Available online at www.sciencedirect.com
SCIENCE DIRECT®
Harmful Algae 5 (2006) 242–250
HARMFUL ALGAE
www.elsevier.com/locate/hal

Molecular quantification of toxic *Alexandrium fundyense* in the Gulf of Maine using real-time PCR

Sonya T. Dyhrman *[,1], Deana Erdner [1], Jane La Du, Madeline Galac, Donald M. Anderson

Biology Department MS #32, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

Received 23 February 2005; received in revised form 5 June 2005; accepted 25 July 2005

Abstract

The toxic dinoflagellate *Alexandrium fundyense* is widespread in the northeastern part of North America, including the Gulf of Maine, and is responsible for seasonal harmful algal blooms in these regions. Even at low cell densities, *A. fundyense* toxins can accumulate in shellfish and result in paralytic shellfish poisoning (PSP). PSP can be debilitating or lethal to humans and other shellfish consumers and is a public health concern. As a result, accurate measurements of *A. fundyense* distributions, particularly at low cell density, are critical to continued PSP monitoring and mitigation efforts. Towards this end we have developed a real-time quantitative PCR (qPCR) method to monitor *A. fundyense*. Laboratory validation indicates that the qPCR assay is sensitive enough to detect 10 cells per sample, and that it does not detect co-occurring dinoflagellates such as *Alexandrium ostenfeldii*. The qPCR methodology was used to quantify *A. fundyense* cell densities in samples collected during a spring 2003 transect in the Gulf of Maine, and the data were compared to those obtained in parallel from light microscope and DNA hybridization-based methods. Results show that *A. fundyense* cell density was low during this period relative to typical cell densities required for PSP contamination of local shellfish, and that qPCR values were comparable to numbers determined by independent methods.

Keywords: *Alexandrium*; Gulf of Maine; Paralytic shellfish poisoning; qPCR

1. Introduction

Harmful algal blooms (HAB) cause serious public health and economic impacts on a global scale (Hallegraeff, 1993; Van Dolah, 2000; Hoagland et al., 2002). For example, some species within the marine dinoflagellate genus *Alexandrium* are responsible for paralytic shellfish poisoning (PSP) along the coastlines of the US, Canada, and many other countries (Hallegraeff, 1993; Anderson, 1997). PSP can result in serious illness, or death if humans ingest sufficient shellfish contaminated with the dinoflagellate toxins (Van Dolah, 2000). Observations of *Alexandrium* and PSP outbreaks in the northeastern US indicate that populations accumulate in a variety of oceanographic

* Corresponding author. Tel.: +1 508 457 4678; fax: +1 508 457 2027.
E-mail address: sdyhrman@whoi.edu (S.T. Dyhrman).
[1] These authors contributed equally to this work.

1568-9883/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.hal.2005.07.005

habitats (Anderson, 1997) and there is considerable interest in the population dynamics of this genus both in the US and globally.

There are a number of challenges to monitoring coastal waters for the presence of potentially dangerous HAB species. To adequately map populations, high-density spatial and temporal sampling is required. Furthermore, HAB taxa can be difficult to identify because of the morphological similarities between toxic and non-toxic strains or genera. In the case of *Alexandrium fundyense*, there are two serious challenges: the fact that *A. fundyense* can result in shellfish contamination even at very low cell densities, and the presence of many non-toxic, co-occurring strains and species that closely resemble toxic *A. fundyense*. These challenges have driven substantial work on developing sensitive, quantitative, and/or high throughput procedures for determining *A. fundyense* cell number in different systems.

There are a number of different enumeration methods that have improved *Alexandrium* detection and monitoring. Several probe-based techniques are available that fluorescently label intact *A. fundyense* cells with either antibody or oligonucleotide probes for subsequent detection with epifluorescence microscopy (Anderson et al., 1999). These approaches have been enormously useful, but drawbacks include the time and labor involved in microscope counts and the potential effects of cell physiology on labeling efficiency (Anderson et al., 1999). Probes have also been used in higher throughput, multi-well plate formats. One example is the development of a semi-quantitative assay involving PCR coupled to radiolabeled probes or in ELISA-type formats (Penna and Magnani, 1999, 2000). "Sandwich" type rRNA hybridization techniques similar to those developed for the toxic diatom *Pseudo-nitzschia* (Scholin et al., 1996) have also been applied to the enumeration of *Alexandrium* (Scholin and Anderson, 1998; Anderson et al., in press). Again, these approaches are all valuable, but have unique challenges in either sensitivity, quantitation, or throughput. Recently, there has been an increasing interest in the ability to quantitatively identify the amount of starting nucleic acid in a sample by real-time observations of PCR kinetics. With the recognition of nucleic acid amplification-based approaches as a valid, sensitive tool for monitoring the abundance of microorganisms, such methods have been developed for a variety of harmful genera including: *Pfiesteria* (Bowers et al., 2000; Saito et al., 2002), *Karenia* (Gray et al., 2003; Casper et al., 2004), *Aureococcus* (Popels et al., 2003), and *Alexandrium minutum* (Galluzzi et al., 2004). These studies highlight the value of PCR-based assays for monitoring toxic algae. In this study, we develop and validate a real-time quantitative PCR (qPCR) assay for enumerating saxitoxin-producing *A. fundyense* in the Gulf of Maine.

2. Methods

2.1. Cultures

Cell cultures were grown in f/2 medium between 15 and 20 °C in cool white fluorescent light (about 200 μmol quanta m^{-2} s^{-1}) on a 14-h light:10-h dark cycle. The f/2 medium was made with 0.2 μm filtered local seawater (salinity of about 31 psu) with silica added only when growing diatoms (Guillard, 1975). Growth in the cultures was evaluated using a fluorometer (Turner Designs), or via cell counts on Utermöhl (1958) preserved samples. For analysis, cells were harvested by centrifugation (3000 × *g* for 5 min) in mid to late log phase and either processed immediately or stored frozen in liquid nitrogen. All cultures used in this study are listed in Table 1.

2.2. Field sampling

Field samples were collected from surface water along an onshore to offshore transect in the western Gulf of Maine during a 29 May–6 June 2003 cruise (see Table 3 for station locations). Two saxitoxin-producing species of *Alexandrium* occur in the Gulf of Maine: *A. fundyense* and *A. tamarense* (Anderson et al., 1994). We consider these to be varieties of the same species (Anderson et al., 1994; Scholin et al., 1995), and neither antibody nor oligonucleotide probes can distinguish between *A. fundyense* and *A. tamarense* from this region. Only detailed analysis of the thecal plates on individual cells can provide this resolution, but this is not practical for large numbers of field samples. Accordingly, for the purpose of this and other field studies we use the name *A. fundyense* to refer to both forms.

Table 1
Specificity of the primer set used in the qPCR assay

Organism	Strain	Location	Detection by PCR
Alexandrium fundyense	GTCA28	Gulf of Maine, USA	+
Alexandrium fundyense	CB301A	Casco Bay Gulf of Maine, USA	+
Alexandrium tamarense	GTTPO1	Perch Pond Cape Cod, USA	+
Alexandrium catenella	ACQH02	Quarter Master Harbor Puget Sound, USA	+
Alexandrium catenella	ACQH01	Quarter Master Harbor Puget Sound, USA	+
Alexandrium tamarense	SP3B5-2	La Coruña Bay, Spain	–
Alexandrium andersoni	GTTC02	Town Cove Cape Cod, USA	–
Alexandrium luscitanicum	AL8T	Trieste, Italy	–
Alexandrium catenella	TN9A	Tanabe Bay, Japan	–
Alexandrium ostenfeldii	NA	Gulf of Maine, USA	–
Prorocentrum minimum	CCMP1329	Great South Bay Long Island, USA	–
Karenia brevis	GBWILSON	NA	–
Amphidinium cartarae	AMPHI	Great Pond Cape Cod, USA	–
Gymnodinium catenatum	CCMP1937	Ria de Vigo, Spain	–
Lingulodinium polyedra	GPES-22	Gullmar Fjord, Sweden	–
Scripsiella trochoidea	SA2	Perch Pond Cape Cod, USA	–
Thalassiosira weisflogii	CCMP1336	Gardiners Island Long Island, USA	–

NA, not available.

At each station, 4 L of surface water was drawn from a Niskin bottle, prescreened through a 64 μm sieve, and collected onto 15 μm Nitex cut to fit a 47 mm filter tower. These samples were immediately stored in liquid nitrogen for subsequent PCR analyses. Additional surface samples for other cell count procedures were drawn from separate Niskin bottles. One sample of 2 L was concentrated, preserved in Utermöhl's solution, and counted immediately. For oligonucleotide-based *A. fundyense* and *A. ostenfeldii* counts, 8 L of water were sieved onto a 20 μm mesh Nitex screen, and the cells retained on the screen were washed back into a 15 mL tube and preserved in 5% formalin. After 20 min, the cells were centrifuged for 10 min at 3000 × *g*, the supernatant was aspirated, and the cell pellet was transferred into methanol for storage. Note that this sampling procedure differed substantially from the procedure used to collect samples for the qPCR analyses.

Cell detection and quantification via the oligonucleotide labeling procedure has been described previously (Anderson et al., 1999) and this cell enumeration technique has been extensively used in field studies of the Gulf of Maine region (Dyhrman and Anderson, 2003; Anderson et al., in press; Gribble et al., in press). In brief, a dual labeling protocol was employed, using the oligonucleotide probe NA1 (Anderson et al., 1999) specific for the North American *A. fundyense/tamarense/catenella* species complex and conjugated to FITC, and the oligonucleotide probe Ao (5′-ATT CCA ATG CCC ACAGGC-3′) specific for *A. ostenfeldii* (John, 2003) and conjugated to CY3. The labeled cells were counted using a Zeiss Axioscope fitted with a FITC/CY3 filter set, excitation 450 nm and emission 750 nm (Chroma Tech.).

2.3. Real-time PCR

All cultures and field samples were extracted using a DNEasy kit (Qiagen) according to the manufacturer's instructions with a minor modification in the lysis procedure. Lysis was performed by digestion with proteinase K for 4 h at 55 °C, followed by two 80 s cycles in a mini-beadbeater (BioSpec Instruments) with 0.5 mm zirconium beads. The resulting lysate was transferred to a clean tube and processed in accordance with the remaining steps of the DNEasy protocol. PCR primers were designed to amplify a 174 base pair sequence of the ribosomal large subunit (LSU) gene. The forward primer (5′-GAATG-CAAAGTGGGTGG-3′) was designed from an alignment of publicly available *Alexandrium* strain sequences. A previously designed oligonucleotide (NA1) was used as a reverse primer (Anderson et al., 1999). The NA1 oligonucleotide is specific to toxic

North American ribotypes of the *Alexandrium fundyense/tamarense/catenella* species complex (Scholin et al., 1994).

PCR amplification was performed in 25 μL reactions with final concentrations of 150 nM for forward and reverse primers, 1.25 μL template (1.25% of the total sample DNA volume), 0.2 mM dNTPs, 1× Taq polymerase buffer (Fisher), and 0.5 μL (2.5 units) of Taq polymerase (Fisher). A modified hot start procedure was employed to maximize amplification, using the following cycle conditions: 5 min at 98 °C, hold at 60 °C to add a master mix including the Taq, followed by 40 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 7 min. Gradient PCR from 50 to 70 °C was used to determine the optimal annealing temperature of 60 °C.

The qPCR analyses were performed using Brilliant SYBR Green QPCR Master Mix (Stratagene), with template and primer concentrations as above with 12.5 μL of Brilliant SYBR Green Master Mix in 25 μL reactions. The cycle conditions were as above with the addition of a melt curve protocol at the end of the program. In all cases, melt curves were used to confirm single amplification products for the different reactions, and in select cases amplification was also confirmed with agarose gel electrophoresis.

The fluorescence threshold was set by the analytical software for the iCycler (BioRad). The PCR cycle during which this threshold was crossed for each sample was designated the C_T. The reported C_T is the average of triplicate field samples, standards or internal controls. Sample C_T can be compared to the C_T of standards with a known cell count to specify the number of cells present in the sample. PCR efficiency (E) was determined using the equation $E = 10^{(-1/\text{slope})}$.

Using qPCR, the number of cells in a field sample was determined through a comparison to a standard curve. Standards of 100, 500, and 1000 cells were prepared via dilution of a concentrated cell stock onto nitex screens. A 10 cell standard was prepared by direct isolation of cells via micropipette. Each standard was separately extracted to account for variable extraction efficiency at different cell concentrations. To account for potential variability in the target copy number between strains, all standards were made from a mixed stock of seven *A. fundyense* isolates from the Gulf of Maine. All of the strains used in the mixed standards were shown to individually amplify with the primer set, and the slopes of standard curves using individual isolates were very similar.

2.4. Cloning and sequencing

The PCR amplicon generated from the station 89 sample was cloned into the pGEM-T-Easy vector (Promega) according to the manufacturer's instructions. Plasmids were screened for the presence of the insert with an EcoR1 digestion and then purified with QIAprep Miniprep kit (Qiagen). DNA sequencing was done using an ABI 3730xl at the Josephine Bay Paul Center of the Marine Biological Laboratory according to the facility's protocols. Briefly, 200–400 ng of plasmid template was combined with 7.5 pmol of vector primer (M13F or R), 1 μL of ABI BigDye Terminator v3.1 cycle sequencing mix, and 1.7 μL of 5× sequencing reaction buffer in a final volume of 6 μL. The cycle sequencing program consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Unincorporated labeled nucleotides were removed by isopropanol precipitation, and the reaction products were resuspended in formamide for analysis.

3. Results

To assess the specificity of the primer set used in the qPCR assay a number of different phytoplankton cultures were tested. Under the conditions used here, amplification only occurred in members of the *A. fundyense/tamarense/catenella* species complex within the North American ribotype, including cultures isolated from the Gulf of Maine and local embayments (e.g. Casco Bay, Perch Pond) or from the Puget Sound (Table 1). Notably, the primers did not amplify DNA from *A. ostenfeldii*, a species that co-occurs with *A. fundyense* in the Gulf of Maine, but does not produce saxitoxin. Analyses of the mixed cell standards indicated as few as 10 cells per sample could be reliably detected (Fig. 1). The standard curve was linear ($r^2 = 0.978$) over two orders of magnitude and exhibited good efficiency ($E = 1.83$ or 92%), and replication between triplicate amplifications of each standard (Fig. 1).

Using qPCR *A. fundyense* was amplified at stations along a surface water transect in the western Gulf of Maine during a Spring 2003 cruise (Table 2). The concentration of cells detected along this transect

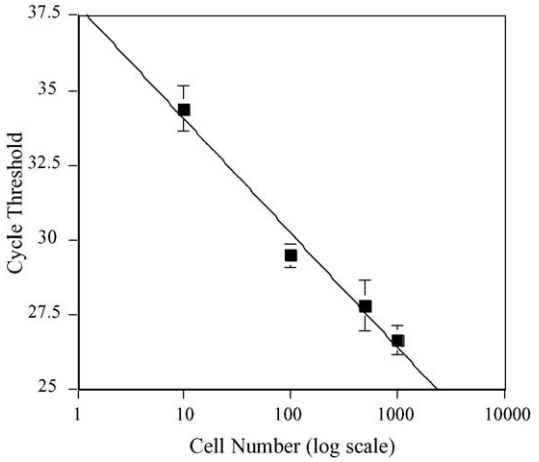

Fig. 1. A standard curve of cycle threshold (C_T) generated for known concentrations of a sample of mixed *A. fundyense* strains. Note that the cells for the 10 cell standard were isolated by micropipette. Error bars denote the standard deviation from the mean C_T of triplicate amplifications of each standard.

ranged from 2.5 to 9.2 cells L^{-1} (Table 3). To assess the potential for PCR inhibition by components of the field sample, a dilution series of DNA from cultured *Alexandrium* cells was added to field sample that contained no *Alexandrium* cells (Table 2). No significant PCR inhibition was detected from the background DNA in the field samples using both qualitative (Fig. 2) and quantitative analyses (Table 2).

Table 2
The average C_T and standard deviation of triplicate PCR amplifications in different samples

Sample	Average C_T	Standard deviation[a]
10 cells	34.4	0.75
100 cells	29.5	0.41
500 cells	27.8	0.85
1000 cells	26.6	0.49
10 cell control[b]	35.9	1.1
100 cell control	29.5	0.5
1000 cell control	27.3	1.0
Station 84	31.9	1.6
Station 85	34.5	1.1
Station 86	32.5	2.4
Station 87	34.1	1.4
Station 89	32.1	0.4
Station 92	32.4	0.6

[a] The standard deviation from the mean of triplicate sample C_T values.

[b] Cell controls consisted of template added to a field matrix without *Alexandrium*.

At one station (89), the PCR product was cloned and sequenced to confirm the specificity of the assay in the field (Fig. 2). The amplified product from both these stations is identical to several known sequences of toxic North American ribotype *A. fundyense* and *A. tamarense* strains (Fig. 3).

Total *Alexandrium* cell numbers were also compared to ship-board counts of newly fixed material and with microscope counts of oligonucleotide labeled *A. fundyense* and *A. ostenfeldii* (Table 3). Regardless of the detection method, cell numbers of *A. fundyense* were low across the transect, ranging from 0 to 78 cells L^{-1}. *Alexandrium* cell numbers determined with direct cell counts were typically higher for a given station when compared to data from the qPCR or oligonucleotide probe methods.

4. Discussion

Alexandrium populations in the Gulf of Maine can result in the contamination of shellfish with PSP toxins even at low cell densities (e.g. 200 cells L^{-1}). These toxic, low density populations in the Gulf of Maine pose challenges to scientists and managers trying to monitor harmful blooms. To address these issues, we have developed and validated a qPCR-based detection assay for *A. fundyense* that is specific and sensitive at low cell density.

The specificity of any amplification-based method is determined by the primer set. The primers used in this study were screened against many dinoflagellates and other potentially co-occurring taxa and found to be specific for the North American ribotype of *Alexandrium* within the *fundyense/tamarense/catenella* species complex (Scholin et al., 1994). Notably, the primers amplified neither Asian isolates within this species complex, nor morphologically similar dinoflagellates such as *Scripsiella* spp. and *A. ostenfeldii*. This is significant, because *A. fundyense* and *A. ostenfeldii* often co-occur in the Gulf of Maine, but *A. ostenfeldii* is not linked to PSP outbreaks. Analysis of culture standards indicated that the method is sensitive at low cell number and over a broad concentration range. The qPCR method reliably and reproducibly detected samples containing only 10 cells, which equates to less than one cell per PCR reaction because only a fraction of the total sample

Table 3
Alexandrium cell numbers at surface stations along a transect in the Gulf of Maine

Station	Long.	Lat.	*A. fundyense* (cell L^{-1}), qPCR	Alexandrium (cell L^{-1}), fixed count[a]	*A. fundyense* (cell L^{-1}), oligo[b]	*A. ostenfeldii* (cell L^{-1}), oligo
84	−68.2165	43.6527	9.2	9	0	0
85	−68.2998	43.7445	2.5	0	0	0
86	−68.3752	43.8403	6.4	39	25	8
87	−68.4162	43.8893	2.9	53	24	6
89	−68.5350	44.0297	8.1	171	78	143
92	−68.9880	44.0070	6.8	47	9	88

[a] Ship-board cell count on freshly fixed sample.
[b] Oligonucleotide probe method on a separately collected and concentrated sample.

volume is used. The ability to detect one cell or less is due to the presence of multiple copies of the ribosomal genes in these organisms; similar results have been observed with other dinoflagellates (e.g. *Pfiesteria*, Bowers et al., 2000). The sensitivity of this method is especially useful for low density populations such as those in the Gulf of Maine.

The qPCR assay works well on field samples, and it can be reliably used to detect *A. fundyense* abundance in natural populations. The specificity of the primer set was confirmed by cloning and sequencing the amplification product from one field station (Station 89). The similarity of the sequence from Station 89 and several *A. tamarense* and *A. fundyense* isolates of the toxic North American ribotype (Fig. 3) indicates

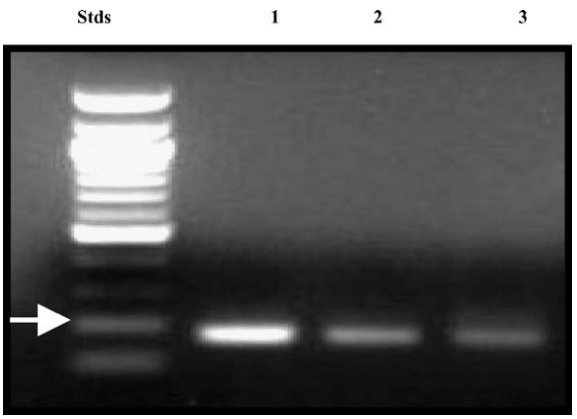

Fig. 2. DNA standards (Stds.) and PCR products from a field sample (Station 89) and a culture of *A. fundyense* strain CA28 imaged in an agarose gel. Lane designations are as follows: (1) 2.5 μL of strain CA28 standard (~50 ng) as template, (2) 1.25 μL of strain CA28 standard in a field matrix, and (3) 1.25 μL of station 89 template. The arrow indicates the 174 bp amplicon.

the specificity of the assay and further highlights the contiguous nature of North American and Northern European *Alexandrium* populations (Medlin et al., 1998; Higman et al., 2001).

Field data generated with the qPCR assay indicated that *A. fundyense* was present, but at low abundance along the transect during the time-frame of the study. Cross confirmation of the presence and low abundance of *A. fundyense* along the Gulf of Maine transect was obtained with two other independent assays; an oligonucleotide probe method and a direct cell count of fixed material. An analysis of a larger sample set would be useful in drawing direct comparisons between methods. Nonetheless, in a comparison between the oligonucleotide-derived cell count and the qPCR-derived cell count, the calculated abundances were within 20 cells L^{-1} except for station 89 where the difference between cell numbers estimated with the two methods was 70 cells L^{-1}. Samples for the different methods were processed from different Niskin bottles, with different concentration techniques (e.g. centrifugation versus filtration) for the two detection methods. Considering the potential patchiness of these low density populations, the data from the qPCR and oligonucleotide methods was strikingly similar.

In a comparison of the qPCR method and the directly counted sample (Utermöhl-fixed, light microscope count) the cell numbers were quite close for several stations. However, the numbers varied more widely in the light microscope counts, due to the presence of *A. ostenfeldii* in those samples (Table 3), as many of the cells of this species cannot be distinguished from *A. fundyense* due to size, shape, and pigment similarities. Here again the qPCR assay

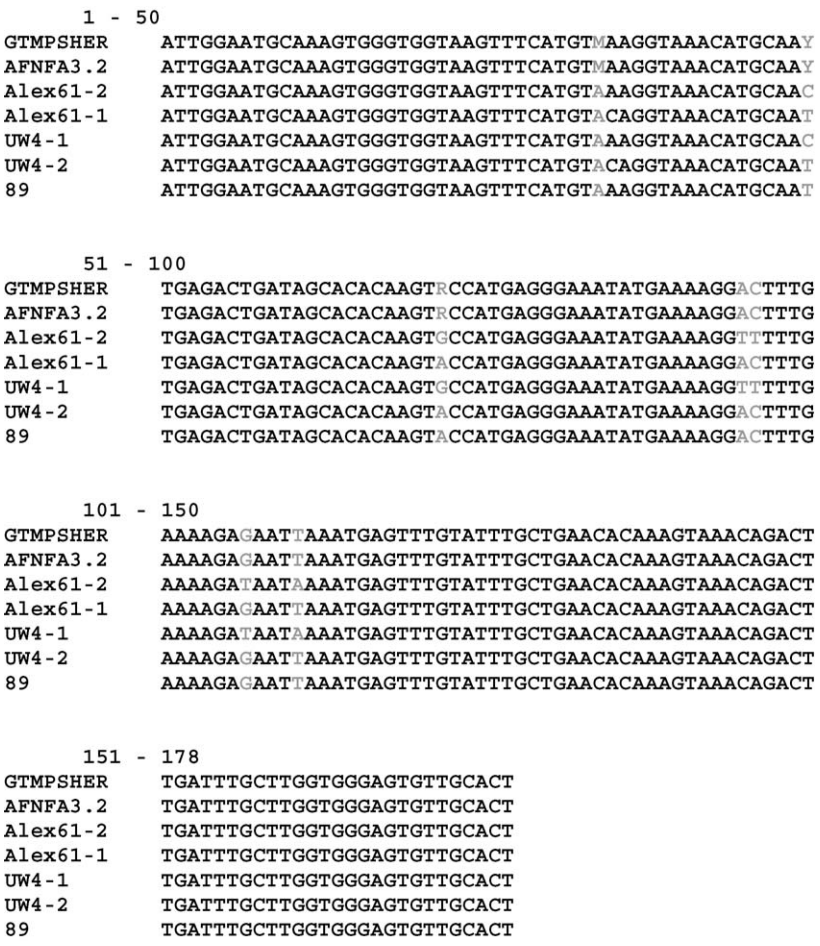

Fig. 3. Sequence alignment comparing known *Alexandrium fundyense/tamarense/catenella* LSU gene sequences and the sequence cloned from Station 89. Strain and sample designations are as follows: GTMPSHER, *Alexandrium fundyense* from Orleans, MA (Scholin et al., 1994); AFNFA3.2, *Alexandrium fundyense* from Newfoundland (Scholin et al., 1994); Alex61-2, Alex61-1, UW4-1, UW4-2, *Alexandrium tamarense* from UK waters (Higman et al., 2001); 89: Station 89 from the Gulf of Maine.

appears robust, as cell numbers were not elevated at stations with high *A. ostenfeldii* concentrations. Moreover, sequence analysis at one of these stations (Station 89) confirmed an *A. fundyense* gene sequence.

Because of the need for widespread spatial and temporal analyses to both protect shellfish consumers and better understand bloom dynamics, the qPCR assay has some advantages over other approaches that require substantial microscopic analysis. Samples can be extracted and analyzed in a 96-well format, minimizing the time required for sample preparation. In addition, the 96-well plate format of the real-time PCR instruments allows for the analysis of standards, controls, and up to 24 unknown samples, all in triplicate, during a single PCR run lasting approximately 4 h. With these advantages also come some challenges inherent to the qPCR methodology. These challenges include accounting for DNA extraction efficiency, variability in the target copy number, and PCR efficiency and inhibition. Difference in extraction efficiency for varying numbers of *Alexandrium* cells was assessed by extracting DNA from individual standards, rather than from one high concentration cell standard that is subsequently diluted. The linearity of

our standard curves indicates that extraction efficiency was consistent for samples containing up to 1000 cells, which is higher than that commonly seen in field samples. To compensate for potential differences in target copy number in field populations, we used a standard containing multiple clonal isolates of *A. fundyense* from the study area. For the application of this assay in other environments, it is important to choose cultured isolates for standard curves that are appropriate to the local population of *Alexandrium* and its diversity. In our application of the method to *Alexandrium* in the Gulf of Maine, we had good PCR efficiency (92%), which is in the range of other studies (Pfaffl, 2001). We also did not see significant PCR inhibition from the background matrix of DNA in our field samples. However, this must be addressed empirically for anyone wishing to utilize this or similar assays for the quantification of field samples. A modified qPCR approach that incorporates an internal DNA standard has been employed by Coyne et al., in press. The internal standard is added prior to DNA extraction and provides an independent assessment of extraction efficiency and PCR inhibition for each sample. The use of an internal standard addresses some primary challenges of these methods and may therefore be advisable for future implementation of qPCR assays. Regardless of the method employed, there are inherent challenges to quantifying cell number, particularly at low abundance and in mixed communities (Freeman et al., 1999; Culverhouse et al., 2003). However, the present data indicate the specificity, sensitivity, and utility of the qPCR assay for the detection and quantification of toxic *A. fundyense* in field populations.

5. Conclusions

There is an ongoing need for sensitive, high throughput methods for screening field populations of harmful algae. Here, we have described the development of a qPCR-based assay for monitoring field populations of toxic *A. fundyense*. This approach does not require taxonomic expertise, is sensitive, and it offers high throughput capabilities. With the current primer set, this method has broad applicability for studying and monitoring populations of *Alexandrium* from the *fundyense/tamarense/catenella* species complex along both the Atlantic and Pacific U.S. coasts.

Acknowledgements

The authors wish to acknowledge the participants of the CODAR cruise and the Captain and crew of the R/V Oceanus. Special thanks also go to Sheean Haley, Kristin Gribble, Claudia Martins, and Bruce Keafer for technical assistance. This research was in part supported by an EPA Star Award through the ECOHAB Program (R-83041501-0), and also by a NSF-REU site grant (OCE-0097498) to the Boston University Marine Program. Further funding was provided by NOAA Grant No. NA96OP0099; NOAA-Sea Grant NA86RG0075 (Project R/B-146); NSF Grant No. OCE-9808173 to D.M.A.[SS]

References

Anderson, D.M., 1997. Bloom dynamics of toxic *Alexandrium* species in the northeastern U.S. Limnol. Oceanogr. 42, 1009–1022.

Anderson, D.M., Kulis, D.M., Doucette, G.J., Gallagher, J.C., Balech, E., 1994. Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Mar. Biol. 120, 467–478.

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

Anderson, D.M., Kulis, D.M., Keafer, B.A., Gribble, K.E., Marin, R., Scholin, C.A. Identification and enumeration of *Alexandrium* spp. from the Gulf of Maine using molecular probes. Deep Sea Research II, in press.

Bowers, H., Tengs, T., Glasgow, H., Burkholder, J., Rublee, P., Oldach, D., 2000. Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl. Environ. Microbiol. 66, 4641–4648.

Casper, E.T., Paul, J.H., Smith, M.C., Gray, M., 2004. Detection and quantification of the red tide dinoflagellate *Karenia brevis* by real-time nucleic acid sequence-based amplification. Appl. Environ. Microbiol. 70, 4727–4732.

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

Culverhouse, P.F., Williams, R., Reguera, B., Henry, V., Gonzalez-Gil, S., 2003. Do experts make mistakes? A comparison of

human and machine identification of dinoflagellates. Mar. Ecol. Prog. Ser. 247, 17–25.

Dyhrman, S.T., Anderson, D.M., 2003. Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. Limnol. Oceanogr. 48, 647–655.

Freeman, W.M., Walker, S.J., Vrana, K.E., 1999. Quantitative RT-PCR: pitfalls and potential. Biotechniques 26, 112–125.

Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garces, E., Magnani, M., 2004. Development of a real-time assay for rapid detection and quantification of *Alexandrium minutum* (a dinoflagellate). Appl. Environ. Microbiol. 70, 1199–1206.

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.

Gribble, K.E., Keafer, B.A., Quilliam, M., Cembella, A.D., Kulis, D.M., Manahan, A., Anderson, D.M. Distribution and toxicity of *Alexandrium ostenfeldii* in the Gulf of Maine, USA. Deep Sea Research II, in press.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum, New York, pp. 29–60.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent increase. Phycologia 32, 79–99.

Higman, W.A., Stone, D.M., Lewis, J.M., 2001. Sequence comparisons of toxic and non-toxic *Alexandrium tamarense* (Dinophyceae) isolates from UK waters. Phycologia 40, 256–262.

Hoagland, P., Anderson, D.M., Kaoru, Y., White, A.W., 2002. The economic effects of harmful algal blooms in the United States: estimates, assessment issues, and information needs. Estuaries 25, 819–837.

John, U., 2003. Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* in co-occurring natural populations from Scottish coastal waters. Eur. J. Phycol. 38, 25–40.

Medlin, L.K., Lange, M., Wellbrock, U., Donner, G., Elbrächter, M., Hummert, C., Luckas, B., 1998. Sequence comparisons link toxic European isolates of *A. tamarense* from the Orkney Islands to toxic North American stocks. Eur. J. Protistol. 34, 329–335.

Penna, A., Magnani, M., 1999. Identification of *Alexandrium* (Dinophyceae) species using PCR and rDNA-targeted probes. J. Phycol. 35, 615–621.

Penna, A., Magnani, M., 2000. A PCR immunoassay method for the detection of *Alexandrium* (Dinophyceae) species. J. Phycol. 36, 1183–1186.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification for real-time RT-PCR. Nucleic Acids Res. 29, 2002–2007.

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.

Saito, K., Drgon, T., Robledo, J.A., Krupatkina, D.N., Vasta, G., 2002. Characterization of the rRNA locus of *Pfiesteria piscicida* and development of standard and quantitative PCR-based detection assays targeted to the nontranscribed spacer. Appl. Environ. Microbiol. 68, 5394–5407.

Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the large subunit ribosomal RNA gene. J. Phycol. 30, 999–1011.

Scholin, C.A., Anderson, D.M., 1998. Detection and quantification of HAB species using antibody and DNA probes: progress to date and future objectives. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. IOC UNESCO, Paris, pp. 253–257.

Scholin, C.A., Buck, K.R., Britschgi, T., Cangelosi, G., Chavez, F.P., 1996. Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. Phycologia 35, 190–197.

Scholin, C.A., Hallegraeff, G.M., Anderson, D.M., 1995. Molecular evolution of the *Alexandrium tamarense* 'species complex' (Dinophyceae): dispersal in the North American and west Pacific regions. Phycologia 34, 472–485.

Utermöhl, H., 1958. Zur vervolkommung der quantitativenphytoplankton-methodik. Mitt. Int. Ver. Theor. Angew. Limnol. 9, 1–38.

Van Dolah, F., 2000. Marine algal toxins: origins, health effects, and their increased occurrence. Environ. Health Perspect. 108, 133–141.