Mar Biol (2010) 157:1161–1169
DOI 10.1007/s00227-009-1383-y

METHOD

Development and environmental application of a genus-specific quantitative PCR approach for *Pseudo-nitzschia* species

Elizabeth Fitzpatrick · David A. Caron · Astrid Schnetzer

Received: 24 June 2009 / Accepted: 23 December 2009 / Published online: 10 January 2010

Abstract Quantitative polymerase chain reaction (qPCR) for the identification and quantification of microbes has become a common tool for the study of harmful algal blooms (HABs). We developed a qPCR method for the diatom genus *Pseudo-nitzschia*. Several species of this genus form toxic blooms through the production of the neurotoxin domoic acid (DA). Outbreaks of toxicity attributed to DA along the US west coast have caused sickness and death of marine mammals and seabirds through food web contamination. The method developed here quantifies *Pseudo-nitzschia* spp. at low abundances in natural samples, thereby, providing a method to improve our understanding of the environmental conditions leading to blooms of these species. This has been accomplished previously by techniques for identification and quantification that are slow and laborious compared to qPCR. The approach was successfully tested and validated using eight species of *Pseudo-nitzschia* and 33 non-target organisms and employed to follow local bloom dynamics.

Introduction

Selected species of the diatom genus *Pseudo-nitzschia* are recognized as producers of domoic acid (DA), a water-soluble neurotoxin that accumulates in filter-feeding organisms such as shellfish and planktivorous fish (Bates et al. 1989; Buck et al. 1992; Lefebvre et al. 2002). Transfer of DA through marine food webs causes sickness and mortality in populations of marine mammals and seabirds after feeding on contaminated prey (e.g., anchovy and sardine; Work et al. 1993; Scholin et al. 2000; Gulland et al. 2002). Humans who ingest DA-containing seafood can experience symptoms of amnesic shellfish poisoning (ASP), which include vomiting, confusion, memory loss, coma or even death (Bird et al. 1988; Perl et al. 1989; Bates and Trainer 2006). The geographical locales and maximal toxin levels associated with toxic blooms of *Pseudo-nitzschia* appear to be increasing (Hasle 2002; Schnetzer et al. 2007; Casteleyn et al. 2008; Trainer et al. 2009). This trend, in combination with the adverse impacts on wildlife and human health, stimulates continued efforts to determine when, where and why *Pseudo-nitzschia* blooms develop.

Paramount for understanding the factors leading to harmful algal blooms (HABs) is the ability to correlate the growth of species with environmental forcing factors. Changes in nutrient regimes (macro and micronutrients) resulting from coastal upwelling and anthropogenic eutrophication have been implicated in HAB formation (Trainer et al. 2000; Parsons et al. 2002; Glibert et al. 2005; Kudela et al. 2005; Anderson et al. 2008). Establishing cause-and-effect between these potential stimulants, growth of *Pseudo-nitzschia* and/or toxin production requires the ability to detect these algae at low cell abundances in conjunction with measurements of pertinent environmental factors. Enumeration of cells in large numbers of field samples, however, is time and labor intensive using traditional microscopy. In addition, detection of *Pseudo-nitzschia* species at the onset of a bloom is difficult because these cells are often obscured by other algae that dominate the phytoplankton assemblage. Quantitative polymerase chain reaction

Communicated by U. Sommer.

E. Fitzpatrick (✉) · D. A. Caron · A. Schnetzer
Department of Biological Sciences,
University of Southern California,
3616 Trousdale Parkway, Los Angeles,
CA 90089-0371, USA
e-mail: efitzpat3@gmail.com

(qPCR) approaches have helped overcome many of the challenges in identifying and quantifying HAB-forming organisms in situ (Bowers et al. 2000; Galluzzi et al. 2004; Coyne et al. 2005; Moorthi et al. 2006), but none is presently available for *Pseudo-nitzschia*.

Here, we introduce a qPCR method for near real-time identification and quantification of members of the genus *Pseudo-nitzschia*. The developed SYBR Green approach was validated with eight different *Pseudo-nitzschia* species, and cross-reactivity tests were conducted using 33 non-target organisms. Finally, we applied our approach to count *Pseudo-nitzschia* in field samples collected throughout different stages of a *P. australis*-dominated bloom in coastal waters off of southern California.

Materials and methods

Pseudo-nitzschia cultures and species identification

Cultures of *Pseudo-nitzschia* were established by micropipetting individual cells or single chains from natural samples collected from the San Pedro Channel using a dissecting microscope. Each cell or chain was transferred through 2–3 washes of F/2 medium prepared using 0.2-μm filtered natural seawater (Guillard 1975). Established cultures were grown in F/2 medium at 15°C and ~165 μEinstein m^{-2} s^{-1} (10:14 h L:D). Aliquots of cultures were preserved with acid Lugol's solution (10% final concentration) and prepared for scanning and transmission electron microscopy to confirm species identities (Miller and Scholin 1998). Cultures from the San Pedro Channel that were used for sequencing and/or specificity tests during method development included *P.* cf. *cuspidata* (SPC0.2; March, 2003), *P. australis* (SPC21; March 2005), *P. pseudo-delicatissima* (SPC22; March 2006) and *P. delicatissima* (SPC3; March 2005). Cultures of *P. seriata* (CCMP 1309 and 1440), *P. multiseries* (CCMP 1660) and *P.*cf. *granii* (CCMP 2093) were also obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (https://ccmp.bigelow.org/). Additionally, several strains of *P. pungens* (Cl172, Cl204 and Cl205; Prince Edward Island, Canada) and *P. fraudulenta* (Cl192; Brunswick, Canada) were kindly provided by Stephen Bates (Gulf of Fisheries Centre, Moncton, New Brunswick, Canada) and *P. australis* (0771B; Drakes Bay, California) by Raphael Kudela.

Cloning and sequencing

18S rDNA sequences were obtained for multiple *Pseudo-nitzschia* species to facilitate the design of group-specific qPCR primers. Culture aliquots were concentrated onto GF/F filters, transferred into 15-ml centrifuge tubes, 2 ml of 2× Lysis buffer (40 mM EDTA, pH 8; 100 mM Trizma-Base, pH 8; 100 mM NaCl; 1% SDS) was added and the samples stored at −20°C until further processing. DNA was extracted using a combination of bead-beating and heating steps, and the DNA in these crude lysates cleaned as described previously (Countway and Caron 2006). Amplification of 18S sequences was conducted using eukaryotic-specific primers Euk A (5′- AACCTGGTTGAT CCTGCCAGT -3′) and Euk B (5′- GATCCTTCTGCAGG TTCACCT AC -3′; Medlin et al. 1988; Countway and Caron 2006). DNA products were run on agarose gels, the DNA recovered, purified and cloned following previously described protocols (Countway and Caron 2006). Sequencing was conducted using a Beckman-Coulter CEQ8000 automated DNA sequencer (Fullerton, CA) using the initial PCR primers Euk A and Euk B (Medlin et al. 1988) as well as 570F (5′- GTAATTCCAGCTCCAATAGC-3′), 570R (5′- GCTATTGGAGCTGGAATTAC-3′), 1055F (5′- GG TGGTGCATGGCCG-3′), 1055R (5′- CGGCCATGCACC ACC-3′; all from Weekers et al. 1994), NS4 (5′-CTTCCG TCAATTCCTTTAAG-3′) and NS5 (5′-AACTTAAAGG AATTGACGGAAG-3′; from White et al. 1990). Sequence reads were obtained for ten strains belonging to seven different species (Table 1) and ranged between 1,304 and 1,841 bases (multiple overlapping reads; at least 3× coverage). Two partial sequences (759 and 529 bases) were acquired for *P.* cf. *granii* (CCMP 2093). Sequence data were deposited in GenBank (Accessions GU373960-GU373970).

Primer design

A genus-specific SYBR Green qPCR assay for *Pseudo-nitzschia* was designed using the software package Allele ID5 (PREMIER Biosoft International, Palo Alto CA). Published 18S sequences for *P. multiseries* (U18241), *P.* sp. (AY485490 = CCMP1309), *P. delicatissima* (FJ222757, FJ222756), *P. australis* (AM235384), *P. pungens* (U18240), *P. turgidula* (FJ222752) and *P. cuspidata* (FJ222754) were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and combined with newly derived sequence data in the Allele ID5 program software. Several genus-specific primer pairs were identified in Allele ID5 (default settings) and, based on assigned quality evaluation scores (thermodynamic characteristics, secondary DNA structures), we selected PnGenusFwd 5′- CTGTGTAGTGCTTCTTAGAGG-3′ and PnGenusRev 5′- AGGTAGAACTCGTTGAATGC-3′ for our approach that predicted a 132-bp product. The forward primer (PnGenusFwd) was located at nucleotide positions 1,360–1,380, and the reverse primer (PnGenusRev) annealed at bases 1,471–1,491 relative to the *P. multiseries* strain sequenced in this study (CCMP1660, GU373964).

Table 1 Alignment of 18S rDNA sequence for multiple *Pseudo-nitzschia* species and non-target organisms showing matches of the forward primer used in the SYBR Green approach

* *P.* cf. *cuspidata* SPC0.2	TATCACTGTG-TAGTGCTTCTTAGAGGGAC
* *P.* cf *granii* CCMP2093	TATCACTGTG-TAGTGCTTCTTAGAGGGAC
* *P. multiseries* CCMP1660	TATCACTGTG-TAGTGCTTCTTAGAGGGAC
P. multiseries U18241	TATCACTGTG-TAGTGCTTCTTAGAGGGAC
* *P. seriata* CCMP1440	TATCACTGTG-TAGTGCTTCTTAGAGGGAC
* *P.* sp. CCMP1309	TATCACTGTG-TAGTGCTTCTTAGAGGGAC
P. sp. CCMP1309 AY485490	TATCACTGTG-TAGTGCTTCTTAGAGGGAC
P. delicatissima FJ222757	TATCACTGTG-TAGTGCTTCTTAGAGGGAC
P. delicatissima FJ222756	TATCACTGTG-TAGTGCTTCTTAGAGGGAC
Primer	CTGTG-TAGTGCTTCTTAGAGG
mismatch: base 1	
* *P. australis* SPC21	TCA T TGTG-TAGTGCTTCTTAGAGGGAC
P. australis AM235384	TATCA T TGTG-TAGTGCTTCTTAGAGGGAC
Primer	C TGTG-TAGTGCTTCTTAGAGG
mismatch: base 8	
* *P. pungens* CL172	TATCACTGTG-TA T TGCTTCTTAGAGGGAC
* *P. pungens* CL204	TCACTGTG-TA T TGCTTCTTAGAGGGAC
* *P. pungens* CL205	TATCACTGTG-TA T TGCTTCTTAGAGGGAC
P. pungens U18240	TATCACTGTG-TA T TGCTTCTTAGAGGGAC
Primer	CTGTG-TA G TGCTTCTTAGAGG
mismatch: base 9	
* *P. pseudodelicatissima* SPC22	TATCACTGTG-TAG A GCTTCTTAGAGGGAC
P. turgidula FJ222752	ACTGTG-TAG A GCTTCTTAGAGGGAC
P. cuspidata FJ222754	ACTGTG-TAG A GCTTCTTAGAGGGAC
Primer	CTGTG-TAC T GCTTCTTAGAGG
AF525665 *Fragilariopsis sublineata*	TATCACTGCATTTGTGCTTCTTAGAGGGAC
AY485467 *Fragilariopsis cylindrus*	TATCACTGCATTTGTGCTTCTTAGAGGGAC
AF374480 *Thalassiosira rotula*	TTTGATTGGTTAAGAGCTTCTTAGAGGGAC
X85396 *Thalassiosira eccentrica*	TTTTCATTGGTAAGAGCTTCTTAGAGGGAC
AJ535167 *Chaetoceros* sp	TCTTCATTGGCAGACATTTCTTAGAGGGAC
M87325 *Bacillaria paxillifer*	TGTTCACTGGGTGAAGCTTCTTAGAGGGAC
AM501996 *Navicula cryptocephala*	TCTCACTGACTGCTGGCTTCTTAGAGGGAC

* Strains sequenced in this study

Sample preparation and qPCR conditions

Culture material and natural samples were collected and extracted, as previously described, to obtain a crude cell lysate. Each qPCR reaction contained 5 µl of diluted crude lysate (1:100 with Milli-Q) and were mixed with Bio-Rad iQ™ SYBR® Green supermix (100 mM KCL, 40nM Tris–HCL, pH 8.4, 0.4 mM of each dNTP, iTaq DNA polymerase, 50units/ml, 6 mM $MgCl_2$, SYBR Green I, 20nM fluorescein, and stabilizers), and with both primers at 400nM final concentration (total reaction volume 25 µl). All samples were amplified for 45 cycles of 30 s at 95°C, 30 s at 61°C and 30 s at 72°C using a Bio-Rad iCycler (Hercules, CA). A final annealing temperature of 61°C was chosen after testing the binding specificity of the designed primers with target and non-target DNA over a temperature gradient of 50–65°C. Melt curves were performed at the end of qPCR runs to check whether amplicons of multiple lengths (not only the desired 132 bp product) were present because the signal detected in a SYBR Green approach derives from the fluorescent stain binding to any double-stranded DNA, which can include primer-dimers and non-specific amplicons (Nolan 2004).

Specificity and sensitivity of qPCR approach

Lysates from 33 different species belonging to several protistan taxonomic groups including ten non-target diatom species were used to test the specificity of the method (Table 2). Between 5 and 10 ml of culture was concentrated for each species after cell densities of at least 1×10^3 cells ml^{-1} were reached (determined using light microscopy) and crude lysates were obtained (Countway and Caron 2006). These lysates were first amplified using universal eukaryotic 18S primers (Euk A in combination with Euk B or 570R), the products run on 1.2% agarose gels and stained with SYBR gold to check for amplicons. Culture samples were then used in cross-reactivity trials to test the genus-specific *Pseudo-nitzschia* primers.

The detection range and sensitivity of the qPCR approach was tested with three different *Pseudo-nitzschia* species. First, Lugol's preserved samples (10% final concentration) were analyzed via light microscopy (Utermöhl 1958) to determine cell abundances of 5.2, 55.5 and 445.2×10^3 cells mL^{-1} in cultures of *P. australis* (0771B), *P. pungens* (Cl172) and *P. delicatissima* (SPC3), respectively. Next, serial dilutions (1:10) were prepared for each culture using autoclaved, filtered (0.2 µm) seawater and unfiltered seawater. The unfiltered seawater was analyzed to ensure that natural constituents (e.g., particulates or non-target organisms) would not interfere with the detection of *Pseudo-nitzschia* species. Finally, the cell abundances of samples within each dilution curve were determined for each species as the number of cells $reaction^{-1}$ factoring in the dilution steps during lysate preparation and information on cell densities and the volume of culture filtered (Fig. 1).

Application of method to natural water samples

The qPCR method was applied to the field samples collected during a local bloom of *Pseudo-nitzschia*. Twenty stations located along the coast of Los Angeles and Orange Counties were sampled monthly between January and April of 2006 as part of a long-term monitoring effort. Water samples were collected from the surface, particulate material in 200 ml subsamples was collected onto GF/F glass fiber filters, and the filters were frozen at −20°C prior to qPCR analysis as described earlier. Subsamples were also preserved in Lugol's fixative for microscopical counts. Surface net tows were conducted at 10 out of the 20 sampling stations using a 20-µm mesh size, and the phytoplankton community examined using electron microscopy to determine the species composition of the *Pseudo-nitzschia* assemblage (Miller and Scholin 1998).

Table 2 Cross-specificity test results for 8 *Pseudo-nitzschia* species and 33 non-target protists. Threshold cycles (C_Ts) are listed when amplification signals were detected using the genus-specific qPCR approach

Taxonomic group	Species	C_T
Stramenopiles		
Bacillariophyceae	*P. delicatissima*	20
	P. australis[a]	23
	P. seriata	23
	P. fraudulenta[b]	25
	P. cf. *granii*	29
	P. pungens[a]	27
	P. cf. *cuspidata*	28
	P. pseudo-delicatissima[a]	28
	Fragilariopsis cylindrus	32
	Minutocellus polymorphus	37
	Thalassiosira weissflogii	37
	T. rotula	38
	Leptocylindrus danicus	39
	Odontella sp.	40
	Chaetoceros socialis	40
	C. neogracile	NA
	Ditylum brightwellii	NA
	Rhizoselenia setigera	NA
Chrysophyceae	*Chromulina nebulosa*	35
	Mallomonas aanulata	39
	Ochromonas tuberculata	NA
	Paraphysomonas bandaiensis	NA
Pelagophceae	*Aureococcus anophagefferens*	37
	Aureococcus sp.	38
	Aureoumbra lagunensis	39
Raphidophyceae	*Heterosigma akashiwo*	NA
Xanthophyceae	*Botrydiopsis interedens*	NA
Alveolata		
Ciliophora	Unknown hymenostome	41
	Uronema marinum	NA
	Uronema sp	NA
Dinophyceae	*Gymnodinium beii*	NA
	Oxyrrhis sp.	37
	Sybiodinium pulchrorum	37
	Scrippsiella nutricula	39
Euglenozoa	*Euglena gracilis*	40
Haptophycae	*Isochrysis galbana*	NA
	Phaeocystis antartica	NA
Chlorophyta	*Nannochloris* sp.	37
	Micromonas pusilla	39
	Chlamydomonas sp.	NA
	Pyamimonas cf. *tychotreta*	NA

Approximately 40% of the non-target species did not produce detectable amplicons (N/A) after 45 cycles

[a] 1-base mismatch with forward primer (PnGenusFwd)

[b] No 18S rDNA information available

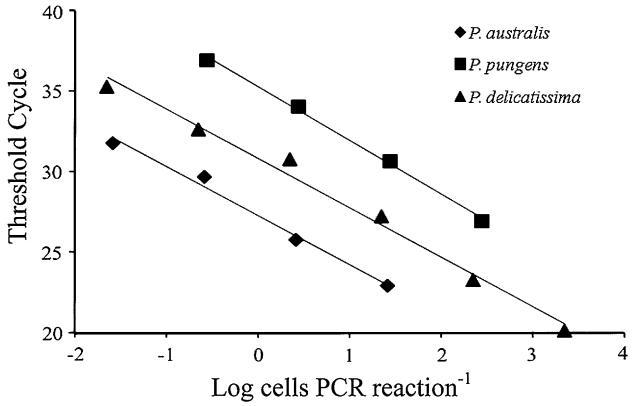

Fig. 1 Standard curves created from serial dilutions of three *Pseudo-nitzschia* cultures plotted as log cells reaction^{-1} versus threshold cycle (C_T)

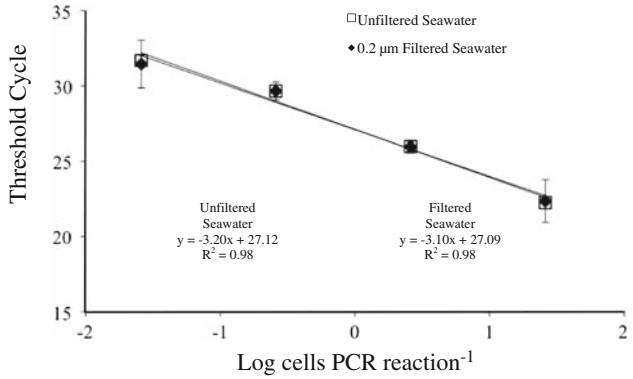

Fig. 2 Standard curves created from serial dilutions of a culture of *P. australis* into filtered seawater (culture dilution) and unfiltered, natural seawater (natural dilution)

Results

qPCR approach

All genus-specific primer pairs that were identified in Allele ID5 for a SYBR Green approach based on 18S sequences resulted in 1-base mismatches for some of the *Pseudo-nitzschia* strains. No species-specific designs were obtained (even when an internal probe was considered) indicating a low level of 18S sequence variability among *Pseudo-nitzschia* species. After evaluating the quality of several genus-specific primer pairs in Allele ID5, we chose the forward primer PnGenusFwd 5′- CTGTGTAGTGCTT CTTAGAGG-3′, which uniquely matched *P.* cf. *cuspidata*, *P.* cf. *granii*, *P. multiseries*, *P. seriata* and *P. delicatissima* but had a 1-base mismatch at position 1 for *P australis*, at position 8 for *P. pungens* and at base 9 for *P. pseudo-delicatissima*, *P. turgidula* and *P. cuspidata* (Table 1). Other diatom genera (e.g. *Fragilariopsis* spp.) exhibited 4 or more mismatches with the forward primer. The corresponding reverse primer PnGenusRev 5′- AGGTAGAACTCG TTGAATGC-3′ was not specific to species within the genus of *Pseudo-nitzschia* alone, but matched a few other diatom genera, mainly *Cylindrotheca* spp. and *Nitzschia* spp.

Specificity and sensitivity of qPCR approach

A total of eight different *Pseudo-nitzschia* species and 33 non-target species were used to test the specificity of the qPCR primers. A *P. australis* culture dilution curve was constructed of known cell concentrations to ensure consistency in qPCR efficiency. Each assay and all amplification signals derived from non-target and other *Pseudo-nitzschia* species were evaluated relative to these internal controls. *Pseudo-nitzschia* species amplified and attained threshold cycles (C_T) at <30 cycles, while non-target species were typically detected after 37 cycles or did not amplify (Table 2). *Fragilariopsis cylindrus* displayed a C_T of 32, although based on available 18S sequence, this species showed 4 mismatches to PnGenusFwd (Table 1). Cell densities in our test cultures were at least 1×10^3 cells ml^{-1}. Cross-reactivity tests for *F. cylindrus* were repeated after obtaining exact cell counts via light microscopy, and it was determined that a C_T of 32 corresponded to 0.08 *Fragilariopsis* cells PCR reaction^{-1}.

Evaluation of the sensitivity of our qPCR approach was conducted for three *Pseudo-nitzschia* species using dilution series that encompassed cell abundances over at least four orders of magnitude. The range was 0.03–25.9 cells reaction^{-1} for *P. australis*, 0.02–2,225.8 cells reaction^{-1} for *P. delicatissima* and 0.28–277.2 cells reaction^{-1} for *P. pungens* (Fig. 1). These results corresponded to detection ranges of 0.005–5.2 $\times 10^3$ cells ml^{-1} for *P. australis*, 0.004–445.2 $\times 10^3$ cells ml^{-1} for *P. delicatissima* and 0.055–55.5 $\times 10^3$ cells ml^{-1} for *P. pungens* in natural samples using sample volumes filtered in this study. Increasing the sample volume (>200 ml) would have allowed for the detection of even lower *Pseudo-nitzschia* abundances in natural samples. No difference was observed in detection efficiency for culture dilutions that were prepared with filtered or unfiltered seawater indicating that there were no inhibitory effects or cross-reactive substances present in the natural seawater (shown for *P. australis* in Fig. 2).

Pseudo-nitzschia abundance in natural samples

Pseudo-nitzschia abundances were determined in coastal waters of the San Pedro Channel during 2006 using qPCR and light microscopy (Fig. 3). Abundance estimates based on microscopical counts revealed only low abundances of *Pseudo-nitzschia* during January and February, with ranges of <5 to 45 and <5 to 234 cells ml^{-1}, respectively (Fig. 3a, b).

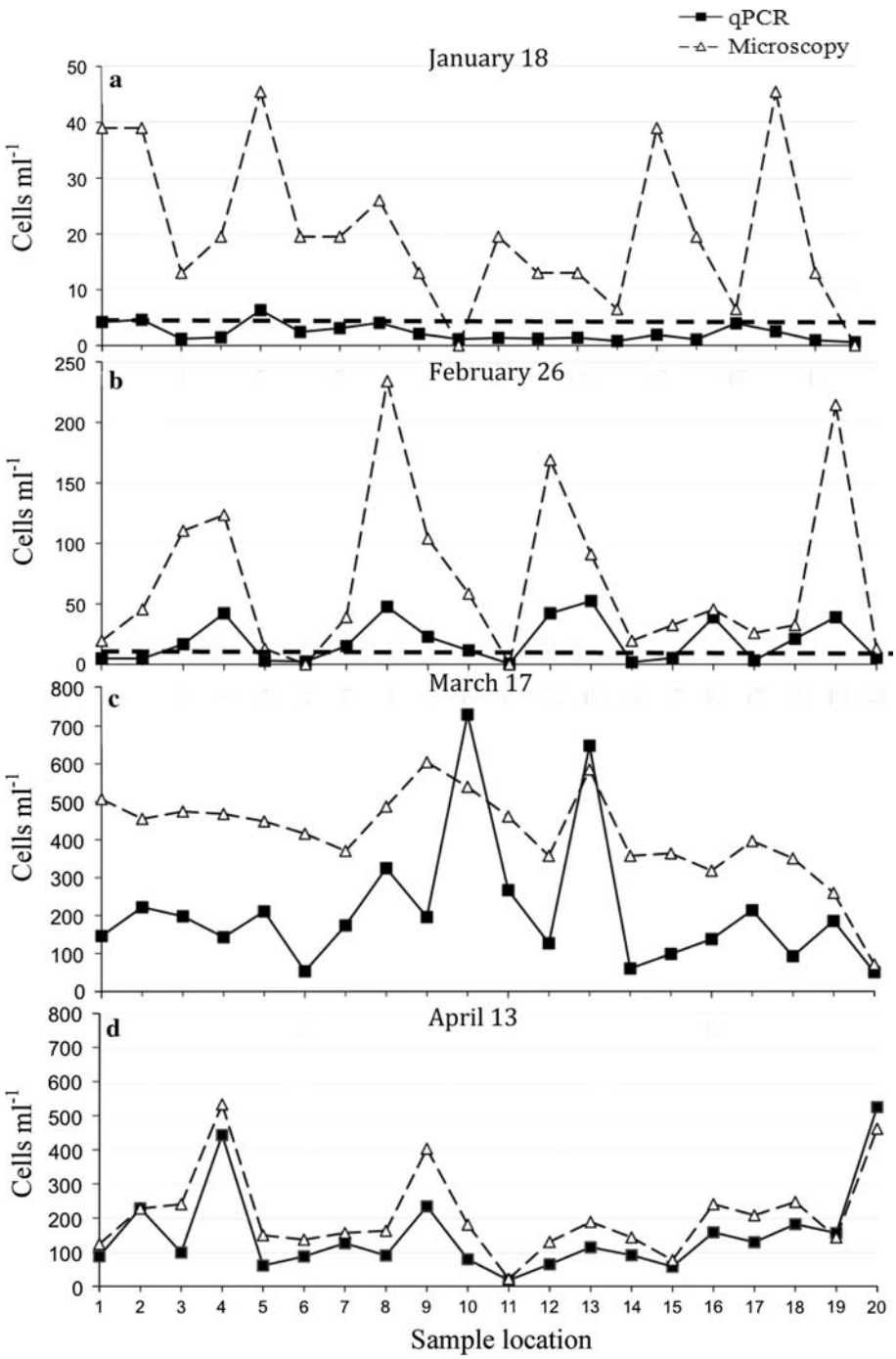

Fig. 3 Environmental samples taken during a bloom event in 2006 in the San Pedro Bight area off Los Angeles. Cell abundances are based on microscopical counts and qPCR-derived estimates. *Dotted line* indicates lower detection limit (5 cells ml^{-1}) derived for the detection of *P. australis*

An approximate lower limit of detection of 5 cells ml^{-1} for the microscopical counts resulted from a total settled volume of 25 ml seawater and counting at least 150 *Pseudo-nitzschia* cells per sample or 20 fields of view at 40× magnification. Abundances increased considerably to 71–604 and 21–533 cells ml^{-1} at the peak of the bloom during March and April, respectively (Fig. 3c, d). Electron microscopy revealed that *P. australis* dominated the *Pseudo-nitzschia* assemblage on all sampling dates with other species only rarely detected (<1% of observed *Pseudo-nitzschia* cells) in January and February. Based on these findings, we approximated the abundances of *P. australis* by using the standard curve obtained for that species (Fig. 1). Enumeration via microscopy and qPCR was in good agreement at high abundances of *Pseudo-nitzschia*. However, microscopical counts generally exceeded abundances determined by qPCR at low-to-moderate abundances of the diatom (Fig. 4).

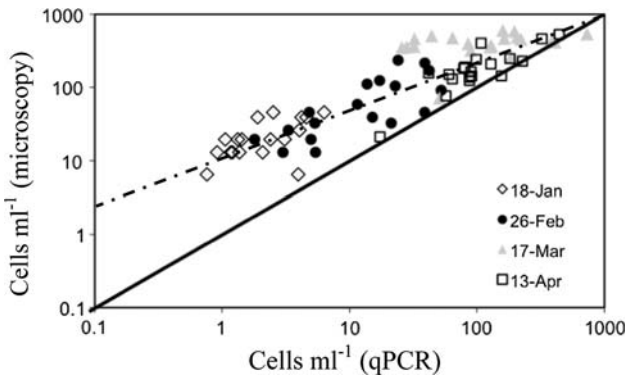

Fig. 4 Correlation between microscopical and qPCR-derived estimates for *Pseudo-nitzschia* abundances. Sampling events are indicated by different symbols. *Solid line* indicates a 1:1 relationship for both methods; *dashed line* indicates regression line ($R^2 = 0.80$). Only, non-zero values are included ($n = 76$)

Discussion

Pseudo-nitzschia blooms are a recurrent phenomenon in waters along the US west coast that adversely impact planktonic food webs and benthic communities (Schnetzer et al. 2007; Bejarano et al. 2008; Kvitek et al. 2008; Sekula-Wood et al. 2009). A clear understanding of the environmental conditions that allow *Pseudo-nitzschia* to proliferate and stimulate species within this genus to produce the neurotoxin domoic acid (DA) is still debated. Indeed, multiple scenarios promoting population growth and toxin production may exist in nature. Here, we introduce a SYBR Green qPCR method that can aid the study of bloom dynamics by identifying and quantifying *Pseudo-nitzschia* in near-real time in a large number of samples over a wide range of cell abundances.

Pseudo-nitzschia abundances have been routinely determined by light microscopy, but this is a method that is time and labor intensive, especially at low abundances of these cells in natural phytoplankton assemblages. Abundance estimates are generated from concentrated samples obtained by settling cells (which takes several hours), and subsequent microscopical analysis requires up to half an hour per sample, depending on the experience of the observer as well as absolute and relative cell abundances (Utermöhl 1958). In comparison, our qPCR assay enables the identification and quantification of *Pseudo-nitzschia* in 40 samples (in duplicate) within ~4 h (including a standard curve) on a 96-well plate. This time estimate encompasses sample processing from preparation of cell lysates to the completion of a qPCR reaction. The qPCR method proved successful in determining cell abundances for 3 different *Pseudo-nitzschia* species, *P. delicatissima*, *P. australis* and *P. pungens,* over a range of at least 4 orders of magnitude.

Overall, a total of eight different *Pseudo-nitzschia* species could be detected at threshold cycles (C_Ts) of 29 or less, which corresponded to 0.4, 5 and 110 cells per PCR reaction for *P. australis*, *P. delicatissima* and *P. pungens*, respectively. *P. australis* showed the highest reactivity. While a mismatch of *P. australis* with the first base of PNGenusFwd seemed negligible for amplification efficiency, a mismatch of *P. pungens* with base 8 of the same primer more likely reduced sensitivity. However, other factors may affect abundance estimates based on qPCR approaches. These factors include differences in number of copies among different *Pseudo-nitzschia* species and variations in copy number as a consequence of differences in physiological status. For these reasons, analyses of a standard curve with known cell abundances side by side with any unknown field samples improve accuracy of the approach.

The specificity of our qPCR approach was tested using a number of non-target algae and protists including ten other diatom species. *F. cylindrus* was the only non-target species that amplified with a C_T similar to those obtained for any of the *Pseudo-nitzschia* species tested. Our test indicated that an abundance of 0.08 *F. cylindrus* cells PCR reaction^{-1}, or natural abundances of 16×10^3 cells ml^{-1} (assuming a sample volume of 200 ml was processed), were required to produce a C_T signal of 32. *F. cylindrus* is a psychrophilic species known to reach bloom abundances in cold water environments of the Southern Ocean but is not known to play any role in temperate waters along the California coast. Therefore, it is unlikely that this species would produce significant interference to this method. A conservative cutoff of $C_T = 35$ could be employed to avoid overestimating *Pseudo-nitzschia* cell abundances in natural samples from environments in which *F. cylindrus* blooms. A C_T of 35 would result in detection limits of 0.22 and 2.77 cells reaction^{-1} for *P. delicatissima* and *P. pungens*, respectively. Our method was not designed to identify *Pseudo-nitzschia* at the species level, which currently requires the use of scanning or transmission electron microscopy (SEM or TEM) or the use of fluorescently labeled in situ hybridization techniques targeting the large subunit ribosomal RNA. The latter approach exists for seven species of *Pseudo-nitzschia* (*P. australis, P. multiseries, P. pungens, P. fraudulenta, P. heimii, P. delicatissima, and P. pseudodelicatissima*; Scholin et al. 1997, 1999), where fluorescently labeled oligonucleotide probes bind to target sequences of the large subunit rRNA. Sample preparation and enumeration via in situ hybridization and electron microscopy are accurate, but require substantial investments of time, labor and expertise. These methods would be highly valuable in conjunction with the genus-specific qPCR protocol reported here. Unfortunately, the development of a qPCR approach based on large subunit

rRNA genes is presently thwarted by insufficient comparative sequence information on non-target species that might enable the design of species-specific primers and probes.

Our qPCR method was employed to monitor *Pseudo-nitzschia* abundances in natural samples throughout different stages of a bloom off the Southern California coast in early 2006. Electron microscopy performed on the same samples confirmed that the *Pseudo-nitzschia* assemblage at that time was dominated by *P. australis* and thus field abundances were calculated from *P. australis* standard curves. Results of the qPCR approach were validated using light microscopy, which generally yielded slightly higher estimates for cell abundances. Close agreement was generally observed between counts based on the two methods, but microscopical counts at low-to-moderate abundances of *Pseudo-nitzschia* were consistently higher than qPCR estimates. We speculate that empty frustules of *Pseudo-nitzschia* may be difficult to identify when the diatom is present at low relative abundance and that the presence of frustules that still contained cell organelles such as chloroplasts but no detectable DNA might have also contributed to this difference. Therefore, our qPCR approach may provide a more accurate estimate of these diatoms at low cell abundance.

We have developed and applied a SYBR Green qPCR method for the identification and quantification of *Pseudo-nitzschia* spp. The reoccurrence of toxic blooms of this diatom genus increases the importance of monitoring tools that can provide rapid quantification of large numbers of samples. Our approach, especially in combination with methods that identify individual species of *Pseudo-nitzschia*, could aid efforts to discern cause-and-effect relationships between environmental conditions and the onset of blooms of this harmful alga.

Acknowledgments This study was supported through funding by the Environmental Protection Agency (EPA: RD-83170501) and the National Oceanographic and Atmospheric Administration (NOAA Monitoring and Event Response for Harmful Algal Blooms (MERHAB) grant: NA05NO54781228 and NOAA Sea Grant: NA06OAR4170012). We thank Peter E. Miller for *Pseudo-nitzschia* species identifications and Stephen Bates and Raphael Kudela for providing us with several strains of *Pseudo-nitzschia*. We also thank Peter Countway for helpful suggestions during method development.

References

Anderson DM, Burkholder JM, Cochlan WP, Glibert PM, Gobler CJ, Heil CA, Kudela RM, Parsons ML, Rensel JEJ, Townsend DW, Trainer VL, Vargo GA (2008) Harmful algal blooms and eutrophication: examining linkages from selected coastal regions of the United States. Harmful Algae 8:39–53

Bates NR, Trainer VL (2006) The ecology of harmful diatoms. In: Graneli E, Turner JT (eds) Ecology of harmful algae. Springer, Heidelberg, pp 81–93

Bates SS, Bird CJ, Defreitas ASW, Foxall R, Gilgan M, Hanic LA, Johnson GR, McCulloch AW, Dodense P, Pocklington R, Quilliam MA, Sim PG, Smith JC, Subba Rao DV, Todd CD, Walter JA, Wright JLC (1989) Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edwards Island, Canada. Can J Fish Aquat Sci 46:1203–1215

Bejarano AC, VanDolah FM, Gulland FM, Rowles TK, Schwacke LH (2008) Production and toxicity of the marine biotoxin domoic acid and its effects on wildlife: a review. Human Ecol Risk Assess 14:544–567

Bird CJ, Boyd RK, Brewer D, Craft AC, de Freitas ASW (1988) Identification of domoic acid as the toxic agent responsible for the PEI contaminated mussel incident. Res Lab Tech Rep 56:1–86

Bowers HA, Tengs T, Glasgow HBJ, Burkholder JM, Rublee PA, Oldach DW (2000) Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl Environ Microbiol 66:4641–4648

Buck KR, Uttal-Cooke L, Pilskaln CH, Roelke DL, Villac MC, Fryxell GA, Cifuentes L, Chavez FP (1992) Autoecology of the diatom *Pseudonitzschia australis*, a domoic acid producer from Monterey Bay, CA. Mar Ecol Prog Ser 84:293–302

Casteleyn G, Chepurnov VA, Leliaert F, Mann DG, Bates SS, Lundholm N, Rhodes L, Sabbe K, Vyverman W (2008) *Pseudo-nitzschia pungens* (Bacillariophyceae): a cosmopolitan diatom species. Harmful Algae 7:241–257

Countway PD, Caron DA (2006) Abundance and distribution of *Ostreococcus* sp in the San Pedro Channel, California, as revealed by quantitative PCR. Appl Environ Microbiol 72:2496–2506

Coyne KJ, Handy SM, Demir E, Whereat EB, Hutchins DA, Portune KJ, Doblin MA, Cary SC (2005) Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. Limnol Oceanogr Methods 3:381–391

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

Glibert PM, Seitzinger S, Heil CA, Burkholder JM, Parrow MW, Codispoti LA, Kelly V (2005) Eutrophication—new perspectives on its role in the global proliferation of harmful algal blooms. Oceanography 18:198–209

Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrate animals. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Publishing Corp, New York, pp 2–60

Gulland FM, Fauquier D, Langlois G, Lander ME, Zabka T, Duerr R (2002) Domoic acid toxicity in Californian sea lions (*Zalophus californianus*): clinical signs, treatment and survival. Vet Rec 150:475–480

Hasle GR (2002) Are most of the domoic acid-producing species of the diatom genus *Pseudo-nitzschia* cosmopolites? Harmful Algae 1:137–146

Kudela RM, Pitcher G, Probyn T, Figueiras F, Moita T, Trainer VL (2005) Harmful algal blooms in coastal upwelling systems. Oceanography 18:184–197

Kvitek RG, Goldberg JD, Smith GJ, Doucette GJ, Silver MW (2008) Domoic acid contamination within eight representative species from the benthic food web of Monterey Bay, California, USA. Mar Ecol Prog Ser 367:35–47

Lefebvre KA, Silver MW, Coale KH, Tjeerdema RS (2002) Domoic acid in planktivorous fish in relation to toxic *Pseudo-nitzschia* cell densities. Mar Biol 140:625–631

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71:491–499

Miller PE, Scholin CA (1998) Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. J Phycol 34:371–382

Moorthi SD, Countway PD, Stauffer BA, Caron DA (2006) Use of quantitative real-time PCR to investigate the dynamics of the red tide dinoflagellate *Lingulodinium polyedrum*. Microb Ecol 52:136–150

Nolan T (2004) Getting started—the basics of setting up a qPCR assay. In: Bustin SA (ed) A–Z of quantitative PCR. International University Line, La Jolla, pp 527–544

Parsons ML, Dortch Q, Turner ER (2002) Sedimentological evidence of an increase in *Pseudo-nitzschia* (Bacillariophyceae) abundance in response to coastal eutrophication. Limnol Oceanogr 47:551–558

Perl TM, Bedard L, Kosatsky T, Hockin JC, Todd EC, McNutt LA, Remis RS (1989) Amnesic shellfish poisoning: a new clinical syndrome due to domoic acid. Can Dis Wkly Rep 16:7–8

Schnetzer A, Miller PE, Schaffner RA, Stauffer B, Jones BH, Weisberg SB, DiGiacomo PM, Berelson W, Caron DA (2007) Blooms of *Pseudo-nitzschia* and domoic acid in the San Pedro Channel and Los Angeles Harbor areas of the Southern California Bight, 2003–2004. Harmful Algae 6:372–387

Scholin CA, Miller PE, Buck KR, 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 CA, Marin R III, Miller PE, Doucette GJ, Powell CL, Haydock P, Howard J, Ray J (1999) DNA probes and a receptor-binding assay for detection of *Pseudo-nitzschia* (Bacillariophyceae) species and domoic acid activity in cultured and natural samples. J Phycol 35:1356–1367

Scholin CA, Gulland F, Doucette GJ, Benson S, Busman M, Chavez F, Cordaro J, Delong EF, Vogelaere AD, Harvey J, Haulena M, Lefebvre K, Lipscomb T, Loscutoff S, Lowenstine LJ, Marin III R, Miller PE, McLellan WA, Moeller PDR, Powell CL, Rowles T, Silvagni P, Silver MW, Spraker T, Trainer VL, Dolah FMV (2000) Mortality of sea lions along the central California coast linked to a toxic diatom bloom. Nature 403:80–84

Sekula-Wood E, Schnetzer A, Benitez-Nelson CR, Anderson C, Berelson WM, Brzezinski MA, Burns JM, Caron DA, Cetinic I, Ferry JL, Fitzpatrick E, Jones BH, Miller PE, Morton SL, Schaffner RA, Siegel DA, Thunell R (2009) Rapid downward transport of the neurotoxin domoic acid in coastal waters. Nat Geosci 2:272–275

Trainer VL, Adams NG, Bill BD, Stehr CM, Wekell JC, Moeller P, Busman M, Woodruff D (2000) Domoic acid production near California coastal upwelling zones, June 1998. Limnol Oceanogr 45:1818–1833

Trainer VL, Hickey BM, Lessard EJ, Cochlan WP, Trick CG, Wells ML, MacFadyen A, Moor SK (2009) Variability of *Pseudo-nitzschia* and domoic acid in the Juan de Furca eddy region and its adjacent shelves. Limnol Oceanogr 54:289–308

Utermöhl H (1958) Zur Vervollkommung der quantitativen Phytoplankton Methodik. Mitteilungen der Internationalen Vereinigung der Limnologen 9:1–38

Weekers PHH, Gast RJ, Fuerst PA, Byers TJ (1994) Sequence variations in small-subunit ribosomal RNAs of *Hartmannella vermiformis* and their phylogenetic applications. Mol Biol Evol 11:684–690

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, Inc, New York, pp 315–322

Work TM, Barr B, Beale AM, Fritz L, Quilliam MA, Wright JLC (1993) Epidemiology of domoic acid poisoning in brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) in California. J Zoo Wildl Med 24:54–62