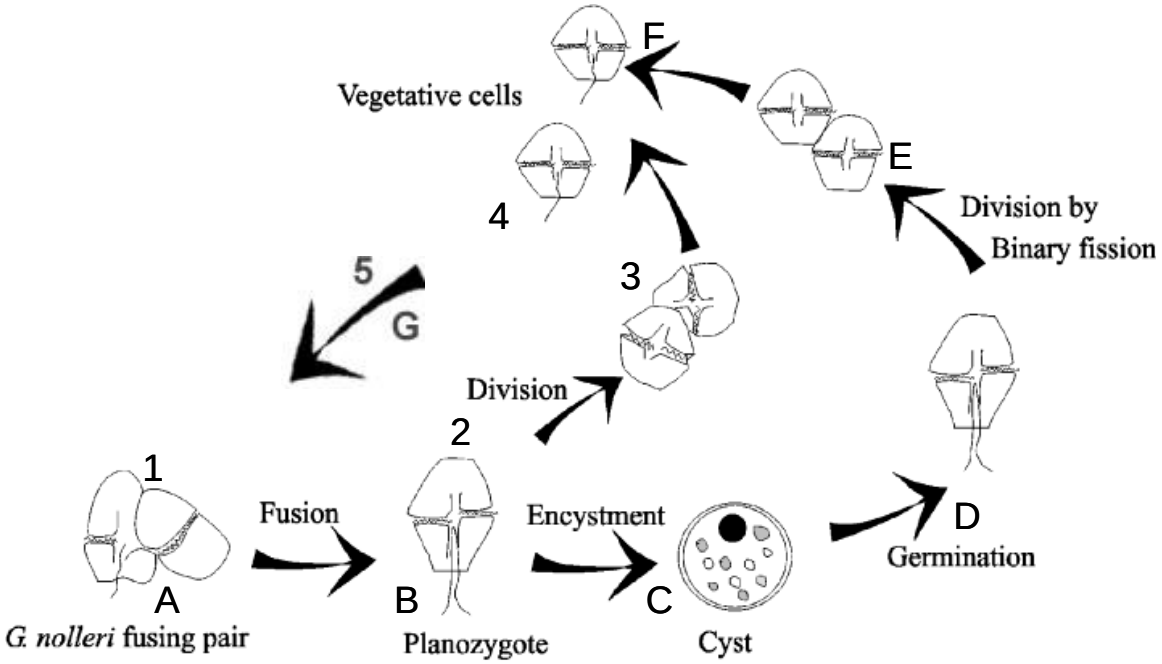

Figure 1. 5. The typical sexual life cycle for the dinoflagellate *Gymnodinium nolleri*. Sexual fusion of two gametes (A); 2N planozygote (B); 2N hypnozygote/cyst (C); planomeiocyte (D); a sexual binary fission (E); vegetative cells (F); gamete formation (G) and then fusing gametes. Forms in culture: fusing formation (1), planozygote (2), a sexual binary fission (3) and individual vegetative cells (4). Taken from Figueroa and Bravo (2005).

1.3.5. Distribution

K. mikimotoi (Miyake and Kominami ex Oda) G. Hansen and Moestrup 2000, is a widely distributed species that proliferates in both the Eastern North Atlantic and Pacific. It was first described in 1935 in western Japan (as *Gymnodinium mikimotoi* Miyake et Kominami ex Oda, 1935) (Oda, 1935) where its type locality was in Gokasho Bay. It was first recorded in European coastal waters from a southern Norwegian fjord in 1966 (Braarud and Heimdal, 1970) and was observed to cause fish kills (Tangen, 1977). Anecdotal observations, however, suggest that it could have bloomed off the southwest of Ireland in 1865 (in Harmful Algal News, no. 40, http://www.ioc-unesco.org/hab/). It is known as widely distributed species and has been reported from regions with a wide range of temperature, salinity and nutrient concentrations (Haywood et al., 1996, Haywood et al., 2004). It forms blooms in Denmark, Norway, Scotland, Ireland, England, France, Japan, Korea, China, Australia, New Zealand and USA (Guillou et al., 2002, Haywood et al., 2004, Miller et al., 2006, Davidson et al., 2009, Taylor et al., 2003, Jones et al., 1982). These blooms are commonly associated with the fish and marine invertebrate mortalities (Taylor et al., 2003). In blooms the cell concentration can reach 48 x 10^6 cells/L (Arzul et al., 1995).

1.3.6. Toxicity

K. mikimotoi is one of the ichthyotoxic species associated with mortality events involving wild or farmed fish, shellfish, such as molluscs, and benthic invertebrates (Table 1.2), including lugworms and sea-urchins (Ballantine and Smith, 1973, Ottaway et al., 1979, Jones et al., 1982, Roberts et al., 1983, Turner et al., 1987, Thain and Watts, 1984). No

effects have yet been noticed on man following the consumption of fish or shellfish from bloom areas, which contrasts to the situation where there are blooms of *Karenia brevis*. *K. mikimotoi* associated mortality has been suggested to be either due to a combination of decreased levels of oxygen saturation, or anoxic conditions, that result from the decomposition of the algae in the latter stages of the bloom and/or the presence of a toxin. Respiration by the algae themselves as well as bacterial respiration at night associated with the breakdown of the bloom consumes oxygen. In estuaries and bays, the bottom waters may become deficient in oxygen and, in extreme cases, anoxic (i.e., totally devoid of oxygen), causing mass mortality of benthic fauna. The process is exacerbated if the water column becomes stratified, either by freshwater inputs to estuaries or through seasonal heating of the surface waters (reviewed by Silke et al. (2005)). Furthermore, Potts and Edwards (1987) showed that the mucilage that is secreted by European *K. mikimotoi* could cause harmful effects by increasing the seawater viscosity: an enhancement of viscosity may cause not only a depletion of oxygen, but also a significant increase of shear stress at gill surfaces during filtration, a physical effect that could tear these fragile tissues (Jenkinson and Arzul, 1998).

K. mikimotoi has been often found to produce cytotoxic and haemolytic compounds associated with ichthyotoxicity (Taylor et al., 2003, Neely and Campbell, 2005) causing extensive damage to gill epithelia in finfish and death in filter feeding benthic fauna (Yamasaki et al., 2004). Moreover, the haemolysins, which are also identified as an exotoxins, were observed to have the capability to lower or inhibit the growth of other algae such as the diatom *Chaetoceros gracilis* (Gentien and Arzul, 1990). Two new cytotoxic marine polyether toxins the Gymnocin-A and Gymnocin-B (Tanaka et al., 2005, Sasaki et al., 2002, Satake et al., 2002, Satake et al., 2005) were recently isolated from cultured *K. mikimotoi* cells which were collected at Kushimoto, Wakayama in Japan.

1.3.7. Native, invasive or cryptic species?

The European *K. mikimotoi* has been widely reported to be a non-indigenous species (also known as an alien, non native, exotic, invasive or introduced species) due to the first description of this species in Japan in 1935 (Minchin, 2007, Pancucci-Papadopoulou et al., 2005, Hickel et al., 1971, Elbrächter, 1998) and its apparent absence elsewhere. As the first sudden European bloom of *K. mikimotoi* occurred off the southern Norwegian coast in 1966, the invasion of this species into the European waters was postulated to be related to either the Japanese oyster transplantations to France from 1966 onwards (van den Bergh et al., 2002) or via ballast water (Gollasch and Nehring, 2006). The second bloom was reported to occur in 1968 in the Southern German Bight (Hickel et al., 1971) and since then it has been found throughout the different parts of the North Sea, North Atlantic (off the southwest of England and France, and around Ireland), which possibly indicates a regional dispersal mechanism (Smayda, 2007), becoming one of the most common bloom forming species in the Northern European waters (Dahl and Tangen, 1993, Partensky and Sournia, 1986).

Gomez addressed the non-indigenous status of *K. mikimotoi* in European waters in 2008 (Gomez, 2008). It was suggested to be a cryptogenic (species whose origin is unknown or unclear) rather than non native species (Gomez, 2008, Davidson et al., 2009). It was pointed out that several reports exist of gymnidonoid-like cells being found in Atlantic waters that could have been *K. mikimotoi* (Gomez, 2008). Smayda and Reynolds (2003) noted that that the gymnodinoid group as a whole is one of the most fast growing, competitive and opportunistic colonist whose natural dispersal capacity is evident from the fact that almost all are cosmopolitan and common HAB species. *K. mikimotoi* could be one

of these species that is globally distributed, occupying both eurythermal and euryhaline habitats (Taylor et al., 2003, Gentien, 1998).

It is also important to note that Partensky et al. (1988) cast doubt on the conspecific classification of the European and Japanese strains, based on genomic and physiological analysis showing that they are dissimilar in their DNA content and life cycle (section 1.3.2). This dissimilarity was also reported for the New Zealand strain (Chang, 1996). It is therefore feasible that both physiological and genetic variation (as has been reported) could be due to the separation of these populations over long time periods through a combination of hydrology and differential selection(Rynearson and Armbrust, 2004).

1.4. Celtic Sea shelf break

The Celtic Sea shelf-break is part of the easterly extension of the Atlantic Ocean, lying off the south coast of Ireland and the St Georges Channel across the continental shelf, with the Bristol and English Channels as its eastern limits (Figure 1.6). It is bounded to the south and west by an extensive, 1000 km shelf edge, which descends rapidly to depths > 1000 m (Cooper, 1967). The shelf region is located in the mid-latitudes, where both the Celtic Sea and the English Channel are subject to a strong seasonal surface heating and cooling, and to energetic (westerly) winds that prevail for much of the year (Mason et al., 2005). This shelf widens dramatically north of the 180 km wide Armoricon Shelf (~48 ºN) of the northern Bay of Biscay, which is an open oceanic bay located at 43.5-48.5 ºN that is characterized by a sharp discontinuity in the coastline orientation from the southern, zonal coastline of Spain to the eastern, meridional coastline of France (Mason et al., 2005).

Celtic Sea shelf-break regions are highly productive areas where seasonal spring, summer and autumn phytoplankton blooms occur (reviewed by Mason et al. (2005)). These are also regions of high fish production (Coombs et al., 1983) and are therefore of significant economic importance.

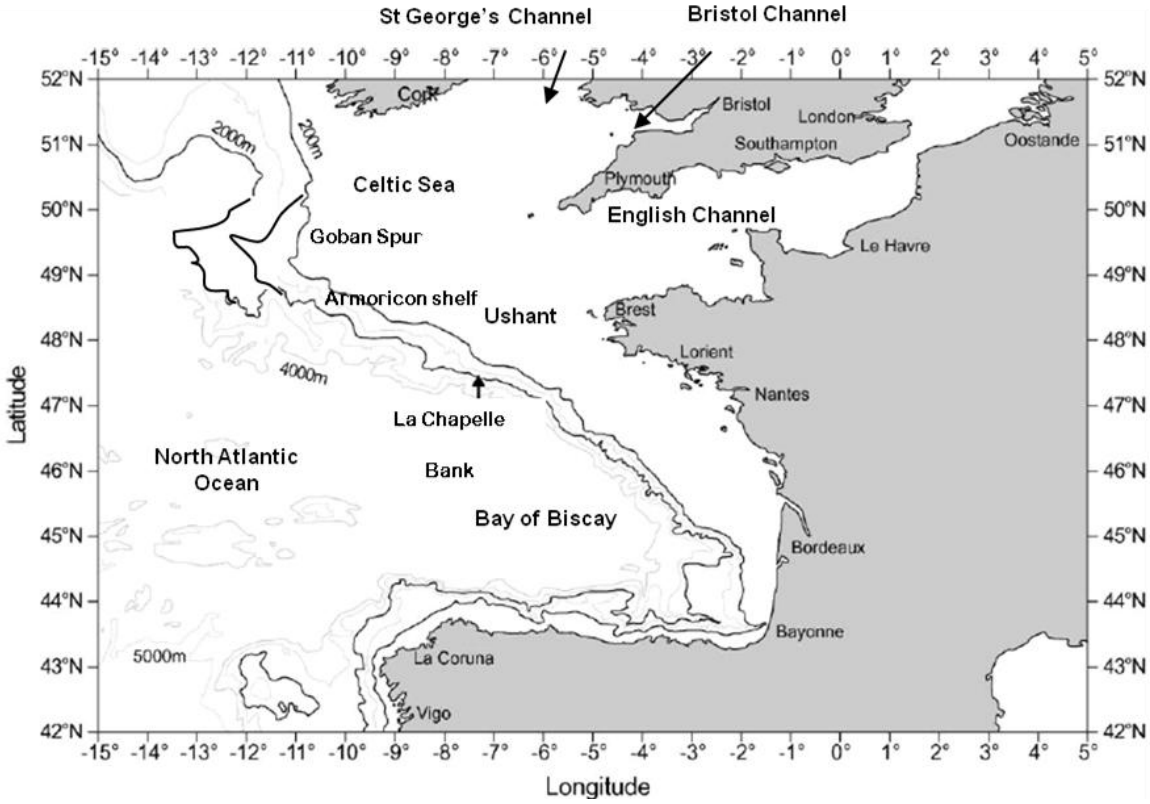

Figure 1. 6. Study area: Celtic Sea shelf-break, 42-52ºN, 0-15ºW. Adapted from Joint et al. (2001a).

1.4.1. Occurrence of seasonal and exceptional blooms

Many studies of the general physical oceanographic features (Pingree, 2005, Cooper, 1967, Pingree and Griffiths, 1980, Pingree and Le Cann, 1989, Mason et al., 2005) and phytoplankton community structure and abundance (Pybus, 2007, Russell et al., 1971, Pingree et al., 1976, Martin-Jezequel and Videau, 1992, Raine and McMahon, 1998, Joint

et al., 2001a, Labry et al., 2001, Pemberton et al., 2004, Fileman et al., 2007, Joint et al., 2001b, Rees et al., 1999) have been performed in the Celtic Sea shelf-break regions. Most of these studies have shown temporal and spatial variation in phytoplankton community distribution. The seasonal variation in concentrations of chlorophyll-a (*chl*-a) and inorganic nutrients, and how these are influenced by tidal mixing, were first described by Pingree et al. (1976). The authors described how tidal mixing influences the physical stability of spring, summer and autumn water columns in the English Channel and the Celtic Sea, which in turn reflects the seasonal changes in the phytoplankton abundance, i.e. the timing and the duration of phytoplankton blooms.

Joint et al. (2001b) studied the seasonal production of phytoplankton communities within two specific regions in the surface water of the Celtic Sea shelf-break: the Goban Spur and La Chapella Bank (Figure 1.6). Others focused on the northern region of the shelf (Fasham et al., 1983, Martin-Jezequel and Videau, 1992, Raine and McMahon, 1998, Joint et al., 2001b, Pemberton et al., 2004, Rees et al., 1999), and on the south-western coast of Ireland (Raine and McMahon, 1998, Ediger et al., 2001, Raine et al., 2001, Gribble et al., 2007). Comparatively speaking the southern region of the shelf, i.e. the La Chapella Bank area, is poorly investigated.

1.4.1.1. Spring Blooms

The spring bloom is important for the productivity of the Celtic Sea shelf-break and is estimated to account for almost half (80 g C m^{-2} yr^{-1}) of the annual primary production (Joint et al., 2001b). The highest primary production has been shown to be associated with the spring bloom by many investigators (Fasham et al., 1983, Martin-Jezequel and Videau,

1992, Joint et al., 2001b, Wollast and Chou, 2001, Hydes et al., 2001, Pemberton et al., 2004, Rees et al., 1999). The duration of the bloom varies from year to year, but generally lasts for about 2 months i.e. April and May, which is a period of rapidly changing conditions in the Celtic Sea shelf-break with increasing stratification, increasing *chl*-a and declining nutrient concentrations (Joint et al., 2001b, Rees et al., 1999).

Pingree et al. (1976) were the first to report that the spring bloom that develops in mid-April in the area to the south of Ireland, where the tidal streams are relatively weak, is related to surface heating. All the conditions at the time of spring phytoplankton outburst in April, including changes in levels of inorganic nutrients, are described in more detail by Pingree et al. (1976).

The overwhelming consensus is that the phytoplankton group dominating spring blooms is the diatoms (Lunven et al., 2005, Fasham et al., 1983, Labry et al., 2001, Fileman et al., 2007, Rees et al., 1999, Joint et al., 1986). However, Martin-Jézéquel and Videau (1992) showed that mixed waters are high in bacterial and ciliate biomass, while mainly diatoms and dinoflagellates dominated the phytoplankton occupying transitional waters. Flagellates, mainly cryptophytes, dominated stratified waters. *Thalassionema nitzschioides* (Grunow) Mereschkowsky 1902 and *Chaetoceros* Ehrenberg 1844 species dominated the diatom assemblages, however, dinoflagellates and small unidentified flagellates were also abundant (Rees et al., 1999). In northern regions of the Celtic Sea, *Pseudo-nitzschia delicatissima* (P.T. Cleve) Heiden and *Thalassiosira* Cleve 1873 sp. were dominant, whereas *Thalassiosira* sp. and *Skeletonema costatum* (Greville) Cleve 1873 occurred in the colder and lower salinity areas (Fasham et al., 1983). Pingree et al. (1976) noted that one feature of the spring outburst was the dominance of chain-forming diatoms, where the most dominant species in the Celtic Sea and the English Channel were *Skeletonema*

costatum (Greville) Cleve, *Thalassiosira* spp., *Lauderia borealis* Gran 1900, *Pseudonitzschia-delicatissima* and *P. seriata* (P.T. Cleve) H. Peragallo.

1.4.1.2. Summer and Autumn Blooms

Dinoflagellates, in general, are the dominant photosynthetic species after the spring diatom bloom, particularly when concentrations of *chl*-a exceed 10 mg m^{-3} (Pingree et al., 1978, Holligan, 1979). Around the British Isles, thermal fronts develop during the summer months (June to July), and occasionally last into the autumn (August to September). They are generally characterized by sharp surface temperature gradients between cold, well mixed waters (rich in inorganic nutrients) and warm, well stratified waters (nutrient depleted) (Pingree et al., 1978). Frontal boundaries are readily detected both by direct measurement at sea and by satellite infrared imagery (Holligan, 1979). Blooms of *Karenia mikimotoi*, have been found associated with these tidal fronts (Holligan, 1979, Pingree et al., 1979, Pingree et al., 1975). Maximum values of *chl*-a concentration were always associated with these frontal blooms and could be more than ten times those found in typical spring bloom conditions (Pingree et al., 1976).

Moore et al. (2006) found that to the west of Irish Shelf Front the phytoplankton community comprised small naked flagellates, mainly *Katodinium* Fott 1957 spp. and other micro flagellates with occasional diatoms. Waters to the east of the front contained a greater species diversity and biomass. These were dominated by dinoflagellates containing many of the important, potentially harmful algal species such as *Dinophysis acuminata* Claparède et Lachmann, 1859, *Gyrodinium aureolum* Hulbert 1957 (now *Karenia*

mikimotoi) and the diatom *Pseudo-nitzschia* H. Peragallo spp., but mainly the non-toxic *P. pungens* (Grunow ex P.T. Cleve, 1897) Hasle, 1993.

Many studies of the Celtic Sea shelf-break have documented the appearance of blooms of the coccolithophore *Emiliania huxleyi* (Lohmann) Hay and Mohler (Holligan and Groom, 1986, Groom and Holligan, 1987, Grepma, 1988, Garcia-Soto et al., 1995, Fileman et al., 2002, Holligan et al., 1983). Whilst this species generally blooms in summer/autumn, it has been observed from February through to September (Holligan et al., 1983) giving a characteristic milky appearance to the water as the calcite plates of the coccolithophores are strongly reflective: in May 1995 a bloom of *E. huxleyi* occurred in the waters off the southwest Irish coast (Raine and McMahon, 1998); extensive patches of water giving high reflectance of visible light were observed in the Celtic Sea and Armorican Shelf regions (Figure 1.6), which indicated the bloom of *E. huxleyi* from the beginning of February to the end of July 1998 (Zeichen and Robinson, 2004). The *E. huxleyi* bloom that occurred off the coast of Devon during July 1999 was associated with diatoms in low reflectance areas while photosynthetic dinoflagellates, mainly *Karenia mikimotoi* (500 cells ml^{-1}), followed by cryptophytes co-occurred in the higher reflectance areas (Fileman et al., 2002). Garcia-Soto et al. (1995) also showed significant variations of phytoplankton community composition inside and outside the *Emiliana huxleyi* blooms.

1.5. *Karenia mikimotoi* blooms

In 1968, *Karenia mikimotoi* was first officially recorded and photographed as a small pigmented and unarmored dinoflagellate in the English Channel. At that time, it was difficult to identify but it was suggested that it might have been also present since 1966

within the recorded "unknown *Gymnodinium*" group (Boalch, 1987). At about the same time a small dinoflagellate bloom in Norwegian waters was associated with fish kills (Braarud and Heimdal, 1970, Boalch, 1987).

Pingree et al. (1975) reported the first red-tide of *K. mikimotoi*, along tidal fronts in the south-western approaches to the English Channel and Ushant front in July 1975. At that time of the year the bloom caused the water to turn reddish-brown to dark brown in colour and *K. mikimotoi* was associated with another red tide bloom of the dinoflagellate *Noctiluca scintillans*. Spectacular and exceptional blooms of *K. mikimotoi* were observed, for the second successive year, in the summer of 1976 at the western entrance to the English Channel (Pingree et al., 1977). In August 1977, Holligan observed large populations of *K. mikimotoi* with two chlorophyll maxima, one in the surface and one at the thermocline associated with Ushant tidal front (Holligan, 1979). Holligan et al. (1983) used satellite sensors to complete the first comprehensive description in the western English Channel of the spatial and temporal distributions of *K. mikimotoi* blooms, for summer 1981.

The first red tide in Ireland occurred in late July and early August in 1976 and was associated with mortalities of littoral and sub-littoral marine organisms (Ottaway et al., 1979). In 1978 another red-tide of the same species was recorded off the south west coast of Ireland (Pybus, 1980), and at the same time a red tide occurred off the south coast of England (Boalch, 1979). More recently, blooms have been detected and documented off the southern coast of Ireland in 1994, 1995, 1998, 1999, 2000 and 2005 (Raine and McMahon, 1998, Ediger et al., 2001, Raine et al., 2001, Silke et al., 2005, Miller et al., 2006, Mitchell and Rodger, 2007) and in the English Channel in 1999, 2000, 2002, 2003, 2004 and 2006 (Groom et al., 2000, Fileman et al., 2002, Kelly-Gerreyn et al., 2004,

Garcia-Soto and Pingree, 2009). The French coast (Brest and Douarnenez Bays) has also experienced *K. mikimotoi* blooms since 1976 (Erard-Le Denn et al., 1990). The biggest bloom in that area was recorded in 1995 and extended further along the south of Brittany, Ushant front and Armorican shelf (Arzul et al., 1995, Gentien, 1998).

1.6. Molecular techniques to discriminate between species within the genus *Karenia*

Molecular techniques have been widely used as diagnostic tools to resolve many of the synonyms and misidentifications that have been assigned to cells of similar morphologies. It has been suggested that molecular-based approaches may indicate taxonomic affiliations better than approaches that only use morphological features and the pigment content. Ribosomal RNA (rRNA) gene sequences have received more attention than others as a means of distinguishing organisms at higher taxonomic levels, and in some cases at the species level. In eukaryotes, rRNA genes are present as tandem repeats containing the 18S rRNA small subunit (SSU), an internal transcribed spacer (ITS1), the 5.8S rRNA, a second internal transcribed spacer (ITS2) and the 28S rRNA large subunit (LSU) encoding regions. Most phylogenetic studies of dinoflagellates have used parts of the rRNA to infer the phylogenetic history of their target species. They include the use of the SSU rDNA (Dolapsakis et al., 2006, Hoppenrath and Leander, 2007, Murray et al., 2005, Murray et al., 2007, Zhang et al., 2007, Hoppenrath et al., 2007), the D1-D6 domains of LSU rDNA (Lenaers et al., 1989, Zardoya et al., 1995, Daugbjerg et al., 2000, Botes et al., 2003, De Salas et al., 2003, de Salas et al., 2004b, de Salas et al., 2004a, Bergholtz et al., 2005, De

Salas et al., 2008, Tang et al., 2008) and ITS regions (Adachi et al., 1995, Guillou et al., 2002, Shao et al., 2004, Litaker et al., 2003).

The different subunits and regions of the rDNA have different degrees of sequence variability, which means they vary in their suitability for use in comparisons at the inter-generic or inter-species level (Adachi et al., 1996). In general, the SSU, 5.8S and LSU genes, which flank the ITS regions, are known to diverge more slowly when compared to the ITS regions, thereby making the ITS regions good candidates for distinguishing unambiguously between even closely related species (Bargues et al., 2000).

The LSU rDNA is comprised of D1-D12 domains (Hassouna et al., 1984, Ellis et al., 1986, Michot and Bachellerie, 1987), with regions D1 and D2 known to span hypervariable regions that constitute one of the fastest evolving portions in DNA-encoded eukaryotic rRNA (Michot et al., 1984, Michot and Bachellerie, 1987, Lenaers et al., 1989, Lenaers et al., 1991). These regions were found to be useful for distinguishing species and strains among dinoflagellates, and for examining sequence heterogeneity among different strains of the same species (Scholin and Anderson, 1996, Scholin et al., 1994, Daugbjerg et al., 2000, Guillou et al., 2002). For example, Scholin et al. (1994) found that the D1 and D2 region of *Alexandrium* Halim 1960 was able to resolve both species and population differences.

Based on LSU rDNA phylogeny, combined with evidence from ultrastructure and pigment analysis, many species within the gymnodinoid group were assigned to the new genera *Karenia*, *Karlodinium* J. Larsen, *Akashiwo* (Daugbjerg et al., 2000) and *Takayama* de Salas, Bolch, Botes et Hallegraff (De Salas et al., 2003), confirming the polyphyletic nature of this group. Three of these genera, *Karenia*, *Karlodinium* (Daugbjerg et al., 2000) and *Takayama* (De Salas et al., 2003), include fucoxanthin-containing species. Bergholtz

et al. (2005) emphasised in their investigation (based on LSU rDNA) that these three genera constitute a separate evolutionary lineage for which the new family Kareniaceae is suggested. Species within the family Kareniaceae have recently been listed and described in more detail for the Gulf of Mexico (Steidinger et al., 2008). Recently, the genus *Brachidinium* F.J.R. Taylor has been found to be related to the genus *Karenia* based on both morphological structures (Gómez et al., 2005) and molecular phylogenies using LSU rDNA, SSU rDNA, ITS and *Cox1* regions (Henrichs et al., 2011).

Recognised *Karenia* species have increased in number in less than five years from two to twelve (Table 1.2). Morphological characteristics, pigment analysis and the partial LSU rDNA sequencing of D1-D3 was used to separate *K. cristata* and *K. bicuniformis* from South Africa (Botes et al., 2003) and *K. umbella* from Australia (de Salas et al., 2004b). LSU rDNA D1-D2 regions were used for *K. papilionacea* from the South China and New Zealand (Haywood et al., 2004, Yeung et al., 2005), and *K. selliformis* and *K. bidigitata* (=*K. bicuniformis*) from New Zealand (Haywood et al., 2004).

LSU rDNA sequences are also useful as biogeographic markers, which separate the same species from different locations. This has been shown within *Gymnodinium aureolum* (Tang et al., 2008), *Karlodinium* spp., *Takayama* spp. and *Karenia umbella* (Bergholtz et al., 2005). The partial LSU rDNA containing D1-D3 regions was used to compare the *K. mikimotoi* sequences from five different geographical areas i.e. England, Norway, Denmark, Australia and Japan (Hansen et al., 2000). The Danish, Australian, and Japanese strains were all identical (group 1), whereas the English and Norwegian strains were very similar showing only two differences within a region of 925 nucleotides (group 2). Similarly, partial LSU rDNA sequences were also used by Daugbjerg et al. (2000) to show that *K. mikimotoi* isolates from Denmark and Japan were identical. Haywood et al. (2004)

also compared all available sequences excluding the Danish strain with two new isolates from New Zealand and another from a different location in Japan. Their results were similar to the previous study by Hansen et al. (2000) as they grouped New Zealand isolate and the new Japanese isolate with group 1. In addition, Guillou et al. (2002) found that one French isolate grouped with Group 1 (Figure 1.7), while another was in Group 2 (England, Norway and a new Australian isolate), but with an additional nucleotide difference.

The LSU rDNA marker with the domains D1 and D2 was selected for the study presented here because: 1) it contains both highly variable and conserved regions, making it suitable for comparisons of organisms from a range of taxonomic levels (Hillis and Dixon, 1991), 2) it is accepted as universal marker (Liao, 1999) that can be used to discriminate between species, including Dinoflagellates, 3) it is known to be tandemly arrayed in hundreds to thousands of copies in most organisms, making it a relatively easy target for amplification, 4) there is a large database of comparable sequences held in GenBank.

The ribosomal DNA internal transcribed spacer (ITS) region, consisting of the ITS-1 and ITS-2 sequences, separates three ribosomal genes in the rRNA operon of eukaryotes (Appels and Honeycutt, 1986), has been used for defining organisms at species level because these regions are known to be evolve faster than the rRNA gene sequences allowing better resolution between closely related species and/or among different strains in one species (Janson and Hayes, 2006). The ITS regions have high base substitution rates and well-documented length variations within various organisms (Shao et al., 2004). These characteristics have lead to the increased use of the ITS sequences for species differentiation in diverse groups such as as the dinoflagellates (Adachi et al., 1997, Baillie et al., 2000, Gottschling and Plötner, 2004, Shao et al., 2004, Litaker et al., 2003). Inter- and intra-ITS region variation has been observed in *Alexandrium* spp. (Adachi et al., 1995)

and *K. mikimotoi* (Guillou et al., 2002). Guillou et al. (2002) observed that the two *K. mikimotoi* strains of French origin that were isolated from the same location, but eight years apart (1987 and 1995), had diverged by only two nucleotides in the intergenic regions. A study by Guofu et al. (2008) made a distinction between two inferred phylogenies using the LSU rDNA (D1-D2) and the ITS region of a *Gymnodinium*-like species from the Chinese coast. They showed that all the strains were better resolved in the ITS tree than in the LSU tree. The ITS region was, therefore, selected as the second molecular marker to investigate whether it is possible to separate *K. mikimoti* strains from different geographical regions

Plastid genes have also been used in phylogenetic and phylogeographic studies of dinoflagellates. They have also been used successfully to test the origin of dinoflagellates based on their photosynthetic pigment content. Photosynthetic dinoflagellates contain several types of plastid. The most common type is a three-membrane bound plastid that contains peridinin as the major carotenoid (Jeffrey, 1989, Dodge, 1989). The second type of plastid, which is also surrounded by three membranes, contains 19'-hexanoyloxy-fucoxanthin and/or butanoyloxy-fucoxanthin and fucoxanthin, but they lack peridinin (Hansen et al., 2000, Jeffrey, 1989). This second type of plastid pigmentation has been found in the genera *Karenia*, *Karlodinium* and *Takayama* (de Salas et al., 2004a, Haywood et al., 2004, Bergholtz et al., 2005). Other species within the genera *Gymnodinium* and *Gyrodinium* have similar pigmentation to the *Karenia* species (Haywood et al., 2004).

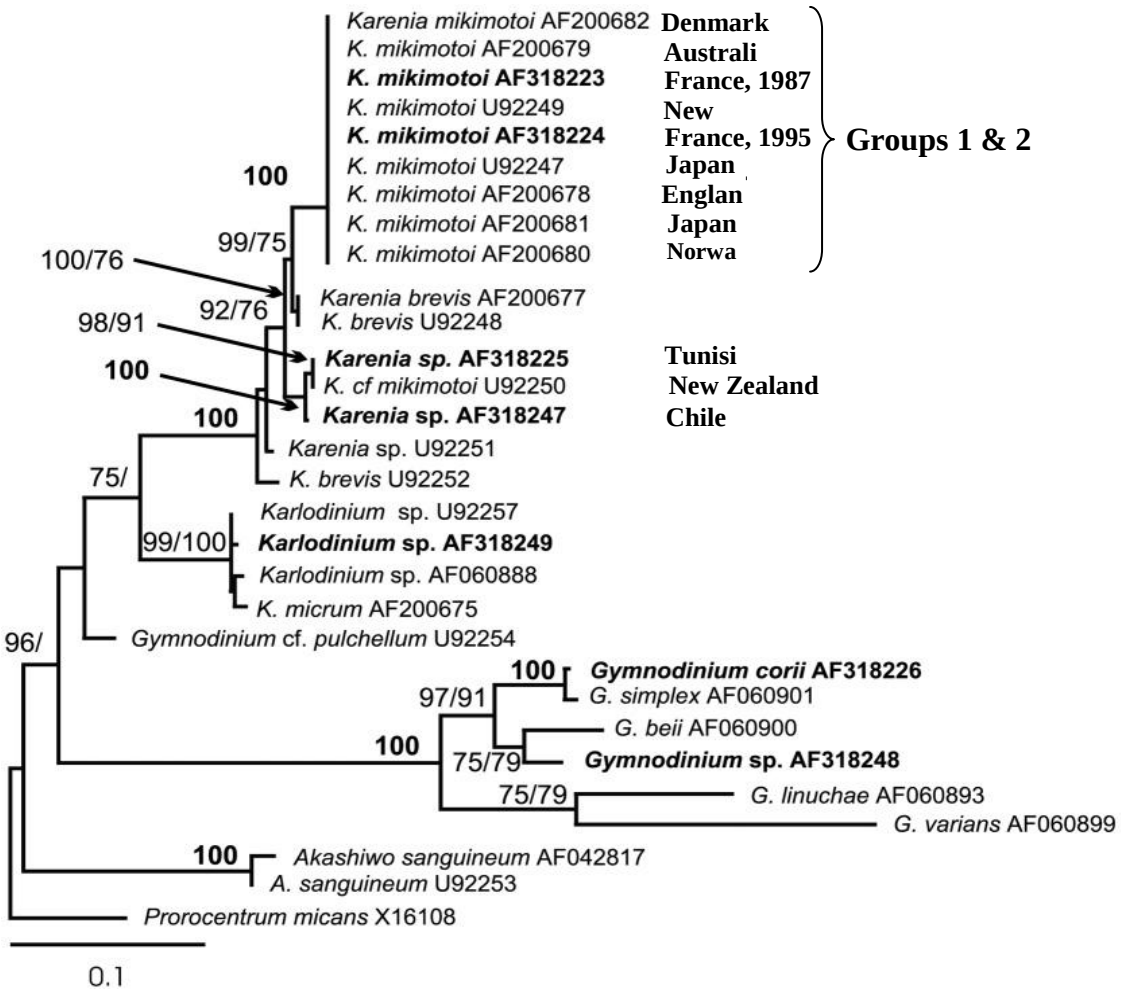

Figure 1. 7. Phylogenetic analyses of 30 different unarmoured dinoflagellates sequences, including the genera *Karenia*, *Karlodinium*, *Akashiwo*, *Gymnodinium* and *Prorocentrum* derived from an alignment of the partial LSU rDNA (D1-D2). Bootstrap values obtained using neighbor-joining and parsimony analyses, respectively, have been marked at the internal branches (500 replicates; values >70% displayed). Bold numbers correspond to identical bootstrap values in both analyses. Scale bar: 0.1%. The tree is rooted using *Prorocentrum micans* (X16108). Taken from Guillou et al. (2002).

Tengs et al. (2000) investigated the origin of 19' hexanoyloyxy-fucoxanthin containing-dinoflagellates, based on the SSU rRNA from the plastid and the nuclear genomes, revealing that these dinoflagellates acquired their plastids via endosymbiosis of a haptophyte. Similarly, Yoon et al. (2002a) carried out investigations based on the *psa*A (photosystem I p700 *chl*-a apoprotein A1), *psb*A (Photosystem II reaction center protein D1) and *rbc*L (ribulose-1,5-biphosphate carboxylase/oxygenase large subunit), showing that both the plastid of peridinin and fucoxanthin-containing dinoflagellates originated from the haptophyte tertiary endosymbiosis that occurred before the split of these lineages. For species and higher taxonomic resolution, the sequences of *rbcL* have also been used to separate dinoflagellates (Gray et al., 2003, Daugbjerg et al., 1995) and this gene was selected as the third molecular marker to be used in the current study to attempt to differentiate between *K. mikimotoi* isolates from different regions.

1.7. Project aims and objectives

This study has answered many of the questions posed in the General Introduction, i.e, do the blooms of *K. mikimotoi* in Celtic Sea shelf break over the past 40 years comprise one or many genotypes? If there are multiple genotypes, where do they come from and what is their relationship with other geographically distinct strains, i.e. could they have been introduced? Only through molecular approaches have we been able to quantify the genetic diversity and the biogeography among the different strains of *K. mikimotoi*. The different timing, location, genetic variability and the biodiversity of associated communities have helped to define the particular conditions that determine when and where *K. mikimotoi* blooms occur.

The application of genetic techniques to the analysis of HAB species has been increasing recently. These techniques can distinguish between morphologically similar species that are difficult to identify and require untrastructural studies and expert taxonomists to identify them: accurate identification of species is necessary if we are to understand their biogeography and ecology, and focus management and control efforts on these problematic algae. Understanding the biology of HAB-forming organisms might ultimately lead to the development of mitigation or control strategies to reduce the impacts of harmful or toxic blooms.

The Oslo and Paris Conventions (OSPAR) provides a mechanism by which 15 governments of the western coasts and catchments of Europe, together with the European Community, cooperate to protect the marine environment of the North-East Atlantic. The UK is obliged to protect marine areas beyond the 12 nautical mile limit of the UK territorial waters to the 200 nautical mile limit of the UK Continental Shelf. In 2002, the UK Government set out a vision of a clean, healthy, safe, productive and biologically diverse oceans and seas. The second commissioned progress report published in 2010, *Charting Progress 2*, provided an assessment of the productivity of UK seas, and the extent to which human uses and natural pressures are affecting their quality and addressing the specific species (including microalgae), habitats and economic issues for the eight UK regions.

The Celtic Sea shelf-break region, one of the eight UK regions, is a highly important productive area, yet little is known about the true inter- and intra- species composition of the microalgae blooms that sustain and/or harm the biodiversity of this region. Moreover, the HAB species *Karenia mikimotoi* is thought to have bloomed in this region since the 1970s, but its true identity and thus origins remain unclear. This study aims therefore to:

1. Determine whether any genetic differences exist between *K. mikimotoi* isolates from different geographical origins.
2. Improve our understanding of the changing genetic structure of *Karenia mikimotoi* blooms over decadal time scales, which has bearing on whether or not *K. mikimotoi* is a native to UK waters.
3. Characterise more broadly the microalgal composition associated with *K. mikimotoi* dominated blooms.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Chemicals, reagents and laboratory consumables

General laboratory chemicals (analytical grade or higher) and consumables were obtained from either Fisher-Scientific (Leicester, UK), Promega (Southampton, UK), Invitrogen (Paisley, UK), Bioline (UK), Applied Biosystem (UK) or Sigma-Aldrich (Poole, UK). All other reagent and consumable suppliers are listed in the text as appropriate.

2.1.2. Commonly used solutions

A list of commonly used solutions is presented in Table 2.1.

Table 2. 1. Composition of commonly used solutions (Sambrook and Russel, 2001).

Solution	Components
10 × TBE	0.89 M Tris-borate; 20 mM EDTA, pH 8.0
10 × TAE	400 mM Tris-acetate; 10 mM EDTA, pH 8.0
TE 10:1 buffer pH 8.0	10 mM Tris HCl; 1 mM EDTA, pH 8.0
10 × Orange G (DNA Loading Dye Buffer)	0.175 g (w/v) Orange G; 50 % 10 × TAE , 50 % Glycerol
SDS	10 % (w/v) sodium dodecyl sulphate
Lysis buffer	10 mM Tris; 0.1M EDTA, pH 8.0; 0.5 % (w/v) SDS; 20 µg ml^{-1} Proteinase K (final concentration)

2.1.3 Growth media

A list of commonly used media is presented in Table 2.2.

Table 2. 2. Composition of growth media used (Sambrook and Russel, 2001).

Medium	Components
F/2- Si medium*	884 µM $NaNO_3$; 36 µM $NaH_2PO_4 \cdot H_2O$ 11.7 µM Fe EDTA·$6H_2O$; 0.9 µM $MnCl_2 \cdot 4H_2O$; 12 µM $Na_2EDTA \cdot 2H_2O$; 0,04 µM $CuSO_4 \cdot 5H_2O$; 0.03 µM $Na_2MoO_4 \cdot 2H_20$; 0.08 µM $ZnSO_4 \cdot 7H_2O$; 0.05 µM $CoCl_2 \cdot 6H_2O$; 0.37 nM vitamin B_{12}; 2 nM biotin; 0.3 µM thiamine HCL
Luria-Bertani (LB) broth- agar medium (per litre)	10 g Tryptone; 5 g Yeast extract; 10 g NaCl; 15 g Agar
SOC medium (per litre)	20 g Tryptone; 5 g Yeast extract; 0.5 g NaCl; 10 ml 1 M $MgCl_2$; 10 ml 1 M $MgSO_4$; 2 ml 20% (w/v) Glucose

*: F/2 – Si medium (Guillard, 1975, Guillard and Ryther, 1962) was made from 30 kD filtered and autoclaved seawater collected from a coastal station L4 (50°15'N, 4°13'W), which lies approximately 10 km off Plymouth in the English Channel.

2.1.4. Dinoflagellate cultures

Thirty five dinoflagellates isolates from various geographical locations were used in this study. These included 11 isolates of *Karenia mikimotoi* (Table 2.3) and 24 isolates of related dinoflagellate species (Table 2.4). The dinoflagellate were selected to include most of the taxa thought to be closely related genera to *K. mikimotoi*, such as other *Karenia* spp.,

Karlodinium, *Takayama*, *Gymnodinium aureolum*. They were collected from different locations and different culture collections as follows: the Provasoli-Guillard National Center for the Culture Collection of Marine Phytoplankton (CCMP, Maine, USA; https://ccmp.bigelow.org/); the Cawthron Institute Culture Collection of Micro-Algae (CICCM) (CAWD, Halifax Street East, NewZealand; http://cultures.cawthron.org.nz/); the Microalgal Culture Collection of the University of Caen Basse (Algobank-Caen (AC), Normandie, France; http://www.unicaen.fr/algobank/accueil/); the Plymouth Culture Collection (Marine Biological Association, (MBA**)**, UK; http://www.mba.ac.uk/); the Scandinavian Culture Collection of Algae and **P**rotozoa (SCCAP) (**K,** University of Copenhagen, Denmark; http://www.sccap.dk/).

Table 2. 3. *Karenia mikimotoi* isolates used in this study.

ID Code	Collection site (country)	Isolated by	Date of isolation or (deposition)
CAWD05 (JP1)	Kushimoto (Japan)	Takashi Ishimaru	1986
MBA561 (JP2)	Unknown (Japan)	Daniel Vaulot	(1996)
CAWD63 (NZ)	Waimangu Point (New Zealand)	A. Haywood	1994
CAWD117 (NZ)	East Bay, Marlb. Sands (New Zealand)	Janet Adamson	2001
CAWD133 (NZ)	Kennedy Bay (New Zealand)	Janet Adamson	2002
CAWD134 (NZ)	Whangaporaoa, (New Zealand)	Janet Adamson	2002
CCMP429 (UK)	Sutton Harbour, Plymouth, (United Kingdom)	D. Harbor	1980
MBA705 (UK)	Cabris Bay, Cornwall (United Kingdom)	Richard Pipe	2006
K0260 (NOR)	Oslo Fjord (Norway)	K. Tangen	1977
CCMP430 (UN)	Unknown	K. Tangen	(1985)
AC213 (UN)	Unknown	Unknown	Unknown

Table 2. 4. List of non-*Karenia mikimotoi* dinoflagellate isolates used in this study.

ID code	Strain name	Collection site
AC215	*Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup 2000	Luc-sur-Mer, France
AC194	*Alexandrium minutum* Halim, 1960	Morlaix, Bretagne, France
CCMP116	*Alexandrium tamarense* (Lebour) Balech	Ria de Vigo, Spain
CCCM592	*Alexandrium tamarense* (Lebour) Balech	Ipswich Bay, MA, USA
AC208	*Amphidinium carterae* Hulburt 1957	Blainville-Normandie, France
CAWD87	*Gymnodinium aureolum* (Hulburt) G. Hansen	Coromandel, NZ
CCMP1937	*Gymnodinium catenatum* Graham 1943	Ria de Vigo, Spain
CCCM900	*Heterocapsa triquetra* (Ehrenberg) F. Stein	Unknown
CAWD81	*Karenia bidigitata* Haywood et Steidinger, 2004	Fouveaux Strait, NZ
CAWD80	*Karenia bidigitata* Haywood et Steidinger, 2004	Fouveaux Strait, NZ
CAWD92	*Karenia bidigitata* Haywood et Steidinger, 2004	Hawkes Bay, NZ
CCMP2228	*Karenia brevis* (Davis) G. Hansen et Moestrup 2000	Mote Marine Laboratory's New Pass Dock, USA
CCMP2820	*Karenia brevis* (Davis) G. Hansen et Moestrup 2000	New Pass Bridge, Sarasota, Florida, USA
CCMP718	*Karenia brevis* (Davis) G. Hansen et Moestrup 2000	Florida, USA
CCMP2281	*Karenia brevis* (Davis) G. Hansen et Moestrup 2000	Gulf of Mexico between Pensacola Beach
CCMP2229	*Karenia brevis* (Davis) G. Hansen et Moestrup 2000	Manasota Key, Florida,USA
CAWD82	*Karenia brevisulcata* (Chang) G. Hansen et Moestrup 2000	Wgtn. Harbour, NZ
CAWD91	*Karenia papilionacea* Haywood & Steidinger, 2004	Hawkes Bay NZ
CAWD79	*Karenia selliformis* Haywood, Steidinger and MacKenzie 2004	Fouveaux Strait NZ
CAWD131	*Karenia umbella* de Salas, Bolch et Hallegraeff, 2004	Port Nelson NZ
CCMP416	*Karlodinium micrum* (Leadbeater et Dodge, 1966) J. Larsen, 2000	Norway
CCCM734	*Karlodinium micrum* (Leadbeater et Dodge, 1966) J. Larsen, 2000	Norway
CCMP2936	*Karlodinium veneficum* (Ballantine, 1956) J. Larsen 2000	Swan Keys, Delaware, Inland Bays,
CAWD115	*Takayama tasmanica* de Salas, Bolch et Hallegraeff 2003	Glenhaven, NZ

2.2. General Methods

2.2.1. Dinoflagellate culturing and harvesting

Cultures were maintained in F/2–Si medium without added silicate (Table 2.2) under controlled conditions at constant temperature (19 ºC) with incident artificial light at 80 µmol photons m^{-2} s^{-1} on a 18:6 hour light-dark cycle. Cultures were checked visually each week and sub-cultured when they reached late exponential phase of growth by transferring 5-10 % (v/v) cultures into fresh F/2-Si medium. Cell density was monitored using a Sedgewick-Rafter cell-counting slide and an inverted light microscope (Rashash and Gallagher, 1995). As required, cells were harvested as pellets from 50 ml aliquots of late exponential phase cultures via gentle centrifugation (4000 × g) for 5 minutes at room temperature. Pellets were stored at -20 ºC for later DNA extraction (section 2.2.6.1).

2.2.2. Combining *K. mikimotoi* cultures for mixing experiment

To test the ability of DNA based assays to distinguish between starins in mixed cultures four strains of *K. mikimotoi*, JP1 (CAWD05), JP2 (MBA561), UK (MBA705) and NZ (CAWD63), were used to prepare two sets of culture preparations with final concentrations of 10^3 and 10^4 cells ml^{-1} for each strain. These were used to prepare mixed samples comprising six different groups (Table 2.5), with each group containing a mixture of two different combinations of strains. A total of 32 2ml aliquots of controls and mixed cells were harvested and stored as described in 2.2.1.

Table 2. 5. Mixed cell cultures of two different combinations of *K. mikimotoi* strains equally and differently prior to DNA extraction. JP1/CAWD05, JP2/MBA561, UK/MBA705 and NZ/CAWD63.

Group	Samples No.	Strains	Cells/ml
Controls	1	JP1	10^3
	2	JP1	10^4
	3	JP2	10^3
	4	JP2	10^4
	5	UK	10^3
	6	UK	10^4
	7	NZ	10^3
	8	NZ	10^4
1	9	JP1 + JP2	$10^3 + 10^3$
	10	JP1 + JP2	$10^4 + 10^4$
	11	JP1 + JP2	$10^3 + 10^4$
	12	JP1 + JP2	$10^4 + 10^3$
2	13	JP1 + UK	$10^3 + 10^3$
	14	JP1 + UK	$10^4 + 10^4$
	15	JP1 + UK	$10^3 + 10^4$
	16	JP1 + UK	$10^4 + 10^3$
3	17	JP2 + UK	$10^3 + 10^3$
	18	JP2 + UK	$10^4 + 10^4$
	19	JP2 + UK	$10^3 + 10^4$
	20	JP2 + UK	$10^4 + 10^3$
4	21	JP1 + NZ	$10^3 + 10^3$
	22	JP1 + NZ	$10^4 + 10^4$
	23	JP1 + NZ	$10^3 + 10^4$
	24	JP1 + NZ	$10^4 + 10^3$
5	25	JP2 + NZ	$10^3 + 10^3$
	26	JP2 + NZ	$10^4 + 10^4$
	27	JP2 + NZ	$10^3 + 10^4$
	28	JP2 + NZ	$10^4 + 10^3$
6	29	UK + NZ	$10^3 + 10^3$
	30	UK + NZ	$10^4 + 10^4$
	31	UK + NZ	$10^3 + 10^4$
	32	UK + NZ	$10^4 + 10^3$

2.2.3. Environmental sample collection

2.2.3.1. The Continuous Plankton Recorder (CPR)

Archived-CPR samples were collected via the CPR-device (Figure 2.1), which is designed to be towed behind a commercial vessel. The CPR samples plankton from the surface layer of the water column (~ 7 m), filtering seawater through a continuously advancing band of a silk (mesh size 270 µm) that moves across the sampling aperture at a rate proportional to the speed of the towing ship. The silk is subsequently immersed in a formalin tank (4 % buffered formaldehyde) (Richardson et al., 2006, Batten et al., 2003, Reid et al., 2003), the samples then being preserved for later analysis. On return to the laboratory, the roll of silk mesh (now containing plankton samples) is removed from the CPR, sprayed with a 4% borate buffered formalin solution and stored prior to dividing the silk into individual samples: each representing 10 nautical miles (3 m^3 of filtered sea water) that are numbered sequentially along the route towed. When the silk is cut into individual samples, they are sprayed with a fungicide consisting of propylene glycol and propylene phenoxetol.

Plankton species are enumerated microscopically by SAHFOS staff using x 450 and x 54 magnification before being sprayed with 4% buffered formalin and then wrapped with plastic bags and stored in plastic containers at room temperature.

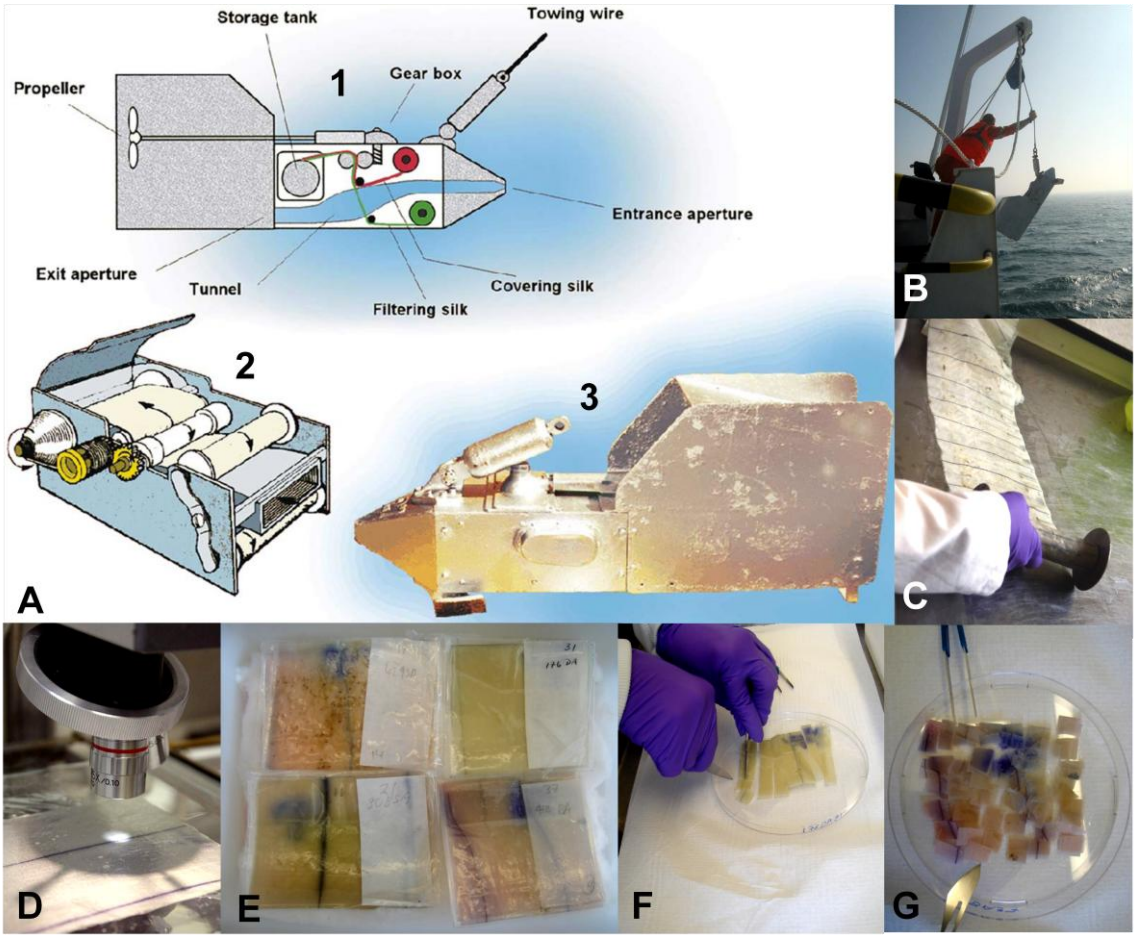

Figure 2. 1. A. CPR device: (1) a cross-section of the device; (2) internal silk collecting mechanism, which is inserted into the CPR body; and (3) an external view of the CPR device. The internal mechanism starts when the water enters the CPR through a square entrance aperture and flows down an expanding tunnel, which effectively reduces the water pressure to minimise damage to the captured plankton, and exits through the rear of the device. The movement of the water past the CPR turns an external propeller at the rear of the device that operates a drive shaft and gear system, which advances the silk filtering mesh. Plankton in the water is filtered onto this constantly moving band of silk. The filtering silk meets a second band of covering silk, effectively sandwiching the plankton, and is then wound onto a spool in a storage tank containing formalin (Richardson et al., 2006). B. The CPR device being deployed behind the vessel; C. Unwinding of the silk ready for cutting; D. Plankton analysis by microscopy; E. Storage of the silk preserved for later analysis; F. Cutting of the silk into small pieces in preparation for DNA extraction; G. Silk pieces ready for DNA extraction.

2.2.3.2. *K. mikimotoi* bloom samples

Two litre volumes of water were collected from six different depths (0, 5, 10, 15, 25 and 40 m) at 3 stations within an *Emiliania huxleyi* and *K. mikimotoi* co-dominated bloom in the English Channel on 26th and 27th of July 2006 (Figure 6.1A and B). The samples were filtered onto 0.45 µm pore size, 47-mm-diameter membrane filter, supor®-450 (Pall life sciences (PALL), Michigan, USA). Filters were then placed in sterile 1.8 ml CryoTube™ vials (Nunc, Thermo Fisher Scientific, Denmark) and stored at -80 ºC prior to DNA extraction (section 2.2.6.1).

2.2.4. Mapping of *K. mikimotoi* blooms and CPR sample selection

2.2.4.1 Collation of historical recordings of *K. mikimotoi* blooms in the Celtic Sea shelf-break region

K. mikimotoi bloom information based on peer-reviewed data and satellite remote sensing imagery within the Celtic Sea shelf-break region (46-52ºN, 0-11ºW) (Figure 1.6) were collected and collated. Remote sensing imagery data were obtained from the Remote Sensing Group at Plymouth Marine Laboratory (PML). These include the Coastal Zone Colour Scanner (CZCS) (9 km monthly *chl*-a 1978-1986), Sea-viewing Wide Field-of-view (SeaWiFS) (9 km monthly *chl*-a 1997-present day), SeaWiFS (1 km weekly true-color 1997-present day) and Moderate Resolution Imaging Spectroradiometer (MODIS) Aqua (9 km monthly *chl*-a 2002-present day) data sets. All the information regarding the *K. mikimotoi* blooms were collated into annual tables (Appendix 2). The timing and the

locations of these blooms were then manually mapped on Geographic Information Systems (GIS) map.

2.2.4.2. Selection of CPR samples

The locations and timing obtained from the mapping of *K. mikimotoi* blooms (section 2.2.4.1) was used to select the most appropriate archived CPR samples for total nucleic acid extraction. This was done firstly for the samples collected earlier than 1979 by checking the longitude and latitude of areas where *K. mikimotoi* frequently blooms, both now and in the past, and using this information to select the appropriate archived CPR samples that fit well within these identified areas. Secondly, all the samples collected later than 1979 were selected to match as closely as possible the regions of high chlorophyll concentration (red to dark red or brown) in the corresponding SeaWiFS images. Then the archived samples were checked carefully to identify CPR samples from routes that fit within the spatial and temporal windows for *K. mikimotoi* blooms.

2.2.5. Accompanying data sets for environmental samples

Climatic data, such as from the advanced very high resolution radiometer (AVHRR) that has been used to determine the 4 km monthly sea-surface temperature (SST) and front images from 1985-present day was provided courtesy of Dr Peter Miller.

2.2.6. Molecular Protocols

2.2.6.1. DNA extraction from dinoflagellate cultures and K. mikimotoi bloom samples

Filters from the environmental bloom samples (section 2.2.3.2) were placed in Petri dishes and rinsed with 2 ml filtered seawater until the cells were clearly dislodged from the filter surface. The cell suspensions were then transferred to 2 ml Eppendorf tubes and were pelleted by centrifugation (4000 ×g) for 5 minutes at room temperature.

Total genomic DNA extracted from the cell pellets of both dinoflagellate cultures (Tables 2.3 and 2.4) and the environmental samples (section 2.2.3.2) (provided by Dr. Andrea Highfield, MBA) using the DNeasy blood and tissue kit (Qiagen) (www.qiagen.com), following the manufacturer's instructions.

2.2.6.2. DNA extraction from mixed strains of *K. mikimotoi* cultures

Pellets from mixed cultures (section 2.2.2) were extracted using a standard chloroform:phenol extraction according to Sambrook and Russell (2001). 500 µl of Lysis buffer (Table 2.1) was added to 500 µl of cell suspension, mixed well, incubated at 55 ° C for 1 hour and then cooled to the room temperature. 1x volume of 24:1 (v/v) chloroform:isoamyl alcohol was added to the mixture and mixed by inversion followed by phase separation by centrifugation at 10,000 rpm for 5 minutes. The upper phase was removed and replaced with 0.25 volume 7.5 M ammonium acetate, left for 30 minutes at room temperature and then centrifuged again at 14,000 rpm for 10 minutes. Then cold 100% Ethanol was added to the supernatant up to the top and kept in freezer for 2hrs-

overnight to precipitate the DNA centrifuged at 14,000 rpm for 30 minutes. Then the supernatant was discarded and 200 µl of 70% of Ethanol was added to the pellet and centrifuged at 14,000 rpm for 15 minutes. Finally the DNA pellet was air dried, re-suspended in 20 µl TE buffer. The DNA concentration was determined and the samples were stored as described in Section 2.2.6.4.

2.2.6.3. DNA extraction from formalin-preserved CPR samples

Total nucleic acids from the selected CPR samples were extracted using the modified chloroform: phenol extraction method described by Ripley et al., (2008). This method entails cutting the CPR silk mesh into small (<1 cm x 1 cm) segments and placing them in a clean tube (Figure 2.1). Sterile TE buffer (Table 2.1) was added to the samples which were then vortexed vigorously for 1 minute to dislodge plankton from the silk mesh. Samples were then incubated, with shaking, at room temperature overnight.

The dislodged plankton was collected by centrifugation and divided into two replicate samples after supernatant removal. Recovered cells were resuspended in a lysis buffer containing 50 µl of 10 % (w/v) SDS (sodium dodecyl sulphate in distilled, deionized water, Promega), 12.5 µl of proteinase K (10 mg/ml) (Thermo Scientific) and 1 ml fresh TE buffer, and incubated with shaking overnight at 55 °C. The next day, a further 50 µl of 10 % SDS and 25 µl of proteinase K were added and the samples were incubated with shaking at 55 °C overnight. A standard phenol:chloroform nucleic acid extraction was then undertaken with an equal volume of TE-saturated phenol followed by TE-saturated phenol and 24:1 (v/v) chloroform:isoamyl alcohol, followed by at least three rounds of extraction with chloroform:isoamyl alcohol (24:1 (v/v)) until the aqueous phase was clear. Any

protein that had not been removed was precipitated out using 0.25 volume 7.5 M ammonium acetate, left for 30 minutes at room temperature and then centrifuged again at 14,000 rpm for 10 minutes. Then cold 100% Ethanol was added to the supernatant up to the top and kept in freezer for 2 hrs-overnight to precipitate the DNA centrifuged at 14,000 rpm for 30 minutes. Then the supernatant was discarded and 200 µl of 70% of Ethanol was added to the pellet and centrifuged at 14,000 rpm for 15 minutes. Finally the DNA pellet was air dried, re-suspended in 20 µl TE buffer. The DNA concentration was determined and the samples were stored as described in Section 2.2.6.4.

2.2.6.4. DNA quantification

A Nanodrop™ 1000 spectrophotometer (Thermo Scientific, Labtech International Ltd, UK) was used the measure absorbance at 260 nm from which the DNA concentration was calculated: purity was assessed using the ratio of the absorbance at 260 and 280 nm. Aliquots of DNA were kept at 4 °C for immediate PCR analysis and replica stocks were held at -20 ºC and -80 ºC for long-term storage.

2.2.6.5. Polymerase Chain Reaction (PCR)

2.2.6.5.1 Primer selection

Eight different sets of oligonucleotide that amplify nuclear and plastid genes were obtained (Eurofins MWG Operon) based on various literature reviews (Table 2.5). These were tested for their efficacy in separating the different *K. mikimotoi* strains (Table 2.3) and closely

related dinoflagellates (Table 2.4) in the culture collection. Several conditions were used in an attempt to optimize the reactions for each primer set: various DNA quantities and both oligonucleotide and magnesium concentrations were trialled.

Ultimately, three sets of primers (with their optimised PCR conditions) were selected and used to genotype *K. mikimotoi* strains. The first set of primers rDNA LSU (D1R-F and D2C-R) amplifies the hyper-variable D1-D2 regions of the nuclear LSU rDNA, which has been successfully used in the past to speciate dinoflagellates (section 3.2.4). This primer set was used here to genotype both the *K. mikimotoi* strains (Table 2.3) and also all the other dinoflagellate species in our culture collection (Table 2.4), both to confirm their identity and to allow comparisons to previously published sequences held in the GenBank (National Center for Biotechnology Information (NCBI)) (www.ncbi.nlm.nih.gov/). The second set of primers (EITS2 DIR and EITS2 REV) was used to allow analyses based on the rDNA the intergenic regions, encompassing the ITS1, 5.8S and ITS2 (section 3.2.5). Finally, the third set of primers (*rbcL* 640F and *rbcL* 1240R, Uni1) was chosen as it targets the plastid gene encoding the large subunit of Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco); a gene previously used to resolve inter- and intra-species variation amongst other dinoflagellates (section 3.2.5). The annealing temperature for the universal nuclear rDNA LSU primers was 60 °C and the rDNA ITS and *rbc*L (*rbc*L 640-F and *rbcL*1240-R) it was optimized to 56 °C.

2.2.6.5.2. Primer design

Three specific primer sets (Table 2.3, Figure 2.2) were designed for use in this study to allow characterisation of intra-specific variation within *K. mikimotoi*. The design of these

primers was informed by the consensus *rbcL* sequence for *K. mikimotoi* isolates from Japan, New Zealand, European and unknown locations. The Kmitrbcl-F1 and Kmitrbcl-R1 (Sp1) primer pair was designed for HRM analysis (Chapter 3.2.7). This primer pair was also used later as universal (Uni2) in nested PCR assays for amplifying DNA fragments from formalin-preserved CPR samples (section 5.2.3). The HRM primers (Kmitrbcl-HRM-F1 and Kmitrbcl-HRM-R1) primer pair was also used for HRM analysis and was designed (Figure 2.2) to amplify a smaller (117 bp) amplicon than Kmitrbcl-F1/Kmitrbcl-R1 pairing. A third forward primer, Manal125bp-F (Sp2) was designed (Figure 2.2) to be used with Uni1 reverse primer (*rbcL* 1240R) for further genotyping the resulted European *K. mikimotoi* strain from the HRM-curve analysis (section 5.2.3, Figure 5.13).

The PCR primer pairs used to amplify the D1 and D2 LSU rDNA domains was redesigned (section 5.2.3) to allow amplification from CPR material based on sequence data of dinoflagellates obtained from our culture collection and sequences deposited in GenBank. Re-designing this primer set was essential as current evidence suggests that for CPR preserved material PCR amplicons of around 200 bp (D. C. Schroeder, pers. comm.) or around 100 bp for older samples (Vezzulli et al., 2011) are necessary to maximize reaction yields. The same forward universal LSU primer (D1R) was used, but the reverse primer was re-designed as D2newR primer (Table 2.5) with an annealing temperature of 50 °C.

2.2.6.5.3. Testing Primer specificity

For the newly designed primers, the oligonucleotides were of ~20 bases long and had a G+C content of ~50 %. The specificity and efficacy were subsequently using Primer 3 and BLAST. They were then synthesized by Eurofins MWG GmbH (EbersbergGermany).

Primers were tested on the dinoflagellate species (Table 2.4) held in culture collection, including those taxa closely related to *K. Mikimotoi*, to ensure their specificity. The optimal annealing temperature for these primers was determined by either manually testing different annealing temperatures or by using the thermal gradient option in PCR thermocycler (CG1-96 Corbett, Life Science, QIAGEN), maximizing their specificity. The PCR was then repeated using the "optimal" annealing temperature determined from the thermal gradient plus/minus 1° C to check the true optimality of the conditions.

Table 2. 6.Primers used in this study.

Primer	Organelle	Target	Specificity	Primer sequence (5'– 3')	Amplicon size (bp)	Reference
D1R-F	Nuclear	28S ribosomal Large Subunit (LSU)	Universal	ACCCGCTGAATTTAAGCATA	760	Lenaers et al. (1989)
D2C-R				CCTTGGTCCGTGTTTCAAGA		
D2newR				TTCCCTCATGGTACTTGT (18)	341	This study
ssu-F	Nuclear	18S ribosomal Small Subunit (SSU)	Universal	CTG(CT)(AG)(AGCT)A(ACT)(AG)GCTCATTA(AGCT)A(AGCT)(AC)	600	This study
ssu-R				TGCCTTCCTT(AG)GA(AT)GT(AG)G(CT)(AC)GC		
EITS2 DIR	Nuclear	ITS-regions (ITS1, 5.8S rRNA, ITS2)^	Universal	GTAGGTGAACCTGC(AGC)GAAGA	710	Guillou et al. (2002)
EITS2 REV				TGGGGATCCTGTTTAGTTTC		
rbcL 640-F (Uni1)	Plastid	RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase)	Universal	ATGATGAAAA(CT)ATTAATTCTCAACC	619	Yoon et al. (2002a)
*rbcL*1240-R (Uni1)				TG(AT)CC(AG)AT(AGT)GTACCACCACC		This study
Kmitrbcl-HRM-F1 (HRM primer)			Specific	CCGTTTATGCGTTA(CT)CGAGA	117	
Kmitrbcl-HRM-R1 (HRM primer)				CTCCATTGTTGCCGCAGTTA		This study
Kmitrbcl-F1 (Sp1 & Uni2)			Optimized	TAACTGCGGCAACAATGGAG	276	
Kmitrbcl-R1(Sp1 & Uni2)				AATTTACCAACAACGGTTCC		This study
Manal125bp-F (Sp2)			Specific	TGGGCTTCTCT(CT)CGTAAG	125	
RbcL090F	Plastid	RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase)	Universal	CCATATGC(CT)AAAATGGGATATTGG	680	Yoon et al. (2002b)
RbcL770R				ATACATTT CTTCCATAGTTGC		
PsaA130F	Plastid	photosystem I P700 chlorophyll *a* apoprotein A1	Universal	AAC(AT)AC(AT)ACTTGGATTTGGAA	800	Yoon et al. (2002b)
PsaA930R				CCCAATTAGTTCTATACAT(AG)T		
PsaA840F	Plastid	photosystem I P700 chlorophyll *a* apoprotein A1	Universal	TT(CT)AAAGGATGG(AGCT)(CT)TAAA(CT)CC	760	Yoon et al. (2002b)
PsaA1600R				GCATGAATATG(AG)TG(AT)ACCAT		
PsbA-F	Plastid	photosystem II reaction center protein D1	Universal	ATGACTGCTACTTTAGAAAGACG	600	Yoon et al. (2002b)
PsbA600R				CCAAATACACCAGCAACACC		
M13-F *			Cloning	GTAAAACGACGGCCAGT	*	TA Cloning kit
M13-R				CAGGAAACAGCTATGAC		

* Size of amplicon depends on size of insert cloned, ^ Internal transcribed spacer 1 (ITS1), 5.8S rRNA and Internal transcribed spacer 2.

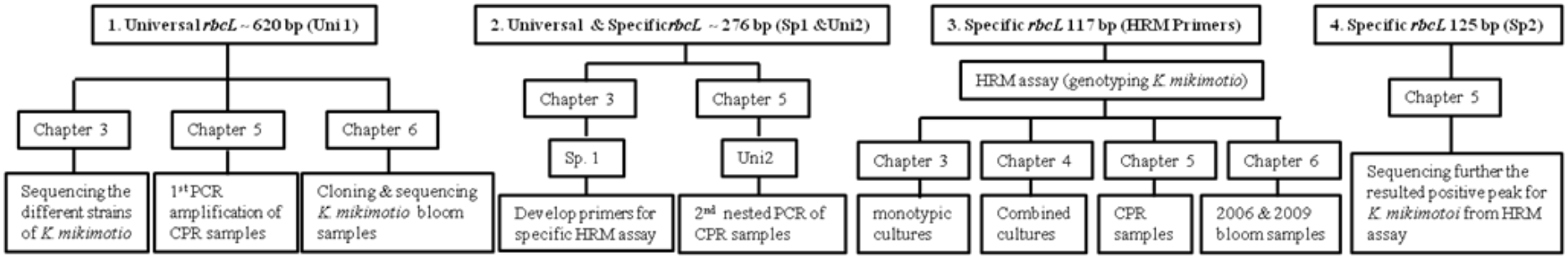

Figure 2. 2. The study plan for the partial *rbcL* sequences: 1. Universal (*rbcL* 640F and *rbcL* 1240R, Uni1), 2. Universal and Specific (Kmitrbcl-F1 and Kmitrbcl-R1, Sp.1 and Uni2), 3. Specific HRM primers (Kmitrbcl-HRM-F1 and Kmitrbcl-HRM-R1) and 4. Specific (Manal125bp-F and *rbcL* 1240R, Sp.2).

2.2.6.5.4. PCR reactions and conditions

All amplification reactions in the present study were performed using a MWG Biotech Primus 96 Plus Thermocycler. Reactions were carried out in 50 µl volumes, containing a range of template DNA (1-5 µl) based on DNA quantification per sample, 1 × *Taq* Buffer (Promega), 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 20 pmol forward primer, 20 pmol reverse primer, 1 Unit *Taq* polymerase (Promega) and molecular biology grade water (Sigma-Aldrich, UK). A typical reaction was as follows: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C - 64 °C (optimized for each pair of primers and also if required for each sample, specifically the different dinoflagellate taxa) according to the primer set (Table 2.5) for 1 minute and extension at 72 °C for 1 minutes, and a final extension step at 72 °C for 5 minutes. Finally, the reactions were cooled down to 15 °C and the PCR products were resolved by electrophoresis (section 2.2.6.6).

2.2.6.5.5. PCR-High Resolution Melting (HRM) analysis

HRM primers (Kmitrbcl-HRM-F1 and Kmitrbcl-HRM-R1) were designed for the analysis of different isolates of *K. mikimotoi* (Table 2.3) and field samples using HRM-curve analysis. For the mixed culture and bloom samples we used either bulk DNA as a direct template or a PCR amplicon obtained using the Uni1 *rbc*L 640-F and *rbcL*1240-R primer pair. For the CPR samples, a PCR amplicon from the Kmitrbcl-F1 and Kmitrbcl-R1 primer set (Table 2.5) was used to ensure enough PCR product for the HRM analysis. In all cases, the 1st round PCR amplicons were cleaned using exonuclease ExoSAP-IT (USB,

Germany) prior to HRM analysis to remove any single-stranded DNA that could have interfered with the HRM analysis. The HRM PCR was performed using the SensiMix HRM kit (Quantace) containing 12.5 µl 1× SensiMix HRM, 1 µl 25× of EvaGreen dye or SyberGreen dye (to test both of them and select the best for this study), 20 pmol each of the forward and reverse primers in a final volume of 25 µl. PCR was carried out using the Corbett Rotor-Gene 6000 (Corbett Life Sciences) under the following cycling conditions: 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 30 seconds, 64 °C for 1 minute and 72 °C for 1 minute, followed by a final extension step at 72 °C for 5 minutes, with fluorescent data being acquired at the end of each extension step using the green channel. Post-PCR, HRM-curve analysis of the amplicons was performed to assess amplification specificity and to identify sequence variation. DNA melting was performed using a temperature ramping rate from 70 °C to 85 °C, rising by 0.1°C each cycle with an acquisition on the HRM channel following each temperature increment. PCR products were also gel verified (section 2.2.6.6).

2.2.6.6. Agarose gel electrophoresis

The quality and characteristics of bulk DNA and PCR products from cultures, field samples and initial amplification reactions prior to HRM analysis were assessed by agarose gel electrophoresis. Hi-Res standard agarose (Bioprodcuts ltd) at a concentration of 1.6 % (w/v) was dissolved in an appropriate volume of 1 × TAE buffer (Table 2.1) by heating to boiling point in a microwave oven until it completely dissolved and became clear. The solution was cooled to approximately 60 °C and Ethidium bromide (EtBr) was added to a final concentration of 0.5 µg ml^{-1}. The solution was then poured into a gel

former and allowed to solidify at room temperature. Samples were loaded in the gel after mixing them with orange G loading buffer (Table 2.1). Electrophoresis was performed in 1 × TAE buffer at 120 volts for 35-60 minutes, until the loading dye has travelled 2/3 of the way through the gel. The gels were visualized on a UV transilluminator, and photographed with a Gel Doc 2000 system (Bio-Rad). Band sizes were estimated using 50 bp DNA ladder (BioLabs, UK), HyperLadder I and II (Bioline, UK).

2.2.6.7. Cloning

Amplicons from dinoflagellate cultures (Table 2.3 and 2.4) that could not be sequenced directly were cloned. The Uni1 *rbcL* amplicons of the archived CPR samples that were identified as being from *K. mikimotoi* blooms using HRM analysis were then subjected to nested PCR using the Sp2 primers Manal125bp-F and *rbcL*1240-R, and the resulting amplicons cloned. Moreover, the nested Uni2 PCR products from the CPR samples that gave negative results in the HRM-curve analysis were also cloned. The selected Uni1 *rbcL* amplicons of *K. mikimotoi* bloom samples for Station 1 (0 m), Station 2 (40 m) and Station 3 (0 m) (Figure 6.1A and B) collected in 2006 was also cloned.

PCR products were separated by electrophoresis and the bands corresponding to the desired product were excised from gels using sterile scalpels and extracted from the agarose using the QiaexII gel extraction kit (Qiagen). These products were ligated into cloning vectors, transformed into competent cells, plated onto LB-agar (Table 2.2) and cloned using the TA cloning kit (Invitrogen) according to manufacturer's instructions. Clones containing inserts were picked with sterile pipette tips and suspended in 5 µl

molecular biology grade water. They were then incubated at 95 °C for 5 minutes and then cooled to 21 °C. Cloned amplicons were identified by PCR using the M13 primer pair (Table 2.5) using the standard PCR conditons (section 2.2.6.5.4) in a 50 µl final volume reactions using 5 µl of crude DNA template. The PCR reactions were performed as follows: 30 cycles of denaturation at 95 °C for 45 seconds, annealing temperature at 56 °C for 45 seconds, extension at 72 °C for 45 seconds and finally 72 °C for 5 minutes.

2.2.6.8. DNA sequencing

PCR amplicons (direct and cloned) were prepared for sequencing using ExoSAP-IT (USB, Germany), following manufacturer's instructions, to remove any single-stranded DNA. PCR products were sequenced, in both directions, using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Manchester, UK) according to manufacturer's instructions, but at $1/8^{th}$ dilution. Sequencing reactions were performed using the MWG Biotech Primus 96 Plus thermocycler, with an initial denaturation at 96 °C for 1 minute, followed by 25 cycles of 96 °C for 10 seconds, 55 °C for 5 seconds and 60 °C for 4 minutes.

Sequencing products were cleaned using EDTA/ethanol precipitation according to Applied Biosystems instructions and were sent to GeneService (www.geneservice.co.uk) (Cambridge) for sequencing. Sequence data was verified manually by eye for base-calling errors and the consensus sequences were assembled and aligned for each of the respective amplicons with similar and/or related DNA sequences held in the GenBank (www.ncbi.nlm.nih.gov/) using the BioEdit program (www.bioedit.com). New sequence

data for the different strains of *K. mikimotoi* were submitted to GenBank under accession numbers (HM807311-HM807347).

2.2.6.9. Phylogenetic analysis

The three sequenced loci (LSU rDNA, ITS and Uni1 *rbc*L) were concatenated for each *K. mikimotoi* isolate (Table 2.1), *K. selliformis* (CAWD79) and two strains of *K. bidigitata* (CAWD81 and 92) (Table 2.2), and used for phylogenetic analysis. Clone libraries for the Uni2 (chapter 5) and Uni1 (chapter 6) *rbc*L sequences of *K. mikimotoi* blooms were also used for phylogenetic analysis. Sequences that occurred only once, when compared to the dominant genotype in the same clone library, were considered to have random substitutions which could have occurred during PCR and/or sequencing. These were then combined together with the dominant genotype. If any individual substitution was repeated in more than one clone then this sequence was treated as new genotype. If any of the substitutions in the clone sequences introduced a stop codon, these were removed from clone libraries. Clone sequence from CPR samples (Chapter 5) were given a name of the primers used (e.g. Sp2 or Uni2), followed by the year of sampling and genotype number. Clone sequence from 2006 bloom samples (chapter 6) were given a name indicative of station number, year of sampling and clone number that retrieved at the time of picking clones. Each analysis included sequences generated in this study together with other related and or similar sequences from GenBank, recognized via using BLAST in the NCBI. These sequence data were then aligned and compared using multiple sequence alignments in BioEdit (www.bioedit.com). Phylogenetic analyses were performed for both the concatenated (*K. mikimotoi* isolates, Figure 3.1) and individual gene alignments

(bloom clone libraries using the *rbcL*) using the various programs in PHYLIP (Phylogeny Inference Package) version 3.68. The robustness of the alignments was tested with the bootstrapping option using 1000 replicates (SeqBoot) and with Bayesian analysis (MrBayes version 3.1.2) (Huelsenbeck and Ronquist, 2001, Ronquist and Huelsenbeck, 2003, Ronquist et al., 2005). Genetic distances, applicable for distance matrix phylogenetic inference, were calculated using the dnadist program in the PHYLIP package. Phylogenetic inferences based on the distance matrix (Neighbor) (N) (1000 replicates) and parsimony (dnapars) (DP) (100 replicates) was applied to the alignments. In all trees, the best tree or majority rule consensus tree was selected using the consensus program (Consense). Trees were visualized and drawn using the TreeView software version 2.1.

Bayesian analysis was done using the command lset nst=6 rates=invgamma, which sets for the evolutionary GTR model (a General Time Reversible model) with gamma (G) distribution to describe rate variation among sites in a sequence. The sampled generation and frequencies numbers were determined based on the number of the sequences that were used to infer the phylogenies. Information about the likelihood of the applied chains was reported for every 100 sample frequencies of 2 x 10^4 generations in case of small number of sequences and every 1000 sample frequencies of a range of 2-7 x 10^5 generations based on the large number of sequences. Consequently, the analyses ran until the convergence diagnostic, the average standard deviation of split frequencies, reached a value below 0.01 (for more precise results) or 0.05 (if the alignment contained the probability of poorly supported groups). After the analysis was completed, the sum*p* sum*t* commands were used to summarize the information and obtain sample trees in the parameter files. The percentage of identity/similarity for any two aligned similar sequences with either short or

long branch length in distance phylogeny was calculated using the pairwise alignment in BioEdit.

2.2.6.10. Rarefaction curve analysis

To assess whether a representative number of clones per clone library generated in the present study had been sequenced a rarefaction analysis was undertaken. Rarefaction compares the taxon richness in the studied samples. It allows the calculation of the taxon richness for a given number of sampled individuals and allows the construction of so called rarefaction curves. This curve is a plot of the number of taxa (clones) as a function of the number of individuals sampled. If the curve approaches an asymptote, a reasonable number of individuals has been sampled, i.e. more intensive sampling is likely to yield only few additional species (Gotelli and Colwell, 2001). Coverage of the clone libraries was estimated using the Good's coverage estimator (Good, 1953), based on the equation by Hulbert (1971): Good's Coverage Estimator = [1-(n/N)] × 100, Where n= Number of singletons and N=Total number of sequences analysed.

Chapter 3: Genotyping the harmful algal bloom species, *Karenia mikimotoi*

3.1. Introduction

It has long been recognised that *K. mikimotoi* constitutes a well-defined taxonomic grouping within the polyphyletic gymnodinoid dinoflagellates that possess fucoxanthin-derived carotenoid pigments rather than peridinin, the typical dinoflagellate carotenoid (Bjørnland and Tangen, 1979, Tangen and Bjørnland, 1981, Steidinger, 1990). The phenotypic plasticity within this group makes them notoriously difficult to assign to species, especially when phenotypic features are used in isolation. Consequently, many species from different geographical areas have been incorrectly classified at both the species and genus level, and this is certainly the case with the European, New Zealand and Japanese isolates of *Karenia mikimotoi* (see section 1.3.2).

There is a need for unambiguous taxonomic markers to underpin studies of differences in the biology and ecology of potentially toxic and non-toxic *K. mikimotoi* isolates/strains. A molecular based assay that reflects the observed biological differences reported in the *K. mikimotoi* species complex (see section 1.6), could be used to screen complex environmental samples in a reliable and rapid manner and would advance our knowledge of the ecology of this HAB species enormously. Molecular assays using both short and long specific oligonucleotides have been developed and are widely used for the monitoring and rapid detection of harmful algae (Godhe et al., 2001, Gray et al., 2003), for example, oligonucleotides have been designed to detect intra- and inter-species variation within a closely related HAB species, *Karenia brevis* (Gray et al., 2003).

High resolution melting (HRM)-curve analysis of amplified gene fragments is a post-PCR technique that is increasingly being used for rapid genotyping, variant scanning and sequence-matching, minimising the requirement for costly labelled probes and sequencing. Target sequence amplification proceeds in the presence of a novel saturating fluorescent dye (e.g. EvaGreen dye) and the resultant PCR products are then subjected to gradual melting. Each unique amplified sequence will have a different melting behaviour and, as such, sequences with only a single base pair difference, may be differentiated (Graham et al., 2005, Odell et al., 2005 , Erali et al., 2008). This technique has been used recently for genotyping harmful and/or toxic micro-algal species such *Pseudo-nitzschia* spp. (Bacillariophyceae) (Andree et al., 2011) and *Cochlodinium polykrikoides* (Dinophyceae) (Park and Park, 2010).

In this chapter I describe newly characterised genetic differences between *K. mikimotoi* strains from different geographical origins and how these have been exploited to develop a molecular assay that can routinely discriminate between them.

3.2. Results

3.2.1. DNA extraction and quantification

Total genomic DNA from the cell pellets of 11 strains of *K. mikimotoi* (Table 2.3) and 24 strains of related dinoflagellate species (Table 2.4) was successfully extracted and quantified (data not shown).

3.2.2. Selecting study primers and PCR conditions

Various primer sets (Table 2.5) were used to screen an initial subset of algal cultures in our culture collection (Table 3.1). Different PCR conditions were trialled to optimise amplicon detection and specificity, including varying the quantity of DNA per reaction and altering the annealing temperature for each primer set. The universal nuclear rDNA LSU primer pair, D1R & D2C, gave a better amplification success rate than the universal nuclear SSU rDNA primer set when amplifying fragments of the rDNA from strains in our culture collection (Table 3.1).

The *Karenia* specific nuclear ITS region (EITS2 DIR & EITS2 REV) and plastid rbcL (*rbcL* 640-F & *rbcL*1240-R) primer sets both gave good amplification specificity for our target *Karenia* species. This was, however, not the case for the four other *Karenia* specific plastid primer gene sets tested (Table 3.1). The *rbcL* 090F & 770R, *psaA* 130F & 930R and *psaA* 840F & 1600R primer sets failed to generate amplification products from any of the strains tested, and although the *psbA* F & 600R primer set did appear to work with *Karenia mikimotoi*, but not *Karenia brevis*, the PCR product was likely non-specific: surprisingly, these primers allowed the generation of an amplicon in the distantly related haptophyte *Emiliania huxleyi* (Table 3.1).

3.2.3. Cloning and sequencing

Because of the specificity of amplification it was possible to sequence most amplicons directly in both directions, however, some required cloning before sequencing. Sequence identities were checked in GenBank (www.ncbi.nlm.nih.gov/) and new sequences were submitted under accession numbers (HM807311-HM807347) (Appendix 1).

Table 3. 1. PCR results for the different primer sets tested on the microalgal culture collection.

Primer set	Target	*Alexandrium minutum*	*Alexandrium tamarense*	*Amphidinium carterae*	*Karlodinium micrum*	*Karlodinium venificum*	*Heterocapsa triquetra*	*Karenia brevis*	*K. mikimotoi* (AC213)	*K. mikimotoi* (MBA561)	*K. mikimotoi* (K260)	*K. mikimotoi* (CCMP429)	*K. mikimotoi* (CAWD63)	*Emiliana huxleyi*
SSU rDNA (18ScomF1&18ScomR)	universal	-	-	-/+	-	-	-	-	-/+	-/+	-/+	-/+	-/+	+
LSU rDNA (D1R & D2C)	universal	+	+	+	+	+	+	+	+	+	+	+	+	+
EITS2 DIR & EITS2 REV	*Karenia* sp.	-	-	-	-	-	-	+	+	+	+	+	+	-
rbcL 640-F & *rbcL*1240-R	*Karenia* sp.	-	-	-	-	-	-	+	+	+	+	+	+	-
*rbcL*090F & *rbcL*770R	*Karenia* sp.	-	-	-	-	-	-	-	-	-	-	-	-	-
*psaA*130F & *psaA*930R	*Karenia* sp.	-	-	-	-	-	-	-	-	-	-	-	-	-
*psaA*840F & *psaA*1600R	*Karenia* sp.	-	-	-	-	-	-	-	-	-	-	-	-	-
psbA-F & *psbA*600R	*Karenia* sp.	-	-	-	-	-	-	-	*	*	*	*	*	+

- = no PCR product, + =PCR product, -/+ = did not work with standard PCR conditions but amplicon produced with optimised PCR conditions, * = multiple amplicons; primer set abandoned. Culture designations: *Alexandrium minutum* = AC194, *A. tamarense* = CAWD592, *Amphidinium carterae* = AC208, *Karlodinium micrum* = CCCM734, *K. venificum* = CCMP2936, *Heterocapsa triquetra* = CCCM 900, *Karenia brevis* = CCMP2229, *Emiliania huxleyi* = CCMP1516.

3.2.4. Genotyping *K. mikimotoi* Strains using the rDNA LSU

Sequence information for an approximately 760 bp rDNA LSU amplicon was obtained for all the strains of *K. mikimotoi* (Appendix 1, Figure 1). A further 12 LSU rDNA partial sequences for *K. mikimotoi* were acquired from GenBank, three of which were re-sequenced in our study (CAWD05, CAWD63 and CCMP429). For ease of comparison, all sequences were compared to the Japanese isolate CAWD05 (Table 3.2). All the sequences from the *K. mikimotoi* strains used in this study were identical to those assigned to Group 1 in previous studies (Hansen et al., 2000, Daugbjerg et al., 2000, Guillou et al., 2002, Haywood et al., 2004). Notably, Hansen et al. (2000) reported CCMP429 to have a single nucleotide substitution and thus classified it to be part of Group 2. Moreover, the strains assigned by other studies to Group 2 (all having different nucleotide substitutions), all have geographic representatives in Group 1 (Table 3.2).

Table 3. 2. Nucleotide substitutions detected in strains of *Karenia mikimotoi* compared to the Japanese isolate CAWD05: based on a 689 bp alignment of the D1 and D2 regions of the LSU rDNA.

Group	Accession number	ID code	Origin	Nucleotide Difference	Reference
1	U92247	CAWD05	Japan	0	Haywood et al. (2004) & This study
	AF200681	None	Japan	0	Haywood et al. (2004)
	AF318224	GA95TIN	France	0	Guillou et al. (2002)
	AF318270	Environmental	France	0	Guillou et al. (2002)
	AF200682	K0579	Denmark	0	Hansen et al. (2000)
	AF200679	K0286	Australia	0	Hansen et al. (2000)
	AY355460	NOAA2	USA	0	Goodwin et al. (2005)
	U92249	CAWD63	New Zealand	0	Haywood et al. (2004) & This study
	HM807325	MBA561	Japan	0	This study
	HM807326	CAWD117	New Zealand	0	This study
	HM807327	CAWD133	New Zealand	0	This study
	HM807328	CAWD134	New Zealand	0	This study
	HM807330	MBA705	UK	0	This study
	HM807331	K0260	Norway	0	This study
	HM807333	CCMP430	Unknown	0	This study
	HM807332	AC213	Unknown	0	This study
	AF200678/	CCMP429/	UK	1	Hansen et al. (2000)
	HM807329	PLY497a		0	This study
2	AF318223	GA87TIN	France	0	This study
	AF200680	KT77b	Norway	1	Hansen et al. (2000)
	EF469238	KMWL01	Australia	1	de Salas et al. (2008)

3.2.5. Genotyping *K. mikimotoi* Strains using the rDNA ITS-regions and *rbcL*

rDNA ITS sequence data was acquired for 11 strains of *K. mikimotoi* (Table 3.3), all of which produced a PCR amplicon of around 710 bp (Appendix 1, Figure 2 and 3). These sequences were compared to the three sequences in GenBank including two *K. mikimotoi* strains (GA87TIN and GA95TIN) collected from the same geographical region in 1987 and 1995 (Bay of Brest, France), respectively, and one isolate (NEPCC 665) from an unknown location (Table 3.3). Using the rDNA ITS sequences, *K. mikimotoi* could be divided into four distinct groups based on the common positions and number of nucleotide changes in comparison to the Japanese strain CAWD05: Group 1 comprised strains of Japanese origin, Group 2 strains from New Zealand, and Groups 3 and 4 strains of European origin. The New Zealand and European strains shared five identical substitutions in the ITS region when compared to the Japanese Group 1, except for the single additional nucleotide substitution in strain GA95TIN.

Sequence data from the 619 bp amplicon of *rbc*L revealed further nucleotide differences between *K. mikimotoi* strains originating from similar geographical regions (Appendix 1, Figure 4 and 5). The *rbc*L sequence comparisons could further separate what was three rDNA ITS groups into five groups (Table 3.4). The Japanese isolate CAWD05 (Group 1) possessed two nucleotide differences in comparison to the Japanese isolate MBA561 (Group 2). The four New Zealand strains in rDNA ITS Group 2 all grouped together in the new *rbc*L Group 3, sharing 43 substitutions when compared to CAWD05. The *rbc*L gene sequence for the AC213 isolate from an unknown location was identical to the strains of UK origin (Group 4), sharing 40 nucleotide substitutions in comparison to CAWD05. The

strains within Group 5 (K0260 and CCMP430), both had one more shared nucleotide substitution compared to Group 4, i.e. 41 substitutions when compared to CAWD05 (Table 3.4).

Table 3. 3. Nucleotide substitutions detected in strains of *Karenia mikimotoi* compared to the Japanese isolate CAWD05: based on a 671 bp alignment of the rDNA ITS regions and the groupings derived from rDNA LSU and ITS sequence analyses.

LSU Group	ITS Group	Accession number	ID code	Origin	Nucleotide Difference	Reference
1	1	HM807311	CAWD05	Japan	0	This study
		HM807312	MBA561	Japan	0	This study
	2	HM807313	CAWD63	New Zealand	6	This study
		HM807314	CAWD117	New Zealand	6	This study
		HM807315	CAWD133	New Zealand	6	This study
		HM807316	CAWD134	New Zealand	6	This study
	3	HM807318	CCMP429	UK	7	This study
		HM807317	MBA705	UK	7	This study
		HM807320	K0260	Norway	7	This study
		HM807321	CCMP430	Unknown	7	This study
		HM807319	AC213	Unknown	7	This study
		AF318223	GA87TIN	France, Bay of Brest, 1987	7	Guillou et al. (2002)
		FJ823564	NEPCC 665	Unknown	7	GenBank
2	4	AF318224	GA95TIN	France, Bay of Brest, 1995	8	Guillou et al. (2002)

Table 3. 4. Nucleotide substitutions detected in strains of *Karenia mikimotoi* compared to the Japanese strain CAWD05: based on a 574 bp alignment of the *rbc*L gene and the groupings derived from rDNA LSU, rDNA ITS and *rbc*L sequence analysis.

LSU Group	**ITS Group**	***rbcL* Group**	**Accession Number**	**ID code**	**Origin**	**Nucleotide Difference**	**Reference**
1	1	1	HM807334	CAWD05	Japan	0	This study
		2	HM807335	MBA561	Japan	2	This study
	2	3	HM807336	CAWD63	New Zealand	43	This study
			HM807337	CAWD117	New Zealand	43	This study
			HM807338	CAWD133	New Zealand	43	This study
			HM807339	CAWD134	New Zealand	43	This study
	3	4	HM807340	CCMP429	UK	40	This study
			HM807341	MBA705	UK	40	This study
			HM807342	AC213	Unknown	40	This study
		5	HM807343	K0260	Norway	41	This study
			HM807344	CCMP430	Unknown	41	This study

3.2.6. Phylogeny

Phylogenetic analysis was performed using the concatenated sequences of the three sequenced regions (rDNA LSU and ITS, and *rbc*L) as described in section 2.2.6.9. This analysis not only confirmed the isolate groupings outlined in Table 3.4, but also demonstrated the clustering of strains originating from European and New Zealand localities (Figure 3.1). Bootstrap values were high (> 97 %), supporting the topography of the tree and suggesting that the distinct phylogenetic clades representing the Japanese, New Zealand and European strains are robust.

3.2.7. Primer design and PCR-High Resolution Melting (HRM) analysis

Two specific HRM compatible PCR primers sets (Table 2.5) derived from the consensus *rbc*L sequence, were designed and used to determine whether HRM could be used as a tool to differentiate *K. mikimotoi* strains from different geographical regions. Kmitrbcl-F1 & Kmitrbcl-R1 (Sp1) was the first primer set tested (Figure 3.2). These primers amplified 276 bp amplicon to allow separation of the Japanese strains from the European and New Zealand strains using the 15 and 16 nucleotide substitutions present within this region. The European strains differed from the New Zealand strains at five positions and the two Japanese strains differed from each other by just a single substitution. The optimal standard PCR annealing temperature (Table 3.5) was 65 °C and the optimal template concentration was 10 ng per reaction (data not shown). However, after many trials of optimizing the HRM PCR reaction, by varying the DNA template, fluorescent dyes (EvaGreen and SyberGreen), amount of PCR cycles, this primer set failed to separate the four different genotypes of *K. mikimotoi* in a HRM-curve analysis (e.g. Figure 3.3).

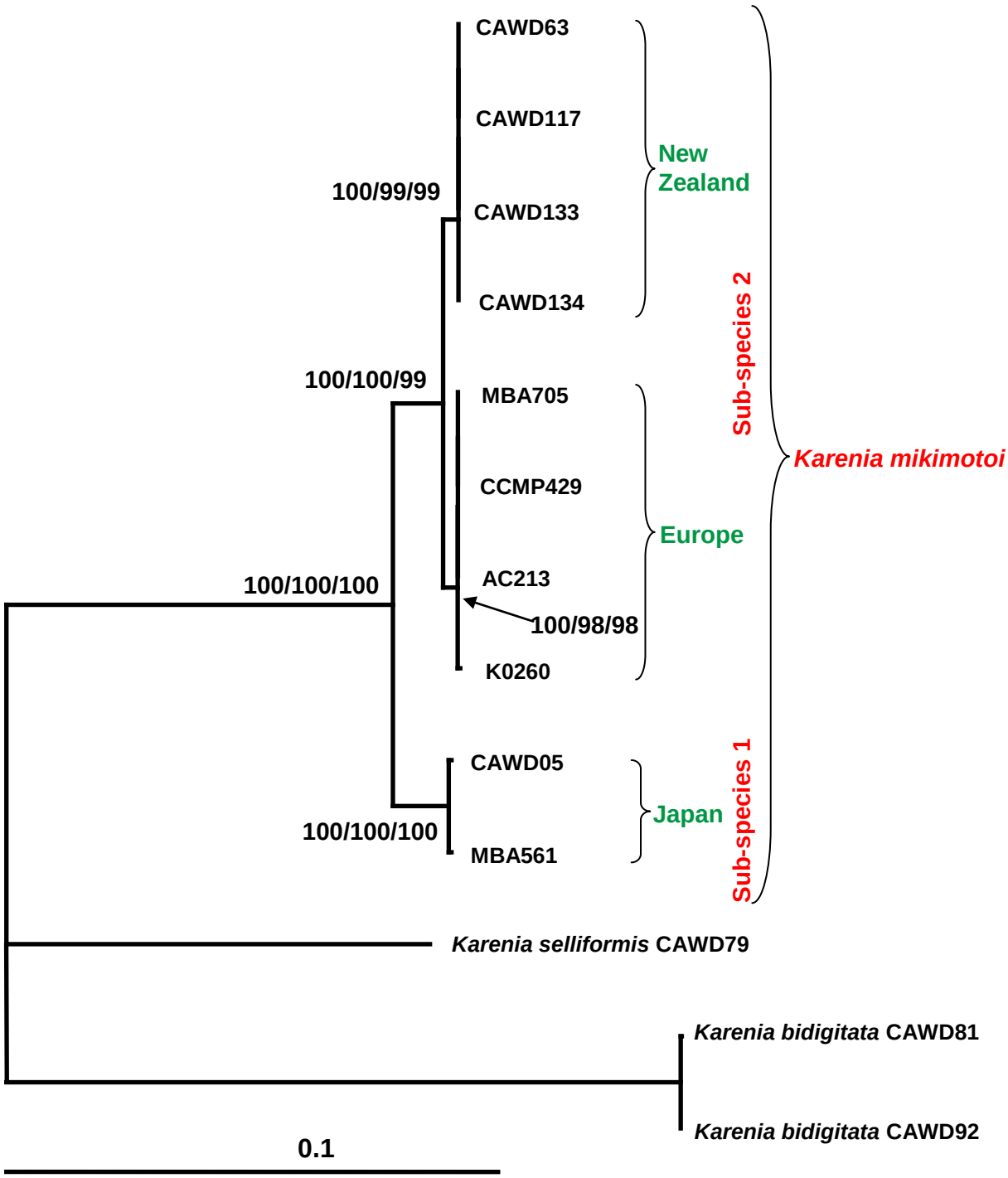

Figure 3. 1. Phylogenetic relationships among *Karenia* isolates revealed through analysis of concatenated sequences from the rDNA D1-D2 LSU, ITS and *rbc*L loci: inference tree based on a distance matrix algorithm (Neighbor, in PHYLIP version 3.69). Numbers at nodes indicate bootstrap values (%) retrieved from 1000 replicates for neighbor-joining, parsimony and maximum likelihood analyses. The bar depicts 1 base substitution per 10 base pairs.

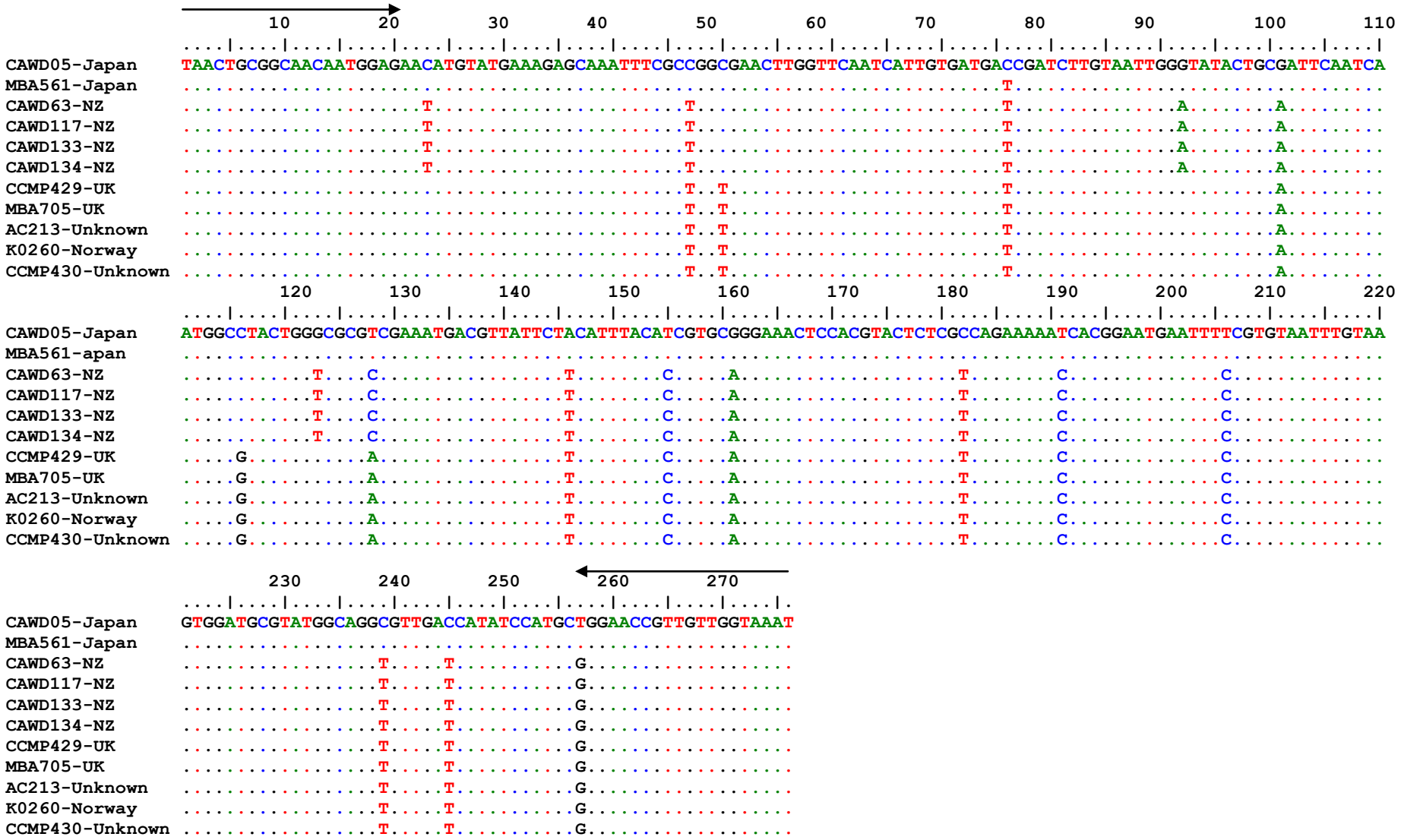

Figure 3. 2. Sequence alignment of a 276 bp fragment of the *rbc*L gene for *K. mikimotoi* strains. Positions of the specific forward (Kmitrbcl F) and reverse (Kmitrbcl R) primers are indicated by arrows. Dots correspond to identical nucleotides.

Table 3. 5. The optimization of the PCR annealing temperature for the *Karenia mikimotoi* specific *rbc*L primer set Kmitrbcl-F1 and Kmitrbcl-R1.

No.	Strain no.	Strain name	PCR Annealing temperature				
			50° C	55 ° C	60 ° C	64 ° C	65° C
1	AC215	*Akashiwo sanguinea*	+	+	+	+	-
2	AC194	*Alexandrium minutum*	+	-	-	-	-
3	CAWD592	*Alexandrium tamarense*	+	-	-	-	-
4	AC208	*Amphidinium carterae*	+	-	+	+	-
5	CAWD87	*Gymnodinium aureolum*	+	+		-	-
6	CCCM 900	*Heterocapsa triquetra*	+	-	-	-	-
7	CAWD92	*Karenia bidigitata*	-	-	-	-	-
8	CCMP2281	*K. brevis*	+	+	-	-	-
9	CCMP2229	*K. brevis*	+	-	-	-	-
10	CCMP718	*K. brevis*	+	-	-	-	-
11	CAWD82	*K. brevisculata*	-	-	-	-	-
12	CAWD81	*K. bidigitata*	-	-	-	-	-
13	CAWD79	*K. selliformis*	+	-	-	-	-
14	CAWD131	*K. umbela*	+	+	+	-	-
15	CAWD115	*Takayama tasmanica*	-	-	-	-	-
16	CCCM734	*Karlodinium micrum*	-	-	-	-	-
17	CCMP2936	*Karlodinium venificum*	-	-	-	-	-
18	CCMP416	*Karlodinium venificum*	-	-	-	-	-
19	CCMP1516	*Emiliania huxleyi*	+	+	+	+	-
20	CAWD05	*K. mikimotoi*	+	+	+	+	+
21	1st Negative	-	-	-	-	-	-
22	2nd Negative	-	-	-	-	-	-

21 and 22 = are negative controls with no template DNA.

The best resolution that could be achieved using this primer set (Figure 3.2) and HRM analysis (Figure 3.3) was the separation of the Japanese strains from the New Zealand and European strains with distinct melting temperatures of 83.17 °C and 82.52 ± 0.05 °C respectively: this primer set was unable to differentiate between the two Japanese strains or between the European and New Zealand strains.

The second primer set, Kmitrbcl-HRM-F1 & Kmitrbcl-HRM-R1, was designed to amplify a 117 bp region that should separate of four out of the five *rbc*L groups (Figure 3.4). This new HRM primer set showed absolute specificity to *K. mikimotoi* (Figure 3.5) when tested under specified PCR conditions and failed to generate amplicons from both closely related dinoflagellates (Table 2.4) and the haptophyte *Emiliania huxleyi*. The annealing temperature range was initially determined by using a thermal gradient PCR machine (Corbett, Life Science) with a 50 ° C to 70° C gradient. The highest amplication specificity was achieved at 64.3 ° C, which was then confirmed by re-testing the primers against other strains from the culture collection by using 62 ° C and 64 ° C annealing temperatures: 64 ° C was confirmed to be the optimum (Figure 3.4). The specificity was further tested on *K. mikimotoi* strains from Japan, New Zealand and UK using HRM-curve analysis. The different *K. mikimotoi* strains could be resolved in HRM-curve analysis when using the EvaGreen dye (Figure 3.6), however, the strains could not be separated when SyberGreen was used. The HRM-curve analysis showed distinct peaks corresponding to the geographic origin of the strain (Figure 3.6): the two Japanese strains, MBA561 and CAWD05, produced melt temperatures of 76.63 ºC and 77 ºC, respectively; melt peaks of 78.27 ± 0.03ºC and 77.94 ± 0.02 ºC represented strains of New Zealand and European origin, respectively. Differences in peak melting temperatures were maintained when the assay was repeated (n=20), confirming that the technique is able to consistently discriminate between different strains.

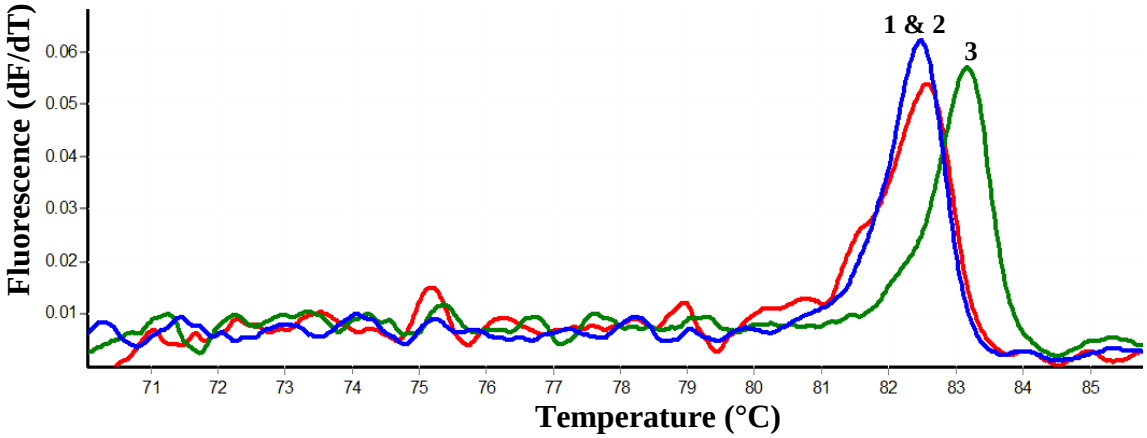

Figure 3. 3. High resolution melt (HRM) curve analysis using Sp1 *rbcL* primers (Kmitrbcl F and Kmitrbcl R) for a representative set of different *K. mikimotoi* strains originating from (1) Europe (CCMP429, blue peak), (2) New Zealand (CAWD134, red peak) and (3) Japan (CAWD05, green peak).

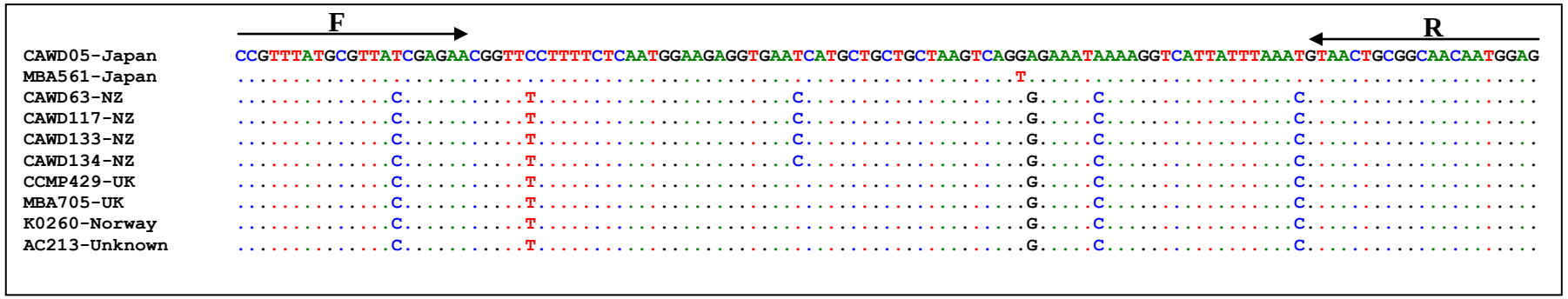

Figure 3. 4. Sequence alignment of a 117 bp fragment of the *rbc*L gene for *K. mikimotoi* strains. Positions of the HRM forward (Kmitrbcl-HRM-F1) and reverse (Kmitrbcl-HRM-R1) primers indicated by arrows.Dots correspond to identical nucleotides and letters correspond to any nucleotide substitutions.

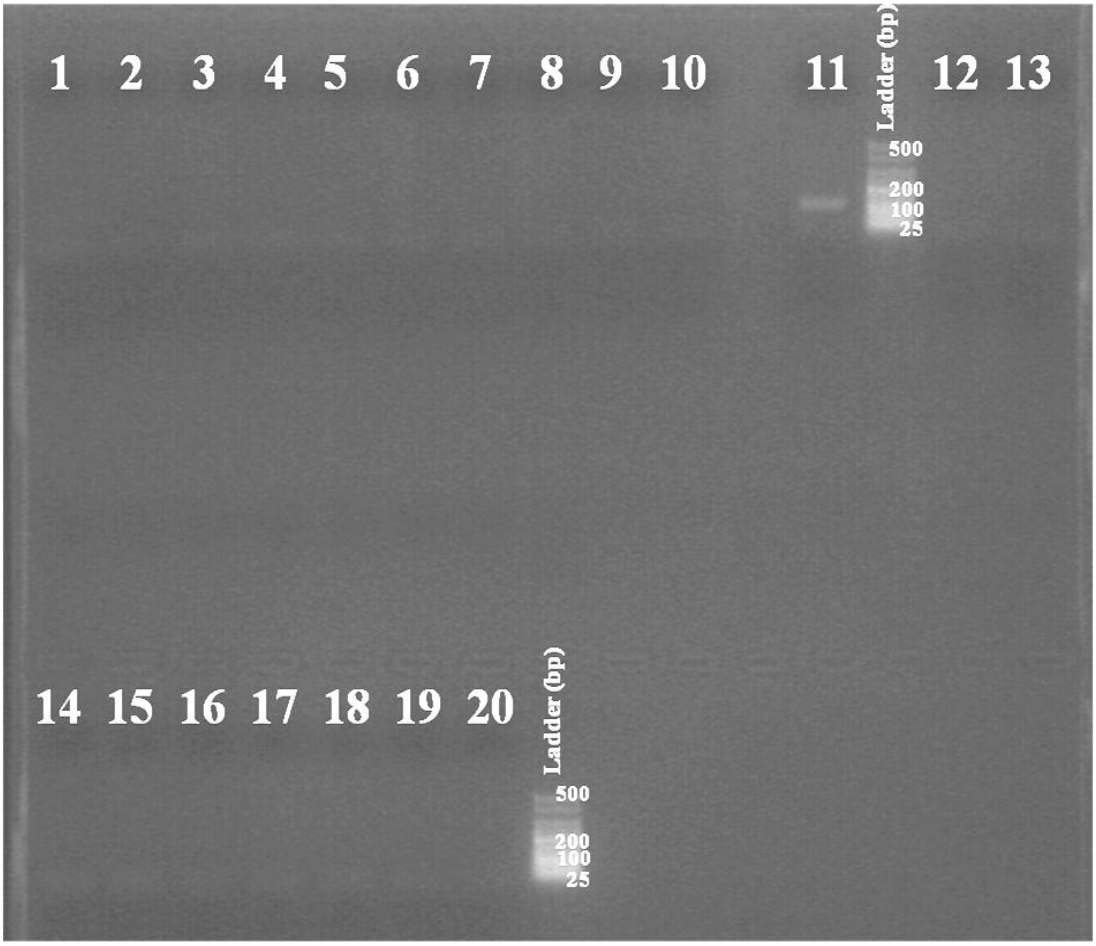

Figure 3. 5. Agarose gel electrophoresis (1.6 % [w/v]) showing amplicon size of 117 bp, produced using the HRM species-specific primers and template DNA from *K. mikimotoi* and other non-target species.1. *Akashiwo sanguínea* (AC215), 2. *Alexandrium minutum* (AC194), 3. *Alexandrium tamarense* (CAWD592), 4. *Amphidinium carterae* (AC208), 5. *Gymnodinium aureolum* (CAWD87), 6. *Heterocapsa triquetra* (CCCM 900), 7. *Karenia bidigitata* (CAWD92), 8. *K. brevis* (CCMP2281), 9. *K. brevis* (CCMP2229), 10. *K. brevisculata* (CAWD82), 11. *K. mikimotoi* (CAWD05), 12 and 13. Negative control, 14. *K. selliformis* (CAWD79), 15. *K. umbella* (CAWD131), 16. *Takayama tasmanica* (CAWD115), 17. *Karlodinium micrum* (CCCM734), 18. *Karlodinium venificum* (CCMP2936), 19. *K. bidigitata* (CAWD81), 20. *Emiliana huxlyei* (CCMP1516).

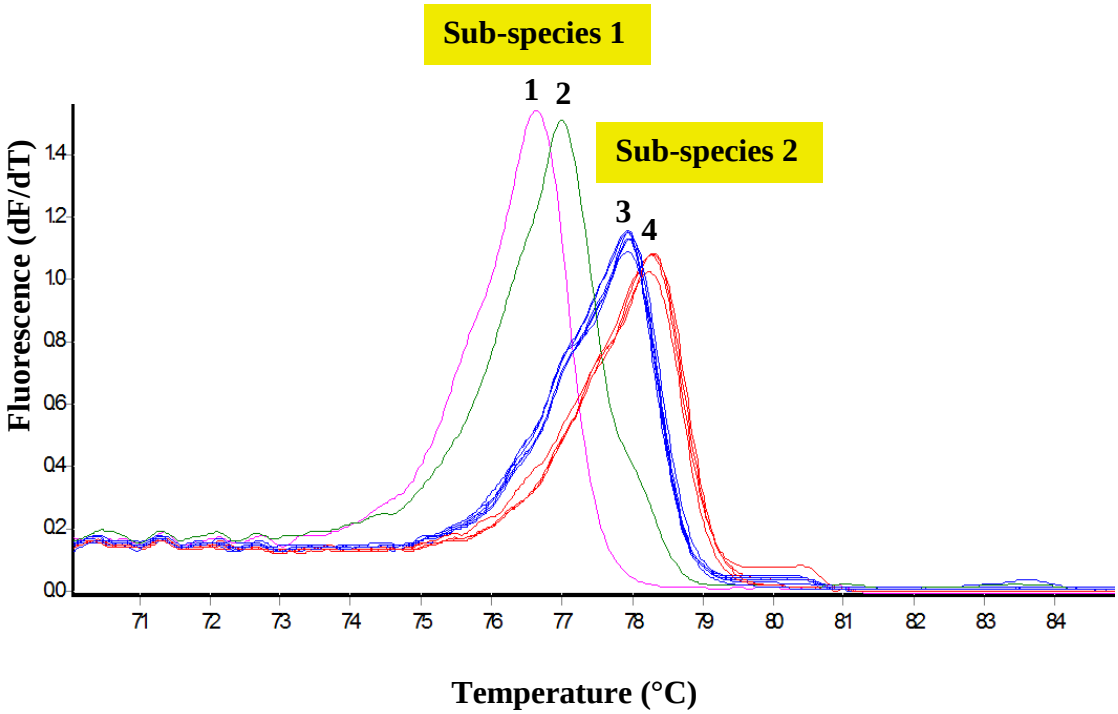

Figure 3. 6. High resolution melt (HRM) curve analysis using the HRM primers (Kmitrbcl-HRM-F1 & Kmitrbcl-HRM-R1) for a representative set of different *K. mikimotoi* strains originating from sub-species 1: (1) Japan (MBA561), (2) Japan (CAWD05) and sub-species2: (3) Europe (CCMP429, MBA705, K-0260 and the predicted European isolate AC213) and (4) New Zealand (CAWD63, CAWD117, CAWD133 and CAWD134).

3.3. Discussion

The sequence analysis of the D1 and D2 regions of the rDNA LSU revealed no differences between any of the *K. mikimotoi* strains. To a large extent, this observation is consistent with observations of Hansen et al. (2000) that the rDNA LSU is highly conserved between strains, however, they and others (Hansen et al., 2000, Haywood et al., 2004, Guillou et al., 2002) reported a single nucleotide substitution in this region supporting the division of *K. mikimotoi* into two groups. Our observations suggest that the D1-D2 region LSU rDNA is likely to be identical for most if not all strains, and that the previously recorded substitutions could be due to PCR or sequencing errors. This is based on the re-sequencing of the same strains, and strains from similar localities, all of which had identical sequences. All analyses of the rDNA LSU confirm that these strains are very similar and share a common ancestor and as such should be considered to belong to the same species, *Karenia mikimotoi*.

Guillou et al. (2002) reported nucleotide differences within the rDNA ITS regions between two French *K mikimotoi* strains collected eight years apart (first in 1987 and second in 1995) from a similar geographical location. Our sequencing analysis included a wider range of *K. mikimotoi* strains and could discriminate between four groups within *K. mikimotoi* on the basis of a number of rDNA ITS nucleotide differences. These groups correlated with geographical origin, however, the two French strains (based on previous study) were placed in different groups. Three strains from unknown locations, including two analyzed in this study and one obtained from GenBank, were found to be identical to the European strains, indicating their likely origin. As anticipated, the rDNA ITS regions revealed more diversity than the rDNA LSU.

The *rbc*L amplicon sequences showed even greater variation than the rDNA ITS and provided further support for geographical differentiation among strains. Interestingly, the *rbc*L gene sequence analysis separated the two Japanese strains MBA561 and CAWD05 on the basis of two nucleotide differences. In addition, the *rbc*L gene sequence for another Japanese isolate deposited in GenBank (Accession number AB034635) showed 21 nucleotide substitutions when compared to CAWD05 over a different but partially overlapping region of the *rbc*L gene (data not shown). No sequence information for the rDNA LSU or ITS loci is available for this isolate, but if it is confirmed to be *K. mikimotoi*, then the type locality for this species appears to support a wider degree of genetic diversity than has been previously recognised. The *rbc*L locus has previously been shown to be suitable for resolving intra- and inter-specific variations within the related red tide organism *Karenia brevis* (Gray et al., 2003), and we report here that it is equally suited to distinguish between *K. mikimotoi* strains.

In addition to the differences seen between the two Japanese strains, the New Zealand and European strains show numerous nucleotide substitutions, 43 and 40-41 respectively, in comparison to CAWD05, which was substantially higher than for the other loci characterised in this survey. Furthermore, the sequence identity for strains CCMP430 and K0260 indicate that these are one and the same. Tangen deposited *K. mikimotoi*, designated CCMP430, in the Provasoli-Guillard National Center for Culture of Marine Phytoplankton in 1985 (Table 2.3). It is highly likely that he submitted the same isolate, collected in 1977 in Norway, in the **S**candinavian **C**ulture **C**entre for **A**lgae and **P**rotozoa as K0260.

Due to the depth of taxonomic confusion surrounding *K. mikimotoi*, the ecological significance and consequences of its presence in various habits cannot yet be understood. One view is that the European *K. mikimotoi* is an alien, invasive species. It was first

misidentified as *Gymnodinium aureolum* due to the morphological similarities that it shared with the North American *Gymnodinium aureolum*, but was later reclassified it as *K. mikimotoi*, which was originally described in Japan, where it is a known fish-killer. The explanation given at the time for the dispersal of this species from Japan was the transplantation of Japanese oysters to France in 1960s, which led to the first bloom occurring in 1966 in Norway, however, it could have arrived as early as 1865 from a completely different location (in Harmful Algal News, no. 40, http://www.ioc-unesco.org/hab/). As a streak of coloured sea water has been reported along the coast off southwest Ireland in 1865 at the end of the American Civil War which is thought to be an early *Karenia* bloom.

K. mikimotoi could, however, be a native and not an alien species at all. Studies have described *K. mikimotoi* as cosmopolitan and able to tolerate a wide range of temperatures and salinities (Gentien, 1998), making it possible that this species has always been in waters other than those found off Japanese coasts. The well documented rapid change in global climate over the past five decades could be the driving force behind the *K. mikimotoi* blooms now seen in European waters since the 1960s. The changing environments in which these different *K. mikimotoi* strains inhabit now allow them to dominate and thus bloom. The mechanism is likely to be complex (removal of prey, pathogens, competitors, etc.), however, molecular and physiological differences could attest to adaptation events being of paramount importance. A combination of the two scenarios presented here is also feasible, with a complex mixture of introduced Japanese genotypes being eroded to just a few through local selections. This could explain the similarities between the European and New Zealand strains as they are more adapted to colder rather than the warmer Japanese waters.

Partensky et al. (1988) were the first to produce compelling evidence that cast doubt upon the conspecificity of all *K. mikimotoi* strains (Miyake and Kominami ex Oda) G. Hansen and Moestrup 2000. The authors suggested that this species should be split with the creation of both new genera and species. However, molecular data, such as that produced by Hansen et al. (2000), appeared to have re-enforced the concept of conspecificity. Our study produces molecular evidence supporting the physiological differences observed by Partensky et al. (1988) and Chang (1996). Phylogeny based on the concatenated rDNA LSU and ITS and *rbc*L genes definitively places the Japanese Group at the base of the *Karenia mikimotoi* clade, with the New Zealand and European Group diverging more recently. Certainly, further effort is required to screen more strains from these and other locations, however, our results are consistent with and support the separation of *K. mikimotoi* into two distinct sub-species: sub-species 1 - *Karenia mikimotoi mikimotoi* (the type species of Japanese origin previously assigned as *Gymnodinium mikimotoi* and *G. nagasakiense*) and sub-species 2 - *Karenia mikimotoi aureolum* (European and New Zealand strains previously assigned as *Gyrodinium aureolum* and *Gymnodinium* cf. *nagasakiense*).

The design of the HRM-curve analysis to separate Japanese, New Zealand and European strains of *K. mikimotoi* could help in resolving this matter. The melt-curve of this analysis showed three discrete melt temperature peaks, one each for the Japanese, New Zealand and UK strains. This HRM-curve analysis can be used as a genotyping screen for environmental samples collected from different oceanographic provinces and over time-scales of decades and can directly address whether the genotypes reported here existed previously in all locations.

Chapter 4: Parameterization of the HRM assay for detecting *K. mikimotoi* in mixed samples

4.1. Introduction

Understanding genetic diversity at the population level is important as this can provide important clues to the habitat or ecological niche of any given species e.g.(Zingone and Enevoldsen, 2000). Furthermore, developments in molecular techniques have provided opportunities to determine intra- and inter-species genetic structure within populations. In the context of HAB species, these technologies are dominated by DNA fingerprinting techniques, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and microsatellite analyses e.g. (Kamikawa et al., 2007, Evans et al., 2004, Alpermann et al., 2009, John et al., 2004, Barreto et al., 2011, Bolch et al., 1999, Medlin, 2007, Medlin et al., 2000).

Mitochondrial based PCR-RFLP assays was used to characterise levels of genetic diversity in the HAB species *Chattonella*, where strains have now been separated into two groups (Kamikawa et al., 2007). These two groups were isolated from two different areas with no correlation observed amongst their phylogeny with any of their morphology or study areas which is likely to be correlated with certain environmental responses. Similarly, both AFLP and microsatellite markers were used to study the toxic armoured dinoflagellate *Alexandrium tamarense* from the North Sea, where the use of both methodologies provided evidence for genetic differentiation (Alpermann et al., 2009). The authors linked the high genetic differentiation to the cyst bank in the sediment that formed over many years and are

able to germinate several years after their encystment. The simulataneous germination of different cohorts combined with sexual reproduction between them are responsible for unexpectedly high genetic diversity.

Microsatellite markers were also used to investigate the genetic structure of distinct populations of the diatoms *Pseudo-nitzschia multiseries* (Hasle) Hasle (Evans et al., 2004) and *Pseudo-nitzschia pungens* (Grunow *ex* P. T. Cleve) Hasle (Evans et al., 2005).

Intra- and inter-specific variation within HAB taxa has also been characterised using single gene loci. A PCR-high resolution melt (HRM) approach has been used to detect the nucleotide polymorphisms known to be present in candidate genes (Andree et al., 2011). In Chapter 3, the HRM melt curve analysis of *rbc*L PCR amplicons was used to differentiate *Karenia mikimotoi* into two distinct geographic sub-species (Al-Kandari et al., 2011). Sub-species 1 included only Japanese genotypes, while sub-species 2 comprised genotypes from both European (UK and Norway) and New Zealand waters. This technology has been applied to detect the toxic *Pseudo-nitzschia* species in environmental samples (Andree et al., 2011), highlighting the benefits of using single gene loci over fingerprinting techniques, as the former does not require the isolation of individuals, thereby making direct analysis of environmental samples feasible. Moreover, by working directly on environmental samples the well-documented bias of working on culturable, fast growing species is avoided (Moreira and López-García, 2002). However, when working with environmental samples other biases associated with both the differential lysis of strains and the preferential amplification of templates from particular strains or genotypes over others, have to be addressed.

This chapter parameterises a HRM assay for separating sub-species of *K. mikimotoi* genotypes in mixed samples. This was achieved by testing mixtures of genomic DNA from different cultured strains of *K. mikimotoi* and by using DNA extracted from cell suspensions where different genotypes had been mixed in known proportions prior to lysis.

4.2. Results

4.2.1. DNA extraction from individual *K. mikimotoi* isolates

DNA was extracted from six *K. mikimotoi* strains, namely the Japanese strains MBA561 (JP1) and CAWD05 (JP2), the British strains CCMP429 (UK1) and MBA705 (UK2), and the New Zealand strains CAWD134 (NZ1) and CAWD63 (NZ2) (Table 2.3). The DNA extraction protocol produced similar amounts of DNA per cell irrespective of the isolate (1.65 - 2.8 ng/10^{-4} cells), suggesting that the extraction protocol did not introduce any marked bias between strains.

4.2.2. HRM melt curve analysis on extracted DNA

K. mikimotoi can be separated into four distinct genotypes: JP1, JP2, UK and NZ (Chapter 3). Each of the four genotypes consistently produced unique melt temperatures (Tm), clearly visible as different melt-curve shapes in both the standard (normal fluorescence, data not shown) and derivative (dT/dF) HRM profiles (e.g. Figure 3.6 and Figure 4.1). Representatives of these 4 genotypes were used in every HRM assay as positive controls and as reference aids in comparing the resultant Tm for the mixed-sample tests.

When the DNA for either of the Japanese strains (JP1 or JP2) were mixed with either the UK1 or NZ1 strains, two distinct peaks corresponding to each particular strain within each sub-species were achieved (Figure 4.1B). Moreover, the Tm position of JP1 only deviated by 0.02 °C when it was combined with either the UK or NZ isolates (76.56 ± 0.02 °C). A similar observation was made with JP2, UK1 and NZ1 as they too had reproducible Tm values of 76.87 ± 0.02 °C, 77.76 ± 0.03 °C and 78.14 ± 0.01 °C, respectively (Figure 4.1B).

When various combinations of DNA from two strains within one sub-species were mixed together, their respective Tm peaks shifted (Figure 4.1C; yellow and green profiles). This also occurred when the two or more strains within one sub-species were mixed with two or more strains from the other sub-species (Figure 4.1C; orange profile). Moreover, the HRM melt curves could no longer distinguish between two strains within the same sub-species (e.g. Figure 4.1; yellow profile). The two Japanese strains merged to produce a new sub-group Tm of 76.72 ± 0.04 °C (Figure 4.1C; purple, red and orange profiles), while the other UK and NZ strains merged to produce a new Tm of 77.88 ± 0.08 °C (Figure 4.1C; yellow, green and orange profiles). The Tm peak for sub-species1 shifted on average +0.16 °C for JP1 and -0.15 for JP2. The Tm peak for sub-species 2 also shifted on average +0.12 for UK and -0.26 °C for NZ.

Despite the inability to separate two strains within one sub-species when they co-occurred in one sample, the HRM could still, nonetheless, reproduce the Tm of either member when they were the only sub-species members present (Figure 4.1C, purple and red profiles). The UK (purple profile) and NZ (red profile) strains still presented Tm's of 77.77 °C and 78.12 °C, respectively.

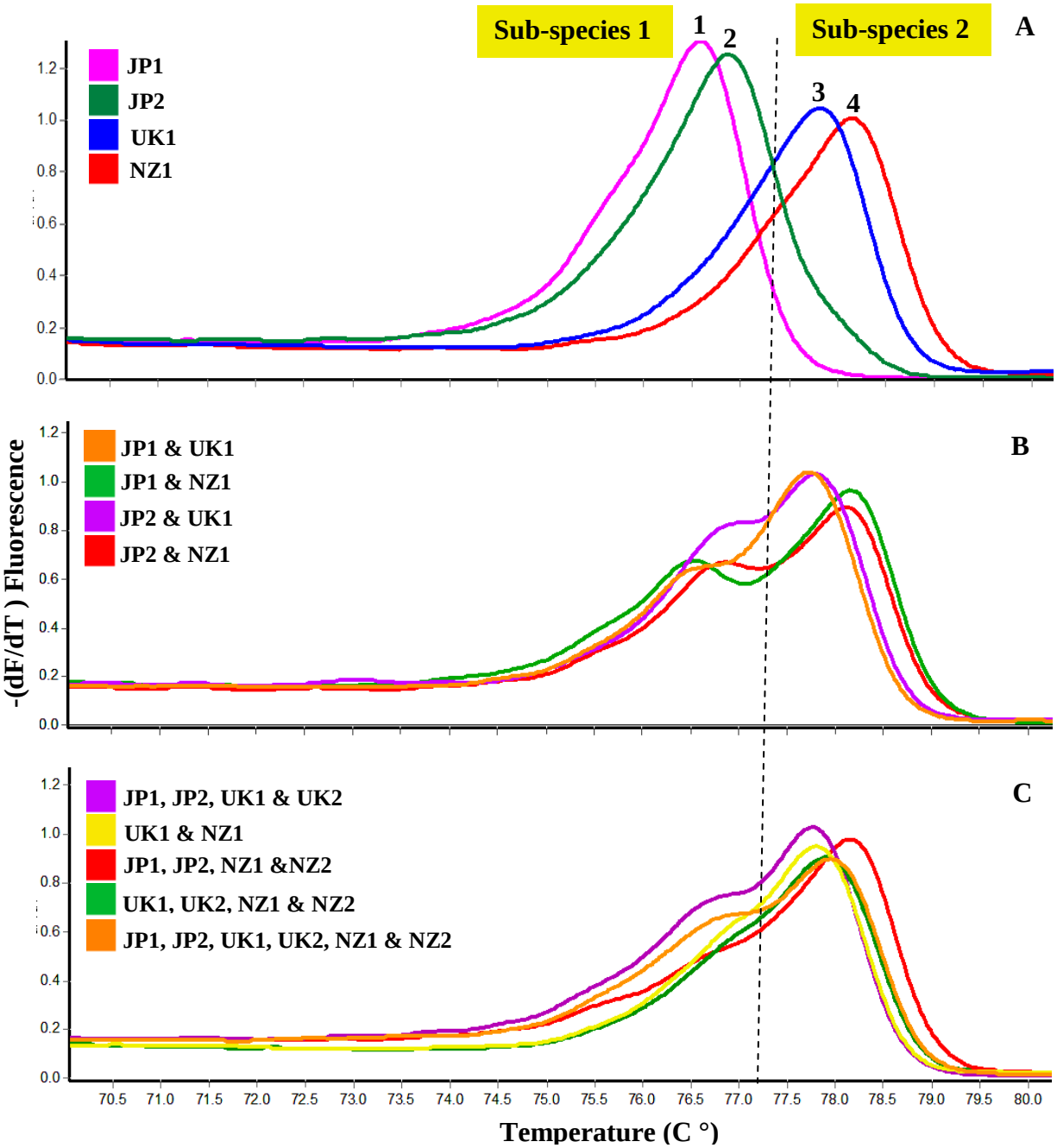

Figure 4. 1. Derivative HRM profiles showing the melt peaks for A) the four distinct genotypes of *K. mikimotoi* strains sub-species 1: 1. MBA561 (JP1/pink) and 2. CAWD05 (JP2/green) and sub-species 2: 3. CCMP429 (UK1/blue) and 4. CAWD134 (NZ1/red); B) the mixed-DNA samples (50:50) of *K. mikimotoi* strains: JP1 & UK1 (orange), JP1 & NZ1 (green), JP2 & UK1 (purple peaks) and JP2 & NZ1 (red); C) the mixed-DNA samples (50:50) of *K. mikimotoi* strains: JP1, JP2, UK1 & MBA705 (UK2) (purple), UK1 & NZ1 (yellow), JP1, JP2, NZ1 & NZ2 (red), UK1, UK2, NZ1 & CAWD63 (NZ2) (green) and JP1, JP2, UK1, UK2, NZ1 & NZ2 (orange).

4.2.3. HRM analysis on DNA extracted from mixed cultures

Four cultures of *K. mikmotoi* strains (JP1, JP2, UK2 and NZ1) were mixed to produce four sets of mixtures (10^3 +10^3, 10^4 + 10^4, 10^3 + 10^4 and 10^4+ 10^3 cells /ml) as described in section 2.2.2. Total genomic DNA was extracted as described in section 2.2.6.1.2 from the cell pellets of both the controls (single *K. mikimotoi* strain cultures) and the mixtures.

The two sets of low and high concentrations (10^3 and 10^4 cells/ml, respectively) for each of the individual genotypes produced standard and derivative HRM-melt curve profiles with unique Tm's consistent with the results obtained in previous section 4.2.2 (data not shown). The high concentration (10^4 cells/ml) was used as the reference for each genotype to compare the peaks from each sample (Figure 4.2A).

Each of the two Japanese strains (JP1 and JP2), with either low or high cell concentrations, when mixed equally with any of the UK or NZ strains showed two distinct Tm peaks that matched well with their corresponding controls (Figure 4.2B). JP1, JP2, UK1 and NZ1 had reproducible Tm positions of 76.75 ± 0.05 °C, 77.05 ± 0.05 °C, 77.94 ± 0.04 °C and 78.30 ± 0.03°C, respectively (Figure 4.1B). However, when the JP1 and JP2 were mixed equally (Figure 4.1B, blue profile), only one peak (76.95 °C) was evident, with a shift of +0.20 °C for JP1 and -0.10 °C for JP2. Similarly, when the UK and NZ strains were mixed equally they also showed only one peak (78.17 ± 0.01° C) between the NZ and UK strains (+0.23 °C to UK and -0.23 °C to NZ) (Figure 4.2B, dark green profile).

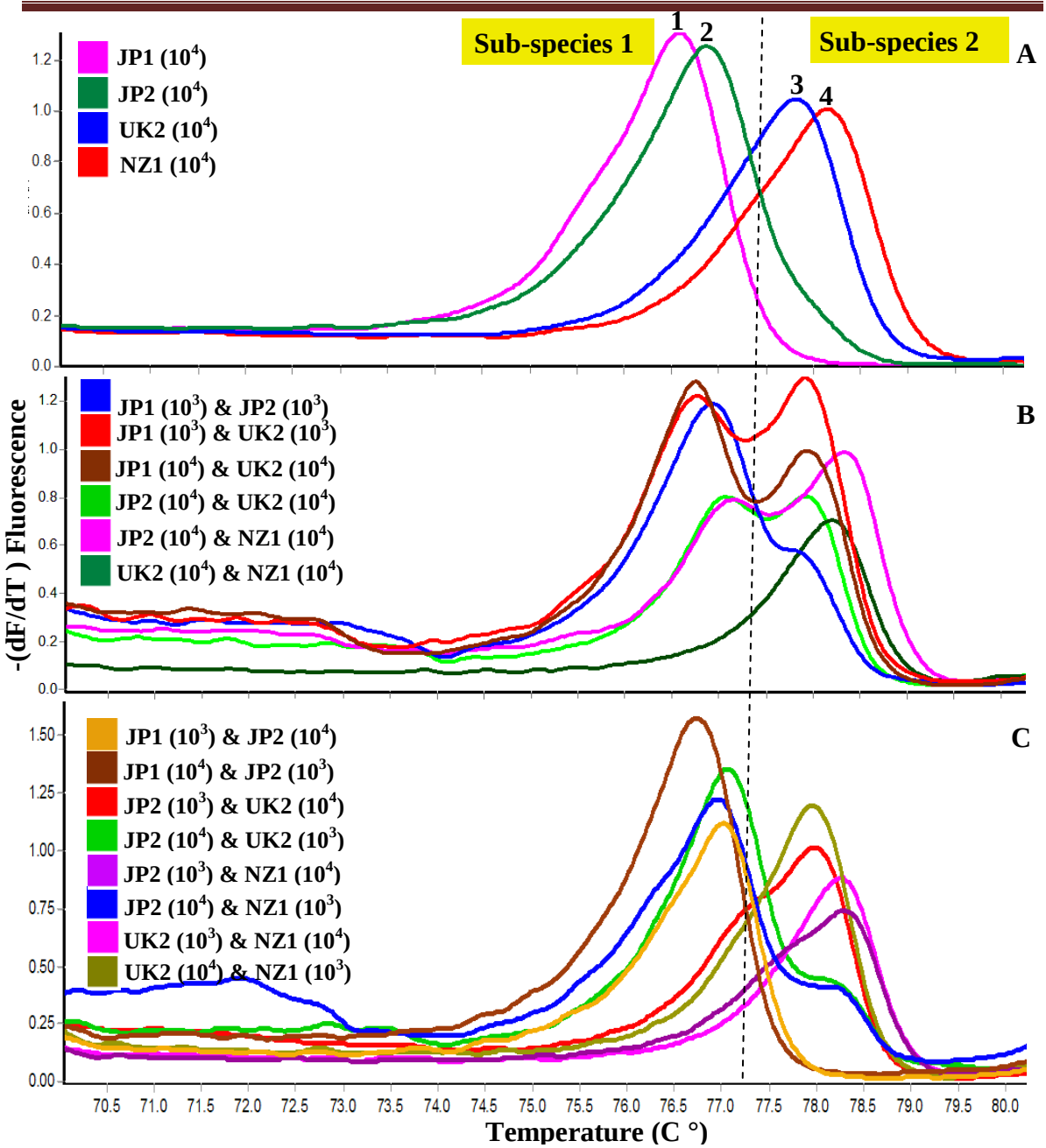

Figure 4. 2. Derivative HRM profiles of A) four distinct *K. mikimotoi* genotypes from DNA extracted at cell concentration of 10^4 cells/ml for sub-species 1: 1. MBA561 (JP1/pink) and 2. CAWD05 (JP2/green) and sub-species-2: 3. MBA705 (UK2/blue) and 4. CAWD134 (NZ1/red); B) DNA samples from mixed cell concentrations (equal numbers of each strain) of *K. mikimotoi* strains as indicated in parenthesis (cells ml^{-1}): JP1 & JP2 (blue), JP1 & UK2 (red), JP1 & UK2 (brown), JP2 & UK2 (green), JP2 & NZ1 (pink) and UK2 & NZ1 (turquoise); C) DNA samples from mixed cell concentrations (unequal numbers of each strain) of *K. mikimotoi* strains in parenthesis (cells ml^{-1}): JP1 & JP2 (light and dark brown), JP2 & UK2 (red and green), JP2 & NZ1 (purple and blue), UK2 and NZ1 (pink and turquoise).

The HRM assay was capable of detecting the dominant strain (the strain at the higher cell concentration) in asymmetric mixtures (Figure 4.2C; red, green, purple and blue profiles). The sub-species 1 (Figure 4.2C, light and dark brown profiles) and sub-species 2 strains (Figure 4.2C, pink and turquoise profiles) showed only one Tm peak, i.e that of the dominant strain.

Whether the HRM assays were performed on mixed DNA or DNA extracted from mixed cultures, the Tm's for individual strains did not differ by more than 0.06 ° C.

4.3. Discussion

HRM-curve analysis has successfully enabled the discrimination of the different strains of *K. mikimotoi* into four distinct genotypes on the basis of their *rbcL* sequence (Al-Kandari et al., 2011). But can this technique be used to unambiguously detect more than one genotype of *K. mikimotoi* in complex samples? Our findings showed that a single nucleotide substitution in the *rbc*L was not sufficient to allow discrimination between genotypes in mixed samples: whenever two members of either sub-species were present in the mixed sample, only a single merged peak was detected. The only exception was when one of the strains was dominant (10 fold more) in the mixed sample. This merged peak phenomenon highlights a major limitation of this HRM assay, at least for *K. mikimotoi* where the *rbc*L amplicon shows limited sequence divergence within a genetic sub-species.

The inter sub-species divergence, however, encompassed more than five base-pair substitutions. These nucleotide differences were sufficient to allow separation of the respective genotypes into their distinctive individual or mixed Tm peaks. Moreover, the

HRM curve analysis can detect the dominant strain (high cell concentration) semi-quantitatively. Knowing the limitations of the HRM assay, important information can still be gleaned when applying this molecular technique to environmental samples.

In conclusion, the HRM technique is a fast, cost-effective molecular tool for identifying the different genotypes of *K. mikimotoi* in culture collections, and can be used to detect more than one genotype when they exist together in the same sample, i.e. at the level of sub-species. The HRM assay therefore remains a valid and promising technique that can be used in HAB monitoring and mitigation programmes for rapid detection and accurate identification of the species that suddenly cause an exceptional harmful and/or toxic bloom.

Chapter 5: *Karenia mikimotoi* in the Celtic Sea shelf-break region

5.1. Introduction

The route of migration or movement of HAB species to new geographical regions and the associated increase in blooms has long been debated (Hallegraeff and Gollasch, 2006, Smayda, 2002, Smayda, 2007, Edwards et al., 2006). The transfer of species between different oceans through ship's ballast water and the importation of living fish, in particular the translocation of aquaculture products such as shellfish and finfish, have been proposed as the main mechanisms (Hallegraeff and Gollasch, 2006, Smayda, 2002, Smayda, 2007). Invasive species, including HAB species, can alter the composition of local phytoplankton communities (Occhipinti-Ambrogi, 2007, Reid et al., 2009) and in turn bring about important economic, and/or ecological challenges.

As discussed in Chapter 1, *Karenia mikimotoi* was first officially recorded in the English Channel in 1968 and was photographed as a small pigmented and unarmored dinoflagellate, however, it could have been present as early as 1966 within the recorded "unknown *Gymnodinium*" group in the Western Channel Observatory (Boalch, 1987). At about the same time a small dinoflagellate bloom in Norwegian waters was associated with fish kills (Braarud and Heimdal, 1970). In addition, Pingree et al. (1975) reported the first red-tide of *K. mikimotoi*, along the tidal fronts in the south-western approaches to the English Channel and Ushant front in July 1975. At that time of the year the bloom caused the water to turn reddish-brown in colour and was associated with another red-tide bloom forming dinoflagellate, *Noctiluca scintillans*.

Spectacular and exceptional blooms of *K. mikimotoi* were observed in the following years, i.e. in the summer of 1976 (Pingree et al., 1977) and again in August 1977 (Holligan, 1979). The first red tide in Ireland occurred in late July and early August in 1976 and was associated with mortalities of littoral and sub-littoral marine organisms (Ottaway et al., 1979). In 1978 another red-tide of *K. mikimotoi* was recorded off the south west coast of Ireland (Pybus, 1980), and at the same time a red tide occurred off the south coast of England (Boalch, 1979).

The European *K. mikimotoi* has been widely reported to be a non-indigenous species (also known as an alien, non-native, exotic, invasive or introduced species), due to the first description of this species in Japan in 1935 (Minchin, 2007, Pancucci-Papadopoulou et al., 2005, Hickel et al., 1971, Elbrächter, 1998) and its apparent absence elsewhere. The invasion of this species into the European waters was thought to be related to either the transplantation of Japanese oysters to France from 1966 onwards (van den Bergh et al., 2002) or to ballast water mediated transport (Gollasch and Nehring, 2006). In the current study, a HRM assay was shown to be able to both detect and separate *K. mikimotoi* isolates from different broad geographical regions into two sub-species (Chapter 3) (Al-Kandari et al., 2011): these findings were verified using extracted DNA from both the single and mixed genotype cultures of *K. mikimotoi* strains (Chapter 4). Deployment of this new technique provides us with an unprecedented opportunity to determine the genetic structure in past and recent *K. mikimotoi* blooms within the Celtic Sea shelf-break region and to examine whether or not the *K. mikimotoi* community has been affected by new introductions.

5.2. Results

5.2.1. Mapping the timing and locations of *K. mikimotoi* blooms

The timing and location of *K. mikimotoi* blooms in the Celtic Sea shelf-break region, from the first reported bloom in 1975 to a more recent bloom in 2006, are listed in Appendix 2, along with the related harmful effects, recorded cell densities, maximum *chl*-a concentration, references and any corresponding satellite images. The blooms differed in terms of the time of year (from June to October), spatial extent (three main areas), cell densities (10^4 to 10^7 cells/L) and whether or not they were recorded as toxic. The spatial variation of *K. mikimotoi* blooms was examined further using series of satellite-derived images of area l *chl*-a distributions (Appendix 3): the CZCS monthly mean *chl*-a images allowed for the spatial evaluation of the recorded blooms from August 1979 to July 1985 (Appendix 3, Figure 1 and 2); the daily SeaWiFS *chl*-a, SeaWiFS pseudo true-colour and MODIS Aqua *chl*-a images were used for blooms occurring from 1998 to 2006 (Appendix 3, Figure 3-10). The dates for these images were selected as examples for each recorded month and year when *K. mikimotoi* bloomed. The pseudo true-colour images were very useful as these were able to positively locate the areas of *K. mikimotoi* blooms on the basis of the typical dark-red to brown discoloration of the surface waters (e.g. Appendix 3, Figures 3-10). From these images, coccolithophore blooms could also be seen, i.e. characteristized by areas high reflectance. An example of this can be seen in Figure 9A where *K. mikimotoi* is a dark red to brown colour and *E. huxleyi* is white. Moreover, the areas with high *chl*-a, seen either by SeaWiFS or MODIS, correspond with the areas where *K. mikimotoi* occurred (Appendix 3). All the blooms in the period spanning 1998-2006 where both *in situ* and satellite data were available confirmed this correlation. The CZCS *chl*-a images showed increased *chl*-a concentration

with *K. mikimotoi* blooms in the satellite images; here to there was a positive correlation between *in situ* sampling data and remote sensing *chl*-a maxima (Appendix 3, Figure 1 & 2).

Historical records have enabled us to confirm the timing and locations of *K. mikimotoi* blooms in the Celtic Sea shelf-break (Chapter 1 and Appendix 2). This data showed that *K. mikimotoi* blooms in three main areas: (A) to the south of Ireland, (B) the western English Channel approaches and (C) the Ushant front and along the nearby French coast (Figure 5.1). The specific coordinates (Figure 5.1, blue dots) indicate the *in situ* observations in the areas where *K. mikimotoi* blooms occurred: A, B and C represent the areas that usually experience mass occurrences of *K. mikimotoi* as detected via satellite images. These *K. mikimotoi* blooms occurred mainly during late July-August (Appendix 2), however, in a few cases they occurred earlier, in June (such as in 2003 in the western English Channel), early July (such as in 1978 in the western English Channel) or continued into September (such as in 1979 and 1984 of southern Irish coast), (Appendix 2).

The timing of the blooms and the *chl*-a concentrations varied between the different areas. The SeaWiFS *chl*-a value data (1997-2008) selected for the three different areas in the Celtic Sea shelf-break region illustrate the significant contribution *K. mikimotoi* makes to primary productivity in the region (Figure 5.2). They were also chosen to show the typical spring (March-May), summer (June-August) and autumn (September-November) phytoplankton blooms that occurred annually. The highest *chl*-a peak in coordinates A and B correspond to the recorded dates for the presence of *K. mikimotoi* blooms (Appendix 2), for example, in the South of Ireland in 1998 and 2000 (Figure 5.2A), and the western English Channel approaches in 2000, 2002, 2003 and 2006 (Figure 5.2B).

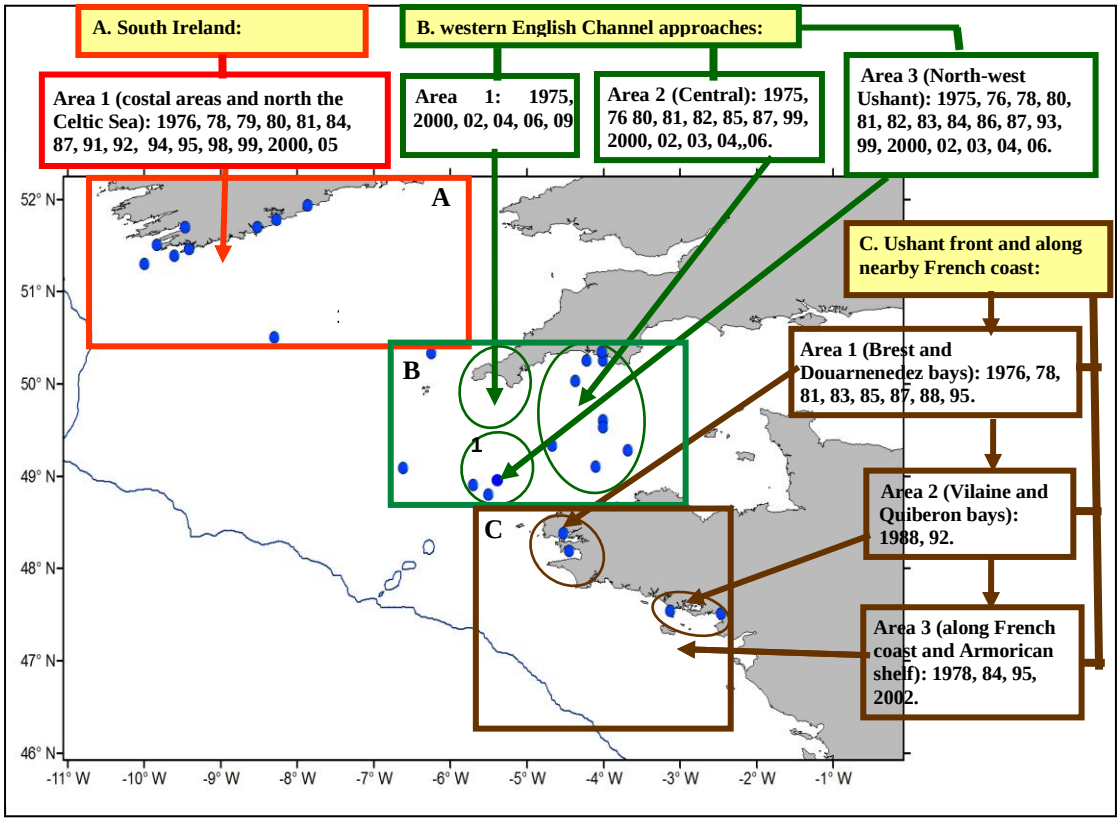

Figure 5. 1. Map of the timing and locations of the recorded *K. mikimotoi* blooms in the Celtic Sea shelf-break from 1975 to 2006. Three regions (highlighted with yellow) with their locations on the map were found to regularly experienced these blooms with the year of a bloom occurrence detailed. A. South Ireland (red box), one area (the coastal areas of the southern Ireland to the northern region of the Celtic Sea); B. western English Channel approaches (green box), including 3 areas: the south west UK/western English Channel (1), central English Channel (2) and the north-west Ushant/ western English Channel (3); C. Ushant front and along nearby French coast (brown box), including 3 areas: the Brest and Douarnenedez bays (1), Vilaine and Quiberon bays (2) and the French coast and Armorican shelf (3). Blue colour dots correspond to *in situ* sampling observations in each area. The circles (labelled 1, 2 and 3) in regions B and C are where regular mass occurrences of *K. mikimotoi* occur. The blue dots with attached dates correspond to one-off blooms within the three defined areas.

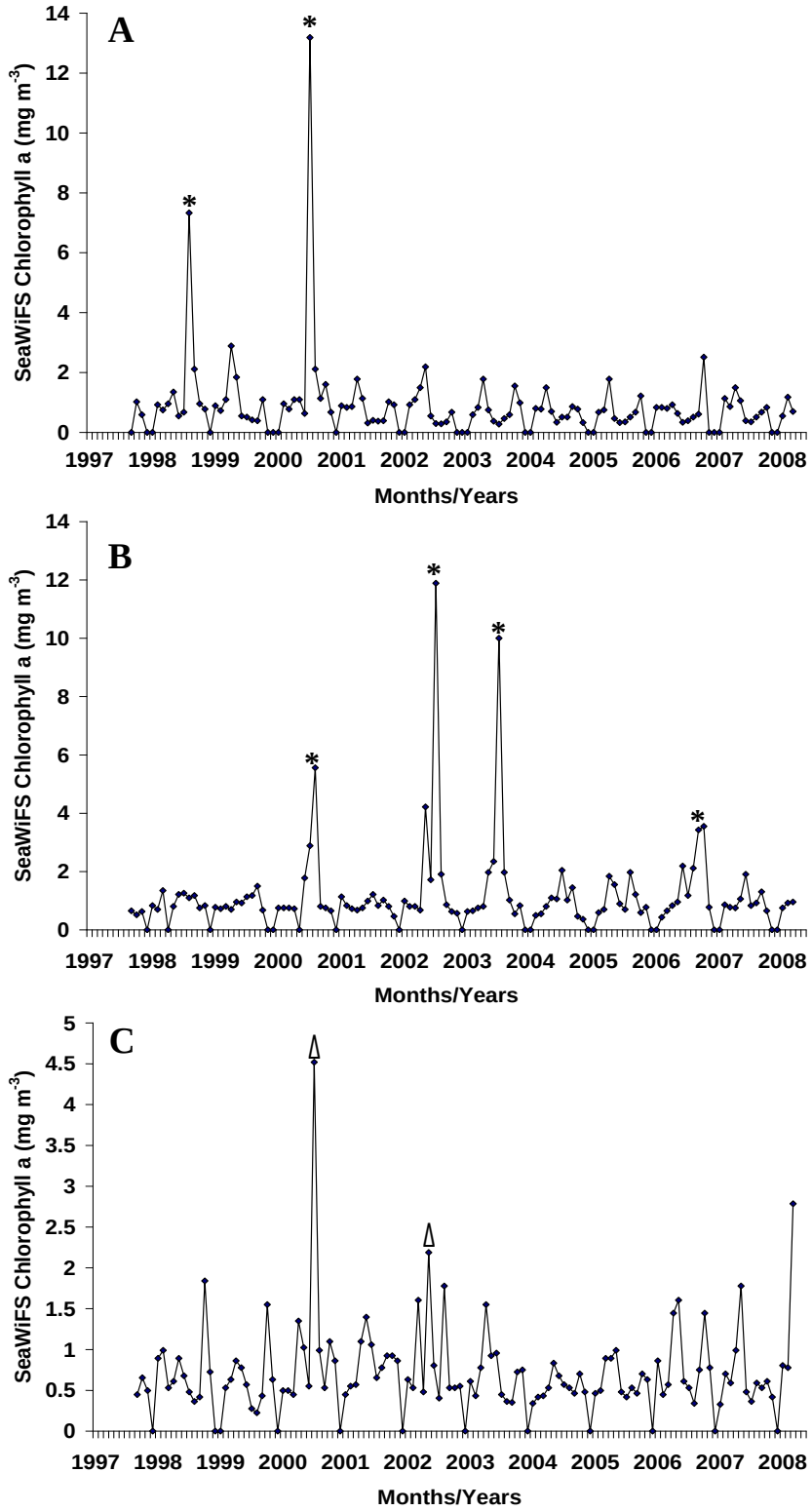

Figure 5. 2. SeaWiFS *chl*-a data (from September 1997 to June 2006) for the coordinates: A, B & C, indicating 50.8°N, -7.9°W; 49.3°N, -3.9°W & 47.9°N, -5.7°W, respectively. The highest *chl*-a peaks corresponding with a documented bloom of *K. mikimotoi* in the area (Appendix 2) are marked with * and Δ indicates only the summer highest *chl*-a peak in the Bay of Biscay.

In addition, high *chl*-a values were also seen in the Bay of Biscay in 2000, 2002 and 2008 (Figure 5.2C). We concluded that these *chl*-a peaks correspond to *K. mikimotoi* blooms as their timing is consistent with previous recordings of *K. mikimotoi* blooms in this region. Satellite *chl*-a maps can also be used to follow the progression of *K. mikimotoi* blooms. The bloom can evolve and change its position within years over the course of days and weeks (Appendix 2, Figures 3-10). Evolution of *K. mikimotoi* blooms has been found to be governed by a combination of physical (advection) and biological (growth, mortality and positive phototaxis) factors (reviewed in Davidson et al. (2009)).

5.2.2. Archived CPR samples

CPR routes within the Celtic Sea shelf-break region (46-52ºN, 0-11ºW) dating from 1963 (the earliest archived CPR silk available in this region) to 2006 are shown in Figure 5.3. Fortunately, these routes correspond well with the regions that regularly experienced *K. mikmotoi* blooms (Figure 5.1). Eight routes totalling 13061 individual samples were available for these areas. Sample coordinates that matched most closely with the historical records of *K. mikmotoi* blooms (time and location) and their corresponding satellite images (Appendix 2 and 3) were selected. These CPR sample coordinates, were mapped using **Geographic Information Systems (GIS)** (Figure 5.3). CPR samples dating before 1979 (1963-1976) were selected from locations where *K. mikimotoi* was thought most likely to occur. CPR samples that were collected later than 1979 were selected to match the high *chl*-a concentration areas in corresponding Coastal Zone Color Scanner (CZCS) data sets (1978-1986) (Figure 5.4 A, B and F; Figure 5.5 A and B), while, CPR samples collected later than 1997 were selected to match the high *chl*-a concentration area in the corresponding SeaWiFS and pseudo-true

colour SeaWiFS (1997-2006), and/or Moderate Resolution Imaging Spectroradiometer (MODIS) (2002-2006) data sets (Figure 5.4 C-E; Figure 5.5 C-F; Figure 5.6 A-F and Figure 5.7 A-B). Incidentally, most of the selected CPR sample co-ordinates which corresponded to the areas of high *chl*-a concentrations were also found to match with both increased Sea Surface Temperature areas (SST) areas and stratified regions identified by tidal front imagery (Appendix 4).

A summary of the data available for each of the CPR samples is shown in Table 5.1. A total of 31 CPR samples dating back to the 1960's were selected for this study, representing different times from three regions of the Celtic Sea shelf-break known to support *K. mikimotoi* blooms. These comprised of 28 samples from areas that were likely to be experiencing a *K. mikimotoi* bloom spanning over 47 years, and 3 samples that could serve as negative controls, i.e. from times when no or very few *K. mikimotoi* should be present. Two of the negative control samples (labelled sample a and b, Figure 5.3) were collected during a diatom (*Thalassiosira* sp. bloom) dominated spring bloom from the western English Channel (David John, SAHFOS, personal communication). The third control sample (sample c, Figure 5.3) was also collected from the western English Channel but during a summer *Emiliania huxleyi* dominated bloom in 2001 (Schroeder et al., 2002). In addition, this sample was collected from an area which matched well with high *chl*-a concentrations in the SeaWiFs satellite images (Figure 5.7 A and B). Total genomic DNA was successfully extracted and quantified from all of these CPR samples (Appendix 5, Table 1).

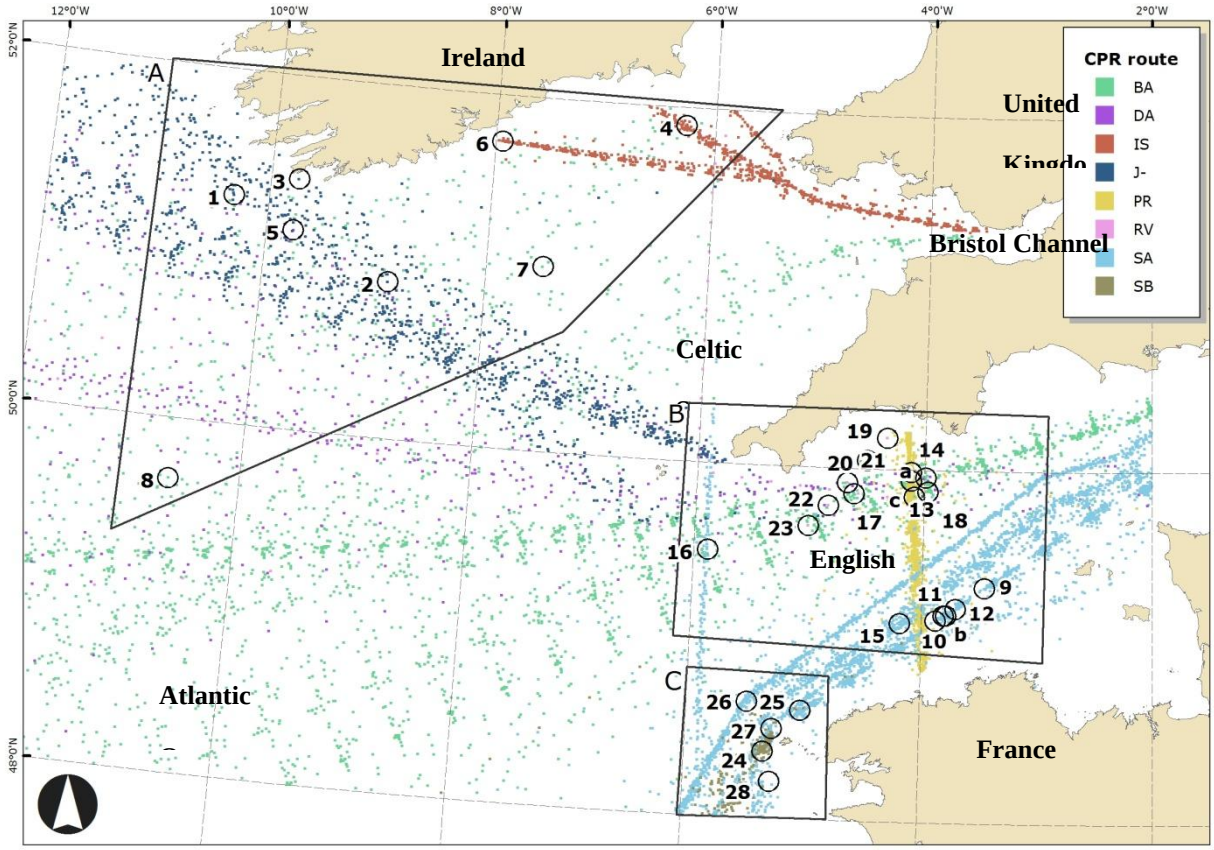

Figure 5. 3. The main CPR routes (colour dots) for the towed CPR device sampling in the Celtic Sea shelf break, the 28 circles indicating the location of Summer and Autumn bloom CPR samples predicted to be positive for *K. mikimotoi* within each route. Those marked a, b & c (area B), were selected during spring bloom events from south-western English Channel in 1968 as negative controls.

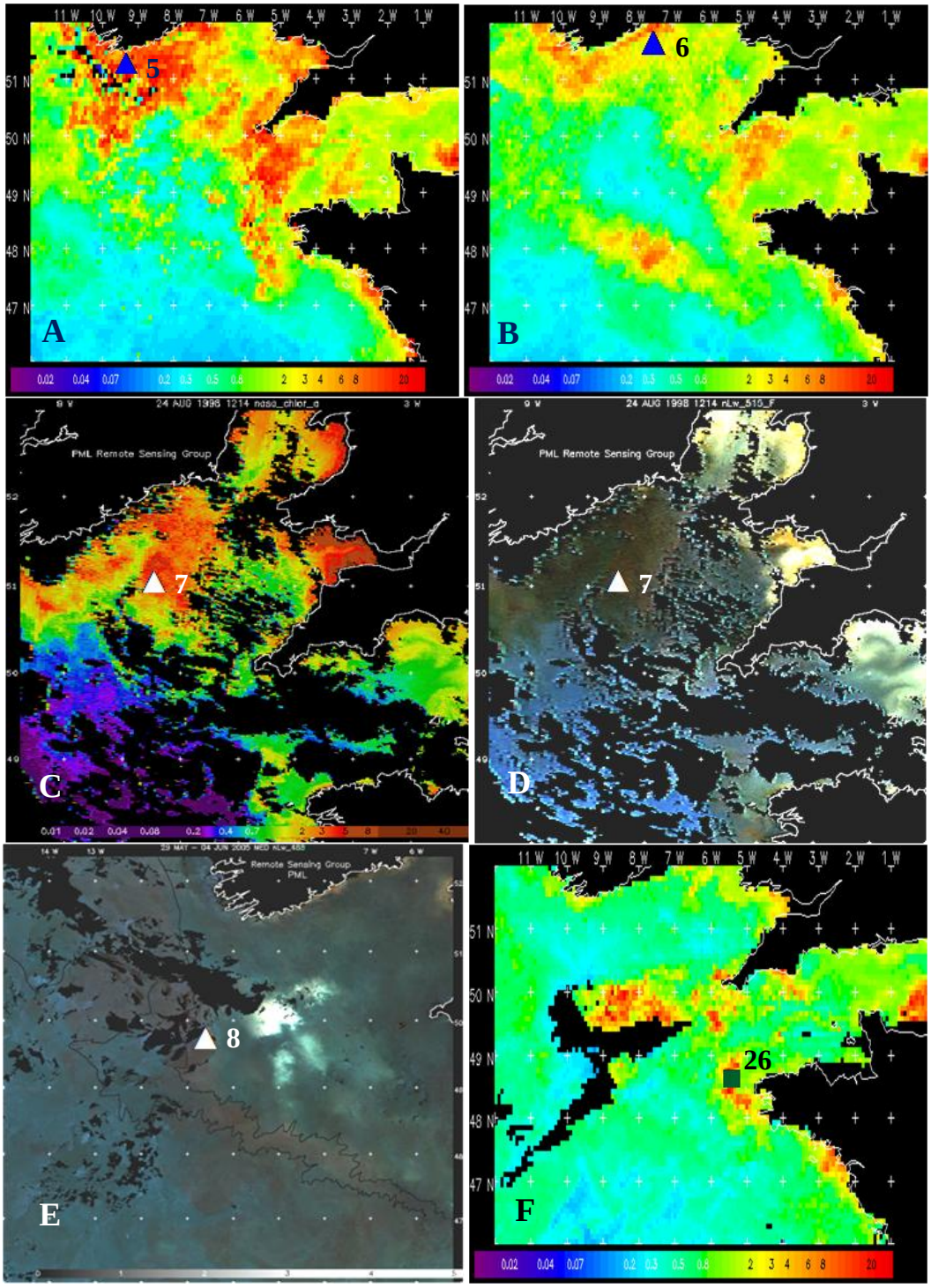

Figure 5. 4. Monthly mean 9 km CZCS (A, B and F), daily SeaWiFs-1 km (C) and pseudo true color SeaWiFs-1 km (D) and weekly SeaWiFs-1 km *chl*-a composite (E) showing the location of the *K. mikimotoi* blooms and CPR samples used in this study from south Ireland (triangles) and along French coast (square). Where A corresponds to September 1979, B corresponds to September 1980 and C and D correspond to 24th August 1998, E corresponds to 29th May 2005-4th June 2005 and F corresponds to July 1985. Numbers near each location (square or triangles) indicated the sample number of selected CPR samples.

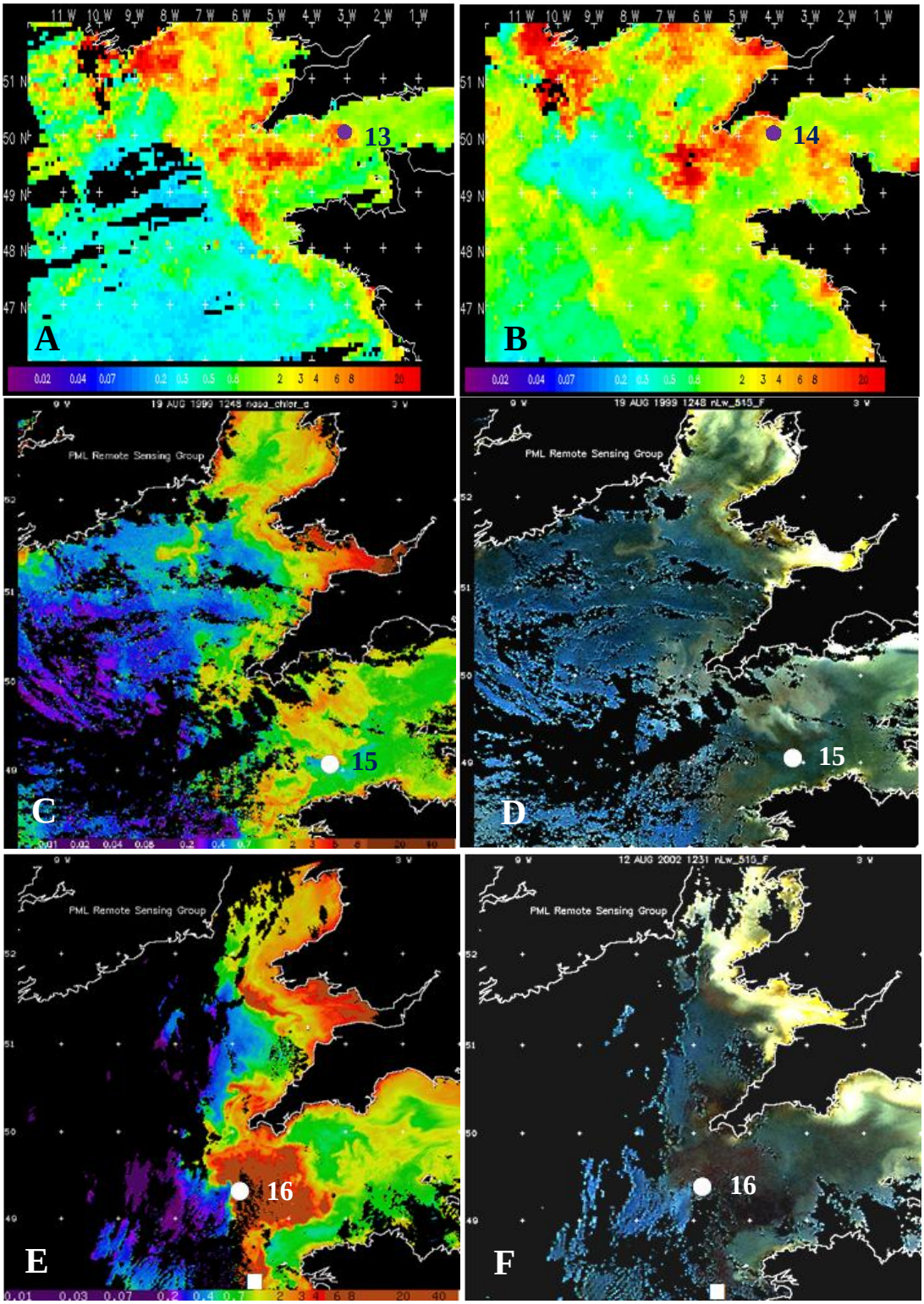

Figure 5. 5. Monthly mean-9 Km CZCS (A and B), daily SeaWiFs-1 Km (C), MODIS Aqua-1 Km (E) and pseudo true-colour SeaWiF-1 Km s *chl*-a (D and F) showing the location of the *K. mikimotoi* blooms and the identified CPR samples used in this study from western English Channel (circles) and along French coast (squares).Where A corresponds to August 1979, B corresponds to August 1981, C and D correspond to 19th August 1999, E and F correspond to 12th August 2002. Numbers near each location (circles) indicated the sample number of selected CPR samples.

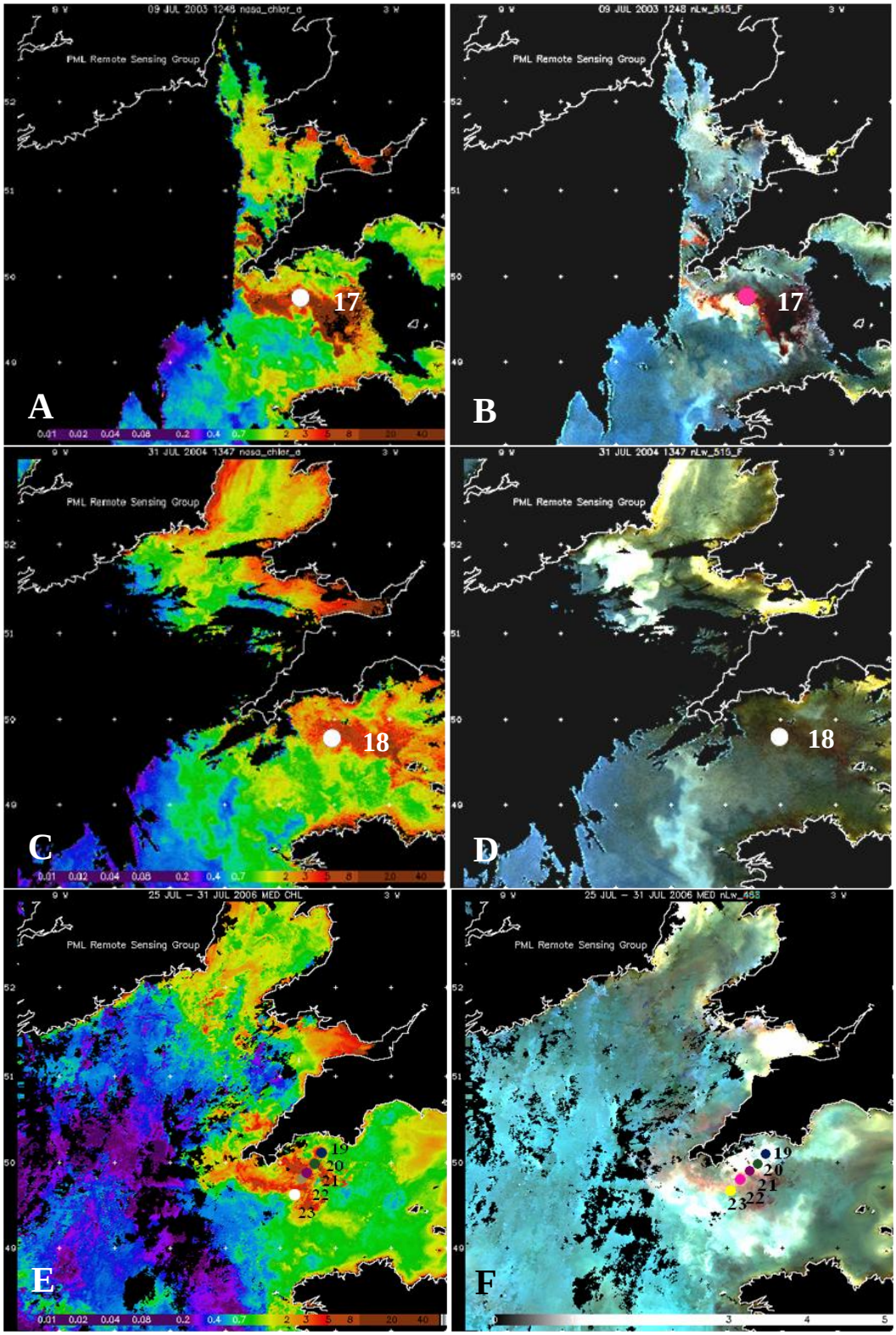

Figure 5.6. Daily SeaWiFs-1 Km (A, C and E) and pseudo true-colour SeaWiFs-1 Km *chl*-a (B, D and F) showing the location of the *K. mikimotoi* blooms and the identified CPR samples used in this study from western English Channel (circles). Where A and B correspond to 9th July 2003, C and D corresponds to 31st July 2004, E and F correspond to 25th-31st 2006. Numbers near each location (circles) indicated the sample number of selected CPR samples.

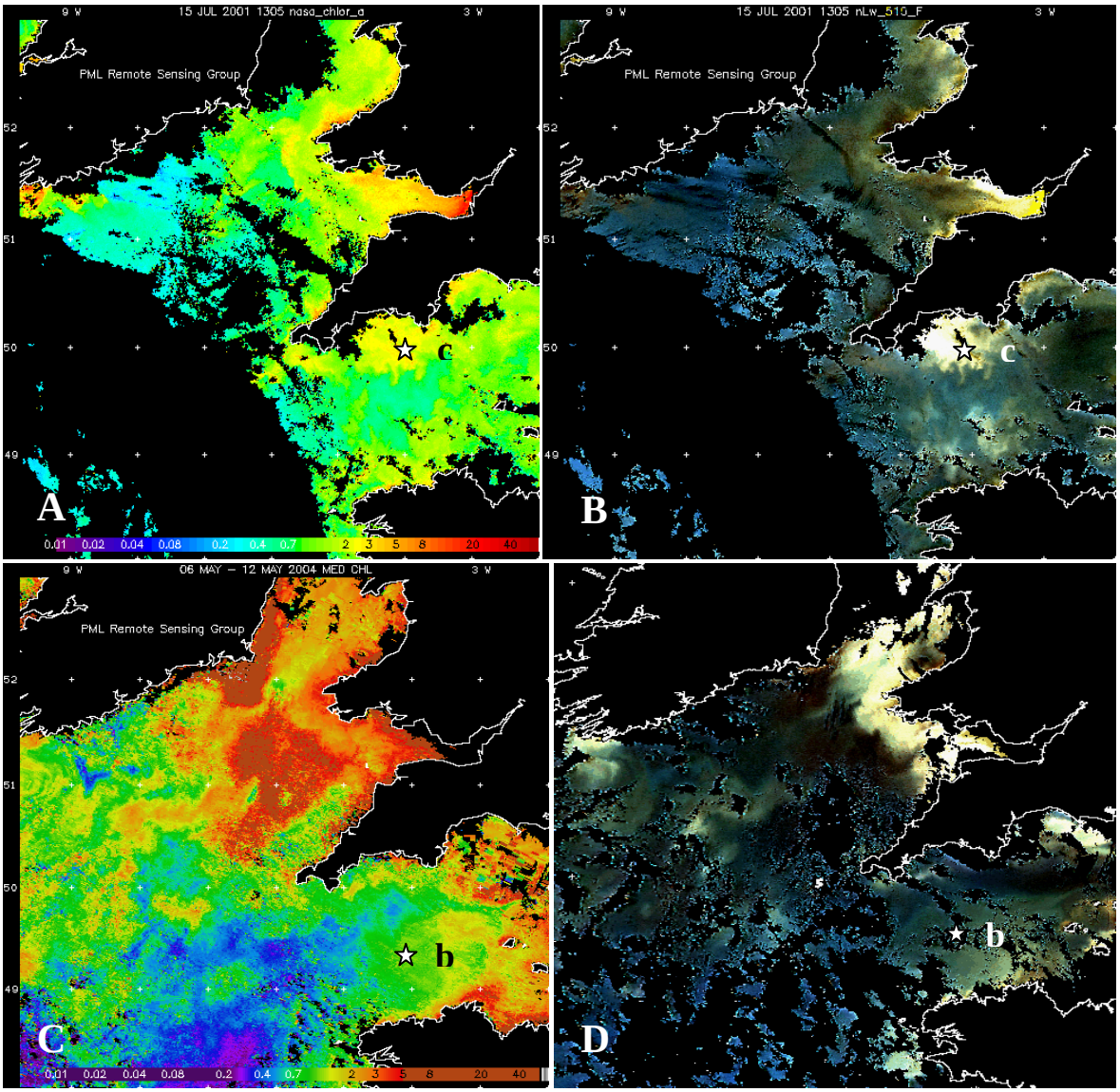

Figure 5.7. Daily SeaWiFs-1 Km (A), weekly SeaWiFs-1 Km *chl*-a composite (C) and daily pseudo true-colour SeaWiFs-1 Km *chl*-a (B and D) showing the locations of where *K. mikimotoi* blooms regularly occurred based on historical records and the identified CPR samples used in this study and employed as the negative controls from western English Channel (stars). Where A and B correspond to 15th July 2001 (during summer bloom), C corresponds to 6th -12th May 2004 and D corresponds to 6th May 2004. Numbers near each location (stars) indicated the sample number of selected CPR samples.

Table 5. 1. Archived CPR samples used in this study from the Celtic Sea shelf-break.

Region (Figure 5.1)	Sample	Date	CPR route	Latitude	Longitude	Bloom confirmed by Historical Record (Appendix 2)	Bloom region identified by SeaWiFS, CZCS or pseudo true-colour
A. South Ireland	1	27.7.1963	79J	51.29	-10.32	None	None
	2			50.89	-8.87		
	3	24.8.1965	90J	51.42	-9.75		
	4	6.8.1976	60IS	51.90	-6.27	Area A: Figure 5.1	
	5	18.9.1979	176DA	51.13	-9.76		Figure 5.4A[C]
	6	5.9.1980	87IS	51.74	-7.94		Figure 5.4B[C]
	7	23.8.1998	332BA	51.05	-7.49		Figures 5.4C[S] & 5.4D[P]
	8	4.6.2005	412BA	49.66	-10.62		Figure 5.4E[P]
B. western English Channel	9	28.7.1963		49.33	-3.46	None	None
	10			49.15	-3.88		
	11	30.7.1966	112SA	49.22	-3.71		
	12	12.7.1968	132SA	49.18	-3.79		
	13	20.8.1979	81PR	49.94	-4.12	Area B: Figure 5.1	Figure 5.5A[C]
	14	10.8.1981	103PR	49.99	-4.12		Figure 5.5B[C]
	15	19.8.1999	473SA	49.13	-4.19		Figures 5.5C[S] & 5.5D[P]
	16	11.8.2002	505SA	49.51	-5.88		Figures 5.5E[S] & 5.5F[P]
	17	9.7.2003	387BA	49.86	-4.62		Figures 5.6A[S] & 5.6B[P]
	18	1.8.2004	401BA	49.88	-3.97		Figures 5.6C[S] & 5.6D[P]
	19	26-27.7.2006	55RV	50.18	-4.34		Figures 5.6E[S] & 5.6F[P]
	20			50.05	-4.51		
	21			49.92	-4.68		
	22			49.79	-4.84		
	23			49.67	-5.01		
	a*	02.5.1968	130SA	49.18	-3.81	None	None
	b*	09.5.2004	398BA	49.85	-4.09		Figures 5.7A[S] & 5.7B[P]
	c*	09.7.2001	365BA	49.96	-3.99		Figures 5.7C[S] & 5.7D[P]
C. French coast	24	19.8.1963	81SB	48.38	-5.33		None
	25	24.7.1976	212SA	48.62	-5.02	Area C: Figure 5.1	
	26	20.7.1985	308SA	48.66	-5.48		Figure 5.4F[C]
	27	3.7.1995	429SA	48.51	-5.26		None
	28	11.8.2002	505SA	48.21	-5.26	None	Figures 5.5E[S] & 5.5F[P]

*** = negative control samples; [C] = CZCS; [S] = SeaWiFS, [P] = pseudo true-colour.**

5.2.3. Genotyping of *Karenia mikimotoi* in the historical archived records

Previously, twenty four dinoflagellate strains (Table 2.4) were genotyped using the universal D1 and D2 regions of LSU rDNA (D1R-F & D2C-R (UniA), Table 2.5) as described in Chapter 2. The PCR length of the D1–D2 LSU rDNA for these different dinoflagellates species varied between 700-916 bp. A typical PCR result for some of these dinoflagellates is presented in Figure 5.8. The different *K. mikimotoi* strains and *K. brevis* species produced a similar sized amplicon at about 780 bp. The other dinoflagellate species *Karlodinium micrum*, *Alexandrium tamarense* and coccolithophore *Emiliania huxleyi* produce amplicons of 795, 730 and 680 bp, respectively. In fact, all of the isolates in the culture collection could be amplified using this primer set. We sequenced 83% of these (Table 2.3 and 2.4), confirming the identity of the amplicon. These and other available GeneBank LSU rDNA sequences from a wide range of dinoflagellate taxa, totalling over 100 sequences, were aligned. This alignment was used to design a new reverse LSU primer D2newR (Table 2.5). LSU rDNA primer pair (D1R-F & D2newR, UniB) was designed to amplify a 341 bp amplicon (examples, Appendix 6). This was predicted to be a good amplicon size for amplifying the DNA from formaldehyde preserved CPR samples. The purified DNA from the thirty one selected archival samples was amplified using the LSU rDNA UniB primers to assess the quality of the extracted DNA (Table 5.2). Four samples of the twenty eight where we expected to find *K. mikimotoi* blooms and one of the three negative control samples failed to generate amplification products. These samples included sample 1 (1963) from south Ireland, samples 16 (2002) and a (1968) from western English Channel and samples 24 (1963) and 27 (1995) from French coast.

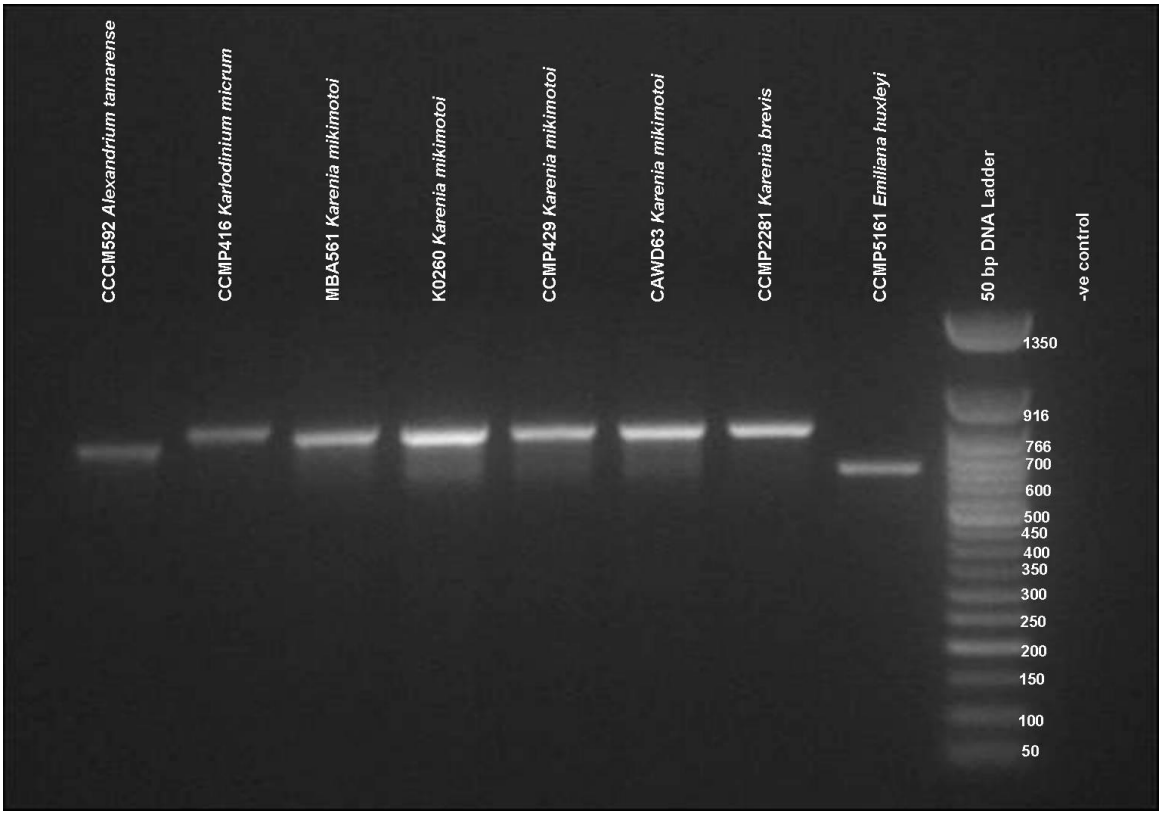

Figure 5.8. Gel image of PCR products generated using the LSU (D1-D2) rDNA primers for different phytoplankton isolates in culture collection.

Table 5. 2. CPR samples used to detect *K. mikimotoi* and the PCR results from different primer sets.

Region	Sample	Date	LSU rDNA UniB (D1-D2newR)	Universal *rbcL* -Uni1	Universal *rbc*L Uni2 (sequencing ID)	HRM Assay	specific *rbc*L - Sp2 (sequencing ID)
A. South Ireland	1	27.7.1963	-	-	-	-	-
	2		+	-	-	-	-
	3	24.8.1965	+	-	-	-	-
	4	6.8.1976	+	+	+	sub-species 2	UK
	5	18.9.1979	+	-	-	-	-
	6	5.9.1980	+	-	-	-	-
	7	23.8.1998	+	-	+	sub-species 2	UK
	8	4.6.2005	+	-	+ (EUP & NEW)	-	-
B. Western English Channel	9	28.7.1963	+	-	-	-	-
	10		+	-	+	sub-species 2	NEW (NOR like)
	11	30.7.1966	+	+	+ (EUP)	-	-
	12	12.7.1968	+	+	+	sub-species 2	UK
	13	20.8.1979	+	-	-	-	-
	14	10.8.1981	+	+	+	sub-species 2	NZ
	15	19.8.1999	+	-	+	sub-species 2	UK
	16	11.8.2002	-	-	-	-	-
	17	9.7.2003	+	-	-	-	-
	18	1.8.2004	+	+	+	sub-species 2	UK, NZ & NOR
	19	26-27.7.2006	+	+	-	-	-
	20		+	+	+	sub-species 2	UK
	21		+	+	-	-	-
	22		+	+	-	-	-
	23		+	+	-	-	-
	a*	02.5.1968	-	-	-	-	-
	b*	09.5.2004	+	-	+ unknown Diatom -	-	-
	c*	09.7.2001	+	-	-	-	-
C. French coast	24	19.8.1963	-	-	-	-	-
	25	24.7.1976	+	+	-	-	-
	26	20.7.1985	+	-	-	-	-
	27	3.7.1995	-	-	-	-	-
	28	11.8.2002	+	-	-	-	-

*** = negative control samples, EUP = European (UK and Norway), NOR = Norwegian strain**

The universal primers *rbcL*640-F and *rbcL*1240-R (Uni1, Table 2.5) were used in an initial screen to confirm the presence of phytoplankton within the CPR material (Figure 5.9). Eleven (40%) of the 28 predicted *K. mikimotoi* bloom samples were positive for the universal *rbc*L (Uni1), each producing a 620 bp amplicon (Table 5.2). All of the Uni1 PCR reactions were used as templates in a nested Uni2 PCR step to further validate the presence of phytoplankton within the CPR samples. Here we used the previously designed primers (Kmitrbcl-F1 and Kmitrbcl-R1 (Sp1), Table 2.5) in Chapter 3, but this time under conditions of greatly reduced stringency (Uni2, section 3.2.7 and Figure 5.9). Five additional positive reactions, including one within the negative *K. mikimotoi* controls (b, Table 5.2) were detected. However, five samples that were previously positive gave negative nested Uni2 PCR results. An overall combined PCR success of 16 out 31 (52%) was achieved for the positive identification of phytoplankton within the CPR samples.

The *K. mikimotoi* HRM assay on the Uni1 PCR products genotyped 5 of the 11 1st round positive Uni1 samples (Table 5.2), as shown in both standard (e.g., Figure 5.10) and derivative (e.g. Figures 5.11 and 5.12) HRM profiles. All five belonged to *Karenia mikimotoi* sub-species 2. Samples 7, 10 and 15 (table 5.2) that originally failed to generate a visible PCR product but then amplified with the Uni2 primers, also was shown by HRM PCR to belong to *K. mikimotoi* sub-species 2 (Table 5.2). The other 6 samples (11, 19, 21, 22, 23, & 25) that produced positive 1st round Uni1 PCR products had undetectable levels of *K. mikimotoi*. The 8 *K. mikimotoi* samples, which dated back to 1963, included samples 4 (1976) and 7 (1998) off southern Ireland and samples 10 (1963), 12 (1968), 14 (1981), 15 (1999), 18 (2004) and 20 (2006) which were located in western English Channel approaches (Table 5.2).

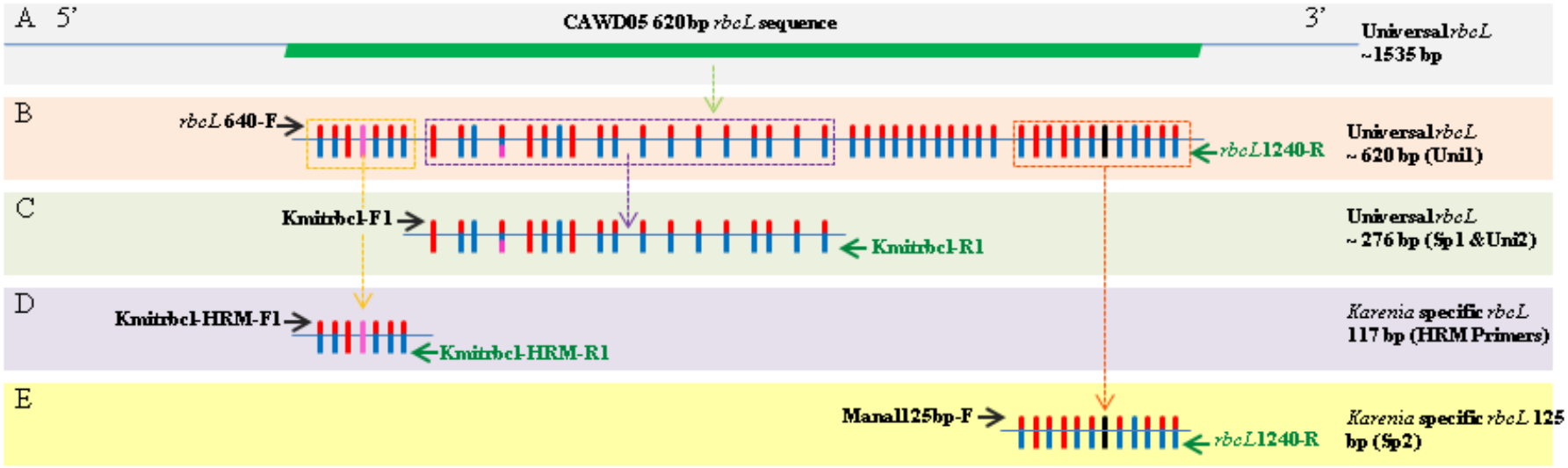

Figure 5.9A-E. The large subunit of *rbcL* gene. A. The whole *rbcL* sequence (~1535 bp) and B-E. partial *rbcL* sequences from different strains of *K. mikimotoi*: B. Universal *rbcL* primers (*rbcL* 640-F and *rbcL*1240-R, Uni1); C. specific (Sp1) *rbcL* primers (Kmitrbcl-F1 and Kmitrbcl-R1) for use in both HRM assays (chapter 3) and for nested *rbcL* (Uni2) for CPR sample amplification and sequencing (chapter 5); D. specific *rbcL* primers (Kmitrbcl-HRM-F1 and Kmitrbcl-R1) for use in HRM assays; E. re-designed specific *rbcL* primers (Manal125bp-F and *rbcL*1240-R) for sequencing the positive CPR samples that had European peak for *K. mikimotoi* in HRM assays (chapter 5). In A the green line = the CAWD05 sequence (JP2). In B to E the small coloured columns represented the nuceotides that differ in comparison to JP2 for JP1 (MBA 561/pink), NZ (CAWD63, CAWD134, CAWD133, CAWD117/red), UK1 & 2 (CCMP429 & MBA705/blue) and Norway (K-0260 and CCMP430/black) strains.

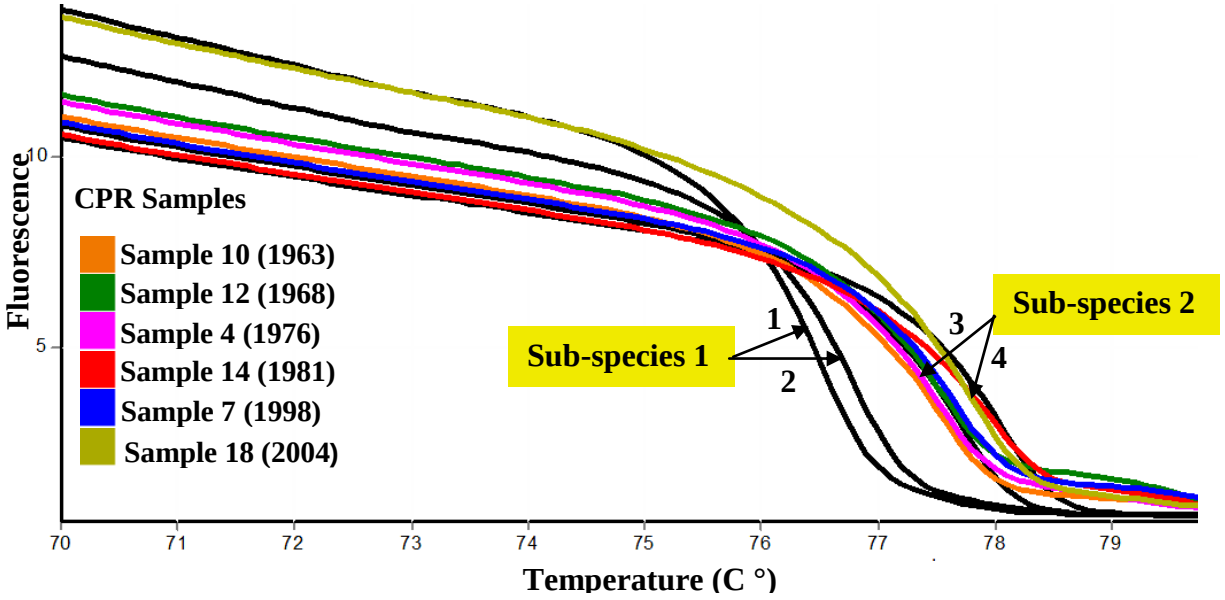

Figure 5.10. Standard HRM profile for the four different genotypes (black) of *K. mikimotoi* sub-species 1: 1. MBA561 (JP1) and 2. CAWD05 (JP2) and sub-species 2: 3. MBA705 (UK2) and 4. CAWD134 (NZ1) (positive controls); and the related CPR samples showing the samples collected in 1963 and 1968 from south-western English Channel and in 1976 and 1998 from south-western Ireland were grouped with corresponded European strain (3), while the samples collected in 1981 and 2004 from south-western English Channel, grouped with the corresponded New Zealand strain (4).

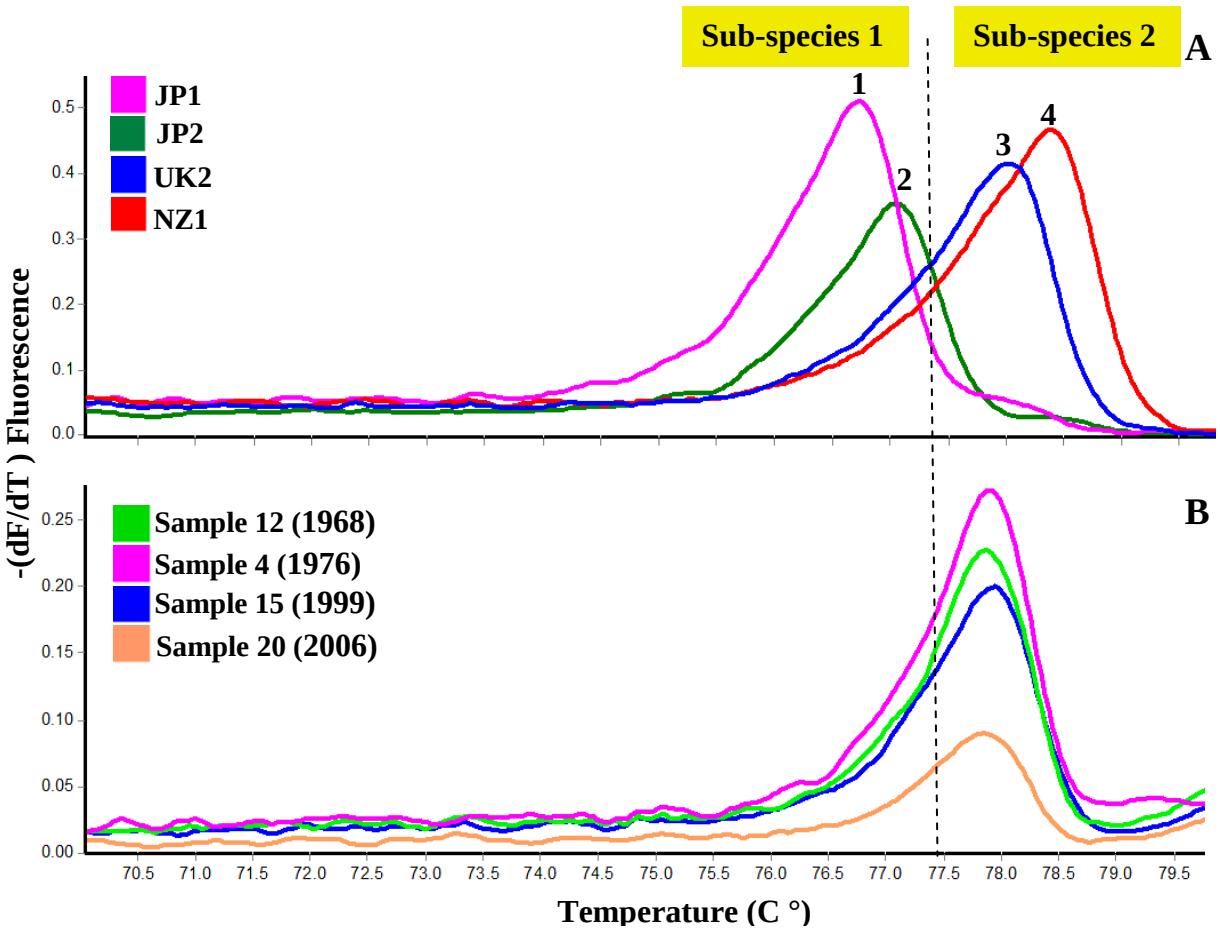

Figure 5.11. Derivative HRM profile for: A. the four different genotypes of *K. mikimotoi* sub-species1: 1. MBA561 (JP1/pink) and 2. CAWD05 (JP2/green) and sub-species 2: 3. MBA705 (UK2/blue), presented Europe genotype and 4. CAWD134 (NZ1/red); B. CPR samples collected in 1968, 1976, 1999 and 2006.

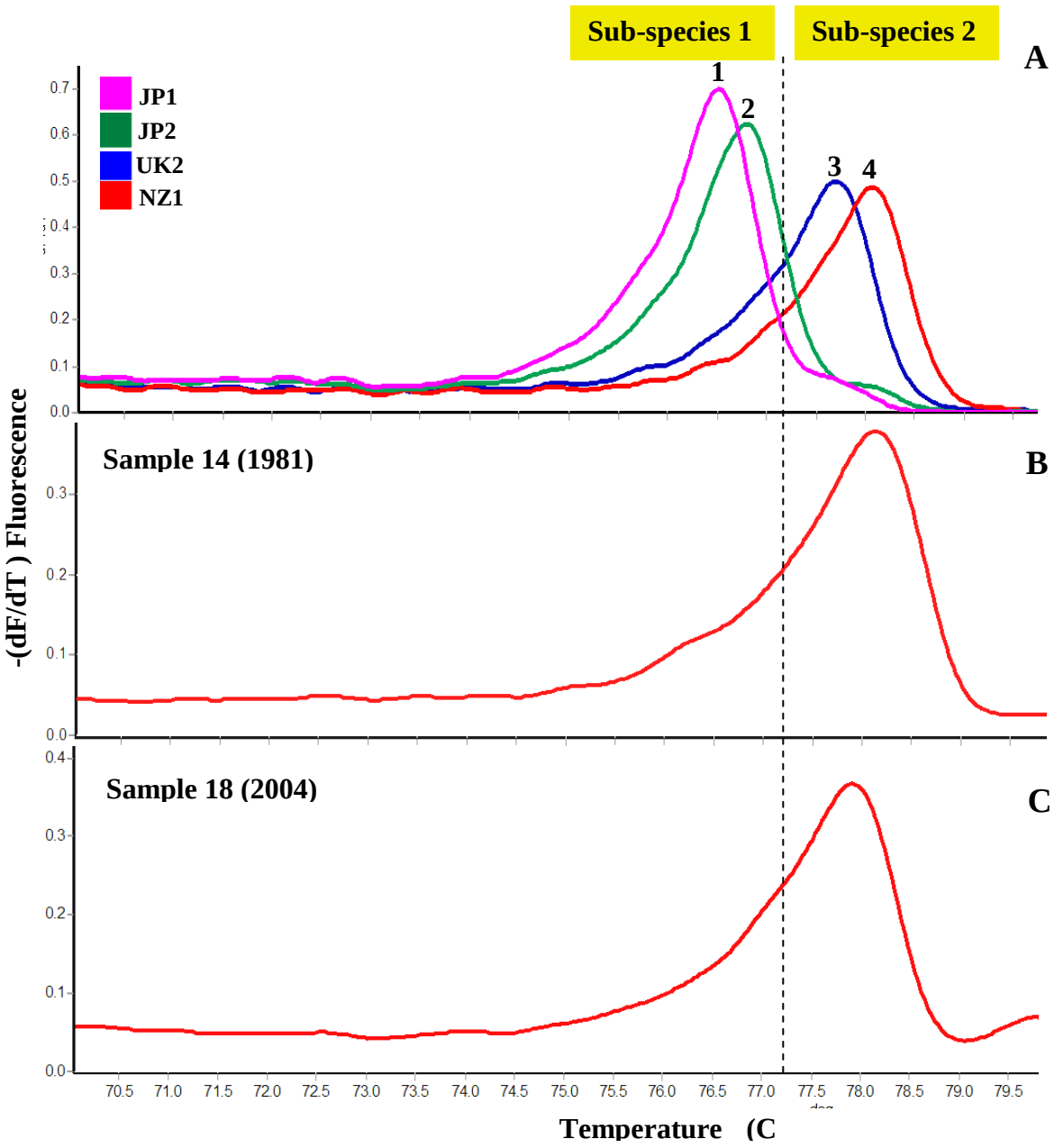

Figure 5.12. Derivative HRM profile for: A the four different genotypes of *K. mikimotoi* sub-species 1: 1. MBA561 (JP1/pink) and 2. CAWD05 (JP2/green) and sub-species 2: 3. MBA705 (UK2/blue) and 4. CAWD134 (NZ1); B CPR sample collected in 1981; C CPR sample collected in 2004.

The HRM analysis was run over 20 times for each control genotype and five times for the environmental samples. There was some variability in the temperature of the melt peaks between runs for the same strains with a range of 0.00 ≤ 0.06 °C, but the majority being ≤ 0.03 °C (data not shown).

The *K. mikimotoi* PCR amplicon Sp2, which was the original 125 bp fragment amplified on the basis of the consensus *rbc*L sequences of *K. mikimotoi* genotypes using the universal primer *rbcL*1240-R of Uni1 as a forward primer and the new primer, Manal125bp-F as a reverse primer (Table 2.5, Figure 5.9) can be used to distinguish between the sub-species 2 (European (UK and Norwegian) and New Zealand) strains and the sub-species 1 (Japanese) strains. This based on the presence of nine and ten nucleotide substitutions in the Sp2 primer set for each of the sub-species 2 and sub-species 1 (Figure 5.13), respectively . In addition, Sp2 can also differentiate between the European and New Zealand strains due to three substitutions (Figures 5.9 and 5.13) and separate the European genotype into the UK and Norway genotypes that differ at one nucleotide position. Therefore, this Sp2 region was developed (Appendix 7, Table 6. 1) and used to confirm the sequence identity of the eight positive *K. mikimotoi* CPR HRM assays. Furthermore, samples 8 (2005), 11 (1966) and b which had positive Uni2 *rbcL* amplicons, but gave no results for the *K. mikimotoi* HRM assays, were also PCR amplified (Uni2), cloned and sequenced. Each clonal sequence was given a reference code, where the letter corresponded to the used primers such as Sp2 for the genotyping Sp2 primers (*rbcL*1240-R and Manal125bp-F) and Uni2 for the Uni2 Primers (Kmitrbcl-F1 and Kmitrbcl-R1) (Table 2.5), followed by the year, clone unique sequence number and number of occurrence in cloning library.

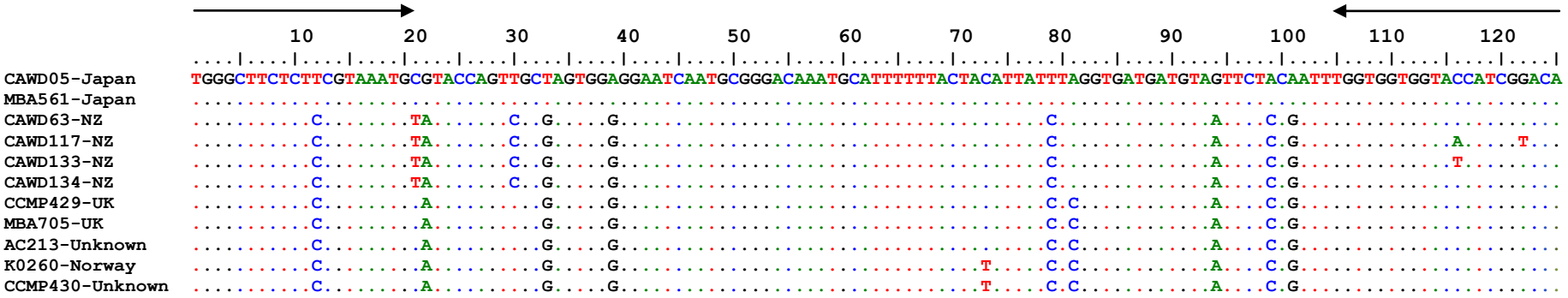

Figure 5.13. Sequence alignment of the 125 bp Sp2 fragment of *rbcL* (*rbcL*1240-R and Manal125bp-F) for *K. mikimotoi* isolates used for the HRM assays. Positions of the specific forward (F) and reverse (R) primers are indicated by arrows. Dots correspond to identical nucleotides and letters show nucleotide substitutions.

Thirty one clones sequenced from sample 10 (1963), indicated the presence of two genotypes Sp263-01 and Sp263-02, representing 94% and 6% of the clone library, respectively. They were both most similar to the characterized Norwegian genotype, sharing 99 % and 98 % sequence identity, respectively (Figure 5.14). The substitutions detected in these sequences were not in the positions of any of the substitutions present in the previously characterised JP, UK and NZ genotypes.

Thirty three, twenty eight, twenty seven and twenty clones were sequenced from Samples 4 (1976) and 7 (1998), 12 (1968), 15 (1999) and 20 (2006), respectively. All samples were found to be dominated by a single genotype which was identical to the CCMP429 UK genotype (Sp276-01 for 1976, Sp268-01 for 1968, Sp299-01 for 1999 and Sp206-01). Samples 14 (1981) and 18 (2004), melt peaks corresponded most closely to the NZ genotype (sub-species 2). Nineteen clones were sequenced from Sample 14, which showed 100% identity to the NZ genotype (Sp281-01, Figure 5.14). Twenty eight clones for Sample 18 were sequencing and 54% of the sequences were identical to the NZ genotype (Sp204-03), while 39% were identical to the UK genotype (e.g. Sp204-01) and 7% were identical to the Norwegian genotype (e.g. Sp204-02).

PCR products from three CPR samples collected in 1966 (112SA-19, Sample 11) and 2004 (398BA-13, Sample b) from English Channel and in 2005 (412BA-37, Sample 8) from southern Ireland (Figures 5.15 and 5.16) were amplified using the 276 bp Uni2 *rbc*L primers. Clone libraries were constructed from each sample and thirty one clones were sequenced for each. Six different sequences were detected from Sample 11 (1966). These included two sequences making up 15% of clone library (Uni266-01 and Uni266-02) that shared 99% sequence identity to the UK genotype of *K. mikimotoi* (Figure 5.15). The other four sequences (Uni266-03, Uni266-04, Uni266-05 and Uni266-06) (Figure 5.16) shared

99-99.6 % sequence identity to each other (Figure 5.16, Appendix 8). Neighbor-Joining and Bayesian analyses on these four sequences produced a well supported (71% and 0.88) novel cluster most closely related to the centric diatoms *Papiliocellulus simplex* and *Minutocellus polymorphus* (Figure 5.16).

Only two genotypes (Uni204-01 and Uni204-02, Appendix 8) were amplified from sample b, both are new members of the class Bacillariophyceae. Neither clustered with any of the known diatoms, therefore, it is likely at they represent an uncharacterised diatom taxa (Figure 5.16).

Sample 8 (2005) sequence data indicated that *K. mikimotoi* was indeed present with four genotypes most closely related to the UK strain (Figure 5.15). This included one sequence identical to the UK genotype (Uni205-01), while the other three sequences had either one (Uni205-02 and Uni205-03) or four (Uni205-04) substitutions, which were not present in either JP or NZ genotypes (Figure 5.15). Sample 8 was dominated by the Uni205-03 sequence (71%).

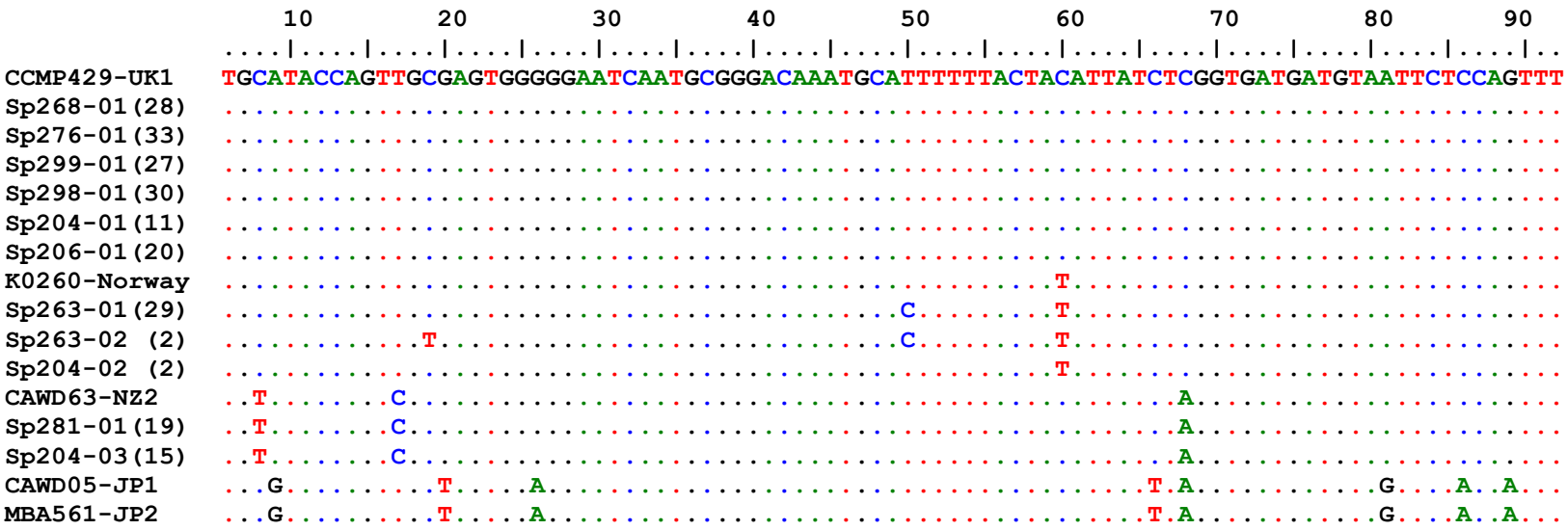

Figure 5.14. Sequence alignment of a 125 bp amplicon (excluding primers) of the genotyping specific Sp2 *rbcL* gene fragment (Manal125bp-F and *rbcL*1240-R) for *K. mikimotoi* clones obtained from CPR samples collected in 1963 (Sp263-11 and Sp263-53), 1968 (Sp268-01), 1976 (Sp276-01), 1999 (Sp299-44), 1998 (Sp298-02 and Sp298-06), 1981 (Sp281-39), 2004 (Sp204-37, Sp204-27 and Sp204-12) and 2006 (Sp206-02). Numbers between brackets correspond to number of occurrence for each sequence, dots correspond to identical nucleotides and letters correspond to nucleotide substitutions.

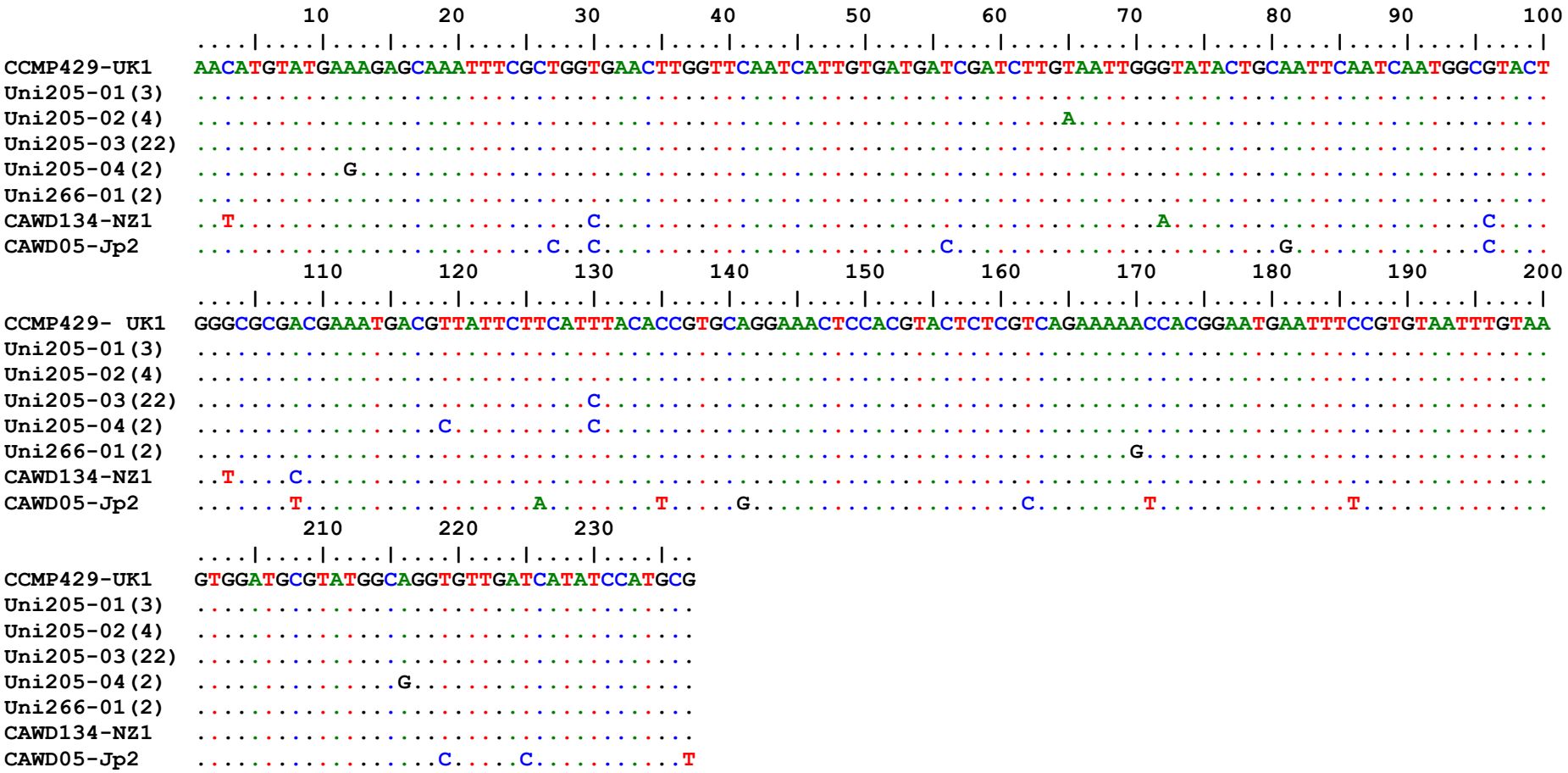

Figure 5.15. Sequence alignment of a 276 bp amplicon (excluding primers) of the Uni2 *rbcL* gene (Kmitrbcl-F1 and Kmitrbcl-R1) fragment for *K. mikimotoi* clones obtained from CPR samples collected in 1966 (Uni266-04) and 2005 (Uni205-06, Uni205-10, Uni205-16 and Uni205-25). Numbers between brackets correspond to number of occurrence for each sequence, dots correspond to identical nucleotides and letters correspond to nucleotide substitutions.

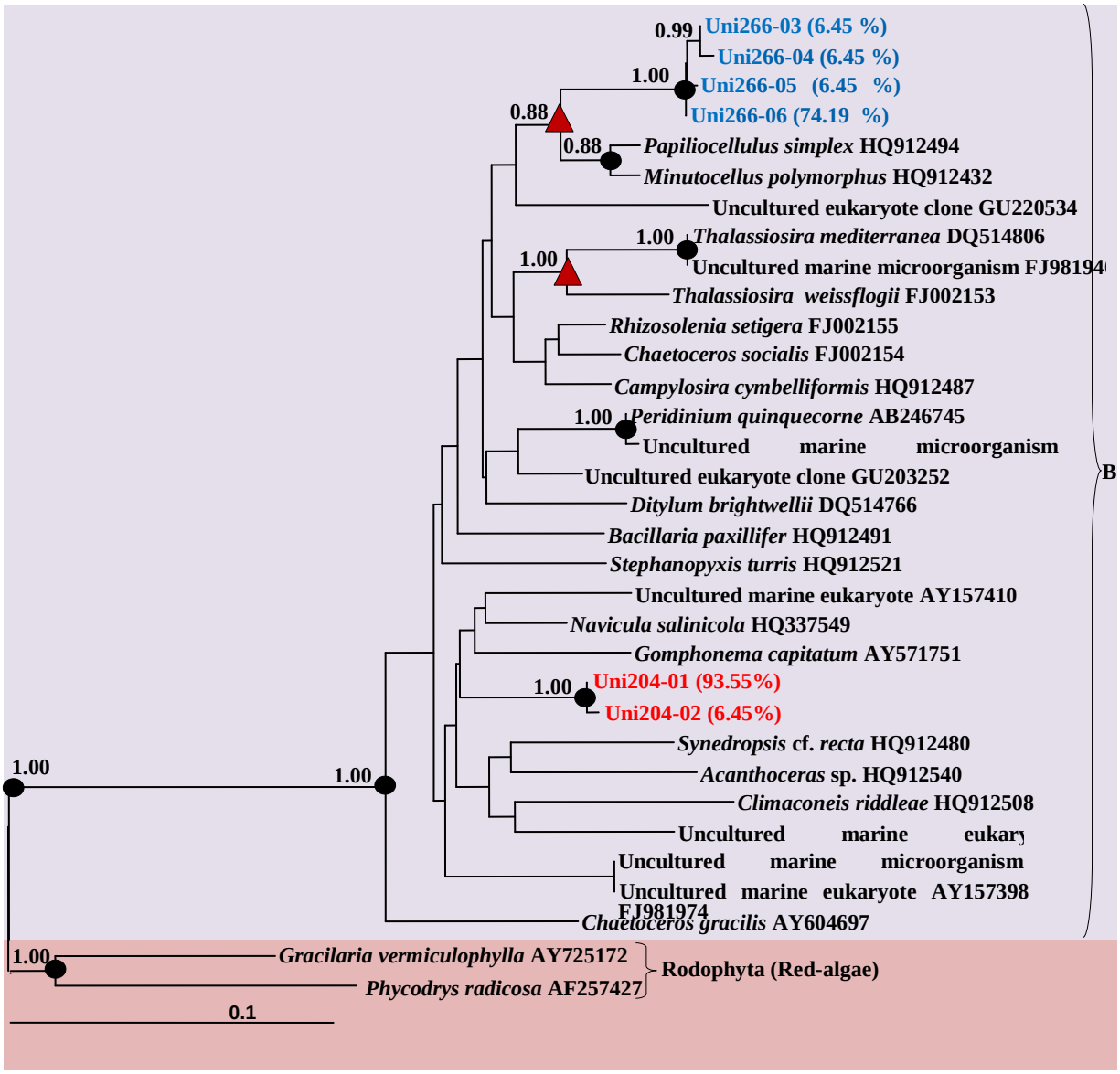

Figure 5.16. Phylogenetic tree of sequenced clones derived from 276 bp of the *rbcL* gene for sample 11 (1966) (blue) and sample b (2004) (red) CPR samples, with the (%) occurrence of each sequence within the clone library. This tree was constructed by using the distance matrix (Neighbor) (NJ), parsimony (dnapars) (DP) and MrBayes (By). Bootstrap values were calculated from 1000 replicates each for NJ and DP. Shapes at nodes indicate bootstrap values greater than 70 % for: both NJ and DP (●), only NJ (▲), only DP (■) and numerals represent By posterior probabilities. Scale bar indicates a distance of 0.1 substitutions per site. The class for each clade were indicated on right side of phylogeny. Sequences derived from red algae were used as out groups. B = Bacillariophyceae.

In summary, of the 28 CPR samples chosen to be likely to include *K. mikimotoi* only 24 generated amplification products using the rDNA LSU primers thus confirming the quality of the extracted DNA. Ten of these 24 samples were positively identified to belong to *K. mikimotoi* sub-species 2 (42 % of total). Of the 28, two sets of samples, namely samples 1 & 2 and 9 & 10, represent two possible bloom events in southern Ireland and western English Channel approaches, respectively. Sample 1 was also one of the four samples thought to have yielded degraded or poor quality DNA as it failed to amplify using rDNA LSU primers. Similarly, five samples from the western English Channel (samples 19 to 23) were taken from a confirmed bloom event in 2006. Therefore, 53 % of the discrete yearly occurrences of *K. mikimotoi* in the shelf break region were found to be positive for *K. mikimotoi* sub-species 2, i.e. of the 19 possible bloom events, 10 were positively confirmed to have *K. mikimotoi* present (Table 5.2).

On a regional basis, three out of a possible seven bloom events in southern Ireland were positive for *K. mikimotoi.* Where as seven out of the nine bloom events in the western English Channel, were positive for *K. mikimotoi.* However, none of the French coast samples (three bloom events in total) were positive for *K. mikimotoi.*

5.3. Discussion

5.3.1. The timing and location of *K. mikimotoi* blooms

In situ data in combination with remote sensing satellite images (CZCS, SeaWiFS and MODIS *chl*-a) enabled us to catalogue the timing and common locations of late summer

and early autumn *K. mikimotoi* blooms. Moreover, the historical records for *K. mikimotoi* enabled us to select samples from the archived CPR silks within the three most common *K. mikimotoi* bloom forming regions, i.e. the western English Channel, southern Ireland and the Ushant front. This study has demonstrated how from a combination of *chl*-a and *in situ* data we can gain a clearer understanding of the distribution and dynamics of *K. mikimotoi* blooms in the Celtic Sea shelf-break region: we confirmed previous observations that satellite-derived maps of *chl*-a can be used to validate the *in situ* occurrences of *K. mikimotoi* blooms (e.g. Miller et al. (2006) and Davidson et al. (2009)).

The previously reported correlation between frontal areas and the occurrence of *K. mikimotoi* blooms was also observed in this study using the remote sensing images (Appendix 4). Furthermore, increased temperature as determined by SST images also matched the timing and locations of *K. mikimotoi* blooms and the high chlorophyll concentration areas in SeaWiFs images. The *K. mikimotoi* bloom in 2003 showed a very high concentration of chlorophyll, reaching up to 100 mg m^{-3}, which was correlated with both the timing of increased SST and the arrival of a low salinity intrusion in western English Channel (Kelly-Gerreyn et al., 2004). Previously, it has been suggested that temperature is one of the important factors in the initiation of *K. mikimotoi* blooms in North European waters (Nielsen and Tønseth, 1991). These findings give evidence that the important factors that trigger the initiation and development of *K. mikimotoi* blooms, are an increase in temperature and stratification associated with tidal fronts and the depth of the pycnocline.

5.3.2. *K. mikimotoi* detection from the archived CPR samples

CPR samples were carefully selected to correspond as closely as possible to areas and times where *K. mikimotoi* blooms were recorded. Our mapping results helped us to locate blooms of our target species in samples dating back to 1963. We selected the CPR survey for use in this study because it is one of the only long-term plankton surveys that has an archive of formaldehyde preserved samples collected from different parts of the North-east Atlantic, where *K. mikimotoi* is known to bloom and cause fish kills and other marine mortality events. Historically, this species has presented many taxonomic problems in the North Atlantic and Pacific Oceans, due to the morphological similarities with many other gymnodinoid species. Specific cell molecular detection methods of the sort developed in this study provide the tools needed to address the temporal and spatial variation within populations of these harmful/or toxic dinoflagellate species: strain, and even genotype specific identification is necessary to address the population biology and ecology of important HAB species.

This present study has generated fresh evidence from past CPR surveys that provides additional insights into the temporal and spatial distribution of the important icthyotoxic dinoflagellate species *K. mikimotoi*. This naked-dinoflagellate species is delicate, lacking a cell wall or an outer covering needed to render it resistant to common fixing or preservative agents and making easily destroyed by net sampling and towing process. These factors make it difficult to visualize and count by the standard methods of CPR plankton analysis. The fixation process for most of the unarmoured dinoflagellates, including gymnodinoid species, causes loss of flagellae and cell deformation, i.e. the disappearance of the main diagnostic characters (Gómez, 2007). The CPR is unable to

survey many of the picoplankton and nanoplankton taxa, such as members of the Bolidomonaceae. Furthermore, whilst sampling with the CPR is a good method for zooplankton, it has been argued that it is inappropriate for the sampling of phytoplankton, particularly the small-sized taxa, due to the large mesh-size (270 µm) employed (Batten et al., 2003). That said, it has also been reported that clogging of the silk filters leads to the collection of samples that are dominated by phytoplankton rather than zooplankton communities (Hunt and Hosie, 2006, Walne et al., 1998). Hunt and Hosie (2006) showed that the phytoplankton which cause the filters to clog is dominated by the larger-size fraction of taxa, mainly diatoms. The clogging of the filters essentially reduces the mesh size, and thus allows smaller species to be captured, such as the diatom *Thalassiosira* and coccolithophores. Moreover, with the aid of molecular techniques, Ripley et al. (2008) and Vezzulli et al. (2011) were able to identify the small, 5µm diameter coccolithophore *Emiliana huxleyi* and even smaller bacterial populations on CPR filters.

In this study, we have successfully used HRM assays to detect *K. mikitmotoi* genotypes from formaldehyde preserved CPR samples. The sub-species 2, European genotype of *K. mikitmotoi* was found to be present in the English Channel as early as 1963, which is earlier than the previously recorded European bloom in 1966 reported off south coast of Norway (Braarud and Heimdal, 1970). At that time, this species was recognized as a non-indigenous species of Japanese origin. Moreover, the transplantation of Japanese oysters to France in 1966 onwards was frequently used as an explanation of the presence of this species in North Atlantic waters (Gómez, 2008b, van den Bergh et al., 2002, Elbrächter, 1998). However, the true identity of this species was unknown and it was unclear whether it was indeed an introduced species or a native that had been previously overlooked; its presence in these waters prior to 1966 could have been missed due to a lack of sampling

and/or it could have been present in low numbers, or it was misidentified and included with other gymnodinoid species (Boalch, 1987). That said, anecdotal evidence has suggested that this species could have actually arrived as early as 1865 from a completely different location (in Harmful Algal News, no. 40, http://www.ioc.unesco.org/hab/). As a streak of red-coloured water has been reported in south-western Ireland in 1865 and thought it might be related to *Karenia* bloom. This present study using new molecular approaches provides, for the first time, data confirming an earlier presence of *K. mikimotoi* in Europe. This finding indicates the presence of the European *K. mikimotoi* strain in 1963 and also in 1966 in the English Channel, but no evidence of sub-species 1 (Japanese strains). These results support the conclusion that *K. mikimotoi* was not an introduction from Japan mediated by the transplantation of Japanese oyster transplantation to France in 1966. The molecular assays detected no genotypes related to Japanese strains in CPR samples that spanned 47 years in both the southern Ireland and the western English Channel. The earliest sample analysed, which was collected in 1963 from the English Channel, demonstrated the presence of a unique sequence. This new sequence/strain differed in only one base pair substitution when compared to the previously characterized Norwegian strains. The samples collected in the following years in 1966, 1968, 1976, 1998, 1999, 2005 and 2006 were all shown to have the UK strain present, which suggests that the dominant strain in southern Ireland and western English Channel is similar or identical to the previously characterized UK strain.

Sequence data also indicated the presence of the New Zealand strain in English Channel in two samples, one collected in 1981 and the other in 2004. The 1981 sample coincides with the first recording of a *K. mikimotoi* bloom in New Zealand (Chang, 1996). The characterized NZ strain was previously found to be more genetically related to the

European strain than the Japanese strains (Al-Kandari et al., 2011). The similarities between the European and New Zealand isolates could be attributed to their likely adaptation to colder waters as opposed to the warmer waters of Japan. Moreover, these isolates have been found to be physiologically similar, with it being reported that both produce two different size populations in their life cycle, which has not been seen for Japanese isolates (Chang, 1996, Partensky and Vaulot, 1989).

The published *rbcL* sequence data (Al-Kandari et al., 2011) of the New Zealand and UK strains, showed them to share 98 % sequence identity despite being isolated from different geographical areas and at different times. For example, with the New Zealand strains, the NZ strain CAWD63 was collected from Hauraki Gulf in 1993, while the other NZ strain CADW134 was collected from Whangaporaoa in 2002 (both from the east coast of NZ), a nine-year gap between their isolation. A similar scenario can be seen in the UK strains with, UK strain CCMP429 collected from Sutton Harbour, Plymouth in 1980, and UK MBA705 collected from Cabris Bay, Cornwall in 2006, a twenty-nine year gap between isolation dates but yeilding identical *rbcL* sequences.

HRM and sequence data could be interpreted as showing a new introduction of the NZ strain into English Channel waters prior to or in 1981 (or vice versa). This introduction might have occurred via ballast water (human-assisted dispersal). This strain could then have been easily able to adapt easily enough to the smilar climatic environment in each area (Gentien, 1998). Moreover, the long term CPR data set showed an exceptional period of unusual climate change in late 1970s and early 1980s that have occurred episodically over a time series of decades in the North Sea (Edwards et al., 2002). This anomalous shift has a dominant effect on marine environment, such as is the case with the abundant dinoflagellate *Ceratium macroceros* which is known to peak in abundance between August

and October in southern North Sea as part of a natural seasonal succession of phytoplankton in the North Sea. This species showed a drop in its recorded frequency of occurance of 40 % in the 1960's to 0.4 % in late 1970's. This event was linked with salinity and temperature minima which were observed in 1968 to 1982 around the North Atlantic. The authors speculate that the *Ceratium* species might have lost its niche temporarily to be replaced by another competitor, which might also be the case with the UK and NZ strains, as the UK strain predominated in most of the study samples and NZ strain bloom suddenly dominant, but only in 1981.

5.3.3 Limits of detection and recording of new species

Cloning and sequencing of 276 bp fragment of *rbcL* amplified using the Uni2 showed the presence of *K. mikimotoi* in a further two samples that were not detected using HRM assay. These two samples were from 1966 (Sample 11), which was selected based on the common locations where *K. mikimotoi* repeatedly bloomed, and 2005 (Sample 8) which was selected due to a huge bloom that occurred in southern Ireland and that was detected by both *in situ* samples and satellite images. In the case of the 1966 sample, it could be that the sample was not from a bloom, but where *K. mikimotoi* was nonetheless indeed present in relatively low numbers. As such it was potentially below the limit of detection for the HRM assay. Moreover, sequence data showed other small diatom taxa dominating the sample and actually only two *K. mikimotoi* sequences were detected out of 31 clones that were sequenced. The two sequences from *K. mikimotoi* in this sample showed one random (a substitution at a different position compared to genotyped strains) nucleotide substitution in each sequence compared with the UK genotype. The phylogenetic analysis

placed the small diatom species from CPR Sample 11 with the small centric diatoms *Papiliocellulus simplex* and *Minutocellus polymorphus* (approximately 5 µm (Gardner and Crawford, 1992) and 2.5-3.5 µm length (Nelson and Siddall, 1988), respectively), which belong to the group Cymatosiraceae. The former species was identified in Britain waters in 1990 and it was recognized only when it grew as colonies in a crude culture from the sample collected at that time (Gardner and Crawford, 1992).

The 2005 sample showed four different genotypes, including one genotype identical to the UK strain (Uni205-01) and three genotypes with 98-99 % identity to the UK strain. It is unclear why the HRM assay failed to detect *K. mikimotoi* in this sample. It is noted, however, that one genotype, Uni205-03, dominated the clone library (71%). This genotype had a single unique nucleotide substitution when compared to UK strain. It is therefore conceivable that the gene region detected by the HRM might have diverged significantly in genotype Uni205-03 for it not to be detected. This potentially indicates the presence of other previously uncharacterized genotypes in southern Ireland and off the French coast, where overall PCR success rate was 43% and 0%, respectively.

Sequence data for Sample b (2004), which was collected during a spring bloom and selected as a negative control, indicated the presence of only one diatom taxon that grouped with the diatom clade and was supported by high bootstrap values. No particular genera or species were found to be closely related to the sequence in the phylogenetic tree which comprised a representative set of diatom species, derived from BLAST searches. This demonstrates the existence of unknown biodiversity within the diatom community and which probably represents those species that are difficult to be isolate/culture from environmental samples.

Our mapping observations resulted in a modest 42 % amplification success rate for *K. mikimotoi* by HRM and sequence analysis. Nevertheless, the actual hit rate is 53 % based on the different period of time and corresponding recorded blooms (Table 5.2). Moreover, when one takes into account the regional biases the highest success rate was 78% in the western English Channel. In addition, these samples demonstrate the unpredictable nature and patchiness of *K. mikimotoi* in well defined blooms. For example, *K. mikimotoi* was found in only one of the five 2006 bloom samples. Analysis of the 2006 bloom satellite images confirmed the patchy chlorophyll *a* distribution across the bloom.

Other reasons might also explain the failure to detect *K. mikimotoi* from bloom events. For example, competing bloom forming phytoplankton species could have dominated at the same time and location of our target species. The pseudo true colour SeaWiFs images for Sample 17 (2003) (Figure 5.7B) and samples 21-23 (2006) (Figure 5.7F) of the 2006 bloom, indicates the presence of a co-occurring *Emiliania huxleyi* bloom. All samples that were positive for *K. mikimotoi* showed no co-occurring *E. huxleyi* bloom, such as in Sample 7 (1998) (Figure 5.5D) and Sample 18 (2004) (Figure 5.7D), or they were clearly spatially separated such as in Sample 8 (2005) (Figure 5.5E) and Sample 15 (1999) (Figure 5.6D). Furthermore, Sample 16 (2002) (Figure 5.6E) which showed no detectable *E. huxleyi* bloom on the pseudo-true colour SeaWiFs image, did, however, include cells of this coccolithopphorid on the CPR filters as revealed by microscopy: the presence of coccolithophore (31 %) might have been due to the end of *E. huxleyi* bloom or the dominance of other taxa, such as unidentified Dinoflagellate cysts (31 %) and the presence of *Noctiluca scintillans* (17 %), might be prevented their reflection from *E. huxleyi* in the satellite image.

Regarding the earlier samples from the 1960s, such as samples 2 (1963), 3 (1965) and 9 (1963), it was only felt that they might have *K. mikimotoi* present because they were from areas where *K. mikimotoi* is known to occur, although no blooms were recorded at these times. If *K. mikimotoi* was present then perhaps the numbers were very low and did not yield enough template DNA for standard PCR amplification.

The most likely reason for the negative PCR results relates to the age and the nature of the sample preservation used. Detection of DNA or RNA from formalin fixed samples (4 % neutral-buffered formalin) using PCR has been reported to be hindered by degradation of nucleic acids during collection, preparation, buffering and long archival storage time, which together lead to the amplification from only a limited number of samples (Gruber et al., 1994, Foss et al., 1994). The formalin fixation procedure has been reported to lower the success of PCR amplification (Ben-Ezra et al., 1991) because of cross-linking between the protein and DNA. Koshib et al. (1993) reported that extensive DNA degradation occurs during formalin fixation because of the presence of formic acid coupled with low pH and low salt concentration at normal room temperature. Although reports of DNA extraction from formalin-fixed specimens are available (Shiozawa et al., 1992; Cano and Poinar, 1993; Shedlock et al., 1997; Chase et al., 1998), in most cases the success rate was not 100 % and highly inconsistent with a large number of specimens. That said, a lower copy number of plastid gene such a *rbc*L will have a lower detection rate compared to higher copy number ribosomal genes for example. However, the five samples which gave negative amplification with universal LSU primers indicate that the DNA was not suitable for PCR.

The positive samples in the present study supported the correlation between the presence of *K. mikimotoi* and the red colours in SeaWiFs *chl*-a satellite images (for those that

satellite images were available). Some negative samples, such as samples 28 (2002) (Figure 5.6E) and 17 (2003) (Figure 5.7A) were related to a dark brown colour in SeaWiFs *chl*-a images. This shows that the anticipated dark red to brown colour does not necessarily correspond with the occurrence of a *K. mikimotoi*, and it may be that other species are blooming at this time resulting in this particular colour detected at the same time as blooms of our target species. High numbers of *Noctiluca scintillans* (which grazes on *K. mikimotoi*) have repeatedly been recorded to coincide with *K. mikimotoi* blooms in the Celtic Sea shelf break, such as in 1975 (Pingree et al., 1975), 1976 (Holligan and Harbour, 1977), 1981 (Holligan et al., 1983) and in July 2000 by Groom et al. (2000). The *N. scintillans* bloom was found to appear at the same time as red patches, or streaks caused by the wind. Other dinoflagellate species are also known to bloom at the same time as *K. mikimotoi* bloom, such as *Prorocentrum micans* in the northern Celtic Sea (Raine and McMahon, 1998) and in the western English Channel in 2006 (data provided by PML).

The timing of sampling is also important to consider as *K. mikimotoi* blooms are known to be associated with the thermally stratified waters (Gentien et al., 2007). This is of relevance as when a pycnocline is established *K. mikimotoi* cells migrate during night into this layer for nutrient uptake. *K. mikimotoi* have diurnal vertical migration speeds of 1-2 m h^{-1} with a range up to 15 m (Koizumi et al., 1996), which helps cells take up nitrate at night (Dixon and Holligan, 1989). The time and rate of cells speed to return to surface varies based on initiation and termination of the bloom. It has been found during a *K. mikimotoi* bloom that occurred in the Seto Inland Sea in Japan in summer 1995, that there were increased cell numbers in the pycnocline layer (15 m) with no cells being detected in the surface during day time until afternoon (Nakamura et al., 1996) when the temperature reached the maximum. Based on migration speed of this species, the authors speculated

that *K. mikimotoi* is present at 10m in the morning then ascends to 5m around noon to photosynthesize actively, and then descends to about 15m at night. Dense populations of *K. mikimotoi* corresponding to sub-surface layers, usually at or just below the pycnocline, in which cell densities achieve several million cells per litre (Gentien et al., 2007), and at frontal regions have been visualized, i.e. in the Kattegat of Denmark (Bjørnsen and Nielsen, 1991), Northern Celtic Sea and south of Cork (inside the Irish shelf front) (Raine and McMahon, 1998, Raine et al., 2001), Fastnet Rock (Roden et al., 1981, Roden et al., 1980, Jenkinson and Connors, 1980) and Bantry Bay (Raine et al., 1993) off southern Ireland, western English Channel and Bay of Biscay (Kelly-Gerreyn et al., 2004). The CPR sampling time varied between samples during the 24 hours and the samples were collected only from the surface at ~7 m depth, therefore if *K. mikimotoi* was migrating diurnally into the pycnocline and the CPR sampling was performed during night or early morning then this would reduce the chance of detecting *Karenia* cells.

5.3.4. Conclusion

Our findings support the notion that *Karenia mikimotoi* is most likely not an introduced species in European waters, at least not in the sense that has been previously reported, as we detected its presence in the western English Channel in 1963, which is five years prior to the first official report of a *K. mikimotoi* bloom. *K. mikimotoi* is known as a widely distributed species that can tolerate a wide range of temperature and salinity and is also known to grow in both rich and poor nitrate areas.

Further effort is, however, still required to screen more isolates from Japan and New Zealand to confirm the true biogeographic history of certain genotypes/strains. This will

clarify if the New Zealand strain has been introduced into European waters (or vice versa) or if it was always present in both regions. It will also allow us to elucidate whether two NZ and European genotypes exist and if either any or both of them bloom only when conditions are favourable. This clarification needs samples from both coastal and oceanic waters.

We have nonetheless demonstrated that the oyster industry was not responsible for introducing *K. mikimotoi* to European waters. None of the Japanese strains were detected in the samples spanning the Celtic Sea shelf-break region. Furthermore, the data presented here show the potential existence of unique genotypes of *K. mikimotoi* near the Irish and French coasts. Recently, CPR survey extended its sampling to now include Japanese waters. This provides future opportunities to screen for more genotypes of *K. mikimotoi* from Japan.

Chapter 6: *K. mikimotoi* and its co-occuring red algal lineage

6.1. Introduction

Little is known about the biodiversity and ecosystem dynamics for the pico- (0.2-2 µm) and nano-fractions (2-20 µm) of the plankton. These organisms are very difficult to study, due to their extremely small size, and require ultrastructural examination by TEM (review by Stockner (1988)). The algal plankton contribute significantly to the total biomass of the phytoplankton community, and in oceans and lakes can responsible for up to 80-90 % of the total daily or annual carbon production (review by Stockner (1988)). The algal plankton is largely comprised of the prokaryotic Cyanobacteria, and Eukaryotic organisms groups including the Chlorophyceae, Prasinophyceae, Eustigmatophyceae, Cryptophyceae, Bolidomonaceae, Pelagophyceae, Bacillariophyceae, Chrysophyceae and Haptophyceae (Andersen et al., 1993, Guillou et al., 1999, Moestrup, 1991, Stockner, 1988).

Photosynthetic organisms differ in their plastids and have different forms of Rubisco (Tabita, 1988, Watson et al., 1999). There are two *rbcL* forms I and II in eukaryotic algae (including cyanobacteria), while forms III and IV are found in Archaea and certain autotrophic bacteria (Tabita, 1999). *rbcL* form I containing organisms can be divided further into four distinct lineages known as forms IA, IB, IC and ID (Ellis, 1979). The ID form of *rbcL* has been targeted in our study (Chapter 3) as it is found to be present in the groups of algae that have red-algal like plastids (including red algae, heterokonts, cryptyphytes, haptophytes and fucoxanthin containing dinoflagellates): for ID is also found in the purple bacteria. This form of *rbc*L is found in most of the above mentioned classes of picoplankton except for the chlorophyceae and Prasinophyceae.

We previously reported a 50 % success rate for the detection of *K. mikimotoi* in CPR samples collected from areas predicted to contain *K. mikimotoi* blooms (Chapter 5). A number of reasons for this relatively low success rate were proposed and discussed, however, one main practical limitation of the CPR sampling method was highlighted. As discussed, the CPR only samples the top ~7 m of the water column (Richardson et al., 2006, Batten et al., 2003, Reid et al., 2003), and as *K. mikimotoi* is known to migrate vertically in mixed and weekly stratified waters (Gentien et al., 2007, Bjørnsen and Nielsen, 1991, Arzul et al., 1993) (Koizumi et al., 1996), the CPR could simply have missed the main population of *K. mikimotoi* if it was at a depth > 7 m. In the case of mixed and weekly stratified water, *K. mikimotoi* has been observed to migrate vertically daily with a range of up to 15 m, between the surface and the pycnocline layer (Bjørnsen and Nielsen, 1991, Arzul et al., 1993).

To address the issue of *K. mikimotoi* migration we analysed samples from depth profiles from three conductivity temperature depth (CTD) casts taken from the 2006 *K. mikimotoi* bloom. In this chapter we report on the results obtained using our HRM assay for the specific detection of *K. mikimotoi* for these samples and on the analysis of sequences types for ‘‘red-type Form I’’ Uni1 *rbcL* genes (*rbcL*640-F and *rbcL*1240-R, Table 2.5) from a subset of samples.

6.2. Results

6.2.1. Analysis of *K. mikmotoi* bloom samples

Eighteen environmental samples were collected from the western English Channel during 2006 (Table 6.1). The 2006 bloom samples were collected from three stations 1, 2 and 3 using depth profiling (conductivity temperature depth-CTD) (Figure 6.1). Each of these stations included samples collected from six different depths (surface, 5, 10, 15, 25 and 40 m). MODIS images that corresponded with the timing and locations of the 2006 *Karenia* bloom showed an area of high *chl*-a concentration (red colour, Figure 6.1A & B). Notably, stations 1 and 3 of 2006 samples occurred in a lower *chl*-a area (green colour) when compared to station 2. Station 1 appeared to be at the edge of the area of high *chl*-a concentration.

Table 6. 1. Field samples details used in this study.

Station	Sample ID	Feature	Date	Location
1	1	Surface	26-27.7.06	50.12º N, 4.2º W
	2	5		
	3	10		
	4	15		
	5	25		
	6	40		
2	7	Surface		49.55º N, 4.4º W
	8	5		
	9	10		
	10	15		
	11	25		
	12	40		
3	13	Surface		49.32º N, 5.15º W
	14	5		
	15	10		
	16	15		
	17	25		
	18	40		

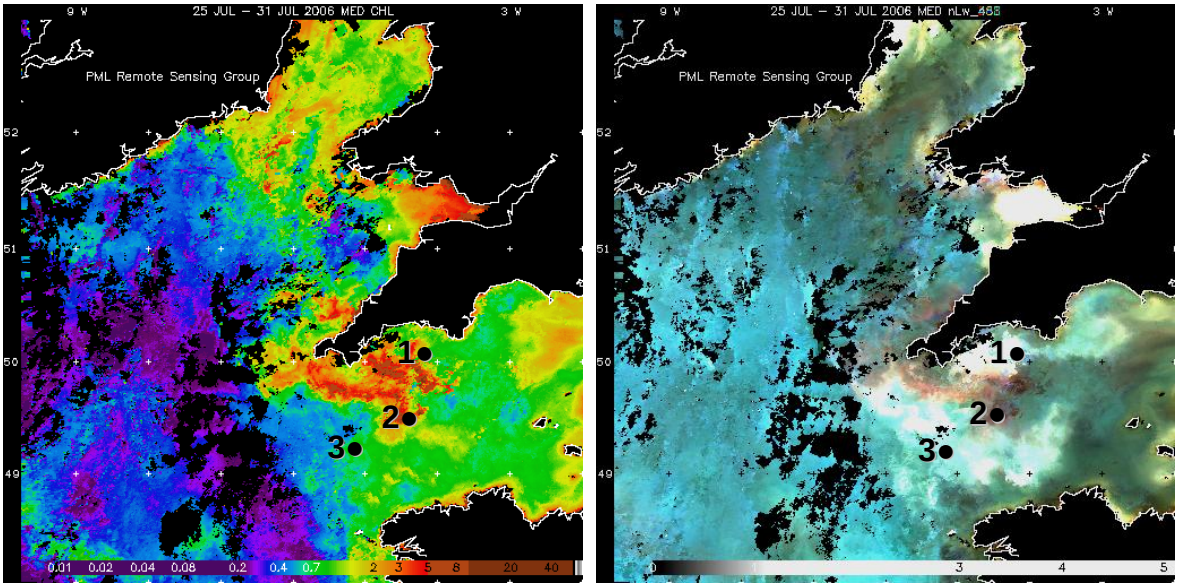

Figure 6. 1. Satellite derived *chl*-a composite (25-31 July 2006) showing the locations (black circle) of water samples collected on 26th/27th July 2006. A, MODIS and B, Pseudo-true colour MODIS images.

The pseudo-true colour MODIS images (Figure 6.1B) indicated the presence of a dark red-brown discolouration close to Station 2. Stations 1 and 3 showed a high reflection signature which is indicative of the presence of an *E. huxleyi* bloom. The D2newR primer (UniB) pair was used to amplify the obtained DNA from the 2006 bloom CTD samples (Table 6.1). All 18 samples produced an amplicon of approximately 346 bp (Figure 6.2). Station 1 samples produced bands of similar molecular size at all depths but they did not correspond with those produced by *K. mikimotoi* and *E. huxleyi*. Most of the Station 2 and 3 samples produced PCR amplicons of a size consistent with *K. mikimotoi* and *Emiliania huxleyi*. Only the 25m sample from Station 2 produced amplicons of unusually small and large size range (Figure 6.2). All the samples generated bands that were smeared in appearance, indicating mixed amplicon sizes (Figure 6.2).

6.2.2. Genotyping *Karenia mikimotoi* in 2006 bloom

PCR-HRM assays were used to analyse the DNA from the 2006 bloom samples (Appendix 5, Table 2). DNA from three different strains of *K. mikmotoi*, UK1 (CCMP429), NZ1 (CAWD134), and JP2 (CAWD05), was used as positive controls in each analysis. 14 out of 18 2006 bloom samples were positive for *K. mikmotoi* and HRM profiling revealed the presence of only sub-species 2 strains (Figure 6.3). The HRM profiles for Station 1 samples were positive for *K. mikmotoi* sub-species 2 in samples from depths of 10, 15 and 25m (Figure 6.3B). For the 15m sample there was a close correlation with the melt temperature (Tm) 77.68 ± 0.00 °C for the UK strain, while the other two samples produced Tm's of 77.83 ± 0.06°C, which is an intermediate Tm typical of a mixed sub-species 2 population (Chapter 4). Station 2 samples were positive for *K. mikmotoi* at all depths except for the surface (Figure 6.3C). The Tm's for all positive samples were 77.84 ± 0.09°C, again typical of *K. mikimotoi* sub-species 2.

Station 3 samples were positive for *K. mikmotoi* sub-species 2 in all depths (Figure 6.3D). The sample from 15 m showed slightly weaker amplification (appeared as light band compared with other bands on gel image, Appendix 9) and a shorter peaks beneath the threshold, which may suggest more cycles are required in the PCR. All positive samples, except the sample from 15 m, corresponded to the UK strain with a Tm of 77.68 ± 0.03 °C.

The PCR-HRM analysis was run in duplicate for all 2006 samples, both using the DNA directly as a template and using the universal *rbcL* (Uni1) amplicon as a template. All of the results were reproducible and were gel-verified (Appendix 9)

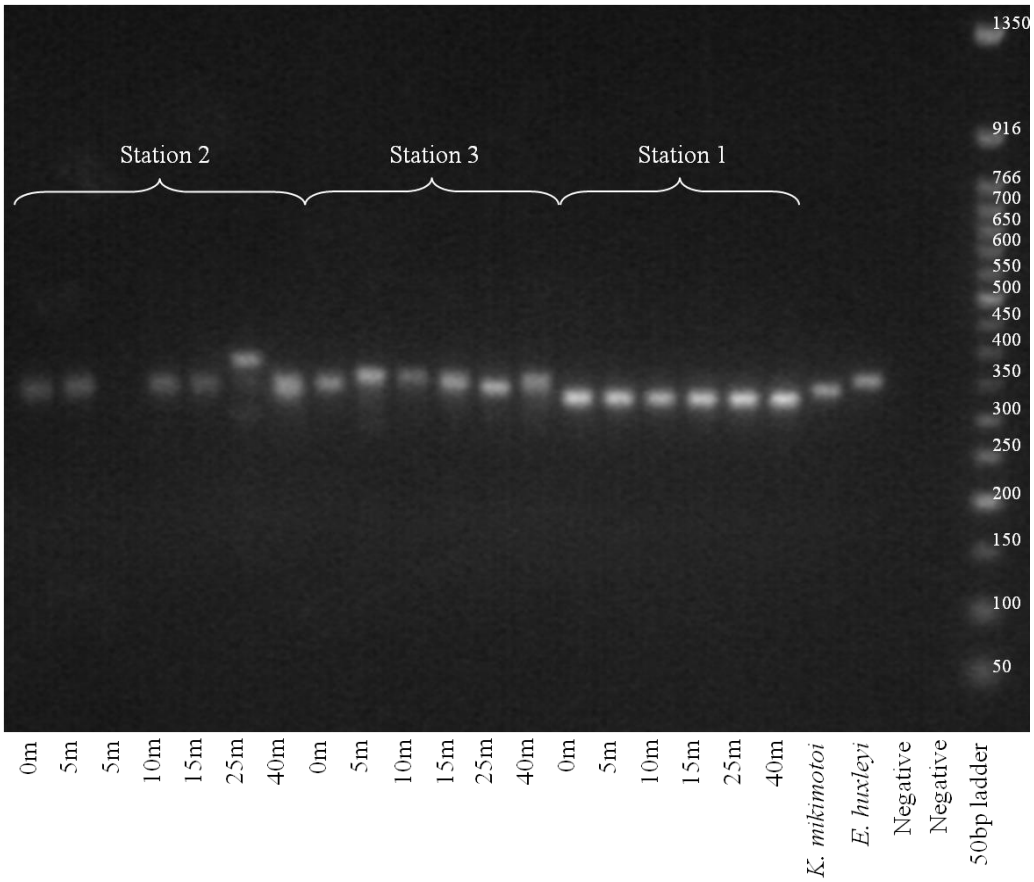

Figure 6. 2. Gel image of PCR products using the LSU (D1R-F & D2newR) rDNA for CTD samples of *K. mikimotoi* and *E. huxleyi* blooms in 2006.

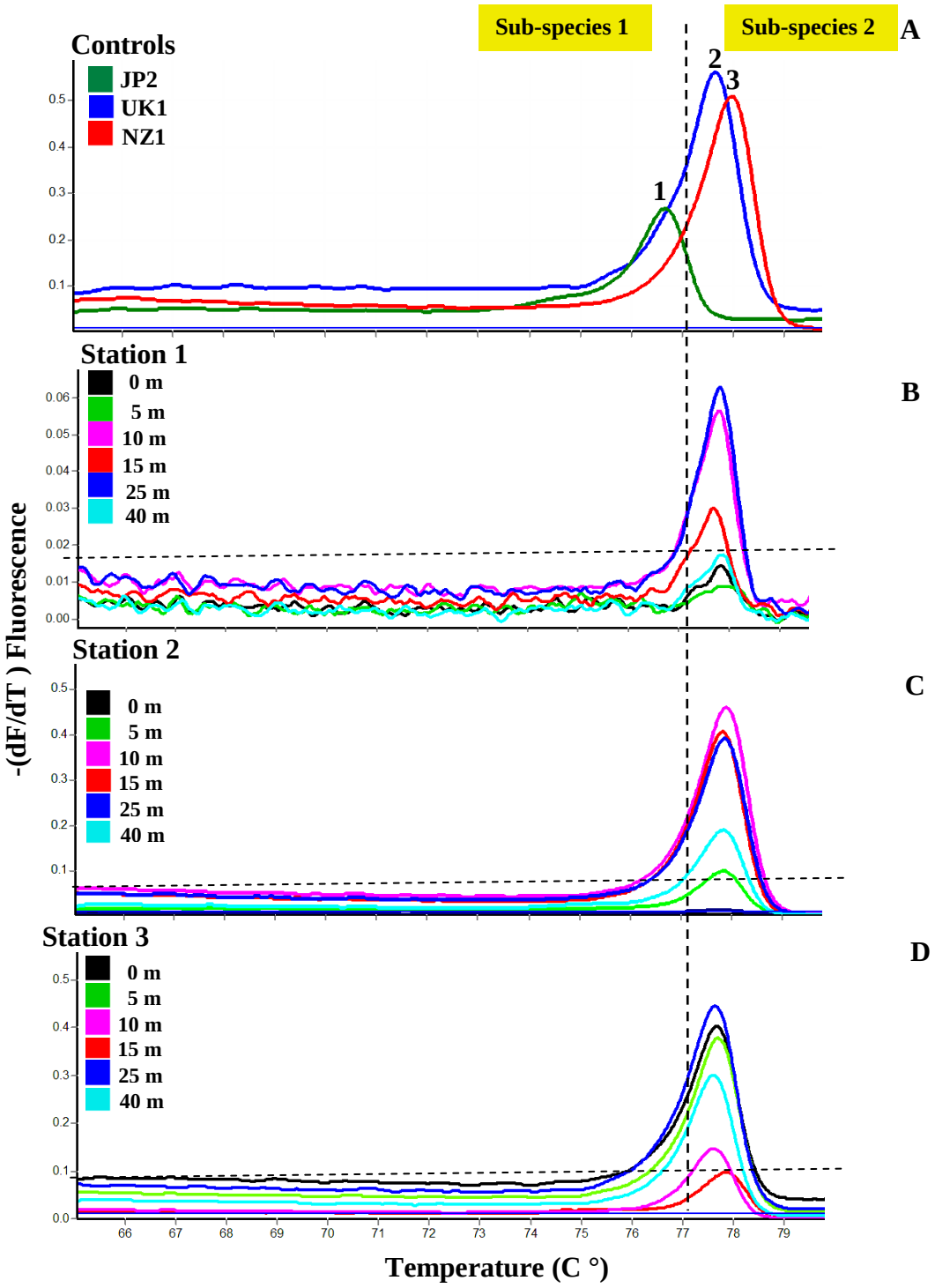

Figure 6. 3. Derivative HRM profile for: A. the three different genotypes of *K. mikimotoi* sub-species 1: 1. CAWD05 (JP2/green) and sub-species 2: 2. CCMP429 (UK1/blue) and 3. CAWD134 (NZ1/red) (positive controls); B-D. amplicons from samples collected in 2006 for: B. Station 1, C. Station 2 and D. Station 3.

6.2.3. CTD samples and *rbcL* gene clone libraries

Three CTD samples, Station 1 (0 m), Station 2 (40 m) and Station 3 (0 m), were selected for clone library construction and sequence analysis to confirm the presence or absence of *K. mikimotoi* and associated taxa in these samples: Stations 2 (40 m) and 3 (surface) samples were positive for *K. mikimotoi* at different depths, and the Station 1 (surface) sample was negative for *K. mikimotoi* as revealed by the HRM assay. The bulk DNA from these three stations was successfully amplified to produce a 620 bp amplicon using the Uni1 *rbcL* primers (*rbc*L 640-F and *rbcL*1240-R, Table 2.5) and ninety eight randomly selected clones from each sample were sequenced. Sequence identity was first determined using the BLAST protocol (Appendix 10, Table 1-3). All of the sequences in each sample library were aligned and manually screened for any sequence calling errors.

6.2.4. Depth samples and *rbcL* gene phylogeny

6.2.4.1. Station 1

A total of eighty nine sequences were obtained from Station 1 (Appendix 11, List 1). All could be assigned to the phylum Heterokontophyta based on BLAST searches (Appendix 10, Table 1) and phylogenic analyses (Figure 6.4). Most of the sequences belonged to the class Bacillariophyceae (99%) (Figure 6.5), with one sequence (S106B24, unknown sp.1) grouping with the class Actinochrysophyceae with high support bootstrap support (100% derived from NJ and DP and P = 1.00 from MrBayes analysis). Its closest neighbour within the class Actinochrysophyceae was the species *Pseudopedinella elastica*, but with only 87 % sequence identity.

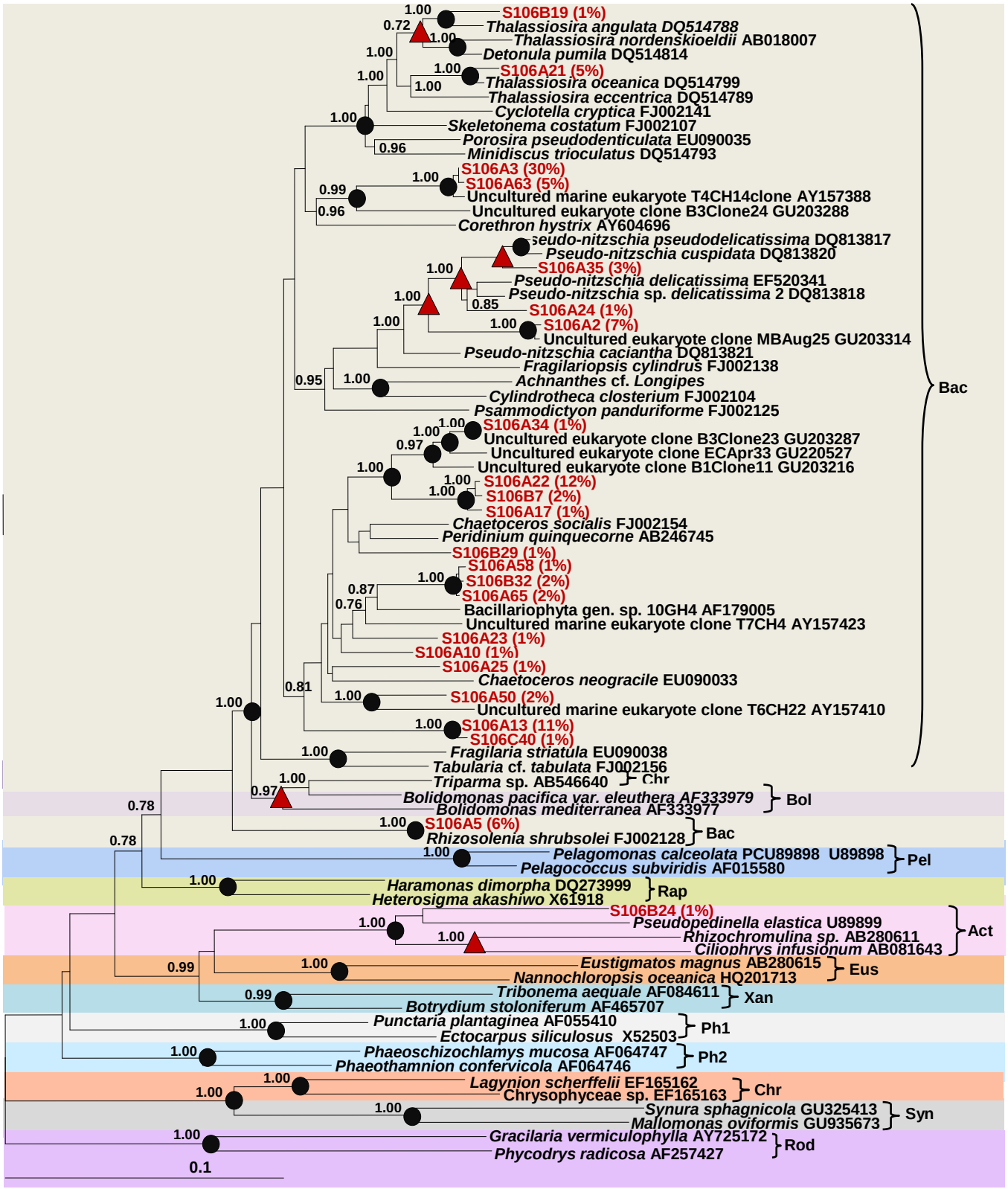
S106B19 (1%)
Thalassiosira angulata DQ514788
Thalassiosira nordenskioeldii AB018007
Detonula pumila DQ514814
S106A21 (5%)
Thalassiosira oceanica DQ514799
Thalassiosira eccentrica DQ514789
Cyclotella cryptica FJ002141
Skeletonema costatum FJ002107
Porosira pseudodenticulata EU090035
Minidiscus trioculatus DQ514793
S106A3 (30%)
S106A63 (5%)
Uncultured marine eukaryote T4CH14clone AY157388
Uncultured eukaryote clone B3Clone24 GU203288
Corethron hystrix AY604696
Pseudo-nitzschia pseudodelicatissima DQ813817
Pseudo-nitzschia cuspidata DQ813820
S106A35 (3%)
Pseudo-nitzschia delicatissima EF520341
Pseudo-nitzschia sp. delicatissima 2 DQ813818
S106A24 (1%)
S106A2 (7%)
Uncultured eukaryote clone MBAug25 GU203314
Pseudo-nitzschia caciantha DQ813821
Fragilariopsis cylindrus FJ002138
Achnanthes cf. Longipes
Cylindrotheca closterium FJ002104
Psammodictyon panduriforme FJ002125
S106A34 (1%)
Uncultured eukaryote clone B3Clone23 GU203287
Uncultured eukaryote clone ECApr33 GU220527
Uncultured eukaryote clone B1Clone11 GU203216
S106A22 (12%)
S106B7 (2%)
S106A17 (1%)
Chaetoceros socialis FJ002154
Peridinium quinquecorne AB246745
S106B29 (1%)
S106A58 (1%)
S106B32 (2%)
S106A65 (2%)
Bacillariophyta gen. sp. 10GH4 AF179005
Uncultured marine eukaryote clone T7CH4 AY157423
S106A23 (1%)
S106A10 (1%)
S106A25 (1%)
Chaetoceros neogracile EU090033
S106A50 (2%)
Uncultured marine eukaryote clone T6CH22 AY157410
S106A13 (11%)
S106C40 (1%)
Fragilaria striatula EU090038
Tabularia cf. tabulata FJ002156
Triparma sp. AB546640
Chr
Bolidomonas pacifica var. eleuthera AF333979
Bolidomonas mediterranea AF333977
Bol
S106A5 (6%)
Rhizosolenia shrubsolei FJ002128
Bac
Pelagomonas calceolata PCU89898 U89898
Pelagococcus subviridis AF015580
Pel
Haramonas dimorpha DQ273999
Heterosigma akashiwo X61918
Rap
S106B24 (1%)
Pseudopedinella elastica U89899
Rhizochromulina sp. AB280611
Ciliophrys infusionum AB081643
Act
Eustigmatos magnus AB280615
Nannochloropsis oceanica HQ201713
Eus
Tribonema aequale AF084611
Botrydium stoloniferum AF465707
Xan
Punctaria plantaginea AF055410
Ectocarpus siliculosus X52503
Ph1
Phaeoschizochlamys mucosa AF064747
Phaeothamnion confervicola AF064746
Ph2
Lagynion scherffelii EF165162
Chrysophyceae sp. EF165163
Chr
Synura sphagnicola GU325413
Mallomonas oviformis GU935673
Syn
Gracilaria vermiculophylla AY725172
Phycodrys radicosa AF257427
Rod
Bac
0.1

Figure 6. 4. Phylogenetic tree of sequenced clones of marine Heterokontophyta taxa derived from a 500 bp amplicon of the *rbcL* gene for Station 1 (surface) sample with the % occurrence for each sequence (bold red). This tree was constructed by using the neighbour-joining (NJ), parsimony (dnapars) (DP) and MrBayes (MB). Bootstrap values were calculated from 1000 trees of NJ and 100 trees of DP. Shapes at nodes indicate bootstrap values greater than 70 % for: both NJ and DP (●), only NJ (▲) and only DP (■) and numerals represent MB. Scale bar indicates a distance of 0.1 substitutions per site. The class for each clade was indicated on the right side of the phylogeny. The corresponding sequences which represent the groups are: Bac = Bacillariophyceae, Bol = Bolidophyceae, Pel = Pelagophyceae, Rap = Raphidophyceae, Act = Actinochrysophyceae, Eus = Eustigmatophyceae, Xan = Xanthophyceae (yellow-green algae), Ph1 = Phaeophyceae (brown algae), Ph2 = Phaeothamniophyceae, Chr = Chrysophyceae (golden algae), Syn = Synurophyceae, Rod = Rodophyta (Red-algae). The corresponding sequences representing the red-algae were employed as an outgroup.

The most abundant sequences (83 % of the clone library) in the class Bacillariophyceae (Diatoms) could not be assigned to any particular genus or species. A sequence similarity cut-off of > 97 % was used to define a "species" (Caron et al., 2009). These genotypes were consequently listed as "unknown" diatoms, 1 to 11 (Table 6.2). Unknown diatom species 1 dominated this class (35%), followed by sp. 4 (16 %) and sp. 11 (12 %). Many clones were found to be most similar to sequences generated from the Western Channel Observatory (Bhadury and Ward, 2009). For example, clone S106A2, or unknown diatom sp. 2, differed by just two base pairs from uncultured marine eukaryote GU203314. Similarly clone S106A34, or unknown diatom sp. 3, shares an identical sequence with uncultured marine eukaryote GU203287 and both were clustered with the unknown marine eukaryotes GU220527 and GU203216 (Figure 6.4).

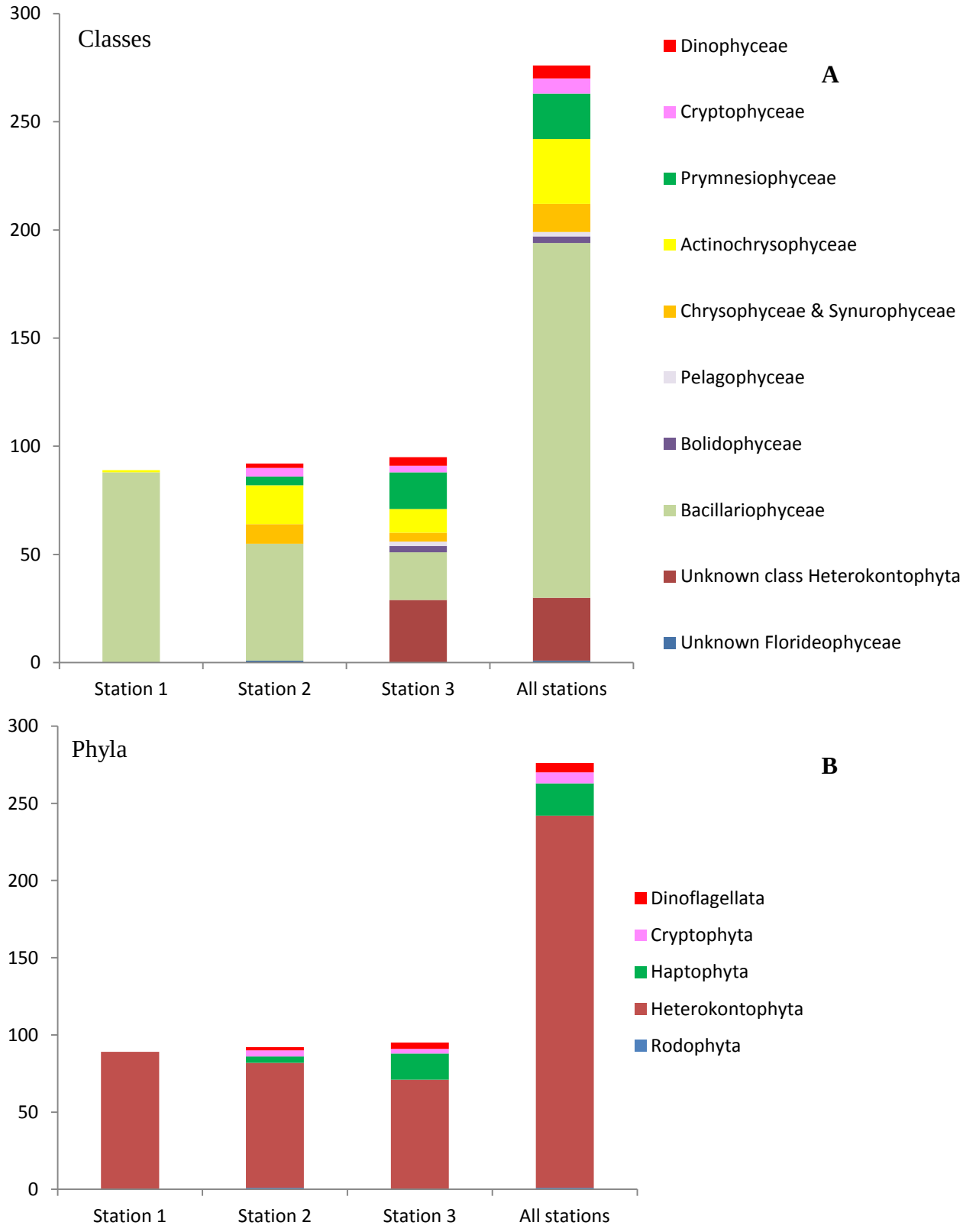

Figure 6.5. The abundance of high-level eukaryotic taxa derived from *rbcL* gene sequences for each of Stations 1, 2 and 3 and for all three stations combined: A, Class level; B, Phylum level.

Many of the clone library sequences have no strong identities to known sequences in GenBank (Appendix 10), however, they were found to be nested within the Bacillariophyceae lineage (Figure 6.4). Clones S106B29 and S106A25, for example, grouped with many unknown uncultured strains and with known diatoms of the *Chaetoceros* genus. They clustered (supported weakly by the Bayesian analysis 0.81, Figure 6.4) with the species *Chaetoceros socialis, C. neogracile* and *Peridinium quinquecorne* (which has the sequence of its *Chaetoceros* endosymbiont (Horiguchi and Takano, 2006)).

Table 6. 2. Taxonomic assignment of red-algal plastid-type phytoplankton found at Stations 1, 2 and 3 based on BLAST similarities and phylogentic groupings.

Taxonomic level	Clone (s)	Station 1	Station 2	Station 3
Division: Rodophyta (Red-algae) Class: Florideophyceae				
Unknown Florideophyceae sp. 1	S206B18		1	
Phylum: Heterokontophyta				
Unknown class Heterokontophyta	S306A42, S306B15, S306A3, S306B33, S306A4			29
Class: Bacillariophyceae (Diatoms)				
Thalassiosira angulata	S106B19	1		
T. oceanic	S106A21	4		
T. eccentric	S206A59		2	
Detonula pumila	S206B22		1	
Minidiscus trioculatus	S206B24, S206A62		2	
Rhizosolenia shrubsolei	S106A5, S206A40	5	10	
Pseudonitzschia sp. 1	S106A35, S206B45	3	9	
P. sp. 2	S106A24, S206A42	1	2	
Psammodictyon panduriforme	S2062A17		1	
Unknown diatom sp. 1	S106A3, S106A63	31		
Unknown diatom sp. 2	S106A2	6		
Unknown diatom sp. 3	S106A34	1		
Unknown diatom sp. 4	S106A22, S106B7, S106A17, S206B48	14	1	
Unknown diatom sp. 5	S106B29	1		
Unknown diatom sp. 6	S106A58, S106B32, S106A65	5		
Unknown diatom sp. 7	S106A23	1		
Unknown diatom sp. 8	S106A10	1		
Unknown diatom sp. 9	S106A25	1		
Unknown diatom sp.10	S106A50	2		
Unknown diatom sp.11	S106A13, S106C40, S306A34, S306A17	11		11
Unknown diatom sp.12	S206A28		3	
Unknown diatom sp.13	S206A6		3	
Unknown diatom sp.14	S206B34		1	
Unknown diatom sp.15	S206A56		1	
Unknown diatom sp.16	S206A7, S206A65, S206B15		12	

Unknown diatom sp.17	S206A33, S206A5		4	
Unknown diatom sp.18	S206B13		1	
Unknown diatom sp.19	S2062A1		1	
Unknown diatom sp. 20	S306A40			2
Unknown diatom sp. 21	S306B26			1
Unknown diatom sp. 22	S306A55			1
Unknown diatom sp. 23	S306A61			3
Unknown diatom sp. 24	S306A12			1
Unknown diatom sp. 25	S306B30			2
Unknown diatom sp. 26	S306B16			1
Class: Bolidophyceae				
Unknown Bolidophyceae sp. 1	S306A7			3
Class: Pelagophyceae				
Unknown Pelagophyceae sp. 1	S306A51			1
Unknown Pelagophyceae sp. 2	S306A54			1
Class: Chrysophyceae & Synurophyceae				
Unknown Parmales sp. 1	S206A39, S306A29		5	1
Unknown Chrysophyceae sp.1	S206A49		1	
Unknown Chrysophyceae sp. 2	S206A60		3	
Unknown Chrysophyceae & Synurophyceae sp. 1	S306A16			3
Class: Actinochrysophyceae				
Verrucophora verruculosa	S306A38, S306A44			2
Unknown Actinochrysophyceae sp. 1	S106B24, S206A4, S206B19, S306A58	1	18	8
Unknown Actinochrysophyceae sp. 2	S306A15			1
Phylum: Haptophyta Class: Prymnesiophyceae				
Phaeocystis pouchetii	S206A23		2	
Emiliania huxleyi & *Gephyrocapsa oceanica*	S306B5			2
Unknown prymneiophyceae sp. 1	S206A61, S306A63		2	1
Unknown prymneiophyceae sp. 2	S306A59, S306B25			4
Unknown prymneiophyceae sp. 3	S306A30			1
Unknown prymneiophyceae sp. 4	S306A19			3
Unknown prymneiophyceae sp. 5	S306A47			1
Unknown prymneiophyceae sp. 6	S306B12			2
Unknown prymneiophyceae sp. 7	S306B4			1

Unknown prymneiophyceae sp. 8	S306A10			1
Unknown prymneiophyceae sp. 9	S306B3			1
Phylum: Cryptophyta Class: Cryptophyceae				
Teleaulax sp. 1	S206A31, S206B20, S306A28, S306A35		3	3
Unknown cryptophyte sp.1	S206B14		1	
Phylum: Dinoflagellata Class: Dinophyceae				
Karenia mikimotoi	S206A32, S306A6		2	4

Shared taxa: Stations 1 & 2 (pink); Stations 2 & 3 (purple); Stations 1 & 3 (brown); Stations 1, 2 & 3 (orange).

Five of the remaining species clusters could be tentatively identified to the species/genus level: Clones S106B19 and S106A21 grouped with similar sequences of *Thalassiosira angulata* and *T. oceanica* sharing sequence identities of 97% and 98%, respectively. Clones S106A35 and S106A24 grouped separately with two different sub-groups of *Pseudo-nitzschia* spp. These subgroups shared 98% and 97% sequence identities, respectively, with one clade containing two different species namely *P. pseudodelicatissima* and *P. cuspidata*, while the other with *P. delicatissima* (Figure 6.4). Finally, clone S106A5 has an identical sequence to *Rhizosolenia shrubsolei*.

6.2.4.2. Station 2

Ninety two sequences were obtained from the 40 m sample from Station 2 (Appendix 11, List 2). The cloned sequences could be assigned to the Heterokontophyta, Haptophyta, Cryptophyta and Dinoflagellata based on both BLAST similarities (Appendix 10, Table 2) and phylogenetic inference (Figures 6.6 and 6.7).

As it was the case of Station 1, the majority of the cloned sequences from the phylum Heterokontophyta belonged to the Bacillariophyceae (59 %) (Figure 6.6), 50 % of which could not be assigned to any particular diatom species of (sp. 4 & sp.12 to 19, Table 6.2). The clones with sequences identical to known diatoms were S206A62, S206B22 and S206A40, which matched *Minidiscus trioculatus*, *Detonula pumila* and *Rhizosolenia shrubsolei*, respectively (Figure 6.6 and Table 6.2). Two clones, S206B24 and S2062A17, had single base mismatchs with *Minidiscus trioculatus* and *Psammodictyon panduriforme*, respectively. Others that could be tentatively assigned to genera or species were *Pseudo-*

nitzschia spp. (S206B45), *Pseudo-nitzschia delicatissima* (S206A42) and *Thalassiosira eccentrica* (S206A59). Clone S206B48 shared 97% sequence identity with other clones from Station 1 (Table 6.2), which in turn grouped with *Chaetoceros socialis* (Figure 6.6). The remainder of the clones, whilst defined here as unknowns, did nonetheless cluster strongly with known diatoms: clones S206A33 and S206A5 with *Hyalodiscus* sp.; clones S206A28 and S206A6 with *Gyrosigma acuminata*; clone S206B34 with *Cylindrotheca* spp.; S206B13 with *Attheya septentrionalis* (Figure 6.6).

The second most abundant heterokont class in the 40 m sample from Station 2 was the Actinochrysophyceae (20%), which comprised two very similar sequences (mismatched by just 2 bp), S206A4 and S206B19 (unknown sp.1). They clustered most closely with *Pseudopedinella elastica* and shared > 97% similarity to the clone sequenced from Station 1 (S106B24). Ten percent of the clones belonged to the classes Synurophyceae and Chrysophyceae, including clone S206A39, which shared 96% sequence identity with of *Triparma* sp. (Parmales) and clustered strongly with the class Bolidophyceae (Figure 6.6). The other two clones, S206A49 and S206A60, were both of unknown origin, but formed a distinct cluster with other Chrysophyceae (Figure 6.6). One clone, S206B18 (unknown sp. 1), clustered within the Rhodophyte Florideophyceae: the best sequence matches were to *Batrachospermum involutum* (71% identity) and *Hildenbrandia rubra* (72% identity) (Figure 6.6).

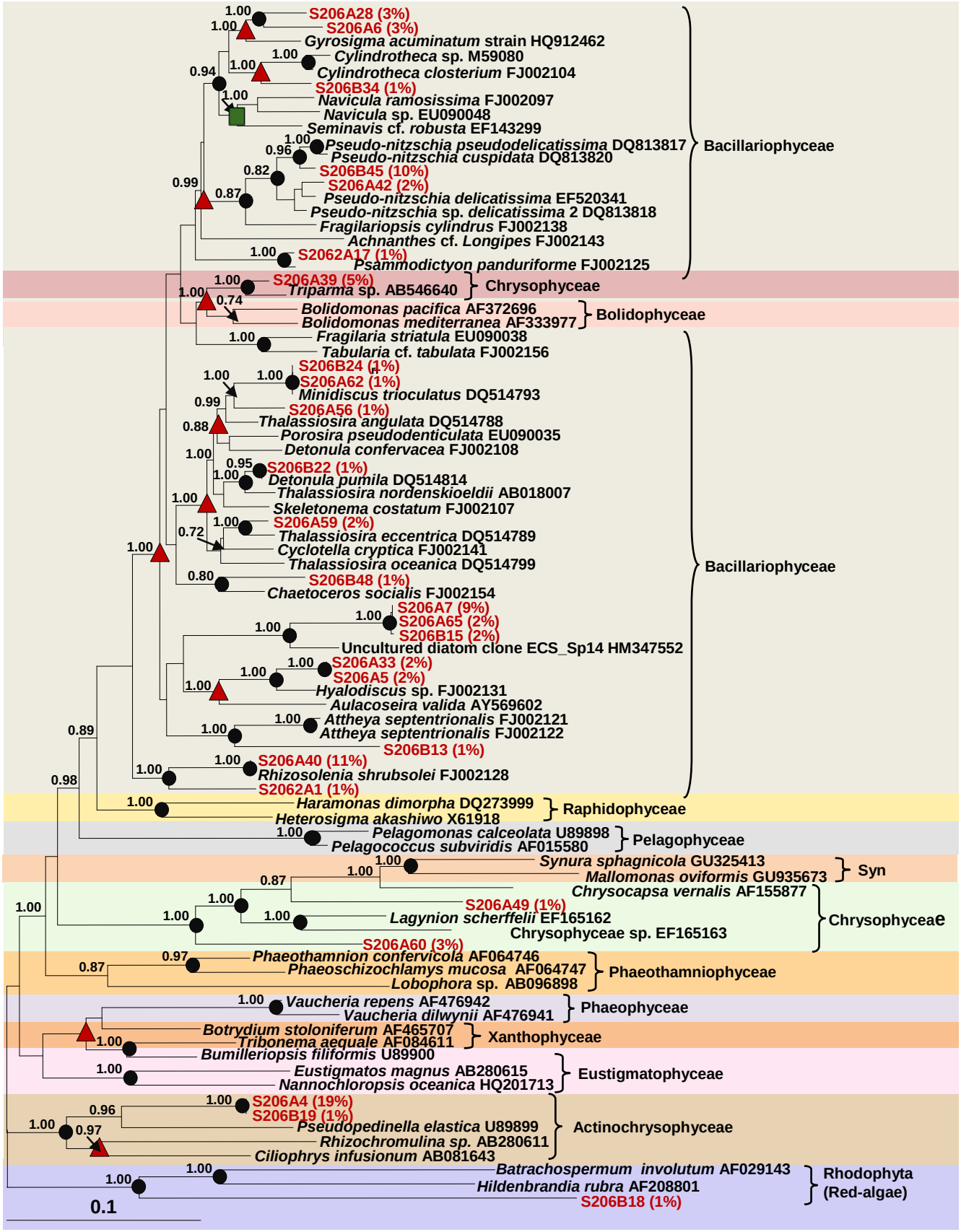
S206A28 (3%)
S206A6 (3%)
Gyrosigma acuminatum strain HQ912462
Cylindrotheca sp. M59080
Cylindrotheca closterium FJ002104
S206B34 (1%)
Navicula ramosissima FJ002097
Navicula sp. EU090048
Seminavis cf. robusta EF143299
Pseudo-nitzschia pseudodelicatissima DQ813817
Pseudo-nitzschia cuspidata DQ813820
S206B45 (10%)
S206A42 (2%)
Pseudo-nitzschia delicatissima EF520341
Pseudo-nitzschia sp. delicatissima 2 DQ813818
Fragilariopsis cylindrus FJ002138
Achnanthes cf. Longipes FJ002143
S2062A17 (1%)
Psammodictyon panduriforme FJ002125
Bacillariophyceae
S206A39 (5%)
Triparma sp. AB546640
Chrysophyceae
Bolidomonas pacifica AF372696
Bolidomonas mediterranea AF333977
Bolidophyceae
Fragilaria striatula EU090038
Tabularia cf. tabulata FJ002156
S206B24 (1%)
S206A62 (1%)
Minidiscus trioculatus DQ514793
S206A56 (1%)
Thalassiosira angulata DQ514788
Porosira pseudodenticulata EU090035
Detonula confervacea FJ002108
S206B22 (1%)
Detonula pumila DQ514814
Thalassiosira nordenskioeldii AB018007
Skeletonema costatum FJ002107
S206A59 (2%)
Thalassiosira eccentrica DQ514789
Cyclotella cryptica FJ002141
Thalassiosira oceanica DQ514799
S206B48 (1%)
Chaetoceros socialis FJ002154
S206A7 (9%)
S206A65 (2%)
S206B15 (2%)
Uncultured diatom clone ECS_Sp14 HM347552
S206A33 (2%)
S206A5 (2%)
Hyalodiscus sp. FJ002131
Aulacoseira valida AY569602
Attheya septentrionalis FJ002121
Attheya septentrionalis FJ002122
S206B13 (1%)
S206A40 (11%)
Rhizosolenia shrubsolei FJ002128
S2062A1 (1%)
Bacillariophyceae
Haramonas dimorpha DQ273999
Heterosigma akashiwo X61918
Raphidophyceae
Pelagomonas calceolata U89898
Pelagococcus subviridis AF015580
Pelagophyceae
Synura sphagnicola GU325413
Mallomonas oviformis GU935673
Syn
Chrysocapsa vernalis AF155877
S206A49 (1%)
Lagynion scherffelii EF165162
Chrysophyceae sp. EF165163
S206A60 (3%)
Chrysophyceae
Phaeothamnion confervicola AF064746
Phaeoschizochlamys mucosa AF064747
Lobophora sp. AB096898
Phaeothamniophyceae
Vaucheria repens AF476942
Vaucheria dilwynii AF476941
Phaeophyceae
Botrydium stoloniferum AF465707
Tribonema aequale AF084611
Xanthophyceae
Bumilleriopsis filiformis U89900
Eustigmatos magnus AB280615
Nannochloropsis oceanica HQ201713
Eustigmatophyceae
S206A4 (19%)
S206B19 (1%)
Pseudopedinella elastica U89899
Rhizochromulina sp. AB280611
Ciliophrys infusionum AB081643
Actinochrysophyceae
Batrachospermum involutum AF029143
Hildenbrandia rubra AF208801
S206B18 (1%)
Rhodophyta
(Red-algae)
0.1

Figure 6. 6. Phylogenetic tree of sequenced clones of marine Heterokonta taxa derived from a 500 bp amplicon of the *rbcL* gene from the Station 2 (40 m) sample with the % occurrence of each sequence (bold red). This tree was constructed by using the neighbour-joining (NJ), parsimony (dnapars) (DP) and MrBayes (MB). Bootstrap values were calculated from 1000 trees of NJ and 100 trees of DP. Shapes at nodes indicate bootstrap values greater than 70 % for: both NJ and DP (●), only NJ (▲) and only DP (■) and numerals represent MB. Scale bar indicates a distance of 0.1 substitutions per site. The class for each clade is indicated on the right side of the phylogeny. The sequences that represent the red-algae were employed as an outgroup.

Genotypes most similar to the phyla Haptophyta, Cryptophyta and Dinoflagellata can be seen in Figure 6.7. Two clones could be assigned to the haptophyte class Prymnesiophyceae. Clone S206A61 (sp.1) shared 94% sequence identity with *Chrysochromulina* sp. and was defined as unknown prymnesiophyte sp. 1 (Table 6.2). The other clone, S206A23, shared 99% sequence identity with *Phaeocystis pouchetii*. The cryptophyte S206A31 shared identical sequence with the uncultured eukaryotes GU203358 and GU2205451 generated from the Western Channel Observatory (Bhadury and Ward, 2009). Both were most likely the cryptophyte endosymbiont sequences of *Dinophysis fortii* and *D. tripos*, which clustered with the known cryptophyte *Teleaulax* sp. (Figure 6.7). Similarly, clone S206B20 shared 99% sequence identity with *Telaulax* sp. Clone S206B14 could not be associated with previously described sequences and was therefore was assigned the name unknown cryptophte sp. 1 (Table 6.2). Finally, only one clone, S206A32, belonging to the class Dinophyceae shared identical sequence with *Karenia mikimotoi* (Figure 6.7).

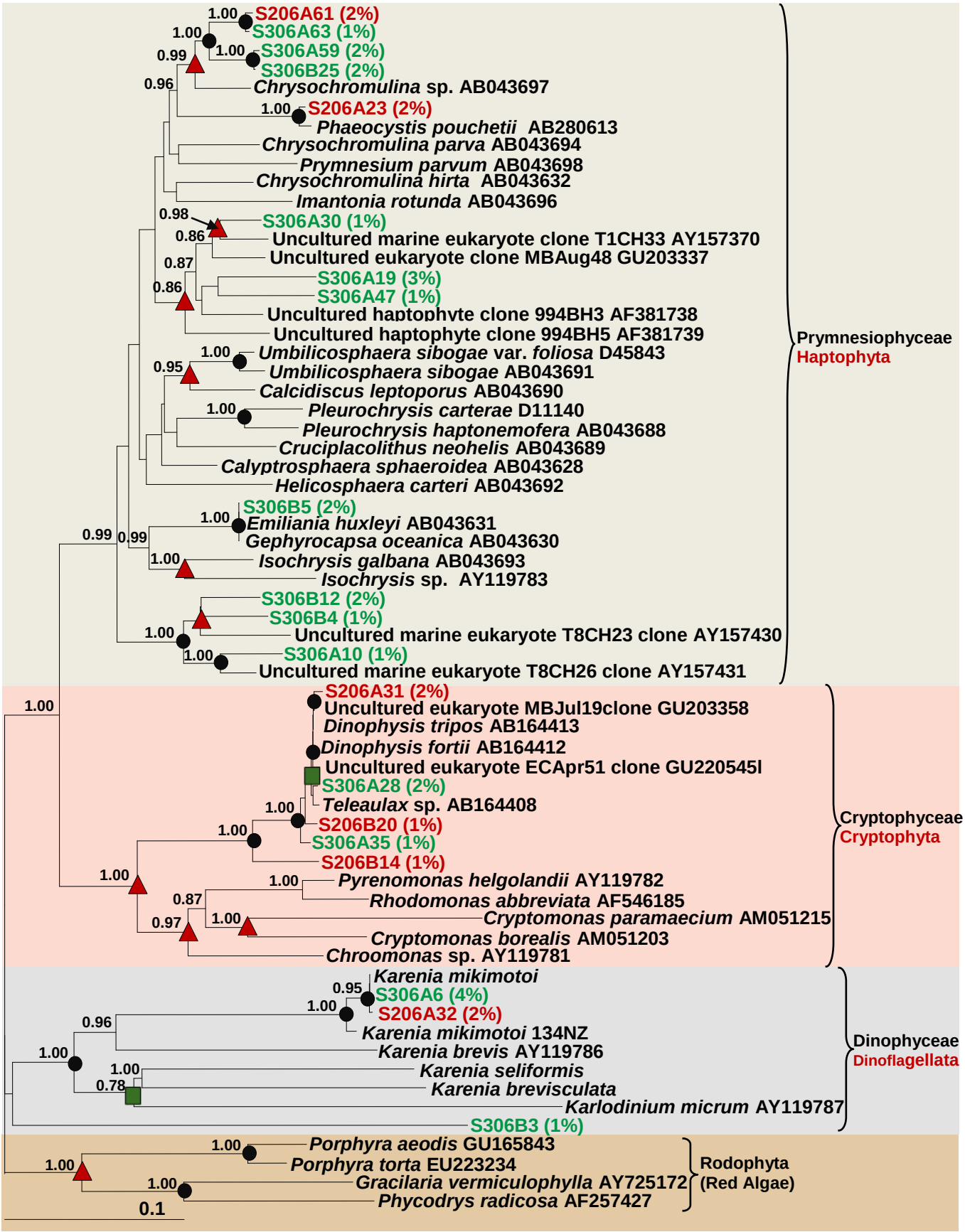
S206A61 (2%)
S306A63 (1%)
S306A59 (2%)
S306B25 (2%)
Chrysochromulina sp. AB043697
S206A23 (2%)
Phaeocystis pouchetii AB280613
Chrysochromulina parva AB043694
Prymnesium parvum AB043698
Chrysochromulina hirta AB043632
Imantonia rotunda AB043696
S306A30 (1%)
Uncultured marine eukaryote clone T1CH33 AY157370
Uncultured eukaryote clone MBAug48 GU203337
S306A19 (3%)
S306A47 (1%)
Uncultured haptophyte clone 994BH3 AF381738
Uncultured haptophyte clone 994BH5 AF381739
Umbilicosphaera sibogae var. foliosa D45843
Umbilicosphaera sibogae AB043691
Calcidiscus leptoporus AB043690
Pleurochrysis carterae D11140
Pleurochrysis haptonemofera AB043688
Cruciplacolithus neohelis AB043689
Calyptrosphaera sphaeroidea AB043628
Helicosphaera carteri AB043692
S306B5 (2%)
Emiliania huxleyi AB043631
Gephyrocapsa oceanica AB043630
Isochrysis galbana AB043693
Isochrysis sp. AY119783
S306B12 (2%)
S306B4 (1%)
Uncultured marine eukaryote T8CH23 clone AY157430
S306A10 (1%)
Uncultured marine eukaryote T8CH26 clone AY157431
Prymnesiophyceae
Haptophyta
S206A31 (2%)
Uncultured eukaryote MBJul19clone GU203358
Dinophysis tripos AB164413
Dinophysis fortii AB164412
Uncultured eukaryote ECApr51 clone GU220545l
S306A28 (2%)
Teleaulax sp. AB164408
S206B20 (1%)
S306A35 (1%)
S206B14 (1%)
Pyrenomonas helgolandii AY119782
Rhodomonas abbreviata AF546185
Cryptomonas paramaecium AM051215
Cryptomonas borealis AM051203
Chroomonas sp. AY119781
Cryptophyceae
Cryptophyta
Karenia mikimotoi
S306A6 (4%)
S206A32 (2%)
Karenia mikimotoi 134NZ
Karenia brevis AY119786
Karenia seliformis
Karenia brevisculata
Karlodinium micrum AY119787
S306B3 (1%)
Dinophyceae
Dinoflagellata
Porphyra aeodis GU165843
Porphyra torta EU223234
Gracilaria vermiculophylla AY725172
Phycodrys radicosa AF257427
Rodophyta
(Red Algae)
0.1

Figure 6. 7. Phylogenetic tree of sequenced clones of marine Haptophyta, Cryptophyta and Dinoflagellata taxa derived from a 500 bp amplicon of the *rbcL* gene for Station 2 (40 m) and Station 3 (surface) samples, with the % occurrence of each sequence (bold red). This tree was constructed by using both the neighbour-joining (NJ) and parsimony (dnapars) (DP) and MrBayes (MB). Bootstrap values were calculated from 1000 trees of NJ and 100 trees of DP. Shapes at nodes indicate bootstrap values greater than 70 % for: both NJ and DP (●), only NJ (▲) and only DP (■) and numerals represent MB. Scale bar indicates a distance of 0.1 substitutions per site. The class for each clade is indicated on the right side of the phylogeny. The corresponding sequences representing the red-algae were employed as an outgroup.

6.2.4.3. Station 3

Ninety five sequences were obtained from the surface sample from Station 3 (0 m) (Appendix 11, List 3). As for Station 2, clones could be assigned to the Heterokontophyta, Haptophyta, Cryptophyta and Dinoflagellata based on BLAST similarities (Appendix 10, Table 3) and phylogenetic inference (Figure 6.7 and 6.8).

The majority (31%) of the clone sequences in the phylum Heterokontophyta (Figure 6.8) were related to an unknown heterokont class or classes. This was followed by those assigned to the class Bacillariophyceae (23%), which like before was dominated by unknown diatoms (sp. 11 and 20-26, Table 6.2). The unknown heterokont class(es) included five sequences (sharing > 97% sequence identity) that did not cluster with any known Heterokontophyta group, although one clone, S306A4, shared 91% sequence identity to an uncultured marine eukaryote AY157374 which generated from Gulf of Mexico (NCBI). These five clones could, however, be assigned with confidence to Heterokontophyta since they shared a branch with members of the classes Xanthophyceae and Actinochrysophyceae with posterior probability of 0.75 (Figure 6.8).

The unknown bacillariophyte clones, S306A34 and S306A17, differed from each other by a single mismatched base pair (sp. 11). They did cluster with *Chaetoceros* spp., but without strong bootstrap or Bayesian support (Figure 6.8). This was also the case for clones S306A55 (sp. 22) and S306A61 (sp. 23), which clustered with *Attheya septentrionalis* (Figure 6.8); and S306B26 (sp. 21) which shared 99% sequence identity with the uncultured eukaryote sequence GU220549 generated from the Western Channel Observatory (Bhadury and Ward, 2009), but clustered with the known species *Rhizosolenia shrubsolei.*

The remainder of unknown Bacillariophyceae (sp. 20 and 24) clustered with other uncultured eukaryote sequences which deposited in GenBank. Clones S306A40 and S306A12 shared 99 % sequence identities with AY157398 which generated from the Gulf of Mexico (NCBI) and GU203288 which generated from the Western Channel Observatory (Bhadury and Ward, 2009) respectively. Clones S306B30 (sp. 25) and S306B16 (sp. 26) clustered with GU203306 which also generated from the Western Channel Observatory (Bhadury and Ward, 2009).

The third most abundant class was the Actinochrysophyceae (12%), which included two sequences S306A38 and S306A44 that were closely related (99% and 98% identity respectively) to *Verrucophora verruculosa*, (Figure 6.8). Clones S306A58 (unknown sp. 1) and S306A15 (unknown sp. 2) clustered with *Pseudopedinella elastica* and *Helicopedinella tricostata*.

The remaining heterokonts could be assigned to the classes Synurophyceae (Parmales), Chrysophceae, Bolidophyceae and Pelagophyceae: clones S306A29 and S306A7 clustered with *Triparma* sp. and *Bolidomonas pacifica*, respectively (Figure 6.8); clone S306A16 clustered with the classes Synurophyceae and Chrysophyceae; Pelagophyceae-like sequences included S306A51 and S306A54 which clustered with *Aureococcus*

anophagefferens and an unnamed pelagophyceae species AF179025. Due to their weak sequence identities to known species in GenBank, all of these clones were assigned as unknown species with their respective classes (Table 6.2).

The clones belonging to the Haptophyta, Cryptophyta and Dinoflagellata are shown in Figure 6.7. Several clones (S306A63, S306A59, S306B25, S306A28, S306A35 and S306A6) shared sequence identity (> 97 %) with sequences obtained from Station 2 (40 m). Two sets of clones within the class Prymnesiophyceae clustered strongly with *Chrysochromulina* sp. and *Emiliania huxelyi/Gephyrocapsa oceanica* strains. The remaining six clones (sp. 3 to 9) could not be assigned to any particular prymnesiophyte group (Table 6.2) and were mostly clustered with uncultured eukaryotes (Figure 6.7). This includes clone S306B3 which clustered within the prymnesiophytes clade when using Bayesian analysis (Figure 6.9).

Cryptophyta-like sequences included S306A28 and S306A35 which shared > 97 % sequence identity with *Teleaulax* sp. Finally, the clone S306A6 was identical to *K. mikimotoi* (class Dinoflagellata).

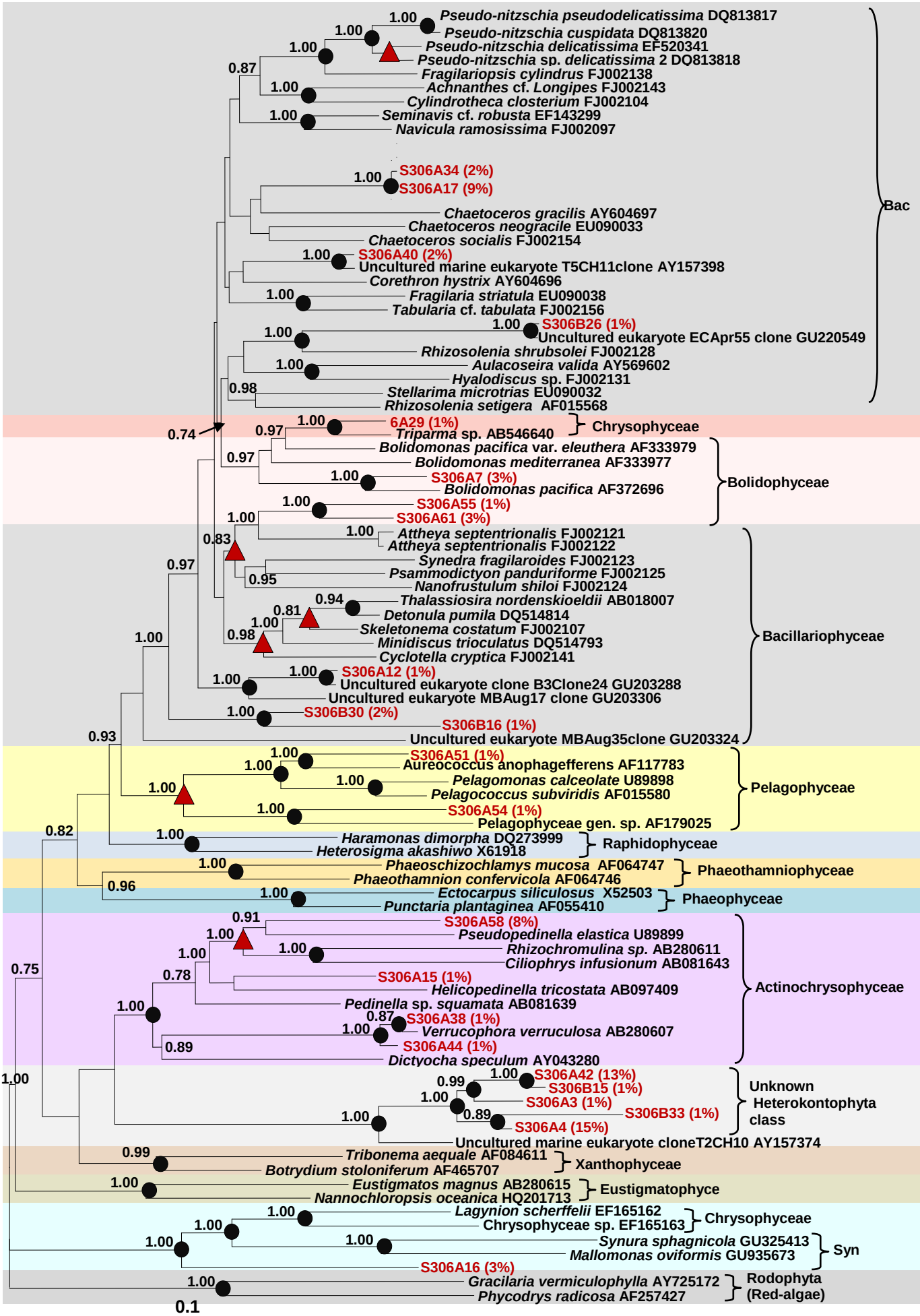
Pseudo-nitzschia pseudodelicatissima DQ813817
Pseudo-nitzschia cuspidata DQ813820
Pseudo-nitzschia delicatissima EF520341
Pseudo-nitzschia sp. delicatissima 2 DQ813818
Fragilariopsis cylindrus FJ002138
Achnanthes cf. Longipes FJ002143
Cylindrotheca closterium FJ002104
Seminavis cf. robusta EF143299
Navicula ramosissima FJ002097
S306A34 (2%)
S306A17 (9%)
Chaetoceros gracilis AY604697
Chaetoceros neogracile EU090033
Chaetoceros socialis FJ002154
S306A40 (2%)
Uncultured marine eukaryote T5CH11clone AY157398
Corethron hystrix AY604696
Fragilaria striatula EU090038
Tabularia cf. tabulata FJ002156
S306B26 (1%)
Uncultured eukaryote ECApr55 clone GU220549
Rhizosolenia shrubsolei FJ002128
Aulacoseira valida AY569602
Hyalodiscus sp. FJ002131
Stellarima microtrias EU090032
Rhizosolenia setigera AF015568
Bac
6A29 (1%)
Triparma sp. AB546640
Chrysophyceae
Bolidomonas pacifica var. eleuthera AF333979
Bolidomonas mediterranea AF333977
S306A7 (3%)
Bolidomonas pacifica AF372696
S306A55 (1%)
S306A61 (3%)
Bolidophyceae
Attheya septentrionalis FJ002121
Attheya septentrionalis FJ002122
Synedra fragilaroides FJ002123
Psammodictyon panduriforme FJ002125
Nanofrustulum shiloi FJ002124
Thalassiosira nordenskioeldii AB018007
Detonula pumila DQ514814
Skeletonema costatum FJ002107
Minidiscus trioculatus DQ514793
Cyclotella cryptica FJ002141
S306A12 (1%)
Uncultured eukaryote clone B3Clone24 GU203288
Uncultured eukaryote MBAug17 clone GU203306
S306B30 (2%)
S306B16 (1%)
Uncultured eukaryote MBAug35clone GU203324
Bacillariophyceae
S306A51 (1%)
Aureococcus anophagefferens AF117783
Pelagomonas calceolate U89898
Pelagococcus subviridis AF015580
S306A54 (1%)
Pelagophyceae gen. sp. AF179025
Pelagophyceae
Haramonas dimorpha DQ273999
Heterosigma akashiwo X61918
Raphidophyceae
Phaeoschizochlamys mucosa AF064747
Phaeothamnion confervicola AF064746
Phaeothamniophyceae
Ectocarpus siliculosus X52503
Punctaria plantaginea AF055410
Phaeophyceae
S306A58 (8%)
Pseudopedinella elastica U89899
Rhizochromulina sp. AB280611
Ciliophrys infusionum AB081643
S306A15 (1%)
Helicopedinella tricostata AB097409
Pedinella sp. squamata AB081639
S306A38 (1%)
Verrucophora verruculosa AB280607
S306A44 (1%)
Dictyocha speculum AY043280
Actinochrysophyceae
S306A42 (13%)
S306B15 (1%)
S306A3 (1%)
S306B33 (1%)
S306A4 (15%)
Uncultured marine eukaryote cloneT2CH10 AY157374
Unknown Heterokontophyta class
Tribonema aequale AF084611
Botrydium stoloniferum AF465707
Xanthophyceae
Eustigmatos magnus AB280615
Nannochloropsis oceanica HQ201713
Eustigmatophyce
Lagynion scherffelii EF165162
Chrysophyceae sp. EF165163
Chrysophyceae
Synura sphagnicola GU325413
Mallomonas oviformis GU935673
S306A16 (3%)
Syn
Gracilaria vermiculophylla AY725172
Phycodrys radicosa AF257427
Rodophyta (Red-algae)
0.1

Figure 6. 8. Phylogenetic tree of sequenced clones of marine Heterokontophyta taxa derived from a 501 bp amplicon of the *rbcL* gene for Station 3 surface water sample with the % occurrence of each sequence (bold red). This tree was constructed by using neighbour-joining (NJ), parsimony (dnapars) (DP) and MrBayes (B). Bootstrap values were calculated from 1000 trees of NJ and 100 trees of DP. Shapes at nodes indicate bootstrap values greater than 70 % for: both NJ and DP (●), only NJ (▲) and only DP (■) and numerals represent MB. Scale bar indicates a distance of 0.1 substitutions per site. The class for each clade was indicated on the right side of the phylogeny. The corresponding sequences from red-algae were employed as out group. Bac = Bacillariophyceae, Syn = Synurophyceae

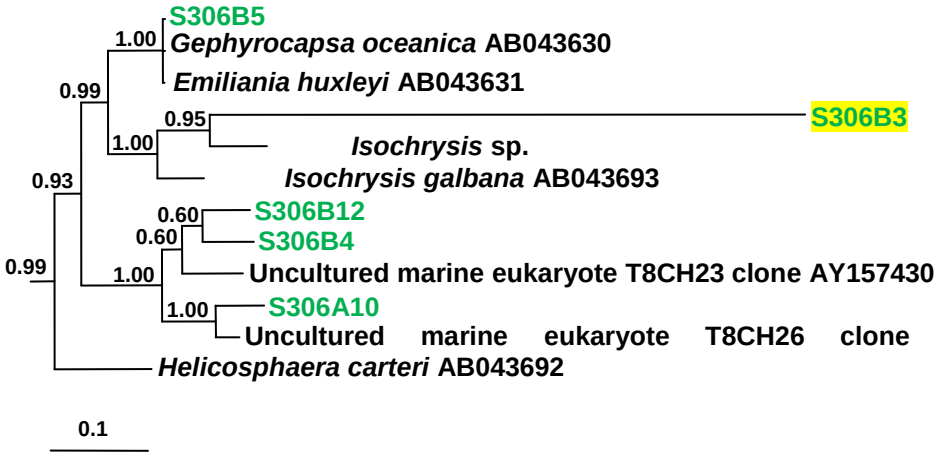

Figure 6. 9. Phylogenetic tree of sequenced clones of marine haptophyta taxa derived from a 500 bp amplicon of the *rbcL* gene sequence from Station 3 surface water sample. This tree was constructed by using MrBayes.

6.2.5. Combining the 2006 CTD bloom samples

6.2.5.2. Rarefaction curves and CTD bloom samples

Four rarefaction curves (Figure 6.10 and 6.11) were created, one for each of the three stations and for the combined dataset. Unsurprisingly, Station 1 (Figure 6.10A), in which members of only two classes were present, showed a straight line when analysing the Operational Taxonomic Units (OTUs) for the different classes. Stations 2 and 3 (Figure 6.10B & C), which included representatives of seven and nine classes, respectively, and the combined data, with 10 classes in total, also plateaued very quickly, after 35 or so clones. The genotypes and species plots in these data sets generated curvi-linear shapes that did not reach a plateau. The Good's coverage estimator indicated that the clone libraries represented 88%, 85% and 75% of the genetic community present at stations 1, 2 and 3, respectively. By combining the data sets, 82% of the community representing the red algal plastid lineage was sampled. While, the species community represented 91%, 90% and 85% of clone libraries for stations 1, 2 and 3, respectively, the total represented 91% of the combining: at the class level, it showed a coverage of 99% for stations 1 and 2, 100 % for station 3 and 99 % when all the clone libraries were combined.

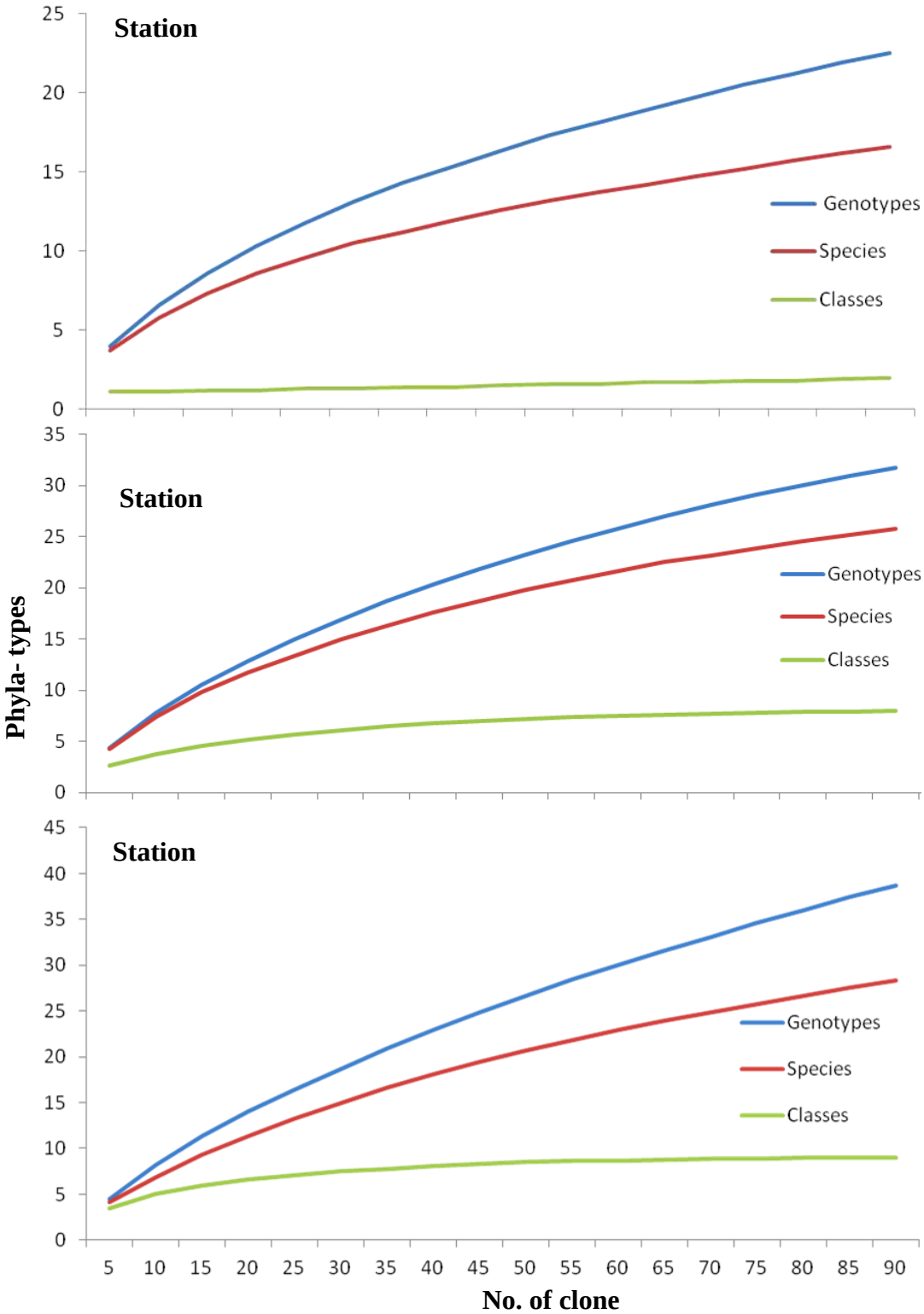

Figure 6. 10. Rarefaction curve of Eukaryotic phylotypes: genotypic (blue), species (red) and classes-level (green) richness obtained from Uni1 *rbcL* gene fragments (*rbcL*640-F and *rbcL*1240-R) amplified from CTD samples collected from the summer 2006 bloom in the Western English Channel. A, Station 1 (surface), B, Station 2 (40 m) and C, Station 3 (surface).

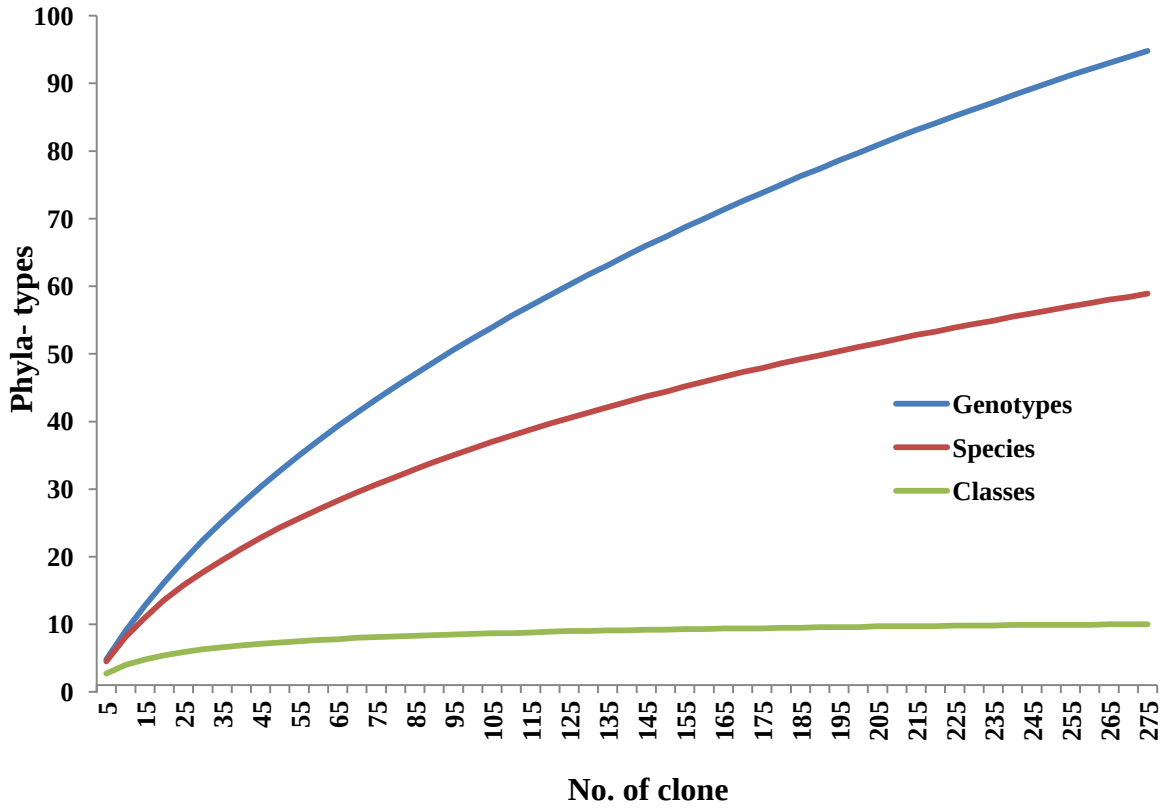

Figure 6. 11. Rarefaction curve of Eukaryotic phyla-types: genotypic (blue), species (red) and class-level (green) richness obtained from Uni1 *rbcL* gene fragments (*rbcL*640-F and *rbcL*1240-R) derived from all three CTD samples: Station 1 (surface), Station 2 (40 m) and Station 3 (surface) collected from the summer 2006 bloom in the Western English Channel.

6.2.5.3. CTD samples and bloom community structure

Combining the obtained data from the *rbcL* gene libraries from the three 2006 bloom samples demonstrated the presence of five eukaryotic phyla, Heterokontophyta (87% of clones), Haptophyta (8% of clones), Cryptophyta (3% of clones), Dinoflagellata (2% of clones) and Rodophyta (0.4 % of clones), with ten different classes being represented (Table 6.2, Figure 6.5). Within the dominant phylum, Heterokontophyta, there were unknowns (11%) and six known classes, with Bacillariophyceae (59%) dominating the community structure (Figure 6.5). Only two of these classes, Bacillariophyceae and Actinochrysophyceae, were found in all the samples (Figure 6.12). The remaining classes including the Chrysophyceae and Synurophyceae (5 %), were found at Stations 2 and 3, and Bolidophyceae (1%) and Pelagophyceae (1%) which were found only in the sample from Station 3 (Figure 6.12).

15 % of the Bacillariophyceae sequences could be assigned to a genus or species (Table 6.2). The diatoms *Rhizosolenia shrubsolei* (8%), the two different species of the genus *Pseudo-nitzschia* (8%) and the unknown diatom sp. 4 (8%) were found at Stations 1 and 2 (Figure 6.12) and the unknown diatom sp.11 (12 %) was found at Stations 1 and 3 (Figure 6.12). The remaining diatoms differed between stations. For example, Station 1 (Figure 6.12) included the centric diatom *Thalassiosira oceanica* (2 %) and different unknown diatom species (55 %), Station 3 (Figure 6.12) had only unknown diatoms (12 %) while Station 2 (Figure 6.12) included sequences related to centric diatoms such as *Thalassiosira eccentrica* (2 %), *Detonula pumila*, (1 %) *Minidiscus trioculatus* (2 %), and the pennate diatom *Psammodictyon panduriforme* (1 %).

The class Actinochrysophyceae included unknown Actinochrysophyceae sp. 1 found in all stations which was supported by high bootstrap values of > 97 % and posterior probabilities of 0.96 and 0.91 for both stations 2 and 3 respectively, while the Station 1 sequence grouped with the similar species but with lower posterior probability (0.64). The remaining Actinochrysophyceae included *Verrucophora verruculosa* and Unknown Actinochrysophyceae sp. 2 which were found at Station 3. The classes Chrysophyceae and Synurophyceae included the unknown Parmales sp. 1, which was found at stations 2 and 3, Unknown Chyrysophyceae sp. 1 and sp. 2, found at Station 2, and Unknown Chrysophyceae and Synurophyceae sp. found at Station 3. The remaining two heterokont classes, the Bolidophyceae, represented by unknown Bolidophyceae sp. 1 and the Pelagophyceae, represented by two unknown species, were only found at Station 3.

The Haptophyta and Cryptophyta were each represented by a single class, the Prymniophyceae (8 %), and Cryptophyceae (3 %) respectively. Unknown haptophytes were found at both stations 2 and 3. Within the Prymnesiophyceae, Unknown Prymniophyceae sp. 1 was found at stations 2 and 3 (B & C, Figure 6.12), *Phaeocystis pouchetii* was found at Station 2 and *Emiliana huxleyi*/*Gephyrocapsa oceanica*-like sequences, Unknown Prymniophyceae species 2-9, were found at Station 3. The Cryptophyceae included *Teleaulax* sp. 1 which found at stations 2 and 3 (B & C, Figure 6.12) and unknown cryptophytes sp. 1 were found at Station 2.

Members of the Dinoflagellata were also found at stations 2 and 3 and contained the UK strain our target study species *K. mikimotoi*.

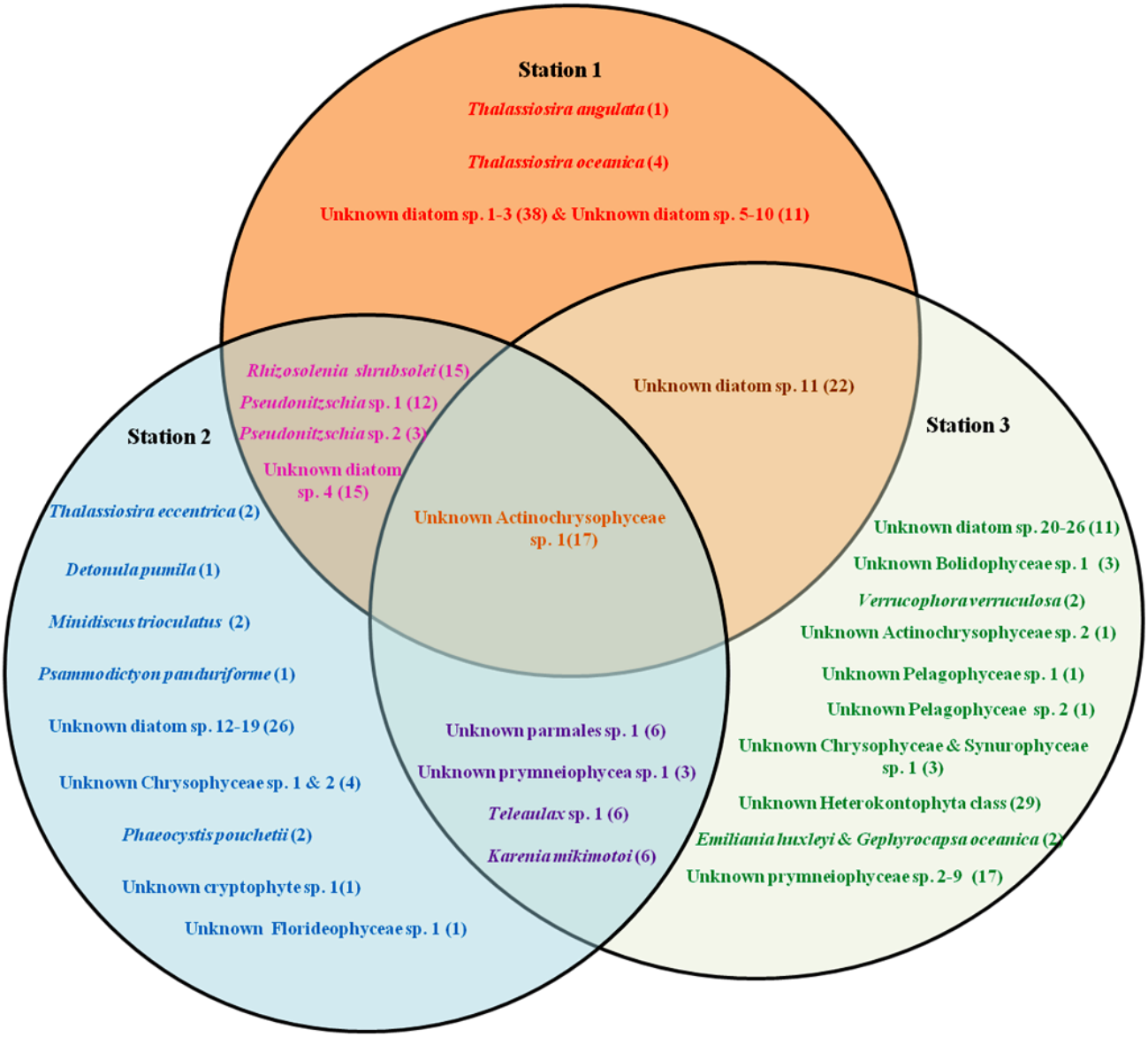

Figure 6.12. Community composition for the three 2006 bloom samples. The numbers in parentheses indicates the number of sequence occurrences for each station or the total number of occurrences where there is an overlap in community composition between stations.

6.3. Discussion

The timing and nature of the Western English Channel 2006 bloom, as revealed by MODIS satellite images, indicated the presence of a high *chl*-a concentration area (red-colour) at Station 2, while stations 1 and 3 showed a comparatively much lower *chl*-a concentration (green colour). The pseudo-true colour of these MODIS images suggested the presence of *Karenia mikimotoi* at Station 2, due to the presence of the dark-red surface discoloration. The presence of *Emiliania huxleyi* at stations 1 and 3 could also be inferred from the white reflective area in these images. *Karenia mikimotoi* was nonetheless found to be present at all the stations and at most depths during this bloom period. The HRM assay found that only *K. mikimotoi* sub-group 2 were present in these samples. This finding is consistent with Chapter 5 results and thus reaffirms the observation that sub-group 1 (the Japanese genotypes of *K. mikimotoi*) is not present in the English Channel.

Although present at all stations, the HRM results did, nonetheless, indicate the absence of *K. mikimotoi* in the surface CTD sample collected at stations 1 and 2, whereas it was present in surface samples at Station 3. Moreover, the 5m depth sample for Station 1 also gave negative results for *K. mikimotoi*, while 10, 15 and 25 m samples were positive. This result highlights the importance of timing when sampling for *K. mikimotoi*. Station 1 samples were collected in morning at 10:12, while stations 2 and 3 were collected in the early and late afternoon at 14:42 and 20:30, respectively. In mixed and weakly stratified water, *K. mikimotoi* was observed to display diurnal vertical migration speeds of 1-2 m h^{-1} with a range up to 15m (Koizumi et al., 1996) between surface and the pycnocline layer. When stratification is greater, *K. mikimotoi* exhibits a non-migrating maximum at the pycnocline (Bjørnsen and Nielsen, 1991, Arzul et al., 1993). *K. mikimotoi* cells migrate

during the night into the pycnocline layer for nitrate uptake (Dixon and Holligan, 1989) and return to the surface by morning for photosynthesis. The salinity and temperature measures for the CTD casts indicated the presence of a thermocline and weak pycnocline at all three stations (Figure 6.13).

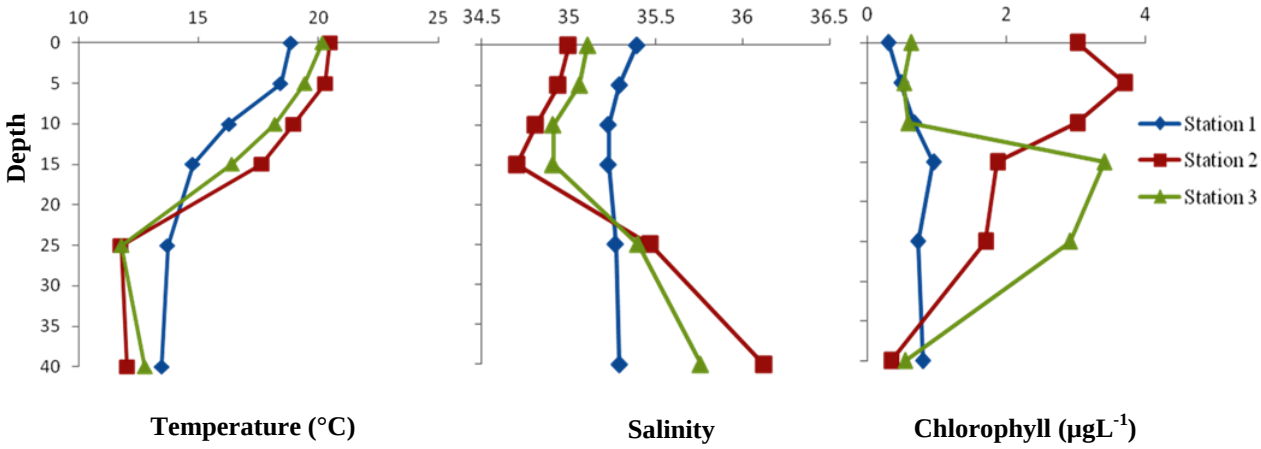

Figure 6. 13. Vertical profile of A temperature (°C), B salinity (ppt), C chlorophyll a (μgL^{-1}) for Station 1 (blue), Station 2 (red) and Station 3 (green) (provided by Tim Smythe, PML).

The surface sample of Station 1 showed a lower temperature and a higher salinity than was the case for stations 2 and 3. The composite satellite image showed that Station 2 had a high *chl*-a concentration area, while stations 1 and 3 were in a region of lower chl-a concentration. This matched the *in situ* chlorophyll measurements (Figure 6.13 C). Station 1, that was closest to the coast, has the characteristics of a weakly stratified to mixed water column, and the shallowness of pycnocline could be due to weakly coastal upwelling

events (McManus et al., 2008). Stations 2 and 3 showed a more dramatic temperature change from the surface down to a depth of 25 m, i.e., typical thermocline and pycnocline between 15 and 25 m. *K. mikimotoi* is one of the HAB dinoflagellates that is associated with frontal regions of stratified water (Pingree et al., 1978, Pingree et al., 1975, Holligan et al., 1983). The chlorophyll maxima showed opposite trends in both stations 2 and 3. Station 2 the chlorophyll peaked at 5 m depth, where *K. mikimotoi* was detected by the HRM assay, but surprisingly it was not detected at the surface despite significant chlorophyll/biomass being present.

Taken together the results are consistent with a *K. mikimotoi* migration behaviour away from the pycnocline into the water surface and where it did not reach the surface waters before the sampling of Station 1 at 10:12 hrs. This is consistent with a migration speed of this species, where *K. mikimotoi* present at 10 m in the morning could ascend to 5 m around noon, and then descend to ca 15 m at night (Nakamura et al., 1996). The absence and presence of *K. mikimotoi* in Station 2 and 3 surface samples, respectively, coupled with the migration speeds could explain the HRM data, i.e., four and a half hours between sampling at stations 1 and 2 is sufficient time to detect *K. mikimotoi* in 5 m depth at Station 2 (but not at the surface) and six hours between stations 2 and 3 is also sufficient to detect *K. mikimotoi* in the surface waters at Station 3.

The chlorophyll maxima detected by both the CTD and satellite images at station 2 is likely to be due to another bloom forming species, e.g. *Emiliania huxleyi*. Many HAB events were undetected because the bloom is initially concentrated in discrete thin sub-surface layers in the water column that are missed by sampling and some of monitoring methods which focus on surface samples (McManus et al., 2008). This highlights the

importance of sub-surface samples for HAB monitoring programs for the early detection of HABs.

The different LSU rDNA amplicon sizes found in the three stations is largely in agreement with the HRM analysis. Station 1 had a dominant amplicon size that did not correspond to either *K. mikimotoi* or *E. huxlyei*, but, stations 2 and 3 generated amplicons of a size that is typical of both *K. mikimotoi* and *E. huxlyei*. These observations, together with HRM and sequencing data, indicated the absence of *K. mikimotoi* in surface waters at Station 1, whereas it was present in samples taken from stations 2 and 3. The Goods Coverage Estimator predicted a high proportion of the genetic diversity was sampled (> 80%), sufficient to assert presence or absence of *K. mikimotoi* with a reasonable degree of confidence. Total coverage, however, was not obtained and therefore we were unable to characterise the full genetic diversity present in the 2006 bloom. Nonetheless, the coverage for species and class diversity was > 90%.

In general, most of the samples were satisfactorily sampled in this study to determine which higher taxonomic groups dominated communities associated with the 2006 *K. mikimotoi* bloom. The *rbcL* clone libraries for the three stations showed the presence of five red plastid-type lineages, i.e. the Heterokontophyta, Haptophyta, Cryptophyta and fucoxanthin-containing Dinoflagellata and an unknown class Florideophyceae of the division Rodophyta. Heterokontophyta was dominant at all stations while the remaining three phyla were found only in stations 2 and 3, which reflected more the nature of the sample and/or the environment sampled, i.e. degree of stratified waters. The most dominant class in the three libraries was the Bacillariophyceae, which comprised 99% of the sequences at Station 1, more than half (59%) at Station 2 and a quarter (23%) of the total diversity at station 3. An over-simplified and likely erroneous interpretation of this

would be that diatoms dominated the summer 2006 bloom and not *K. mikimotoi* and *Emiliania huxleyi*. This unexpected high number of sequences of Bacillariophyceae occurrence in the study libraries during summer bloom could be related to biases in DNA extraction method and PCR for diatoms. The physical data, however, revealed that the timing of sampling and the physical conditions of sampling area would favour diatoms over haptophytes and dinoflagellates in the specific samples analysed. As mentioned above the physical parameter measurements (Figure 6.14) of the temperature and salinity for these CTD samples indicated that Station 1 was in a region of weakly stratified to mixed water from a coastal area which was shallower than the body of water sampled at stations 2 and 3. Station 1 was affected by vertical mixing and turbulence, an environment suited to diatoms. Stations 2 and 3 were deeper, which explains the dominance of diatoms at 40 m depth sample of station 2 and less so in the surface sample of station 3.

The Bacillariophyceae sequences included many unknown diatoms: 83% for Station 1, 29% for Station 2 and 23% for Station 3. This suggests that the mixed water at Station 1 favoured the growth of many unknown (molecularly) Bacillariophyceae species. These unknown Bacillariophyceae sequences constitute 45% of the total biodiversity of the combined samples, and they were either found to be closely related to other uncultured environmental clone sequences deposited in GenBank. The lack of known sequence identity for these sequences could be either due to their tiny sizes and lack of distinctive taxonomic characters which make them difficult to be isolated, cultured and identified based only on phenotypic approaches (Potter et al., 1997), or simply a reflection of the poor representation of *rbcL* sequences from known species in GenBank when compared with ribosomal genes, or probably, a combination of both. This case of unknown representative sequences was also observed with other classes in this study. All the

unknown lower taxonomic level sequences from these different classes encompassed 79 % of the total obtained sequence diversity at the three stations, with many belonging to the classes which contain the picoplanktonic organisms, which are difficult to culture and identify by simple visual analysis (LM) methods. Two of the four new picoplanktonic classes that have been described in the last twenty years i.e. Pedinophyceae (Moestrup, 1991), Pelagophyceae (Andersen et al., 1993), Bolidophyceae (Guillou et al., 1999) and Pinguiphyceae (Kawachi et al., 2002) were found in our samples. Additional sampling and sequencing would no doubt have helped us to uncover many more novel taxa and this sort of information will be needed to fully resolve the important relationships between organisms/communities and their physico- chemical environment.

Recently, *rbcL* clone library targeting the red-type plastid form was also generated for spring (April) and summer (July) 2004 bloom samples collected from the L4 station in western English Channel (Bhadury and Ward, 2009). Our 2006 Station 1 sample was collected from the surface depth during the same month (July). The L4 station has the co-ordinate (50.15 ° N, 4.13 ° W), i.e. close to our Station 1 (50.12 ° N, 4.20 ° W). Furthermore, *K. mikimotoi* and *E. huxleyi* blooms were recorded based on *in situ* and satellite data observation in both 2004 and 2006 samples (Rees et al., 2009, Garcia-Soto and Pingree, 2009). As with this study, the summer 2004 bloom library showed the dominance of Bacillariophyceae (72%) and included 52% unknown Bacillariophyceae species. Some of these sequences (Table 6.3) were similar to our unknown diatom species. In addition, sequences of known taxonomic affinity were detected in both samples, i.e. *Thalassiosira angulat*a and *Pseudo-nitzschia* ap. 2. The authors reported the presence of 7% of Dinophyceae in their library, but these sequences are more than likely unknown Bacillariophyceae endosymbionts of these Dinophyceae (discussed below). The clustering

of some of these unknown diatom sequences with *Chaetoceros* spp. was also observed in both blooms (2004 and 2006), such as in the case with unknown diatom sp. 3 of Station 1 and unknown diatom sp. 21 of Station 3. No *K. mikimotoi* sequence was detected, which could be again related to the timing of sampling, but an *E. huxleyi* sequence was detected. Our Station 3 sample showed the presence of 18% of unknown Prymnesiophyceae, which is similar to the 2004 L4 sample which showed 10% of Prymnesiophyceae, comprising many unknown species that clustered with different *Chrysochrumolina* sp. Using electron microscopy the coccolithophorid species, *Gaarderia corolla*, *Syracosphaera* sp. and other unidentified coccolithophores were identified in the 2006 bloom samples and it is possible that these sequences correspond to these species (Unpublished data by Dr. Alison Taylor, MBA), however, as no sequence data currently exist, this cannot be proven. The remaining sequences from 2004 bloom samples represented the unknown Bolidophyceae (10%) and Dictyophyceae (1%). At Station 3 we also observed 3% of unknown Bolidophyceae.

From above resulted sequence similarities it appears that there is a strong correspondence in the community structure between the surface samples (2004 bloom, stations 1 and 3). In this study stations 2 and 3 included the cryptophyte *Teleaulax* sp. which grouped with related *Dinophysis* spp. endosymbiant-like sequences and the previously published sequences (GU203358 and GU2205451). These published sequences obtained from spring (April) 2004 bloom sample (Bhadury and Ward, 2009).

Table 6. 3. Taxonomic assignment of red algal plastid lineage phytoplankton found in 2006 bloom samples (stations 1, 2 and 3) and in a summer 2004 bloom sample using *rbcL* gene sequences.

Taxonomic level	Station 1	Station 2	Station 3	2004
Phylum: Rodophyta Class: Florideophyceae				
Unknown Florideophyceae sp. 1		1		-
Phylum: Heterokontophyta				
Unknown class Heterokontophyta			29	-
Class: Bacillariophyceae (Diatoms) (%)				
Thalassiosira angulata	1			
T. oceanica	4			+
T. eccentric		2		-
Detonula pumila		1		-
Minidiscus trioculatus		2		-
Rhizosolenia shrubsolei	5	10		-
Pseudo-nitzschia sp. 1	3	9		-
P. sp. 2 (delicatssima group)	1	2		+
Psammodictyon panduriforme		1		-
Unknown diatom sp. 1	31			*
Unknown diatom sp. 2	6			+
Unknown diatom sp. 3	1			+/*
Unknown diatom sp. 4	14	1		*
Unknown diatom sp. 5	1			-
Unknown diatom sp. 6	5			-
Unknown diatom sp. 7	1			-
Unknown diatom sp. 8	1			-
Unknown diatom sp. 9	1			-
Unknown diatom sp.10	2			-
Unknown diatom sp.11	11		11	-
Unknown diatom sp.12		3		-
Unknown diatom sp.13		3		-
Unknown diatom sp.14		1		-
Unknown diatom sp.15		1		-
Unknown diatom sp.16		12		-
Unknown diatom sp.17		4		-
Unknown diatom sp.18		1		-
Unknown diatom sp.19		1		-
Unknown diatom sp. 20			2	-
Unknown diatom sp. 21			1	+
Unknown diatom sp. 22			1	-
Unknown diatom sp. 23			3	-
Unknown diatom sp. 24			1	+/*
Unknown diatom sp. 25			2	-
Unknown diatom sp. 26			1	-
Class: Bolidophyceae				
Unknown Bolidophyceae sp. 1			3	-
Class: Pelagophyceae				-
Unknown Pelagophyceae sp. 1			1	-
Unknown Pelagophyceae sp. 2			1	
Class: Chrysophyceae & Synurophyceae				
Unknown Parmales sp. 1		5	1	-
Unknown Chrysophyceae sp.1		1		-
Unknown Chrysophyceae sp. 2		3		-

Unknown Chrysophyceae & Synurophyceae sp. 1			3	-
Class: Actinochrysophyceae				
Verrucophora verruculosa			2	-
Unknown Actinochrysophyceae sp. 1	1	18	8	-
Unknown Actinochrysophyceae sp. 2			1	-
Phylum: Haptophyta Class: Prymnesiophyceae				
Phaeocystis pouchetii		2		-
Emiliania huxleyi & *Gephyrocapsa oceanica*			2	+
Unknown prymneiophyceae sp. 1		2	1	-
Unknown prymneiophyceae sp. 2			4	-
Unknown prymneiophyceae sp. 3			1	*
Unknown prymneiophyceae sp. 4			3	-
Unknown prymneiophyceae sp. 5			1	-
Unknown prymneiophyceae sp. 6			2	-
Unknown prymneiophyceae sp. 7			1	-
Unknown prymneiophyceae sp. 8			1	-
Unknown prymneiophyceae sp. 9			1	-
Phylum: Cryptophyta Class: Cryptophyceae				
Teleaulax sp. 1		3	3	-
Unknown cryptophyte sp.1		1		-
Phylum: Dinoflagellata Class: Dinophyceae				
Karenia mikimotoi		2	4	-

Stations 1 & 2 (pink), stations 2 & 3 (purple), stations 1 & 3 (brown) and stations 1, 2 & 3 (orange). * clustered with.

Our results were consistent with (Takahashi et al., 2005) phylogenies which were based on using both SSUrDNA and *rbcL* gene for those published sequences and showed that the sequences of *Dinophysis* spp. contain endosymbionts which were more closely related to the *Teleaulax* sp. than to other cryptophyte species and mentioned that the reason for this is that *Teleaulax* sp. forms an endosymbiotic association with *Dinophysis* spp.

The sequences from the Actinochrysophyceae, Bolidophyceae, Chrysophyceae, Synurophyceae, Pelagophyceae, Haptophyceae (except the coccolithophore group) and Cryptophyceae are not detected in formaldehyde preserved material i.e, the CPR samples, and these taxa were missed in CPR survey phytoplankton list. This again is almost certainly due to the tiny size of the the members of these classes and, in some cases, due

to their cells being naked (without calcified scales) at some stages of their life history (Olson et al., 1989) and fragile, making them easily deformed and destroyed in unsuitable preservative materials (Hotzel and Croome, 1999, Tomas, 1997, Tomas et al., 1993).

200 clones were sequenced (Appendix 12) using the LSU rDNA primers (D2C-F and D2-NewR (UniB), Table 2.5) on a CPR sample (station 20, chapter 5), which was sampled at the same time as the 2006 CTD bloom. This clone library included a wide diversity of eukaryotic representatives, including members of the Kingdom Plantae, Opisthokonta and Chromalveolata. This library was dominated (56%) by *E. huxleyi* –like sequences and included two sequences from *K. mikimoto*i. However, a low diversity of Bacillariophyceae was detected with only one genotype sequence (3%) of an unknown diatom sp., with no respresentatives of other Heterokontophyte classes such as Pelagophyceae, Bolidophyceae, and Actinochrysophyceae. Neither the cryptophyte nor other haptophytes (other than *E. huxleyi*) were detected.

Despite the advantages of the nuclear rRNA genes for detecting a wide variety of eukaryotic taxonomic groups, and the large numbers of sequences available in GenBank, the large subunit of the plastid borne *rbcL* gene can be better for assessing phytoplankton diversity: has become a key marker for phylogenetic purposes for all photosynthetic, and also some chemosynthetic, organisms (Medlin et al., 2006). This gene has several conserved regions, although to a lesser degree than the rRNA genes (Medlin et al., 2006, Watson and Tabita, 1997), for phylogenetic studies. The ID form of *rbcL* is important for the detection of groups that are missed in many studies, possibly due to their small size. Clone library characterisation should be considered as complementary to microscopic descriptions and culturing studies, because all three need to be combined to produce a wider view of the community structure and dynamics. The former approach is less time-

consuming, independent of the choice of the molecular method for further studies but may depend on the needs of the researcher and the amount of samples in the study. The clone libraries allow the description of any target study group biodiversity based on suitable molecular marker in a sample down to the species level. Conversely, clone libraries are not suitable for comparing large numbers of samples, but better reflect their diversity due to the screening capabilities and the larger information content of the cloned fragments. However, as prices for sequencing services drop it and many molecular assisted equipment for covering many samples are developed, these techniques may be easier to apply in the future.

Chapter 7: Summary and future work

7.1. Overall Discussion

A paucity of diagnostic morphological characters for identification, and high morphological plasticity within the Gymnodinoid group has led to confusion about the identity, distribution and spread of many species, such as in the case of the cryptogenetic genus *Karenia*. The use of molecular tools to aid identification of such algal species has been promising in other morphologically challenging taxa such as *Gymnodinium* spp., *Gyrodinium* spp., *Karlodinium* spp. *Takayama* spp. *Pfiesteria* spp. and *Heterocapsa* spp. (Thoha, 2010, Seaborn et al., 2006, De Salas et al., 2005, Gómez, 2008a, Botes et al., 2002). The main focus of this study centred on the development of a diagnostic molecular method to allow unambiguous recognition of *K. mikimotoi* in European waters. This has been the first study to discriminate between different geographic strains of *K. mikimotoi* using a combination of three molecular markers. The rDNA LSU D1 and D2 domains were successfully amplified in all dinoflagellates of our culture collection including the *K. mikimotoi* isolates, however, it was unable to differentiate between the different strains of *K. mikimotoi*, at sub-population level, which is consistent with previous studies (Hansen et al., 2000, Haywood et al., 2004, Guillou et al., 2002, Daugbjerg et al., 2000). The same region has also been found to be unable to resolve other dinoflagellate taxa, such as *Alexandrium minutum* (Hansen et al., 2003), at the sub-specific level: the LSU rDNA is only really discriminating at generic and specific levels e.g. (Kim and Kim, 2007). The rDNA ITS region, which is preferred for discrimination at lower taxonomic levels, e.g. sub-species or ecotype level (Rodríguez et al., 2005), was found to be able to resolve variation between *K. mikimotoi* strains, however, the *rbc*L gene was to be the most useful in this respect due to the high sequence variability between strains from different localities.

Therefore, the *rbcL* gene was used as the basis of our diagnostic HRM assay using specifically designed primers sets. The phylogeny constructed using concatenated sequences of the three markers supported the separation of *K. mikimotoi* into two sub-species: sub-species 1 comprising Japanese isolates and sub-species 2 comprising European and New Zealand isolates.

The HRM assay based on the *rbcL* gene was able to successfully discriminate between the four different *K. mikimotoi* isolates, even when separated by a single nucleotide difference, i.e, between the two Japanese strains and between the UK and NZ strains. For rapid detection of whether a bloom contains *K. mikimotoi* or not, the HRM assay is useful, particularly when monitoring a large number of samples, and could be used for other HAB species. For example, this method has already been used for identifying strains of the dinoflagellate *Symbiodinium* species (Granados-Cifuentes and Rodriguez-Lanetty, 2011) and to differentiate between the toxic and non-toxic species of the diatom *Pseudo-nitzschia* spp. (Andree et al., 2011). Limitations of the HRM assay were, however, evident when analysing samples containing more than one *K. mikimotoi* genotype, although it was able to distinguish between the two Sup-species of *K. mikimotoi* (the Japanese strains and the European/NZ strains). This study highlighted the need for the target amplicon to include enough substitutions to separate different strains if it is to be able to reliably detect more than one strain in mixed environmental samples: one substitution was found to be not enough to discriminate between two different strains using this method in mixed samples. As such we suggest that the HRM assay is a good and rapid molecular technique to be used for monitoring HAB program and for analyzing large numbers of samples, but that it is not suited for sub-species level discrimination in mixed samples.

Sampling with the CPR is a good method for zooplankton, it has been argued that it is inappropriate for the sampling of phytoplankton, particularly the small-sized taxa, due to the large mesh-size (270 µm) employed (Batten et al., 2003). That said, it has also been reported that clogging of the silk filters leads to the collection of samples that are dominated by phytoplankton rather than zooplankton communities (Hunt and Hosie, 2006, Walne et al., 1998). Hunt and Hosie (2006) showed that the phytoplankton which cause the filters to clog is dominated by the larger-size fraction of taxa, mainly diatoms. The clogging of the filters essentially reduces the mesh size, and thus allows smaller species to be captured, such as the diatom *Thalassiosira* and coccolithophores. Moreover, with the aid of molecular techniques, Ripley et al. (2008) and Vezzulli et al. (2011) were able to identify the small, 5µm diameter coccolithophore *Emiliana huxleyi* and even smaller bacterial populations on CPR filters. Furthermore, naked-dinoflagellate species a delicate, lacking a cell wall or an outer covering needed to render it resistant to common fixing or preservative agents and making easily destroyed by net sampling and towing process. These factors make it difficult to visualize and count by the standard methods of CPR plankton analysis. The fixation process for most of the unarmoured dinoflagellates, including gymnodinoid species, causes loss of flagellae and cell deformation, i.e. the disappearance of the main diagnostic characters (Gómez, 2007). The CPR is unable to survey many of the picoplankton and nanoplankton taxa, such as members of the Bolidomonaceae.

The CPR is increasingly being exploited for molecular analysis and the potential uses for this long time series are great (Ripley et al., 2008, Al-Kandari et al., 2011). Since first being available, remote sensing data has been useful in identifying the possible areas where surface blooms of *K. mikimotoi* occurred, but such data cannot identify where potentially

sub-surface populations occur e.g. phytoplankton population maxima at the pycnocline during the period of summer sratification. Notwithstanding this limitation, remote sensing can differentiate between different types of bloom, such as the dinoflagellate/diatom blooms (with a red to dark-red colour of high chlorophyll concentration) and coccolithophore blooms (with a white calcite reflection signature). By employing remote sensing data to map when and where blooms had occurred we could then correlate this to the the timings and locations of CPR transects. For earlier samples we were reliant on historical records on the occurrence of blooms. One drawback of this approach is that one cannot always ensure that a CPR transect will correspond with a location/time where a bloom has been identified. As such it is often necessary to select the closest possible transect, which will not necessarily be suitable. Species such as *K. mikimotoi* are known to migrate vertically between the pycnocline at night, where they access nutrients and the surface, particularly during the afternoon, where they photosynthesize, and this also raises an issue. The CPR is set to tow at a constant depth ~ 7 m therefore if a species has migrated above or below this depth it may not be sampled. Regardless of these limitations the CPR, has proved itself as a valuable tool for the molecular study of *K. mikimotoi*; this in itself was a momentous result as *K. mikimotoi* is not identified microscopically in the CPR due to it being damaged and thus rendered unrecognisable during towing/preservation. This opens the CPR survey for further exploitation as molecular studies can be used for other such species that may not be easily visualised.

The earliest samples analysed in this study dated back to 1963, at which time no *K. mikimotoi* blooms had been recorded, however, the European isolate of *K. mikimotoi* was found to dominate the study samples collected from south-west English Channel and south-west Ireland. The NZ strain was found solely in 1981 and at the same time as the UK

and Norwegian strains in 2004, a sample in which the UK strain was dominant. No Japanese strains were detected in any of the study samples, which is a strong indication that the European *K. mikimotoi* was not introduced from Japan: it is proposed that *K. mikimotoi* is native to European waters. The presence of UK and NZ strains in south-western English Channel might be explained by their similarities. Physiologically they are likely to be similar as they both produce two sub-generations of small and large cells and also they are likely to be both adapted to colder waters than the Japanese strains. Moreover, *K. mikimotoi* is now known to be a widely distributed species and known to tolerate a wide range of temperatures and salinity. Thus combining these characters it is feasible to postulate that both the UK and NZ strains could adapt and bloom simultaneously.

Preserved samples from biological archival collections may provide a rich source of study material. These archival collections of preserved samples are now very important as a precious source of biological data especially as DNA extraction from these samples is now possible. Genotyping these samples with suitable molecular markers for any study target species can address many questions, including those regarding the history and identity of this species. The present study highlighted the usefulness of archival material by detection the important HAB species *K. mikimotoi*, which is known to cause fish and marine mortalities in North Atlantic and Pacific oceans, using the HRM assay based on suitable molecular markers on long term of past and recent CPR samples.

Approximately 47 % of CPR samples were negative for *K. mikimotoi* even though they were selected on the basis of where and when this species was likely to be present. To explore what might be contributing to these negative results, eighteen samples of fresh (unpreserved) depth profiles samples from a *K. mikimotoi* bloom in 2006 were collected

from three stations in the Western English Channel and examined for the presence/absence of *K. mikimotoi* at the water surface. The HRM assay showed the presence of *K. mikimotoi* (sub-species 2) in all except four samples, including two surface samples, one sample from 5 m and on from 40 m. These results also confirm the importance of timing (24 hours/day) and location (weakly/strongly stratified or mixed) when sampling *K. mikimotoi* blooms and explain at least some of the negative CPR results. Sampling through the water column is recommended when looking for blooms, but in cases where only the water surface is accessible, then consideration should be given to the timing of sample collection, i.e, around noon (Nakamura et al., 1996) when the sun reached a maximum, and the location whether mixed, weakly stratified or stratified when *K. mikimotoi* could be confined to pycnocline layer (Bjørnsen and Nielsen, 1991, Arzul et al., 1993): presence/absence of *K. mikimotoi* at the water surface during blooms is influenced by a combination of biological (diurnal migration pattern) (Dixon and Holligan, 1989) and physical factors (wind strength, speed and direction which drive the water system circulation i.e, upwelling, mixing and advection) (Raine and McMahon, 1998, Raine et al., 2001). An understanding of the influence of wind stress on water circulation and population with respect to organism behaviour its necessary for forcasting HAB bloom events.

The microbial community associated with *K. mikimotoi* blooms was also investigated as part of the current study. Little is known currently about the small and delicate phytoplankton species that are associated with *K. mikimotoi* blooms, knowledge of which can help in understanding bloom dynamics. It is possible that other *Karenia* species might co-occur with *K. mikimotoi*, as is known to be the case in the Gulf of Mexico (Steidinger et al., 2008) and New Zealand (Haywood et al., 2007). The surveys in Western English Channel e.g., the CPR and PML monitoring stations showed the limitations in identifying

many of the small phytoplankton taxa, particularly the pico and nano sized species which constitute many of recently discovered groups (Kim et al., 2011), where most of them contain the red type of *rbcL* gene and are responsible of for up to 80-90 % of primary production (review by Stockner (1988)), Such microscopy based surveys require further data, such as linked molecular analysis to characterise the pico- and nano- fractions: identifications based on only on morphology requires expertise in taxonomy and still often fail to distinguish differences amongst cryptic species and minute, morphologically depauperate planktonic organisms. Molecular techniques, which have revealed new insights into bacterial and picoplankton communities, may also enhance our knowledge of the diversity among communities of larger plankton.

We compared phytoplankton identifications and community assessments based on CTD samples collected from sampling stations in the south-west English Channel during a *K. mikimotoi* bloom in 2006, with CPR and PML LM data. The Molecular analysis revealed the presence of a diverse community. Molecular phylogenetic analysis indicated the presence of many novel organisms, several of which were most closely related to other unidentified sequences from diverse marine environments. For delicate and picoplanktonic organisms molecular techniques can enhance our understanding of plankton diversity, particularly by detecting new taxa and revealing the presence of previously unrecorded organisms. Our results support the idea that we are still just beginning to uncover the diversity of eukaryotic marine organisms and that there may be many more ubiquitous, microeukaryotic plankton than previously realised.

Eukaryotic assemblages were analyzed from CTD samples of *K. mikimotoi* and *E. huxleyi* blooms in south-western English Channel in 2006 by sequencing the cloned red-type *rbcL* gene fragment of heterokonts, haptophytes, cryptophytes, fucoxanthin dinoflagellates and

red algae in three libraries: two from the surface and one from 40 m depth. The application of environmental gene sequencing is important for investigating the microbial community structures, as it helps to identify new groups and the data obtained contributes to the growth of gene sequence databases, allowing global distribution studies (Vaulot et al., 2008). The sequencing of *K. mikimotoi* bloom samples in this study generated many unknown sequences, which either have no closely related sequences in GenBank or which are related to sequences unassigned to specific taxa and collected from a variety of unrelated locations and/or times.

Photosynthetic groups are often underrepresented in clone libraries constructed using universal ribosomal RNA gene primers, despite the fact that the small eukaryotic community is dominated by phototrophs (Not et al., 2004, Romari and Vaulot, 2004) . To solve this problem it is necessary to use multiple sets of primers (Stoeck et al., 2006) or primers that are specific for certain phylogenetic groups (Bass and Cavalier-Smith, 2004) to assess the full microeukaryotic diversity in environmental samples. Using primers or probes, such as for the rRNA genes, targets a wide eukaryotic groups, but make it difficult to assess community structure because of the wide variation of the number of rRNA gene copies among species (Zhu et al., 2005). The use of combinations of primers or probes targeting specific phylogenetic group, genera or species offers a more promising approach to assessing community structures (Countway and Caron, 2006).

Our sequencing showed the dominance of Heterokontophyta in the three stations with members of the Bacillariophyceae dominant at the surface and at 40 m depth: they were present whether or not *K. mikimotoi* was found. Furthermore, the samples (stations 2 and 3) with *K. mikimotoi* cells also contained members of the Haptophyta and Cryptophyta, which were undetected in samples without *K. mikimotoi*. The three *rbcL* gene clone

libraries showed station-specific taxon variation of about 50 % of the total diversity, which again raises the issue about the different timing and location of the sampling area. Phytoplankton biomass and diversity in upwelling regions are in general, influenced by many physical, chemical and biological factors. The extent of the summer bloom depends on the wind stress, which is the main driving force in such systems. In upwelling systems, the nutrient input into the euphotic zone leads to high productivity and consequently, the upwelling circulation overrides both the nutrient limitation of stratified waters and the light limitation of well-mixed waters.

Rarefaction curve with "Good Estimator" showed that we recovered about 82 % of the total diversity from the three *rbcL* clone libraries.

We conclude that environmental clone library construction is sufficient to compare samples, but the total diversity will probably always be underestimated and relative abundance estimates should be treated with caution (Potvin and Lovejoy, 2009).

Finally, although the current study provides a comprehensive molecular description of phytoplankton assemblages that can be linked to environmental conditions, we have no idea about the morphology of the typical cells of these sequences. Therefore, the use of complementary approaches such as fluorescent *in situ* hybridization (FISH; Not et al. 2002) are needed to add detail to studies of the distribution and seasonal dynamics of each group. Representatives of uncultivated groups, especially heterotrophic ones, which represent many of the nano and picoplankton species, must be brought into culture to assess their biology and role in the ecosystem.

7.2. Future work

HRM assays based on suitable molecular samples and long term sampling surveys, such as the CPR survey, give the opportunity for more research aimed at detecting and investigating the identity and history for the other HAB species in North Atlantic and the Pacific. With regards to the study of *K. mikimotoi* strains and their geographical dispersal/range, investigating more isolates and *K. mikimotoi* bloom samples from Japan and New Zealand, would be advantageous. This type of biogeographic study should develop and apply the specific *rbcL* molecular marker for toxic *K. mikimotoi* strains from Europe, New Zealand and Japan, and could be expanded into further regions of the world to give new insights and to develop well defined global molecular markers. Understanding strain differences is important in the investigation of ballast water mediated invasions and the global redistribution of genotypes. Our characterisation of *K. mikimotoi* isolates was limited by their availability in culture collections across the world. *K. mikimotoi* can be notoriously hard to maintain in culture, however, future work should include the isolation of more strains from different geographical regions so we can fully determine the spatial range of different sub-species.

The CPR survey is now extending its sampling range to include Japanese waters, which gives the opportunity for screening more samples from the CPR based on the timing and location of *K. mikimotoi* blooms in Japan. This will be limited to more recent years, however, the data will still be of use for characterising the genetic structure of *K. mikimotoi* blooms. Information from this will establish whether the UK strain was also present in New Zealand and if there are more as of yet unseen genotypes that exist in Japanese or New Zealand waters. Any novel sequences should be then used for molecular marker development.

Studies based in Kuwait will aim to characterise more isolates of *K. mikimotoi* from middle-eastern countries and to establish whether any they differ from other already characterised strains. The HRM technique developed in this study is a promising technique for future rapid detection of many HAB causative bloom species. In combination with this method, toxin analysis is required for future isolates of *K. mikimotoi* to detect whether the *rbcL* gene marker is able to differentiate between the toxic or non-toxic strains. This will be of great significance for the monitoring of HABs allowing for the rapid detection of a toxic bloom and permitting a quick response for mitigating bloom impacts, including protecting human health through quick enforcement of temporary fisheries closures, closures of public beaches.

7.3. HAB incidence in Kuwait

In September and October 1999, Kuwait water's experienced an extensive red tide bloom that caused a widespread fish kill (Glibert, 2007, Heil et al., 2001). The causative species was initially identified as *Gymnodinium* sp. based solely on light microscopy. Efforts to correctly identify the causative bloom species at specific level led the local research agency's (EPA and KISR) in Kuwait to acquire expertise from other countries. Nonetheless, even with expert identification, there was confusion about the likely candidate with either *G. mikimotoi* or *G. selliformis* being implicated, until an in-depth study using electron microscopy and toxin analysis confirmed it to be *G. selliformis* (now *Karenia selliformis*). Another common toxic species *Prorocentrum rathymum*, was also found to bloom at the same time. Many other harmful species were also recorded at that time, but they did not reach the bloom conditions. The results of the above red tide and fish

kill phenomenon served as a warning sign that more HAB outbreaks could occur in the future. Recently, the Arabian Gulf and the northen Gulf of Oman have experienced several harmful algal blooms (HABs) e.g. during August 2008 – May 2009, caused mainly by *Cochlodinium polykrikoides* species with accompanying massive fish kills (Richlen et al., 2010). *Karenia* species are found in Kuwaiti samples, but without species level diagnosis there has been confusion between the *K. mikimotoi* and *K. selliformis* and between *K. brevis* and *K. papillioneca*. Recently, the use of PCR assays confirmed that these different species of *Karenia* co-occur together in the same bloom, such as the case in USA and New Zealand (Haywood et al., 2007, Steidinger, 2009).

The Oceanographic department in KISR lack the molecular expertise to resolve such taxonomic problems within *Karenia* species and other planktonic taxa. Moreover, the Oceanographic department in KISR, have many short and long term projects that require monitoring a large number of samples for zooplankton, phytoplankton, icthyoplankton, bacteria and viruses. These different groups have different analysts who base their identification only on microscopic analysis and preserved samples. Therefore, many of the relevant and important taxa cannot be identified to species level or, sometimes to genus level. Furthermore, the nano and pico plankton fractions were missed altogether in the data and group level information can be detected by HPLC. These taxonomic problems can only be resolved with molecular techniques, such as cloning and barcoding, followed by other technique, such as FISH to identify those species related to previously published sequences.

The techniques and expertise developed during the course of the current study will now be used to assist in developing our understanding of HAB communities and dynamics in the Gulf region.

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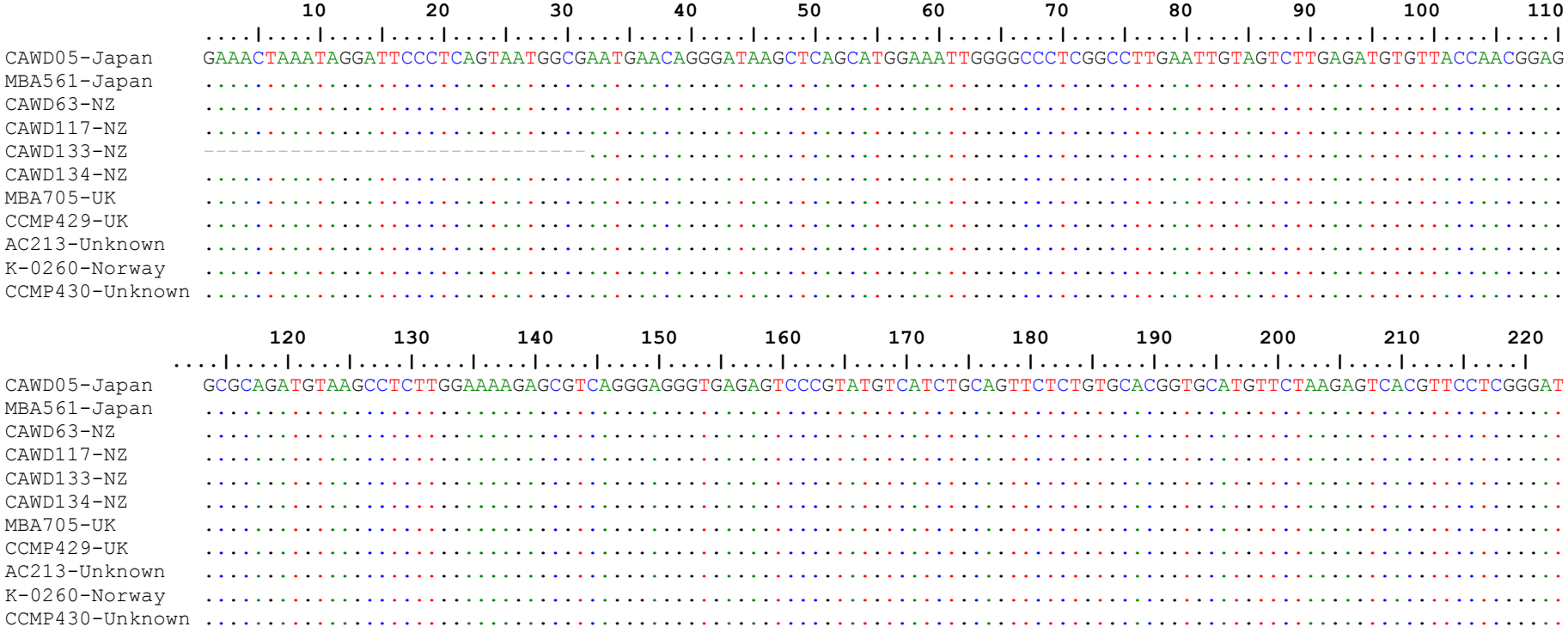
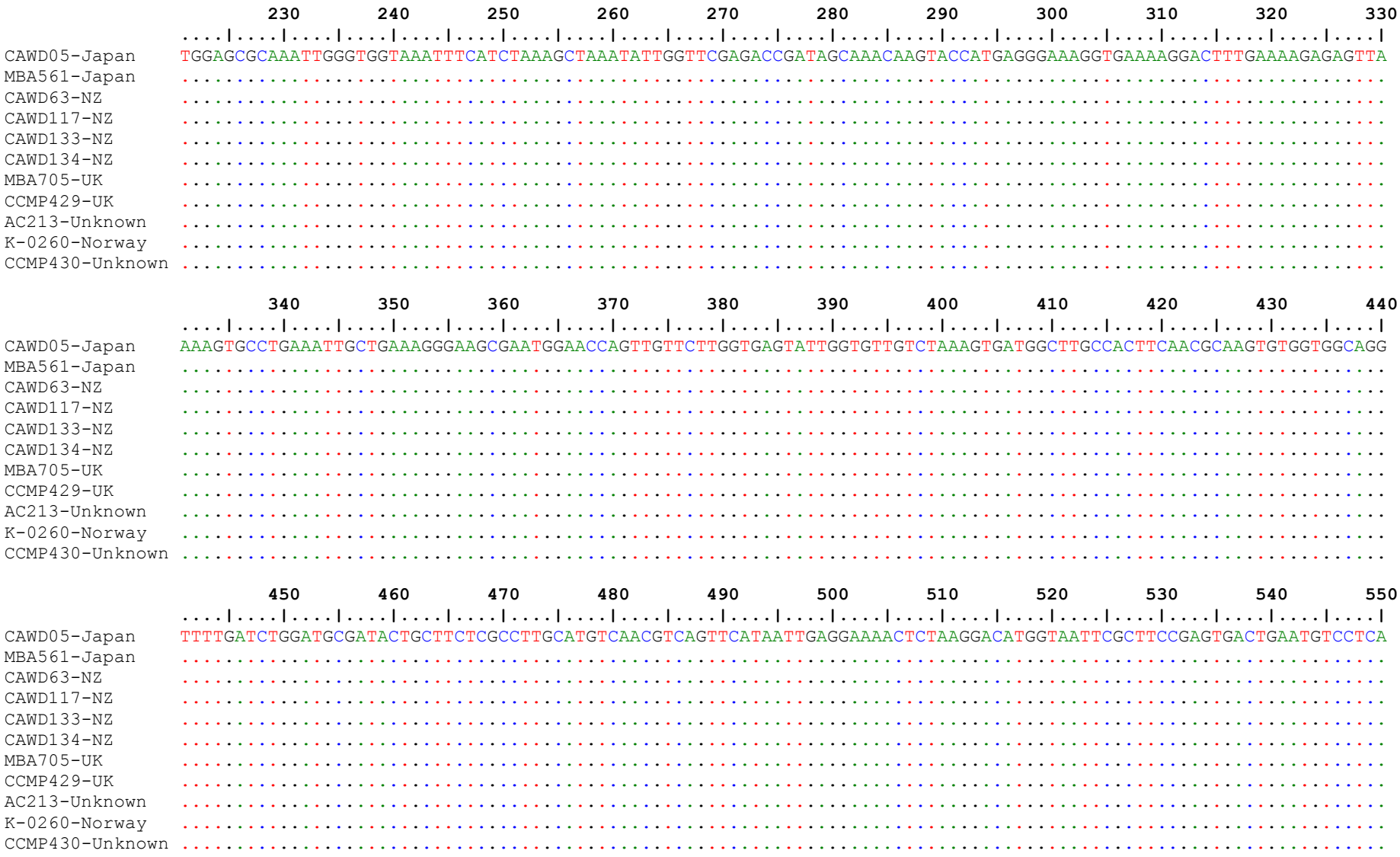
List of Appendixes

Appendix 1

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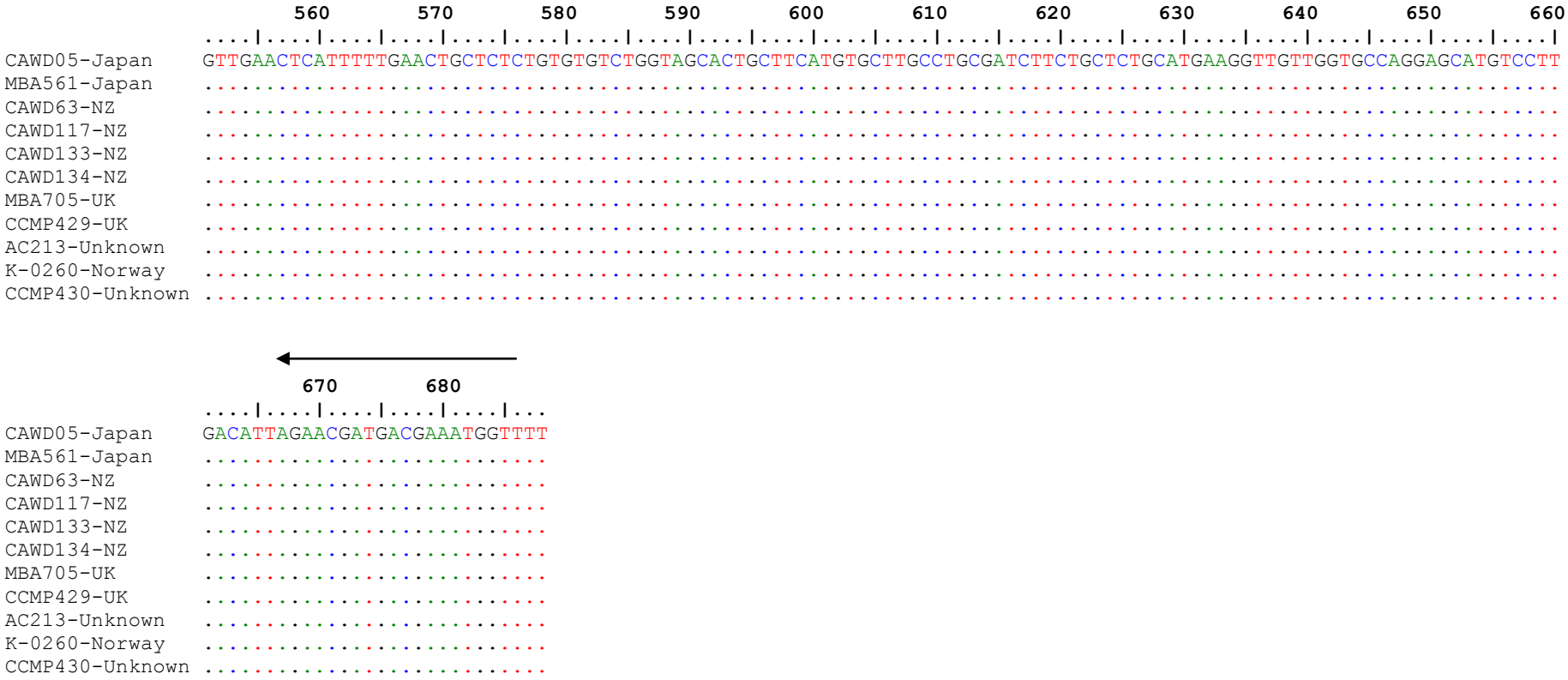
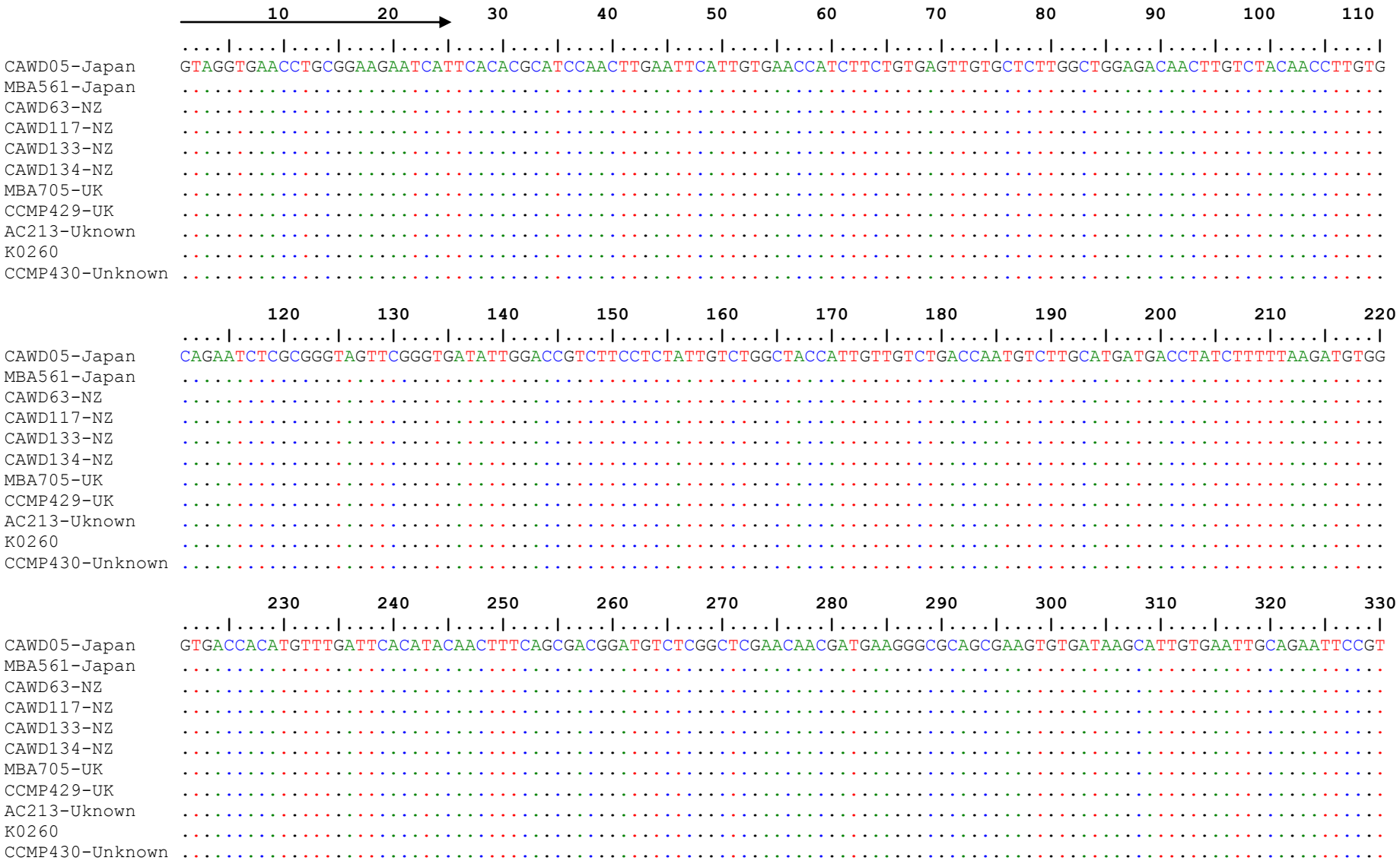


Figure 1. Sequence alignment of a 688 bp fragment of the LSU (D1 and D2 regions) rDNA gene (D1R-F & D2C-R) for *K. mikimotoi* isolates. Positions of the universal forward (F) and reverse (R) primers indicated by arrows. Dots correspond to identical nucleotides and letters correspond to any nucleotide substitutions.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CAWD117-NZ
CAWD133-NZ
CAWD134-NZ
MBA705-UK
CCMP429-UK
AC213-Uknown
K0260
CCMP430-Unknown
120 130 140 150 160 170 180 190 200 210 220
CAWD05-Japan CAGAATCTCGCGGGTAGTTCGGGTGATATTGGACCGTCTTCCTCTATTGTCTGGCTACCATTGTTGTCTGACCAATGTCTTGCATGATGACCTATCTTTTTAAGATGTGG
MBA561-Japan
CAWD63-NZ
CAWD117-NZ
CAWD133-NZ
CAWD134-NZ
MBA705-UK
CCMP429-UK
AC213-Uknown
K0260
CCMP430-Unknown
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MBA561-Japan
CAWD63-NZ
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AC213-Uknown
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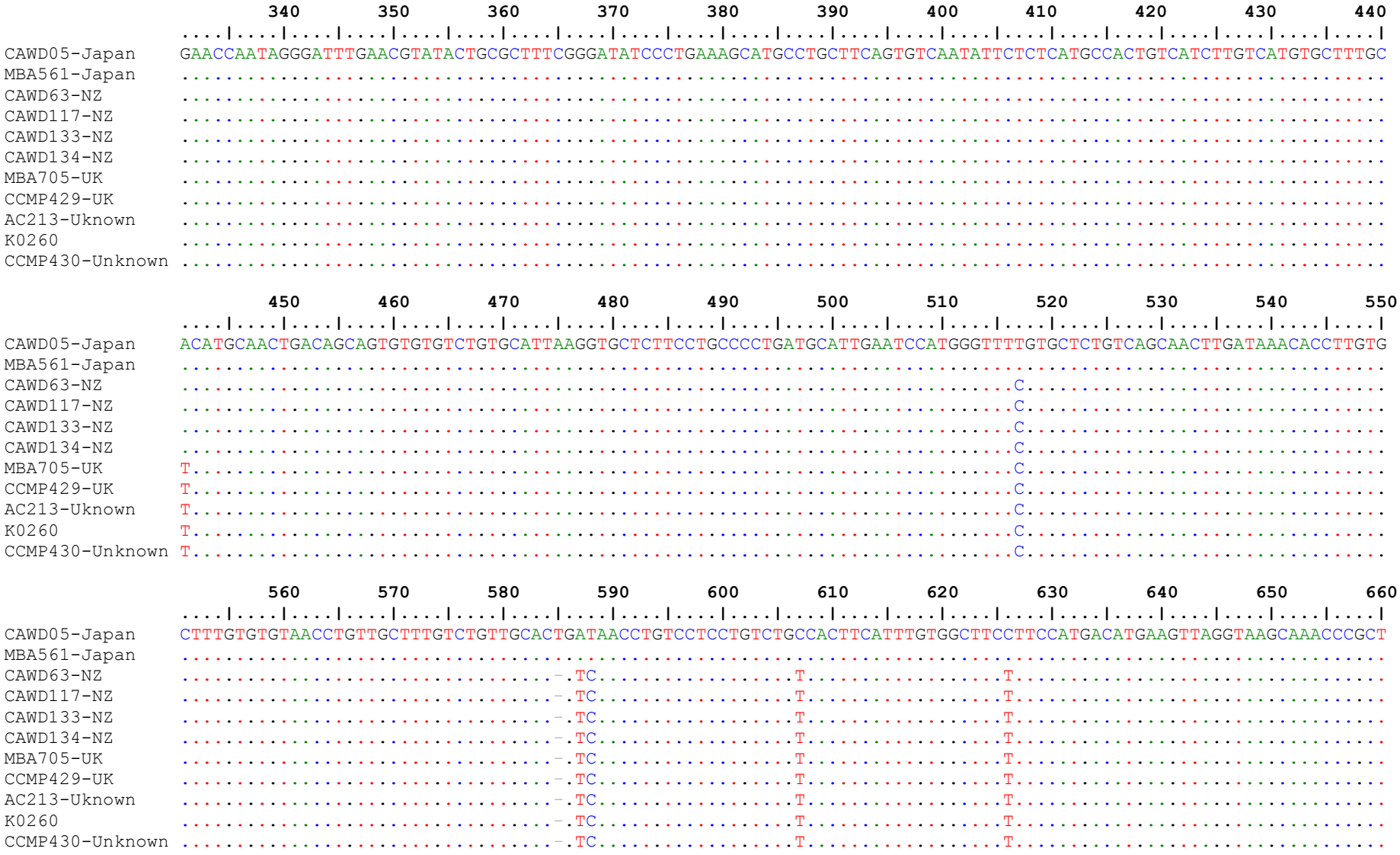
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AC213-Uknown
K0260
CCMP430-Unknown
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MBA561-Japan
CAWD63-NZ C
CAWD117-NZ C
CAWD133-NZ C
CAWD134-NZ C
MBA705-UK T C
CCMP429-UK T C
AC213-Uknown T C
K0260 T C
CCMP430-Unknown T C
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MBA561-Japan
CAWD63-NZ -TC T T
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CAWD134-NZ -TC T T
MBA705-UK -TC T T
CCMP429-UK -TC T T
AC213-Uknown -TC T T
K0260 -TC T T
CCMP430-Unknown -TC T T

Figure 2. Sequence alignment of a 710 bp fragment of the ITS-regions rDNA gene (EITS2 DIR & EITS2 REV) for *K. mikimotoi* isolates. Positions of the universal forward (F) and reverse (R) primers indicated by arrows. Dots correspond to identical nucleotides and letters correspond to any nucleotide substitutions.

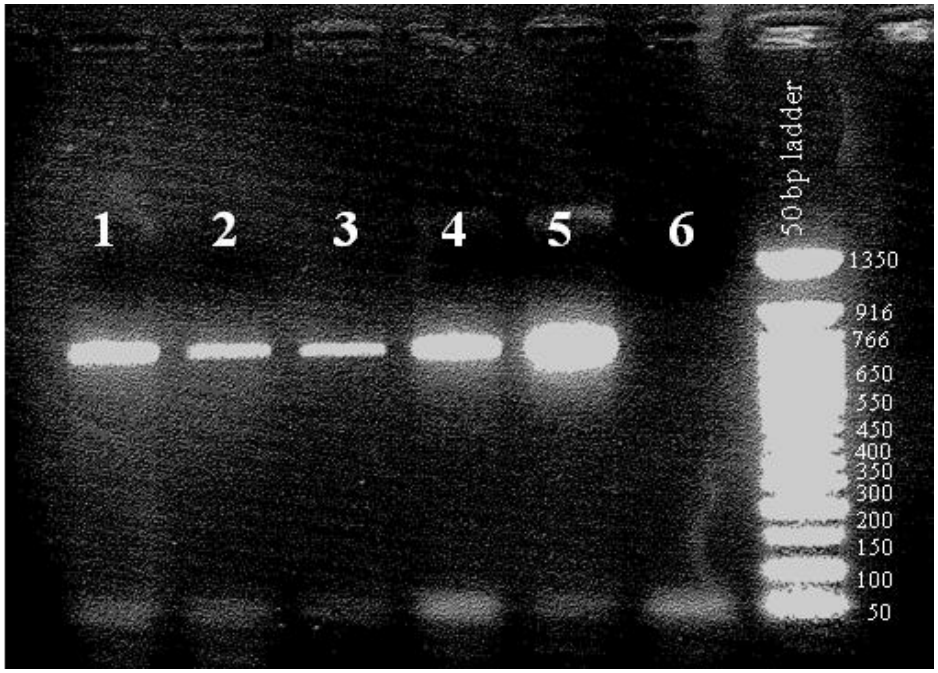
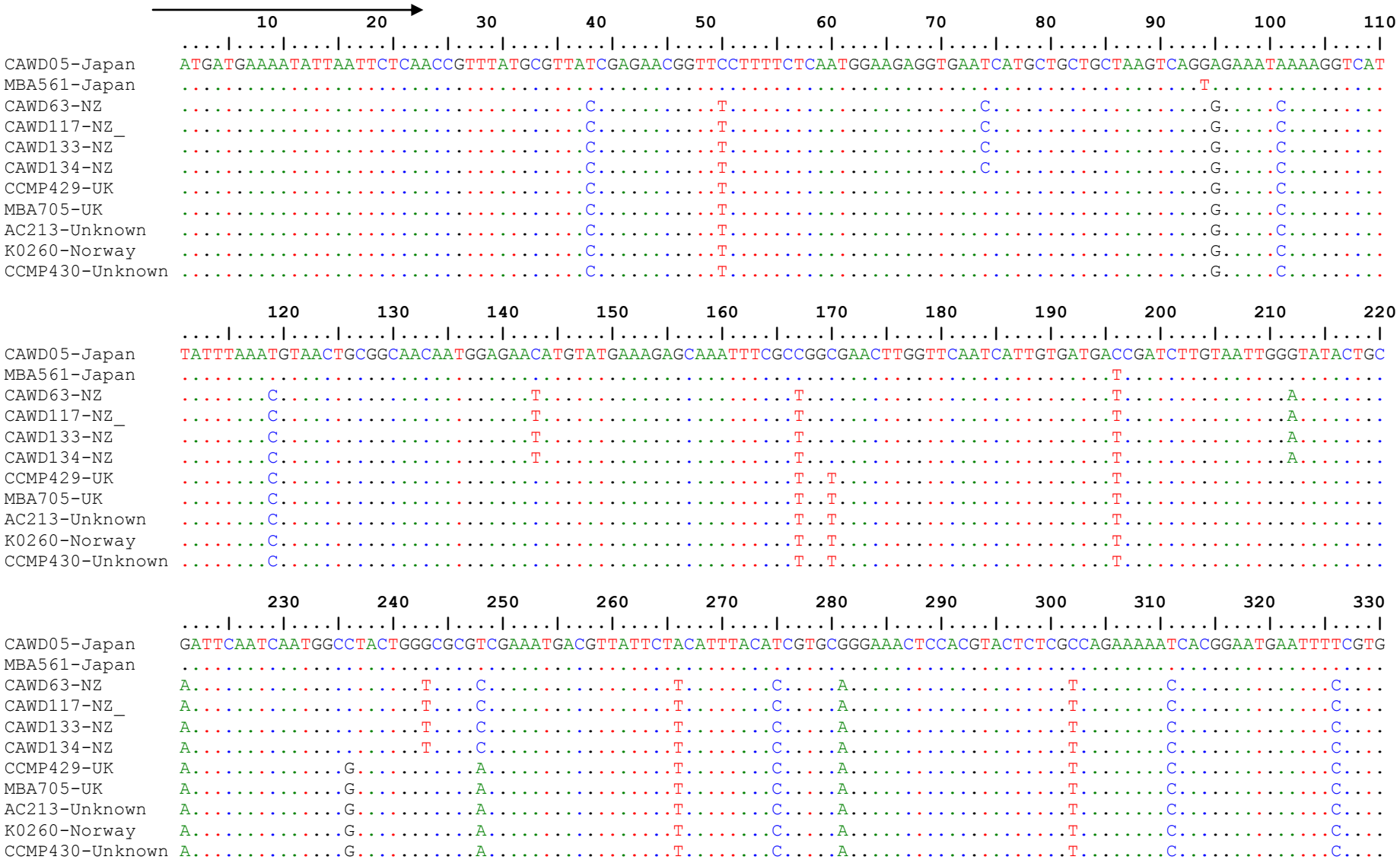


Figure 3. Gel image for *K. mikimotoi* strains using ITS-region gene (EITS2 DIR & EITS2 REV) of : 1. AC213 , 2. MBA561, 3. K-260, 4. CCMP429, 5. CAWD63, 6. Negative control.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CAWD117-NZ_
CAWD133-NZ
CAWD134-NZ
CCMP429-UK
MBA705-UK
AC213-Unknown
K0260-Norway
CCMP430-Unknown
120 130 140 150 160 170 180 190 200 210 220
CAWD05-Japan TATTTAAATGTAACTGCGGCAACAATGGAGAACATGTATGAAAGAGCAAATTTCGCCGGCGAACTTGGTTCAATCATTGTGATGACCGATCTTGTAATTGGGTATACTGC
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CAWD117-NZ_
CAWD133-NZ
CAWD134-NZ
CCMP429-UK
MBA705-UK
AC213-Unknown
K0260-Norway
CCMP430-Unknown
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MBA561-Japan
CAWD63-NZ
CAWD117-NZ_
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CAWD134-NZ
CCMP429-UK
MBA705-UK
AC213-Unknown
K0260-Norway
CCMP430-Unknown

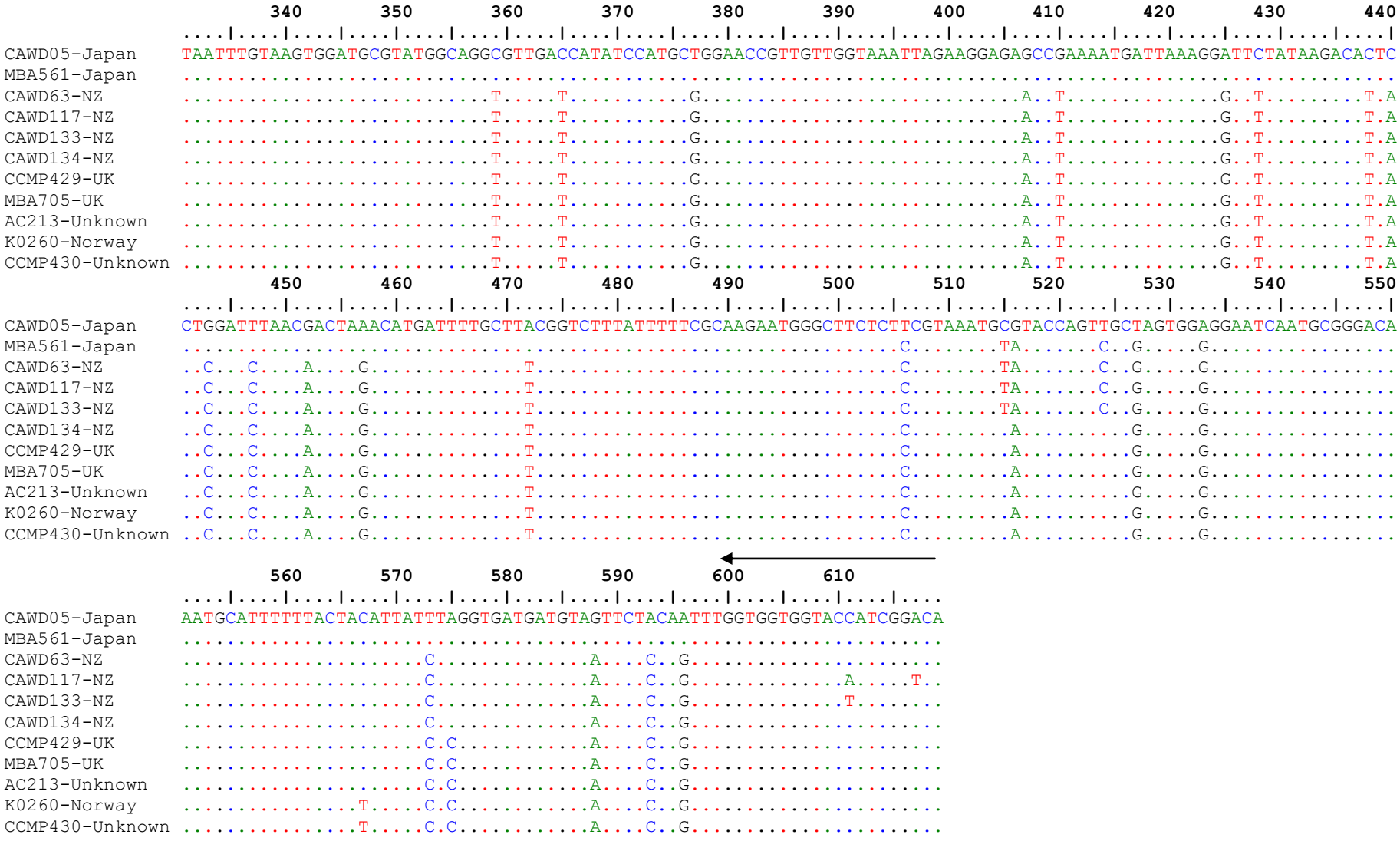


Figure 4. Sequence alignment of a 620 bp fragment of the Uni 1 (*rbcL* 640-F & *rbcL*1240-R) *rbcL* gene for *K. mikimotoi* isolates. Positions of the universal forward (F) and reverse (R) primers indicated by arrows. Dots correspond to identical nucleotides and letters correspond to any nucleotide substitutions.

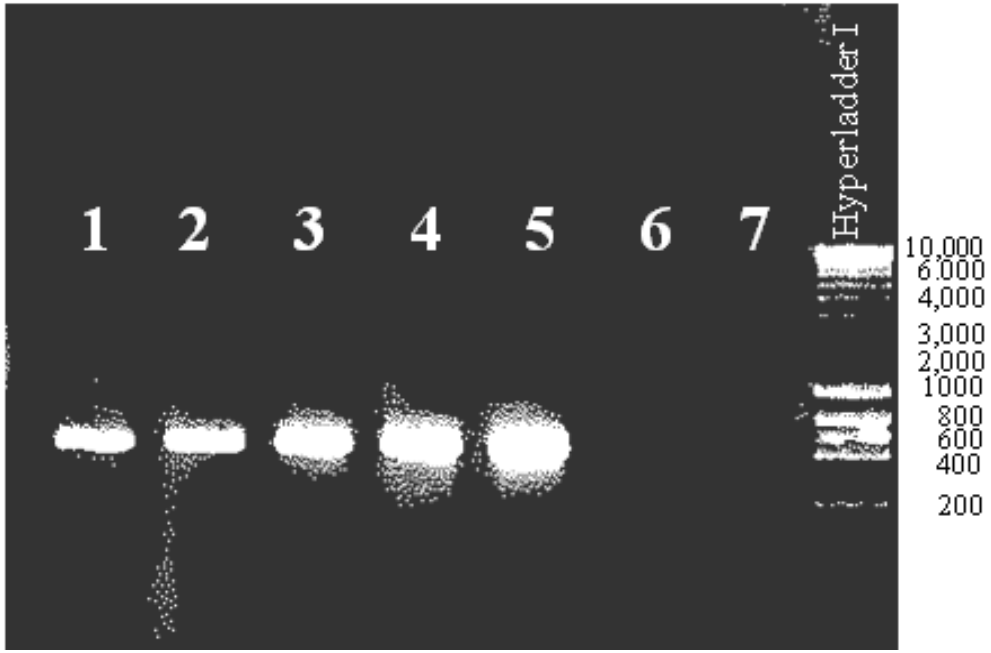


Figure 5. Gel image for *K. mikimotoi* strains using Uni1 *rbcL* gene (*rbcL* 640-F & *rbcL*1240-R) of : 1. AC213 , 2. MBA561, 3. K-260, 4. CCMP429, 5. CAWD63, 6 & 7. Negative controls.

Appendix 2

Western English channel											
Date	Location	CZCS *chl* a	Sea WiFS *chl* a	MODIS *chl* a	Confirmed *K. mikimotoi*	Max *chl* a (mg m^{-3})	Max cell no. (cells/L)	Associated species	Harmful effect	Source	Figure
26 July - 7 Aug 1975	49-49.5º N, 5.5-5.9º W; 50º N, 4º W; E1 (50º 02' N, 4º 22' W); 48.8 ºN, 5.5º W (North-west Ushant)				+	100	$5x10^6$	*N. scintillans*		Pingree et al. (1975); Pingree et al. (1977); Holligan and Harbour (1977); Holligan et al. (1984)	
27 July - 26 Aug1976	Western entrance to English Channel: 48.9-49.5 º N, 5.5-5.9º W; E1 (50º 02' N, 4º 22' W); 49º 16' 50 N, 3º40' 50 W; 48.8 ºN, 5.5º W (North-west Ushant)				+	128	$9.3x10^5$	*N. scintillans*		Grall (1976); Holligan and Harbour (1977); Pingree et al. (1977); Pingree et al. (1978)	
14 July - Aug 1977	Offshore from Plymouth & in Islay front 48.8º N, 10º W				+		abundant			Pingree et al. (1978); Holligan (1979)	
July 1978	48.72º N, 5 .74º W (North-west Ushant); Offshore from Plymouth & English south coast				+	200	$1x10^7$		Wild fishes & benthic invertebrates mortalities	Boalch (1979); Pingree et al. (1979)	
Aug - Oct 1979		+			Suspected					This survey	1A-C
July -18 Aug 1980	49.5-50.4º N, 4-4.1º W	+			+	9				Jordan and Joint (1984)	1D & E
23 July - 2 Aug 1981	Between Plymouth & Roscoff, North-wesr Roscoff & central the English channel	+			+	50 to 70		*N. scintillans*		Holligan et al. (1983, 1984); Jordan and Joint (1984)	1F & 2A
Aug1982	Plymouth sound				+		$2.6x10^6$	*N. scintillans*		Boalch (1987b)	2B
July - Aug 1983	Plymouth Sound	+			Suspected		$1.2x10^6$			This survey & Boalch (1987b)	2C
Aug - Sept 1984		+			Suspected					This survey	2D & E
29 July - 2 Aug 1985	49º N, 4º W	+			+					Dixon and Holligan (1989)	2F
July 1986	48.9º N, 5.7º W & Plymouth Sound				+		$9x10^5$			Boalch (1987); Le Corre et al. (1993)	
26 - 30 July 1987	49º 20' N, 4º 40'				+	12.8	$>2.5x10^6$			Garcia and Purdie (1994)	
June 1992	49 º 80' N, 6º 15' W				+		$> 2.5x10^5$	*E. huxleyi*		Garcia-Soto et al. (1995)	
July 1993	Plymouth Sound (50º 15'N, 04º 13'W)				+	9				Rodríguez et al. (2000)	
23-24 July 1999	Celtic Sea & Western English Channel		+		Suspected			*E. huxleyi*		This survey	4A-D
30 July 1999	Off the coast of Devon & Cornwall (50º N, 4ºW)		+		+		$5x10^5$	*E. huxleyi*		Fileman et al., 2002	4E & F
5 August 1999	Western English Channel		+		Suspected			*E. huxleyi*		This survey	4G & H
15-18 July 2000	Western English Channel		+		Suspected			*E. huxleyi*		This survey	5A-D

20– 25 July 2000	Western English Channel (central) & Cornwall		+	+	+		$1x10^7$	*N. scintillans* & *E. huxleyi*		Groom et al. (2000); This survey	5E & F
30 Aug 2000	Western English Channel		+		Suspected			*E. huxleyi*		Garcia-Soto and Pingree (2009)	5G & H
20 - 27 July 2002	Western English Channel (central) (49.5º N, 4º W)		+	+	+	23		*E. huxleyi*		Kelly-Gerreyn et al. (2004); Miller et al. (2006); Garcia-Soto and Pingree (2009)	6A-C
28 July - 12. Aug. 2002	Western English Channel		+	+	Suspected			*E. huxleyi*		Miller et al. (2006)	6D-H & 7A-D
26 June -19 July 2003	49.1°N,4.1°W & large spatial extent in Western English Channel		+	+	+	100	$1x10^6$	*E. huxleyi*		Kelly-Gerreyn et al. (2004); Vanhoutte-Brunier et al. (2008)	7E-H & 8A-B
6-23 July 2004	Western English Channel		+	+	Suspected			*E. huxleyi*		This survey	8C-H & 9A-F
31 July 2004	Western English Channel		+	+	+			*E. huxleyi*		Garcia-Soto and Pingree (2009)	9G & H & 10A
31 Aug 2004			+	+	Suspected			*E. huxleyi*		This survey	10B-D
14 – 30 July 2006			+	+	Suspected			*E. huxleyi*		This survey	11D-H & 12A-D
31 July - 9 Aug 2006			+	+	+			*E. huxleyi*		Gracia-Soto and Pingree (2009)	12E-H & 13A

ushant and French Coasts											
Date	Location	CZCS *chl* a	Sea WiFS *chl* a	MODIS *chl* a	Confirmed *K. mikimotoi*	Max *chl* a (mg m^{-3})	Max cell no. (cells/L)	Associated species	Harmful effect	Source	Figure
July 1975	Ushant Front				+					Pingree et al., (1975,1976); Holligan, 1979; Holligan et al. (1984); Garcia-Soto and Purdie (1994)	
Aug 1976	Ushant Front Brest & Douarnenez Bays				+		$6 x10^5$		Shellfish mortalities	Pingree et al. (1986); Holligan and Harbour (1977); Erard-Le Denn et al. (1990)	
12 July 1977	Ushant Front				+	10	$3.69x10^5$			Pingree et al. (1968)	
1978	Ushant Front ,Brest & Douarnenez Bays				+		$6 x10^5$		Shellfish mortalities	Holligan (1979); Erard-Le Denn et al. (2001)	
July - Aug 1980	Ushant Front	+			+					This survey Pingree et al. (1982)	1D 1E
July 1981	Brest & Douarnenez Bays	+			+		$6 x10^5$		Shellfish mortalities	Erard-Le Denn et al. (2001)	1F
July 1983	Brest & Douarnenez Bays	+			+		$6 x10^5$	*Dinophysis* spp.	Shellfish mortalities	Belin et al. (1989); Erard-Le Denn et al. (2001)	2C
1984	French coasts				+			*Dinophysis* spp.		Belin et al. (1989)	2E
1985	Brest & Douarnenez Bays	+			+		$8 x10^5$	*Dinophysis* spp.	Closure of scalop nurseries	Belin et al. (1989) ; Erard-Le Denn et al. (2001)	2F
July 1986	Ushant front				+					Le Corre et al. (1993)	
18-22 July 1987	Brest & Douarnenez Bays				+		$3x10^6$	*Dinophysis* spp.		Belin et al. (1989); Gentien and Arzul (1990)	
1988	Somme, Brest, Douarnenez & Vilaine Bays				+					Belin et al. (1989)	
1992	Vilaine & Quiberon Bays				+		$7 x10^5$		Shellfish mortalities	Erard-Le Denn et al. (2001)	
1995	South Brittany, French Atlantic coast, near the Ushant & along the Armorican Shelf				+		$4.8x10^7$		invertebrates , shellfish & fish mortalities	Arzul et al. (1995); Gentien (1998)	
21 July 2002	Near the Ushant		+	+	Suspected					Gracia-Soto and Pingree (2009) & This survey	6D-F
12 July 2004	Near the Ushant		+	+	Suspected					This survey	9A-C
14-30 July 2006	Near the Ushant		+	+	suspected					This survey	11F-H & 12A & D

Celtic Sea, South of Ireland											
Date	Location	CZCS *chl* a	Sea WiFS *chl* a	MODIS *chl* a	Confirmed *K. mikimotoi*	Max *chl* a (mg m^{-3})	Max cell no. (cells/L)	Associated species	Harmful effect	Source	Figure
21 July-Aug 1976	South coast of Youghal.				+				Marine life mortality	Ottway et. Al. (1979)	
26 Aug –Sept 1978	Cork Harbour, vicinity of Youghal., Off Kinsale, fastnet Rock (Roaring water Bay) & Dunmanus Bay				+	32	$1x10^6$		Many invertebrates & caged fish mortalities	Leahy (1980); Pybus (1980); Roden et al. (1980)	
15 July-27 Aug 1979	Around Sherkin Isl&, Roaring water Bay,	+			+		$> 1x10^6$		Massive mortalities of farmed rainbow trout	Jenkinson and Connors (1980); Rhoden et al. (1981)	1A-B
1-6 Sept 1979	Fastnet Rock & Dunmanus Bay				+						
Oct 1979	South western Ireland	+			Suspected						1C
July 1980	South western coasts	+			Suspected					This survey	1D
Aug 1980	Dunmanus Bay	+			+		$2.6 x10^6$		No mortalities	Blake and Walker (1981)	1E
Aug 1981	Around Fastnet Rock	+			+		$0.5 x 10^6$			Raine at al. (1990)	2A
Aug - Sept 1984	Around Fastnet Rock (Roaring water Bay)	+			+		$5x10^5$			Raine at al. (1990)	2D & E
Aug 1987	Bantry Bay				+		$5.5x10^5$			Raine at al. (1990)	
5-8 Aug 1991	Bantry Bay				+		$7x10^5$			Raine et al. (1993)	
1992	South-west				+				Farmed clams mortalities	Joyce (1995)	
14 Aug 1994	Fastnet Rock				+		$>5x10^5$			Raine and McMahon (1998)	
14-15 Aug1995	Fastnet Rock, Northern Celtic Sea & south of Cork Harbour				+		$4x10^6$			Raine and McMahon (1998)	
4-15 Aug. 1998	South-west of Ireland		+		+	23	$9.4x10^5$			Ediger et al. (2001); Raine et al. (2001)	3A-H
29 July 1999	Rosses Point in Co Sligo, shelf water & Aran Islands.				+		$7.5x10^5$			O'Boyle (2002)	
Aug 1999	North-west coast				+		$8.6x10^4$			O'Boyle (2002)	
July 2000	South west & Celtic Sea		+		Suspected					Miller et al. (2006)	5A & 5C-F
June-Aug 2005	South west		+		+				Wild & cultured fish & shellfish mortalities	Silke et al. (2005); Mitchell and Rodger (2007)	10E-H & 11A-C

Appendix 3

1. Monthly mean Satellite CZCS chl-a images for the exceptional increased chl-a areas that have been found to be corresponding with *K. mikimotoi* blooms in the earlier years (1979-1985) in the Celtic Sea shelf-break regions (Appendix 2 Tables).

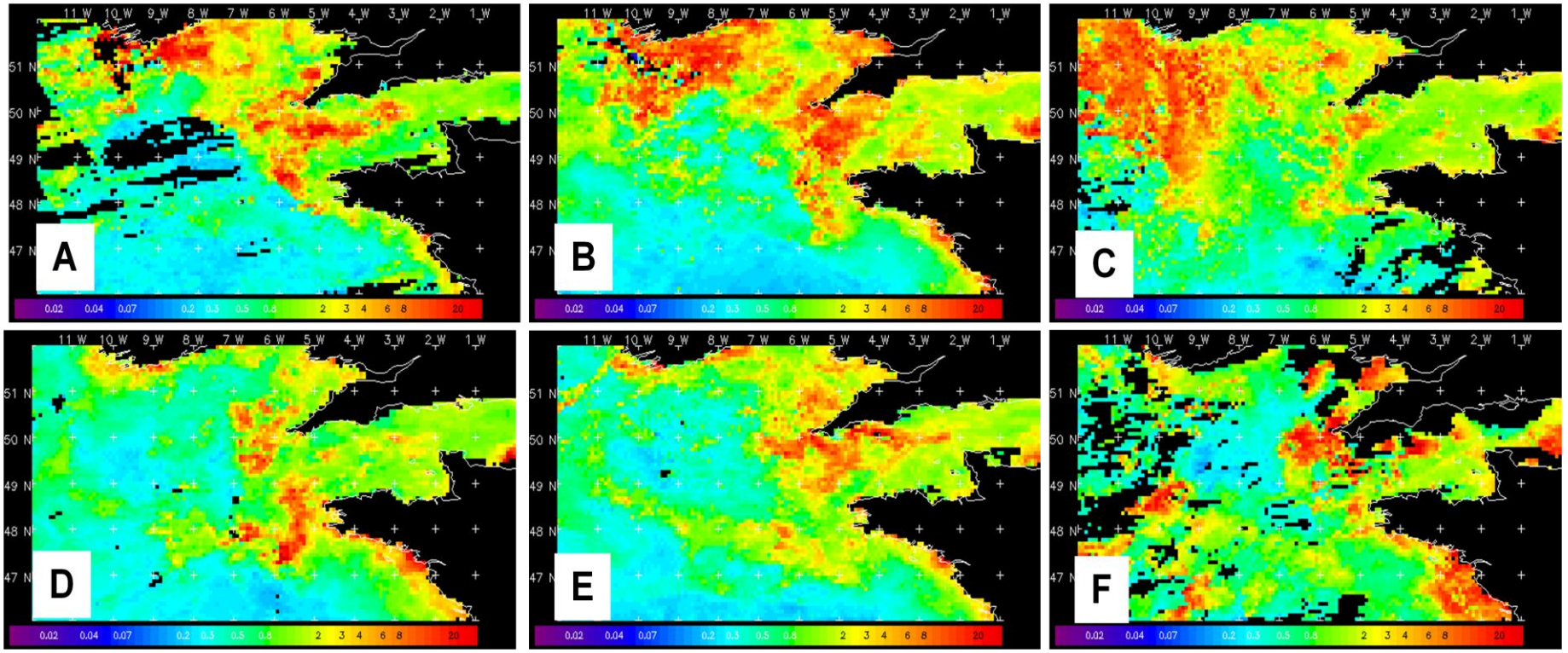


Figure 1. CZCS Chl-a (monthly mean-9 Km) of: A. August 1979; B. September 1979; C. October 1979; D. July 1980; E. August 1980; F. July 1981.

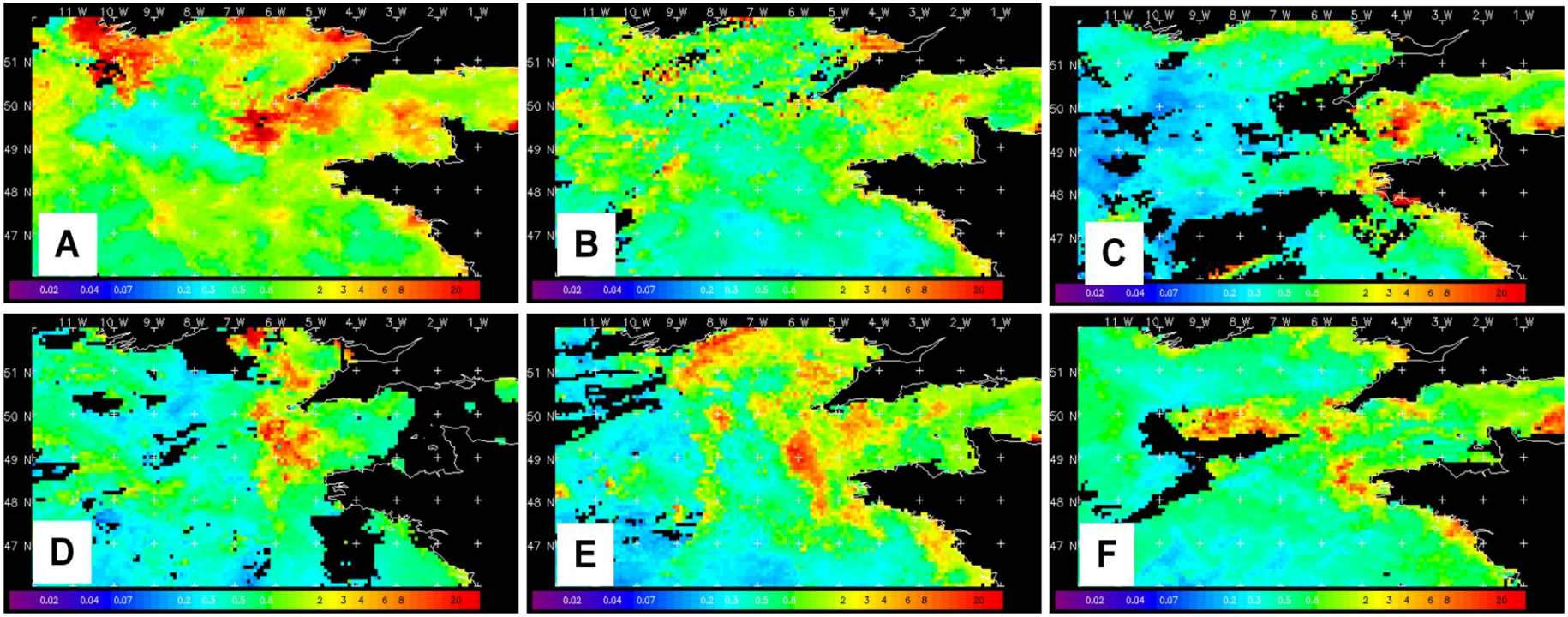


Figure 2. CZCS chl-*a* (monthly mean-9 Km) of: A. August 1981; B. August 1982; C. July 1983; D. August 1984; E. September 1984; F. July 1985.

2. Daily Satellite chl-a images (SeaWiFS, MODIS and pseudo true-colour images) (1997-2006) corresponding to *K. mikimotoi* blooms in the Celtic Sea shelf-break.

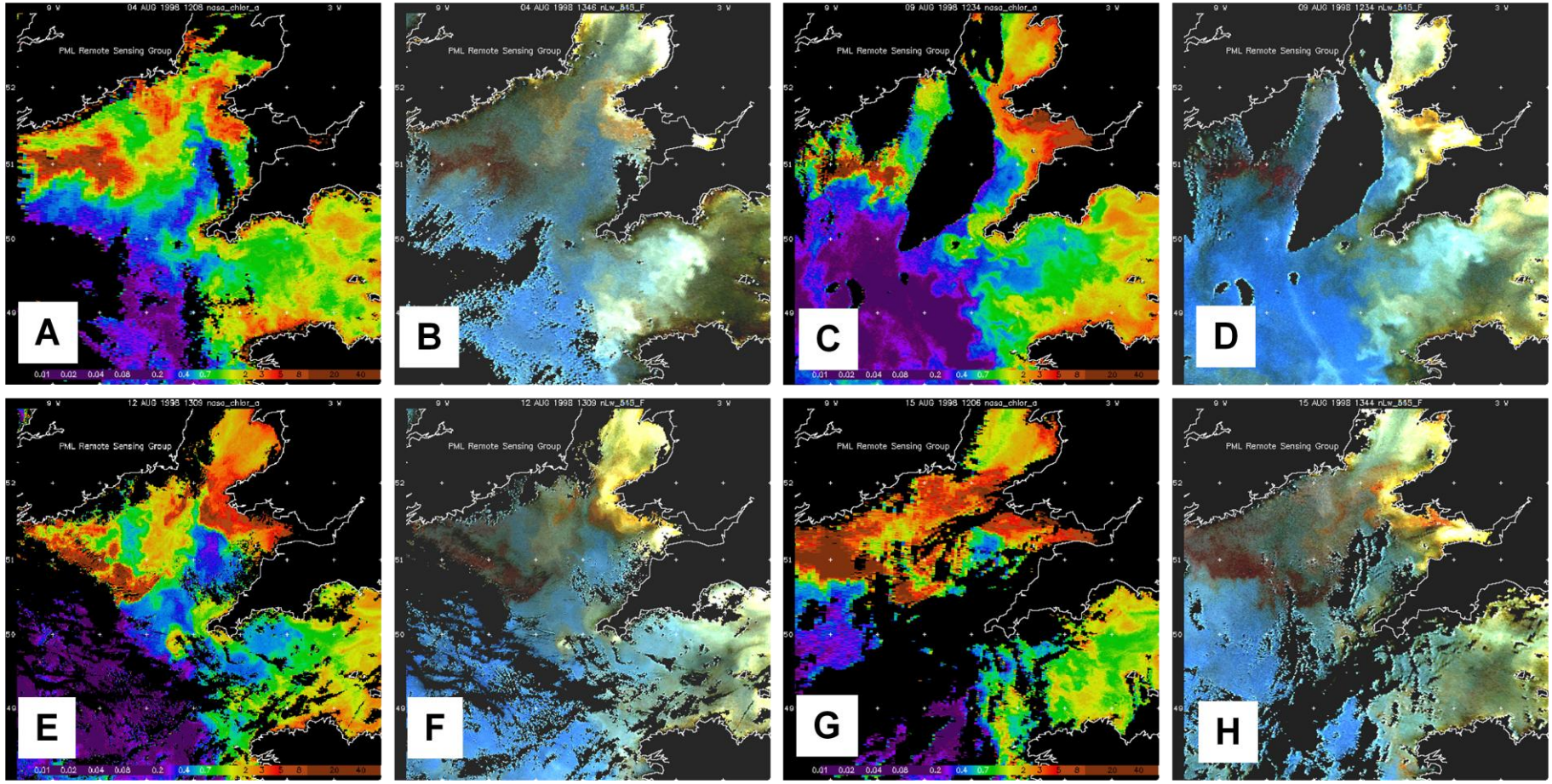


Figure 3. Satellite SeaWiFS chl-a images corresponding to *K. mikimotoi* bloom in 1998 for: 4 August 98, A. chl-a, B. pseudo true-color ; 9 August 98, C. chl-a, D. pseudo true-color; 12 August 98, E. chl-a, F. pseudo true-color; 15 August 98, G. chl-a, H. pseudo true-color.

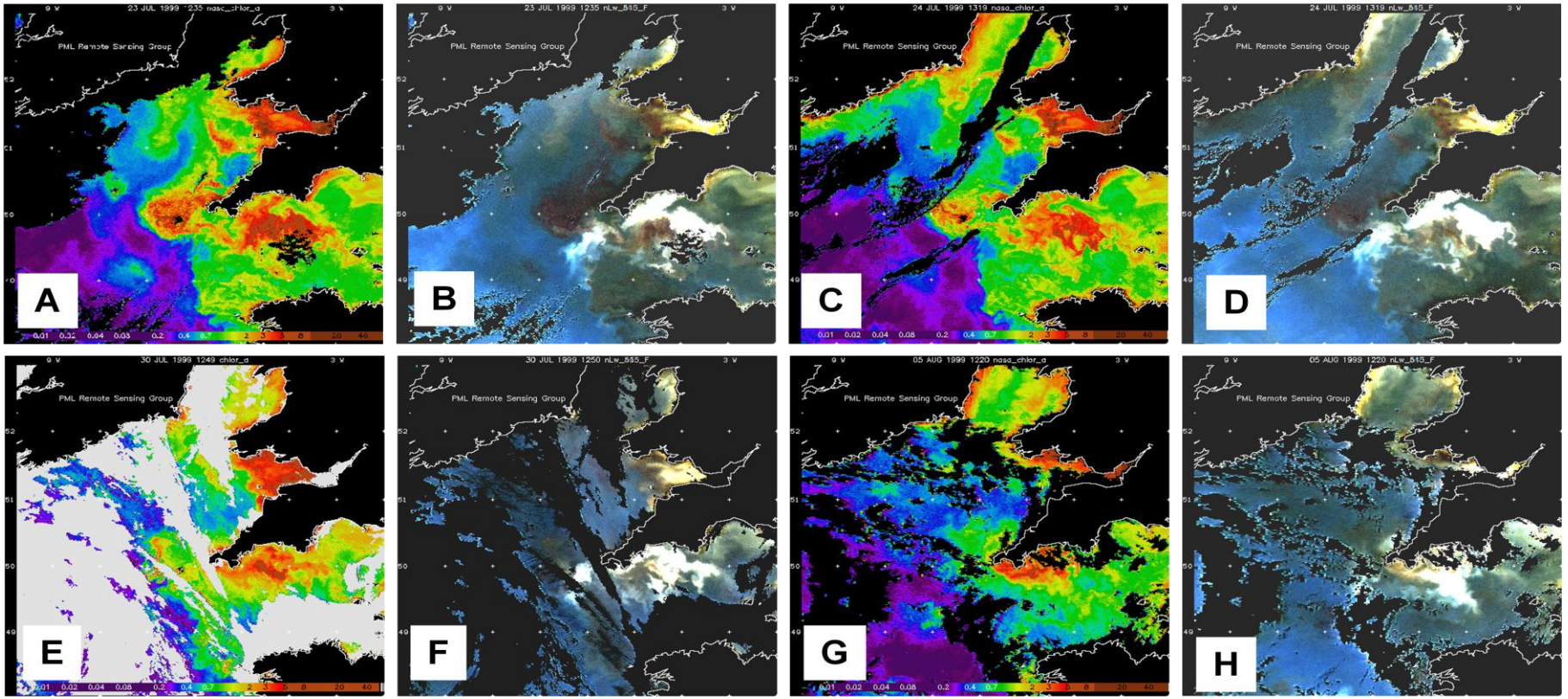


Figure 4. Satellite SeaWiFS chl-a images corresponding to *K. mikimotoi* bloom in 1999 for: 23 July 99, A. chl-a, B. pseudo true-color ; 24 July 99, C. chl-a, D. pseudo true-color; 30 July 99, E. chl-a, F. pseudo true-color; 5 August 99, G. chl-a, H. pseudo true-color.

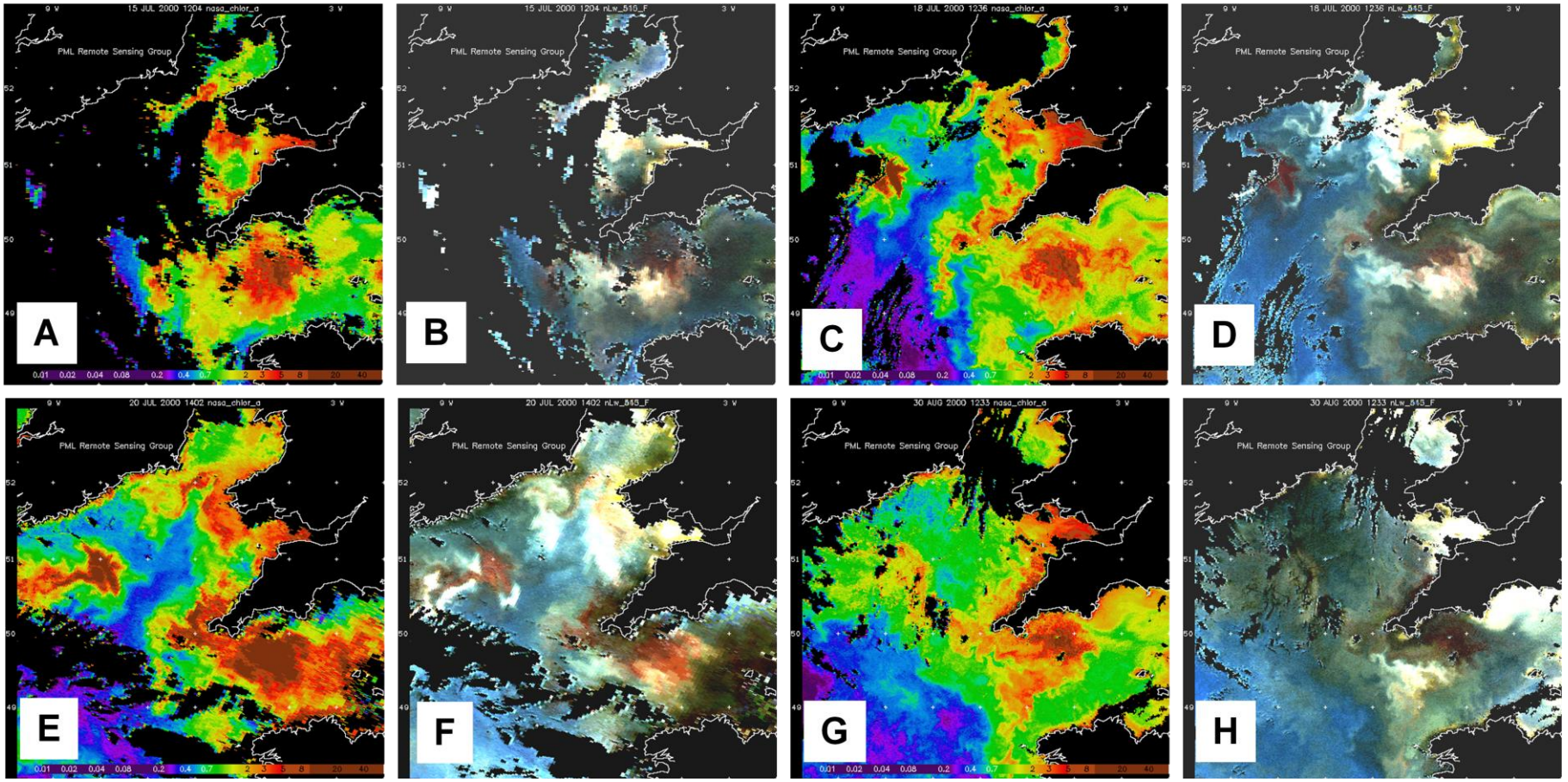


Figure 5. Satellite SeaWiFS chl-a images corresponding to *K. mikimotoi* bloom in 2000 for: 15 July 00, A. chl-a, B. pseudo true-color; 18 July 00, C. chl-a, D. pseudo true-color; 20 July 00, E. chl-a, F. pseudo true-color; 30 August 00, G. chl-a, H. pseudo true-color.

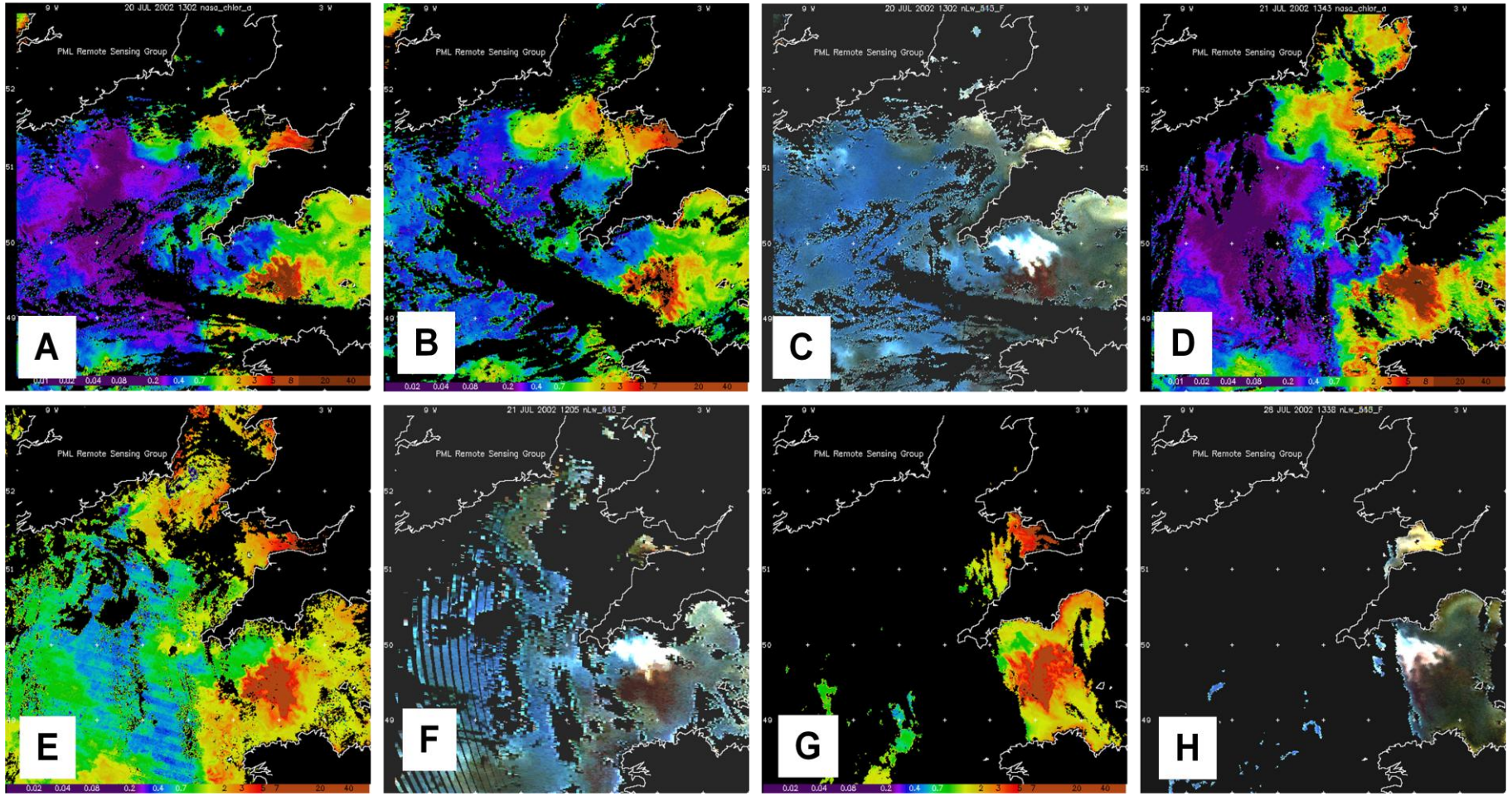


Figure 6. Satellite chl-a colour images corresponding to *K. mikimotoi* bloom in 2002 for: 20 July 02, A. SeaWiFS, B. MODIS Aqua, C. SeaWIFS pseudo true-color; 21 July 02, D. SeaWiFS, E. MODIS Aqua, F. pseudo true-color; 28 July 02, G. MODIS Aqua, H. pseudo true-color.

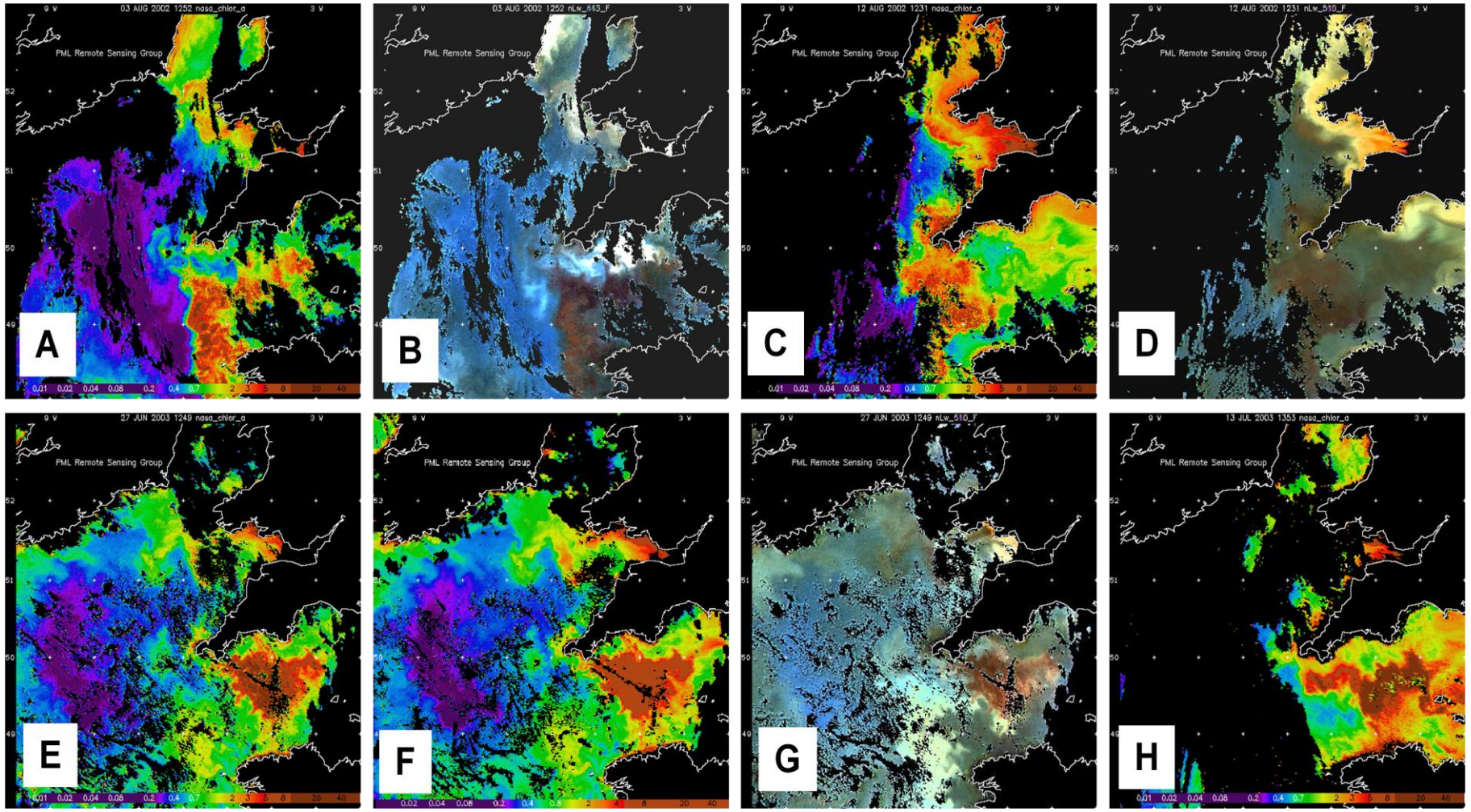


Figure 7. Satellite chl-a colour images corresponding to *K. mikimotoi* bloom in 2002 for: 3 August 02, A. SeaWiFS, B. SeaWiFS pseudo true-color; 12 August 02, C. SeaWiFS, D. SeaWiFS pseudo true-color. *K. mikimotoi* bloom in 2003 for: 27 June 2003, E. SeaWIFS, F. MODIS Aqua, G. SeaWiFS pseudo true-color; 13 July 03, H. SeaWiFS.

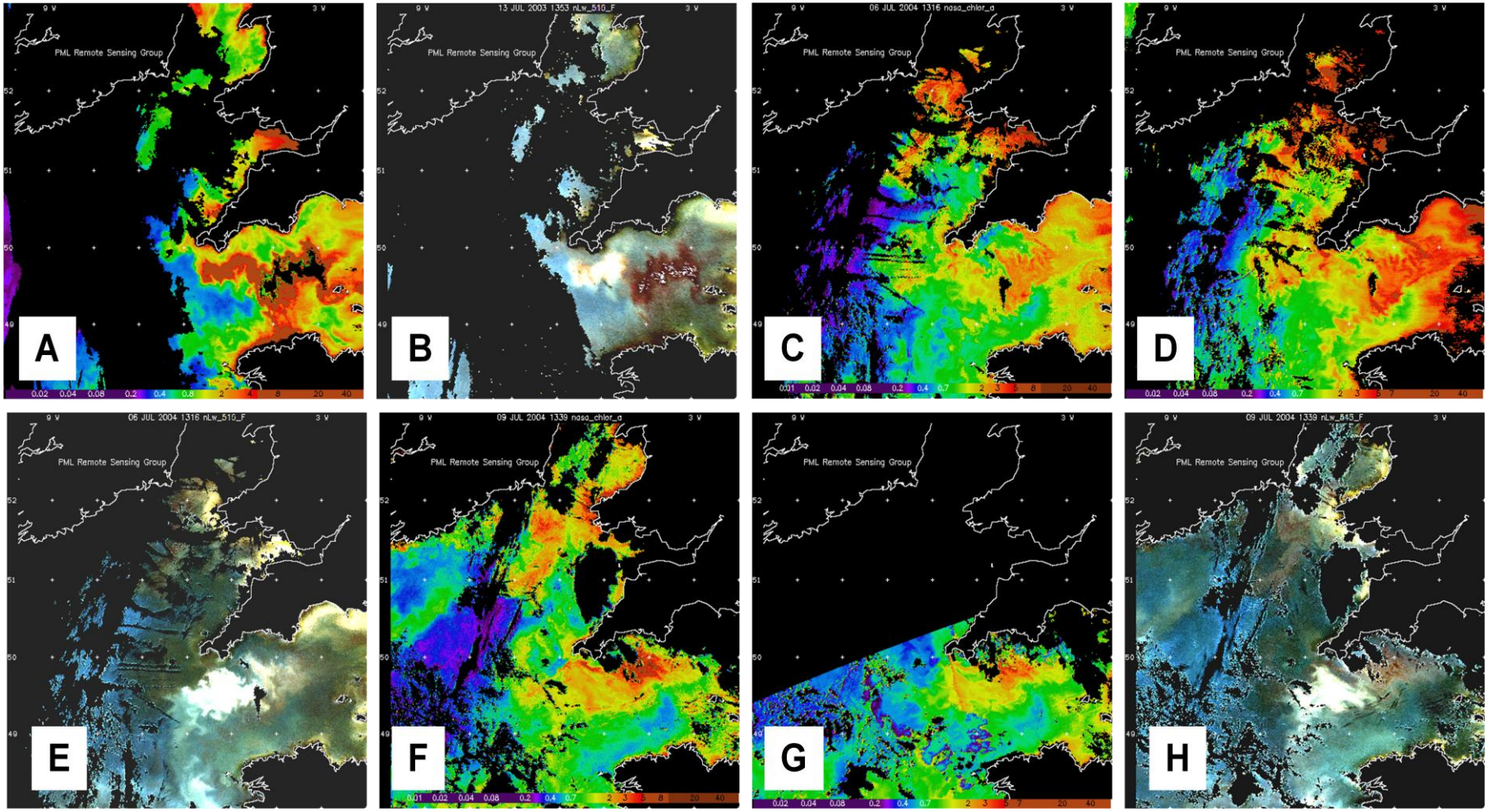


Figure 8. Satellite chl-a colour images corresponding to *K. mikimotoi* bloom for: 13 July 03, A. MODIS Aqua, B. SeaWiFS pseudo true-color; 6 July 04, C. SeaWiFS, D. MODIS Aqua, E. SeaWiFS pseudo true-color; 9 July 04, F. SeaWiFS, G. MODIS Aqua, H. SeaWiFS pseudo true-color.

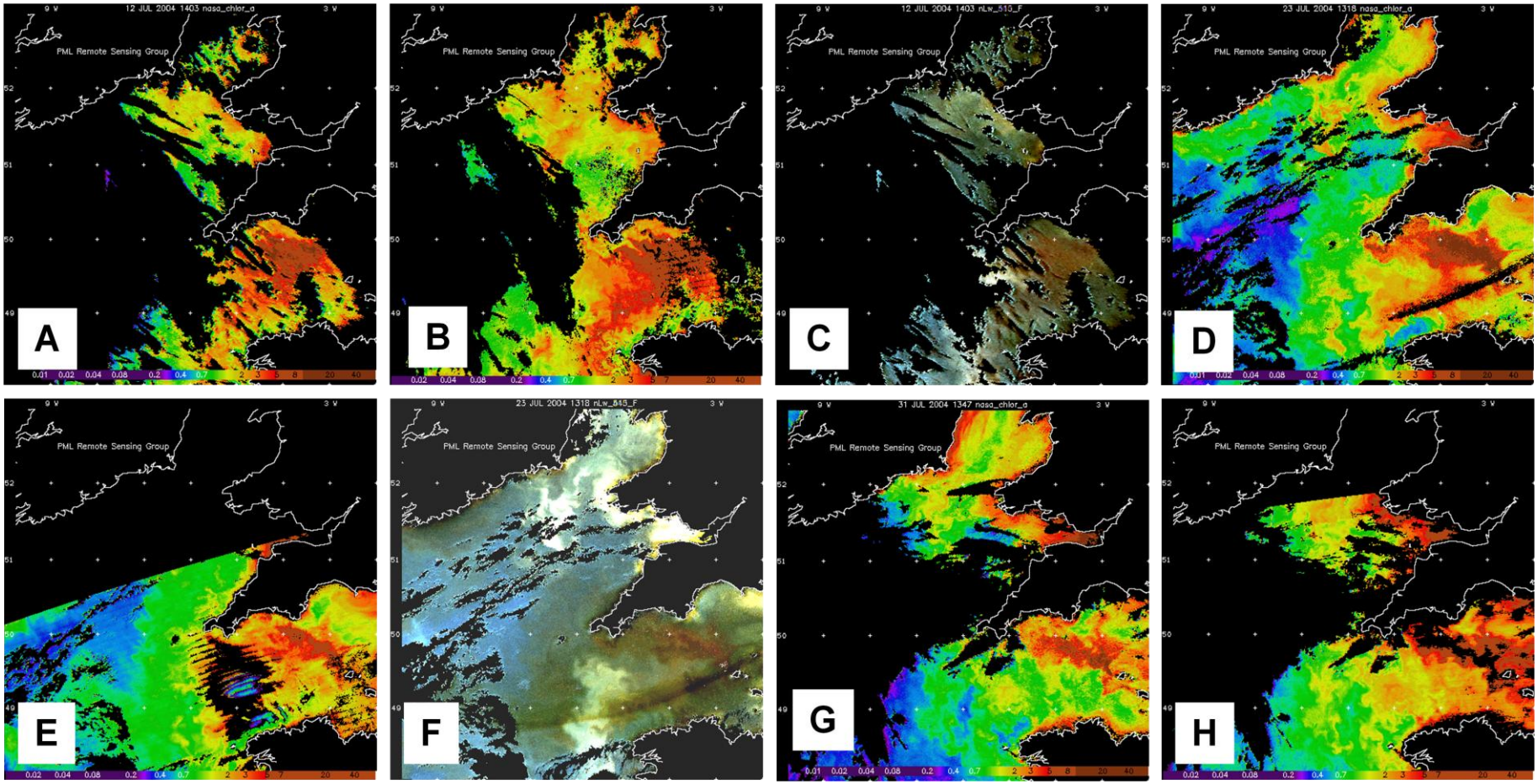


Figure 9. Satellite chl-a colour images corresponding to *K. mikimotoi* bloom for: 12 July 04, A. SeaWiFS, B. MODIS Aqua, C. SeaWiFS pseudo true-color; 23 July 04, D. SeaWiFS, E. MODIS Aqua, F. SeaWiFS pseudo true-color; 31 July 04, G. SeaWiFS, H. MODIS Aqua.

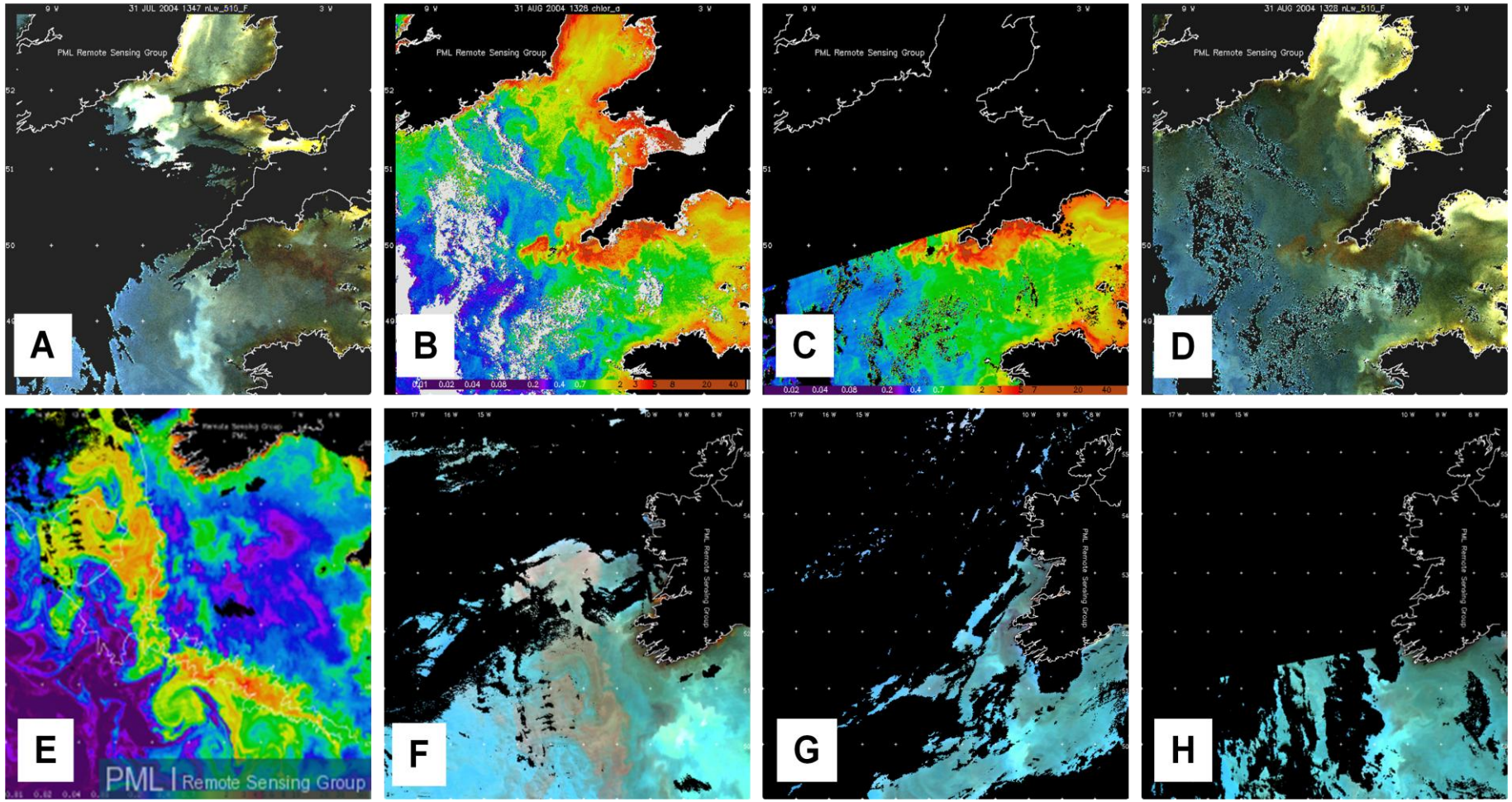


Figure 10. Satellite chl-a colour images corresponding to *K. mikimotoi* bloom for: 31 July 04, A. SeaWiFS pseudo true-color; 31 August 04, B. SeaWiFS, C. MODIS Aqua, D. SeaWiFS pseudo true-color; 9 June 05, E. SeaWiFS, F. SeaWiFS pseudo true-color; 26 July 05, G. SeaWiFS pseudo true-color; 1 August 05, H. SeaWiFS pseudo true-color.

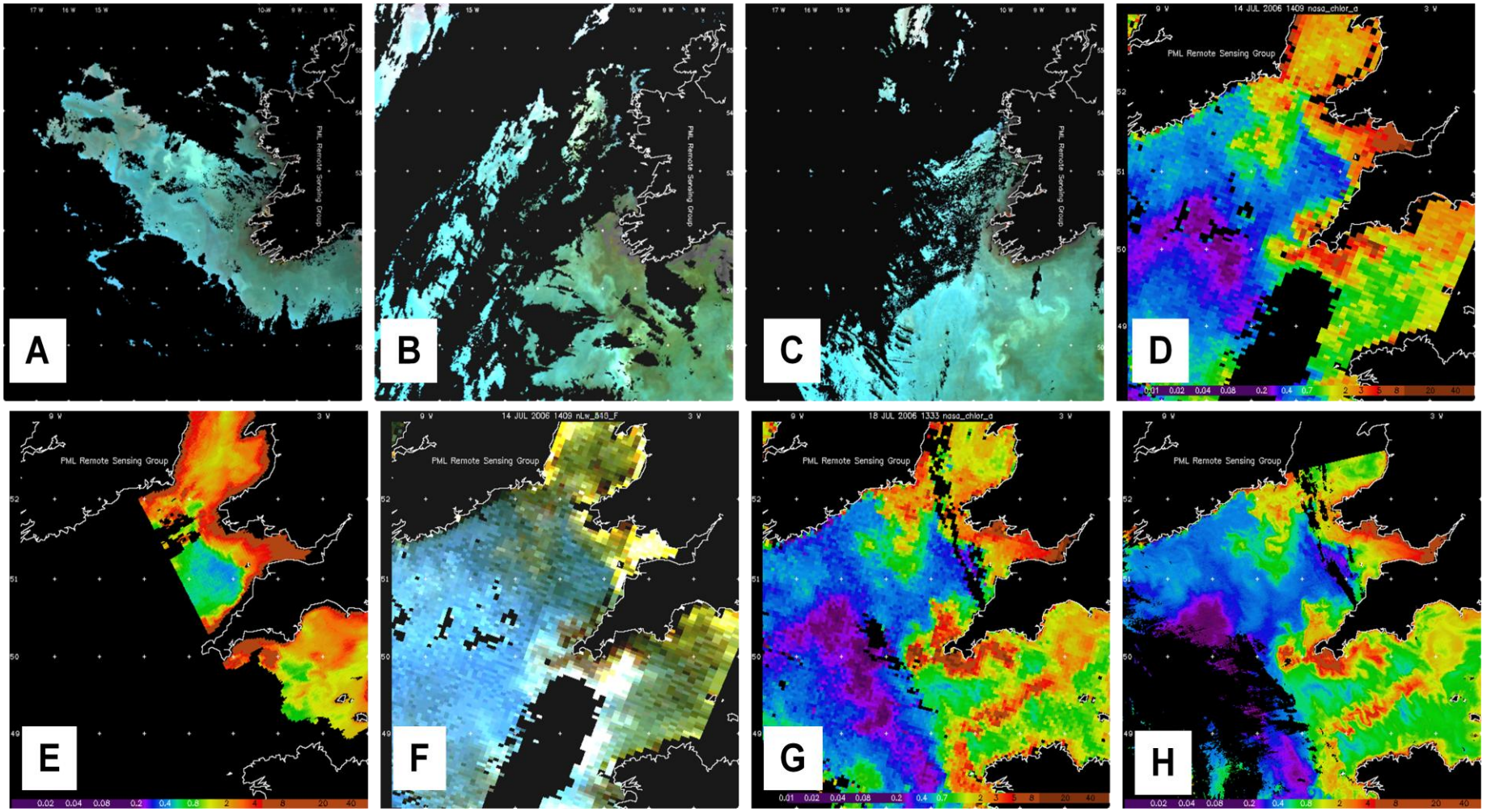


Figure 11. Satellite chl-a colour images corresponding to *K. mikimotoi* bloom for: 5 August 05, A. SeaWiFS pseudo true-color; 9 August 05; B. SeaWiFS pseudo true-color; 10 August 05, C. SeaWiFS pseudo true

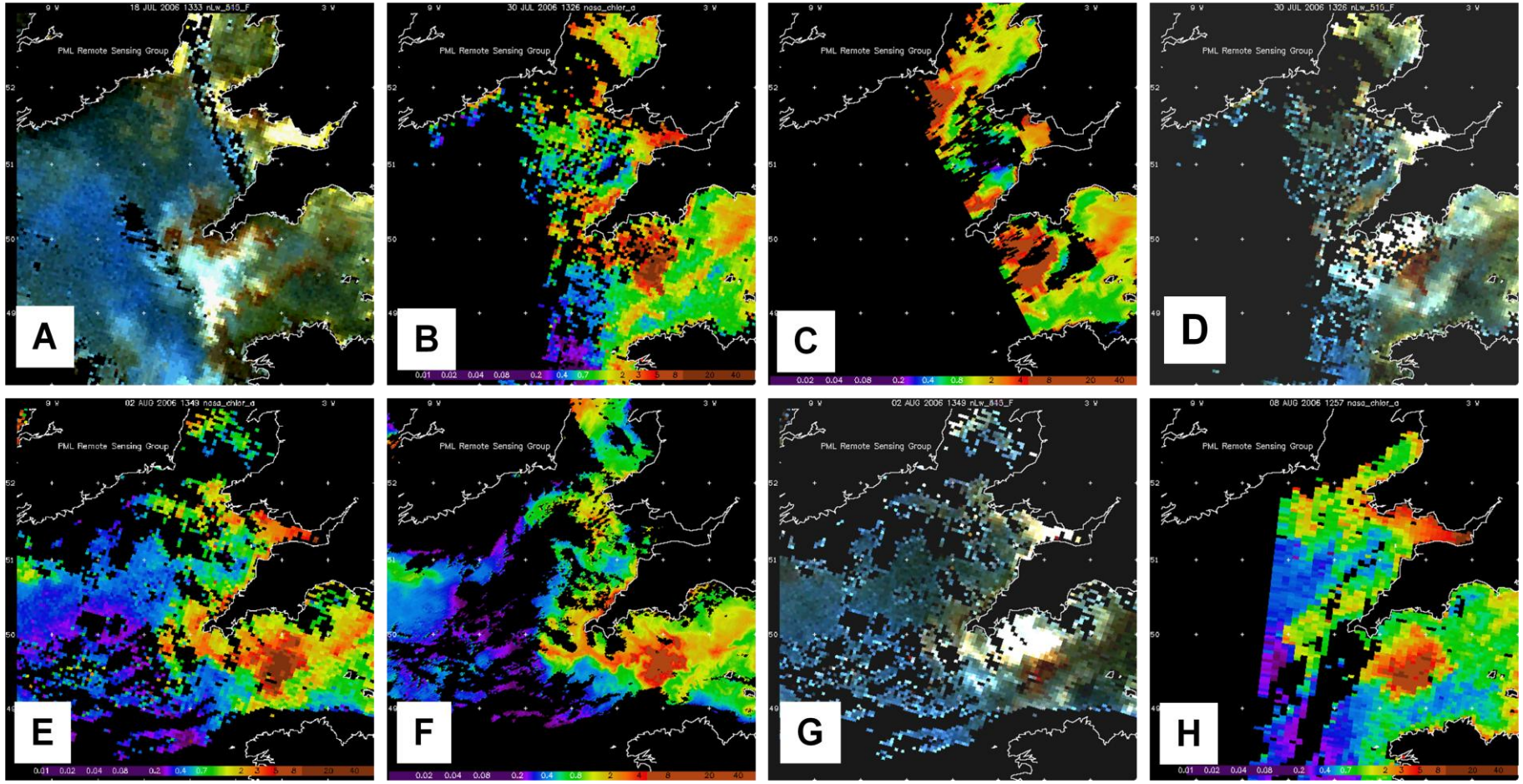


Figure 12. Satellite chl-a colour images corresponding to *K. mikimotoi* bloom for: 18 July 06, A. SeaWiFS pseudo true-color; 30 July 06, B. SeaWiFS, C. MODIS Aqua, D. SeaWiFS pseudo true-color, 2 August 06, E. SeaWiFS, F. MODIS Aqua, G. SeaWiFS pseudo true-color; 6 August 06, H. SeaWiFS.

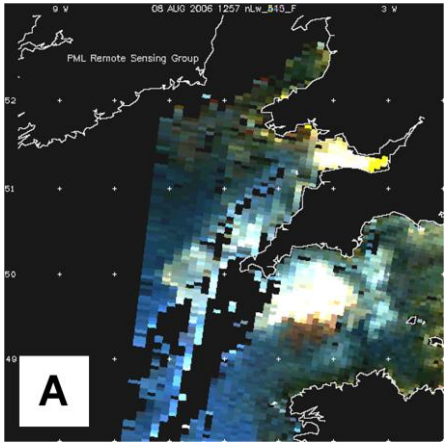


Figure 13. Satellite chl-a colour images corresponding to *K. mikimotoi* bloom for: 6 August 06, A. SeaWiFS pseudo true-color .

Appendix 4

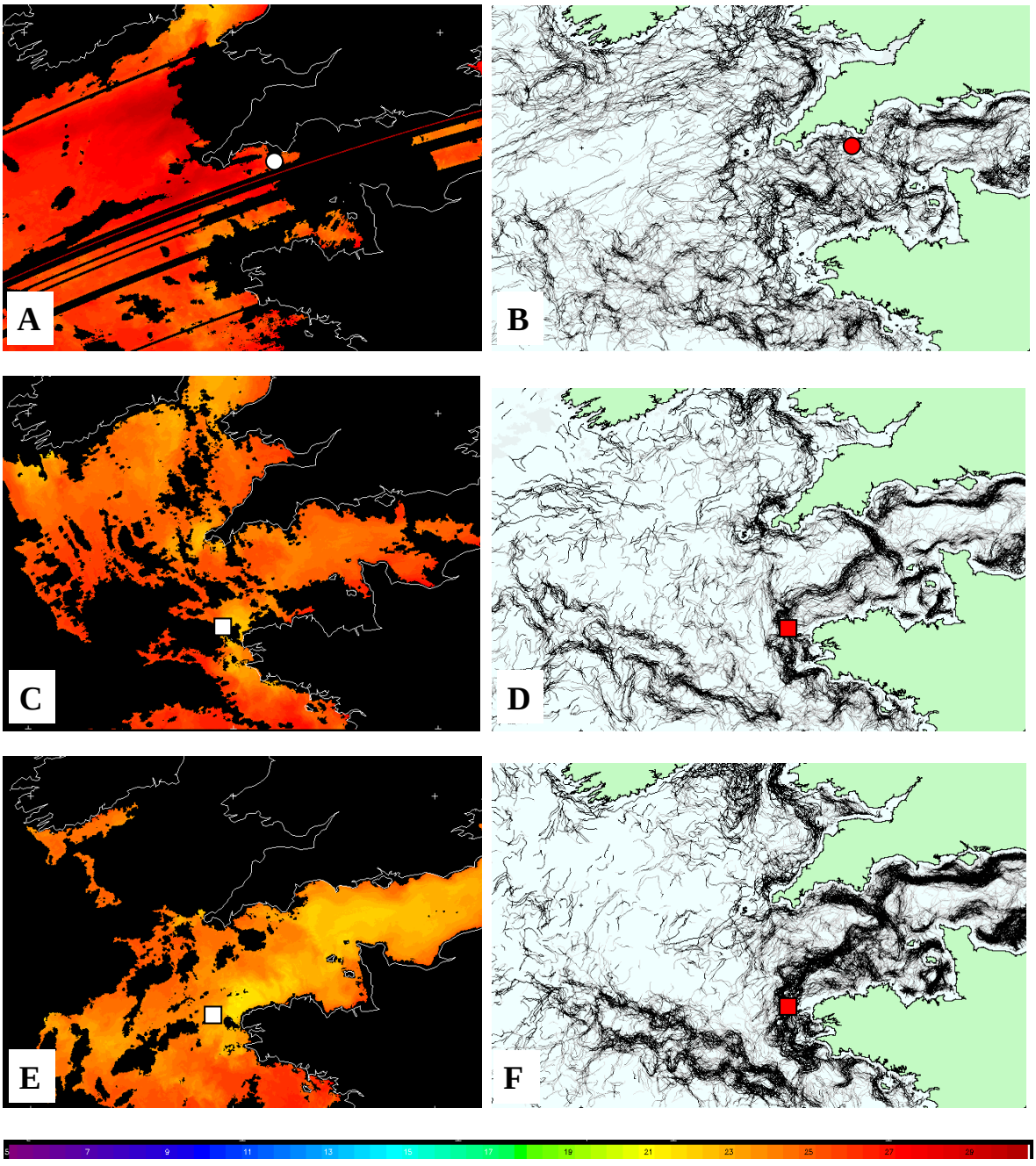


Figure 1. Satellite images of SST and associated tidal fronts corresponded with selected CPR samples that matched the locations and timing of *K. mikimotoi* bloom for: A. SST (17.8.81); B. front (1.8.81-31.8.81); C. SST (14.7.85); D. front (1.7.85-31.7.85); E. SST (4.7.95); F. front (1.7.95-31.7.95). Numbers = stations numbers for CPR samples coordinates, circles = locations in south-western English Channel, squares = locations along French coast, bottom scale for the SST images.

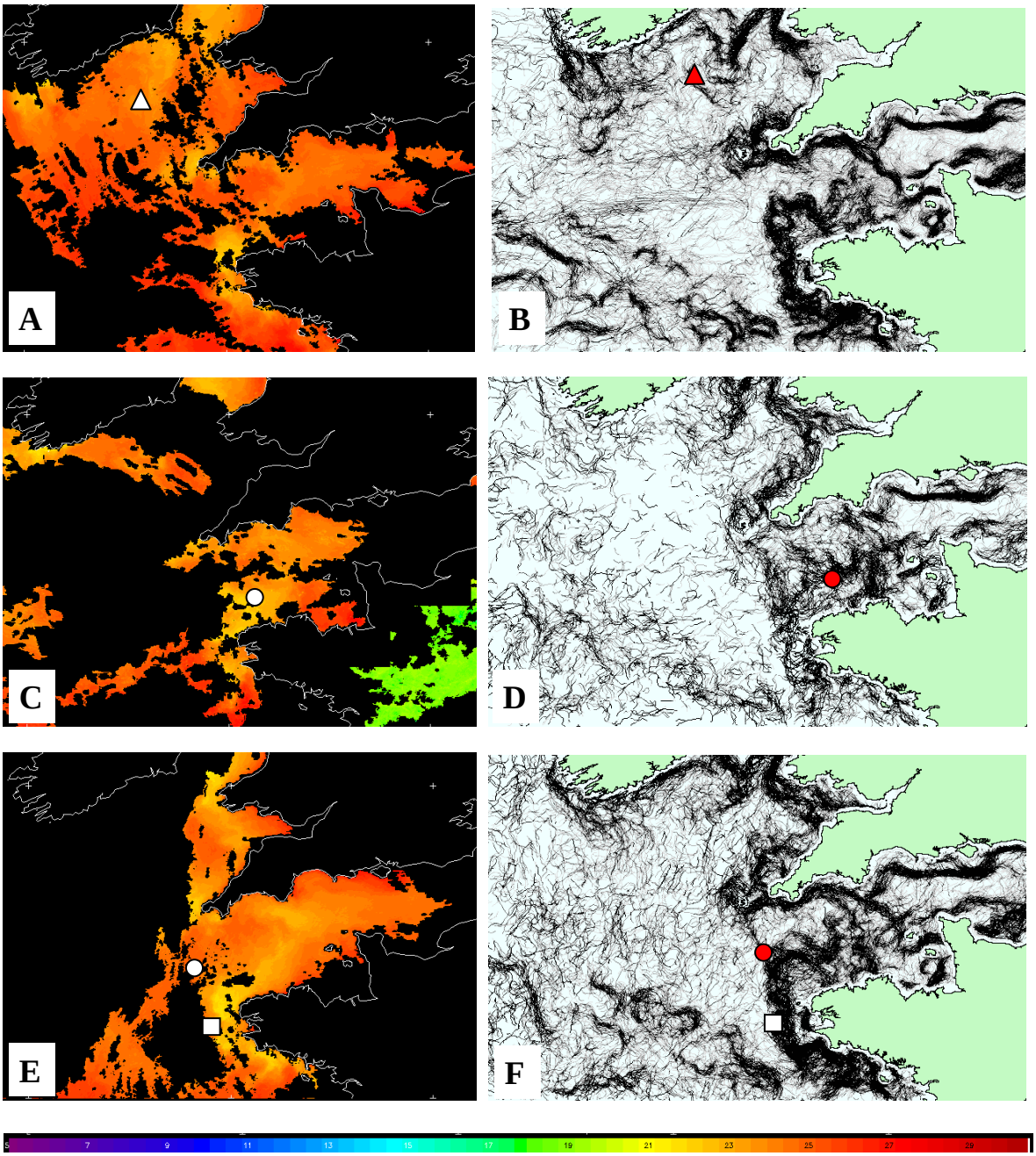


Figure 2. Satellite images of SST and associated tidal fronts corresponded with selected CPR samples that matched the locations and timing of *K. mikimotoi* bloom for: A. SST (24.8.98); B. front (1.8.98-31.8.98); C. SST (19.8.99); D. front (1.9.99-31.9.99); E. SST (12.8.02); F. front (1.8.02-31.8.02). Numbers = stations numbers for CPR samples coordinates, triangle = locations in south-western Ireland, circles = locations in south-western English Channel, squares = locations along French coast, bottom scale for the SST images.

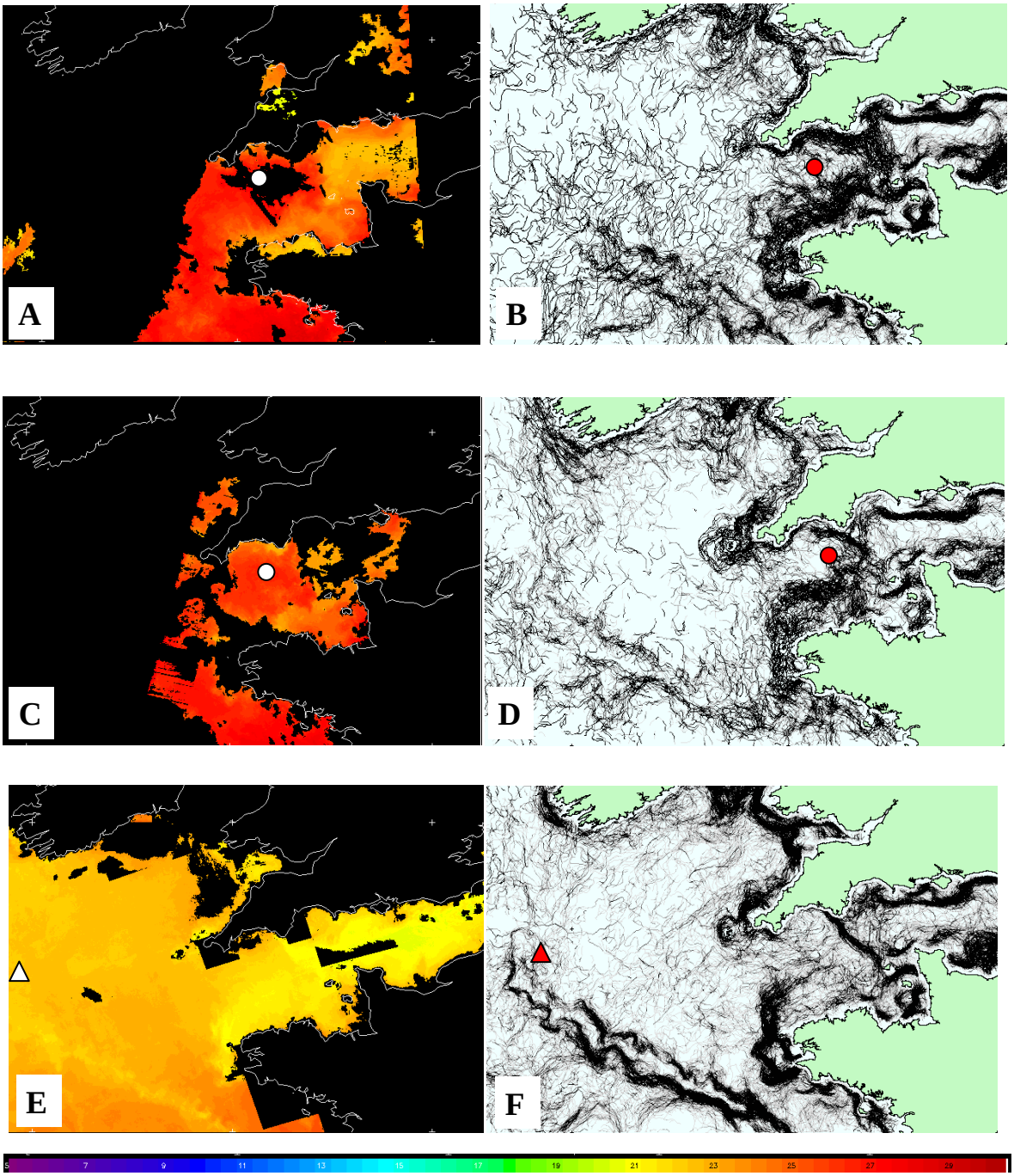


Figure 3. Satellite images of SST and associated tidal fronts corresponded with selected CPR samples that matched the locations and timing of *K. mikimotoi* bloom for: A. SST (9.7.03); B. front (1.7.03-31.7.03); C. SST (1.8.04); D. front (1.8.04-31.8.04); E. SST (4.6.05); F. front (1.6.05-31.6.05). Numbers = stations numbers for CPR samples coordinates, triangle = locations in south-western Ireland, circles = locations in south-western English Channel, bottom scale for the SST images.

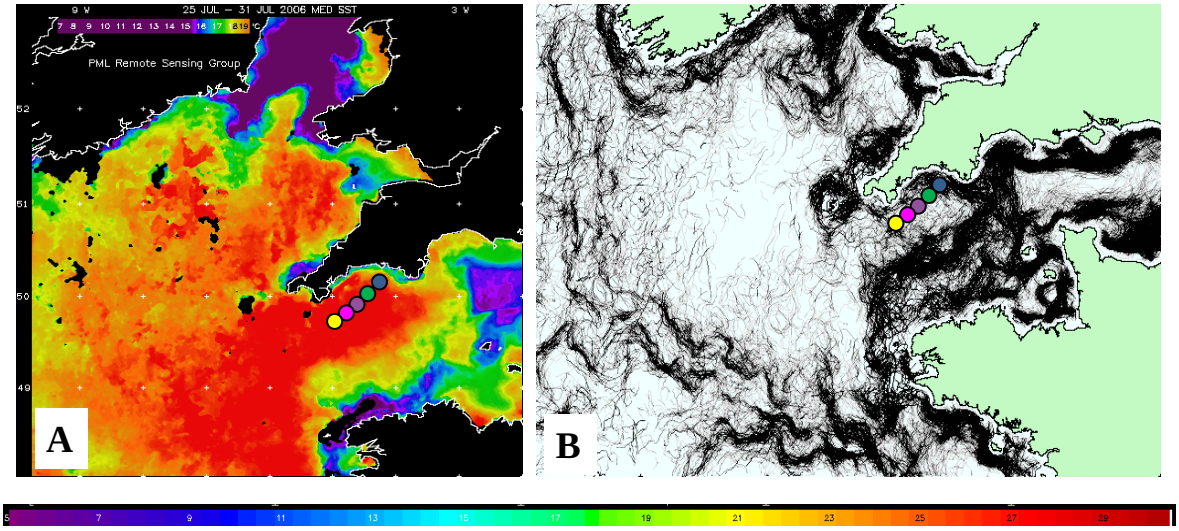


Figure 4. Satellite images of SST and associated tidal fronts corresponded with selected CPR samples that matched the locations and timing of *K. mikimotoi* bloom for: A. SST (composite 25-31.7.06); B. front (1.7.06-31.7.06). Numbers (19-23) = stations numbers for CPR samples coordinates, circles = locations in south-western English Channel, bottom scale for the SST images.

Appendix 5

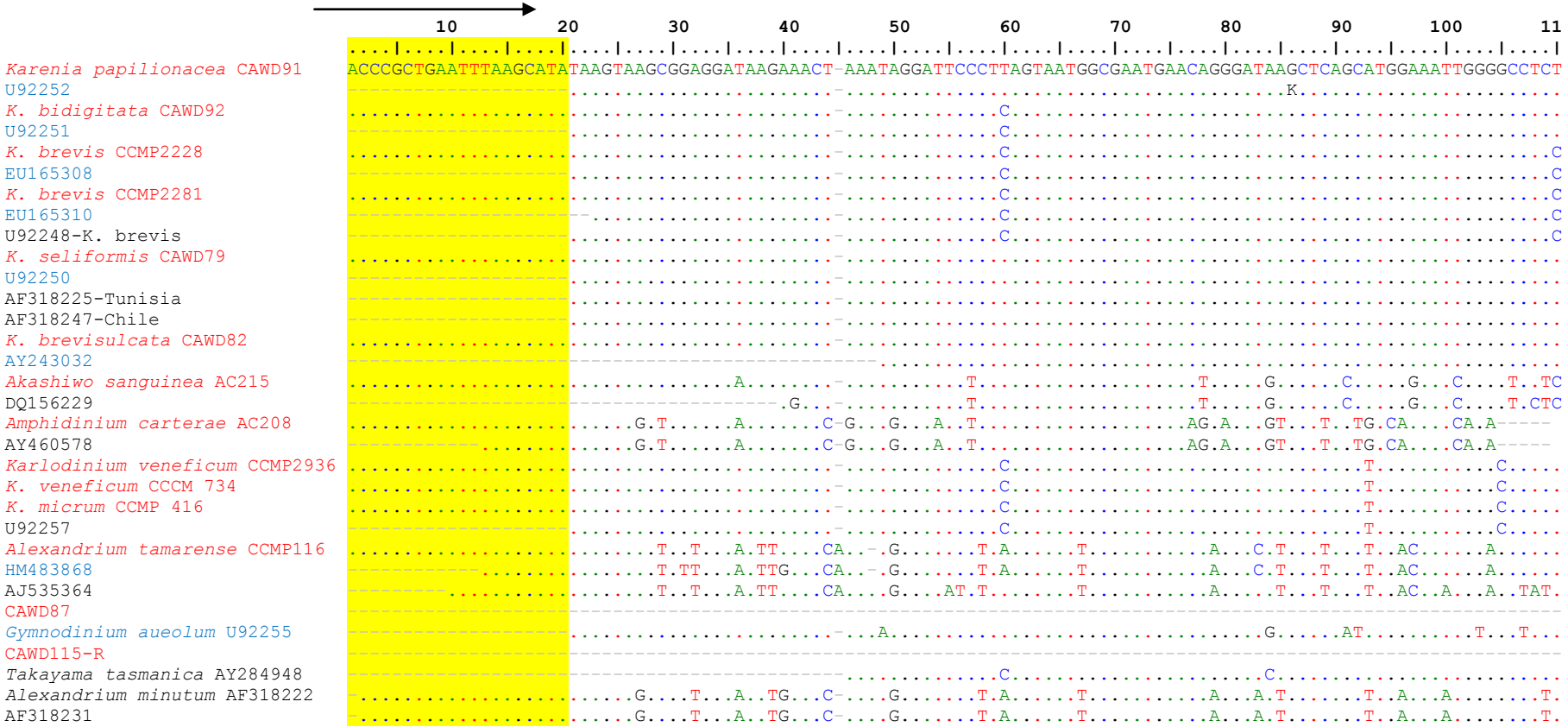
Table 1. The total genomic DNA obtained from study CPR samples.

Regions	Date	Sample	CPR[$] ref.	CPR number	Latitude	Longitude	DNA (ng/µl)
A. South-west Ireland	27.7.1963	1	79J	7	51.29	-10.32	21.48
	27.7.1963	2	79J	1	50.89	-8.87	11.45
	24.8.1965	3	90J	17	51.42	-9.75	275
	6.8.1976	4	60IS	11	51.90	-6.27	376
	18.9.1979	5	176DA	31	51.13	-9.76	19.82
	5.9.1980	6	87IS	10	51.74	-7.94	61.62
	23.8.1998	7	332BA	15	51.05	-7.49	138.9
	4.6.2005	8	412BA	37	49.66	-10.62	104.42
B. English Channel	28.7.1963	9	80SA	17	49.33	-3.46	43.43
	28.7.1963	10	80SA	19	49.15	-3.88	25.9
	30.7.1966	11	112SA	19	49.22	-3.71	142
	12.7.1968	12	132SA	19	49.18	-3.79	17.97
	20.8.1979	13	81PR	2	49.94	-4.12	173
	10.8.1981	14	103PR	2	49.99	-4.12	9.78
	19.8.1999	15	473SA	13	49.13	-4.19	145
	11.8.2002	16	505SA	3	49.51	-5.88	2374.5
	9.7.2003	17	387BA	14	49.86	-4.62	104
	1.8.2004	18	401BA	11	49.88	-3.97	8.4
	26-27.7.2006	19	55RV	1	50.18	-4.34	21.1
		20		2	50.05	-4.51	9.2
		21		3	49.92	-4.68	23.0
		22		4	49.79	-4.84	181.1
		23		5	49.67	-5.01	0.92
C. French coast	19.8.1963	24	81SB	27	48.38	-5.33	62.11
	24.7.1976	25	212SA	25	48.62	-5.02	133.1
	20.7.1985	26	308SA	21	48.66	-5.48	306.1
	3.7.1995	27	429SA	19	48.51	-5.26	3253.8
	11.8.2002	28	505SA	11	48.21	-5.26	444.2
Spring bloom*	02.5.1968	a	130SA	19	49.18	-3.81	26.19
	09.5.2004	b	398BA	13	49.85	-4.09	54.76
Summer bloom*	09.7.2001	c	365BA	11	49.96	-3.99	60.0

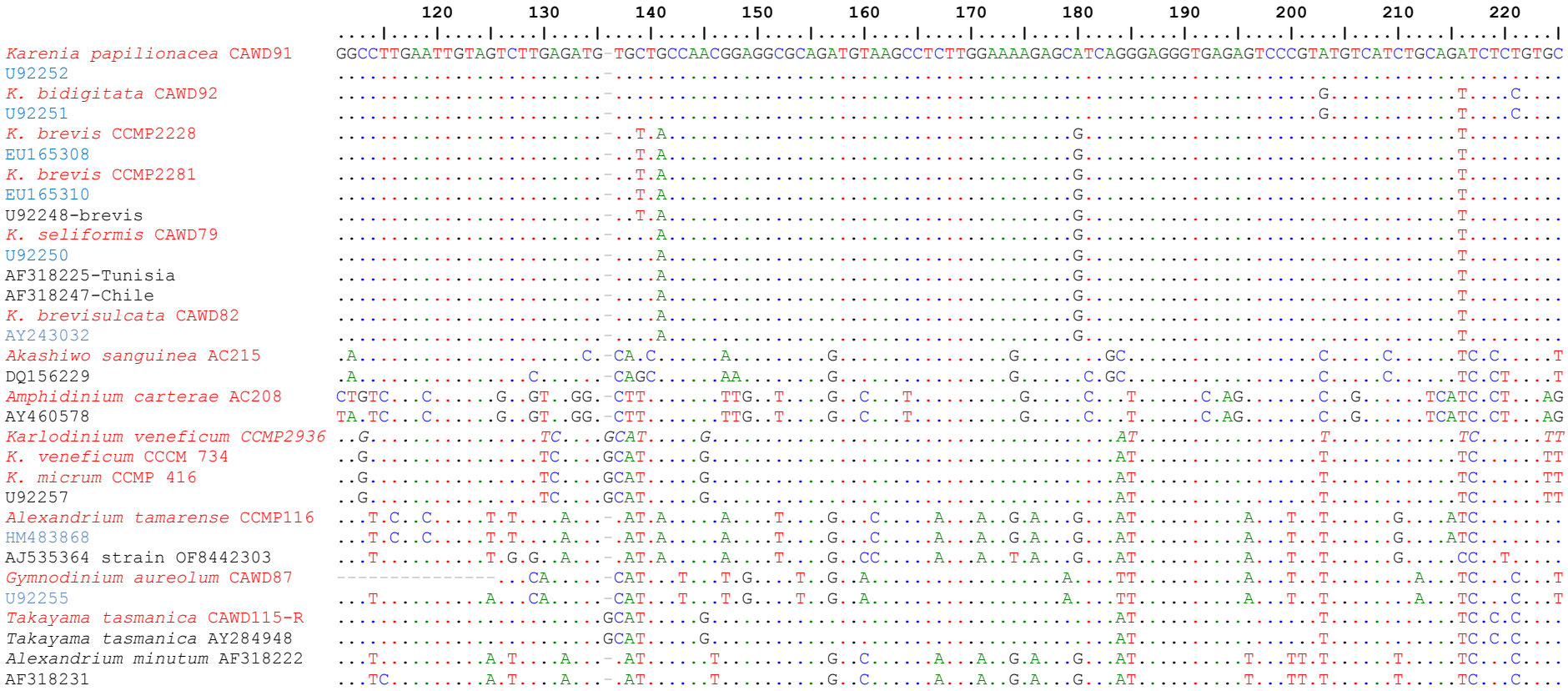
Table 2. The total genomic DNA obtained from study 2006 and 2009 *K. mikimotoi* bloom samples.

Date	Station	Location	Depth (m)	DNA (ng/µl)
26-27/07/2006	1	50.12º N, -4.2º W	surface	4.65
			5	17.30
			10	13.29
			15	14.25
			25	10.16
			40	20.02
	2	49.55º N, -4.4º W	surface	6.36
			5	12.66
			5	5.83
			10	7.60
			15	7.74
			25	36.22
			40	8.78
	3	49.32º N, -5.15º W	surface	5.58
			5	3.62
			10	9.12
			15	3.91
			25	4.08
			40	2.82

Appendix 6:


10
20
30
40
50
60
70
80
90
100
11
Karenia papilionacea CAWD91
U92252
K. bidigitata CAWD92
U92251
K. brevis CCMP2228
EU165308
K. brevis CCMP2281
EU165310
U92248-K. brevis
K. seliformis CAWD79
U92250
AF318225-Tunisia
AF318247-Chile
K. brevisulcata CAWD82
AY243032
Akashiwo sanguinea AC215
DQ156229
Amphidinium carterae AC208
AY460578
Karlodinium veneficum CCMP2936
K. veneficum CCCM 734
K. micrum CCMP 416
U92257
Alexandrium tamarense CCMP116
HM483868
AJ535364
CAWD87
Gymnodinium aueolum U92255
CAWD115-R
Takayama tasmanica AY284948
Alexandrium minutum AF318222
AF318231

Continued


120 130 140 150 160 170 180 190 200 210 220
Karenia papilionacea CAWD91
U92252
K. bidigitata CAWD92
U92251
K. brevis CCMP2228
EU165308
K. brevis CCMP2281
EU165310
U92248-brevis
K. seliformis CAWD79
U92250
AF318225-Tunisia
AF318247-Chile
K. brevisulcata CAWD82
AY243032
Akashiwo sanguinea AC215
DQ156229
Amphidinium carterae AC208
AY460578
Karlodinium veneficum CCMP2936
K. veneficum CCCM 734
K. micrum CCMP 416
U92257
Alexandrium tamarense CCMP116
HM483868
AJ535364 strain OF8442303
Gymnodinium aureolum CAWD87
U92255
Takayama tasmanica CAWD115-R
Takayama tasmanica AY284948
Alexandrium minutum AF318222
AF318231

Continued

Figure 1. Sequence alignment of a 346 bp fragment of the re designed LSU (D1 and D2 regions) rDNA gene for *K. mikimotoi* isolates. Dots correspond to identical nucleotides and letters correspond to any nucleotide substitutions. Strains sequenced in this study indicated red text name followed by blue text GenBank accession number showing the similar strain sequence in the GenBank and black text GenBank accession number showing other related sequence for different strain in GenBank. The forward (D1R-F) and re designed reverse primers (D1-D2newR) were highlighted with yellow.

Appendix 7

Design the Sp2 primer

Since the new forward primer (Manal125bp-F) arrived, they were tested on the culture collection (Tables 2.3 & 2.4) using the thermal gradients from 60 °C to 70 °C, and verified as described in section 2.2.6.2.2. The resulted annealing temperature was re-tested again on the culture collection in 66 ° and 68 ° C and was optimized to be specific at 68 °C. Only one species AC215 *Akashiow sanguínea* was amplified with the Positive control *K. mikimotoi* from 60 °C up to 67 ° C.

Table 1. The optimization of the PCR annealing temperature for the *Karenia mikimotoi* species-specific *rbc*L primer (Sp2) set (Manal125bp-F and *rbcL*1240-R).

No.	Strain no.	Strain name	60 ° C	64 ° C	66 ° C	68° C
1	AC194	*Alexandrium minutum*	-	-	-	-
2	CAWD592	*Alexandrium tamarense*	-	-	-	-
3	AC208	*Amphidinium carterae*	-	-	-	-
4	CAWD87	*Gymnodinium aureolum*	-	-		-
5	CCCM 900	*Heterocapsa triquetra*	-	-	-	-
6	CAWD92	*Karenia bidigitata*	-	-	-	-
7	CCMP2281	*K. brevis*	-	-	-	-
8	CCMP2229	*K. brevis*	-	-	-	-
9	CCMP718	*K. brevis*	-	-	-	-
10	CAWD82	*K. brevisculata*	-	-	-	-
11	CAWD05	*K. mikimotoi*	+	+	+	+
12	AC215	*Akashiow sanguinea*	+	+	+	-
13	CAWD81	*K. bidigitata*	-	-	-	-
14	CAWD79	*K. selliformis*	-	-	-	-
15	CAWD131	*K. umbela*	-	-	-	-
16	CAWD115	*Takayama tasmanica*	-	-	-	-
17	CCCM734	*Karlodinium micrum*	-	-	-	-
18	CCMP2936	*Karlodinium venificum*	-	-	-	-
19	CCMP416	*Karlodinium venificum*	-	-	-	-
20	CCMP1516	*Emiliania huxleyi*	-	-	-	-
21	1st Negative	-	-	-	-	-
22	2nd Negative	-	-	-	-	-

21 and 22 = are negative controls with no template DNA.

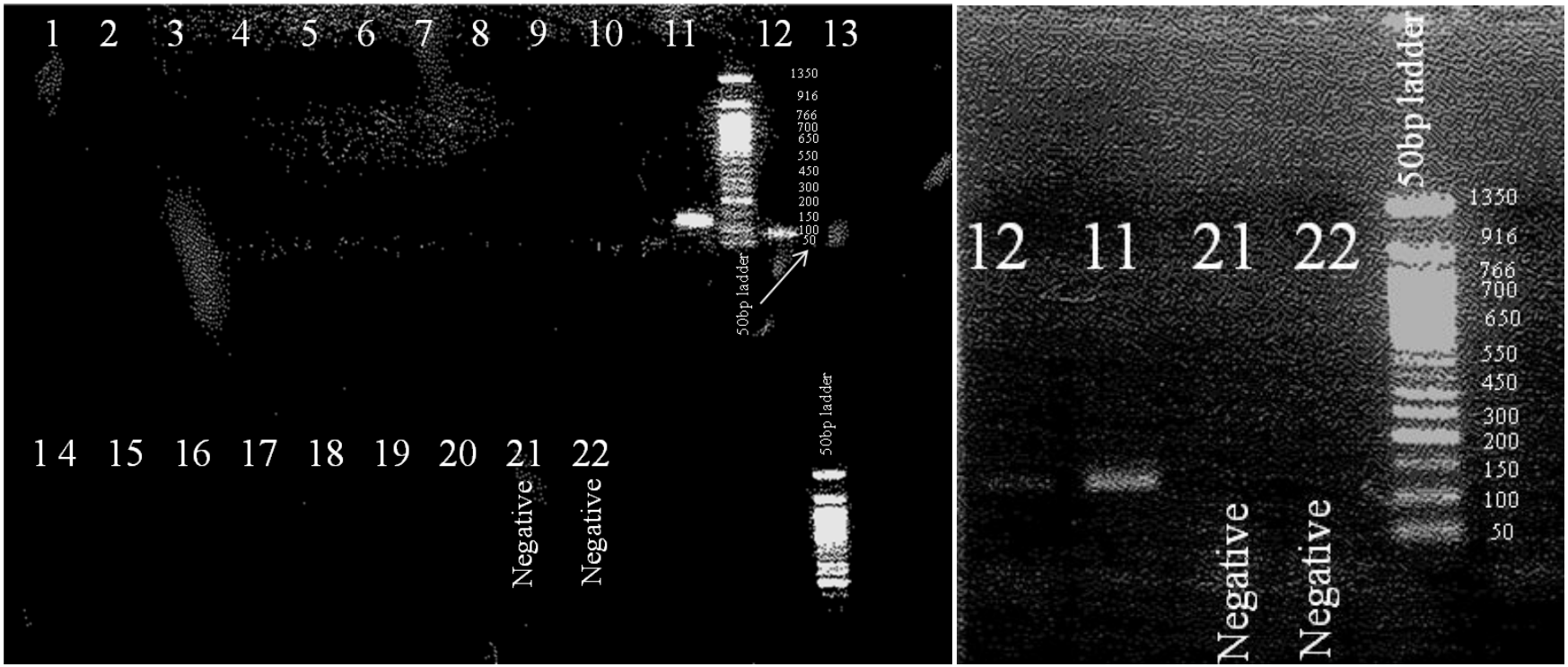


Figure 1. Agarose gel electrophoresis (1.6 % [w/v]) of PCR products (~ 125 bp) using the Sp2 *rbcL* species-specific primers (Manal125bp-F and *rbcL*1240-R) and template DNA from *K. mikimotoi* and other non-target species. 1. *Alexandrium minutum* (AC194), 2. *Alexandrium tamarense* (CAWD592), 3. *Amphidinium carterae* (AC208), 4. *Gymnodinium aureolum* (CAWD87), 5. *Heterocapsa triquetra* (CCCM 900), 6. *Karenia bidigitata* (CAWD92), 7. *K. brevis* (CCMP2281), 8. *K. brevis* (CCMP2229), 9. *K. brevis* (CCMP718), 10. *K. brevisculata* (CAWD82), 11. *K. mikimotoi* (CAWD05), 12. *Akashiow sanguínea* (AC215) 13. *Karenia bidigitata* (CAWD81), 14. *K. selliformis* (CAWD79), 15. *K. umbella* (CAWD131), 16. *Takayama tasmanica* (CAWD115), 17. *Karlodinium micrum* (CCCM734), 18. *K. venificum* (CCMP2936), 19. *K. venificum* (CCMP416), 20. *Emiliana huxlyei* (CCMP1516), 21. first Negative control and 12. second Negative control. PCR annealing temperature: A. 64 ° C and B. 66 ° C.

Appendix 8

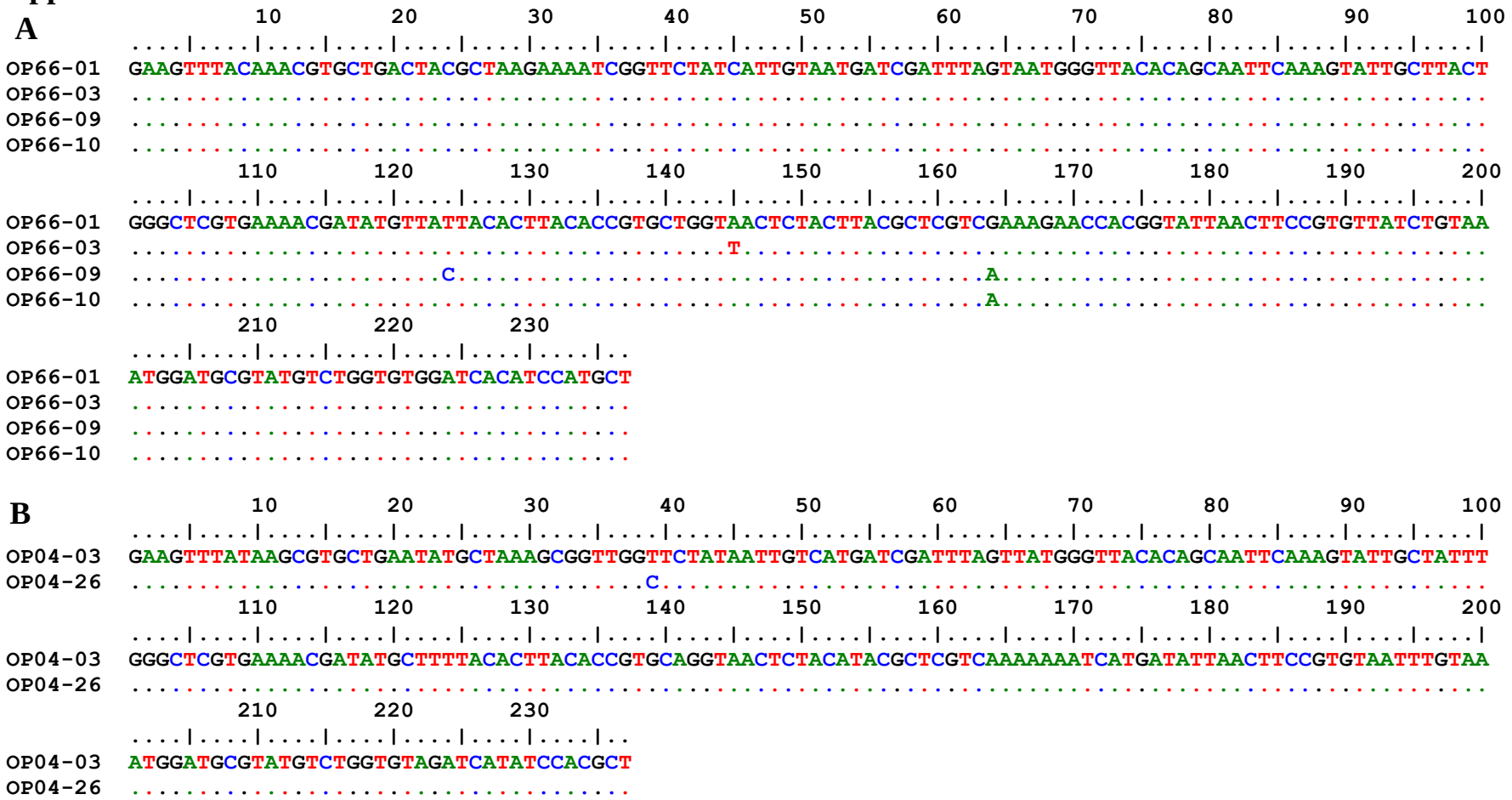


Figure 1. Sequence alignment of a 276 bp fragment of the optimized *rbcL* gene (Kmitrbcl-F1 and Kmitrbcl-R1) for diatom clones obtained from CPR samples collected in: A. 1966 (OP66-01, OP66-03, OP66-09 and OP66-10) and B. 2004 (OP04-03 and OP04-26). Positions of the universal forward (F) and reverse (R) primers indicated by arrows. Dots correspond to identical nucleotides and letters correspond to any nucleotide substitutions.

Appendix 9

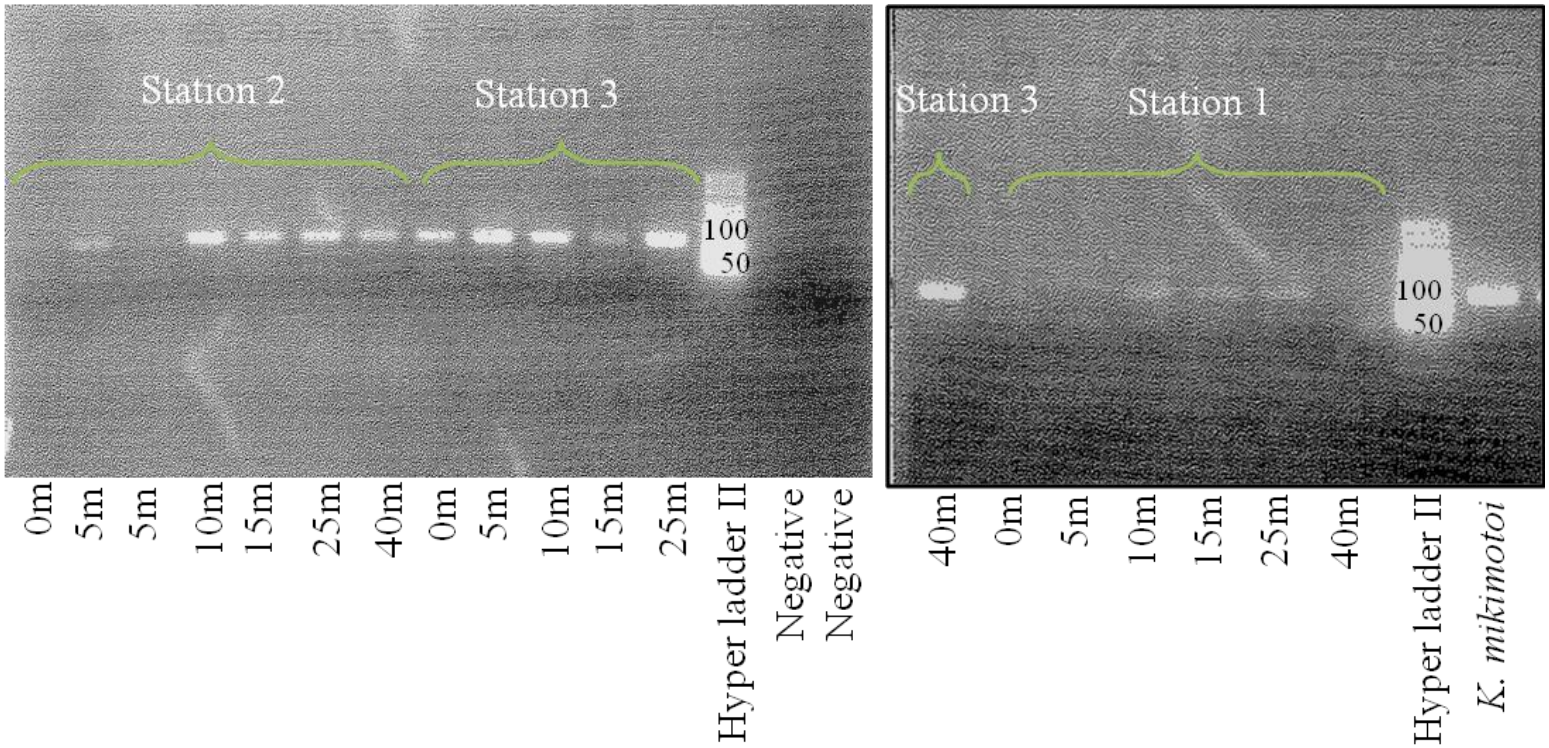


Figure 1. Gel image of PCR products (~ 117 bp) using the *rbcL* HRM primers (Kmitrbcl-HRM-F1 and Kmitrbcl-HRM-R1) for CTD samples of *K. mikimotoi* and *E. huxleyi* blooms in 2006.

Appendix 10

Table 1. BLASTed eukaryotic taxa sequences obtained from *K. mikimotoi* and *E. huxleyi* blooms for the CTD sample station1 (surface) which collected from the Western English Channel in 2006, using the Uni1 *rbcL* gene sequences (~620 bp).

Clone Code	Obtained sequences	Top BLAST species (GenBank accession no.)	Query coverage (%)	Max identity (%)
S106B19	1	*Thalassiosira angulata* strain BEN02-35	100	97
S106A21	4	*Thalassiosira oceanica* CCMP1005	99	98
S106A3	27	Uncultured marine eukaryote clone T4CH14	88	99
S106A63	4	Uncultured marine eukaryote clone T4CH14	88	99
S106A35	3	*Pseudo-nitzschia pseudodelicatissima* strain AL-15 (DQ813817)	100	98
S106A24	1	*Pseudo-nitzschia delicatissima* strain CLA1.A2 (EF520341)	100	97
S106A2	6	Uncultured eukaryote clone MBAug25 (GU203314)	91	99
S106A34	1	Uncultured eukaryote clone B3Clone23	91	99
S106A22	11	*Cerataulina pelagica* strain ECT3845 (HQ912533)	100	98
S106B7	2	*Cerataulina pelagica* strain ECT3845 (HQ912533)	99	99
S106A17	1	*Cerataulina pelagica* strain ECT3845 (HQ912533)	99	97
S106B29	1	*Chaetoceros socialis* isolate C4 (FJ002154)	99	94
S106A58	1	*Peridinium quinquecorne* (AB246745)	100	94
S106B32	2	*Peridinium quinquecorne* (AB246745)	100	94
S106A65	2	*Peridinium quinquecorne* (AB246745)	100	94
S106A23	1	*Chaetoceros radicans* (AB430666)	100	93
S106A10	1	*Chaetoceros socialis* isolate C4 (FJ002154)	100	94
S106A25	1	*Hemiaulus sinensis* strain CCH-2 (HQ912488)	100	95
S106A50	2	*Peridinium quinquecorne* (AB246745)	100	93
S106A13	10	*Hemiaulus sinensis* strain CCH-2 (HQ912488)	99	91
S106C40	1	*Hemiaulus sinensis* strain CCH-2 (HQ912488)	99	91
S106A5	5	*Rhizosolenia shrubsolei* isolate C75 (FJ002128)	100	100
S106B24	1	*Pseudopedinella elastica* culture-collection CCMP:716 (HQ710600)	99	89

Table 2. BLASTed eukaryotic taxa sequences obtained from *K. mikimotoi* and *E. huxleyi* blooms for the CTD sample station 2 (40 m) which collected from the Western English Channel in 2006, using the Uni1 *rbcL* gene sequences (~620 bp).

Clone Code	Obtained sequences	Blast species	Query coverage (%)	Max identity (%)
S206B45	9	*Pseudo-nitzschia pseudodelicatissima* strain AL-15 (DQ813817)	99	97
S206A42	2	*Pseudo-nitzschia delicatissima* strain CLA1.A2 (EF520341)	99	97
S2062A17	1	*Psammodictyon constrictum* (AB430697)	99	99
S206B34	1	*Cylindrotheca closterium* isolate C27 (FJ002104)	99	94
S206A28	3	*Gyrosigma acuminatum* strain UTEX FD317 (HQ912462)	99	94
S206A6	3	*Gyrosigma acuminatum* strain UTEX FD317 (HQ912462)	99	93
S206B48	1	*Cerataulina pelagica* strain ECT3845 (HQ912533)	98	99
S206B22	1	*Detonula pumila* strain NB48 (DQ514814)	100	99
S206A56	1	*Thalassiosira angulata* strain BEN02-35 (DQ514788)	98	96
S206A59	2	*Thalassiosira eccentrica* strain BER02-09 (DQ514789)	100	97
S206B24	1	*Minidiscus trioculatus* strain CCMP495 (HQ912427)	99	99
S206A62	1	*Minidiscus trioculatus* strain CCMP495 (HQ912427)	100	99
S206B13	1	*Attheya septentrionalis* strain CCMP2084 (HQ912482)	100	88
S206A39	5	*Triparma* sp. TOY-0807 (AB546640)	100	96
S206A40	10	*Rhizosolenia shrubsolei* isolate C75 (FJ002128)	99	99
S2062A1	1	Uncultured eukaryote clone ECApr27 (GU220521)	89	99
S206A7	8	Uncultured diatom clone ECS_Sp14 (HM347552)	96	92
S206A65	2	Uncultured diatom clone ECS_Sp14 (HM347552)	95	92
S206B15	2	Uncultured diatom clone ECS_Sp14 (HM347552)	95	92
S206A33	2	*Hyalodiscus scoticus* (AB430660)	100	95
S206A5	2	*Hyalodiscus scoticus* (AB430660)	100	94
S206A4	17	*Pseudopedinella elastica* culture-collection CCMP:716 (HQ710600)	99	89
S206B19	1	*Pseudopedinella elastica* culture-collection CCMP:716 (HQ710600)	100	88
S206A49	1	*Epipyxis pulchra* (AF015571)	98	88
S206A60	3	Uncultured bacterium GRIST05 genomic sequence (EU795146)	100	87
S206B18	1	*Vaucheria terrestris* (AJ874702)	98	78
S206A61	2	*Chrysochromulina* sp. TKB8936 (AB043697)	98	93
S206A23	2	*Phaeocystis pouchetii* (AB280613)	100	98
S206A31	2	*Dinophysis tripos* (AB164413)	99	99
S206B20	1	*Dinophysis tripos* (AB164413)	99	98
S206B14	1	*Dinophysis tripos* (AB164413)	98	95
S206A32	2	*Karenia mikimotoi* (HM807342)	100	90

Table 3. BLASTed eukaryotic taxa sequences obtained from *K. mikimotoi* and *E. huxleyi* blooms for the CTD sample station 3 (surface) which collected from the Western English Channel in 2006, using the Uni1 *rbcL* gene sequences (~620 bp).

Clone Code	Obtained sequences	Blast species	Query coverage (%)	Max identity (%)	E-vlaue
S306A34	1	*Hemiaulus sinensis* strain CCH-2 (HQ912488)	99	91	0.0
S306A36	1	*Hemiaulus sinensis* strain CCH-2 (HQ912488)	99	91	
S306A20	1	*Hemiaulus sinensis* strain CCH-2 (HQ912488)	99	91	
S306A17	7	*Hemiaulus sinensis* strain CCH-2 (HQ912488)	99	91	
S306B37	1	*Hemiaulus sinensis* strain CCH-2 (HQ912488)	99	91	
S306A40	2	Uncultured marine microorganism clone FormID_114 (FJ981974)	91	99	
S306B26	1	Uncultured eukaryote clone ECApr55 (GU220549)	91	99	
S306A29	1	*Triparma* sp. TOY-0807 (AB546640)	100	96	
S306A7	3	*Bolidomonas pacifica* strain CCMP1866 (HQ912421)	100	95	
S306A55	1	*Nitzschia palea* (FN557024)	99	91	
S306A61	3	*Attheya septentrionalis* isolate C102 (FJ002121)	100	92	
S306A12	1	Uncultured eukaryote B3Clone24 (GU203288)	91	99	
S306B30	2	*Cymatosira* cf. *belgica* (AB430667)	100	92	
S306B16	1	*Thalassiosira mediterranea* (DQ514806)	97	84	
S306A51	1	*Aureococcus anophagefferens* (HQ710615)	100	93	
S306A54	1	*Aureococcus anophagefferens* strain CCMP1706 (AF117783)	100		
S306A58	8	*Pseudopedinella elastica* CCMP716 *(*HQ710600)	99	89	
S306A15	1	*Apedinella radians* CCMP1767 (HQ710599)	100	88	
S306A38	1	*Pseudochattonella verruculosa* (AB280607)	100	99	
S306A44	1	*Pseudochattonella verruculosa* (AB280607)	100	98	
S306A42	12	*Umbilicosphaera sibogae* (AB043691)	99	89	
S306B15	1	*Umbilicosphaera sibogae* (AB043691)	99	88	
S306A3	1	*Umbilicosphaera sibogae* var. *Foliosa* (AB043629)	100	89	
S306B33	1	Uncultured marine eukaryote clone T2CH10 (AY157374)	70	87	1e-126
S306A4	14	Uncultured marine eukaryote clone T2CH10 (AY157374)	87	93	
S306A16	3	Uncultured marine microorganism clone FormID_88 (FJ981948)	91	98	
S306A63	1	*Chrysochromulina* sp. TKB8936 (AB043697)	99	94	
S306A59	2	Uncultured marine eukaryote clone T3ACH24 (AY157381)	87	99	
S306B25	2	Uncultured marine eukaryote clone T3ACH24 (AY157381	87	99	
S306A30	1	Uncultured eukaryote clone MBAug48 (GU203337)	91	95	
S306A19	3	Uncultured eukaryote clone MBAug48 (GU203337)	91	92	
S306A47	1	Uncultured eukaryote clone MBJul1 (GU203340)	91	97	
S306B5	2	*Emiliania huxleyi* strain CCMP 373 (AY741371)	100	100	

S306B12	2	Uncultured marine eukaryote clone T8CH23 (AY157430)	88	92	
S306B4	1	Uncultured marine eukaryote clone T8CH23 (AY157430)	88	92	
S306A10	1	Uncultured marine eukaryote clone T8CH26 (AY157431)	88	95	
S306A28	2	*Dinophysis tripos* (AB164413)	100	99	
S306A35	1	*Dinophysis tripos* (AB164413)	100	99	
S306A6	4	*Karenia mikimotoi* (HM807342)	100	100	
S306B3	1	*Phycodrys* sp. (AF257430)	88	78	2e-74

Appendix 11

List 1. The obtained unique sequences of Uni1 *rbcL* gene (*rbcL* 640-F & *rbcL*1240-R) from CTD sample station 1 (surface) which collected during *K. mikimotoi* and *E. huxleyi* blooms in south-western English Channel in 2006.

>S106B19

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AGTTAACTTACCATTCGGTATCTTCTTCGAAATGGATTGGGCTAGTTTACGTCGTTGTATGCCAGTAGCTTCTGGTGGTATTCACTG
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>S106A21

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CACGCCGGTACAGTTGTTGGTAAATTAGAAGGTGATCCTTTAATGATTAAAGGTTTCTACGACATCTTACGTTTAACTGAATTAGA
AGTTAACTTACCTTTTGGTGTATTCTTCGAAATGGATTGGGCTAGTTTACGTCGTTGTATGCCAGTAGCTTCTGGTGGTATTCACTG
TGGTCAAATGCACCAATTAATTCACTATTTAGGTGATGATGTTGTATTACAATTT

>S106A3

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>S106A63

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>S106A35

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>S106A24

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>S106A2

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>S106A34

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>S106A22

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>S106B7

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>S106A17

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>S106B29

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>S106A58

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>S106B32

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>S106A65

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>S106A23

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>S106A10

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>S106A25

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>S106A50

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>S106A13

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>S106C40

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>S106A5

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>S106B24

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List 2. The obtained *rbcL* gene unique sequences of Uni1 *rbcL* gene (*rbcL* 640-F & *rbcL*1240-R) from CTD sample station 2 (40 m) which collected during *K. mikimotoi* and *E. huxleyi* blooms in south-western English Channel in 2006.

>S206A28

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>S206A6

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List 3. The obtained *rbcL* gene unique sequences of Uni1 *rbcL* gene (*rbcL* 640-F & *rbcL*1240-R) from CTD sample station 3 (0 m) which collected during *K. mikimotoi* and *E. huxleyi* blooms in south-western English Channel in 2006.

>S306A34

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>S306A17

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>S306A16

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>S306A63

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>S306A59

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>S306A6

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Appendix 12

Table 1. Eukaryotic species composition associated with *K. mikimotoi* 2006 bloom obtained from the archived CPR sample CPR55RV2 (station 20) of the Western English Channel using the universal LSU rDNA sequences (~264 bp)

Clone Code	Obtained sequences	Blast species	Query coverage (%)	Closest known phylogeny taxa/group	Phylogenetic Bootstraps		
					N (%)	DP (%)	B (P)
CPR206C32	5	*Musa acuminata* subsp. *burmannicoides*	100	*Musa acuminata* subsp. *burmannicoides*	100	98	1.00
CPR206E10	2	*Musa acuminata* subsp. *burmannicoides*	100	*Musa acuminata* subsp. *burmannicoides*	100	98	1.00
CPR206A28	6	*Musa acuminata* subsp. *burmannicoides*	100	*Musa acuminata* subsp. *burmannicoides*	100	98	1.00
CPR206B18	3	*Musa acuminata* subsp. *burmannicoides*	100	*Musa acuminata* subsp. *burmannicoides*	100	98	1.00
CPR206B37	1	*Oikopleura* sp.	100	*Oikopleura* sp.	100	100	1.00
CPR2065A7	1	*Oikopleura* sp.	100	*Oikopleura* sp.	100	100	1.00
CPR206D47	1	*Lensia conoidea*	100	*Lensia conoidea*	96	99	1.00
CPR206A17	1	*Mnemiopsis leidyi*	100	*Mnemiopsis leidyi*	91	89	1.00
CPR206A50	4	*Stereum sp.*, *Stereum subtomentosum*, Unidentified basidiomycete, etc.	100	*Stereum*	78	71	0.77
CPR206C43	6	*Malassezia restricta,* Uncultured Basidiomycota clone	100	*Malassezia restricta*	100	100	1.00
CPR206F35	1	*Malassezia restricta,* Uncultured Basidiomycota clone, Uncultured fungus clone, etc.	100	*Malassezia restricta*	90	77	0.82
CPR206D38	18	Uncultured fungus clone, Uncultured Basidiomycota clone, etc.	100	*Malassezia restricta*	90	77	0.82
CPR206B5	7	*Bryobia* sp., *Tylencholaimus mirabilis*, *Paravulvus hartingii* etc.	10	Unknown Animalia▪	45	73	0.98
CPR206D21	2	*Bryobia* sp., *Tylencholaimus mirabilis*, *Paravulvus hartingii* etc.	10	Unknown Animalia▪	45	73	0.98
CPR206B38	3	*Bryobia* sp., *Tylencholaimus mirabilis*, *Paravulvus hartingii* etc.	10	Unknown Animalia▪	45	73	0.98
CPR206A1	74	*Chrysochromulina kappa*, *C. ericina*, *Prymnesium faveolatum* etc /*Emiliana huxlyei*	100/65*	*Emiliana huxlyei* ▲	100	100	0.95
CPR206B17	2	*Chrysochromulina kappa*, *C. ericina*, *Prymnesium faveolatum* etc. /*Emiliana huxlyei*	100/65*	*Emiliana huxlyei* ▲	100	100	0.95
CPR206F31	2	*Chrysochromulina kappa*, *C. ericina*, *Prymnesium faveolatum* etc. /*Emiliana huxlyei*	100/65*	*Emiliana huxlyei* ▲	100	100	0.95
CPR206B26	5	*Chrysochromulina kappa*, *C. ericina*, *Prymnesium faveolatum* etc. /*Emiliana huxlyei*	100/65*	*Emiliana huxlyei* ▲	100	100	0.95
CPR206F1	2	*Chrysochromulina kappa*, *C. ericina*, *Prymnesium faveolatum* etc. /*Emiliana huxlyei*	100/65*	*Emiliana huxlyei* ▲	100	100	0.95
CPR206A5	8	*Chrysochromulina kappa*, *C. ericina*, *Prymnesium faveolatum* etc. /*Emiliana huxlyei*	100/65*	*Emiliana huxlyei* ▲	100	100	0.95
CPR206D44	2	*Chrysochromulina kappa*, *C. ericina*, *Prymnesium faveolatum* etc. /*Emiliana huxlyei*	100/65*	*Emiliana huxlyei* ▲	100	100	0.95
CPR206B8	5	*Chaetoceros lorenzianus*, *Asteroplanus karianus*, *Stephanopyxis turris*, etc.	100	unknown diatom^	67	67	1.00
CPR206D9	7	*Gymnodinium catenatum*, *Chrysolepidomonas dendrolepidota*, *Mallomonas asmundae*, *Chrysolepidomonas dendrolepidota*, etc.	100	*Gymnodinium**	75	69	0.74
CPR2065A13	2	*Karenia mikimotoi*	100	*Karenia mikimotoi*	99	95	0.98

*= only the first 200 bp, ▲ = sequence obtained from the Joint Genome Institute (JGI), ▪ = bootstraps for grouping obtained clones with the Arthropoda and Myxozoa, ^ = bootstraps for grouping this sequenced clone with the Bacillariophyceae and Bolidophyceae.