Harmful Algae 37 (2014) 92–99

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Effects of growth phase, diel cycle and macronutrient stress on the quantification of *Heterosigma akashiwo* using qPCR and SHA

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ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form 21 May 2014
Accepted 21 May 2014

Keywords:
Cell cycle
Diel cycle
Heterosigma akashiwo
Nutrient stress
qPCR
SHA

ABSTRACT

The development of molecular probe technologies over the last several decades has enabled more rapid and specific identification and enumeration of phytoplankton species compared to traditional technologies, such as light microscopy. Direct comparisons of these methods with respect to physiological status, however, are sparse. Here we directly compare quantitative real-time PCR (qPCR) and sandwich hybridization assay (SHA) for enumerating the raphidophyte *Heterosigma akashiwo* at several points during its growth phase, over a diel cycle and with macronutrient stress in laboratory cultures. To ensure consistency between comparisons, a single cellular homogenate was generated from each culture and split for analysis by qPCR and SHA. Since the homogenate was generated from the same number of cells during each experiment, results reflect changes in nucleic acid content (rRNA and DNA) at each time point or in response to environmental conditions relative to a reference sample. Results show a greater level of precision in SHA results which contributed to significant (2–3 fold) differences in rRNA content per cell in several of these analyses. There was significantly greater rRNA content during lag and exponential phases compared to stationary phase cultures, and a significant decrease in rRNA content during the light cycle compared to cells harvested in the dark. In contrast, there were no significant differences in DNA content per cell as determined by qPCR over a diel cycle or during different growth phases. There was also no decrease in either rRNA or DNA content for cultures under low P conditions compared to nutrient replete conditions. However, both rRNA and DNA content were significantly lower under N stress when compared to nutrient replete conditions. Results of this study suggest that growth stage, nutrient stress and cell cycle may impact molecular analyses, and that physiological status should be taken into account when using these methods for HAB monitoring.

1. Introduction

A fundamental requirement for any harmful algal bloom (HAB) monitoring program is the accurate determination of both species and abundance. Light microscopy has historically been the method of choice for detection, identification, and enumeration of HAB species, but this technique can be time consuming and may require specialized training for accurate identification between species or strains. In addition, the use of microscopy for monitoring HAB species may not be sensitive enough to detect organisms that are a minor component of the assemblage (Anderson et al., 2005; Godhe et al., 2007). Further identification to the species level may also require the use of electron microscopy (Anderson, 1995), which is not amendable to high-throughput analysis (Culverhouse et al., 2003). Over the past couple of decades, development of molecular probe technologies have enabled rapid and specific identification of phytoplankton species while also providing increased sensitivity of detection (Coyne et al., 2001, 2005; Greenfield et al., 2006; Godhe et al., 2007). As a result, the use of molecular approaches in HAB research and monitoring programs has increased (Rhodes et al., 2001; Anderson et al., 2005, 2012; Haywood et al., 2009).

Quantitative real-time PCR (qPCR) and sandwich hybridization assay (SHA) are two molecular approaches that have been developed for detection and quantification of HAB species (Tyrrell et al., 2002; Coyne et al., 2005; Haywood et al., 2007; Greenfield et al., 2008). Each approach uses targeted molecular probes or non-specific fluorescent dyes for detection and quantification. The techniques differ in that qPCR typically targets DNA and requires

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http://dx.doi.org/10.1016/j.hal.2014.05.014
1568-9883/

isolation and purification of DNA from samples, whereas SHA uses direct detection of ribosomal RNA (rRNA) from unpurified and unamplified samples (Greenfield et al., 2006; Scholin et al., 2003). Both technologies have been used extensively in the marine environment for quantitative detection of HAB species including *Pseudo-nitzschia* (Scholin et al., 1997), *Aureococcus anophagefferens* (Popels et al., 2003), *Alexandrium fundyense* (Godhe et al., 2007), *Karlodinium veneficum* (Handy et al., 2008), *Karenia brevis* (Gray et al., 2003; Haywood et al., 2007), *Cochlodinium polykrikoides* (Mikulski et al., 2008), *Ostreopsis* spp. (Hariganeya et al., 2013) and several raphidophyte species, including *Heterosigma akashiwo* (Tyrrell et al., 2002, 2001; Coyne et al., 2005; Bowers et al., 2006; Handy et al., 2006; Demir et al., 2008; Greenfield et al., 2008; Portune et al., 2009).

Heterosigma akashiwo is a globally distributed harmful alga (Li and Smayda, 2000; Tyrrell et al., 2002; O'Halloran et al., 2006; Kempton et al., 2008; Portune et al., 2009) that has been implicated in fish killing blooms in China (Tseng et al., 1993), Japan (Honjo, 1992), New Zealand (Chang et al., 1990), South Africa (Bates et al., 2004) and estuaries on the east (Kempton et al., 2008) and west (Rensel et al., 2010) coasts of the United States. Although both qPCR (Coyne et al., 2005; Handy et al., 2006; Bowers et al., 2006; Demir et al., 2008; Portune et al., 2009) and SHA (Tyrrell et al., 2002, 2001; Greenfield et al., 2006, 2008) have been developed and independently validated for this HAB species, direct comparison of these methods has only recently been carried out (Doll et al., 2014). In this recent study, Doll and co-authors directly compared the accuracy and sensitivity of each method for enumeration of *H. akashiwo* over a range of cell concentrations. In addition, several strains of *H. akashiwo* representing various geographic origins were assessed using qPCR and SHA, with results showing a high degree of correlation between methods. These results also highlighted the capabilities and limitations of each method. For example, qPCR accurately enumerated cells over a wide range of cell concentrations but was less able to distinguish differences in small (~2-fold) changes in cell abundance. By comparison, SHA was capable of detecting small changes in cell concentrations, particularly at pre-bloom levels, but became oversaturated at higher levels of cell abundance (Doll et al., 2014).

The physiological status of the cell may also affect the accuracy of qPCR and SHA to enumerate cell abundances (e.g. Miller et al., 2002; Tyrrell et al., 2002; Haywood et al., 2007; Dittami and Edvardsen, 2012), but rigorous comparisons between these methods with respect to physiological status of HAB species have not been conducted. Changes in growth rate during bloom progression, for example, are likely to impact both DNA (Berdalet et al., 1992) and rRNA content (Ayers et al., 2005), and can contribute to an overestimation of cell abundance during exponential growth compared to stationary phase when using SHA (Tyrrell et al., 2001; Dittami and Edvardsen, 2012). Diel changes in the cell cycle are also accompanied by changes in cellular DNA and rRNA content (Zachleder and Šetlík, 1988; Berdalet et al., 1992). In *Heterosigma akashiwo*, replication of DNA occurs during the daylight (Satoh et al., 1987; Lee et al., 2012; Tobin et al., 2013), suggesting that enumeration of cell densities from samples collected during the light and dark cycles may yield different results by either qPCR or SHA. Finally, nutrient stress can affect both growth rates and rRNA synthesis. In photosynthetic organisms, RNA is a major source of non-storage phosphorus (Raven, 2013), and the concentrations of rRNA have been shown to decrease under phosphorus limitation in a number of phytoplankton species (Elser et al., 2000, 2003; Flynn et al., 2010). Nitrogen limitation, on the other hand has a direct impact on protein content, which can result in lowered growth rates and adversely affecting rRNA content per cell (Rhee, 1978; Miller et al., 2002; Sterner and Elser, 2002; Dittami and Edvardsen, 2012). Although this has been demonstrated for *H. akashiwo* (e.g. Blanco et al., 2013), the effect of nutrient stress on DNA-based analysis such as qPCR have not been investigated for this species.

The objective of this study was to examine the potential effects of physiological status on the accuracy of qPCR compared to SHA. This research extends the study of Doll et al. (2014), by directly comparing qPCR and SHA methods for enumerating cell density with respect to (1) diel cycle, (2) growth phase, and (3) macronutrient (nitrogen and phosphorus) stress, using *Heterosigma akashiwo* as a model organism. Results of this study are broadly applicable to research and monitoring efforts for *H. akashiwo* and other HAB species.

2. Materials and methods

2.1. Culture conditions

Heterosigma akashiwo, Center for Applied Aquatic Ecology (CAAE) strain 1663, previously isolated from an estuary in Hilton Head, South Carolina, USA (32.18° N, 80.74° W), was used for all experiments. Batch cultures of *H. akashiwo* were grown in 500 mL *f*/2 medium (−Si) at a salinity of 25 (Guillard and Ryther, 1962). Cultures were kept at 25 °C with 140 μE m^{-2} s^{-1} irradiance on a 12:12 h light:dark cycle. For all experiments, cell densities were determined from replicate (5–9) counts using a light microscope and a Sedgewick-Rafter chamber, as a reference for results produced by qPCR and SHA according to previously described methods (Doll et al., 2014; Greenfield et al., 2008). The Sedgewick-Rafter chamber detects lower concentrations of cells and replicate counts have smaller deviations than either a Palmer-Malony slide or a hemocytometer (Godhe et al., 2007).

2.2. Cellular homogenization

For each experiment described below, cultures were filtered using a gentle vacuum (380 mmHg) onto 25 mm, 0.65 μm pore size hydrophilic Durapore filters (Millipore) with a minimum of 10 filters per sample. Filters were immediately placed into 2 mL cryovials (Nalgene, Rochester, NY, USA), sample facing inward, and then stored in liquid nitrogen until processing. For direct comparison of qPCR and SHA, a single cellular homogenate was generated then split for use in both assays as described in Doll et al. (2014). Briefly, frozen filters ($n = 5$) were briefly thawed, then incubated with 2 mL of 0.2 μm filtered lysis buffer containing guanidinium thiocyanate (3 M, pH 8.9, Saigene Biotech Corporation, Marina, CA, USA) at 85 °C for 5 min (Goffredi et al., 2006). Homogenate within each cryovial was then combined and syringe-filtered through a 0.22 μm Millex filter (Fisher). A 2 mL aliquot of filtered homogenate was removed and stored at −80 °C until shipped overnight on dry ice to the University of Delaware (Lewes, DE, USA) for qPCR analyses as described below. A second portion of the cellular homogenate (250 μL per sample well) was analyzed for SHA as described below at the University of South Carolina (Charleston, SC, USA).

2.3. Growth phase and diel cycle

Batch cultures ($n = 3$) were sampled at the start of the light cycle over four growth phases (lag, exponential, stationary and decline, Fig. 1). Growth phases were determined by cell counts of batch culture as described above. At each phase, 40,000 cells from each replicate were collected by gentle vacuum onto 0.65 μm Durapore filter. Additional sampling occurred during exponential stage for comparison of changes during the diel cycle. Sampling for diel cycle began at the start of the light cycle (T_0), and additional samples were collected at 4, 8, 12 and 20 h after T_0. Samples were collected, filtered and stored in liquid nitrogen as above.

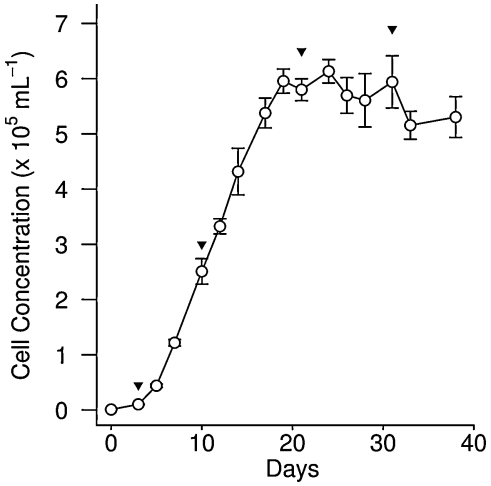

Fig. 1. Growth curve of *Heterosigma akashiwo*. Arrow heads (▼) represent collection dates for growth phase experiment: lag phase, Day 3; exponential phase, Day 10; stationary phase, Day 22; and decline phase, Day 31.

2.4. Macronutrient stress

To examine the effects of low levels of nitrogen (N) and phosphorus (P) on quantification of *Heterosigma akashiwo*, initial batch cultures were grown to exponential stage. A pre-treatment sample in nutrient replete *f*/2 medium (−Si, 883 μM $NaNO_3$ and 36.3 μM $NaH_2PO_4{\cdot}2H_2O$) consisting of ~60,000 cells from the batch culture was collected and used for establishment of cultures in low nitrogen (low N, 1:1 N:P, 36.3 μM $NaNO_3$ and 36.3 μM $NaH_2PO_4{\cdot}2H_2O$) or low phosphorus (low P, 100:1 N:P, 883 μM $NaNO_3$ and 8.83 μM $NaH_2PO_4{\cdot}2H_2O$) media ($n = 3$). Control cultures were nutrient replete in *f*/2 (−Si) seawater medium. Batch cultures were grown to exponential phase in low N or P media or nutrient-replete *f*/2 (control) medium and transferred a second time to minimize carryover from nutrient replete conditions. Cell counts were conducted as above, and acclimatized cultures were sampled during exponential phase at the start of the light cycle for qPCR and SHA analyses. Sample volumes containing 40,000 cells were collected, filtered and stored in liquid nitrogen as described above.

The physiological status of *Heterosigma akashiwo* was assessed in tandem with sampling for qPCR and SHA analyses. The efficiency of photosystem II, Fv/Fm, was measured as an indicator of stress (Parkhill et al., 2001) using a Phyto-PAM fluorometer (Walz, Germany) after a 25 min dark adaptation. Chlorophyll *a* (chl *a*) concentrations were measured after extraction with 90% acetone (Arar and Collins, 1992). In addition, samples for particulate carbon (PC) and nitrogen (PN) analysis were collected to detect changes in C:N. Culture (1 mL) was concentrated onto a pre-combusted GFF and stored desiccated until analysis using a Costech Elemental Combustion System CHNS-O 4010 (Costech Analytical Technologies Inc., Valencia, CA, USA). An aliquot of culture was syringe-filtered through a pre-combusted GFF for dissolved nutrient (N and P) concentrations performed using a Lachat Quik-Chem-8000 autoanalyzer (Loveland, CO, USA; Johnson and Petty, 1983; Hansen and Koroleff, 1999). An aliquot of 2 mL was collected in triplicate from each culture for analysis of protein and carbohydrate content. Cell pellets were obtained by centrifuging for 5 min at 10,600 × *g*. The supernatant was carefully removed and cell pellets were frozen immediately and stored at −80 °C until shipping over night for analyses at the University of Delaware. Proteins were extracted from pelleted algal cells by sonication in KPi buffer (200 mM potassium phosphate monobasic and 140 mM potassium hydroxide, pH 7.9). Total protein content was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to manufacturer instructions. For total carbohydrate content, algal cells were digested in phenol and sulfuric acid (final concentration 0.66% and 13 M respectively). Samples were incubated in a room temperature water bath for 30 min and absorbance was read at 482 nm on a FLUOstar Omega (MG LABTECH GmbH, Germany; DuBois et al., 1956). Concentrations of each constituent (protein and carbohydrate) were determined by linear regression analysis.

2.5. DNA extraction

Prior to extraction of DNA for qPCR analysis, 50 ng mL^{-1} (2 μL) of pGEM plasmid (pGEM-3Z Vector, Promega, Fitchburg, WI, USA) was added to samples as an internal standard (Coyne et al., 2005). To provide concentrations of background DNA comparable to those found in environmental samples, 5 μg mL^{-1} of sheared herring sperm (Invitrogen, Grand Island, NY, USA) was also added. DNA was extracted from each cellular homogenate using a DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA), eluted in 100 μL of elution buffer and diluted 1:5 with LoTE (3 mM Tris–HCl, 0.2 mM EDTA, pH 7.5) prior to qPCR analysis.

2.6. qPCR

qPCR was performed using an Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Grand Island, NY, USA). Abundances of pGEM and *Heterosigma akashiwo* 18S rDNA were determined in separate qPCR reactions. DNA was amplified in 10 μL reactions for each target sequence, consisting of 5 μL of TaqMan Universal Master Mix (Applied Biosystems), 1 μL diluted template and appropriate concentrations for primers and probe. For amplification of *H. akashiwo* rDNA gene, final concentrations were 0.9 μM of each primer (Hs 1350F and Hs 1705R; Coyne et al., 2005) and 50 nM Hs Probe (Coyne et al., 2005), modified with a 5′-HEX reporter dye, an internal ZEN quencher and 3′-IBFQ1 quencher molecule (Integrated DNA Technologies, Coralville, IA, USA). For amplification of the internal standard pGEM plasmid, final concentrations were 0.9 μM of each primer (M13F and pGEMR; Coyne et al., 2005), and 0.2 μM pGEM Probe (Coyne et al., 2005). Cycling parameters for amplification of each target gene consisted of 2 min at 50 °C, 10 min at 95 °C for activation of the polymerase, followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C, and 1 min at 72 °C. Relative abundance of *H. akashiwo* was determined using a standard curve generated from *H. akashiwo* 18S rRNA plasmid and normalized to the relative abundance of pGEM in each sample. Results of each analysis were then normalized to a reference sample for ease of comparison between methods.

2.7. SHA

Detailed methodologies for using SHA with a 96-well plate and robotic processor are described elsewhere (Goffredi et al., 2006). Briefly, homogenized samples (250 μL) were loaded in triplicate to pre-made 96-well plates (Saigene Biotech Corporation) containing the capture probe Het 1 and signal probe Raph BI DIG (Dioxygenin; Tyrrell et al., 2001, 2002; Greenfield et al., 2008). Hybridization reactions were conducted in triplicate at 30 °C with biotinylated prongs (Saigene Biotech Corporation) on an Affirm robotic processor (Microprobe Corporation, Southbury, CT, USA; Goffredi et al., 2006), and 0.2 μm-filtered lysis buffer only was used as a control. When the reaction was complete, optical density was measured using a Synergy HT plate reader (Biotek, Winooski, VT, USA), and results were recorded at 450 nm using Gen 5 software

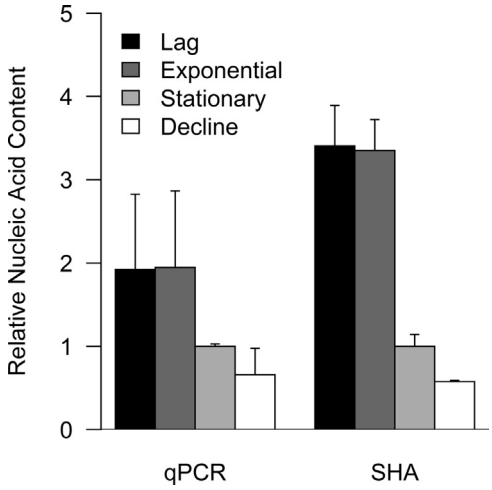

Fig. 2. qPCR and SHA analysis during growth of *Heterosigma akashiwo*. Results were normalized to stationary phase for ease of comparison. Error bars represent one standard deviation.

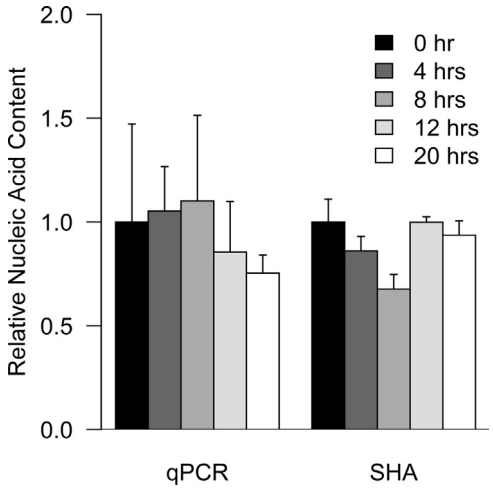

Fig. 3. qPCR and SHA analysis during the diel cycle for *Heterosigma akashiwo*. Results were normalized to T_0 (lights on). Error bars represent one standard deviation.

(Biotek). Results of each analysis were then normalized to a reference sample as above.

2.8. Statistics

Statistical comparisons were performed using the R statistical package (R Core Team, 2012). Outliers were determined using the Dixon's Q test, with removal of outliers if the p-value was <0.05. Data from growth phase, diel cycle and nutrient stress experiments were examined for significant relationships between treatment groups using a 1-way ANOVA. A difference between procedures or treatments was considered significant if the p-value was <0.05. If a significant relationship was detected, further examination was carried out using a Tukey honest significant differences (Tukey HSD) post hoc test to determine relationships.

3. Results

3.1. Growth phase

Results for qPCR and SHA analyses of samples containing the same number of cells collected from lag, exponential, stationary and decline growth phases were normalized to average results of the stationary phase cultures for comparison. qPCR results were not significantly different over the four collection periods (Fig. 2), due in part to the large standard deviations among replicate samples. Mean optical densities from SHA analyses were not significantly different between lag and exponential phases but were significantly different ($p < 0.001$) for all other comparisons. For SHA analysis, calculated cell densities (expressed as relative rRNA content) during lag and exponential phase growth were 3.1 and 3.3 times greater, respectively, than stationary phase for SHA analysis, while cultures in stationary phase were 1.7 times greater than that of decline phase cultures.

3.2. Diel cycle

qPCR and SHA results were normalized to results at T_0 for the diel cycle experiment (Fig. 3). Results for qPCR analysis were not significantly different between sampling time points. Cell abundance as determined by SHA analysis was significantly different ($p < 0.001$) between results obtained at T_0 and T_8, T_8 and T_{12}, and T_8 and T_{20}. Calculated cell densities during T_0, T_{12}, and T_{20} were 1.4, 1.5 and 1.3 times greater, respectively, than cell densities at T_8.

3.3. Macronutrient stress

Nutrient concentrations for low N and P cultures and control cultures are shown in Table 1. Several additional measurements were used to evaluate physiological status. Fv/Fm measures the quantum yield efficiency of photosystem II and has been previously shown to indicate physiological status of phytoplankton in natural and cultured samples (Jakob et al., 2005). In the current study, Fv/Fm was significantly lower ($p < 0.05$) in low N cultures compared to nutrient replete conditions (Table 2). Chl *a* concentrations and protein content were lower in low N and P cultures, but only low N cultures were significantly lower ($p < 0.05$) than nutrient replete conditions (Table 3). Carbohydrate content for low N cultures was significantly greater ($p < 0.001$) than controls, with 9.4 times more carbohydrates than nutrient replete conditions, whereas low P cultures were not significantly different than controls. Finally, pg N cell^{-1} were significantly higher ($p < 0.001$) in low P cultures than replete conditions (Table 3) and pg C cell^{-1} were significantly higher in both low N and P cultures ($p < 0.001$ and $p < 0.05$ respectively) when compared to replete cultures (Table 3). However, the C:N ratio of low P cultures was not significantly different from nutrient replete cultures.

Results of qPCR and SHA analyses were normalized to nutrient replete (control) samples (Fig. 4). qPCR results for low N cultures were significantly lower ($p < 0.05$) than nutrient replete (control)

Table 1
Mean nutrient concentrations and nitrogen to phosphorus (N:P) ratio (±sd) after transfer during the nutrient-stress experiment. Sampling occurred during exponential phase growth.

Treatment	Nitrite (μM)	Nitrate (μM)	Orthophosphate (μM)	Ammonium (μM)	N:P
Control	0.64 ± 0.01	774.40 ± 95.06	25.95 ± 0.66	4.01 ± 0.53	30.1 ± 2.64
Low N	0.33 ± 0.03	6.76 ± 11.40	38.60 ± 5.31	2.29 ± 0.52	0.220 ± 0.259
Low P	0.74 ± 0.25	820.90 ± 27.44	1.85 ± 2.05	2.95 ± 0.18	892 ± 617

Table 2
Mean Fv/Fm and chl *a* content (±sd) for nutrient-stress experiments. Sampling occurred during exponential phase.

Treatment	Fv/Fm	Chl *a* (pg cell^{-1})
Control	0.65 ± 0.01	$5.05 \times 10^{-6} \pm 0.09 \times 10^{-6}$
Low N	0.59 ± 0.02*	$3.51 \times 10^{-6} \pm 0.59 \times 10^{-6}$*
Low P	0.67 ± 0.01	$3.87 \times 10^{-6} \pm 0.65 \times 10^{-6}$

* $p < 0.05$.

cultures, which were 1.98 times greater than low N cultures. Similar results were obtained from SHA, where replete cultures were 1.36 times greater than the low N cultures.

4. Discussion

In this study we examined the effects of growth phase, diel cycle, and macronutrient stress on quantification of *Heterosigma akashiwo* by qPCR and SHA in order to determine whether physiological factors may contribute to variability in results obtained by these methods. Ribosomal RNA sequences typically contain conserved and variable regions that allow for differentiation of species (Diaz et al., 2010), and both 18S rRNA and its gene sequence (rDNA) within the genome are abundant in eukaryotes, making this an ideal target for molecular methods. However, rDNA copy number within the cell changes during cell division (due to genome duplication), which may affect qPCR results during periods of rapid growth. Cellular content of 18S rRNA may also vary over the diel cycle (Berdalet et al., 1992) and can be affected by nutrient stress (Vrede et al., 2004), potentially impacting the accuracy of SHA results.

Both SHA and qPCR methods have been used to enumerate *Heterosigma akashiwo* in laboratory culture experiments (Tyrrell et al., 2001, 2002; Bowers et al., 2006; Greenfield et al., 2008; Handy et al., 2006, 2008), and in field samples (Ayers et al., 2005; Coyne et al., 2005; Demir et al., 2008; Greenfield et al., 2008; Handy et al., 2005, 2008; Portune et al., 2009; Ryan et al., 2011), but rigorous comparisons between these two methods have never been carried out across a range of physiological conditions. In this study, the number of cells filtered for analysis at each sampling point was kept constant to minimize errors due to extraction efficiencies or range of detection. Results were then normalized to a reference sample to facilitate comparison between the two methods. Since the same number of cells was filtered for each time point or treatment, the results presented here are indicative of the relative nucleic acid content of DNA (for qPCR) or rRNA (for SHA) per cell.

In general, standard deviations between replicate samples were greater for qPCR compared to SHA, indicating a greater level of precision by SHA. A number of factors may contribute to errors by each of these methods. Quantification of nucleic acid content by qPCR can be influenced by the quantity and quality of DNA extracted (Boström and Simu, 2004; Coyne et al., 2005). Inhibitory compounds that co-precipitated with DNA during extraction may also affect the efficiency of the PCR reaction. Addition of exogenous reference standard DNA to the extraction buffer can control for variability in extraction and amplification efficiencies, as both target and reference standard DNA are equally affected (Coyne et al., 2005). In Coyne et al. (2005), the reference standard plasmid pGEM was added to the bulk extraction buffer, resulting in a constant concentration of the reference standard between samples. In this study, we found that addition of pGEM to the buffer interfered with SHA analysis (data not shown), requiring us to add the plasmid to each split sample before extraction for qPCR analysis. Errors in pipetting when adding the exogenous reference standard to individual samples, however, may have contributed to greater standard deviation between replicates when analyzed by qPCR. In addition, PCR amplification is exponential, so that small differences in reference standard DNA content can result in greater standard deviations between replicates. In contrast, SHA measures RNA content within the lysate and does not require further amplification of the target for detection and quantification, resulting in lower standard deviation between replicates. While the results obtained by SHA showed greater precision, Doll et al. (2014) demonstrated that qPCR had a greater range and sensitivity of detection compared to SHA without adjusting sample volume or diluting the homogenate.

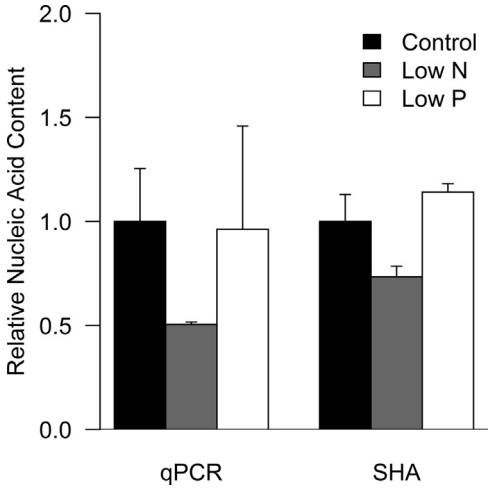

Fig. 4. qPCR and SHA analysis for *Heterosigma akashiwo* cultures during low nutrient concentration conditions. Results were normalized to nutrient replete (control) cultures. Error bars represent one standard deviation.

For the growth phase study, results obtained by both qPCR and SHA were not significantly different between earlier growth stages for either method (Fig. 2). The trend to higher nucleic acid content during the early growth stages for qPCR analysis may be attributed to an increase in DNA content when a greater proportion of cells were undergoing cell division. rRNA content has also been shown to be positively related to specific growth rate (Elser et al., 2000; Sterner and Elser, 2002; Vrede et al., 2004), which would contribute to the greater signal by SHA during lag and exponential

Table 3
Protein (pg cell^{-1}), carbohydrate content (pg cell^{-1}), protein:carbohydrate ratio, nitrogen and carbon per cell (pg cell^{-1}), and C:N ratio (±sd) for nutrient-stress experiment. Sampling occurred during exponential phase.

Treatment	Proteins	Carbohydrates	Protein:carbohydrate ratio	Nitrogen cell^{-1}	Carbon cell^{-1}	C:N ratio
Control	48.66 ± 6.47	12.10 ± 9.51	3.99 ± 2.31	52.64 ± 4.76	177.91 ± 13.99	3.95 ± 0.20
Low N	28.76 ± 9.85*	114.56 ± 101.20**	0.13 ± 0.10	NA	512.85 ± 192.40**	NA
Low P	39.04 ± 1.81	16.94 ± 10.02	2.51 ± 0.47	79.55 ± 6.49**	240.64 ± 43.07*	3.52 ± 0.48

NA represents below detection limits.
* $p < 0.05$.
** $p < 0.01$.

growth phases compared to stationary phase. These results are consistent with those of Tyrrell et al. (2001) who previously demonstrated using SHA that *Heterosigma akashiwo* rRNA content declined by a factor of approximately 2 between exponential and stationary stages. A decline in SHA signal associated with growth phase was also noted for *Karenia* spp. (Haywood et al., 2007), where the variability in results was attributed to cell lysis of unhealthy cells during filtration, or the possible inhibition of rRNA synthesis during senescence.

Previous research demonstrated that the diel cell cycle in *Heterosigma akashiwo* is light dependent, with DNA synthesis occurring during the light cycle, and cell division occurring at night (Kohata and Watanabe, 1986; Satoh et al., 1987; Lee et al., 2012). In the data presented here, qPCR and SHA data generated over a diel cycle was normalized to T_0 (lights on). Although results obtained by qPCR were not significantly different over the course of the day, the trend toward higher nucleic acid content during the light phase may be due to the higher proportion of cells that were synthesizing DNA (Lee et al., 2012). A discrepancy in qPCR results over a diel period has also been noted in other species. In the dinoflagellate *Lingulodinium polyedrum*, for example, cell division caused an asynchrony between microscopic counts and abundances determined by qPCR (Moorthi et al., 2006). In contrast, results obtained by SHA showed a decrease in rRNA during the daylight which returned to levels similar to the initial time point at T_{12} and T_{20} (Fig. 3). This may be due to circadian patterns of transcription, with greater rRNA content required at the beginning of light and dark phases (T_0 and T_{12}; e.g. Doran and Cattolico, 1997). A similar pattern of diel variability in rRNA content was observed in *Synechococcus* cultures entrained in a 12:12 h light:dark cycle (Lepp and Schmidt, 1998). In this case, a decrease in rRNA content during daylight hours due to the circadian control of transcription was not considered plausible, because of the large number of genes that were differentially expressed during the light compared to dark phases. Instead, the diel pattern was attributed to a rate of cell division that exceeded rRNA transcription. Results presented here agree with the pattern observed in *Synechococcus*, but not with the explanation. Instead, rRNA synthesis in *Heterosigma* appears to keep pace with cell division, so that rRNA levels at T_{12} and T_{20} are similar to levels at T_0. The decrease in rRNA content during the day may be a consequence of cells managing internal resources. *H. akashiwo* has a very large nuclear genome ($\sim$2.9 pg DNA cell^{-1}; Cattolico et al., 1976), suggesting that shared cellular resources such as P, that are typically incorporated into RNA may be diverted to dNTP synthesis, resulting in lower rRNA content.

Nitrogen (N) and phosphorus (P) concentrations are potential limiting nutrients for primary producers in aquatic environments (Beardall et al., 2001), and their concentrations can vary substantially over the course of a bloom. Low concentrations of N and/or P can have an effect on cellular DNA and rRNA content. Several studies, for example, demonstrate that regulation of rRNA synthesis is coupled to nutrient availability in yeast and mammalian cells (reviwed by Grummt, 2003). Although little is known about the molecular regulation of rRNA synthesis in phytoplankton, several studies have shown a relationship between N and P limitation and decreases in rRNA content in phytoplankton species. In *Alexandrium fundyense*, for example, N and P limitation resulted in a 4-fold decrease of rRNA fluorescence intensity when compared to nutrient replete conditions (Anderson et al., 1999). Similar results were noted in a recent study on *Heterosigma akashiwo* (Blanco et al., 2013), where N depletion significantly lowered cellular rRNA content after 72 h when compared to controls. N and P are also required for DNA synthesis, and limitation of either or both of these nutrients will result in lower growth and average DNA content per cell (Berdalet et al., 1996). In work presented here, we evaluated cell status at N:P ratios that deviated considerably from the Redfield ratio of 16:1 as a metric for assessing whether cells were stressed by N or P availability. Fv/Fm values for cultures with low concentrations of N were significantly lower when compared to nutrient replete cultures (Table 2). Chl *a* measurements have often been used as a metric of cell density, but chl *a* content per cell can vary with nutrient status (Geider and La Roche, 2002; Geider et al., 1993; Moore et al., 2008). In this study, chl *a* content of cells in low N cultures of *H. akashiwo* was significantly lower than controls (Table 2). Finally, protein to carbohydrate ratios may be used as indicators of nutrient limitation (Pick, 1987), with ratios > 1.2 indicative of no deficiency while ratios < 0.7 as an indicator of extreme deficiency. In our study, the protein to carbohydrate ratio of low N cultures was 0.13, confirming that N stress had a significant effect on carbon partitioning in *Heterosigma* (Table 3). There was no indication from the protein to carbohydrate ratios that the low P cultures were under P-stress. The half-saturation constant (K_s) for growth on orthophosphate for other strains of *H. akashiwo* (Zhang et al., 2006) were lower than the concentrations used here, suggesting that these cultures may not have been truly P limited. Likewise, cellular carbon to nitrogen ratios were not significantly different for low P cultures compared to controls whereas particulate N in low N cultures was below the level of detection (Table 3).

The level of nutrient stress as demonstrated by Fv/Fm, C:N and carbohydrate:protein ratios proved to be a good indicator for qPCR and SHA results. Calculated cell abundances in low N cultures were significantly lower than controls for both qPCR and SHA analyses, whereas results for low P cultures were similar to nutrient replete conditions. For qPCR, the decrease in nucleic acid content under low N conditions was likely due to decreased growth rates, as noted above. It was surprising, however, that SHA results were more significantly affected by low N concentrations than low P concentrations. RNA is a major source of non-storage P in photosynthetic organisms, and therefore a decline in RNA content would be expected under low or limiting P conditions (Raven, 2013). The data presented here do not demonstrate this, suggesting further that cultures were not truly P-stressed. Instead, it was likely that decreased rRNA under low N conditions was a function of decreased growth, as proposed by the Growth Rate Hypothesis (GRH; Sterner and Elser, 2002). GRH suggests that rRNA content is linked to cellular requirements for protein synthesis, which is reduced during periods of low growth (Elser et al., 2000). While there is some controversy about that application of GRH to phytoplankton taxa (Flynn et al., 2010), the general assumption that decreases in rRNA content under N-stress are a consequence of low growth rate is supported by a number of investigations. Several studies, (e.g. Rhee, 1978; Worden and Binder, 2003) for example, have demonstrated that RNA content is reflective of growth rate in phytoplankton. Zachleder and Šetlík (1988) also demonstrated a decline in the abundance of RNA for nitrate starved cells of the freshwater alga *Scenedesmus quadricauda*. In *Hymenomonas carterae* and *Thalassiosira weissflogii*, RNA content during nitrogen limitation decreased to one third of replete conditions (Olson et al., 1986). Finally, Berdalet et al. (1994) showed that N starvation led to parallel decreases in RNA and protein concentrations in *Heterocapsa* sp.

Previous results examining qPCR and SHA methods individually indicated that these methods could be used for accurate identification and quantification of *Heterosigma akashiwo* in laboratory and field samples (Tyrrell et al., 2002, 2001; Greenfield et al., 2008; Handy et al., 2006, 2008; Ryan et al., 2011). However, this investigation demonstrates that growth stage, nutrient stress, and cell cycle may also impact results of molecular analysis, suggesting that resource managers should consider these factors when evaluating results from either method. Our results suggest

that "calibrator" samples, consisting of a sample collected under similar conditions and for which a cell count has been done, may be necessary to accurately ground-truth molecular methods (Coyne et al., 2005). However, exact cell abundances may not be required by resource managers, since often the 2–3 fold difference in cell abundances shown here may be within acceptable limits of variability. Batch cultures used here were not exposed to environmental conditions such as changes in temperature or nutrient flux, or inter- or intraspecific competition for resources, which may also impact results. Future work will examine these factors using field collected samples in order to determine their effects on qPCR and SHA results.

Acknowledgements

This work was supported by the National Oceanic and Atmospheric Association (NOAA) Monitoring and Event Response for Harmful Algal Blooms (MERHAB) research program (Grant # NA10NOS4780141 to KJC and DIG). We would like to thank E. Smith (USC) for use of his CHN analyzer. This is contribution # 178 from the NOAA MERHAB program, 422 from the Grice Marine Lab, 725 from MRRI, and 1696 from the University of South Carolina.

References

Anderson, D.M., 1995. Identification of harmful algal species using molecular probes: an emerging perspective. In: Harmful Marine Algal Blooms. Lavoisier, Paris, France, pp. 3–13.

Anderson, D.M., Kulis, D.M., Keafer, B.A., Berdalet, E., 1999. Detection of the toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae) with oligonucleotide and antibody probes: variability in labeling intensity with physiological condition. J. Phycol. 35, 870–883.

Anderson, D.M., Kulis, D.M., Keafer, B.A., Gribble, K.E., Marin III, R., Scholin, C.A., 2005. Identification and enumeration of *Alexandrium* spp. from the Gulf of Maine using molecular probes. Deep Sea Res. Part II 52, 2467–2490.

Anderson, D.M., Cembella, A.D., Hallegraeff, G.M., 2012. Progress in understanding harmful algal blooms: paradigm shifts and new technologies for research, monitoring, and management. Annu. Rev. Mar. Sci. 4, 143–176.

Arar, E., Collins, G., 1992. In vitro determination of chlorophyll a and pheophytin a in marine and freshwater phytoplankton by fluorescence. USEPA Method 445.0. USEPA Methods Determ. Chem. Subst. Mar. Estuar. Environ. Samples. Cincinnati, OH.

Ayers, K., Rhodes, L.L., Tyrrell, J., Gladstone, M., Scholin, C.A., 2005. International accreditation of sandwich hybridisation assay format DNA probes for microalgae. N.Z. J. Mar. Freshw. Res. 39, 1225–1231.

Bates, S.S., Gaudet, J., Kaczmarska, I., Ehrman, J.M., 2004. Interaction between bacteria and the domoic-acid-producing diatom *Pseudo-nitzschia multiseries* (Hasle) Hasle; can bacteria produce domoic acid autonomously? Harmful Algae 3, 11–20.

Beardall, J., Young, E., Roberts, S., 2001. Approaches for determining phytoplankton nutrient limitation. Aquat. Sci. 63, 44–69.

Berdalet, E., Latasa, M., Estrada, M., 1992. Variations in biochemical parameters of *Heterocapsa* sp. and *Olisthodiscus luteus* grown in 12:12 light:dark cycles. I. Cell cycle and nucleic acid composition. Hydrobiologia 238, 139–147.

Berdalet, E., Latasa, M., Estrada, M., 1994. Effects of nitrogen and phosphorus starvation on nucleic acid and protein content of *Heterocapsa* sp. J. Plankton Res. 16, 303–316.

Berdalet, E., Marrasé, C., Estrada, M., 1996. Microbial community responses to nitrogen- and phosphorus-deficient nutrient inputs: microplankton dynamics and biochemical characterization. J. Plankton Res. 18, 1627–1641.

Blanco, E.P., Hagström, J., Salomon, P.S., Granéli, E., 2013. Detection of *Heterosigma akashiwo* (Hada) using specific RNA probes: variability of RNA content with environmental conditions. Harmful Algae 24, 80–88.

Boström, K.H., Simu, K., Hagström, Å., Riemann, L., 2004. Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. Limnol. Oceanogr. Methods 2, 365–373.

Bowers, H.A., Tomas, C., Tengs, T., Kempton, J.W., Lewitus, A.J., Oldach, D.W., 2006. Raphidophyceae [Chadefaud ex Silva] systematics and rapid identification: sequence analyses and real-time PCR assays. J. Phycol. 42, 1333–1348.

Cattolico, R.A., Boothroyd, J.C., Gibbs, S.P., 1976. Synchronous growth and plastid replication in the naturally wall-less alga *Olisthodiscus luteus*. Plant Physiol. 57, 497–503.

Chang, F.H., Anderson, C., Boustead, N.C., 1990. First record of a *Heterosigma* (Raphidophyceae) bloom with associated mortality of cage-reared salmon in Big Glory Bay, New Zealand. N.Z. J. Mar. Freshw. Res. 24, 461–469.

Coyne, K.J., Hutchins, D.A., Hare, C., Cary, S.C., 2001. Assessing temporal and spatial variability in *Pfiesteria piscicida* distributions using molecular probing techniques. Aquat. Microb. Ecol. 24, 275–285.

Coyne, K.J., Handy, S.M., Demir, E., Whereat, E.B., Hutchins, D.A., Portune, K.J., Doblin, M.A., Cary, S.C., 2005. Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA. Limnol. Oceanogr. Methods 3, 381–391.

Culverhouse, P., Williams, R., Reguera, B., Herry, V., González-Gil, S., 2003. Do experts make mistakes? A comparison of human and machine identification of dinoflagellates. Mar. Ecol. Prog. Ser. 247, 17–25.

Demir, E., Coyne, K.J., Doblin, M.A., Handy, S.M., Hutchins, D.A., 2008. Assessment of microzooplankton grazing on *Heterosigma akashiwo* using a species-specific approach combining quantitative real-time PCR (QPCR) and dilution methods. Microb. Ecol. 55, 583–594.

Diaz, M.R., Jacobson, J.W., Goodwin, K.D., Dunbar, S.A., Fell, J.W., 2010. Molecular detection of harmful algal blooms (HABs) using locked nucleic acids and bead array technology. Limnol. Oceanogr. Methods 8, 269–284.

Dittami, S.M., Edvardsen, B., 2012. Culture conditions influence cellular RNA content in ichthyotoxic flagellates of the genus *Pseudochattonella* (Dictyochophyceae). J. Phycol. 48, 1050–1055.

Doll, C., Main, C.R., Bianco, C., Coyne, K.J., Greenfield, D.I., 2014. Comparison of sandwich hybridization assay and quantitative PCR for the quantification of live and preserved cultures of *Heterosigma akashiwo* (Raphidophyceae). Limnol. Oceanogr. Methods 12, 230–243.

Doran, E., Cattolico, R.A., 1997. Photoregulation of chloroplast gene transcription in the chromophytic alga *Heterosigma carterae*. Plant Physiol. 115, 773–781.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Elser, J.J., Sterner, R.W., Gorokhova, E., Fagan, W.F., Markow, T.A., Cotner, J.B., Harrison, J.F., Hobbie, S.E., Odell, G.M., Weider, L.W., 2000. Biological stoichiometry from genes to ecosystems. Ecol. Lett. 3, 540–550.

Elser, J.J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T.A., Watts, T., Hobbie, S., Fagan, W., Schade, J., Hood, J., Sterner, R.W., 2003. Growth rate–stoichiometry couplings in diverse biota. Ecol. Lett. 6, 936–943.

Flynn, K.J., Raven, J.a., Rees, T.A.V., Finkel, Z., Quigg, A., Beardall, J., 2010. Is the growth rate hypothesis applicable to microalgae? J. Phycol. 46, 1–12.

Geider, R.J., Roche, J., Greene, R.M., Olaizola, M., 1993. Response of the photosynthetic apparatus of *Phaeodactylum tricornutum* (Bacillariophyceae) to nitrate, phosphate or iron starvation. J. Phycol. 29, 755–766.

Geider, R., La Roche, J., 2002. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. Eur. J. Phycol. 37, 1–17.

Godhe, A., Cusack, C., Pedersen, J., Andersen, P., Anderson, D.M., Bresnan, E., Cembella, A., Dahl, E., Diercks, S., Elbrächter, M., Edler, L., Galluzzi, L., Gescher, C., Gladstone, M., Karlson, B., Kulis, D., LeGresley, M., Lindahl, O., Marin III, R., McDermott, G., Medlin, L.K., Naustvoll, L.-J., Penna, A., Töbe, K., 2007. Intercalibration of classical and molecular techniques for identification of *Alexandrium fundyense* (Dinophyceae) and estimation of cell densities. Harmful Algae 6, 56–72.

Goffredi, S.K., Jones, W.J., Scholin, C.A., Marin III, R., Vrijenhoek, R.C., 2006. Molecular detection of marine invertebrate larvae. Mar. Biotechnol. 8, 149–160.

Gray, M., Wawrik, B., Paul, J., Casper, E., 2003. Molecular detection and quantitation of the red tide dinoflagellate *Karenia brevis* in the marine environment. Appl. Environ. Microbiol. 69, 5726–5730.

Greenfield, D.I., Marin III, R., Jensen, S., Massion, E., Roman, B., Feldman, J., Scholin, C.A., 2006. Application of environmental sample processor (ESP) methodology for quantifying *Pseudo-nitzschia australis* using ribosomal RNA-targeted probes in sandwich and fluorescent in situ hybridization formats. Limnol. Oceanogr. Methods 4, 426–435.

Greenfield, D.I., Marin III, R., Doucette, G.J., Mikulski, C., Jones, K., Jensen, S., Roman, B., Alvarado, N., Feldman, J., Scholin, C.A., 2008. Field applications of the second-generation environmental sample processor (ESP) for remote detection of harmful algae. Limnol. Oceanogr. Methods 6, 667–679.

Grummt, I., 2003. Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. Genes Dev. 17, 1691–1702.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. Can. J. Microbiol. 8, 229–239.

Handy, S.M., Coyne, K.J., Portune, K.J., Demir, E., Doblin, M.A., Hare, C., Cary, S.C., Hutchins, D.A., 2005. Evaluating vertical migration behavior of harmful raphidophytes in the Delaware Inland Bays utilizing quantitative real-time PCR. Aquat. Microb. Ecol. 40, 121–132.

Handy, S.M., Hutchins, D.A., Cary, S.C., Coyne, K.J., 2006. Simultaneous enumeration of multiple raphidophyte species by quantitative real-time PCR: capabilities and limitations. Limnol. Oceanogr. Methods 4, 193–204.

Handy, S.M., Demir, E., Hutchins, D.A., Portune, K.J., Whereat, E.B., Hare, C., Rose, J., Warner, M., Farestad, M., Cary, S.C., 2008. Using quantitative real-time PCR to study competition and community dynamics among Delaware Inland Bays harmful algae in field and laboratory studies. Harmful Algae 7, 599–613.

Hansen, H.P., Koroleff, F., 1999. Determination of nutrients. In: Methods of Seawater Analysis. John Wiley & Sons, Ltd., Weinheim, Germany, pp. 159–228.

Hariganeya, N., Tanimoto, Y., Yamaguchi, H., Nishimura, T., Tawong, W., Sakanari, H., Yoshimatsu, T., Sato, S., Preston, C.M., Adachi, M., 2013. Quantitative PCR method for enumeration of cells of cryptic species of the toxic marine dinoflagellate *Ostreopsis* spp. in coastal waters of Japan. PLoS One 8, e57627.

Haywood, A.J., Scholin, C.A., Marin III, R., Steidinger, K.A., Heil, C., Ray, J., 2007. Molecular detection of the brevetoxin-producing dinoflagellate *Karenia brevis* and closely related species using rRNA-targeted probes and a semiautomated sandwich hybridization assay. J. Phycol. 43, 1271–1286.

Haywood, A.J., Scholin, C., Marin III, R., Petrik, K., Pigg, R., Garrett, M., Steidinger, K., Heil, C., 2009. Detection of *Karenia brevis* in Florida coastal waters using sandwich hybridization assays in two formats. In: Proceedings of the 6th International Conference on Molluscan Shellfish Safety. pp. 95–100.

Honjo, T., 1992. Harmful red tides of *Heterosigma akashiwo*. Natl. Ocean. Atmos. Adm. Tech. Rep. Natl. Mar. Fish. Serv. 111, pp. 27–32.

Jakob, T., Schreiber, U., Kirchesch, V., Langner, U., Wilhelm, C., 2005. Estimation of chlorophyll content and daily primary production of the major algal groups by means of multiwavelength-excitation PAM chlorophyll fluorometry: performance and methodological limits. Photosynth. Res. 83, 343–361.

Johnson, K., Petty, R., 1983. Determination of nitrate and nitrite in seawater by flow injection analysis. Limnol. Oceanogr. 28, 1260–1266.

Kempton, J., Keppler, C.J., Lewitus, A., Shuler, A., Wilde, S., 2008. A novel *Heterosigma akashiwo* (Raphidophyceae) bloom extending from a South Carolina bay to offshore waters. Ecology 7, 235–240.

Kohata, K., Watanabe, M., 1986. Synchronous division and the pattern of diel vertical migration of *Heterosigma akashiwo* (Hada) Hada (Raphidophyceae) in a laboratory culture tank. J. Exp. Mar. Biol. Ecol. 100, 209–224.

Lee, J., Katano, T., Chang, M., Han, M., 2012. Application of tyramide signal amplification-fluorescence in situ hybridisation and flow cytometry to detection of *Heterosigma akashiwo* (Raphidophyceae) in natural waters. N.Z. J. Mar. Freshw. Res. 46, 137–148.

Lepp, P.W., Schmidt, T.M., 1998. Nucleic acid content of *Synechococcus* spp. during growth in continuous light and light/dark cycles. Arch. Microbiol. 170, 201–207.

Li, Y., Smayda, T.J., 2000. *Heterosigma akashiwo* (Raphidophyceae): on prediction of the week of bloom initiation and maximum during the initial pulse of its bimodal bloom cycle in Narragansett Bay. Plankton Biol. Ecol. 47, 80–84.

Mikulski, C.M., Park, Y.T., Jones, K.L., Lee, C.K., Lim, W.A., Lee, Y., Scholin, C.A., Doucette, G.J., 2008. Development and field application of rRNA-targeted probes for the detection of *Cochlodinium polykrikoides* Margalef in Korean coastal waters using whole cell and sandwich hybridization formats. Harmful Algae 7, 347–359.

Miller, P.E., Marin III, R., Scholin, C., Goldman, J.C., Doucette, G., Powell, C., 2002. Variation in reactivity of rRNA-targeted probes towards *Pseudo-nitzschia multiseries* grown in nitrate- and silicate-limited continuous cultures. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), Proceedings of the Xth International Conference on Harmful Algae, Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, St. Petersburg, Florida, USA, pp. 270–272.

Moore, C.M., Mills, M.M., Langlois, R., Milne, A., Achterberg, E.P., La Roche, J., Geider, R.J., 2008. Relative influence of nitrogen and phosphorus availability on phytoplankton physiology and productivity in the oligotrophic sub-tropical North Atlantic Ocean. Limnol. Oceanogr. 53, 291–305.

Moorthi, S.D., Countway, P.D., Stauffer, B.A., Caron, D.A., 2006. Use of quantitative real-time PCR to investigate the dynamics of the red tide dinoflagellate *Lingulodinium polyedrum*. Microb. Ecol. 52, 136–150.

O'Halloran, C., Silver, M.W., Holman, T.R., Scholin, C.A., 2006. *Heterosigma akashiwo* in central California waters. Harmful Algae 5, 124–132.

Olson, R.J., Vaulot, D., Chisholm, S.W., 1986. Effects of environmental stresses on the cell cycle of two marine phytoplankton species. Plant Physiol. 80, 918–925.

Parkhill, J.-P., Maillet, G., Cullen, J.J., 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J. Phycol. 37, 517–529.

Pick, F.R., 1987. Carbohydrate and protein content of lake seston in relation to plankton nutrient deficiency. Can. J. Fish. Aquat. Sci. 44, 2095–2101.

Popels, L.C., Cary, S.C., Hutchins, D.A., Forbes, R., Pustizzi, F., Gobler, C.J., Coyne, K.J., 2003. The use of quantitative polymerase chain reaction for the detection and enumeration of the harmful alga *Aureococcus anophagefferens* in environmental samples along the United States East Coast. Limnol. Oceanogr. Methods 1, 92–102.

Portune, K.J., Coyne, K.J., Hutchins, D.A., Handy, S.M., Cary, S.C., 2009. Quantitative real-time PCR for detecting germination of *Heterosigma akashiwo* and *Chattonella subsalsa* cysts from Delaware's Inland Bays, USA. Aquat. Microb. Ecol. 55, 229–239.

R Core Team, 2012. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Raven, J.A., 2013. RNA function and phosphorus use by photosynthetic organisms. Front. Plant Sci. 4, 536.

Rensel, J.E.J., Haigh, N., Tynan, T.J., 2010. Fraser river sockeye salmon marine survival decline and harmful blooms of *Heterosigma akashiwo*. Harmful Algae 10, 98–115.

Rhee, G.-Y., 1978. Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. Limnol. Oceanogr. 23, 10–25.

Rhodes, L.L., Mackenzie, A.L., Kaspar, H.F., Todd, K.E., 2001. Harmful algae and mariculture in New Zealand. ICES J. Mar. Sci. 58, 398–403.

Ryan, J., Greenfield, D.I., Marin III, R., Preston, C., Roman, B., Jensen, S., Pargett, D., Birch, J., Mikulski, C.M., Doucette, G., Scholin, C.A., 2011. Harmful phytoplankton ecology studies using an autonomous molecular analytical and ocean observing network. Limnol. Oceanogr. 56, 1255–1272.

Satoh, E., Watanabe, M.M., Fujii, T., 1987. Photoperiodic regulation of cell division and chloroplast replication in *Heterosigma akashiwo*. Plant Cell Physiol. 28, 1093–1099.

Scholin, C.A., Miller, P.E., Buck, K., Chavez, F., Harris, P., Haydock, P., Howard, J., Cangelosi, G., 1997. Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. Limnol. Oceanogr. 42, 1265–1272.

Scholin, C., Vrieling, E., Peperzak, L., Rhodes, L., Rublee, P., 2003. Detection of HAB species using lectin, antibody and DNA probes. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris, pp. 131–163.

Sterner, R.W., Elser, J.J., 2002. Ecological Stoichiometry. The Biology of the Elements from Molecules to the Biosphere Princeton University Press, Princeton, New Jersey.

Tobin, E.D., Grünbaum, D., Patterson, J., Cattolico, R.A., 2013. Behavioral and physiological changes during benthic-pelagic transition in the harmful alga, *Heterosigma akashiwo*: potential for rapid bloom formation. PLoS One 8, e76663.

Tseng, C.K., Zhou, M.J., Zou, J.Z., 1993. Toxic phytoplankton studies in China. In: Smayda, T.J., Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York, pp. 347–352.

Tyrrell, J.V., Bergquist, P.R., Bergquist, P.L., Scholin, C.A., 2001. Detection and enumeration of *Heterosigma akashiwo* and *Fibrocapsa japonica* (Raphidophyceae) using rRNA-targeted oligonucleotide probes. Phycologia 40, 457–467.

Tyrrell, J.V., Connell, L.B., Scholin, C.A., 2002. Monitoring for *Heterosigma akashiwo* using a sandwich hybridization assay. Harmful Algae 1, 205–214.

Vrede, T., Dobberfuhl, D.R., Kooijman, S.A.L.M., Elser, J.J., 2004. Fundamental connections among organisms C:N:P stoichiometry, macromolecular composition, and growth. Ecology 85, 1217–1229.

Worden, A.Z., Binder, B.J., 2003. Growth regulation of rRNA content in *Prochlorococcus* and *Synechococcus* (marine cyanobacteria) measured by whole-cell hybridization of rRNA-targeted peptide nucleic acids. J. Phycol. 39, 527–534.

Zachleder, V., Šetlík, I., 1988. Distinct controls of DNA replication and of nuclear division in the cell cycles of the chlorococcal alga *Scenedesmus quadricauda*. J. Cell Sci. 91, 531–540.

Zhang, Y., Fu, F.-X., Whereat, E.B., Coyne, K.J., Hutchins, D.A., 2006. Bottom-up controls on a mixed-species HAB assemblage: a comparison of sympatric *Chattonella subsalsa* and *Heterosigma akashiwo* (Raphidophyceae) isolates from the Delaware Inland Bays, USA. Harmful Algae 5, 310–320.