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Detection And Quantification Of Karenia Brevis By Carbon Fixation Gene Expression Analysis

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Detection And Quantification Of Karenia Brevis By Carbon Fixation Gene Expression Analysis

by

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Michael Gray

ABSTRACT

Karenia brevis (Davis cf. Hansen & Moestrup = *Gymnodinium breve*) is the non-peridinin containing dinoflagellate responsible for many harmful algal blooms (red tides) in the Gulf of Mexico. These recurrent blooms can have significant negative ecological, economic, and human health impacts including fish kills, tainting of shellfish, poisoning of marine mammals, loss of tourism revenue due to beach closures, and respiratory distress and food poisoning in humans.

A method for detection of *Karenia brevis* was developed based upon amplification of the mRNA for the plastid-encoded gene of the carbon fixing enzyme ribulose 1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit (*rbcL*). Using sequence information from a primer set targeting a 554-bp region of the *Karenia rbcL* gene, a small (91 bp amplicon) primer and probe set was created for TaqMan® real time RT-PCR of *K. brevis rbcL.* The primer/probe set is sensitive to as little as 0.1 fg of target transcript and as little as 1 pg of total cellular *K. brevis* RNA extract,

corresponding to less than 1 cell reaction^{-1}. The primer/probe set did not amplify *rbcL* transcript from any of the non-target algae tested.

Bloom samples analyzed by this method have shown the assay to be a reliable method, with effective enumeration and a linear relationship showing good correlation to the cell counts by microscopy (r^2= 0.8344). The assay has been shown to be robust and perform well even in non-ideal conditions, with pre-extraction RNA from unialgal culture stable at room temperature for up to 3 days and up to a month at –80º C in Stratagene's lysis buffer.

The transcription of the *rbcL* gene demonstrated minor variation throughout the diel period, however the variation was not linked to the diel cycle or to carbon fixation, which showed a distinct diel signal. Due to the relatively constant expression of the *rbcL* gene, the real-time RT-PCR assay developed should be able to reliably enumerate *K. brevis* populations in the natural environment, as long as the sample is placed in Stratagene's lysis buffer and processed within one or two days or frozen at –80º C and processed within a month.

Chapter 1: Introduction

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

Throughout the world, harmful algal blooms (HABs) are chronic and often costly problems that have been a nuisance throughout history. The above quote from the Bible is believed to be the earliest written record of a HAB (22); however coastal communities from much earlier times likely encountered occasional bloom events. The Hebrew dietary rules include an injunction against consuming shellfish, perhaps partly due to their potential to carry HAB contamination. Dale and Yentsch (9) and Carson (6) note that early explorers of North America frequently found Native American tribes with taboos against eating shellfish, as well as some tribes that kept a nightly watch for bioluminescence and daily a watch for discolored water (which can be indicators of a dinoflagellate bloom) in order to avoid harvesting and consuming potentially toxic seafood.

In the Gulf of Mexico, waters stained red, presumably by algal blooms, have been described as early as in the ship's logs of the Spanish explorers. A massive local bloom was brought to the notice of Gunther (20) who described fish kills from north of the Dry Tortugas as far north as Boca Grande. It was estimated that as many as 50 million fish

including a Goliath Grouper were killed as well as turtles. A patch of water stained yellow, once investigated revealed a large population of *Gymnodinium* sp. (*Karenia* sp.) along with an acrid gas that escaped from the water when boiled, later to be identified as aerosolized brevetoxin. This species was initially described in detail by Davis (10) as an unarmored dinoflagellate, with a width and height ranging from 25 to 32 μm, a thickness of about 12 μm, containing many plastids and a nucleus placed below the girdle and slightly to the left of the sulcus and named *Gymnodinium brevis*. The name has been changed over the years to more closely reflect evolutionary descent community of the group, with the currently accepted name of *Karenia brevis* in recognition of Karen Steidinger's significant contribution to the knowledge about this organism.

The characterization of *K. brevis* has been further expanded to its unusual pigment characteristics. *Karenia brevis* does not contain peridinin, the accessory pigment found in the plastids of most autotrophic dinoflagellates. The primary chemotaxonomic pigments are fucoxanthin, 19'-hexanoyloxyfucoxanthin and 19'-butanyloxyfucoxanthin with smaller concentrations of the accessory pigments β, ε-carotene, 19'-hexanoyloxyparacentrone 3-acetate and gyroxanthin diester. The pigment complement found in *K. brevis, K. mikimotoi* and *Karlodinium micrum* are very similar and almost identical to the Haptophyte *Pelagomonas calceolata* (4). The unusual plastid pigment complement is due to the incorporation of a plastid from a Haptophyte through tertiary endosymbiosis (30), which it shares with other closely related dinoflagellates, such as *K. mikimotoi* (59). When blooming, *K. brevis* tends to aggregate near the surface of the water (54) and this has been attributed to a combination of positive

phototaxis and negative geotaxis (31), with non-directional dispersal at night. *Karenia brevis* is not constrained to the surface of the water and unless there is a significant pycnocline through which it cannot swim, it has been found throughout the shallow water column. *Karenia brevis* is capable of swimming at a speed of ca. 1 m h^{-1} (55).

Blooms of *K. brevis* in the Gulf of Mexico that typically affect the west Florida shelf initiate in the mid-shelf waters (61). Blooms of this organism are not thought to be due to an increased growth rate, but rather the concentration of the organisms through physical processes, thus the term bloom refers to the aggregation of cells, not to accelerated growth (61). Shoreward movement of water masses associated with the Loop Current can carry early bloom populations of *K. brevis* into nearshore waters where they may remain and become more dense, affecting the residents of the nearshore waters. Blooms may move into local estuaries as the water masses in which they are entrained are moved by wind and currents (38).

Throughout the past century, *K. brevis* blooms have been recurrent problems, especially for the west coast of Florida. A chronology of *K. brevis* HAB events compiled by Mote Marine Lab indicates blooms occurring in 30 of the last 57 years and every year since 1991 (14 of 14 years). One of the events in 1952 was so severe that Clearwater Mayor Herbert Brown sought approval to fire-bomb the dead fish with napalm to prevent their arrival onshore. More than 150 tons of dead fish killed by a bloom washed ashore and had to be removed in 1967. While the most often affected animals are fish, there are occasionally other animals affected, including waterfowl (Cormorants (34), Mergansers

and lesser Scaups (12)), Dolphins (15), Manatees, (7 in 1963, (36), 39 in 1982, (46), 149 in 1996, (5), and 98 in 2003 (FMRI press release)) and Sea Turtles.

The effects of *K. brevis* on humans are dependent on the type of exposure. Most commonly this exposure occurs as a result of inhalation of aerosolized toxin, which can lead to irritation of the eyes, nose and throat, and is most likely at or near the beach/shore with an accompanying onshore breeze (48). Less frequent, but more troublesome are cases of ingestion via contaminated seafood. Filter-feeding seafood such as mussels, clams, and oysters can potentially concentrate brevetoxins to levels that are dangerous to humans. Intoxication from contaminated shellfish can result in neurotoxic effects caused by brevetoxin's action on voltage sensitive sodium channels resulting in repeated uncontrolled depolarization of the cell (63). Symptoms can include chills, headache, diarrhea, weakness, muscle and joint pain, paraesthesia, trouble breathing, double vision, and trouble talking or swallowing.

While it is difficult to determine the economic impact of HABs on a yearly basis for the United States of America, Hoagland et al. (27) estimated that the cost is in the range of $24 to $83 million per year, including treatment of illnesses, monitoring by state agencies, and tourism and fisheries losses. In the review, Hoagland et al. estimated an annual cost of $184,000 for monitoring and management in the state of Florida alone based on data from 1987 to 1992.

As *K. brevis* can have such dramatic impacts on human health and the environment and economies of Florida, nearby regions of the Gulf of Mexico and the southern Atlantic coast of the United States, it is clearly important to be able to detect and

quantify these organisms or their toxins from environmental or shellfish samples. The most conventional method to detect and enumerate *K. brevis* from the marine environment is counting by direct microscopic observation. This technique is, unfortunately, tedious, slow and while not technically difficult, requires significant expertise in order to correctly identify *K. brevis* and not count other, less problematic species that may have similar appearances, such as *K. mikimotoi*. *Karenia brevis* identification is further complicated by natural variation of cell size and shape that has been documented at least in culture (69). Even among scientists familiar with identification of dinoflagellates, identification and enumeration by microscopy can include errors. In experiments with pictures of *Dinophysis* species, only 84 to 95% accuracy was returned by experts routinely involved in certain identification tasks (8). Newer direct optical detection techniques have been developed based on flow cytometry and digital imaging technologies and systems have been designed to automatically detect and enumerate certain cell types continuously in land-deployed situations (52).

As the most important impact of *K. brevis* blooms, from a human health perspective, is the contamination of shellfish, there are many methods aimed at detection of brevetoxins in food, water and air samples. The earliest techniques involve detection of toxicity only, using a mouse bioassay. Essentially, a mouse is injected with a sample and toxicity is determined through observation of symptoms and time to death of the mouse. More advanced techniques have been developed involving chromatographic techniques (e.g. HPLC). Other techniques involve antibody immunoassays (for example, (3)) and have been extended to ELISA assays (41) for detection of brevetoxins in various

sample matrices (seawater, shellfish and mammalian body fluid). Concentrations of brevetoxins in marine derived aerosols have been studied using high volume air samplers and HPLC (48).

In addition to the detection of HAB organisms or their effects through toxin analysis and microscopy, methods have been developed based on photopigments to determine the presence and relative abundance of HAB species. Gyroxanthin-diester, relatively rare and very minor photopigment found in *K. brevis*, haptophytes and other *Karenia* species, was targeted for an HPLC-based pigment detection approach (45). Chlorophyll anomalies in near shore waters as detected by satellites have also been used as an indication of HABs (56).

In addition to methods designed to detect the toxins and pigments associated with HABs, there have been developments aimed at the detection of the HAB organisms based on their genetic sequences. One method that could increase the ability of the researcher to correctly identify HAB species (*Heterosigma akashiwo* and *Fibrocapsa japonica*) uses a combination of microscopy and fluorescence in-situ hybridization (FISH) with oligonucleotide probes targeting ribosomal RNA (rRNA) (64). Tyrrell et al. (64) also developed a microtiter plate-based sandwich hybridization assay as an extension of their FISH study, to eliminate the need for microscopy.

There have been several assays designed around amplification and detection of specific sequences such as that of Guillou et al. (19) who targeted the rRNA large subunit for a semi-nested PCR detection method for *Alexandrium, Dinophysis* and *Karenia*. This approach has several major drawbacks for the enumeration of HAB species, due to the

non-quantitative nature of endpoint PCR analysis. Godhe (17) also used an approach targeting the SSU rRNA of *Gymnodinium mikimotoi* (recently reclassified as *Karenia mikimotoi*) and the *Alexandrium minutum* species-group using PCR, although, as with the assay of Guillou et al. it was not a quantitative assay; rather, it indicated presence or absence only.

While endpoint analysis of PCR is inherently non-quantitative, it is possible to use a PCR-based assay to determine the concentration of target nucleic acid that was originally in the sample. As the sample progresses through the PCR amplification process, the target sequence accumulates and eventually begins a logarithmic amplification. The time of entry into logarithmic amplification corresponds to the initial concentration of the target sequence. If this entry into logarithmic amplification can be detected, the PCR based assay can be quantitative. There are several approaches to this type of assay. The least technologically demanding method to accomplish quantitative PCR is to use a time-step process. Zhang and Lin (71) developed a time-step PCR procedure to detect *Pfiesteria piscicida* by targeting the mitochondrial cytochrome b gene. In time-step PCR, as defined in the study, 5 μl of the PCR reaction was removed at intervals throughout the cycling (at 25 and 30 cycles) and after all 35 cycles had been completed, the reactions were visualized using agarose gel electrophoresis. From this information, approximate cell concentrations in the original sample could be calculated based on the dilution curve generated. This method was able to detect between 0.2 and 15,625 cells per reaction, though saturation of the signal (optical density of the band on the gel) was a significant problem and required dilution of samples or reduction of the

number of amplification cycles. While this method has the potential to be quantitative, it remains a labor-intensive method, requiring careful electrophoresis and analyses at each of the time points in order to accurately and precisely quantify *P. piscicida*.

One technological step further than time-step PCR is "real-time" PCR using fluorescent dye such as SYBR green, which becomes highly fluorescent when bound to DNA. Amplification can then be detected as the reaction is progressing; as more DNA is produced, more SYBR green binds to the DNA, resulting in an increase in fluorescence. This approach was used by many investigators to detect and enumerate a wide variety of organisms and can be a reliably quantitative method.

Another approach to quantitative, real-time PCR is to introduce a third oligonucleotide to the reaction. This oligo serves as a probe and binds to target sequences between the two primers. To this probe are bound two fluorochromes, a reporter and a quencher. When both are bound to the probe, fluorescence is quenched through resonant energy transfer. Through the use of a Taq polymerase with 5'-3' exonuclease activity (which degrades the bound probe as it amplifies the target sequence) the reporter fluor is released into solution and can fluoresce upon excitation (23). This method, dubbed a Taq nuclease assay (TNA, marketed by Applied Biosystems as TaqMan® PCR) has the potential to be quantitative by detection of the time of a sample's entry into log phase PCR amplification (which is directly proportional to the initial concentration of the target). Figure 1 shows an example of the amplification curves that are generated in the course of a real-time RT-PCR assay with the time of a samples entry into log phase amplification through a threshold level indicative of the initial

concentration of the target RNA. This method also has the potential to be more specific than standard PCR, due to the introduction of a third oligonucleotide that must also match in sequence the target. See Figure 2 for a graphical representation of the TNA process.

Similar to the assay of Godhe et al., Tengs et al. (60) developed a PCR based assay to detect plastid small subunit rRNA from *Gymnodinium galatheanum*. The critical difference in this assay is that it has the potential to be quantitative, as Tengs et al. used a Taq Nuclease assay.

The study presented here represents a molecular detection and quantitation method for the Florida HAB organism *K. brevis*. The study is presented in two chapters, the first is a description of the development of the real time RT-PCR assay, with sensitivity and specificity testing. The second chapter describes expanded testing of the assay as applied to natural bloom samples, field applicability and robustness as well as testing of the diel variability of *rbcL* gene expression in *K. brevis*.

Figure 1: Theoretical amplification curves that would be generated through real-time RT-PCR. Shown are curves representing ten-fold dilutions of target between each of the samples (solid curves) and the threshold level that indicates a sample's entry into log phase amplification (dashed line).

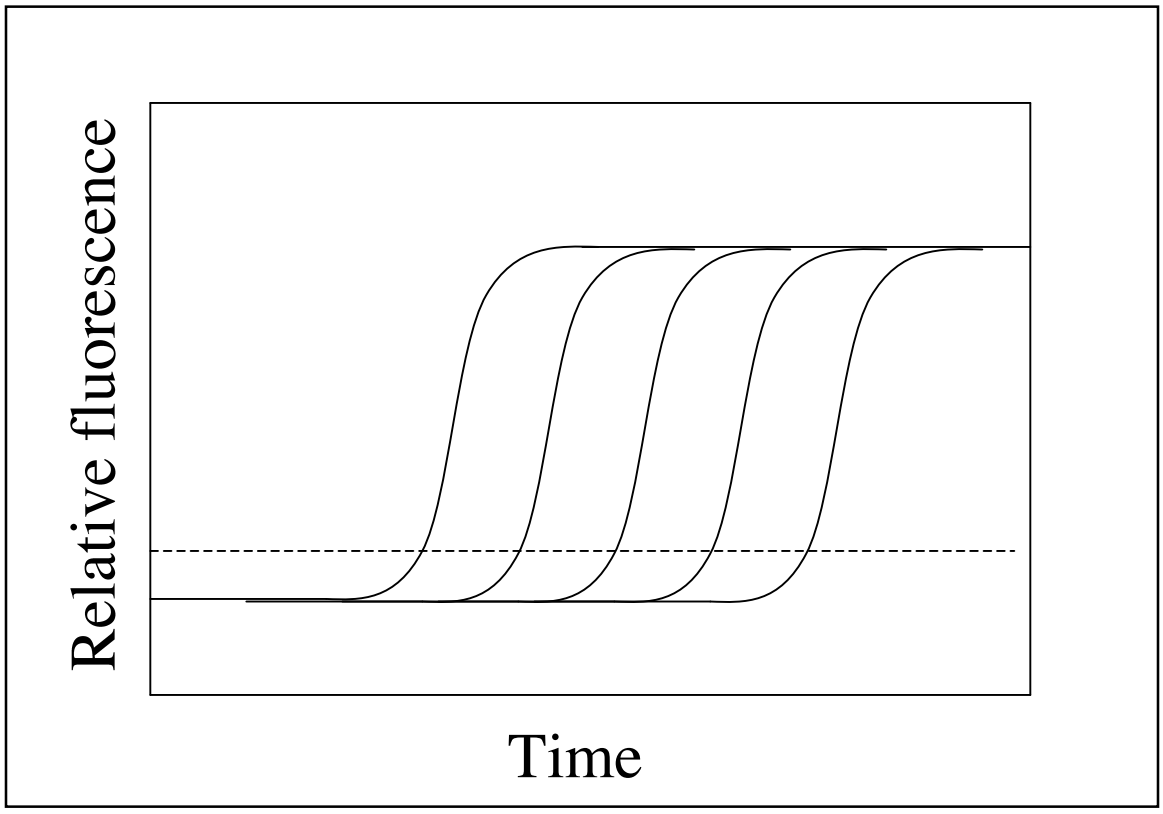
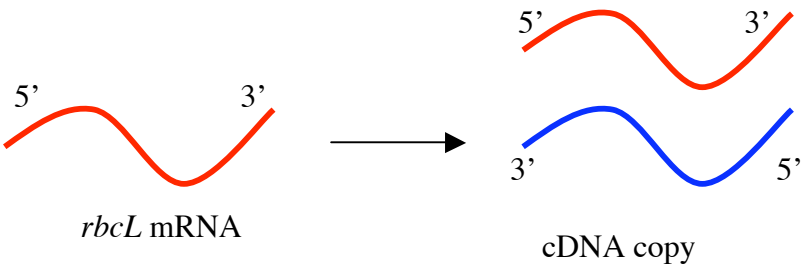

Relative fluorescence
Time

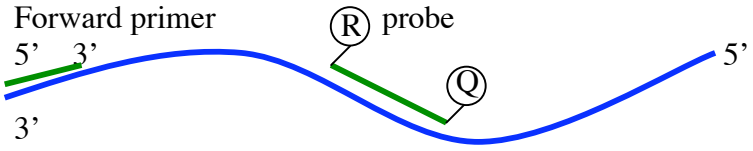
Figure 2: The Taq nuclease assay (TNA) process.

Step 1, reverse transcription

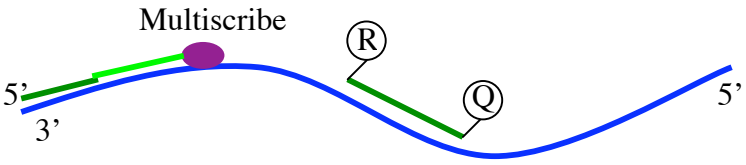


Random hexamers are used as primers for reverse transcriptase or Applied Biosystems’ Multiscribe® which makes a cDNA copy of all RNA in the reaction. The RT step takes place at 45° C for 30 minutes, followed by initial denaturation at 95° C for 10 minutes.

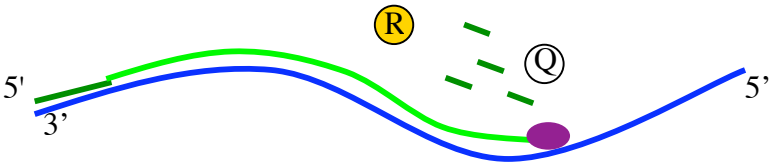
Step 2, amplification



The forward primer and probe (with its reporter (R) and quencher (Q)) bind to the target sequence. This occurs at 52°C for one minute.



Amplification begins at the forward primer site where Taq DNA polymerase or Multiscribe® binds and begins synthesis of new DNA. This occurs at 72° C for one minute.



Amplification continues and as the Multiscribe® encounters the probe, the probe is degraded by the enzyme’s 5’, 3’ exonuclease activity. This releases the reporter from the quencher, allowing it to fluoresce and report amplification. After the extension step, the cycle begins again with a 95° C denaturation for one minute.

The detection method chosen for this assay is real time RT-PCR using Applied Biosystems' TaqMan® technology. TaqMan® technology has been demonstrated to be a very reliable and quantitative detection system in a wide range of applications: food microbiology and safety (2, 26), environmental monitoring (21, 43), marine ecosystem research (65), biodefence and clinical pathogen detection (7, 11, 13, 28, 51), cancer research (29, 70), genetic research (50) and others.

In order to have an assay that is sensitive only to viable cells, we targeted mRNA as RNA is rapidly degraded in the environment. The rapid degradation of the RNA in the environment ensures that, if mRNA is detected, it has likely recently been transcribed and therefore the population detected is viable. DNA is a much more stable molecule and it is possible that detection of a DNA target does not reflect the viability of the population detected.

The ribulose 1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit gene (*rbcL*) was chosen as the target of this assay for several reasons. RuBisCO catalyzes the rate-limiting step of photosynthetic carbon fixation and it is notoriously inefficient and slow, therefore a large quantity of enzyme must be made, so the mRNA should be abundant. In addition to catalyzing the addition of CO_2 to ribulose 1, 5-bisphosphate, it can catalyze the addition of O_2 in a competing oxygenase reaction. As RuBisCO is an inefficient enzyme, the cell must maintain a sufficient pool of enzyme to survive, consequently, in land plants, as much as 50% of the nitrogen in leaves is devoted to RuBisCO (see review (53)). Therefore the cell must transcribe many copies of *rbcL* in

order to maintain the RuBisCO pool. This high transcription rate will help to greatly increase the sensitivity of the assay as the *rbcL* mRNA will be in significantly higher concentrations than the DNA of the gene. The *rbcL* gene is encoded in the DNA of the plastid, of which *K. brevis* usually has many, further increasing the transcript levels in the cell and thus our limit of detection. Again due to the plastid location of the gene it is likely that *K. brevis* will have an *rbcL* gene sequence that is divergent from that of other dinoflagellates due to the unusual nature of the Haptophyte-derived plastid that *K. brevis* shares with very few other dinoflagellates. As *rbcL* is a functional gene, its evolution may be tightly constrained, however there may be portions of the gene that have more freedom to change in the second or third codon positions without yielding a non-functional enzyme. This high transcription rate, the potential uniqueness, the likelihood that it will not vary within the species due to the relatively recent acquisition of the Haptophyte-derived plastid and the possibility of variable regions within this gene combine to make *K. brevis' rbcL* a promising target for a molecular detection and quantitation assay.

Chapter 2: Development of assay, sensitivity and specificity testing

Introduction

Karenia brevis (Davis cf. Hansen & Moestrup = *Gymnodinium breve*) is an unarmored, non-peridinin containing dinoflagellate that grows to ca. 20-40 μm in diameter. The organism is positively phototactic, negatively geotactic (24, 32), swims at a speed of ca. 1 mh^{-1} (55) and is thought to be an obligate photoautotroph (1). *Karenia brevis* is the causative agent of the recurring red tide blooms (21 of 22 years from 1975-1997) observed in the Gulf of Mexico and Southeastern Atlantic Coast of the United States (61), which have been reported since the Spanish conquests (20). Lipophilic brevetoxins (37) produced by *K. brevis* can result in massive fish kills and have been implicated in the mortality of 700 bottlenose dolphins off the east coast of the U.S.A. in 1987 (25) and the mysterious deaths of 149 Florida manatees in 1995 and 1996 (63). In human exposure cases, brevetoxin can cause respiratory distress by inhalation and food poisoning by consumption of tainted shellfish.

Current methods for the detection of *K. brevis* depend on microscopy or pigment analysis that are time consuming, and require a considerable amount of expertise and skill (38). Consequently, rapid molecular methods to detect *K. brevis* in the environment are needed. To this end, we have been investigating the ribulose-1, 5-bisphosphate

carboxylase/oxygenase large subunit gene (*rbcL*) as a potential molecular marker for this organism.

The molecular phylogeny of the *rbcL* gene in dinoflagellates is complex. Almost all eukaryotic oxygenic phototrophs contain a functional RuBisCO enzyme composed of both large and small subunits (L_8S_8), known as Form I (33) (57)). A second, evolutionarily distant lineage of the enzyme (Form II) composed of large subunits only (L_2) is found in certain form I-containing proteobacteria (i.e. *Rhodobacter* and *Rhodopseudomonas*; (16) (58) and interestingly in some dinoflagellates (*Amphidinium carterae, Symbiodinium* sp.*;* (40)). *Karenia brevis* and the other fucoxanthin containing dinoflagellates have a Form ID *rbcL* enzyme and genetic evidence suggests that they contain plastids of Haptophyte origin acquired through tertiary endosymbiosis (30) (59). Sequence information for the *rbcL* genes of many of the dinoflagellates is unavailable, however, due to the apparent ability of dinoflagellates to acquire plastids through retention of the plastids of their prey or some other endosymbiosis mechanism, it is unlikely that further sequencing of *rbcL* genes will elucidate the phylogenetic relationships of Dinophyceae. Evolutionary constraints on the functionality of RuBisCO (giving rise to little sequence variability) also will most likely yield little resolution of phylogenetic relationships. It would be interesting to sequence *rbcL* from many other groups of dinoflagellates in order to possibly determine the ancestral trophic strategies of the dinoflagellates. The presence of a form II RuBisCO in many dinoflagellates (*Amphidinium carterae*, and *Symbiodinium* sp., (68), and *Gonyaulax polyedra*, (40)) is interesting, especially considering that all of the species above contain chloroplasts or

kleptochloroplasts. These chloroplasts may still contain a form I RuBisCO, though it is common for plastids, even if relatively recently acquired, to drastically reduce their genome to the bare minimum for functionality in their new intracellular environment (39). The function of a nuclear encoded, possibly redundant RuBisCO would be interesting to further explore, but is beyond the scope of this study.

As a functional gene, *rbcL* is highly expressed in viable cells. Therefore we targeted the mRNA, which can be orders of magnitude higher in concentration than the DNA of the gene, in order to increase the sensitivity of our assay. As RNA is rapidly degraded in the environment, an RNA target will give an indication of a viable population, compared to DNA based methods, which would detect dead cells as well.

Methods

Design of Real-Time RT-PCR Primer and Probe Set

Culture Conditions

Cultures of *K. brevis* were provided courtesy of Karen Steidinger of the Florida Fish and Wildlife Conservation Commission's Florida Marine Research Institute (FMRI). Strains were isolated by her lab from the following locations around the Florida coast: Apalachicola, Charlotte Harbor, Mexico Beach, Jacksonville, and Piney Island. Strains used in this analysis were named for their isolation location and the plate well into which they were isolated. Several non-target algal strains of diverse lineage were obtained from either the Provasoli-Guillard Center for Culture of Marine Phytoplankton (West

Boothbay Harbor, ME) or from the Steidinger lab (see Table 1). All strains were under a 12/12 light regime at 26 μmol s^{-1} m^{-2} and were incubated at 20° C or 14° C in F/2 media (18), modified for each strain's needs, according to CCMP's directions (media recipes and the requirements of each of the tested strains can be found in Appendix 1).

DNA Extraction, Amplification, Cloning and Sequencing

Karenia brevis cells were harvested by centrifugation (10 minutes at 5000 x G) and the DNA was extracted using a modified phenol/chloroform method (14). A previously designed PCR primer set that amplifies a 554 base pair region of *Karenia*'s *rbcL* gene was used to amplify the gene fragment from *K. brevis*. (Forward primer: GATGATGARAAYATTAACTC; reverse primer: ATTTGTCCCGCATTGATTCCT. International Union for Pure and Applied Chemistry degeneracy symbols used.) Polymerase chain reaction (PCR) amplification was conducted with final concentrations of 1 μM primers, 3 mM $MgCl_2$, 0.4 mM each dNTP, 2.5 units of Taq polymerase (Promega Corp., Madison, WI). Cycling conditions were 40 repetitions of 95°C for 1 minute; 50°C for 1 minute; 72°C for 1.5 minutes with a final extension step at 72°C for 15 minutes. Amplification was confirmed by agarose gel electrophoresis. PCR amplicons were purified using a QiaQuick PCR purification kit (Qiagen, Valencia, CA), ligated into the pCR® II vector and TOP10 cells were transformed according to the manufacturer's instructions (Invitrogen Corp., Carlsbad CA). Transformations were plated onto 2x YT plates containing 50 μg/ml kanamycin and ampicillin (see Appendix 1). White colonies were screened for insert size by PCR amplification. Positive clones

were grown in 2x YT broth with kanamycin and ampicillin. One milliliter of the culture was frozen at -80º C with a final concentration of 25% glycerol and from the remaining 9 ml, plasmid DNA was extracted using a Wizard Plus SV miniprep spin kit (Promega Corp., Madison, WI). These plasmid extracts were sent to the University of Florida's DNA Core Sequencing laboratory to be sequenced.

Sequence Analysis

Sequences were received from the UF Core laboratory via email and a BLAST search was conducted using GenBank to reveal the orientation of the sequences. The BLAST search also indicated related sequences that would be used in phylogenetic analysis, including the closely related *K. mikimotoi*. Once the orientation was determined, the sequences were imported into the KODON software package version 1.0 (Applied Maths, Inc. Austin, Texas). The sequences were aligned in KODON, which uses a Clustal W-based alignment algorithm (62). The complete multiple sequence alignment from selected algal species covering the 554 base pair (184 amino acid) portion of the *rbcL* gene appears in Appendix 2. Multiple sequence alignments were exported and analyzed using the MEGA 2.0 program (35) for both the amino acid and nucleotide sequences.

The sequence data from the *K. brevis rbcL* clones showed a short (91 bp) region that was markedly different from *K. mikimotoi rbcL* sequence. This portion of the *rbcL* gene of *K. brevis* was selected as the target for a primer and probe set for TaqMan® Taq nuclease assay (Real Time RT-PCR). A primer set and an internal fluorogenic probe

were designed to amplify the 91 bp region (Forward primer: TGAAACGTTATTGGGTCTGT; Reverse primer: AGGTACACACTTTCGTAAACTA; Internal probe: FAM – TTAACCTTAGTCTCGGGTA – TAMRA).

Testing of the Real Time RT-PCR Primer and Probe Set

Optimization of Reaction Conditions

The concentration of the primers was the first parameter to be optimized. RT-PCR reactions were set up with the following primer concentrations: 2000 nM, 1000 nM, 500 nM, and 250 nM. In subsequent experiments, the times of the cycle steps (denaturing, annealing and extension) as well at the temperature of the annealing step were varied to determine the optimal cycle conditions. The concentrations of the other reagents in the reaction solution were not optimized, as the one step Real Time RT-PCR master mix kit (Applied Biosystems, Valencia, CA) is pre-mixed for ease of use and increased reproducibility.

Sensitivity and specificity testing

One of the sequenced clones carrying the 554-bp insert from *K. brevis* APC6 (clone 15) was selected for use in sensitivity testing. Non-target *rbcL* clones from a cruise to the Mississippi River plume in the Gulf of Mexico, known to be amplifiable with other *rbcL* primers surrounding the same region of the gene were used to initially test specificity (See Table 3) (Wawrik, personal communication). Based on the direction of the insert, the vector was linearized by digesting the plasmid with either HinDIII or

EcoRV and sense transcript was made by in vitro transcription using the T7 or SP6 promoter site. The transcripts were purified using the Qiagen RNeasy RNA extraction kit with the DNAse digestion step according to the manufacturer's instructions (Qiagen, Valencia, CA). These transcripts were quantified using the Ribogreen RNA quantification kit according to the manufacturer's instructions (Molecular Probes Inc., Eugene, OR), mixed 1:1 with RNA storage buffer (8 M guanidinium isothiocyanate, 80 mM Tris-HCl pH 8.5, 24 mM $MgCl_2$, 140 mM KCl), aliquoted and frozen at –80°C. The APC6-15 transcript was used to generate Real Time RT-PCR standard curves, while the others were used to test the specificity of the primer/probe set.

RNA was extracted using the Qiagen RNeasy spin kit with the following modifications. One ml of culture was filtered onto a 0.45 μm-pore-size HV polyvinylidene difluoride filter (Millipore Durapore®). The filters were placed into 2 ml screw-cap microcentrifuge tubes containing 750 μl of RLT lysis buffer with 2-mercaptoethanol (10 μl ml^{-1}). The filters were incubated for 10 minutes at room temperature and 500 μl were removed into a 1.5 ml microcentrifuge tube and RNA extraction continued according to the manufacturer's instructions (Qiagen, Valencia, CA). The extracted RNA was quantified using the Ribogreen RNA quantification kit according to the manufacturer's instructions. All non-target algal strains were tested for amplification with the real-time primer/probe set with 10 pg of non-target RNA per reaction.

Cell counts for all non-target algal strains as well as *K. brevis* strains were carried out by filtering an appropriate amount of diluted culture onto 0.22 μm black

polycarbonate Poretics filters (Osmonics Inc., Minnetonka, MN). Cells were counted using epifluorescence microscopy on an Olympus BX-60 microscope using appropriate objectives and blue excitation (filter set U-MNIB).

Results

Phylogenetic Analysis

A 554 bp *rbcL* amplification product was used for the phylogenetic analysis, which indicated tight clustering of *Karenia* within the Haptophytes at both the protein level and at the nucleotide level (see Figure 3). All *Karenia* were deeply rooted within the form ID *rbcL* group. The *K. brevis* strains were quite different from *K. mikimotoi* ranging from 85% to 86% amino acid similarity and only 76% nucleotide similarity. *K. brevis* strains were very different from other Haptophytes (82% to 85% amino acid similarity), Rhodophytes (79% to 80% amino acid similarity), diatoms (72% to 74% amino acid similarity), *Prochlorococcus* (47% to 49% amino acid similarity) and higher plants (51% to 54% amino acid similarity to Spinach). Table 1 shows the percent identity of *rbcL* sequences from several selected organisms based on at both the amino acid (top) and DNA (bottom) level. Table 2 lists accession numbers for all sequences generated during the course of this study as well as those for the clones used and all other taxa used in the phylogenetic analysis.

Table 1: Percent identity of selected *rbcL* sequences at both the amino acid (top) and nucleotide level (bottom).

Amino Acid / Nucleotide	Appalachicola C6	Piney Island B4	Mexico Beach C5	Charlotte Harbor A2	Charlotte Harbor C2	Karenia brevis	Mexico Beach B3	Karenia mikimoitoi	Pleurochrysis	Isochrysis	Prymnesium	Guillardia	Porphyridium	Mazzaella	Gelidium	Rhizosolenia	Heterosigma	Spinacia	Prochlorococcus
Appalachicola C6		98%	98%	97%	98%	97%	96%	85%	83%	82%	83%	76%	80%	79%	79%	72%	76%	51%	47%
Piney Island B4	99%		100%	99%	100%	99%	98%	86%	85%	84%	84%	78%	82%	80%	80%	73%	77%	53%	48%
Mexico Beach C5	99%	99%		99%	100%	99%	98%	86%	85%	84%	84%	78%	82%	80%	80%	73%	77%	53%	48%
Charlotte Harbor A2	98%	99%	99%		99%	98%	97%	86%	84%	83%	84%	77%	81%	80%	80%	73%	77%	52%	48%
Charlotte Harbor C2	98%	99%	99%	99%		99%	98%	86%	85%	84%	84%	78%	82%	80%	80%	73%	77%	53%	48%
Karenia brevis	98%	99%	99%	99%	99%		97%	86%	85%	84%	85%	78%	82%	81%	81%	74%	78%	54%	49%
Mexico Beach B3	98%	99%	99%	98%	98%	98%		85%	83%	82%	83%	76%	80%	79%	79%	72%	76%	52%	48%
Karenia mikimoitoi	76%	76%	76%	76%	76%	75%	76%		83%	82%	84%	76%	78%	77%	77%	72%	75%	56%	49%
Pleurochrysis	73%	73%	73%	73%	73%	73%	73%	73%		92%	92%	85%	86%	84%	84%	78%	83%	52%	50%
Isochrysis	72%	73%	72%	72%	72%	72%	72%	73%	87%		88%	82%	83%	82%	83%	77%	80%	53%	50%
Prymnesium	72%	73%	72%	72%	72%	72%	72%	73%	84%	85%		83%	84%	83%	83%	76%	79%	52%	50%
Guillardia	68%	69%	69%	69%	69%	69%	68%	71%	76%	77%	79%		86%	83%	84%	74%	80%	50%	50%
Porphyridium	73%	73%	74%	73%	73%	74%	73%	73%	78%	79%	78%	82%		85%	85%	78%	85%	52%	51%
Mazzaella	74%	75%	74%	74%	74%	75%	74%	72%	74%	75%	77%	78%	80%		88%	78%	82%	51%	50%
Gelidium	69%	70%	70%	70%	70%	70%	69%	71%	74%	73%	75%	77%	77%	81%		77%	82%	52%	50%
Rhizosolenia	69%	70%	70%	70%	70%	70%	69%	68%	73%	74%	74%	75%	77%	75%	73%		88%	52%	49%
Heterosigma	70%	71%	70%	70%	70%	71%	70%	70%	78%	77%	77%	79%	79%	77%	77%	85%		50%	50%
Spinacia	57%	58%	58%	58%	58%	58%	58%	56%	58%	59%	59%	58%	60%	58%	59%	60%	59%		69%
Prochlorococcus	54%	55%	55%	54%	54%	55%	55%	55%	54%	55%	58%	58%	59%	61%	59%	58%	58%	63%	

Figure 3: Phylogenetic tree based on *rbcL* amino acid sequences. The tree was obtained from Neighbor Joining analysis using a Poisson distribution of a 148 residue deduced amino acid sequence covering approximately 1/3 of the RuBisCO large subunit. The tree shows RuBisCO form ID diversity and the tight grouping of *K. brevis* and *K. mikimotoi* (both the sequence generated in this study and one from GenBank) within the Haptophyte clade. Several sequences from form IA and IB carrying organisms were used as outgroups. Bootstrap values for 1000 replicates are shown when the value is 40 or higher.

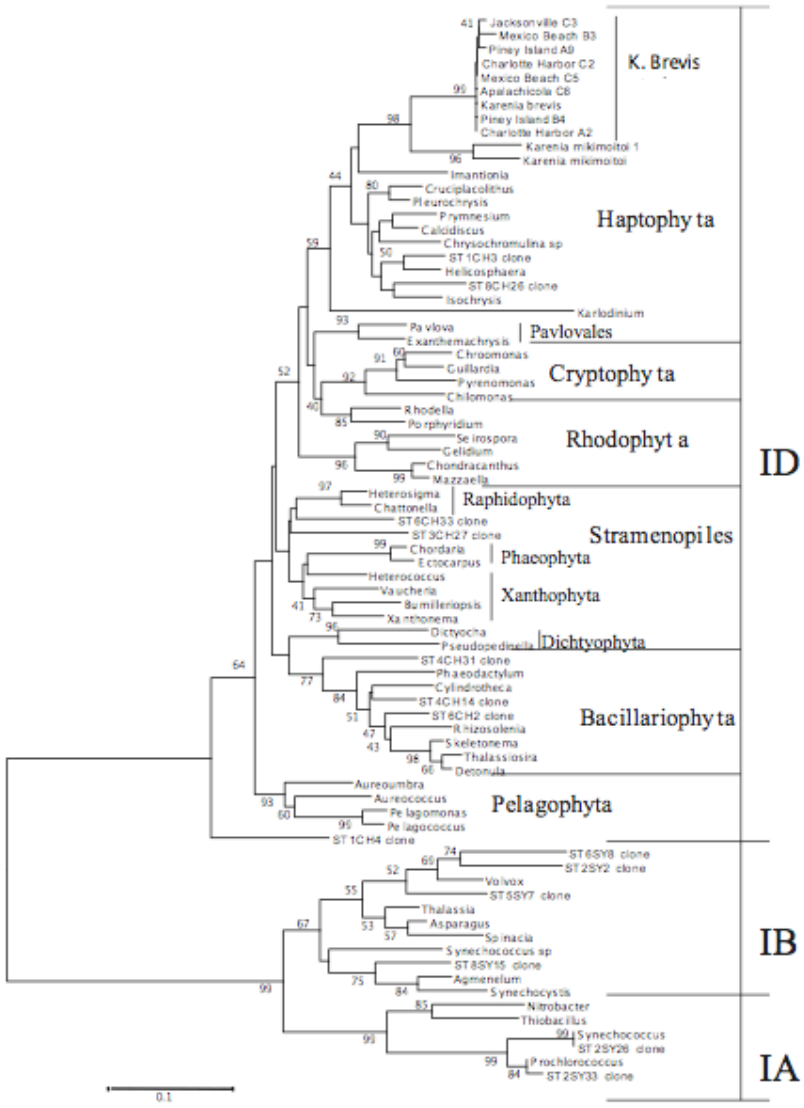

Jacksonville C3
Mexico Beach B3
Piney Island A9
Charlotte Harbor C2
Mexico Beach C5
Apalachicola C8
Karenia brevis
Piney Island B4
Charlotte Harbor A2
K. Brevis
Karenia mikimotoi 1
Karenia mikimotoi
Imantonia
Cruciplacolithus
Pleurochrysis
Prymnesium
Calcidiscus
Chrysochromulina sp
ST1CH3 clone
Helicosphaera
ST8CH26 clone
Isochrysis
Haptophyta
Karlodinium
Pavlova
Exanthemachrysis
Pavlovales
Chroomonas
Guillardia
Pyrenomonas
Cryptophyta
Chilomonas
Rhodella
Porphyridium
Seirospora
Gelidium
Chondracanthus
Mazzaella
Rhodophyta
ID
Heterosigma
Chattonella
Raphidophyta
ST6CH33 clone
ST3CH27 clone
Stramenopiles
Chordaria
Ectocarpus
Phaeophyta
Heterococcus
Vaucheria
Bumilleriopsis
Xanthonema
Xanthophyta
Dictyocha
Pseudopedinella
Dichtyophyta
ST4CH31 clone
Phaeodactylum
Cylindrotheca
ST4CH14 clone
ST6CH2 clone
Rhizosolenia
Skeletonema
Thalassiosira
Detonula
Bacillariophyta
Aureoumbra
Aureococcus
Pelagomonas
Pelagococcus
Pelagophyta
ST1CH4 clone
ST6SY8 clone
ST2SY2 clone
Volvox
ST5SY7 clone
Thalassia
Asparagus
Spinacia
Synechococcus sp
ST8SY15 clone
Agmenellum
Synechocystis
IB
Nitrobacter
Thiobacillus
Synechococcus
ST2SY26 clone
Prochlorococcus
ST2SY33 clone
IA
0.1

Table 2: Accession numbers for sequences used in phylogenetic analyses.

Species	Strain	GenBank Accession Number
Sequences generated in this study		
K. brevis	APC6	AY154877
K. brevis	CHA2	AY154878
K. brevis	MBC3	AY154879
K. brevis	PIB4	AY154880
K. brevis	CHC2	AY154881
K. brevis	PIA9	AY154882
K. brevis	MBB3	AY154883
K. mikimotoi	N/A	AY251791
Other Sequences used		
Thalassia		AB004897
Detonula		AB018006
Thalassiosira		AB018007
Asparagus		AB029849
Karenia mikimotoi		AB034635
Pleurochrysis		AB043688
Helicosphaera		AB043692
Isochrysis		AB043693
Imantionia		AB043696
Chrysochromulina sp		AB043697
Prymnesium		AB043698
Exanthemachrysis		AB043701
Volvox		AB076099
Rhizosolenia		AF015568
Skeletonema		AF015569
Pelagococcus		AF015580
Chattonella		AF015581
Heterococcus		AF084610
Xanthonema		AF084612
Aureococcus		AF117906
Aureoumbra		AF118136
Mazzaella		AF146214
Phaeodactylum		AF195952
Vaucheria		AF207527
Agmenellum		D13971
Dictyocha		AY043280
Rhodella		AY119776
Chilomonas		AY119780
Chroomonas		AY119781
Pyrenomonas		AY119782
Pavlova		AY119785
Karenia brevis		AY119786
Karlodinium		AY119787
Cylindrotheca		M59080
Prochlorococcus		D21833
Nitrobacter		L22885
Synechocystis		NC 000911
Spinacia		NC 002202
Synechococcus sp		D13539
Synechococcus		U46156
Thiobacillus		X70355
Cruciplacolithus		AB043689
Calcidiscus		AB043690
Ectocarpus		X52503
Heterosigma		X61918

Continued on next page.

Table 2: Accession numbers for sequences used in phylogenetic analyses, continued.

Species	Strain	GenBank Accession Number
Trichodesmium		AF136182
Amphidinium		AF312209
ST6SY8 clone		AY157473
ST8CH26 clone		AY157431
ST6CH2 clone		AY157408
ST4CH31 clone		AY157392
ST4CH14 clone		AY157388
ST3CH27 clone		AY157382
ST1CH3 clone		AY157369
ST5SY7 clone		AY157469
ST2SY26 clone		AY157446
ST2SY33 clone		AY157449
ST2SY2 clone		AY157444
ST8SY15 clone		AY157479
ST6CH33 clone		AY157412
ST1CH4 clone		AY157371

Real-Time RT-PCR

The TaqMan® probe-based RT-PCR assay (91 bp amplicon) only yielded positive results with *K. brevis* strains (Table 3). All other dinoflagellates (including *K. mikimotoi*) and algal strains resulted in no amplification. All strains tested were present in sufficient concentration to allow for amplification based on the lowest detectable concentration of *K. brevis*.

Standard curves using in vitro transcript from APC6 clone 15 showed linear sensitivity over a range of concentrations spanning 7 orders of magnitude, ranging from 10^{-1} to 10^{5} fg, as shown in Figure 4. Standard curves using whole-cell extract from *K. brevis* culture were sensitive to as little as 1 pg of total RNA (less than 1 cell reaction^{-1}, based on cell counts and dilution) and also showed a linear relationship from 1 cell to 2000 cells reaction^{-1} (highest concentration tested). Figure 5 shows an example of the cell standard curves, demonstrating linearity. In addition to showing linearity with both the transcript and whole cellular RNA within each reaction experiment, the assay was relatively consistent between assays. Figure 6 shows a composite average of the transcript standard curves, demonstrating good agreement between amplification events. The composite average of the cell standards curves appears in Figure 7, again demonstrating good agreement between experiments.

Table 3: Positive and negative controls for amplification by real-time RT-PCR

Species	Strain/Clone	Detection by Real-Time PCR
Positive Controls		
K. brevis	Apalachicola B5	+
K. brevis	Apalachicola C6	+
K. brevis	Charlotte Harbor A2	+
K. brevis	Charlotte arbor C2	+
K. brevis	Mexico Beach B3	+
K. brevis	Mexico Beach C5	+
K. brevis	Jacksonville C3	+
K. brevis	Piney Island A3	+
K. brevis	Piney Island B4	+
K. brevis	Wilson	+
Negative Controls		
Dinoflagellates		
K. mikimotoi	CCMP430	-
Amphidinium carterae	CCMP1314	-
Akashiwo sanguinea	CCMP1321	-
Alexandrium tamarense	CCMP1493	-
Glenodinium foliacrum	N/A	-
Gymnodinium catenatum	CCMP1937	-
Gyrodinium sp.	N/A	-
Kryptoperidinium foliaceum	N/A	-
Lingulodinium polyedra	CCMP1738	-
Prorocentrum micans	N/A	-
Scrippsiella trochoidea	N/A	-
Scrippsiella precaria	N/A	-
Diatoms		
Phaeodactylum tricornutum	CCMP1327	-
Cylindrotheca sp.	ST6CH2 clone	-
Skeletonema sp.	ST4CH31 clone	-
Skeletonema sp.	ST4CH14 clone	-
Raphidophyte		
Heterosigma akashiwo	N/A	-
Praisinophytes		
Tetraselmis sp.	850001	-
Tetraselmis sp.	CCMP961	-
Unidentified sp.	CCMP1536	-
Prymnesiophytes		
Isochrysis sp.	3C	-
Pavlova lutheri	CCMP1325	-
Prymnesium parvum	N/A	-
Unidentified sp.	ST8CH26 clone	-
Unidentified sp.	ST1CH3 clone	
Chlorophyte		
Chlamydomonas euryale	CCMP219	-
Unidentified sp.	ST5SY7 clone	-
Chlamydomonas sp.	ST2SY2 clone	-
Pycnococcus sp.	ST6SY8 clone	-
Cyanophytes		
Synechococcus sp.	CCMP836	-
Synechococcus sp.	WH7803	-
Synechococcus sp.	ST2SY26 clone	-
Prochlorococcus sp.	ST2SY33 clone	-

Continued on next page.

Table 3: Positive and negative controls for amplification by real-time RT-PCR, continued

Species	Strain/Clone	Detection by Real-Time PCR
Trichodesmium sp.	ST8SY15 clone	-
Trebouxiophyte		
Chlorella autotrophica	CCMP243	-
Coscinodicsophyte		
Thalassiosira pseudonana	CCMP 1335	-
Eustigmatophyte		
Nannochloropsis sp.	ST3CH27 clone	-
Nannochloropsis sp.	ST1CH4 clone	-
Xanthophyte		
Heterococcus sp.	ST6CH33 clone	-

Figure 4: An example standard curve generated using in vitro transcript. The curve is plotted with the trendline indicating the precision of the real-time RT-PCR assay.

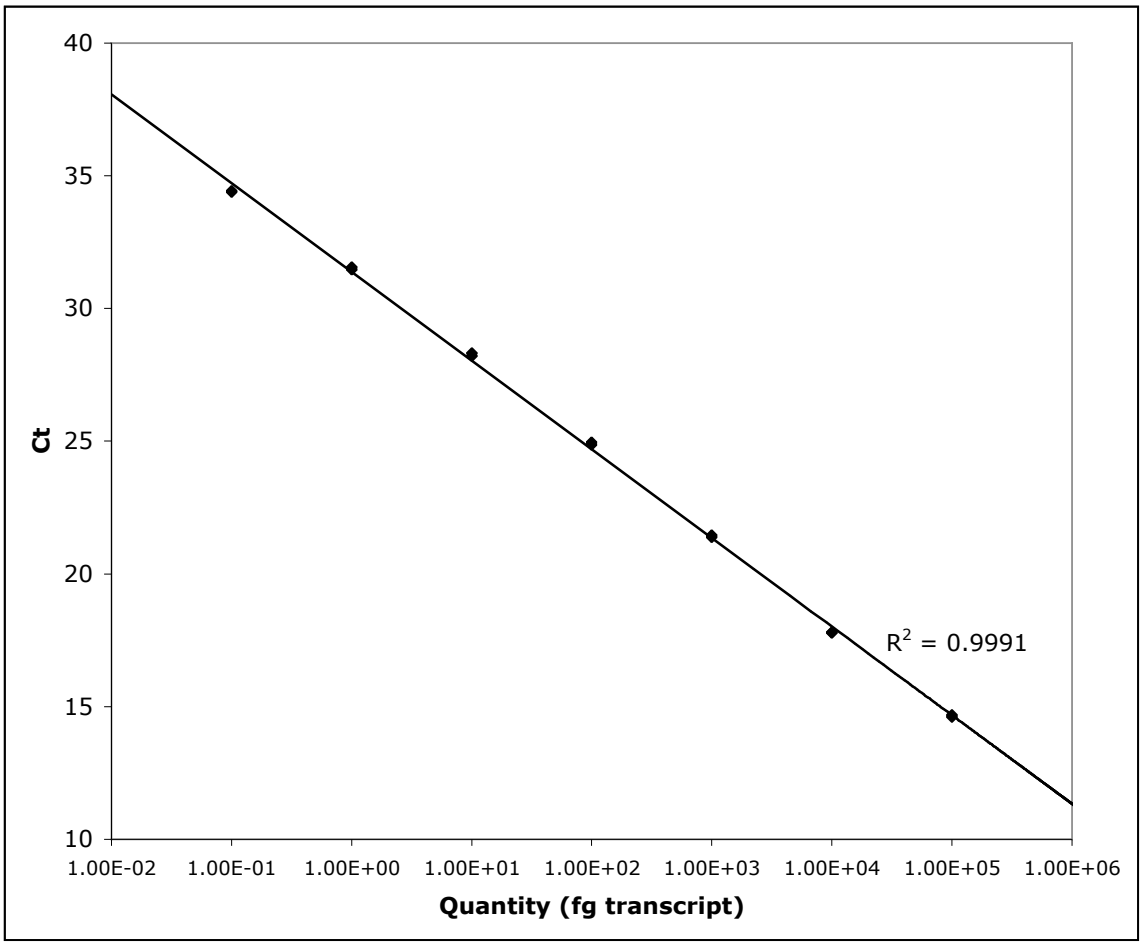

40
35
30
25
20
15
10
Ct
R^2 = 0.9991
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
1.00E+04
1.00E+05
1.00E+06
Quantity (fg transcript)

Figure 5: An example standard curve generated using whole cell RNA extract. The curve is plotted with a trendline indicating the precision of the real-time RT-PCR assay.

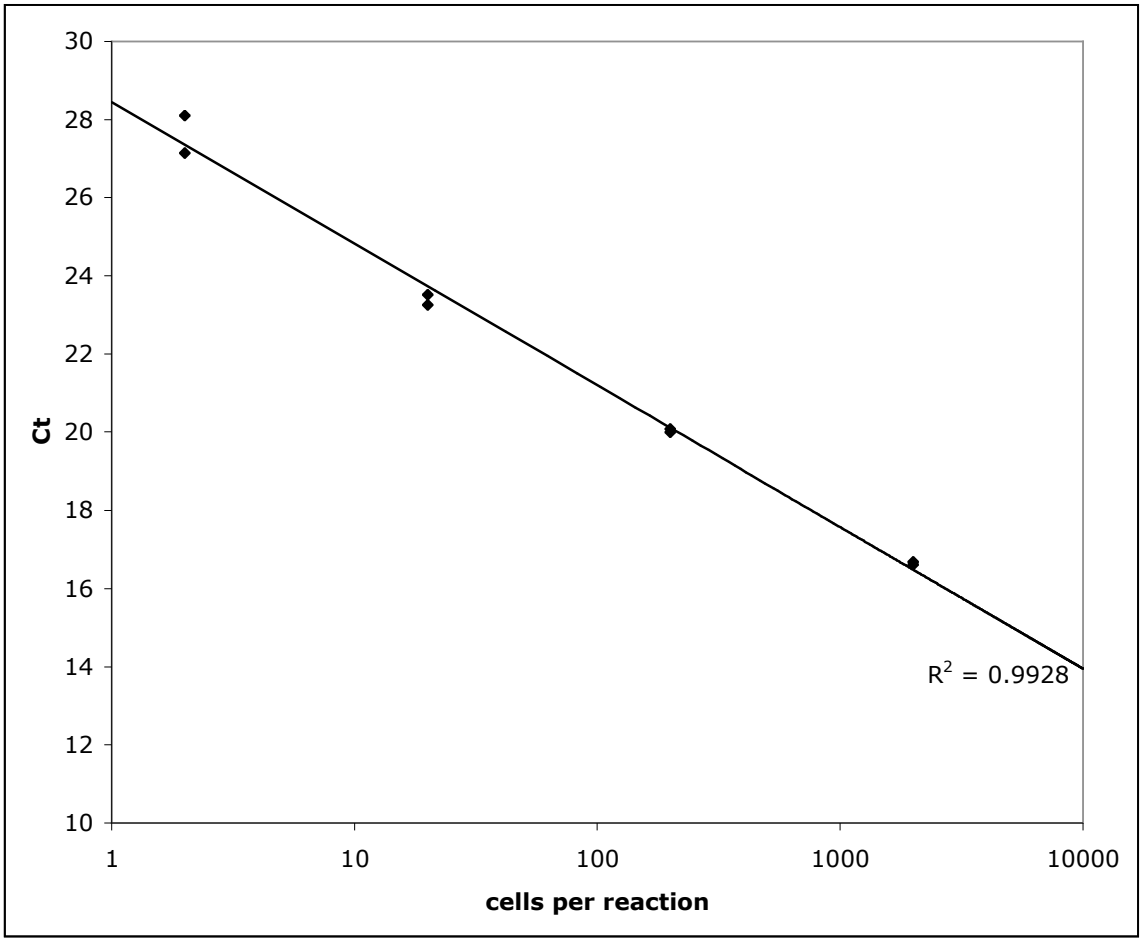

30
28
26
24
22
20
18
16
14
12
10
Ct
1
10
100
1000
10000
cells per reaction
R2 = 0.9928

Figure 6: Composite of all transcript standard curves. Error bars represent the standard deviation of the Ct values for each of the transcript concentrations (n=30).

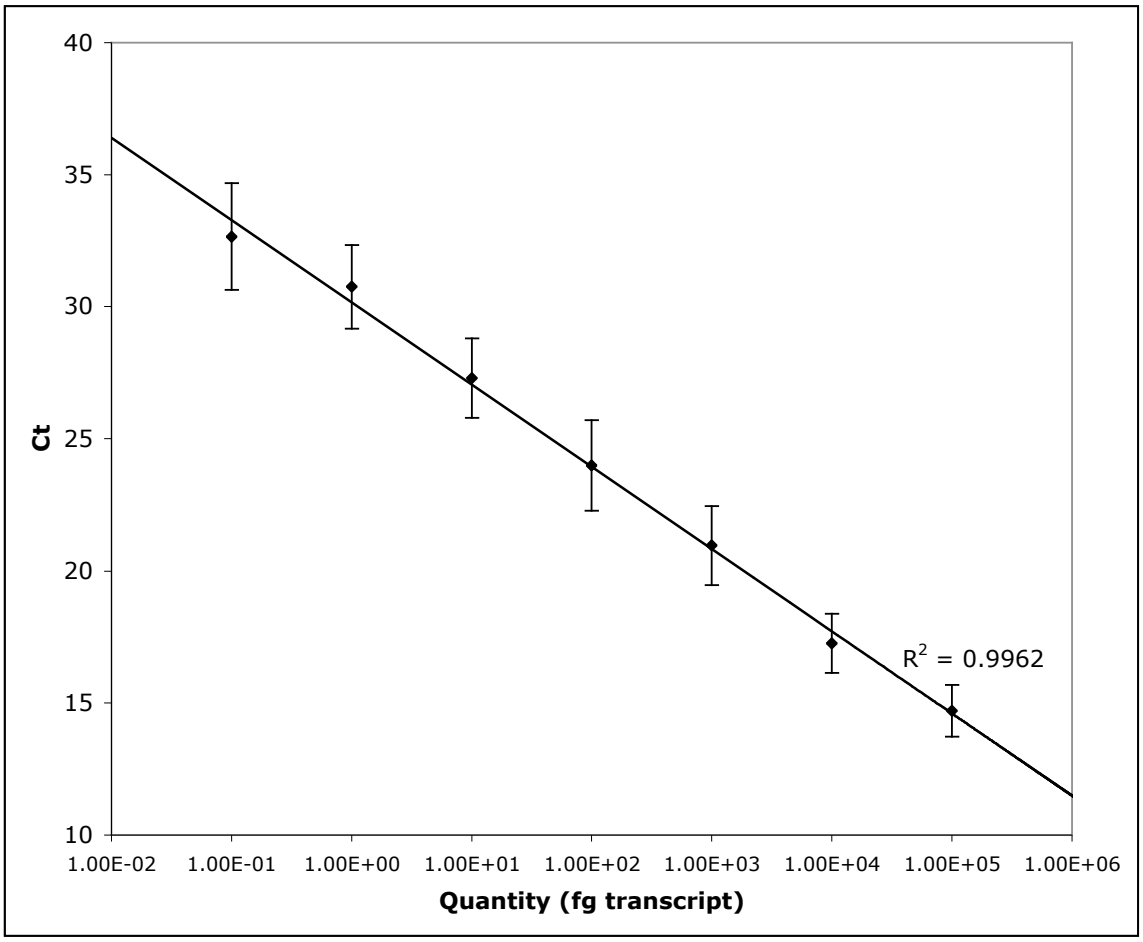
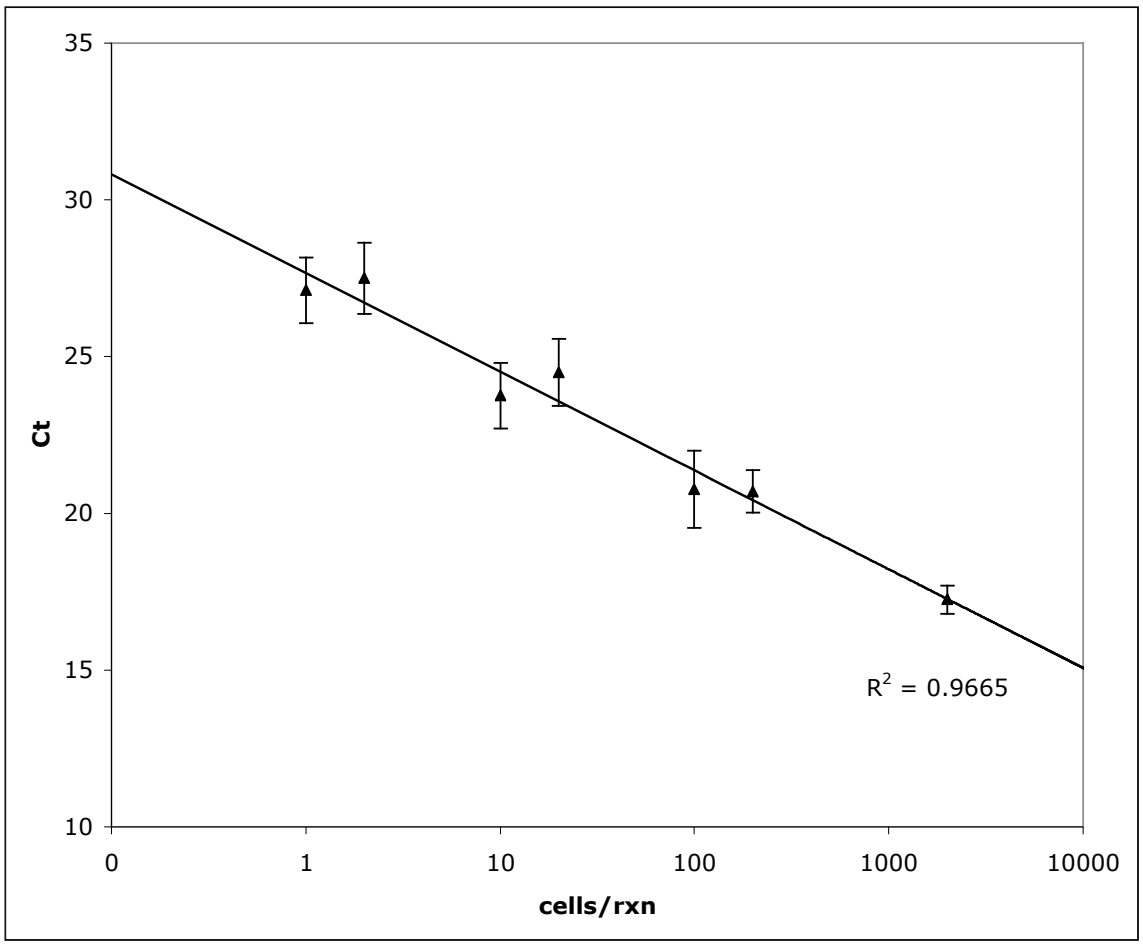

40
35
30
25
20
15
10
Ct
1.00E-02
1.00E-01
1.00E+00
1.00E+01
1.00E+02
1.00E+03
1.00E+04
1.00E+05
1.00E+06
Quantity (fg transcript)
R2 = 0.9962

Figure 7: Composite of all cell standard curves. Error bars represent the standard deviation of the Ct values for each of the cell concentrations (n=10).


35
30
25
20
15
10
Ct
0
1
10
100
1000
10000
cells/rxn
R2 = 0.9665

Discussion

A real-time RT-PCR method was developed to detect and quantify cell abundance based upon *rbcL* transcription in the red tide forming dinoflagellate *K. brevis* with good specificity and sensitivity. Using our TaqMan® probe, we were able to amplify and detect a wide range of concentrations of *K. brevis* to the exclusion of all non-target DNA and RNA tested. The dynamic range over which this technique is effective covers the range of natural *K. brevis* blooms in the environment. Populations of *K. brevis* with <1000 cells L^{-1} (as determined by microscopy cell counts) is considered "present" but poses no risk of adverse health effects or shellfish contamination. Samples with <5000 cells L^{-1} are considered to have a "Very Low" level bloom, carrying a slight risk of respiratory irritation. When concentrations of >5000 cells L^{-1} occur, shellfish harvesting is closed. The highest level of a bloom has been reached when there are >1 million cells L^{-1}. A bloom of this magnitude can result in massive fish kills, respiratory distress, discoloration of the water and may have effects on higher animals such as dolphins and manatees. These ranges are well within the range of detection ability of this assay when as little as 1-5 ml of sample water is filtered and extracted.

It is essential that a probe designed to detect an important bloom forming organism not detect other closely related (or unrelated) organisms. Bloom detection and tracking relies heavily on being sure of the identity of the blooming organism. The TaqMan® probe created in this study showed high specificity by only amplifying *K. brevis* and not amplifying any of the non-target samples tested, as listed in Table 3.

This method represents the first molecular detection strategy for *K. brevis*, and it may be well suited for detection and monitoring of red tide blooms caused by *K. brevis* in the Gulf of Mexico and the southern Atlantic coast of the United States. It may provide an easy and relatively rapid procedure that could be employed as an alternative to the more difficult and time-consuming methods currently used by red tide monitoring and management programs in the state of Florida and other states affected by *K. brevis*.

Chapter 3: Testing of the assay, field samples, diel studies and method evaluation

Introduction

The real-time RT-PCR detection and enumeration strategy outlined in the previous section performed well under preliminary testing conditions. However, an assay is only as good as its applicability to natural environmental samples. For a *K. brevis* detection assay to have full applicability, it has to accurately and reliably detect and enumerate populations of *K. brevis* from natural samples and it must be robust enough for much of the assay to be conducted in non-laboratory conditions. Specifically, the RNA purification has to be complete enough to allow for reliable detection of *K. brevis* regardless of the other constituents of the water sample to be analyzed, such as other phytoplankton species and organic detritus. The extraction procedure must also allow for the possible lack of adequate facilities that may be encountered either shipboard or in other field situations. At this time, unfortunately, the real-time RT-PCR step is constrained mostly to the lab due to the required instrumentation, such as the Applied Biosystems model 7700 Sequence Detection System used in this study; however there are systems that are beginning to show some field deployable capabilities.

In addition to being able to reliably enumerate *K. brevis* from natural samples despite possible interference, the assay may be sensitive to cell phase. Dinoflagellates

have been shown to exhibit diel cell cycling with certain genes and enzymes. In *K. brevis,* cell cycling over diel periods had been demonstrated and linked to cyclin-dependant kinase (66), with the dark to light transition shown to be the dominant cue. Expression of some genes in some dinoflagellates has been shown to be regulated over a diel cycle. Approximately 3% of the genes in *Pyrocystis lunula* were shown to be circadian regulated at the transcriptional level (44), notably several genes responsible for translation demonstrated diel regulation, but regulation of carbon fixation gene expression has so far not been shown in dinoflagellates. RuBisCO appears not to be differentially expressed at the mRNA level nor do protein levels change in the dinoflagellate *Lingulodinium polyedrum* over the diel cycle (44) and differences in photosynthetic capacity appear to be controlled by differential distribution of RuBisCO within the chloroplasts of *Gonyaulax,* with protein levels remaining constant (42). It has, however been shown that *rbcL* expression can exhibit great diel variability in other organisms, such as diatoms (*Phaeodactylum tricornutum*) (67), where transcript levels varied by a factor of approximately 3. It would be important to know for this assay if correction needs to be made based on time of day that the sampling was carried out, in order for the counts inferred from the real-time RT-PCR to more accurately represent the actual cell concentrations, should there be any diel variation in transcription of *rbcL.*

Methods

RNA extraction and amplification from environmental samples

Environmental bloom and non-bloom samples were received from Earnest Truby at the Florida Fish and Wildlife Conservation Commission's Florida Marine Research Institute (FMRI). *Karenia brevis* abundance in samples was determined by microscopy by FMRI personnel (Table 4).

Ten to 20 ml of the samples were filtered onto sterile 0.45 μm pore-size Millipore™ Durapore® type HVLP filters and placed in lysis buffer (buffer RLT, Qiagen, Valencia CA or Lysis buffer, Stratagene, La Jolla, CA) and processed immediately if possible. If immediate processing was not possible, the tubes were frozen at –80º C. Samples were processed using the RNA extraction kits from either Qiagen (RNeasy™) or Stratagene (Absolutely RNA™) according to the manufacturer's instructions. From the extraction (50 μl final eluent), 5 μl were added to the amplification reaction and real-time RT-PCR was carried out as described in the previous section with a standard curve of transcript spanning seven orders of magnitude (0.1 to 10^5 fg of target transcript reaction^{-1}) and a standard curve of whole cell extracts spanning 3-4 orders of magnitude (i.e. 1 to 1000 cells per reaction). Real-time RT-PCR data generated from these amplifications was analyzed preliminarily using the Sequence Detection software (versions 1.6 to 1.9) supplied by Applied Biosystems. Further analysis was carried out using Excel (Microsoft Corporation, Redmond, WA).

Diel experiments

Cultures and culture conditions

For all diel experiments the same isolate, Piney Island B4 was used. The cultures were acquired from Bill Richardson at FMRI and placed in an incubator in our lab at 22º C under a 12-hour light-12-hour-dark regimen at 26 μmol s^{-1} m^{-2} in F/2-Si medium (18) for at least 3 days to acclimate before any experimentation.

Sampling schedule, cell counts and RNA extraction

Four non-consecutive 24-hour diel studies were conducted as follows (using a new culture each time): starting at 4:00 AM, before the lights of the incubator were turned on (at 6:30 AM) and every two hours thereafter, the culture was removed from the incubator, gently swirled to homogenize the culture without lysis of the fragile *K. brevis* cells and approximately 30 ml of culture were poured into a 50 ml disposable centrifuge tube. All further sampling (cell counts, RNA extraction and carbon fixation) was carried out using sub-samples from this 30 ml sample. Before every removal of sub-samples from the 30 ml aliquot, the 50 ml centrifuge tube was capped and gently inverted to ensure even distribution of the cells and wide-bore pipette tips or serological pipettes were used to decrease turbulence and accidental cell rupture.

Cell counts were carried out as described in the last section (briefly, filtering onto a 0.22 μm pore-size black membrane and counted by epifluorescence microscopy, in triplicate).

Duplicate or triplicate 1 ml culture samples were filtered onto sterile Durapore® filters and placed in Qiagen's RLT or Stratagene's Lysis Buffer and immediately frozen

in liquid nitrogen and placed in the freezer at –80º C for extraction and real-time RT-PCR analysis once the diel sampling period was over. These samples were extracted, amplified and analyzed as described above.

Carbon fixation

Two 10 ml aliquots of culture were placed into acid washed, sterile scintillation vials, one clear glass and the other covered with black electrical tape to seal out light. To each of these aliquots, 5 μl of ^{14}C labeled HCO_3 (10 μCi) were added. Two 2 ml samples from each vial were immediately filtered onto 0.22 μm pore-size Millipore type GS filters, rinsed with filtered seawater and placed in scintillation vials with 0.5 ml of 0.5 N HCl. The clear vial was placed in a lighted incubator at room temperature with 30 μmol s^{-1} m^{-2} light and the taped (dark) vial was placed in a dark drawer at room temperature. The vials were incubated for 30 minutes. After the incubation, two 2 ml aliquots were again filtered from each sample, rinsed with filtered seawater and placed in scintillation vials with 0.5 ml of 0.5 N HCl. Once all of the time points had been sampled, the vials were allowed to remain at room temperature for 8 to 10 hours to allow unincorporated ^{14}C (as CO_2) to off-gas. To each of the samples, 1 ml of ethyl acetate was added and the filter was allowed to dissolve for 1 hour. Then 10 ml of Ecoscint A scintillation fluid (National Diagnostics, Atlanta, GA) was added, and the samples were placed into the scintillation counter to be counted for 0.5 minutes each.

Counting efficiency was determined as follows: a Millipore GS filter was placed in 3 scintillation vials and dissolved with 1 ml ethyl acetate. Ten ml of Ecoscint A and 1

ml of 0.01 N NaOH was added to each vial and mixed well. To two of these vials, 5 μl of diluted ^{14}C was added (diluted 1:1000, 0.01 μCi, 22,200 DPM) and mixed well, the third vial served as a blank for background comparison. These vials were also counted for 0.5 minutes each. Efficiency (e) was calculated using the following equation: $e=(CPM_{sample}-CPM_{blank})/22,200$. Counting efficiency ranged from 0.30 to 0.70. These data were analyzed either by the C14 program (Qbasic, in-house software) or using an Excel spreadsheet based on that program.

Alkalinity Determination

After the initial sampling, 100 ml of culture were removed and analyzed for culture alkalinity as follows. From the 100 ml sample, the temperature, salinity and pH were determined. The sample was placed on a stir-plate with a stir-bar and 25 ml of 0.01 N HCl were added and the pH was measured again. If the pH was not below 4.0, an additional 5 ml of 0.01 N HCl were added and the pH was measured. The data from these measurements were analyzed by the ALK1 program (Qbasic, in-house software) for an estimated alkalinity and amount of dissolved inorganic carbon. These data were used in the ^{14}C incorporation calculations.

Evaluation of real-time assay reagents and extraction methods

Room temperature and freezer (-80º C) stability of *K. brevis* RNA lysates

In order to determine the field applicability, convenience and flexibility inherent in the real-time RT-PCR assay, a series of experiments were carried out based on anticipated field extraction conditions. To determine the stability of the target RNA in

cases where liquid nitrogen or –80º C freezers are not readily available, and to evaluate the performance of the guanidine thiocyanate-based lysis buffers from both Qiagen and Stratagene, two separate experiments were carried out. In the first, 20 1-ml aliquots of culture were filtered onto Durapore® filters and placed in RLT buffer with 2-mercaptoethanol. Two of those samples were frozen immediately at –80º C and the rest were placed in a dark drawer at room temperature. Each day, 2 of the samples were removed from the drawer, labeled with the day and frozen. Once all 10 days worth of samples had been frozen, the samples were thawed and extracted following Qiagen's instructions. The samples were then analyzed by real-time RT-PCR as described above. The second experiment was conducted as above using Stratagene's Lysis Buffer and extraction kit, but the sampling was done in quintuplicate.

To evaluate the samples' stability in Stratagene's Lysis buffer at –80º C, 25 1 ml aliquots were filtered onto Durapore® filters and placed into lysis buffer. Twenty of the samples were placed immediately into the –80º C freezer, while the remaining 5 were extracted and analyzed using the real-time RT-PCR assay. At the following intervals 5 of the samples were thawed, extracted and analyzed using the real-time RT-PCR assay: 1 week, 2 weeks, 4 weeks and 6 weeks.

Alternative extraction methods

In order to evaluate and minimize variation due to the RNA extraction process, several approaches were tested. As the *K. brevis* cell is very fragile, two methods were tested to minimize breakage and subsequent loss of RNA and one method was tested to

reduce sample handling and possibly lead to a more easily automated extraction protocol. In the first method, rather than filtering onto Durapore® membranes, 1 ml of culture was centrifuged at 5000 rpm (2300 X g), the supernatant was removed and the cells were resuspended in 100 μl of lysis buffer. Extraction was continued according to the manufacturer's instructions. In the second method, rather than manipulating the cells at all, 1 ml of culture was added directly to 1.5 ml of lysis buffer and vortexed vigorously. To this mixture, 2.5 ml of 70% ethanol was added and the samples were run through the RNA extraction column either through multiple centrifugation steps or pulled through by placing the column in a centrifuge tube with the bottom cut off and placed into a rubber stopper that was placed on a vacuum manifold and light vacuum was applied (80 to 100 mm Hg). Extraction was continued according to the manufacturer's instructions. The third method tested was designed to facilitate future automation of the extraction process and was carried out by filtering the *K. brevis* culture directly onto the silica matrix of the extraction column using a light vacuum as above. To the column, 200 μl of a 1:1 mixture of lysis buffer and 70% ethanol were added, incubated for 10 minutes and removed by centrifugation. Extraction was continued according to the manufacturer's instructions. The standard method using the Durapore® membrane was also conducted for comparison. All culture samples were diluted so that 1000 cells were extracted into the final eluent, and extractions were performed with the Stratagene columns with five replicates of each extraction method.

Results

Field samples

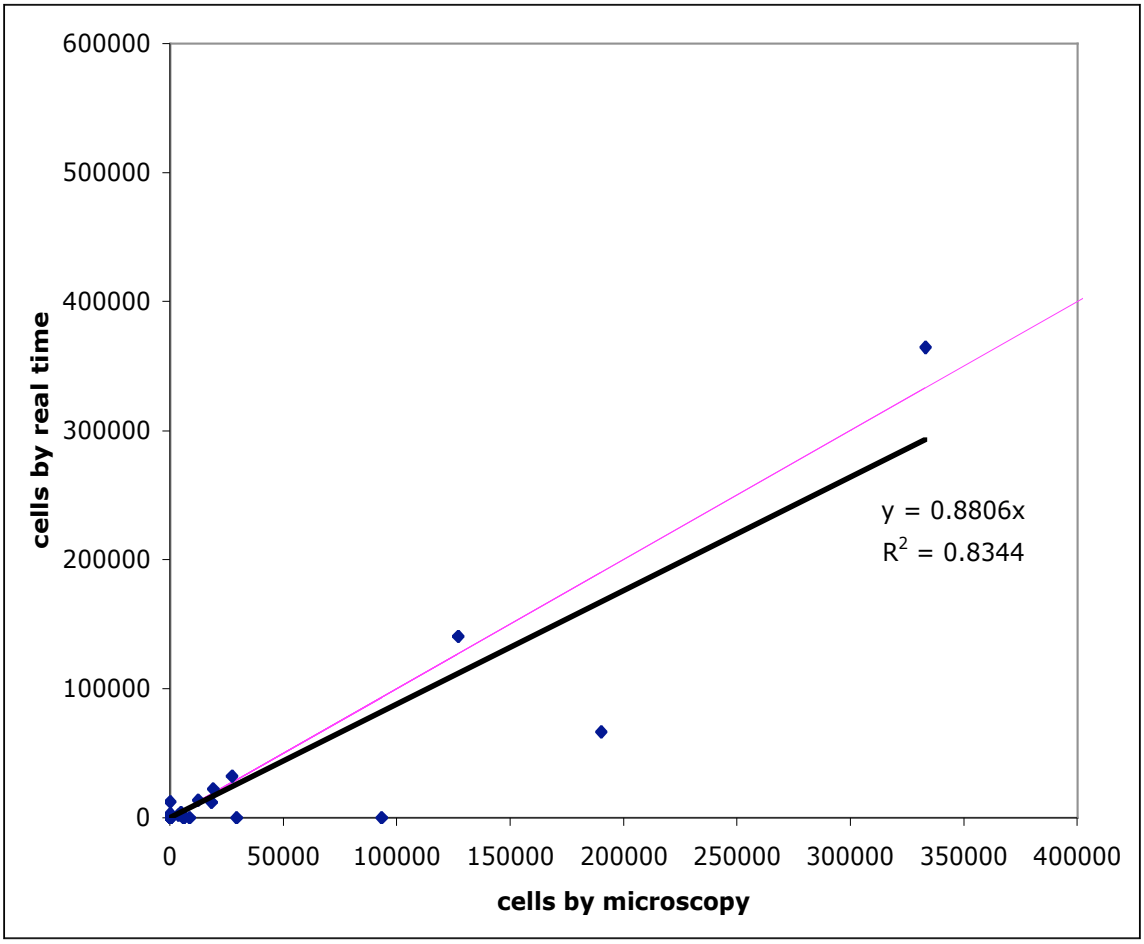
Twenty-eight field samples from FMRI were analyzed by the real time RT-PCR assay and the calculated cell counts based on the cell and transcript standard curves as well as the microscopy counts are listed in Table 4. Figure 8 shows a graphical comparison of the microscopy-based counts and the real-time RT-PCR assay, with a 1:1 line plotted for reference. The correlation (r^2) between microscopy counts and real-time RT-PCR counts was 0.8344, with a slope of 0.88. Samples that were negative by microscopy (limit of detection approximately 300 cells $liter^{-1}$) often were positive by RT-PCR (ranging from 0 to 12476 cells $liter^{-1}$, average: 1646 cells $liter^{-1}$, n= 12). The samples that were stored in the freezer for over a month (bloom dates 7/1/03 and 8/27/03) yielded dramatically lower calculated cell concentrations than expected, indicating background concentrations of *K. brevis* despite the microscopy counts that were in the 6,000 to 100,000 cells L^{-1} range as counted prior to filtering and freezing in lysis buffer

Table 4: Bloom samples counted by microscopy and inferred counts by real-time RT-PCR.

Site ID	Sampling date	Cell Counts (Cells/L)	Real Time PCR (Cells/L)	Bin agreement
Naples Pier	3/26/03	0	219	Y
Clam Pass	3/26/03	12300	13752	Y
Barefoot Beach	3/26/03	333000	364645	Y
Naples Pier	4/2/03	190000	66540	N
South Marco	4/2/03	0	1791	N
951 Launch ramp	4/2/03	0	1152	N
Clam Pass	4/2/03	127000	140758	Y
Good Land	4/2/03	0	3167	N
Marco Pass	4/2/03	0	12476	N
Howard Park	7/1/03	0	0	Y
Honeymoon Island	7/1/03	6000	12	N
Pier 60	7/1/03	6330	634	N
Reddington Pier	7/1/03	0	2133	N
Bowditch Park	8/27/03	29300	133	N
Bonita Beach Park	8/27/03	0	4	Y
Lover's Key	8/27/03	93300	122	N
Little Hickory Island Park	8/27/03	8670	49	N
Lely Barefoot Beach	10/16/03	4670	4089	Y
Seagate	10/16/03	27300	32311	Y
Naples Pier	10/16/03	0	0	Y
Vanderbilt Beach	10/16/03	19000	22484	Y
South Marco Beach	10/16/03	18300	12050	Y
Naples Pier	10/27/03	3670	2295	Y
Seagate	10/27/03	667	294	Y

Bin Key	*K. brevis* cells/l
Present	<1000
Very Low a	>1000 to 5000
Very Low b	5000 to 10,000
Low a	10,000 to 50,000
Low b	50,000 to 100,000
Medium	100,000 to 1,000,000
High	>1,000,000

Figure 8: Cell counts microscopy and the real-time RT-PCR assay. Comparison of counts by microscopy and inferred counts by real-time RT-PCR. Plotted are both a trendline for the data (thick line) and a one-to-one line (thin line) that would indicate perfect agreement, for comparison.


600000
500000
400000
300000
200000
100000
0
cells by real time
0
50000
100000
150000
200000
250000
300000
350000
400000
cells by microscopy
y = 0.8806x
R2 = 0.8344

Diel Studies

Carbon fixation showed a typical diel pattern of increased production during the day, with a peak of production occurring around noon. Production increased rapidly after the start of the light period, beginning with the 8:00 AM sampling period for all of the diel studies, and had reached maximal activity by the noon sampling. In 2 of the 4 diel periods studied, *K. brevis* displayed a depression in carbon fixation at the 2:00 PM sample, but had returned to a higher production level at the 4:00 PM or 6:00 PM sampling. In the other two diel studies, the *K. brevis* cultures did not express this decline in production during the light period. Carbon fixation returned to nighttime levels by 8:00 PM, 1.5 hours after the start of the dark period. Figure 9 shows carbon fixation over the four non-consecutive 24-hour periods analyzed.

Cell counts during the 24 hour sampling periods of the four diel studies oscillated, varying by as much as 2000 cells ml^{-1}, most likely due to incomplete mixing of the culture before the initial sampling of the 30 ml aliquot. The cultures did not display significant growth over the 24-hour periods studied, and there was no trend to the variation in cell counts.

Expression of the *rbcL* gene as analyzed by real-time RT-PCR showed some variability over the diel period in each of the 24-hour periods studied, however these variations do not fall at the same time periods in all of the 4 diel periods studied. Figure 10 shows the *rbcL* RNA signal as detected by real-time RT-PCR, corrected by cell counts at each time point, where possible. Figure 11 shows an overlay of all of the diel RNA

Figure 9: Carbon fixation over one diel cycle in four experiments. Diel experiments 1 through 4 are represented by diamonds, squares, triangles and x marks, respectively.

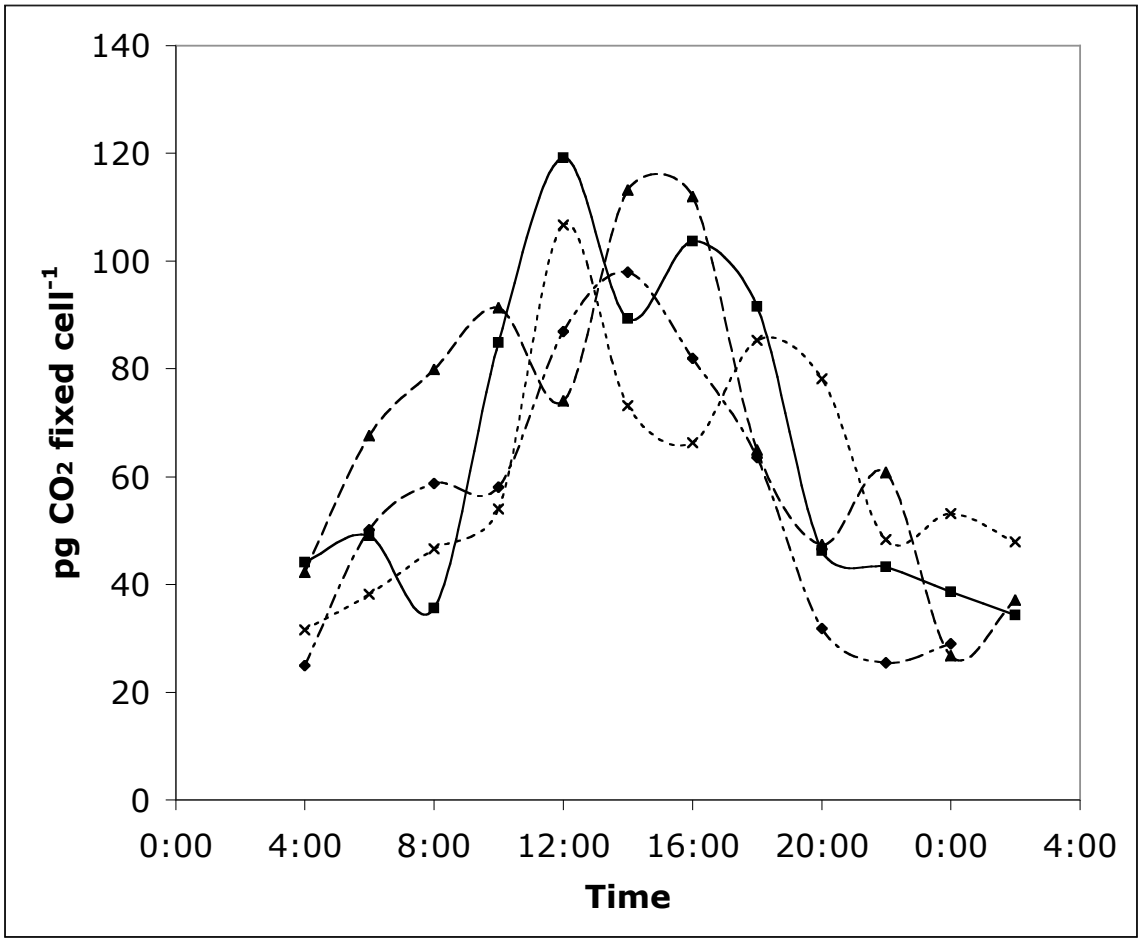

140
120
100
80
60
40
20
0
pg CO2 fixed cell-1
0:00
4:00
8:00
12:00
16:00
20:00
0:00
4:00
Time

Figure 10: RNA transcription over the diel periods. Diel experiments 1 through 4 are represented by diamonds, squares, triangles and x marks, respectively.

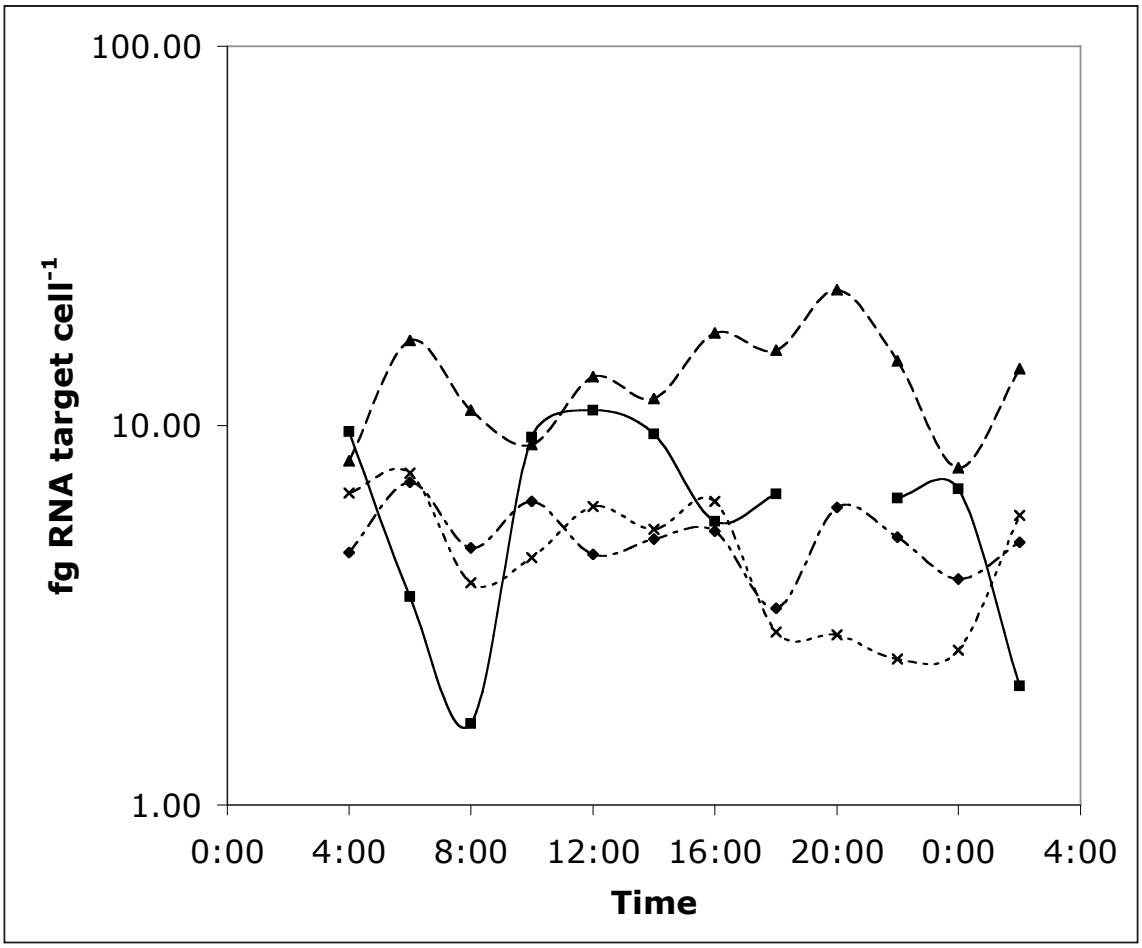
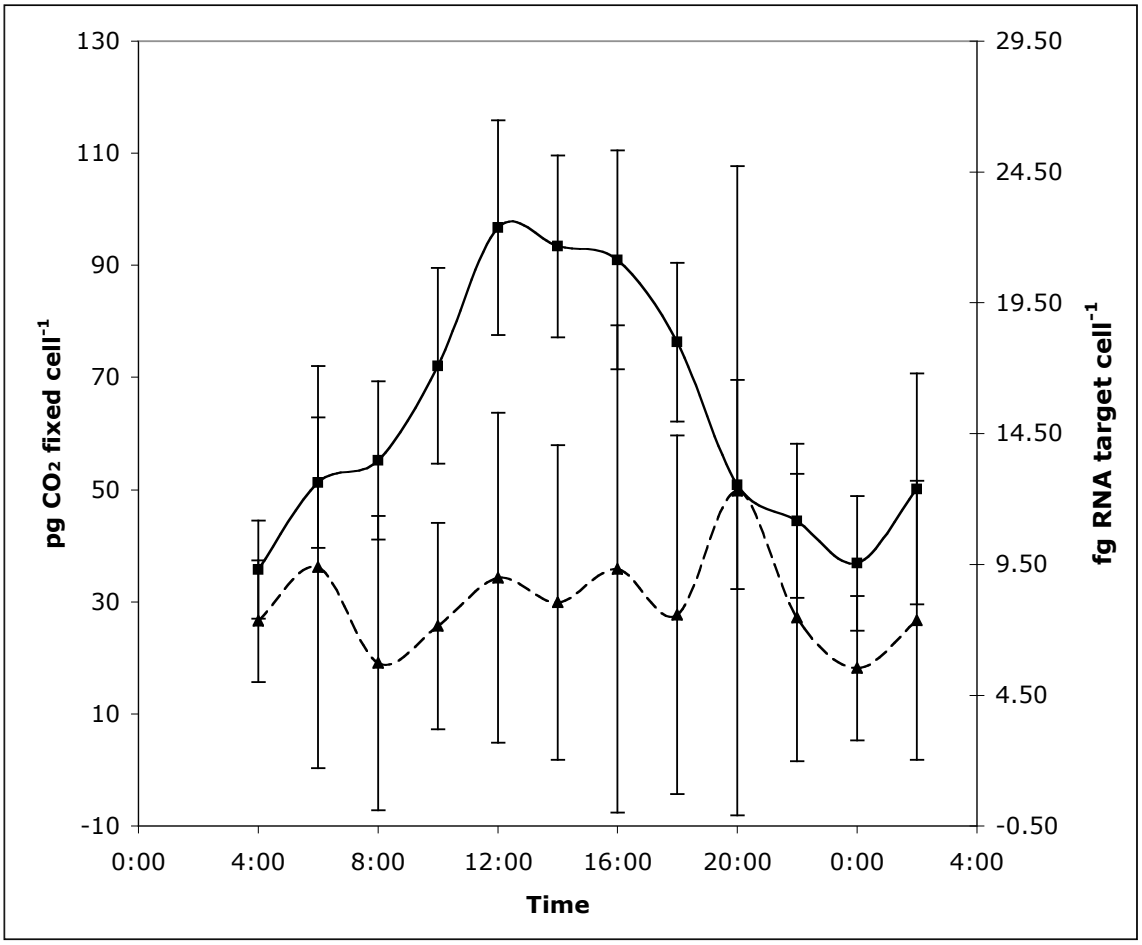

100.00
10.00
1.00
fg RNA target cell-1
0:00
4:00
8:00
12:00
16:00
20:00
0:00
4:00
Time

Figure 11: Average carbon fixation (squares, solid line) and average *rbcL* transcription (triangles, dashed line) for all of the diel experiments.


130
110
90
70
50
30
10
-10
pg CO2 fixed cell-1
29.50
24.50
19.50
14.50
9.50
4.50
-0.50
fg RNA target cell-1
0:00
4:00
8:00
12:00
16:00
20:00
0:00
4:00
Time

results and the carbon fixation results. The minima and maxima in RNA transcript levels did not consistently occur at any specific time in the diel cycle and ranged from 3.30 to 7.09 fg cell^{-1}, 1.64 to 10.96 fg cell^{-1}, 7.72 to 22.79 fg cell^{-1} and 2.43 to 7.48 fg cell^{-1} in each of the diel experiments, with an average range of 5.53 to 12.31 fg cell^{-1} and an average cellular *rbcL* target concentration of 7.50 fg cell^{-1}.

Room Temperature Stability in Lysis Buffer

Samples retained much of their RNA when stored in lysis buffer from Stratagene when stored at room temperature in the dark for a much as 6 days, however the replicates became more variable in terms of their RNA content. On average, the RNA content did not dramatically change, however, by day seven, there were samples in which very little (two orders of magnitude less) RNA remained. Samples stored in Qiagen's lysis buffer lost approximately half of the RNA after one day stored at room temperature, but remained at that lower level for 6 more days before continuing to degrade. Figure 12 shows the RNA present in samples stored at room temperature in the dark in Qiagen's lysis buffer (Figure 12(a)) and Stratagene's lysis buffer (Figure 12(b)) and the stability of the target over 10 days. Figure 13 compares the change in RNA signal in the two lysis buffers, normalized as average percent change of RNA concentration from day 0.

Freezer stability in lysis buffer

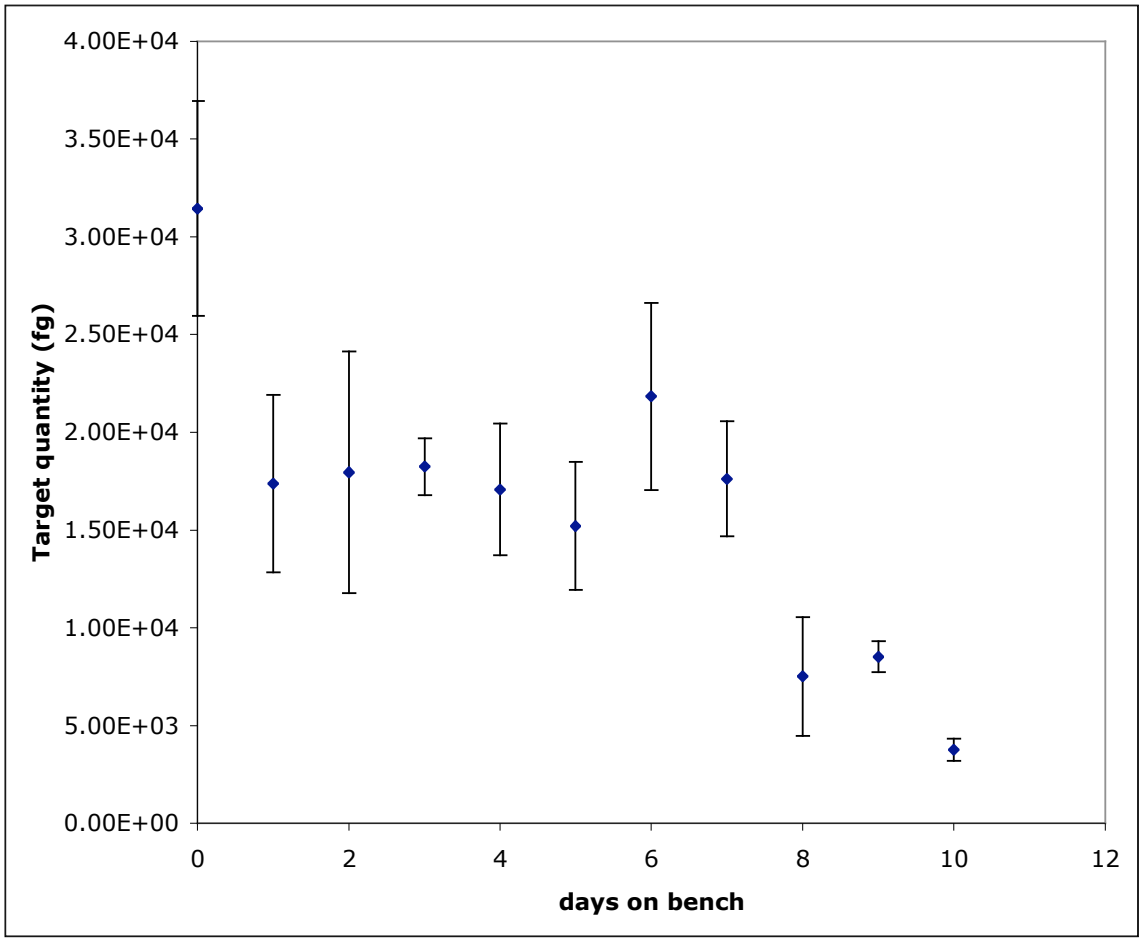
Samples stored in Stratagene's lysis buffer at –80º C began to lose some target RNA content after two weeks, though with less variability than was seen associated with the room temperature-stored samples after 7 days. The loss of RNA seen in the samples at three weeks did not continue as a trend and at the end of the experiment there was not a significant loss of RNA in the samples stored for as much as six weeks in the –80º C freezer. Figure 14 shows the detected amount of RNA from samples stored at –80º C over the course of six weeks.

Alternative extraction methods

Samples analyzed with the alternative extraction methods, though representing the same cell numbers, did show differences in their RNA signal. Samples that were filtered directly onto the extraction column, without prior filtration or concentration steps returned on average the most RNA of the methods tested, however the column filtration method also exhibited the most variability of RNA signal. The two methods which used a concentration mechanism (filtration onto a Durapore® membrane or concentration by centrifugation) displayed very similar RNA signals, with the centrifugation method showing less variability than the filtering method. The extraction directly from the liquid culture returned the lowest RNA signal, as well as the lowest variability. The results of this experiment are presented in Figure 15.

Figure 12: RNA stability at room temperature. (A) RNA stability at room temperature in Qiagen's RLT buffer. Plotted are the mean values and the error bars represent the standard deviation (n=3). (B) RNA stability at room temperature in Stratagene's Lysis buffer. Plotted are the mean values and the error bars represent the standard deviation (n=5).

A)


4.00E+04
3.50E+04
3.00E+04
2.50E+04
2.00E+04
1.50E+04
1.00E+04
5.00E+03
0.00E+00
Target quantity (fg)
0
2
4
6
8
10
12
days on bench

B)

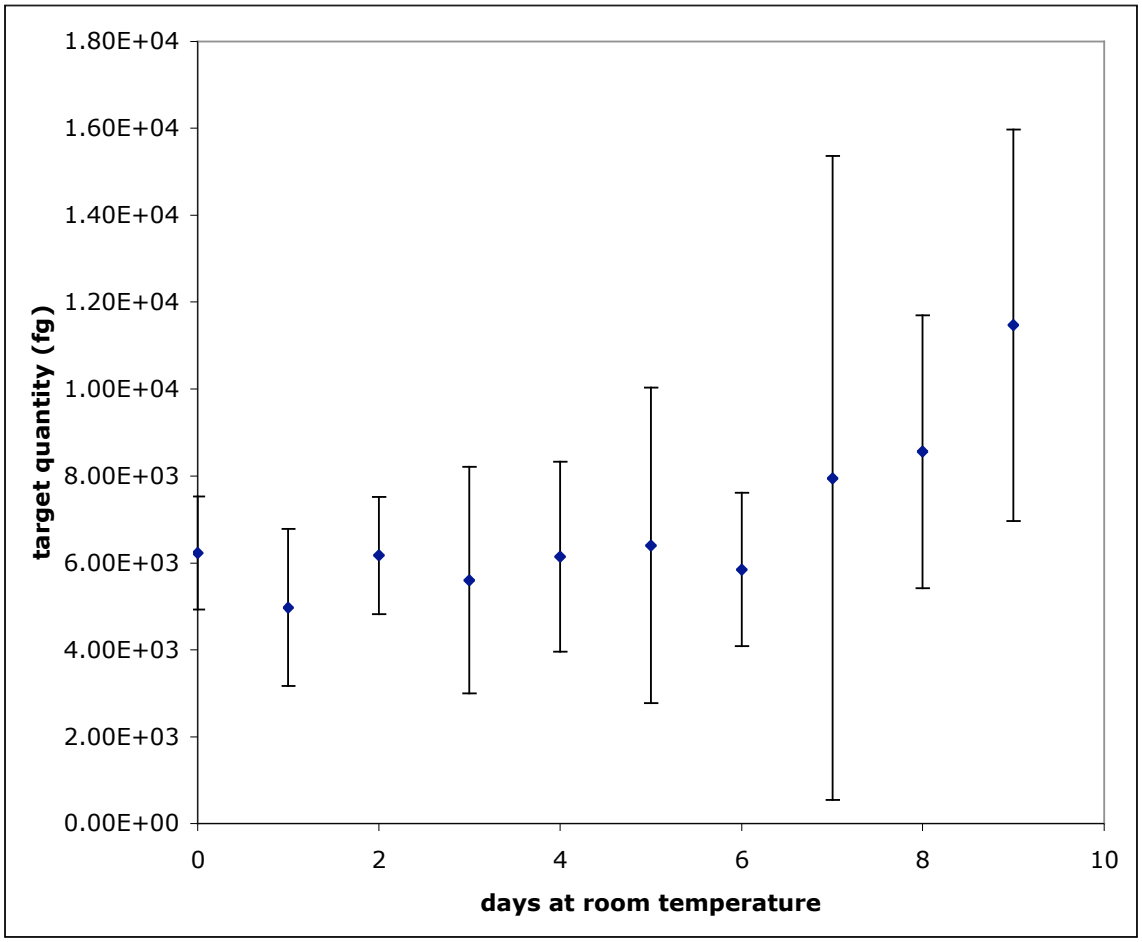

1.80E+04
1.60E+04
1.40E+04
1.20E+04
1.00E+04
8.00E+03
6.00E+03
4.00E+03
2.00E+03
0.00E+00
target quantity (fg)
0
2
4
6
8
10
days at room temperature

Figure 13: Average percent of initial RNA signal after storage at room temperature. Shown are the average percents of the detected *rbcL* RNA signal after storage at room temperature in RLT (squares) and Lysis buffer (circles).

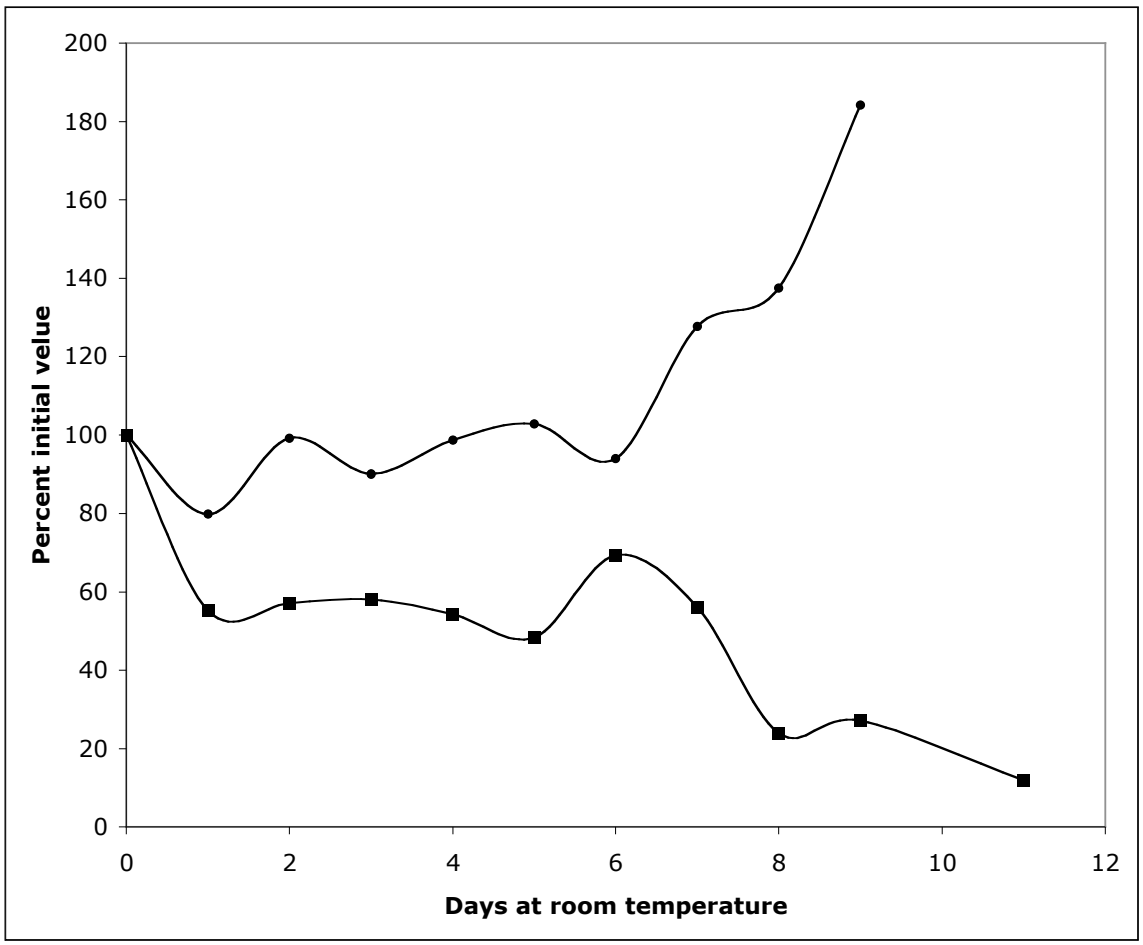

200
180
160
140
120
100
80
60
40
20
0
Percent initial velue
0
2
4
6
8
10
12
Days at room temperature

Figure 14: RNA stability in Stratagene's Lysis buffer after storage at –80º C.

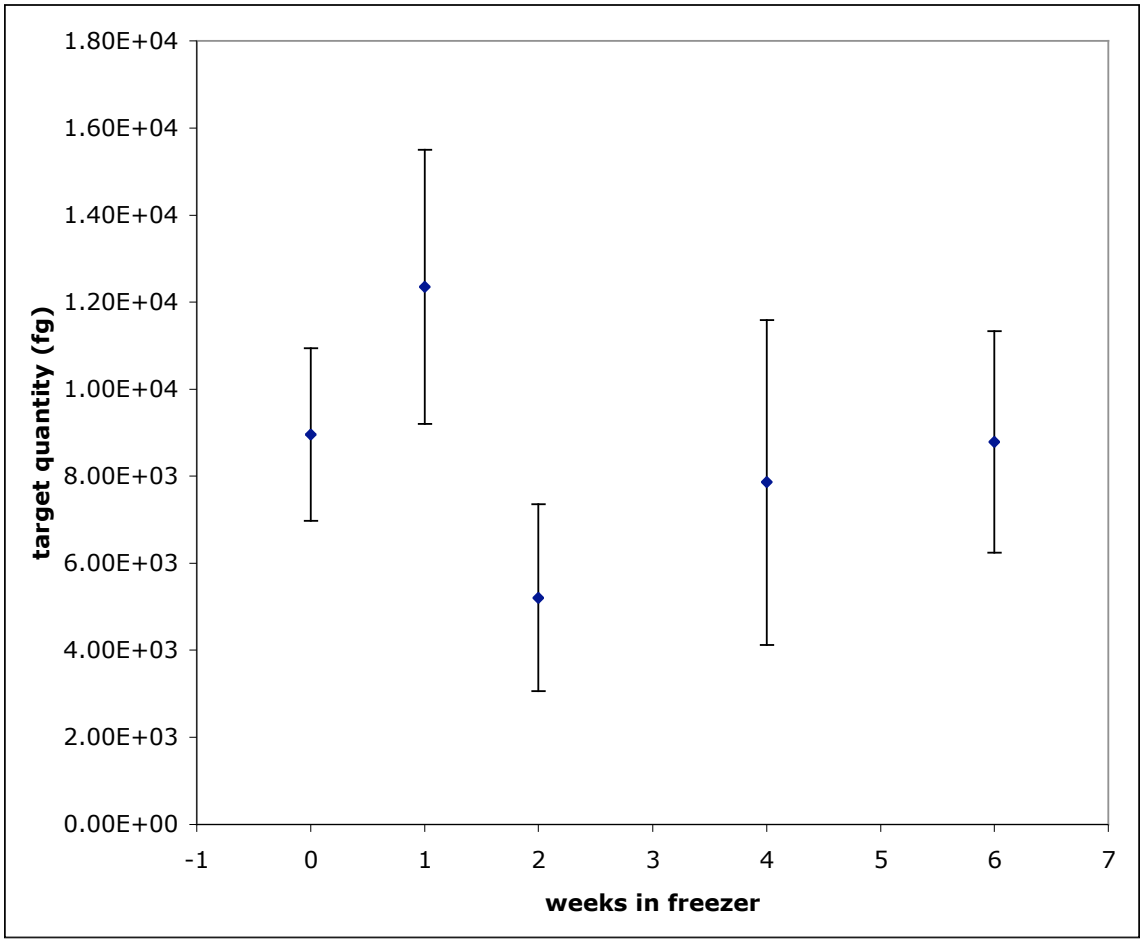
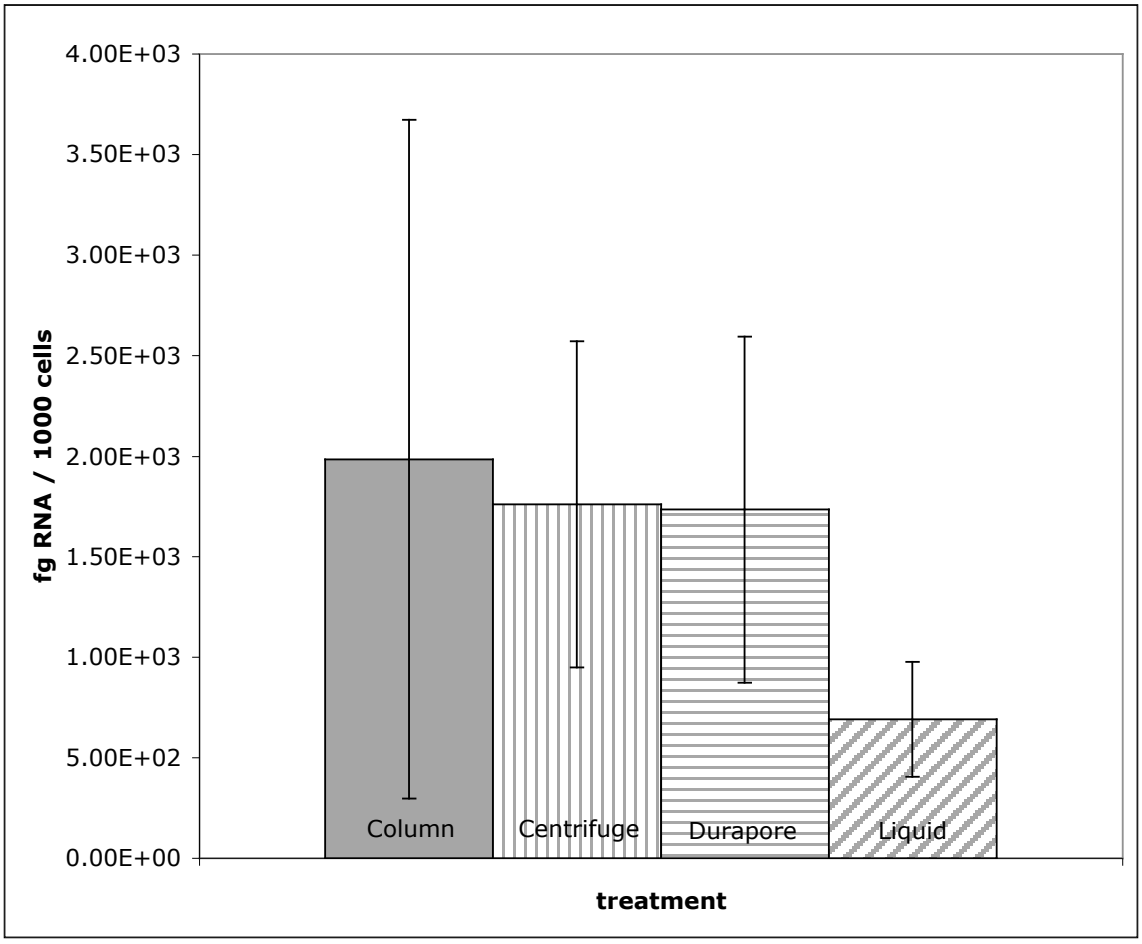

1.80E+04
1.60E+04
1.40E+04
1.20E+04
1.00E+04
8.00E+03
6.00E+03
4.00E+03
2.00E+03
0.00E+00
target quantity (fg)
-1
0
1
2
3
4
5
6
7
weeks in freezer

Figure 15: Detected *rbcL* RNA signal following alternative extraction methods. Plotted are the average values and the error bard represent the standard deviation (n=5 per treatment).


4.00E+03
3.50E+03
3.00E+03
2.50E+03
2.00E+03
1.50E+03
1.00E+03
5.00E+02
0.00E+00
fg RNA / 1000 cells
Column
Centrifuge
Durapore
Liquid
treatment

Discussion

The real-time RT-PCR assay developed has performed well when applied to field samples. The assay was able to quantitate cellular abundance of *Karenia brevis* from natural populations without interference from other phytoplankton or other contamination inherent in natural marine samples for most of the samples analyzed. The assay was effective over a wide range of cell concentrations, ranging from below the direct microscope detection method limit of 300 cells liter^{-1} to the highest sample analyzed at over 3X10^{5} cells liter^{-1}. Based on the cell standard curves, it would be possible to detect as many as 10^{4} cells reaction^{-1}, which corresponds to 10^{7} cells liter^{-1} if 20 ml is filtered, and 5 μl of the 50 μl column elution is used in the RT-PCR reaction. This assay, then, is able to detect and quantify the entire range of commonly seen *K. brevis* bloom concentrations.

The variation of *rbcL* transcript concentration in the cells of *K. brevis* do not appear to drastically or regularly change over a diel cycle, in contrast with carbon fixation. It is possible that RuBisCO activity in *K. brevis* is controlled at the translation or post translation level, or by changes in distribution within the chloroplast rather than at the transcription level as in *Gonyaulax* or *Lingulodinium* or as in synechococcus or a prymnesiophyte (47). There were some changes in the transcript signal at different times of the day and these changes did not appear to be a strictly regulated diurnal signal, as the changes did not occur at the same times in all of the diel experiments. These changes in

transcript levels could be a response to the cell's need for RuBisCO, natural fluctuations of a gene that is not strictly controlled at the transcriptional level, the inherent variability of the RT-PCR assay or the uneven distribution and fragility of cells in culture. Overall, changes between time points were relatively minor and often fell within the variability between replicates, as variability between replicates was large.

The variability of the *rbcL* transcript levels in *K. brevis* could lead to some error in the calculation of cell concentration from bloom samples by the real time RT-PCR assay, but the variation is minor and the assay is accurate enough to serve a useful purpose in the enumeration of *K. brevis* in the environment. Current guidelines use a binned system from categorization of blooms ranging from "not present" through "very high" (see Table 4). The ability of the real-time RT-PCR assay to reliably identify the category level of a bloom based on the system currently used by the FMRI is 54%. Without including the samples that were stored for a long period, this increases to 69%, indicating that long-term storage is not advisable when quantitative data are needed due to the increase of variability and potential loss of RNA as seen in Figure 14. Storage of samples should be kept to a minimum with samples processed as soon as possible. While storage at –80º C of lysed culture samples demonstrated some level of stability, the RNA signal from bloom samples that were stored for a month or more was not detectable.

The developed RT-PCR assay, while effective for the detection and enumeration of *K. brevis*, appears to be a poor tool for analysis of gene expression due to both its variability and the variability of cell density in *K. brevis* culture, which may swamp the signal of transcriptional regulation. Its sensitivity is very high (as little as one copy of

target (49)) compared to that of other gene expression methods, such as dot-blotting and probing, but the signal of amplification becomes saturated at fairly low concentrations of target. Hybridization type assays, such as blotting and probing, have a much lower sensitivity, but are less likely to exhibit the variability of real time RT-PCR when sufficient target is present (67).

The method developed appears to be well suited to limited field use. The samples were stable at room temperature in Lysis buffer for at least 3 days, though the RNA target was not as stable in RLT. Therefore it would be preferable to use the RNA microprep extraction kit from Stratagene for the increased stability of the RNA target at room temperature as well as the decreased variability that was apparent in the room temperature stability experiment. If it is not possible to immediately extract and amplify the RNA from an environmental sample, it would be advisable to freeze them at –80º C, rather than leave them at room temperature. The stability of the filtered samples was far greater when frozen at –80º C than at room temperature. The samples that were frozen maintained most of their RNA throughout the experiment only losing RNA beginning at one month. Most of the RNA remained, however and even after 6 weeks, there was enough of the RNA to still detect, though quantitative representation of cell counts as inferred from real-time RT-PCR would be difficult due to the increased variability between samples. As the RNA began to degrade and the samples exhibited increased variability, it would be advisable to process samples that are to be quantitative within two weeks. If the samples have been in the freezer for more than 6 weeks, the assay may be unreliable as a quantitative method and should be only used as a presence/absence

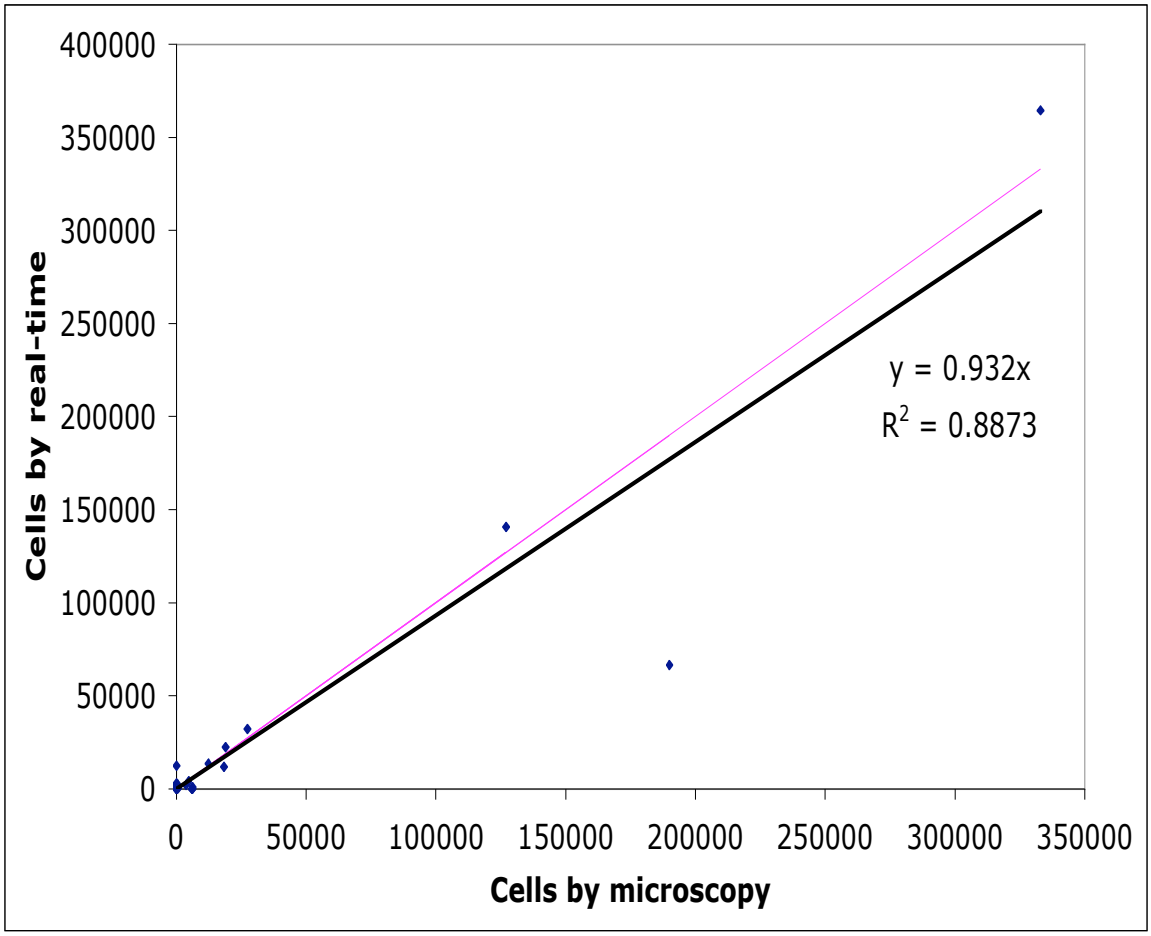
indicator, though even that may be too unreliable after samples had been frozen much longer than 6 weeks.

The bloom samples that were analyzed included some samples that had been frozen for over one month. According to the freezer stability experiment, these samples quantitative ability should have been reliable, however these samples inferred counts by real-time RT-PCR did not reflect the counts by microscopy. For comparison, those samples have been removed from the calculations in Figure 15(a) with the entire data set represented for comparison in Figure 15(b). The correlation between inferred counts by RT-PCR and microscopy counts has increased (r^2=.8873, vs. r^2=.8344) and the slope of the linear regression trendline is closer to a slope of 1 (y=.932(x), vs. y=.881(x)) that would indicate perfect agreement. It is unclear why the bloom samples that had been stored at –80 ºC did not amplify in agreement with the microscopy counts, though there may have been compounds in the samples that inhibited the guanidine thiocyanate protection of the RNA. For this reason, samples from natural waters should not be frozen for extended periods if quantitative data are needed.

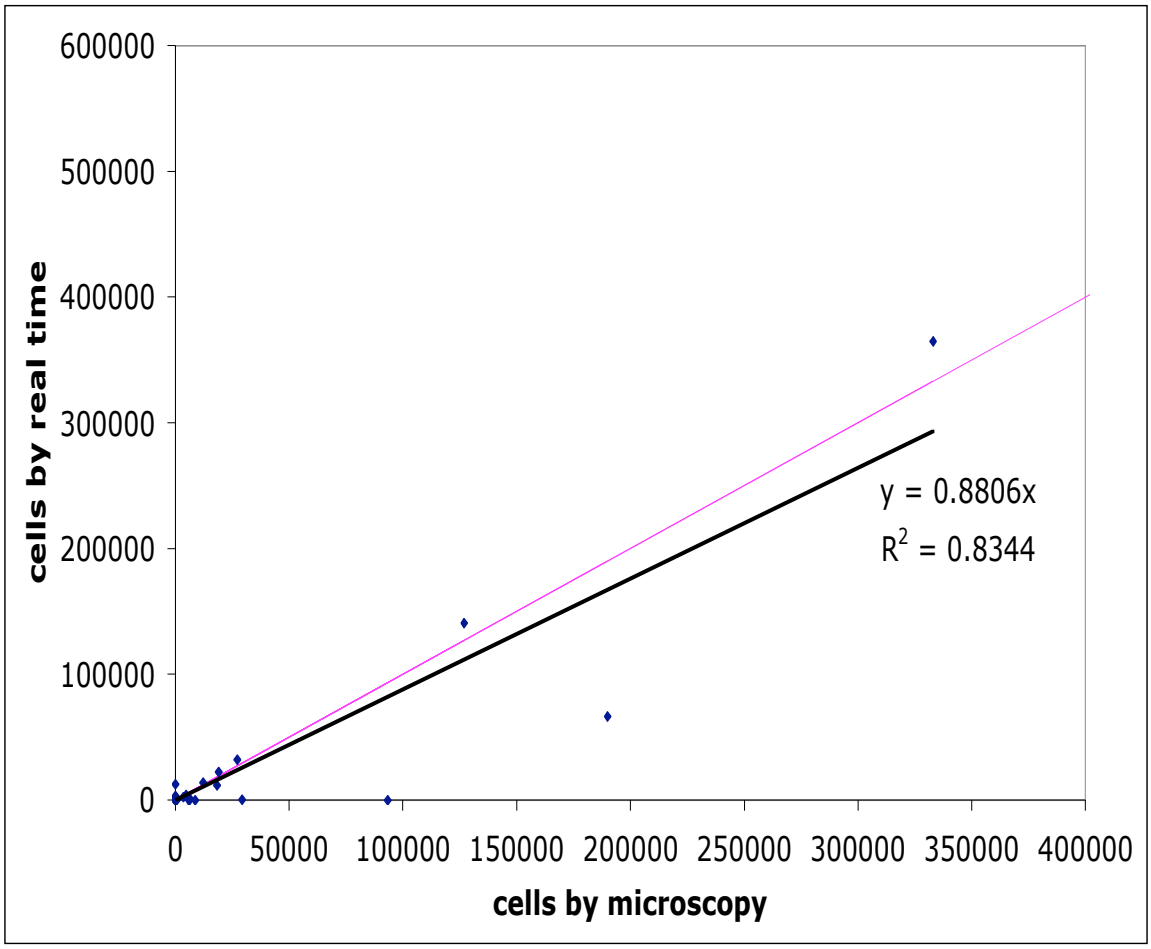
Experiments with the alternative extraction methods demonstrated distinct differences in target RNA content between the different methods. The increased variability of the column extraction method, despite its high yield of RNA, makes it a method that should be employed only if easier automation is required, as in a deployed system where the extra handling involved in a separate filtration or centrifugation step would be difficult to accomplish due to engineering difficulties and power requirements. Both the Durapore® filtration method and the centrifugation method, with their associated

Figure 16: Cell counts by microscopy and the real-time RT-PCR assay. A) Cell counts by microscopy and the real-time RT-PCR assay not including samples that had been stored at –80º C for over one month. B) Cell counts by microscopy and the real-time RT-PCR assay including samples that had been stored at –80º C for over one month.

A)


400000
350000
300000
250000
200000
150000
100000
50000
0
Cells by real-time
y = 0.932x
R2 = 0.8873
0
50000
100000
150000
200000
250000
300000
350000
Cells by microscopy

B)


600000
500000
400000
300000
200000
100000
0
cells by real time
0
50000
100000
150000
200000
250000
300000
350000
400000
cells by microscopy
y = 0.8806x
R2 = 0.8344

moderate variability and good RNA recovery are well suited to a procedure that would be used in the laboratory for quantitative evaluation of *K. brevis* abundance. Both methods have drawbacks inherent in the methods, however, when applied to natural bloom samples. For the Durapore® filtration method, filtration of samples, even the relatively small amount of 20 ml, can be difficult with some estuarine samples if there is a high load of detritus or suspended sediment. With the centrifugation method the volume needed to detect bloom populations may exceed the capacity of available centrifuges, though this is not a likely scenario in a relatively well-equipped laboratory. The liquid extraction protocol is the least likely to be useful, despite demonstrating the lowest variability. While it may be valuable for comparative studies using cultures, where extracted volumes are small, it would quickly become exceedingly cumbersome and expensive with higher volumes of sample to be extracted. For example, for 20 ml of a bloom sample to be extracted, 30 ml of lysis buffer would be required, as well as 50 ml of 70% ethanol, totaling 100 ml of lysate that must be loaded onto the extraction column, either using a vacuum manifold or 200 consecutive 30 second centrifugation steps. For these reasons, unless automation is a requirement it is recommended that either the Durapore® or centrifugation method be used for reproducible extraction and subsequent quantification of RNA from *K. brevis*.

These calculations indicate that when all of the recommendations made regarding processing of the samples, the assay can be very accurate in returning concentrations of *K. brevis* in the marine environment. Briefly, these recommendations include: process samples as soon as possible; if immediate processing is not possible, samples may be

frozen for a maximum of 3-4 weeks; if freezing is not possible, samples should be filtered, placed in Stratagene's lysis buffer and kept in the dark for a maximum of 3 days before freezing or processing. With these recommendations implemented, the assay performs very well, with a tendency to slightly overestimate cell concentrations in seawater samples. As a tool for managing and monitoring harmful algal blooms caused by *K. brevis* this trend of slight overestimation is preferable to underestimation and should not be cause for concern regarding the use of this method.

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Appendices

Appendix 1

Media Recipes:

F/2 medium

To 950 ml of filtered seawater, add:

Quantity	Compound	Stock Solution (in deionized water)
1.0 ml	$NaNO_3$	75.0 g L^{-1}
1.0 ml	$NaH_2PO_4 \cdot H_2O$	5.0 g L^{-1}
1.0 ml	$Na_2SiO_3 \cdot 9H_2O$	30.0 g L^{-1}
1.0 ml	F/2 Trace Metal Solution	(See Recipe Below)
0.5 ml	F/2 Vitamin Solution	(See Recipe Below)

Adjust final volume to 1.0 L with filtered seawater, autoclave

Trace Metal Solution

To 950 ml deionized water, add:

Quantity	Compound	Stock Solution (in deionized water)
3.15 g	$FeCl_3 \cdot 6H_2O$	--
4.36 g	$Na_2EDTA \cdot 2H_2O$	--
1.0 ml	$CuSO_4 \cdot 5H_2O$	9.8 g L^{-1}
1.0 ml	$Na_2MoO_4 \cdot 2H_2O$	6.3 g L^{-1}
1.0 ml	$ZnSO_4 \cdot 7H_2O$	22.0 g L^{-1}
1.0 ml	$CoCl_2 \cdot 6H_2O$	10.0 g L^{-1}
1.0 ml	$MnCl_2 \cdot 4H_2O$	180.0 g L^{-1}

Adjust final volume to 1.0 L, autoclave.

Vitamin Solution
To 950 ml deionized water, add:

Quantity	Compound	Stock Solution (in deionized water)
1.0 ml	Vitamin B12 (Cyanocobalamin)	1.0 g L^{-1}
10.0 ml	Biotin	0.1 g L^{-1}
200 mg	Thiamine HCl	--

Adjust final volume to 1.0 L with deionized water. Filter sterilize into plastic vials and store in refrigerator.
*Note that f/2 media is high in Silicon and should only be used for culture of diatoms. For cultures of non-diatoms, use f/2-Si (omit the Na_2SiO_3 $9H_2O$)

2XYT

to 950 ml deionized water, add:
16.0 g Tryptone
10.0 g Yeast Extract
5.0 g NaCl
adjust volume to 1.0 L with deionized water and autoclave.
For agar plates, add 15.0 g Bacto Agar, boil to dissolve, then autoclave.
If antibiotics are needed, add 1.0 ml Kanamycin (50 mg/ml) and/or 1.0 ml Ampicillin (50 mg/ml).

SN medium

Quantity	Compound	Stock solution
2.5 ml	$NaNO_3$	300.0 g L^{-1}
2.6 ml	K_2HPO_4	6.1 g L^{-1}
5.6 ml	$Na_2EDTA•2H_2O$	1.0 g L^{-1}
2.6 ml	Na_2CO_3	4.0 g L^{-1}
1.0 ml	Vitamin B12	1.0 mg L^{-1}
1.0 ml	Trace metal solution	(see below)

Add ingredients aseptically to 1 L autoclaved 75% seawater in a Teflon bottle.

Appendix 1 (Continued)

Trace Metal Solution

Quantity	Compound
6.25 g	Citric acid • H_2O
6.0 g	Ferric Ammonium Citrate
1.4 g	$MnCl_2 • 4H_2O$
0.39 g	$Na_2MoO_4 • 2H_2O$
0.025 g	$Co(NO_3)_2 • 6H_2O$
0.222 g	$ZnSO_4 • 7H_2O$

Dissolve each metal compound individually in 100 ml deionized water. Combine and mix the solutions, then bring final volume to 1.0 L with deionized water.

Appendix 1 (Continued)

Strains cultured and their media requirements:

Organism	strain	media required	growth temperature
Dinoflagellates			
K. brevis	various	f/2 -Si	22º C
K. mikimotoi	CCMP430	f/2 -Si	16º C
Amphidinium carterae	CCMP1314	f/2 -Si	22º C
Akashiwo sanguinea	CCMP1321	f/2 -Si	22º C
Alexandrium tamarense	CCMP1493	f/2 -Si	22º C
Glenodinium foliacrum	N/A	f/2 -Si	22º C
Gymnodinium catenatum	CCMP1937	f/2 -Si	22º C
Gyrodinium sp.	N/A	f/2 -Si	22º C
Kryptoperidinium foliaceum	N/A	f/2 -Si	22º C
Lingulodinium polyedra	CCMP1738	f/2 -Si	22º C
Prorocentrum micans	N/A	f/2 -Si	22º C
Scrippsiella trochoidea	N/A	f/2 -Si	22º C
Scrippsiella precaria	N/A	f/2 -Si	22º C
Diatom			
Phaeodactylum tricornutum	CCMP1327	f/2	22º C
Raphidophyte			
Heterosigma akashiwo	N/A	f/2 -Si	22º C
Praisinophytes			
Tetraselmis sp.	850001	f/2 -Si	22º C
Tetraselmis sp.	CCMP961	f/2 -Si	22º C
Unidentified sp.	CCMP1536	f/2 -Si	22º C
Prymnesiophytes			
Isochrysis sp.	3C		
Pavlova lutheri	CCMP1325	f/2 -Si	16º C
Prymnesium parvum	N/A	f/2 -Si	22º C
Chlorophyte			
Clamydomonas euryale	CCMP219	f/2 -Si + NH4	22º C
Cyanophytes			
Synechococcus sp.	CCMP836	SN	22º C
Synechococcus sp.	WH7803	SN	22º C
Trebouxiophyte			
Chlorella autotrophica	CCMP243	f/2	16º C
Coscinodicsophyte			
Thalassiosira pseudonana	CCMP 1335	f/2	16º C

Appendix 2

Multiple sequence alignment of *rbcL* DNA sequences used in phylogenetic analysis

```
      Emiliana_huxleyi: GGTGGTCTAGACTTCCTTAAGGATGATGAGAACATTAACTCACAGCCATT
         ST8CH26_clone: ----------------------------------------C---CATT
            Isochrysis: GGTGGTCTAGACTTCCTTAAGGATGATGAGAACATTAACTCACAACCATT
          Calcidiscus: GGTGGTCTAGACTTCCTTAAGGATGATGAGAACATTAACTCACAACCATT
      Cruciplacolithus: GGTGGTCTAGACTTCCTTAAGGATGATGAGAACATTAACTCACAGCCGTT
         Pleurochrysis: GGTGGTCTAGATTTCCTTAAGGACGACGAGAACATTAACTCACAACCTTT
               Pavlova: GGTGGTCTGGACTTCCTTAAGGATGACGAAAACATCAACTCACAACCATT
      Exanthemachrysis: GGTGGTCTAGACTTCCTTAAGGATGACGAGAACATTAACTCACAACCATT
           Pyrenomonas: GGCGGTCTTGACTTCCTTAAGGATGATGAAAACATCAACTCTCAACCATT
            Chroomonas: GGTGGTTTAGACTTCCTTAAAGATGATGAAAACATTAACTCTCAACCATT
              Rhodella: GGTGGATTAGACTTCTTAAAGGATGATGAAAACATTAACTCTCAACCGTT
             Dictyocha: GGTGGTCTTGATTTCCTTAAAGATGATGAGAATATTAACTCACAAGCGTT
         ST6CH33_clone: ----------------------------------------C---CATT
           Heterosigma: ATTTTTTCTTAGACTGTACACTAACGTACAAAAATTAAAAAGGAGGAATA
           Chattonella: GGTGGTCTTGACTTCTTAAAAGATGATGAGAACATTAACTCTCAACCATT
         ST4CH31_clone: ----------------------------------------C---CATT
          ST1CH4_clone: ----------------------------------------CAACCATT
         ST3CH27_clone: ----------------------------------------CAAGCATT
            Ectocarpus: GGTGGTCTTGACTTCCTTAAAGATGATGAAAATATTAATTCACAACCATT
            Xanthonema: GGTGGTCTTGACTTCTTAAAAGATGATGAAAACATTAACTCTCAACCATT
             Vaucheria: GGTGGTTTAGATTTCTTAAAAGATGATGAGAATATTAATTCTCAACCATT
          Heterococcus: GATATGCGAATTCCTTTTGCTTACCTAAAAACATTCCAAGGTCCTGCAAC
            Aureoumbra: GGTGGTTTAGACTTTTTAAAGGATGATGAAAACATTAACTCGCAACCTTT
           Aureococcus: GGTGGTTTAGACTTCTTAAAGGATGATGAGAACATTAACTCACAACCATT
          Pelagococcus: GGTGGTTTAGACTTCTTAAAGGATGACGAGAACATTAACTCACAACCATT
         Cylindrotheca: GGTGGTTTAGACTTTTTAAAAGATGATGAAAACATTAACTCACAACCATT
         Phaeodactylum: GGTGGTTTAGACTTCTTAAAAGATGATGAGAATATTAACTCACAACCCTT
         ST4CH14_clone: ----------------------------------------C---CATT
          ST6CH2_clone: ----------------------------------------C---CATT
          Rhizosolenia: GGTGGTTTAGACTTCTTAAAAGATGATGAAAACATTAACTCACAACCATT
           Skeletonema: GGTGGTTTAGACTTCTTAAAGGATGATGAAAATATTAACTCTCAACCATT
         Thalassiosira: GGTGGTTTAGACTTCTTAAAAGATGATGAAAACATTAACTCTCAACCATT
               Detonula: CGTCGTTGTATGCCAGTAGCTTCTGGTGGTATCCACTGTGGTCAAATGCA
            Chilomonas: GGTGGCTTGGACTTCCTTAAGGATGATGAAAACATTAATTCTCAGCCTTT
Mazzaella_membranacea: GGTGGTTTAGACTTCTTAAAAGATGATGAGAATATTAATTCTCAGCCTTT
       Karenia_brevis: GGGGGATTAGATTTTCTTAAAGATGATGAAAATATTAATTCGCAGCCTTT
       Jaxonville C3a: ----------------------GATGATGAAAATATTAACTCGCAGCCTTT
      Mexico_Beach_C5: ----------------------GATGATGAAAATATTAACTCGCAGCCTTT
      Piney_Island_A9: ----------------------GATGATGAAAATATTAACTCGCAGCCTTT
  Charlotte_Harbor_C2: ----------------------GATGATGAAAATATTAACTCGCAGCCTTT
  Charlotte_Harbor_A2: ----------------------TATGATGAAAATATTAACTCGCAGCCTTT
      Piney_Island_B4: ----------------------GATGATGAGAATATTAACTCGCAGCCTTT
      Mexico_Beach_B3: ------------------------GATGAGAACATTAACTCGCAGCCTTT
     Appalachicola_C6: ----------------------GATGATGAGAATATTAACTCGCAGCCTTT
  Karenia_mikimotoi_1: ----------------------GATGATGAGAATATTAACTCACAACCGTT
    Karenia_mikimotoi: GGAGGCTTGGATTTCTTAAAGGATGATGAGAACATTAACTCACAACCGTT
          Karlodinium: GGCGGTTTGGATTTTCTTAAAGATGATGAAAATATCAATTCTCAACCTTT

        Amphidinium: CTTGCTAACATGTCAAGCGAAGCCCTGTCGACGGTCTTCACCATAGGTGC
       ST2SY33_clone: GGTGGTCTCGATTTAACGAAGGATGACGAGAATATAAACTCTCAGCCATT
     Prochlorococcus: GGCGGTCTTGATTTAACGAAGGATGATGAGAATATTAATTCTCAACCATT
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Appendix 2 (Continued)

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          Thiobacillus: CTACGTCAAGACCTGCGGTGGGCCACCCCACGGGATTCAGGTCGAACGCG
      Synechococcus_sp: GGTGGTTTGGACTTCACCAAAGATGACGAAAACATCAACTCCCAGCCTTT
         ST2SY26_clone: GGTGTTTTGNACTTCACCAAAGACGACGAGAACATCAACTNTCAGCCCTT
         Synechococcus: GTTCATCAAGAGCTGCTACGGCCCGCCGAACGGCATCCAGGTCGAGCGCG
           Nitrobacter: TTACGTGAAGACCTGCGGCGGCCCTCCGCATGGTATCCAGGTCGAGCGCG
         Synechocystis: GGTGGTTTGGACTTCACCAAAGACGACGAAAACATCAACTCCCAGCCCTT
Synecchococcus_PCC7002: GGTGGTCTTGACTTCACCAAAGATGACGAAAACATCAACTCTCAGCCTTT
         ST8SY15_clone: GGAGGTCTAGACTTCACCAAGGATGACGAAAATATTAACTCTCAACCTTT
         Trichodesmium: GGAGGTCTAGACTTCACCAAGGATGACGAAAATATTAACTCTCAACCTTT
          ST5SY7_clone: GGTGGTTTAGATTTTACTAAGGATGACGAGAACGTAAATTCTCAACCTTT
          ST2SY2_clone: ------------------------GATGAGAACGTAAACTCACAACCCTT
          ST6SY8_clone: GGTGGTCTTGACTTCACTAAGGATGATGAGAACGTAAACTCCCAGCCTTT
        Volvox_carteri: GATCTTCGTATTCCACCTGCTTACGTTAAAACATTCCAAGGTCCACCACA
               Spinacia: GGTGGACTTGATTTTACCAAAGATGATGAAAACGTGAACTCCCAGCCGTT
             Asparagus: GGTGGGCTTGATTTTACCAAGGATGATGAAAACGTGAACTCACAACCTTT
              Thalassia: GATTTGCGAATTCCCCCTTCCTATTCCAAAACTTTCCAAGGTCCACCTCA
            Prymnesium: GGTGGTCTTGATTTCCTTAAGGATGATGAGAACATTAACTCTCAACCATT
   Chrysochromulina_sp: GGTGGTCTTGACTTCCTAAAGGATGATGAGAACATTAACTCTCAGCCATT
           Imantionia: GGTGGTCTTGACTTCCTAAAGGATGACGAGAACATTAACTCGCAGCCTTT
         ST1CH3_clone: ----------------------------------------C---CATT
        Helicosphaera: GGAGGTCTAGATTTCCTTAAGGATGATGAGAACATCAACTCACAACCATT

      Emiliana_huxleyi: CATGCGTTACCGTGAGCGTTTCCTTTACTCAATGGAAGGTGTTAACCATG
         ST8CH26_clone: CATGCGTTACAGAGAGCGTTTCTTATACTCAATGNAAGGTGTTAACCACG
             Isochrysis: TATGCGTTACCGTGAGCGTTTCCTTTACTCAATGGAAGGTGTTAACAACG
          Calcidiscus: TATGCGTTACAGAGAGCGTTTCCTTTACTCAATGGAAGGTGTAAACCACG
      Cruciplacolithus: CATGCGTTACAGAGAGCGTTTCCTTTACTCAATGGAAGGTGTAAACCACG
         Pleurochrysis: CATGCGTTACCGCGAGCGTTTCCTTTACTCAATGGAAGGTGTAAACCATG
               Pavlova: TATGCGTTGGAGAGAGCGTTTCCTATTCTCGATGGAAGGTGTTAACCGTG
      Exanthemachrysis: CATGCGTTGGAGAGAGCGTTTCCTATTCTCAATGGAAGGTGTTAACCGTG
           Pyrenomonas: CATGAGATGGAAAGAGCGTTTCTTATTCGGTATGGAAGGTGTTAACAGAG
            Chroomonas: CATGAGATGGAAAGAGCGTTTCTTATTTGGTATGGAAGGTGTTAACCGTG
              Rhodella: TATGCGTTACAGAGAGCGTTTCTTATTCTCTATGGAAGGTGTAAACCGTG
             Dictyocha: CATGCGTTGGAGAGAACGTTTCCTTTACTGTCAAGAAGGTATTCAACGTG
         ST6CH33_clone: CATGCGTTGGAGAGAGCGTTTCTTATACGTAATGGAAGGTGTTAACCGTG
           Heterosigma: CATGTCTAACAACGTATACGAACGTAACCGAATCAAAAATGATCGTTATG
           Chattonella: CATGCGTTGGAGAGAGCGTTTCCTTTACTGTATGGAAGGTATTAATCGTG
         ST4CH31_clone: CATGCGTTGGAGAGAACGTTTCCTATACTGTATGGAAGGTATTAATCGTG
          ST1CH4_clone: CATGAGATGGCGTGAAAGATACTTATACGTTATGGAAGGAATTAACAAAG
         ST3CH27_clone: CATGAGATGGAGAGAAAGATTCTCTTACGTAATGGAAGGTGTTAACCGTG
            Ectocarpus: TATGCGTTGGAAAGAACGTTTCTTATACTGTATGGAAGGTGTTAACCGTG
            Xanthonema: CATGCGTTGGAGAGAACGTTTCCTTTACTGTATGGAAGGTATTAACCGTG
             Vaucheria: TATGCGTTGGAGAGAGCGTTTTCTTTACTGTATGGAAGGTGTTAATCGTG
          Heterococcus: TGGTGTTATTGTAGAAAGAGAAAGAATGGACA---AATTCGGTCGCCCTT
            Aureoumbra: CATGCGTTGGCGTGAGCGTTTCTTATACTGTATCGAAGGTATTAATCGTG
           Aureococcus: CATGCGTTGGCGTGAGCGTTTCATCTACTGTGTAGAGGGTATCAACCGTG
          Pelagococcus: CATGCGTTGGCGTGAGCGTTTCTTATACTGTGTAGAAGGTATTAACCGTG
         Cylindrotheca: TATGCGTTGGAGAGAACGTTTCTTAAACTGTATGGAAGGTATTAACCGTG
         Phaeodactylum: CATGCGTTGGAGAGAACGTTTCTTATACTGTATGGAAGGTATTAATCGCG
         ST4CH14_clone: CATGCGTTGGAGAGAGCGTTTCTTAAACTGTATGGAAGGTATCAACCGTG
          ST6CH2_clone: CATGCGTTGGAGAGAGCGTTTCTTATACTGTATGGAAGGTATTAACCGTG
          Rhizosolenia: CATGCGTTGGAGAGAACGTTTCTTATACTGTATGGAAGGTATTAACCGTG
           Skeletonema: CATGCGTTGGAGAGAACGTTTCTTAAACTGTATGGAAGGTATTAACCGTG
         Thalassiosira: TATGCGTTGGAGAGAACGTTTCTTAAACTGTTTAGAAGGTATCAACCGTG
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Appendix 2 (Continued)

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            Detonula: TCAATTAATTCATTACTTAGGTGATGATGTTGTATTACAATTCGGTGGTG
          Chilomonas: TATGAGATGGAGAGAACGCTTCCTATTTGGGATTGAAGGAGTTAGCAGAG
Mazzaella_membranacea: CATGCGTTGGAAAGAAAGATACCTATATTCAATGGAAGGTGTAAACCGTG
       Karenia_brevis: TATGCGGTATCGTGAACGTTTCCTATACTCTATGGAAGGTGTCAATCATG
       Jaxonville C3a: TATGCGGTATCGTGAACGTTTCCTATACTCTATGGAAGGTGTCAATCATG
      Mexico_Beach_C5: TATGCGGTATCGTGAACGTTTCCTATACTCTATGGAAGGTGTCAATCATG
      Piney_Island_A9: TATGCGGTATCGTGAACGTTTCCTATACTCTATGGAAGGTGTCAATCATG
  Charlotte_Harbor_C2: TATGCGGTATCGTGAACGTTTCCTATACTCTATGGAAGGTGTCAATCATG
  Charlotte_Harbor_A2: TATGCGGTATCGTGAACGTTTCCTATACTCTATGGAAGGTGTCAATCATG
      Piney_Island_B4: TATGCGGTATCGTGAACGTTTCCTATACTCTATGGAAGGTGTCAATCATG
      Mexico_Beach_B3: TATGCGGTATCGTGAACGTTTCCTATACTCTATGGAAGGTGTCAATCATG
     Appalachicola_C6: TACGCGGTATCGTGAACGTTTCCTATACTCTATGGAAGGTGTCAATCATG
  Karenia_mikimotoi_1: TATGCGTTACCGAGAACGGTTTCTTTTCTCAATGGAAGAGGTGAATCATG
    Karenia_mikimotoi: TATGCGTTATCGAGAACGGTTCCTTTTCTCAATGGAAGCGGTGAATCATG
          Karlodinium: TATGAGGTATAGAGAAAGGTTTTTATTCTCCATGGAAGGGGTGAATCATG
          Amphidinium: CGGTGATGCAACGCTCACCAGAGCGCACCCAGGGATAAAGGCTGGGCAGC
        ST2SY33_clone: CCAACGTTGGAGAGAAAGATTTGAGTTTGTTGCAGAAGCAGTTAAGCTTG
       Prochlorococcus: CCAACGTTGGAGAGAAAGATTTGAGTTTGTTGCAGAAGCAGTTAAGCTTG
          Thiobacillus: ACATCATGAACAAGTATGGTCGCCCATTGCTGGGCTGCACCATCAAACCC
     Synechococcus_sp: CCAACGCTGGCGCGATCGCTTCCTGTTTGTTGCTGATGCCATTCACAAAG
        ST2SY26_clone: CNAGCGTTGGCAGAACCGCTTCGAATTCGTTGCGGAAGCCATCAAGCTGT
       Synechococcus: ACCGGATGAACAAGTACGGCCGTCCCCTGCTGGGTTGCACCATCAAGCCG
         Nitrobacter: ACAAGCTTAACAAGTACGGCCGCCCGCTGTTGGGCTGCACCATCAAGCCG
       Synechocystis: CATGCGTTGGCGCGATCGTTTCCTCTTCGTTCAAGAGGCGATCGAAAAAG
Synecchococcus_PCC7002: CATGCGTTGGCGCGATCGCTTCCTGTTCGTTCAAGAAGCTATCGAAAAAT
        ST8SY15_clone: CATGCGTTGGCGCGATCGCTTCCTATTCGTTCAAGAAGCCATTGAAAAGG
        Trichodesmium: CATGCGTTGGCGCGATCGCTTCCTATTCGTTCAAGAAGCCATTGAAAAGG
         ST5SY7_clone: TATGAGATGGAGAGATAGATTCTTATTTGTTTCTGAAGCAATTTATAAAG
         ST2SY2_clone: TCAGCGTTGGCGTGATCGTTTCCTATTCATTGCTGAGGCTACACACAAGT
         ST6SY8_clone: CATGCGTTGGCGTGATCGTTTCCTTTTCTGTGCTGAGGCTATTTACAAGG
       Volvox_carteri: CGGTATTCAGGTTGAACGTGACAAACTAAACA---AATATGGTCGTGGTC
             Spinacia: TATGCGTTGGAGAGACCGTTTCCTATTTTGTGCCGAAGCTCTTTATAAAG
           Asparagus: TATGCGTTGGCGAGACCGTTTCGTATTTTGTGCTGAAGCTCTTTATAAAG
           Thalassia: TGGAATCCAAGTGGAAAGAGATAGATTGAACA---AATACGGCCGCCCTC
          Prymnesium: CATGCGTTACAGAGAGCGTTTCCTTTATTCAATGGAAGGTGTTAACCACG
 Chrysochromulina_sp: CATGCGTTACAGAGAGCGTTTCCTTTACTCAATGGAAGGTGTTAACCACG
           Imantionia: CATGCGTTACAGAGAGCGTTTCCTTTACTCAATGGAAGGTGTTAACAACG
        ST1CH3_clone: TATGCGTTTCAGAGAGCGTTTCCTATACTCTATGGAAGGTGTAAACCACG
       Helicosphaera: CATGCGTTACAGAGAGCGTTTCCTATACTCAATGGAAGGTGTAAACCACG

     Emiliana_huxleyi: CAGCTTGTCTAACTGGTGAAGTTAAAGGTCACTACCTTAACACAACTGCG
        ST8CH26_clone: CTGCTTGTCAGACTGGTGAAGTTAAAGGTCACTACCTTAACGCAACAGCT
           Isochrysis: CTGCTGCAACAACTGGTGAGGTTAAGGGTCACTACCTTAACACAACTGCA
         Calcidiscus: CTGCTGCAACAACTGGTGAGGTTAAAGGTCACTACCTTAACGCAACAGCT
     Cruciplacolithus: CTGCTGCAATGTCTGGTGAAGTTAAGGGTCACTACCTTAATGCAACAGCT
        Pleurochrysis: CAGCGGCAGTAAGTGGGGAAGTAAAAGGGCACTACCTTAACGCAACGGCA
              Pavlova: CTGCAGCTGGTTCTGGAGAGGTTAAAGGTCACTACCTAAATGCAACAGCT
     Exanthemachrysis: CAGCGGCAGCTTCTGGAGAAGTTAAAGGTCACTACCTAAACGCTACAGCT
          Pyrenomonas: CTGCTGCTGGTACTGGTGAGATTAAAGGTCACTACTTCAACATTACTGCA
           Chroomonas: CTGCTGCTGCTGCAGGTGAAGTTAAAGGACATTACTTTAACGTTACAGCT
             Rhodella: CTTCTGCAAGTTCTGGTGAAGTTAAAGGTCATTACCTTAACGTCACAGCA
            Dictyocha: CCGCAGCAGCTACTGGTGAAGTTAAAGGTNNNTACCTTAATGGTACTGCT
        ST6CH33_clone: CAGCAGCTGCTTCTGGTGAAGTTAAAGGTTCTTACTTAAATGCTACTGCA
          Heterosigma: AATCAGGTGTAATCCCTTACGCTAAAATGGGTTATTGGGATGCTAATTA-
          Chattonella: CTTCTGCTGCTTCAGGTGAAGTTAAAGGTTCTTACTTAAACTGTACAGCT
        ST4CH31_clone: CTTCAGCTGCAACTGGTGAAACAAAAGGTTCATACCTAAACATTACAGCA
         ST1CH4_clone: CAGCAGCTAAAACTGGTGCAGTTAAAGGTTCTTACTTAAACGTAACGGCT
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Appendix 2 (Continued)

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          ST3CH27_clone: CTGCTACTGCAAGTGGTGAAGTTAAAGGTTCTTACTTAAACGTAACTGCT
             Ectocarpus: CTGCAGCTGCAACAGGTGAAGTTAAAGGTTCTTATCTTAACATTACAGCC
             Xanthonema: CTGCTGCTGCAACAGGTGAAGTTAAAGGTTCTTACTTAAACGTTACAGCT
              Vaucheria: CAGTAGCAGCTAGTGGTGAAGTTAAAGGTTCATACCTTAATACAACTGCA
           Heterococcus: TATTAGGTGCAACGGTAAAACCAAAATTAGGTCTTTCAGGTAAAAACTAC
             Aureoumbra: CCGTTTCAGTAACAGGTGAGGTTAAGGGTTCTTACTTAAACATTACTGCT
            Aureococcus: CCGCTGCTGCAACTGGTGAAGTTAAAGGTTCTTACTTAAACATTACTGCT
           Pelagococcus: CTGCTGCTGCAACTGGTGAAGTTAAGGGTTCTTACTTAAACATGACTGCT
          Cylindrotheca: CTTCTGCTGCTACAGGTGAAGTTAAAGGTTCTTATTTAAACGTTACAGCT
          Phaeodactylum: CTTCTGCAGCTACAGGTGAAACTAAAGGTTCATACCTAAACATCACAGCT
          ST4CH14_clone: CTGCTGCTGCTACTGGTGAAATTAAAGGTTCTTACTTAAACATCACTGCA
           ST6CH2_clone: CTTCAGCAGCTACTGGTGAAGTTAAAGGTTCTTACTTAAACGTAACTGCT
           Rhizosolenia: CTGCTGCCGCTACTGGTGAAGTTAAAGGTTCTTACTTAAACATTACTGCT
            Skeletonema: CATCAGCTGCAACTGGTGAAGTTAAAGGTTCTTACTTAAACATTACTGCT
          Thalassiosira: CAGCTGCTGCAACTGGTGAAGTTAAAGGTTCTTACTTAAACATTACTGCT
               Detonula: GTACTATTGGTCACCCTGATGGTATTCAAGCTGGTGCTACAGCAAACCGT
             Chilomonas: CTTCTGCTTCTTCTGGTGAAGTAAAAGGTCACTACTTTAACATCACTGCG
 Mazzaella_membranacea: CTATTGCTGCTGCTGGTGAAACAAAGGGGCATTATTTAAATGTAACAGCA
         Karenia_brevis: CTGCCGCAAAAACCGGTGAAATCAAAGGACATTATTTAAATGTCACGGCA
        Jaxonville C3a: CTGCCGCAAAAACCGGTGAAATCAAAGGACATTATTTAAATGTCACGGCA
       Mexico_Beach_C5: CTGCCGCAAAAACCGGTGAAATCAAAGGACATTATTTAAATGTCACGGCA
       Piney_Island_A9: CTGCCGCAAAAACCGGTGAAATCAAAGGACATTATTTAAATGTCACGACA
   Charlotte_Harbor_C2: CTGCCGCAAAAACCGGTGAAATCAAAGGACATTATTTAAATGTCACGGCA
   Charlotte_Harbor_A2: CTGCCGCAAAAACCGGTGAAATCAAAGGACATTATTTAAATGTCACGGCA
       Piney_Island_B4: CTGCCGCAAAAACCGGTGAAATCAAAGGACATTATTTAAATGTCACGGCA
       Mexico_Beach_B3: CTGCCGCAAAAACCGGTGAAATCAAAGGACATTATTTAAATGTCACGGCA
      Appalachicola_C6: CTGCCGCAAAAACCGGTGAAATCAAAGGACATTATTTAAATGTCACGGCA
   Karenia_mikimotoi_1: CTGCTGCTAAGTCAGGGGAAATCAAAGGTCATTATTTAAACGTAACTGCG
     Karenia_mikimotoi: CTGCTGCTCAGTCAGGAGAAATAAAAGGTCATTACCTAAACATAACTGCG
           Karlodinium: CTTCTTGTTTAACGGGTGAGGTTAAAGGACATTATTTAAATGTTACTGGG
            Amphidinium: TTCGTGGCGTTCCCATGAGTATGGACGCTGCCGAACTTTTGCCCACCGCA
          ST2SY33_clone: CTCAGCAAGAAACTGGTGAAGTTAAAGGTCACTACCTAAACTGTACTGCC
       Prochlorococcus: CTCAGCGAGAAACTGGAGAAGTTAAAGGTCACTATCTAAATTGTACTGCT
          Thiobacillus: AAGCTGGGTTTGTCGGCCAAGAACTATGGTCGCGCTTGCTATGAGGGTCT
      Synechococcus_sp: CCCAGGCGGAAACCGGTGAAATCAAAGGGCACTATTTGAACGTCACCGCT
         ST2SY26_clone: CCGAGCAGGAGACCGGCGAGCGCAAGGGTCACTACCTCAACGTGACCGCC
         Synechococcus: AAGCTCGGCCTGAGCGGCAAGAACTACGGCCGTGTGGTCTATGAGTGCCT
           Nitrobacter: AAGCTTGGTCTGTCCGCGAAGAACTACGGCCGCGCCTGCTATGAAGCGCT
         Synechocystis: CCCAGGCTGAGACCAACGAAATGAAAGGTCACTACCTGAACGTCACCGCT
Synecchococcus_PCC7002: CCCAAGCTGAAACCAACGAAGTTAAGGGTCACTACCTTAACGTCACCGCT
          ST8SY15_clone: CCCAAGCAGAAACAGGTGAAATTAAAGGTCACTACCTCAACGTAACTGCC
          Trichodesmium: CCCAAGCAGGAACAGGTGAAATTAAAGGTCACTACCTCAACGTAACTGCC
           ST5SY7_clone: CTCAAGCAGAAACAGGAGAAATTAAAGGTCATTACTTAAATGCTACTGCA
           ST2SY2_clone: CTCAAGCAGAGACTGGTGAGATCAAGGGTCACTACCTAAACGCTACTGCT
           ST6SY8_clone: CACAAGGTGAAACTGGTGAGATTAAGGGTCACTACCTTAACGCAACTGCT
         Volvox_carteri: TTTTAGGTTGTACAATCAAACCTAAATTAGGTCTTTCAGCTAAAAACTAC
               Spinacia: CACAAGCCGAAACAGGCGAAATCAAAGGGCATTACTTGAATGCTACCGCG
              Asparagus: CACAAGCAGAAACAGGTGAAATCAAAGGCCATTACTTGAATGCAACTGCA
              Thalassia: TACTAGGATGTACTATTAAACCAAAATTGGGATTATCCGCGAAAAACTAT
             Prymnesium: CAGCAGCAATGACTGGTGAAGTTAAAGGTCACTACCTAAATGCAACGGCA
   Chrysochromulina_sp: CATCTGCACTAACTGGTGAAGTTAAGGGTCACTACCTAAACTCTACTGCG
             Imantionia: CAGCTGCTCACAGTGGTGAGGTTAAAGGTCACTACCTAAACACTACTGGT
           ST1CH3_clone: CAGCAGCAATGTCTGGTGAGGTTAAAGGTCACTACCTAAACACTACTGCT
         Helicosphaera: CAGCTGCAATGACAGGTGAAGTTAAAGGTCACTACCTTAACACAACTGCT

      Emiliana_huxleyi: GCAACAATGGAAGATATGTACGAACGTGCTAACTTCGCAAGAGATCTAGG
         ST8CH26_clone: TCAACTATGGAAGACATGTACGAGCGTTCTGAGTTCTGTTACGAATTAGG
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Appendix 2 (Continued)

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              Isochrysis: GCTACAATGGAAGATATGTACGAGCGTGCTGACTTCGCTAAGGAGTTAGG
             Calcidiscus: GCAACAATGGAAGATATGTATGAGCGTGCAGAATTCTGTAAAGAGCTTGG
       Cruciplacolithus: GCAACAATGGAAGATATGTATGAGCGCGCAGAATTCTCTAAGGAGCTTGG
          Pleurochrysis: GCGACGATGGAAGATATGTACGAGCGTGCTGAATTCGCTAAGGATTTAGG
                Pavlova: TCTACTATGGAAGAAATGTATACGCGTGCTGACTACGCTAAGGAGCTAGG
       Exanthemachrysis: GGTACTATGGAAGACATGTACGAGCGTGCTGACTACTCTAGAGATCTAGG
            Pyrenomonas: GGTACAATGGAAGATATGTACGAGCGTGCTGAATTCTGTAAAGAAATCGG
             Chroomonas: GGTACAATGGAAGAAATGTATGCTCGTGCTGAATTCTGTTCAGATTTAGG
               Rhodella: GCAACTATGGAAGATATGTACGAGCGTGCTGAATTCTCTAAAGCAGTTGG
              Dictyocha: GGTACTATGGAAGNNGTTTACGAACGTGCTGAATATGCTAAGGAACTTGG
          ST6CH33_clone: GCGACTACGGAAGAAATGTACGTTCGTGCAGATTACGCTAAGTCTGTTGG
            Heterosigma: --TTCTGTTAAAGATACAGATTTATTAGCTTTATTCCGTCTTACACCACA
             Chattonella: GCTACAATGGAAGAAATGTACGAACGTGCAGAATATGCTAAGTCTGTTGG
          ST4CH31_clone: GCTACAATGGAAGAAGTTTACAAACGTGGTGATTATGCGAAAGCAGTTGG
           ST1CH4_clone: GCAACTATGGACGAAATGTATGAAAGAGCTGAATTCGCAAAAGAAGTTGG
          ST3CH27_clone: GGTACTATGGAGCAAATGTACGAACGTGCTGAATTCGCTAAAATCATTGG
             Ectocarpus: GCAACTGTAGAGCAAATGTATGAACGTGCAGAGTATGCTCATGCAATTGG
             Xanthonema: GGTAACATGGAAGACATGTACGAACGTGCTGAATACGCTAAAACAATTGG
              Vaucheria: GGTACAATGGAAGAGATGTATGAACGTGCAGAATATGCTAAAAGTATAGG
           Heterococcus: GGTCGTGTAGTATATGAAGGTCTTCGTGGTGGTCTTGATTTCTTAAAAGA
             Aureoumbra: GGTACTATGGAAGAAATGTACACACGTGCAGAGTACGCTAAGGAACTTGG
            Aureococcus: GCAACAATGGAAGAAATGATCATTCGTGCAGAGTACGCTAAAGAGTTAGG
           Pelagococcus: GGTAACAACGATGAAATGATCACTCGTGGTGAGTACGCTAAAGAGATCGG
         Cylindrotheca: GCTACTATGGAAGAAGTATACAAACGTTCTGAGTATGCTAAAGAAGTAGG
         Phaeodactylum: GGTACTATGGAAGAAGTTTACAAACGTGCAGAATATGCTAAAACAGTAGG
         ST4CH14_clone: GGTACAATGGAAGAAGTTTACAAACGTGCAGAATATGCTAAATCTGTAGG
          ST6CH2_clone: GCCACTATGGAAGAAGTTTACAAACGTGCAGAATATGCTAAACAAGTTGG
          Rhizosolenia: GCAACATACGAAGAAGTAGAAAAACGTGCTGACTATGCTAAAGCTGTTGG
           Skeletonema: GCTACAATGGAAGAAGTATACAAACGTGCTGAGTATGCTAAAGCTGTTGG
         Thalassiosira: GCTACTATGGAAGAAGTATACAAACGTGCTGAGTATGCTAAAGCTATTGG
              Detonula: GTTGCTTTAGAAGCAATGGTTTTTTCTCGTAACGAAGGTGCTGACTACTT
            Chilomonas: GGAACAATGGAAGACATGTATGAGAGGGCTAATTTTGCTAAAGAAATTGG
 Mazzaella_membranacea: GCTACAATGGAACAAATATATGAAAGAGCTGAATTTGCTAAAGAGCTTGG
        Karenia_brevis: GCGACGATGGAAGACATGTATGAAAGAGCGAATTTCGCTACGGAACTAGG
       Jaxonville C3a: GCGACGATGGAAGACATGTATGAAAGAGCGAATTTCGCTACGGAACTAGG
      Mexico_Beach_C5: GCGACGATGGAAGACATGTATGAAAGAGCGAATTTCGCTACGGAACTAGG
       Piney_Island_A9: GCGACGATGGAAGACATGTATGAAAGAGCGAATTTCGCTACGGAACTAGG
   Charlotte_Harbor_C2: GCGACGATGGAAGACATGTATGAAAGAGCGAATTTCGCTACGGAACTAGG
   Charlotte_Harbor_A2: GCGACGATGGAAGACATGTATGAAAGAGCGAATTTCGCTACGGAACTAGG
       Piney_Island_B4: GCGACGATGGAAGACATGTATGAAAGAGCGAATTTCGCTACGGAACTAGG
      Mexico_Beach_B3: GCGACGATGGAAGACATGTATGAAAGAGCGAATTTCGCTACGGAACTAGG
      Appalachicola_C6: GCGACGATGGAAGACATGTATGAAAGAGCGAATTTCGCTACGGAACTAGG
   Karenia_mikimotoi_1: GCAACAATGGAGAACATGTATGAAAGAGCAAATTTCGCTGGTGAACTTGG
     Karenia_mikimotoi: GCAACAATGGAGGACATGTATGAAAGAGCAGATTTCGCCGCCGAACTTGG
           Karlodinium: GCAACCATGGAAGATCTTTATGAACGTTCAGACCTATGTTATCAACTAGG
           Amphidinium: GAGAAGGGTGCGAGTTCTTCTTCTGTCGTTCTTGGATCTGTGGCCGTTGG
         ST2SY33_clone: AACACTCCTGAAGAACTTTACGAAAGAGCTGAATTTGCAAAAGAGCTAGA
       Prochlorococcus: AACACTCCTGAAGAACTCTATGAAAGAGCTGAATTTGCAAAAGAGCTCGA
          Thiobacillus: GCGTGGTGGACTCGACTTCACTAAAGATGACGAAAACGTCAATAGCCAGC
      Synechococcus_sp: CCCACCTGTGAAGAAATGTTGAAACGGGCAGAGTTCGCTAAAGA---CTG
         ST2SY26_clone: AACACTCCCGAAGAGATGTACGAGCGCGCTGAGTTCGCCAAGGAACTCGG
         Synechococcus: GCGTGGCGGTCTGGACTTCACCAAGGACGACGAGAACATCAACTCCCAGC
           Nitrobacter: ACGTGGCGGTCTGGACTTCACCAAGGACGACGAGAACATCAACAGCCAGC
         Synechocystis: GGCACCTGCGAAGAAATGATGAAACGGGCCGAGTTTGCCAAGGAAATTGG
Synecchococcus_PCC7002: GGCACTTGCGAAGAAATGCTCAAGCGGGCTGAATTCGCTAAGGAAATCGG
         ST8SY15_clone: CCTACCTGTGAAGAAATGTTAGAGCGTGCAGATTTTGCTAAGGAAATTGG
         Trichodesmium: CCTACCTGTGAAGA---ATGTAGACGTGCAGTTTTTGCTAAGGAAATTGG
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Appendix 2 (Continued)

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          ST5SY7_clone: GGTACTTGTGAAGAGATGTTAAAAAGAGGTGAATGTGCTAAAGAATTAGG
          ST2SY2_clone: GGTCACTGTGATGAGATGCTTAAGCGTGCAGCATTTGCTCGTGAGATCGG
          ST6SY8_clone: GGTACGAATGAGGAGATGATGAAGCGTGCTGAGTTCGCTGTTGATCTTGG
        Volvox_carteri: GGTCGTGCAGTTTACGAATGTTTACGTGGTGGTTTAGACTTTACTAAAGA
              Spinacia: GGTACATGCGAAGATATGATGAAAAGGGCTGTATTTGCCAGAGAATTGGG
             Asparagus: GGTACATGTGAAGAAATGATGAAAAGGGCCATATTTGCCAGAGAATTGGG
             Thalassia: GGTAGAGCAGTTTATGAATGTCTACGTGGTGGACTGGATTTTACTAAAGA
            Prymnesium: GCTACTATGGAAGATATGTACGAACGTGCAGAGTTCTGTAAAGAGCTAGG
  Chrysochromulina_sp: GCTACTATGGAAGATATGTACGAACGTGCTGATTTCGCTAAAGAGCTAGG
           Imantionia: GCTACGATGGAAGATATGTACGAGCGTGCAAACTTCGCGAAAGATCTAGG
         ST1CH3_clone: TCTACAATGGAAGATATGTATGAGCGTGCTGATTTAGCATGTGAATTAGG
        Helicosphaera: GCAACTATGGAAGATATGTACGAGCGTGCAGATTTTGCAAAAGAACTTGG

     Emiliana_huxleyi: TTCAGTTATTGTAATGATTGACCTTGTAATTG---GTTATA--------C
        ST8CH26_clone: TTCAGTTATTGTAATGATTGACCTTGTAA---TCGGTTATA--------C
           Isochrysis: TTCAGTTATTGTTATGATCGACCTTGTAATCG---GTTATA--------C
         Calcidiscus: TTCTGTAATTGTGATGATCGACCTTGTAATTG---GTTATA--------C
     Cruciplacolithus: TTCAGTAATTACAATGATTGACCTTGTAATTG---GTTACA--------C
        Pleurochrysis: TTCAGTTATTGTTATGATTGACCTTGTGATTG---GTTATA--------C
              Pavlova: TTCAATCATTGTTATGATTGACCTTGTTATTGGA---TACA--------C
     Exanthemachrysis: TTCAATCATTGTTATGATCGACCTTGTAATCG---GATACA--------C
          Pyrenomonas: TACTGTTATTTGTATGATCGACCTTGTAATCGGT---TACA--------C
           Chroomonas: CTCTGTAATTTGTATGATCGACCTTGTAATTGGT---TATA--------C
             Rhodella: AAGCATCATTTGTATGATCGACCTTGTAATTGGT---TATA--------C
            Dictyocha: TACTATTGGTATTATGAATGACCTTGGTATGG---GTTATA--------C
        ST6CH33_clone: TACTATCATTATCATGATTGACCTTGTAA---TTGGTTACA--------C
          Heterosigma: ACCTGGAGTAGACCCTGTGGAAGCTGCAGCGGCAGTAGC--AGGTGAATC
          Chattonella: TTCTGTAATTATCATGATTGACCTTGTAATTG---GTTACA--------C
        ST4CH31_clone: ATCTGTAATTGTCATGATTGATTTAGTAA---TGGGTTACA--------C
         ST1CH4_clone: ATCAGTTATTATCATGATTGACTTAGTAA---TTGGTTACA--------C
        ST3CH27_clone: ATCTGTAATTATTATGATCGATTTAGTAA---TTGGTTATA--------C
           Ectocarpus: TTCAGTTATTGTTATGATCGATTTAGTTATTGGTTATAC--AGCAATTCA
           Xanthonema: TTCAATCATTGTTATGATCGACCTTGTAATTG---GTTACA--------C
            Vaucheria: CTCAATTATTGTAATGATTGACCTTGTAATTGGTTATAC--TGCTATTCA
         Heterococcus: TGATGAAAACATTAACTCACAACCATTCATGCGTTGGAGAG--------A
           Aureoumbra: TTCAATTATTGTAATGATCGATTTAGTTATCG---GTTACA--------C
         Aureococcus: TTCTATCATTATCATGATTGACTTAGTTATTG---GTTACA--------C
         Pelagococcus: TTCTGTAATCGTAATGATTGACTTAGTAATTG---GTTACA--------C
        Cylindrotheca: TTCTATCATTATCATGATCGATTTAGTTATGG---GTTACA--------C
        Phaeodactylum: TTCTATCGTTGTTATGATCGATTTAGTTATGG---GTTATA--------C
        ST4CH14_clone: TTCTATCATTGTAATGATCGATTTAGTTA---TGGGTTACA--------C
         ST6CH2_clone: TTCTGTAATTATCATGATCGATTTAGTAA---TGGGTTATA--------C
         Rhizosolenia: TTCTGTAGTTGTAATGATCGATTTAGTAATGG---GTTACA--------C
          Skeletonema: TTCTATCGTTGTTATGATCGATTTAGTAATGG---GTTACA--------C
        Thalassiosira: TTCTGTCCTTGTAATGATCGATTTAGTAATGGGTTACAC--TGCAATTCA
             Detonula: TAATAACCAAGTAGGTCCTCAAATCTTACGTGACGCAGCTAAGACATGTG
           Chilomonas: TAGTGTTATCTGTATGATCGATCTTGTTATTGGA---TATA--------C
Mazzaella_membranacea: TAGTATCATTGTAATGATTGACCTTGTAATTG---GTTATA--------C
       Karenia_brevis: TTCCATAATTGTAATGATAGATCTTGTAATTGGTT---ATA--------C
       Jaxonville C3a: TTCCATAATTGTAATGATAGATCTTGTAA---TTGGTTATA--------C
      Mexico_Beach_C5: TTCCATAATTGTAATGATAGATCTTGTAA---TTGGTTATA--------C
      Piney_Island_A9: TTCCATAATTGTAATGATAGATCTTGTAA---TTGGTTATA--------C
  Charlotte_Harbor_C2: CTCCATAATTGTAATGATAGATCTTGTAA---TTGGTTATA--------C
  Charlotte_Harbor_A2: TTCCATAATTGTAATGATAGATCTTGTAA---TTGGTTATA--------C
      Piney_Island_B4: TTCCATAATTGTAATGATAGATCTTGTAA---TTGGTTATA--------C
      Mexico_Beach_B3: TTCCATAATTGTAATGATAGATCTTGTAA---TTGGTTATA--------C
    Appalachicola_C6: TTCCATAATTGTAATGATAGATCTTGTAA---TTGGTTATA--------C
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Appendix 2 (Continued)

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   Karenia_mikimotoi_1: TTCAATCATTGTGATGATCGATCTTGTAA---TTGGGTATA--------C
     Karenia_mikimotoi: TTCGGTCATTGTGATGATCGACCTTGTAATTGGGT---ATA--------C
           Karlodinium: GTCGGTTATTTTAATGATTGATCTTGTTATTGGTT---ATA--------C
           Amphidinium: TCTTGTGGGTGCTGGTTTGCGCTCACGGAACTCGCGCTTGG--------C
         ST2SY33_clone: TATGCCAATCATCATGCATGATTATATAACTGGCGGTTTTA--------C
       Prochlorococcus: TATGCCAATCATCATGCATGATTACATAACTGGTGGTTTTA--------C
          Thiobacillus: CTTTCATGCGCTGGAGGCAGCGTTTCGACTTCGTCATGG--AGGCCATCC
      Synechococcus_sp: GAATGCCATCATCATGCACGACTTTCTCACCGCTGGCTTCA--------C
         ST2SY26_clone: CATGCCGATCATCATGCACGACTTCATCACCGGTGGCTTCA--------C
         Synechococcus: CCTTCCAGCGTTGGCAGAACCGCTTCGAATTCGTTGCGG--AAGCCATCA
          Nitrobacter: CCTTCATGCGCTGGCGCGATCGTTTCGATTTCGTGATGG--AGGCTGTTC
        Synechocystis: CACCCCCATCATCATGCATGACTTCTTCACCGGCGGTTTCA--------C
Synecchococcus_PCC7002: CACTCCCATCATCATGCACGACTTCTTAACTGGTGGTTTCA--------C
        ST8SY15_clone: CACTCCAATCATTATGCACGACTACCTAACTGGTGGTTTTA--------C
        Trichodesmium: CACTCCAATCATTATGCACGACTATCTCACAGGTGGTTTTA--------C
         ST5SY7_clone: TGTTCCTATTATTATGCATGACTATTTAGCAGGTGGATTTA--------C
         ST2SY2_clone: TGCACCCATTATTATGCATGACTACCTAACAGGTGGTTTCA--------C
         ST6SY8_clone: TATGCCCATTATTATGCACGACTACCTTACTGGTGGTTTTA--------C
       Volvox_carteri: CGATGAAAACGTTAACTCACAACCATTCATGCGTTGGAGAG--------A
             Spinacia: CGTTCCTATTGTAATGCATGACTACTTAACAGGGGGATTCA--------C
           Asparagus: AGTTCCCATCGTAATGCATGACTACTTAACTGGAGGATTTA--------C
           Thalassia: TGATGAAAACGTAAATTCCCAGCCATTTATGCGTTGGAGAG--------A
           Prymnesium: TTCTGTTATCGTAATGATTGACTTAGTAATCG---GTTACA--------C
   Chrysochromulina_sp: TTCGATTATTGTAATGATTGACCTTGTAATTG---GTTATA--------C
           Imantionia: TTCAATCATTGTAATGATTGACCTTGTAATCG---GTTACA--------C
         ST1CH3_clone: TTCAATCATTGTAATGATTGACCTTGTAA---TCGGTTATA--------C
        Helicosphaera: TTCAGTTATTGTAATGATTGACCTTGTAATTG---GTTACA--------C

      Emiliana_huxleyi: AGCTATCCAATCAATGGGTAAGTGGTCACGTGATAACGATGTGATCCTTC
         ST8CH26_clone: AGCAATTCAAACAATGGGTAAGTGGTGTCGTAAGAGAGATATGATCCTTC
            Isochrysis: TGCTATCCAGACAATGGGTCGTTGGGCTCGTAAGTCTGATGTTATCCTTC
          Calcidiscus: AGCTATTCAATCAATGGCTAAATGGTCACGTAAGTATGATATGATCCTGC
      Cruciplacolithus: AGCTATCCAATCAATGGCTAATTGGGCACGTAAGTCTGATATGATTCTTC
         Pleurochrysis: AGCAATCCAATCGATGGCTATCTGGGCACGTAAGACAGATATGATTCTTC
               Pavlova: TGCTATTCAAACAATGGGTATCTGGGCTCGTAAGAATGATACAATTCTTC
      Exanthemachrysis: AGCTATTCAAAGTATGGCTAAGTGGGCTCGTAAGTACGATATGATCCTTC
           Pyrenomonas: AGCTATCCAAAGTATGGGTATCTGGGCTCGTAAGAACAGTATGATCTTAC
            Chroomonas: AGCTATCCAATCTATGGGTATTTGGGCTCGTAAGAACAGTATGATTCTTC
              Rhodella: TGCAATTCAAAGTATGGCTATTTGGGCTCGTAAAAACGATATGATCTTAC
             Dictyocha: AGCAATTCAATCAAATGCGTATTGGGCTCGTAAGAATGATATGATTCTTC
         ST6CH33_clone: AGCTATCCAATCTATGGCTGTTTGGGCTCGTGAAAACGATATGATTTTAC
           Heterosigma: TTCTACCGCTACTTGGACTGTAGTATGGACAGATTTATTAACTGCAT---
           Chattonella: AGCTATCCAAAGTATGGCTATCTGGTCTCGTAAAAACGACATGATTTTAC
         ST4CH31_clone: AGCAATTCAAAGTATTGCACTTTGGGCTCGTGAGAATGATATGCTTTTAC
          ST1CH4_clone: TGCTATTCAAACTATGGCATTCTGGGCTCGTAAGAATGATATGATTCTTC
         ST3CH27_clone: TGCAATTCAATCAATGGCTATTTGGGCTCGTAACAACGATATGATCTTAC
            Ectocarpus: AAGTATGGCAATCTGGGCACGAAAAGCTGAAGTGATTTTACATTTAC---
            Xanthonema: AGCGATCCAATCAATGGGTATTTGGGCTCGTAAAAATGACATGATTCTTC
             Vaucheria: ATCTATGTCAATTTGGGCTAGAAAAGCTGATATGATTTTACATTTAC---
          Heterococcus: ACGTTTTCTTTACTGTATGGAAGGTGTAAACCGTGCGGCTGCAGCAACTG
            Aureoumbra: AGCTATTCAAAGTATGGCTATTTGGGCTCGTAATAACGATATGGTTTTAC
           Aureococcus: AGCTATCCAAACTATGGCTATCTGGTCTCGTGAGAACGATATGCTTTTAC
          Pelagococcus: TGCTATCCAAACTGCTGCAATCTGGGCTCGTTCAAACGACATGATTTTAC
        Cylindrotheca: AGCAATTCAAAGTATGGCTTTATGGGCTCGTGAAAATGATATGCTTTTAC
        Phaeodactylum: TGCAATCCAATCAGCTGCGATTTGGGCGCGTGATAATGATTTAATTTTAC
         ST4CH14_clone: AGCTATTCAATCAATTGCAATCTGGGCACGTGAAAACGATATGCTTTTAC
          ST6CH2_clone: TGCGATTCAAAGTATTGCATTATGGTCTCGTGAAAACGATATGGTTTTAC
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Appendix 2 (Continued)

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          Rhizosolenia: AGCGATTCAAAGTATTGCTCACTGGGCTCGTGACAACGATATGGTATTAC
           Skeletonema: TGCAATTCAATCAATTGCATACTGGGCTCGTGAAAACGATATGTTATTAC
         Thalassiosira: ATCAATTGCATACTGGGCTCGTGAAAACGATATGTTATTACATTTAC---
              Detonula: GTCCTTTACAAACAGCTTTAGATCTATGGAAAGATATTAGTTTCAACTAT
            Chilomonas: TGCCATTCAGTCTATGGCTATTTGGGCTCGTAAAAATAGTATGATATTAC
 Mazzaella_membranacea: AGCTATTCAATCAATGGCTGTTTGGGCTCGTAAAAATGATATGATTCTTC
        Karenia_brevis: AGCGATACAATCAATGGCTTACTGGGCTCGCAATAATGATACTATGCTTC
       Jaxonville C3a: GGCGATACAATCAATGGCTTATTGGGCTCGCAATAATGATACTATGCTTC
      Mexico_Beach_C5: AGCGATACAATCAATGGCTTACTGGGCTCGCAATAATGATACTATGCTTC
      Piney_Island_A9: AGCGATACAATCAATGGCTTACTGGGCTCGCAATAATGATACTATGCTTC
  Charlotte_Harbor_C2: AGCGATACAATCAATGGCTTACTGGGCTCGCAATAATGATACTATGCTTC
  Charlotte_Harbor_A2: AGCGATACAATCAATGGCTTACTGGGCTCGCAATAATGATACTATGCTTC
      Piney_Island_B4: AGCGATACAATCAATGGCTTACTGGGCTCGCAATAATGATACTATGCTTC
      Mexico_Beach_B3: AGCGATACAATCAATGGCTTACTGGGCTCGCAATAATGATACTATGCTTC
     Appalachicola_C6: AGCGATACAATCAATGGCTTACTGGGCTCGCAATAATGATACTATGCTTC
  Karenia_mikimotoi_1: TGCAATTCAATCAATGGCGTACTGGGCGCGACGAAATGACGTTATTCTTC
    Karenia_mikimotoi: TGCGATTCAATCAATGGCCTACTGGGCGCGTCGAAATGACATTCTTCTAC
          Karlodinium: TGCAATTCAATCAATTACACAATGGTCGAGAAAACATGATATGCTATTAC
          Amphidinium: AAAGAAGTCAGGCAAGCGCAGTATTGTTGCACGCAAGGCATTGGACCAGT
        ST2SY33_clone: TGCAAATACTGGATTAGCAAACTGGTGTCGTAAAAATGGCATGCTTCTAC
       Prochlorococcus: TGCAAATACTGGATTAGCTAATTGGTGTCGTAAAAATGGCATGCTTCTGC
          Thiobacillus: AGAAGGCCGAGGCCGAGACCGGAGAGCGTAAGGGGCACTACCTGAACGTT
     Synechococcus_sp: GGCCAACACCACCCTCTCGAAAGGGTGCCGTGACAACGGCATGTTGCTAC
        ST2SY26_clone: GGCCAACACCGGTTTGTCGAAGTGGTGCCGCAAGAACGGCATGTTGCTGC
       Synechococcus: AGCTGTCCGAGCAGGAGACCGGTGAGCGCAAGGGTCACTACCTCAACGTG
         Nitrobacter: AAAAAGCCGAACACGAGACCGGCGAGCGGAAAGGTCACTATCTCAACGTG
       Synechocystis: TGCCAACACCACCCTCGCTCGTTGGTGTCGGGACAACGGCATTTTGCTCC
Synecchococcus_PCC7002: TGCGAATACTACCCTTGCGAAGTGGTGTCGTGATAACGGCGTTCTGCTCC
        ST8SY15_clone: AGCTAATACAACTTTAGCGAAATGGTGCCGTCGTAATGGTGTATTATTAC
        Trichodesmium: AGCTAATACAACTTTAGCGAAATGGTGCCGTCGTAATGGTGTATTATTAC
         ST5SY7_clone: TGCAAACACTACATTAGCTAAATACTGTAGAGATACTGGATTATTATTAC
         ST2SY2_clone: TGCTAACACTACGCTAGCAATGTACTCTCGTGATAACGGTCTACTTCTAC
         ST6SY8_clone: ATCTAACACTACTCTTTCTAACTACTGTCGTGACAACGGTCTTCTCCTCC
       Volvox_carteri: CCGTTTCCTTTTCGTAGCTGAAGCTATTTACAAAGCACAAGCAGAAACAG
            Spinacia: TGCAAATACTACCTTGTCTCATTATTGCCGAGATAATGGTCTACTTCTTC
          Asparagus: TGCAAATACCACTTTGGCTCATTATTGCCGCGACAATGGTCTACTTCTTC
          Thalassia: CCGTTTCCTATTTTGTGCCGAAGCCATTTTTAAAGCGCAAGACGAAACAG
         Prymnesium: AGCTATCCAGTCAATGGCTAAATGGTCACGTAAAACTGATATGATCCTTC
  Chrysochromulina_sp: TGCTATTCAATCAATGGCTAAGTGGTGTCGTAAGACTGATGTAATCCTTC
           Imantionia: TGCTATTCAGTCAATGTCTCACTGGGCGCGTGATAACGATGTGATCCTTC
        ST1CH3_clone: TGCTATCCAATCAATGGCTAAGTGGTCTCGTAAGAAAGATATGCTTCTTC
       Helicosphaera: GGCTATCCAATCAATGGGTAAATGGTCTCGTAAGTACGATGTGCTTCTTC

      Emiliana_huxleyi: ACCTTCACCGTGCGGGTAACTCAACTTACTCACGTCAGAAGAACCACGGT
        ST8CH26_clone: ACTTACACCGTGCTGGTAACTCAACTTACTCACGTCAGAAGAACCACGGT
           Isochrysis: ACCTTCACCGTGCAGGTAACTCAACTTACTCTCGTCAAAAGAGCCACGGT
         Calcidiscus: ACCTTCACCGTGCTGGTAACTCAACTTATTCTCGTCAGAAGAATCATGGT
      Cruciplacolithus: ACCTTCACCGTGCAGGTAACTCAACTTACTCGCGTCAAAAGAGTCACGGT
        Pleurochrysis: ACCTTCACCGTGCGGGTAATTCAACTTACTCACGTCAAAAGACTCATGGT
              Pavlova: ACCTTCACCGTGCTGGTAACTCTACTTACTCTCGTCAAAAGAACCACGGT
      Exanthemachrysis: ACCTTCACCGTGCTGGTAACTCAACTTACTCTCGTCAAAAGAACCACGGT
          Pyrenomonas: ACTTACACCGTGCTGGTAACTCTACTTACTCTCGTCAAAAGACTCACGGT
           Chroomonas: ACCTACACCGTGCAGGTAACTCTACATATTCCCGTCAAAAGACACATGGT
             Rhodella: ACTTACACCGTGCAGGAAACTCTACTTATTCTCGTCAAAAGAATCATGGT
            Dictyocha: ACTTACACCGTGCAGGTAACTCTACATATGCGCGTCAAAAGAACCATGGA
        ST6CH33_clone: ATTTACACCGTGCTGGTAACTCTACTTATGCTCGTCAAAAGAATCACGGT
          Heterosigma: ---------GTGATGTTTATCGTGCAAAAGCTTACCGTGTGGACCCTGTA
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Appendix 2 (Continued)

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            Chattonella: ACTTACACCGTGCAGGTAACTCTACTTACGCTCGTCAAAAGAACCACGGT
          ST4CH31_clone: ATTTACACCGTGCAGGTAACTCTACATATGCTCGTCAAAAAAATCATGGC
           ST1CH4_clone: ACTTACACCGTGCTGGTAACTCTACTTACGCTAGACAAAAAGAACATGGT
          ST3CH27_clone: ACTTACACCGTGCTGGTAACTCTACTTATGCACGTCAAAAAAACCATGGT
             Ectocarpus: ------ATCGTGCTGGTAACTCTACTTATGCTCGTCAAAAAAACCATGGT
             Xanthonema: ACTTACACCGTGCCGGTAACTCAACTTACGCACGTCAAAAAAACCACGGT
              Vaucheria: ------ACCGTGCAGGTAATTCTACATATGCACGTCAAAAAAATCATGGG
           Heterococcus: GTGAAGTAAAAGGTTCTTACCTAAACGCTACTGCTGGAAACATGGAAGAA
             Aureoumbra: ACTTACACCGTGCTGGTAACTCAACATATGCACGTCAAAAGACTCATGGT
            Aureococcus: ATTTACACCGTGCTGGTAACTCAACTTACGCTCGTCAAAAGAGTCACGGT
           Pelagococcus: ATTTACACCGTGCTGGTAACTCAACTTACGCTCGTCAAAAGAACCACGGT
          Cylindrotheca: ATTTACACCGTGCAGGTAATTCTACTTATGCTCGTCAAAAAAATCATGGG
          Phaeodactylum: ATTTACACCGTGCTGGTAACTCAACTTACGCGCGTCAAAAAAATCATGGT
          ST4CH14_clone: ATTTACACCGTGCAGGTAACTCTACATACGCTCGTCAAAAGAACCACGGT
           ST6CH2_clone: ATTTACACCGTGCTGGTAACTCTACATATGCACGTCAAAAGAACCACGGT
           Rhizosolenia: ATTTACACCGTGCTGGTAACTCTACTTATGCTCGTCAAAAGAATCATGGT
            Skeletonema: ACTTACACCGTGCTGGTAACTCTACATACGCTCGTCAAAAGAATCATGGT
          Thalassiosira: ------ACCGTGCTGGTAACTCTACTTACGCTCGTCAAAAGAATCATGGT
               Detonula: ACTTCTACGGATACAGCTGATTTCGCTGAAACAGCTACTGCAAACAGATA
             Chilomonas: ATTTACACCGTGCTGGTAACTCCACATACTCTAGGCAAAAGATTCATGGA
 Mazzaella_membranacea: ATTTGCATCGTGCAGGTAACTCTACTTATTCTCGTCAAAAAATTCATGGT
        Karenia_brevis: ACTTGCATCGTGCCGGTAATTCTACGTATTCTCGACAAAAAAATCATGGT
       Jaxonville C3a: ACTTGCATCGTGCCGGTAATTCTACGTATTCTCGACAAAAAAATCATGGT
      Mexico_Beach_C5: ACTTGCATCGTGCCGGTAATTCTACGTATTCTCGACAAAAAAATCATGGT
      Piney_Island_A9: ACTTGCATCGTGCCGGTAATTCTACGTATTCTCGACAAAAAAATCATGGT
  Charlotte_Harbor_C2: ACTTGCATCGTGCCGGTAATTCTACGTATTCTCGACAAAAAAATCATGGT
  Charlotte_Harbor_A2: ACTTGCATCGTGCCGGTAATTCTACGTATTCTCGACAAAAAAATCATGGT
      Piney_Island_B4: ACTTGCATCGTGCCGGTAATTCTACGTATTCTCGACAAAAAAATCATGGT
      Mexico_Beach_B3: ACTTGCATCGTGCCAGTAATTCTACGTATTCTCGACAAAAAAATCATGGT
     Appalachicola_C6: ACTTGCATCGTGCCGGTAATTCTACGTATTCTCGACAAAAAAATCATGGG
  Karenia_mikimotoi_1: ATTTACACCGTGCAGGAAACTCCACGTACTCTCGTCAGAAAAACCACGGA
    Karenia_mikimotoi: ATTTACATCGTGCGGGAAACTCCACGTACTCTCGCCAGAAAAATCACGGA
           Karlodinium: ATTTGCATCGTGCTGGTAATTCGGCGTATGCAAGGCAAAAAGATCATGGT
            Amphidinium: CCAGCAGATACGCAGACCTGTCCCTCACCGAAGAGCAGCTCATCCAACAG
          ST2SY33_clone: ATATTCATAGAGCGATGCATGCTGTTATTGATAGACATCCAAAACACGGT
       Prochlorococcus: ATATTCACAGAGCTATGCATGCTGTTATTGATAGACATCCAAAGCATGGT
          Thiobacillus: ACCGCCCCGACTCCGGAGGAAATGTACAAGCGTGCGGAGTACGCCAAGGA
      Synechococcus_sp: ACATTCACCGTGCTATGCACGCCGTGATGGACCGTCAGAAAAACCACGGC
          ST2SY26_clone: ACATCCACCGCGCCATGCACGCGGTGATCGACCGTCACCCCAAGCACGGC
          Synechococcus: ACCGCCAACACTCCCGAAGAGATGTATGAGCGCGCTGAGTTCGCCAAGGA
            Nitrobacter: ACCGCGCCAACGCCTGAGGAAATGTACAAGCGCGCCGAGTATGCCAAGGA
          Synechocystis: ATATTCACCGGGCAATGCACGCCGTAGTTGACCGTCAGAAAAACCACGGG
Synecchococcus_PCC7002: ACATCCACCGGGCAATGCACGCGGTAATCGACCGTCAGAAGAACCACGGT
          ST8SY15_clone: ACATCCACCGGGCAATGCACGCCGTTATTGACCGTCAGAAAGCCCACGGT
          Trichodesmium: ACATCCACCGGGCAATGCACGCCGTTATTGACCGTCAGAAAGCCCACGGT
           ST5SY7_clone: ATATTCATCGTGCGATGCATGCGGTTATTGATAGACAAAAAAATCATGGT
           ST2SY2_clone: ACATTCACCGTGCAATGCACGCGGTAATTGACCGTCAGCGTAACCACGGT
           ST6SY8_clone: ACATTCACCGTGCAATGCACGCGGTAATTGACCGTCAGCGTAACCACGGT
         Volvox_carteri: GTGAAGTAAAAGGTCATTATTTAAACGCTACAGCTGGTACATGCGAAGAA
              Spinacia: ACATCCACCGTGCAATGCACGCAGTTATTGATAGGCAGAAGAATCATGGT
            Asparagus: ACATCCACCGCGCAATGCATGCTGTTATTGATAGACAGAAAAATCATGGT
             Thalassia: GTGAAATCAAAGGACATTACTTGAATGCTACTGCAGGTACGTGTGAAGAA
           Prymnesium: ACTTACACCGTGCTGGTAACTCAACTTACTCACGTCAAAAAAACCACGGT
  Chrysochromulina_sp: ACTTACACCGTGCAGGTAACTCAACTTACTCACGTCAGAAGAACCACGGT
            Imantionia: ACTTACACCGTGCAGGTAACTCAACTTATTCACGTCAGAAAAACCACGGT
           ST1CH3_clone: ACTTACACCGTGCAGGTAACTCAACTTACTCACGTCAGAAGAACCACGGT
         Helicosphaera: ACCTACACCGTGCAGGTAACTCTACTTACTCTCGTCAAAAGTCACATGGT
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Appendix 2 (Continued)

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      Emiliana_huxleyi: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGTCAGGTTGTGACCA
         ST8CH26_clone: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGTCAGGTTGTGATCA
            Isochrysis: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGTCAGGTTGTGACCA
          Calcidiscus: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGTCAGGTGTTGACCA
      Cruciplacolithus: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGTCAGGTGTTGATCA
         Pleurochrysis: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGTCAGGTGTTGACCA
               Pavlova: ATGAACTTCCGTGTTATCTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
      Exanthemachrysis: ATGAACTTCCGTGTAATTTGTAAGTGGATGCGTATGTCAGGTGTTGACCA
           Pyrenomonas: ATGAACTTCCGTGTTATCTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
            Chroomonas: ATGAACTTCCGTGTAATTTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
              Rhodella: ATGAACTTCCGCGTAATCTGTAAATGGATGCGTATGGCTGGTGTTGACCA
             Dictyocha: ATTAACTTCCGTGTAATTTGTAAGTGGATGCGTATGGCTGGTGTTGATCA
         ST6CH33_clone: ATTAACTTCCGTGTAATTTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
           Heterosigma: CCAAGTGCAGCTGACCAGTACTTCGCATACATCGCATACGAGTGTGACCT
           Chattonella: ATTAACTTCCGTGTAATTTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
         ST4CH31_clone: ATGAATTTCCGTGTAATTTGTAAGTGGATGCGTATGTCAGGAGTTGACCA
          ST1CH4_clone: ATTAACTTCCGTGTAATTTGTAAATGGATGCGTATGGCTGGTGTTGACCA
         ST3CH27_clone: ATTAACTTCCGTGTAATTTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
            Ectocarpus: ATTAATTTCAGAGTTATCTGTAAATGGATGCGTATGTGTGGTGTAGACCA
            Xanthonema: ATTAACTTCCGTGTAATTTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
             Vaucheria: ATCAATTTCCGTGTAATTTGTAAATGGATGCGTATGGCAGGTGTAGATCA
          Heterococcus: ATGTACACACGTGCTGAATACGCAAAAGAAATTGGTTCAATCATTGTGAT
            Aureoumbra: ATTAATTTCCGTGTAATTTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
           Aureococcus: ATTAACTTCCGTGTAATCTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
          Pelagococcus: ATCAACTTCCGTGTAATCTGTAAGTGGATGCGTATGGCTGGTGTTGATCA
         Cylindrotheca: ATTAACTTCCGTGTAATTTGTAAATGGATGCGTATGTCAGGTGTAGATCA
         Phaeodactylum: ATCAACTTCCGTGTTATTTGTAAATGGATGCGTATGTGTGGTGTGGATCA
         ST4CH14_clone: ATCAACTTCCGTGTTATCTGTAAATGGATGCGTATGTCTGGTGTAGATCA
          ST6CH2_clone: ATTAACTTCCGTGTTATCTGTAAATGGATGCGTATGTCTGGTGTAGATCA
          Rhizosolenia: ATTAACTTCCGTGTTATCTGTAAATGGATGCGTATGTCTGGTGTAGATCA
           Skeletonema: ATTAACTTCCGTGTTATCTGTAAATGGATGCGTATGTCTGGTGTAGATCA
         Thalassiosira: ATTAACTTCCGTGTTTTCTGTAAATGGATGCGTATGTCAGGTGTGGATCA
              Detonula: ATTAATTACTTTTATATATATTTAAGGAGTATTTGAATAGTGAGACTTAC
          Chilomonas: ATGAACTTCCGTGTCATTTGTAAGTGGATGCGCATGGCTGGTGTTGATCA
Mazzaella_membranacea: ATGAATTTCCGAGTAATATGTAAATGGATGCGTATGGCAGGTGTTGATCA
       Karenia_brevis: ATGAATTTCCGCGTCATCTGTAAATGGATGCGAATGGCTGGTGTTGATCA
       Jaxonville C3a: ATGAATTTCCGCGTCATCTGTAGATGGATGCGAATGGCTGGTGTTGATCA
      Mexico_Beach_C5: ATGAATTTCCGCGTCATCTGTAAATGGATGCGAATGGCTGGTGTTGATCA
      Piney_Island_A9: ATGAATTTCCGCGTCATCTGTAAATGGATGCGAATGGCTGGTGTTGATCA
  Charlotte_Harbor_C2: ATGAATTTCCGCGTCATCTGTAAATGGATGCGAATGGCTGGTGTTGATCA
  Charlotte_Harbor_A2: ATGAATTTCCGCGTCATCTGTAAATGGATGCGAATGGCTGGTGTTGATCA
      Piney_Island_B4: ATGAATTTCCGCGTCATCTGTAAATGGATGCGAATGGCTGGTGTTGATCA
      Mexico_Beach_B3: ATGAATTTCCGCGTCATCTGTAAATGGATGCGAATGGCTGGTGTTGATCA
     Appalachicola_C6: ATGAATTTCCGCGTCATCTGTAAATGGATGCNAATGGCTGGNGTTGATCA
  Karenia_mikimotoi_1: ATGAATTTCCGTGTAATTTGTAAGTGGATGCGTATGGCAGGTGTTGATCA
    Karenia_mikimotoi: ATGAATTTTCGTGTAATTTGTAAGTGGATGCGTATGGCAGGCGTTGACCA
          Karlodinium: ATCAATTTTCGTGTAATATGTAAGTGGATGAGAATGGCGGGCGTAGACCA
          Amphidinium: GGTCAGCATGTGCTTGTGGCATACATCATGAAGCCAAAGGCTGGCTACGA
         ST2SY33_clone: ATCCATTTCAGGGTTCTAGCAAAATGTTTGAGACTCTCCGGAGGAGATCA
       Prochlorococcus: ATTCACTTCAGAGTTCTTGCAAAATGTTTGAGACTATCTGGAGGAGACCA
          Thiobacillus: AATCGGCGCACCCATCATCATGCACGATTACATCACCGGCGGCTTCTGCG
      Synechococcus_sp: ATCCACTTCCGTGTCTTGGCCAAATGTTTGCGGATGTCTGGCGGTGACCA
         ST2SY26_clone: ATTCACTTCCGCGTTCTCGCCAAGTGTCTGCGTCTGTCCGGTGGTGACCA
         Synechococcus: ACTCGGCATGCCGATCATCATGCACGACTTCATCACCGGTGGCTTCACGG
           Nitrobacter: AATCCGCGCTCCGATCATCATGCACGATTACCTCGCTGGCGGGCTTTGCG
         Synechocystis: ATCCACTTCCGGGTTTTGGCCAAGTGTCTGCGTCTGTCCGGCGGTGACCA
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Appendix 2 (Continued)

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Synecchococcus_PCC7002: ATTCACTTCCGCGTTCTCGCTAAGTGTCTCCGCCTCTCTGGTGGTGACCA
         ST8SY15_clone: ATTCACTTCCGGGTTTTAGCTAAGTGCTTGAGAATGTCTGGTGGTGACCA
         Trichodesmium: ATTCACTTCCGGGTTTTAGCTAAGTGCTTGAGAATGTCTGGTGGTGACCA
          ST5SY7_clone: ATTCACTTCCGTGTTTTAGCTAAGGCTTTACGTTTATCTGGTGGTGATCA
          ST2SY2_clone: ATTCACTTCCGTGTACTTGCTAAGGCTCTACGTATGTCAGGTGGTGACCA
          ST6SY8_clone: ATTCACTTCCGTGTTCTCGCGAAGGCTCTCCGTCTTTCTGGTGGTGACCA
        Volvox_carteri: ATGTTAAAACGTGCTCAATGTGCTAAAGAACTAGGTGTACCAATTATCAT
               Spinacia: ATGCACTTCCGTGTACTAGCGAAAGCGTTACGTCTATCTGGTGGAGATCA
             Asparagus: ATGCATTTTCGTGTACTAGCTAAAGCATTACGTATGTCTGGTGGAGATCA
              Thalassia: ATGATCAAAAGAGCTGTATGTGCCAGAGAATTGGGAGTTCCTATCGTAAT
            Prymnesium: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
   Chrysochromulina_sp: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGGCTGGTGTTGACCA
             Imantionia: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGGCTGGTTGTGACCA
          ST1CH3_clone: ATGAACTTCCGTGTAATCTGTAAGTGGATGCGTATGGCTGGTTGTGACCA
        Helicosphaera: ATGAACTTCCGAGTAATTTGTAAGTGGATGCGTATGGCTGGATGTGACCA

      Emiliana_huxleyi: CATTCACGCAGGTACTGTAGTAGGTAAGCTAGAAGGTGATCCACTAATGA
         ST8CH26_clone: CATTCACGCTGGTACAGTAGTTGGTAAGCTAGAAGGTGATCCATTAATGA
            Isochrysis: CATTCACGCAGGTACAGTAGTTGGTAAGCTAGAAGGTGATCCTTTAATGA
          Calcidiscus: TATCCACGCTGGTACAGTAGTAGGTAAGCTAGAAGGTGATCCTCTAATGA
      Cruciplacolithus: CATTCATGCAGGTACGGTAGTAGGTAAGTTAGAAGGTGATCCTTTAATGA
         Pleurochrysis: CATCCACGCGGGTACAGTAGTAGGTAAGTTAGAAGGTGACCCTCTAATGA
               Pavlova: CATTCACGCAGGTACCGTAGTAGGTAAGCTTGAAGGGGATCCACTAATGG
      Exanthemachrysis: CATTCACGCTGGTACTGTTGTAGGTAAGCTTGAAGGTGATCCTCTAATGG
           Pyrenomonas: TATTCACGCTGGTACAGTTGTAGGTAAATTAGAGGGAGATCCTCTAATGG
            Chroomonas: TATCCACGCTGGTACAGTAGTTGGTAAGCTTGAAGGAGATCCTTTAATGG
              Rhodella: TATTCACGCTGGTACAGTAGTAGGAAAATTAGAAGGGGATCCTTTAATGA
             Dictyocha: TATCCATGCGGGTACAGTTGTTGGTAAATTAGAAGGTGATCCTTTAATGG
          ST6CH33_clone: TATCCACGCTGGTACTGTAGTAGGTAAATTAGAAGGTGATCCATTAATGG
            Heterosigma: TTTCGAAGAAGGTTCTTTAGCTAACATGACTGCATCTATCATTGGTAACG
            Chattonella: CATTCACGCAGGTACTGTTGTAGGTAAATTAGAAGGTGACCCTCTAATGG
          ST4CH31_clone: TATTCACGCGGGTACTGTAGTAGGTAAGCTTGAGGGTGATCCTCTAATGA
           ST1CH4_clone: TATCCACGCTGGTACAGTTGTAGGTAAATTAGAAGGAGATCCAAACACAG
          ST3CH27_clone: TATTCACGCTGGTACTGTTGTAGGTAAATTAGAAGGTGATCCATTAATGG
             Ectocarpus: TATCCATGCAGGTACTGTAGTTGGTAAACTAGAAGGAGATCCCTTAATGG
             Xanthonema: CATTCACGCAGGTACTGTTGTTGGTAAATTAGAAGGTGACCCTTTAATGG
              Vaucheria: CATTCATGCTGGTACTGTTGTAGGTAAATTAGAAGGTGACCCTTTAATGG
           Heterococcus: GATCGATT---TAGTTATTGGGTACACAGCCATTCAATCCATGGCTATTT
             Aureoumbra: TATCCACGCAGGTACTGTTGTAGGTAAGTTAGAAGGTGACCCATTAATGG
            Aureococcus: CATTCACGCTGGTACTGTTGTAGGTAAGTTAGAAGGTGATCCATTAATGG
           Pelagococcus: CATTCACGCTGGTACAGTTGTAGGTAAGTTAGAAGGTGATCCATTAATGA
          Cylindrotheca: TATTCACGCTGGTACAGTTGTAGGTAAATTAGAAGGGGATCCTTTAATGA
          Phaeodactylum: TATCCATGCTGGTACAGTTGTAGGTAAATTAGAAGGCGATCCTTTAATGA
          ST4CH14_clone: TATTCACGCTGGTACAGTTGTTGGTAAATTAGAAGGTGATCCTTTAATGA
           ST6CH2_clone: CATCCACGCTGGTACAGTAGTAGGTAAATTAGAAGGTGATCCTTTAATGA
           Rhizosolenia: TATCCACGCAGGTACAGTTGTTGGTAAATTAGAAGGTGATCCTTTAATGA
            Skeletonema: CATCCACGCTGGTACAGTTGTTGGTAAGTTAGAAGGTGATCCTTTAATGA
          Thalassiosira: TATCCATGCTGGTACAGTTGTTGGTAAATTAGAAGGTGATCCTTTAATGA
               Detonula: ACAAGGTTGCTTCTCTTTCTTACCTGATTTAACTGACGAACAAATTGAAA
            Chilomonas: CATTCATGCTGGTACTGTTGTGGGTAAATTAGAAGGCGATCCTTTGATGG
 Mazzaella_membranacea: TATTCATGCAGGTACTGTTGTTGGTAAACTAGAAGGCGATCCTCTAATGA
        Karenia_brevis: TATTCATGCCGGAACCGTTGTGGGTAAACTAGAAGGAGATCCAAAAATGA
       Jaxonville C3a: TATTCATGCCGGAACCGTTGTGGGTAAACTAGAAGGAGATCCAAAAATGA
      Mexico_Beach_C5: TATTCATGCCGGAACCGTTGTGGGTAAACTAGAAGGAGATCCAAAAATGA
       Piney_Island_A9: TATTCATGCCGGAACCGTTGTGGGTAAACTAGAAGGAGATCCAAAAATGA
  Charlotte_Harbor_C2: TATTCATGCCGGAACCGTTGTGGGTAAACTAGAAGGAGATCCAAAAATGA
  Charlotte_Harbor_A2: TATTCATGCCGGAACCGTTGTGGGTAAACTAGAAGGAGATCCAAAAATGA
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Appendix 2 (Continued)

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        Piney_Island_B4: TATTCATGCCGGAACCGTTGTGGGTAAACTAGAAGGAGATCCAAAAATGA
        Mexico_Beach_B3: TATTCATGCCGGAACCGTTGTGGGTAAACTAGAAGGAGATCCAAAAATGA
     Appalachicola_C6: TATTCATGCCGGAACCGTTGTGGGTAAACTAGAAGGAGATCCAAAAATGA
  Karenia_mikimotoi_1: TATCCATGCGGGAACCGTTGTTGGTAAATTAGAAGGAGAACCTAAAATGA
    Karenia_mikimotoi: TATCCATGCTGGAACCGTTGTTGGTAAATTAGAAGGAGAGCCGAAAATGA
          Karlodinium: TTTACATGCTGGAACGGTAGTTGGAAAACTTGAGGGCGATCCTTACATGG
          Amphidinium: CTACCTCGCAACGGCTGCGCACTTTGCAGCTGAATCATCCACTGGTACCA
       ST2SY33_clone: ACTACATACTGTAACTGTTGTTGGAAAACTAGAAGGTGATCGTCAAACAA
     Prochlorococcus: ATTACATACTGGAACCGTGGTTGGAAAACTAGAAGGTGATCGTCAAACAA
        Thiobacillus: CCAACACGGGTCTGGCCAACTGGTGCCGCGACAACGGCATGCTCCTGCAC
     Synechococcus_sp: CATCCACACCGGTACCGTTGTTGGTAAGCTCGAAGGGGATAAAGCCGTTA
       ST2SY26_clone: GCTCCACACCGGCACCGTGGTCGGAAAGCTGGAAGGTGATCGTCAGACCA
       Synechococcus: CCAACACCGGTCTGTCGAAGTGGTGCCGCAAGAACGGCATGTTGCTGCAC
         Nitrobacter: CCAACGCGGGTCTGGCGAACTGGTGCCGCAACAACGGAATGCTGCTGCAC
       Synechocystis: CCTCCACTCCGGTACCGTGGTTGGTAAATTGGAAGGGGAACGGGGTATCA
Synecchococcus_PCC7002: CCTCCACTCCGGTACGGTTGTTGGTAAGCTCGAAGGCGATCGCGCCGCCA
       ST8SY15_clone: CCTCCACTCTGGTACTGTGGTAGGAAAGCTCGAAGGTGAAAAAGGCATCA
       Trichodesmium: CCTCCACTCTGGTACTGTCGTGGGCAAGCTCGAAGGA-------------
        ST5SY7_clone: CTTACATAGTGGTACTGTTGTAGGAAAACTAGAAGGTGATAAAGATATCA
        ST2SY2_clone: CCTTCACTCAGGTACTGTAGTAGGTAAGCTTGAGGGTGAGCGTGAAGTAA
        ST6SY8_clone: CCTTCACTCTGGTACTGTAGTAGGTAAGCTTGAGGGTGAGCGTGAAGTAA
      Volvox_carteri: GCACGACTACTTAACTGGTGGTTTTACAGCTAACACATCATTAGCTTCTT
            Spinacia: TATTCACTCTGGTACCGTAGTAGGTAAGCTTGAAGGAGAAAGAGATATTA
          Asparagus: TATTCACGCTGGTACAGTAGTAGGTAAACTGGAAGGGGAACGTGAGATGA
          Thalassia: GCATGACTACTTAACGGGGGGATTCACTGCAAATACTAGCTTGGCTCGTT
         Prymnesium: TATCCACGCAGGTACTGTAGTTGGTAAGCTAGAAGGTGATCCTCTAATGA
   Chrysochromulina_sp: CATTCACGCTGGTACTGTAGTAGGTAAGCTAGAAGGTGATCCTCTAATGA
          Imantionia: CATCCACGCCGGTACTGTAGTAGGTAAGTTAGAAGGTGATCCTCTAATGA
        ST1CH3_clone: CATTCACGCCGGTACAGTAGTAGGTAAGCTTGAAGGTGATCCACTAATGA
        Helicosphaera: CATTCACGCAGGTACAGTAGTAGGAAAGTTAGAAGGTGATCCTCTAATGA

     Emiliana_huxleyi: TTAAAGGTTTCTACAACACTCTACTTGATACTAAGACTGAAGTTAACCTT
        ST8CH26_clone: TTAAAGGTTTCTACAACACTTTATTAGATACTAAGACTGATATTAACCTA
           Isochrysis: TCAAAGGTTTCTACAACACTCTACTAGATACTAAGACTGATATTAACCTT
         Calcidiscus: TTAAAGGTTTCTACAACACTCTACTAGATTTCAAGACTGATGTTAACTTA
     Cruciplacolithus: TTAAAGGTTTCTACAACACTCTATTAGATTTCAAGACTGATATCAATCTG
        Pleurochrysis: TTAAAGGTTTCTACAACACTCTACTTGACTTCAAGACTGATATTAACCTA
              Pavlova: TTAAAGGTTTCTATAACACTCTTCTAGAAAACAAAACTGATATCAACTTA
     Exanthemachrysis: TTAAAGGATTCTACAACACTCTACTTGAGAACAAGACTGACATTAACCTA
          Pyrenomonas: TTAAAGGTTTCTACGATACATTATTAGAAGTTAAAACAGAAGTTAACTTA
           Chroomonas: TTAAAGGTTTCTACAATACATTACTAGAAGCTAAGACTGATGTAAACCTT
             Rhodella: TTAAAGGTTTCTACAACGTTCTATTAGAAACTAGTCTTGACATCAACTTA
            Dictyocha: TTAAAGGTTTCTATCATACATTACTTGATGTTAAGACTGATGTTAACCTT
        ST6CH33_clone: TTAAAGGTTTCTACAACACATTATTAGACACTGAAAATAAGATCAACTTA
          Heterosigma: TATTCGGTTTCAAAGCTGTAGCTGCATTACGTTTAGAAGATATGCGTATT
          Chattonella: TTAAAGGTTTCTACGACACTTTACGTGAATGTGAGTTAAGTATCAACTTA
        ST4CH31_clone: TTAAAGGTTTCTACAATACACTTTTAGCCACAAAATCAGAGACTTCTCTG
        ST1CH4_clone: TTAAAGGATTCTACGATACACTATTATTACCTGCATTAAAAGAAGATCGT
        ST3CH27_clone: TTCGTGGTTTCTATAGAACATTATTAGATAACAGTTTATCTGTTAACTTA
           Ectocarpus: TTAAAGGATTCTACAACAGTTTATTATTAACTCATTTAAAAATTAATTTA
          Xanthonema: TTAAAGGTTTCTACAACACTTTATTACAAAGTGAATCTGAAATCAACCTT
           Vaucheria: TTAAAGGCTTTTATAATACATTATTATTAACGAAATTAGAAATTAATCTT
        Heterococcus: GGGCTCGCCAAGCAGACATGATCTTACATTTACACCGTGCAGGTAACTCT
          Aureoumbra: TTCAAGGTTTCTACGATACTTTATTATTAACTCACTTAAAGATTGATTTA
         Aureococcus: TTCAAGGTTTCTACGATACTTTATTAAAAACTAAGTTAGCAATCGATTTA
        Pelagococcus: TCCAAGGTTTCTACGACACATTATTAAAGACTAAGTTAGCTATCGATTTA
      Cylindrotheca: TTAAAGGTTTTTATCATACTTTACGGTTAACAACATTAGATGTTAACTTA
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Appendix 2 (Continued)

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          Phaeodactylum: TTAAAGGTTTCTACGATACTTTATTACTAACTCATTTAAATGTTAATTTA
          ST4CH14_clone: TTAAAGGTTTCTACGATACTTTACGTTTAACAAACTTAGATGTTAACTTA
           ST6CH2_clone: TTAAAGGTTTCTACGATATTTTACGTTTAACAACATTAGATGTAAACTTA
           Rhizosolenia: TTAAAGGTTTCTACGATGTATTACGTTTACCAGTTTTAGACGTTAACTTA
            Skeletonema: TTAAAGGTTTCTACGATATCTTACGTTTAACTGAATTAGAAGTAAACTTA
          Thalassiosira: TTAAAGGTTTCTACGACATCTTACGTCTAACTGAATTAGAAGTTAACTTA
               Detonula: AACAAGTAGCTTGCGCTATGTCTAGAGGTTTAGCGATGAACGTAGAGTGG
             Chilomonas: TTAAAGGGTTTTATAATGTTTTACTACGAACTAAGCTTGATGTAAACCTA
 Mazzaella_membranacea: TCCGAGGTTTCTATAACACACTACTACTACCATATTTAGAAGTGAATTTA
        Karenia_brevis: TTAAAGGGTTCTATGAAACGTTATTGGGTCTGTGTACACGAATTAACCTT
       Jaxonville C3a: TTAAAGGGTTCTATGAAACGTTATTGGGTCTGTGTACACGAATTAACCTT
       Mexico_Beach_C5: TTAAAGGGTTCTATGAAACGTTATTGGGTCTGTGTACACGAATTAACCTT
       Piney_Island_A9: TTAAAGGGTTCTATGAAACGTTATTGGGTCTGTGTACACGAATTAACCTT
   Charlotte_Harbor_C2: TTAAAGGGTTCTATGAAACGTTATTGGGTCTGTGTACACGAATTAACCTT
   Charlotte_Harbor_A2: TTAAAGGGTTCTATGAAACGTTATTGGGTCTGTGTACACGAATTAACCTT
       Piney_Island_B4: TTAAAGGGTTCTATGAAACGTTATTGGGTCTGTGTACACGAATTAACCTT
       Mexico_Beach_B3: TTAAAGGGTCCTATGAAACGTTATTGGGTCTGTGTACACGAATTAACCTT
      Appalachicola_C6: TTAAAGGGTTCTATGAAACGTTATTGGGTCTGTGTACACGAATTAACCTT
   Karenia_mikimotoi_1: TTAAAGGGTTTTATAAGACATTACTCGATCTAACAACTAGACATGATTTT
     Karenia_mikimotoi: TTAAAGGATTCTATAAGACACTCCTGGATTTAACGACCGAACATGATTTC
           Karlodinium: TCAAAGGGTTTTATGCTACTCTTCTTACAACAAAGGCAGCAGCAAACATA
            Amphidinium: ACGTGAACGTGTGCACCACCGACGACTTCACCAAGTCAGTCGATGCACTG
          ST2SY33_clone: CTCTTGGTTACATTGACAACTTAAGAGAGTCATTTGTTCCCGAAGATAGA
        Prochlorococcus: CTCTTGGTTACATTGACAACTTAAGAGAGTCATTTGTTCCTGAAGATAGA
           Thiobacillus: ATTCACCGCGCCATGCATCGGGTGCTGGACCGCAACCCTCACCACGGCAT
       Synechococcus_sp: CCCTCGGCTTTGTCGATCTGCTGCGGGAAAACTACATTGAGCAGGATCGC
          ST2SY26_clone: CCCTCGGCTACATCGACCAGCTGCGCGAATCCTTCGTGCCCGAAGACCGC
          Synechococcus: ATCCACCGCGCCATGCACGCGGTGATCGACCGTCACCCCAAGCACGGCAT
            Nitrobacter: ATCCACCGCGCTATGCATGCGGTGATCGATCGCAACCCGCATCACGGTAT
          Synechocystis: CCATGGGCTTCGTTGACCTCATGCGCGAAGATTACGTTGAGGAAGATCGC
Synecchococcus_PCC7002: CCCTCGGTTTCGTAGACCTGATGCGTGAAGACTACGTTGAAGAAGATCGT
          ST8SY15_clone: CAATGGGCTTTGTAGACTTAATGCGGGAAGACCATGTTGAGCAAGATCGC
          Trichodesmium: --------------------------------------------------
           ST5SY7_clone: CTTTAGGTTTTGTAGACTTAATGAGAGATGATTATGTCAAATTAGACCGT
           ST2SY2_clone: CTCTAGGTTTCGTAGATCTAATGCGTGATAACTTCATTGAGAAGGATCGT
           ST6SY8_clone: CTCTTGGTTTCGTAGACCTTATGCGTGACGACTTTGTTGAGAAGGATCGT
         Volvox_carteri: ACTGTCGTGATAATGGTCTTTTATTACACATTCACCGTGCTATGCACGCA
               Spinacia: CTTTAGGCTTTGTTGATTTACTACGTGATGATTATACTGAAAAAGACCGA
             Asparagus: CTTTAGGTTTTGTTGATTTATTACGTGATGATTATATTGAAAAAGACCGA
              Thalassia: ATTGCCGAGACAACGGATTACTTCTTCACATTCACCGCGCAATGCATGCT
            Prymnesium: TTAAAGGTTTCTACAACACACTTCTAGACTTTAAATCTGAGATGTGCCTA
   Chrysochromulina_sp: TTAAAGGTTTCTACAACACTCTTCTTGACTTCAAGTCAGATGTTAGTCTA
             Imantionia: TTAAAGGTTTCTACAACACTCTTCTAAACTGTAAGACTGATGTAAACCTA
           ST1CH3_clone: TCAAGGGCTTCTACAACACTCTACTTGACTTCAAGACCGACGTAAACCTA
          Helicosphaera: TTAAAGGTTTCTACAACACGCTTCTAGACTTCAAGACAGATGTAAACTTA

       Emiliana_huxleyi: CCTCAAGGTTTATTCTTCGCTCAAGATTGGGCTTCACTTCGTAAGTGTGT
          ST8CH26_clone: CCTCAAGGTTTATTCTTCGCTCAGGATTGGGCTTCATTACGTAAGTGTAT
              Isochrysis: CCTCAAGGTTTATTCTTCGCTCAAGATTGGGCTTCTCTTCGTAAGTGTGT
           Calcidiscus: CCTCAAGGTCTATTCTTTGCACAAGATTGGGCTTCTTTACGTAAGTGTGT
       Cruciplacolithus: CCTCAAGGTCTATTCTTCGCGCAAGATTGGGCTTCTTTACGTAAGTGTGT
          Pleurochrysis: CCTCAAGGTTTATTCTTCGCACAGGATTGGGCTTCTCTACGTAAGTGTGT
                Pavlova: CCGGAAGGTCTATTCTTTGAGCAAGATTGGGCTTCTTTACGTAAGTGTAT
       Exanthemachrysis: CCAGAAGGTCTATTCTTCGAGCAAGATTGGGCTTCTCTACGTAAGTGTAT
            Pyrenomonas: GTTGAAGGTTTATTCTTCGCTCAAGACTGGGCTTCTTTAGCTAAGTGTAT
             Chroomonas: GTTCAAGGTTTATTCTTCGCTCAAGATTGGGCTTCTTTAGCTAAGTGTCT
               Rhodella: CCTCAAGGTATATTCTTTGCACAAGATTGGGCTTCTTTACGTAAGTGTAT
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Appendix 2 (Continued)

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                Dictyocha: CCACAAGGTTTATTCTTCGCCCAAGATTGGGCTTCTCTACGTAAATGTCT
           ST6CH33_clone: CCTCAAGGTATCTTCTTCGATATGGACTGGGCTTCTTTACGTCGTTGTGT
             Heterosigma: CCTTACGCTTACTTAAAAACATTCCAAGGTCCAGC---------------
             Chattonella: CCACAAGGTATCTTCTTTGAAATGGATTGGGCTTCTTTACGTAAATGTTG
           ST4CH31_clone: CCCGAAGGCTTATTCTTCGTGCAAGACTGGGCTTCTTTACGTAAATGCGT
            ST1CH4_clone: ACTTTAGGATTATTCTTCGATATGGATTGGGCTTCATTACGTAAATGTAT
           ST3CH27_clone: CCAGAAGGTATTTTCTTCGAAATGGACTGGGCTTCTTTACGTAAAACATT
              Ectocarpus: GCTGAAGGCTTATTCTTTGATATGGATTGGGCATCTCTTAGAAAATGTGT
              Xanthonema: CCTCAAGGTATCTTCTTCGCAATGGATTGGGCTTGTTTACGTAAAACTGT
               Vaucheria: TCTCAAGGTGTATTTTTTGAAATGGATTGGGCAGCATTACGTAAAACTGT
            Heterococcus: ACATATGCGCGTCAAAAAAATCATGGTATTAACTTCCGTGTAATTTGTAA
              Aureoumbra: CCTAAGGGTATTTTCTTCGATATGGATTGGGCATCTTTACGTAAGTGTAT
             Aureococcus: CCTAAGGGTATCTTCTTCGACATGGACTGGGCTGCTTTACGTAAGTGTAT
            Pelagococcus: CCTAAGGGTATCTTCTTCGATATGGACTGGGCTTCTTTACGTAAGTGTTT
           Cylindrotheca: CCTTATGGTTTGTTCTTCGAAATGTCATGGGCTAGTTTACGTCGTTGTAT
           Phaeodactylum: CCATACGGTATTTTCTTCGAAATGACTTGGGCAAGTTTACGTAAATGTAT
           ST4CH14_clone: CCTTACGGTTTATTCTTCGAAATGGACTGGGCTAGTTTACGTAAGTGTAT
            ST6CH2_clone: CCATTCGGTATTTTCTTCGAAATGGATTGGGCTAGTTTACGTAAGTGTAT
            Rhizosolenia: CCATTCGGTATTTTCTTCGAAATGGATTGGGCTAGTTTACGTAAATGTAT
             Skeletonema: CCTTTCGGTATCTTCTTCGAAATGGATTGGGCTAGTTTACGTCGTTGTAT
           Thalassiosira: CCTTTTGGTGTGTTCTTCGAAATGGATTGGGCTAGTTTACGTCGTTGTAT
                 Detonula: ACAGATGATCCACATCCTCGTAATAACTACTGGGAATTATGGGGTTTACC
              Chilomonas: CCACAAGGGTTGTTCTTTTCGCAAGATTGGGCTTCGCTTGCTAAATGTAT
  Mazzaella_membranacea: CCACAAGGTATATTCTTTGAACAAGATTGGGCATCTCTTAGAAAAGTTAC
         Karenia_brevis: AGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGT
        Jaxonville C3a: AGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGT
       Mexico_Beach_C5: AGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGT
       Piney_Island_A9: AGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGT
    Charlotte_Harbor_C2: AGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGT
    Charlotte_Harbor_A2: AGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGT
        Piney_Island_B4: AGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGT
       Mexico_Beach_B3: AGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGT
      Appalachicola_C6: AGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGT
    Karenia_mikimotoi_1: GCTTTCGGTCTTTATTTTTCGCAAGAATGGGCTTCTCTCCGTAAATGCAT
      Karenia_mikimotoi: GCTTACGGTCTTTACTTTTCGCAAGACTGGGCTTCTCTTCGTAAATGCGT
             Karlodinium: AAAAATGGTTTATTTTTTCCGCAAGATTGGGCATCGTTGCGCCGATGTGT
              Amphidinium: GTGTACTACATCGACCCAGAGAATGAGGAGTGCAAGATCGCCTACCCAAA
          ST2SY33_clone: TCAAGAGGTAACTTCTTTGATCAAGATTGGGGTTCAATGCCAGGAGTATT
        Prochlorococcus: TCAAGAGGTAACTTCTTTGATCAAGATTGGGGTTCAATGCCCGGAGTATT
           Thiobacillus: CCACTTCCGTGTGCTGACCAAGATTCTCCGTCTGTCCGGCGGCGATCACC
       Synechococcus_sp: TCCCGTGGTATTTACTTCACCCAAGACTGGGCCTCGATGCCCGGAGTGAT
          ST2SY26_clone: AGCCGCGGCAACTTCTTCGATCAGGACTGGGGCTCCATGCCCGGCGTGTT
          Synechococcus: CCACTTCCGCGTTCTCGCGAAGTGTCTGCGTCTGTCCGGTGGTGACCAGC
            Nitrobacter: CCACTTCCGCGTATTGACCAAGATCTTGCGTCTGTCGGGCGGCGACCATC
          Synechocystis: TCCCGGGGTATTTTCTTCACCCAAGACTATGCCTCCATGCCTGGCACCAT
Synecchococcus_PCC7002: TCTCGCGGTGTATTCTTCACCCAAGACTACGCTTCTCTCCCCGGCACCAT
          ST8SY15_clone: GAACGTGGTATTTACTTTACTCAAGATTGGGCTTCTATGCCTGGTGTAAT
          Trichodesmium: ----------------------------------------------------
           ST5SY7_clone: TCAAGAGGTATCTATTTTACTCAAGACTGGGCATCAATGGGTGGTGTAAT
           ST2SY2_clone: TCTCGTGGTATTTACTTTACTCAGGATTGGGTATCAATGGGTGGTACAAT
           ST6SY8_clone: AACCGTGGTATCTACTTTACTCAGGAATGGTGTTCTATGGGTGGTACAAT
         Volvox_carteri: GTAATTGACCGTCAACGTAACCATGGTATTCACTTCCGTGTTCTAGCAAA
                Spinacia: AGTCGCGGTATTTATTTCACTCAATCTTGGGTTTCCACACCAGGTGTTCT
              Asparagus: AGTCGCGGTATTTTTTTCACTCAAGATTGGGTTTCTATGCCAGGTGTTAT
              Thalassia: GTTATTGATAGACAGAAGAATCATGGTATGCATTTCCGTGTACTAGCTAA
             Prymnesium: GCAGAAGGTCTATTCTTTGCACAAGATTGGGCTTCGCTACGTAAGTGTGT
    Chrysochromulina_sp: CCTGAAGGTATGTTCTTCGCACAAGATTGGGCTTCTCTACGTAAGTGTGT
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Appendix 2 (Continued)

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            Imantionia: CCTGAAGGTCTATTCTTTGCACAAGATTGGGCTTCTCTACGTAAGTGTGT
          ST1CH3_clone: CCTCAAGGTTTATTCTTTGCTCAAGATTGGGCTTCACTACGTAAGTGTGT
         Helicosphaera: CCTCAAGGTTTATTCTTCGCACAGGATTGGGCTTCACTACGTAAGTGTGT

     Emiliana_huxleyi: ACCAGTTGCTTCAGGTGGT-------ATCCACTGTGGTCAAATGCACCAG
        ST8CH26_clone: TCCAGTAGCTTCTGG-----------------------------------
           Isochrysis: ACCAGTAGCTTCTGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
         Calcidiscus: ACCAGTAGCTTCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
     Cruciplacolithus: ACCAGTAGCTTCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
        Pleurochrysis: ACCGGTAGCATCTGGCGGT-------ATCCACTGTGGTCAAATGCACCAA
              Pavlova: GCCAGTAGCTTCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
     Exanthemachrysis: GCCAGTAGCTTCTGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
           Pyrenomonas: GCCAGTTGCTTCAGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
            Chroomonas: ACCAGTTGCTTCTGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
              Rhodella: GCCTGTTGCTTCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
             Dictyocha: GCCAGTAGCCTCTGGTGGT-------ATTCATTGTGGTCAAATGCACCAA
        ST6CH33_clone: ACCTGTAGCTTCTGG-----------------------------------
          Heterosigma: ------AACTGGTATTATT-------GTAGAACGTGAGCGTCTTGATACA
          Chattonella: TCCAGTTGCTTCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
        ST4CH31_clone: ACCGGTAGCCTCCGG-----------------------------------
         ST1CH4_clone: GCCAGTAG---------------------------------------------
        ST3CH27_clone: ACCAGTAGC-------------------------------------------
           Ectocarpus: TCCGGTAGCTTCTGGGGGA-------ATCCATTGTGGTCAAATGCACCAA
           Xanthonema: ACCAGTAGCTTCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
            Vaucheria: ACCTGTAGCATCTGGTGGT-------ATTCACTGTGGACAATTACATCAA
         Heterococcus: GTGGATGCGTATGGCAGGT-------GTGGATCACATTCACGCAGGTACA
           Aureoumbra: GCCTGTTGCATCGGGTGGT-------ATCCATTGTGGTCAAATGCATCAA
          Aureococcus: GCCAGTAGCTTCTGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
         Pelagococcus: ACCAGTAGCTTCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
        Cylindrotheca: GCCTGTTGCTTCTGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
        Phaeodactylum: GCCTGTTGCTTCTGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
        ST4CH14_clone: GCCTGTAGCTTCAGG-----------------------------------
         ST6CH2_clone: GCCAGTAGCTTCTGG-----------------------------------
         Rhizosolenia: GCCTGTAGCTTCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
          Skeletonema: GCCAGTAGCTTCTGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
        Thalassiosira: GCCAGTAGCTTCTGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
              Detonula: ATTATTCGACATTAAAGATCCTGCAACAGTAATGTTTGAATTAAATGAAG
            Chilomonas: GCCAGTTGCTTCTGGTGGT-------ATTCACTGCGGGCAGATGCATCAA
Mazzaella_membranacea: GCCTGTTGCTTCTGGTGGT-------ATTCATTGTGGACAAATGCATCAA
       Karenia_brevis: ACCTGTCGCTAGCGGAGGA-------ATTCATGCAGGACAAATGCACTAC
       Jaxonville C3a: ACCTGTCGCTAGCGGAGGA-------ATCAATGCGGGACAAAT-------
      Mexico_Beach_C5: ACCTGTCGCTAGCGGAGGA-------ATCAATGCGGGACAAAT-------
      Piney_Island_A9: ACCTGTCGCTAGCGGAGGA-------ATCAATGCGGGACAAAT-------
  Charlotte_Harbor_C2: ACCTGTCGCTAGCGGAGGA-------ATCAATGCGGGACAAAT-------
  Charlotte_Harbor_A2: ACCTGTCGCTAGCGGAGGA-------ATCAATGCGGGACAAAT-------
      Piney_Island_B4: ACCTGTCGCTAGCGGAGGA-------ATCAATGCGGGACAAAT-------
      Mexico_Beach_B3: ACCTGTCGCTAGCGGAGGA-------ATCAATGCGGGACAAAT-------
    Appalachicola_C6: ACCTGTCGCTAGCGGAGGA-------ATCAATGCGGGACAAAT-------
  Karenia_mikimotoi_1: ACCAGTTGCGAGTGGAGGA-------ATCAATGCGGGACAAAT-------
    Karenia_mikimotoi: ACCAGTTGCTAGTGGAGGA-------ATCAATGCGGGACAAATGCATTTT
          Karlodinium: ACCAGTAGGTTCAGGTGGT-------ATTCATTGTGGTCAAATGCATAAA
           Amphidinium: CTTGCTCTTTGACCGCAAC-------ATCACCGATGG-------------
        ST2SY33_clone: TGCTGTCGCATCAGGTGGT-------ATCCATGTCTG-------------
       Prochlorococcus: TGCAGTCGCATCGGGTGGT-------ATCCATGTTTGGCATATGCCTGCA
          Thiobacillus: TGCACTCGGGTACCGTGGTCGGCAAGCTCGAAGGCGACCGTGAGGCGACC
     Synechococcus_sp: GGCCGTGGCCTCCGGTGGG-------ATTCACGTCTGGCACATGCCTGCC
        ST2SY26_clone: CGCCGTTGCTTCCGGCGGT-------ATCCACGTCTG-------------
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Appendix 2 (Continued)

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          Synechococcus: TCCACACCGGCACCGTGGTCGGAAAGCTGGAAGGTGATCGTCAGACCACC
            Nitrobacter: TGCACACCGGCACCGTCGTGGGCAAGCTCGAAGGCGATCGTGCTTCCACC
          Synechocystis: GCCCGTAGCTTCCGGTGGT-------ATCCACGTATGGCACATGCCCGCG
Synecchococcus_PCC7002: GCCTGTGGCTTCCGGTGGT-------ATCCACGTATGGCACATGCCTGCC
          ST8SY15_clone: GCCAGTAGCTTCTGGTGGT-------------------------------
          Trichodesmium: ------------------------------------------------
           ST5SY7_clone: GCCAGTTGCTTCAGGTGGT-------------------------------
           ST2SY2_clone: TCCTGTAGCATCAGGTGGT-------ATCCACGTCTG-------------
           ST6SY8_clone: GCCCGTAGCATCTGGTGGT-------------------------------
        Volvox_carteri: AGCTCTTCGTATGTCTGGT-------GGTGACCACCTTCACTCAGGTACT
               Spinacia: GCCTGTTGCTTCAGGCGGT-------ATTCACGTTTGGCATATGCCTGCT
             Asparagus: TCCCGTGGCTTCAGGGGGT-------ATTCATGTTTGGCATATGCCTGCC
             Thalassia: AGCATTACGTATGTCTGGT-------GGGGATCACATTCACGCTGGTACG
            Prymnesium: ACCAGTTGCTTCGGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
   Chrysochromulina_sp: ACCAGTAGCATCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
             Imantionia: ACCAGTTGCTTCTGGTGGT-------ATTCACTGTGGTCAAATGCACCAA
           ST1CH3_clone: ACCAGTAGCTTCTGG-----------------------------------
          Helicosphaera: ACCAGTAGCTTCAGGTGGT-------ATCCACTGTGGTCAAATGCACCAA
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