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Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard

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Abstract

Quantitative real-time PCR (QPCR) is a powerful and sensitive method for quantitative detection of microorganisms. Application of this methodology for enumeration of harmful algal bloom (HAB) species has the potential to revolutionize our approach to HAB research, making it possible to identify correlations between cell abundances and factors that regulate bloom dynamics. Its application to ecological studies, however, has produced mixed results. QPCR assays typically rely on the generation of standard curves from plasmids or laboratory cultures that may be unrealistic when compared to amplification of DNA extracted from field samples. In addition, existing methods often fail to incorporate controls to assess variability in extraction and amplification efficiencies, or include controls that are sequence-specific and preclude the investigation of multiple species. Here, we describe the development and rigorous analysis of QPCR assays for two HAB species, *Chattonella subsalsa* and *Heterosigma akashiwo*, in which we introduce a known concentration of exogenous DNA plasmid into the extraction buffer as a reference standard. Since the target DNA is extracted in the presence of the reference standard, inherent variability in extraction and amplification efficiencies affect both target and standard equally. Furthermore, the reference standard is applicable to QPCR analysis of any microbial species. Using environmental bloom samples as calibrators, we evaluated the accuracy of the comparative Ct method for enumeration of target species in several field samples. Our investigation demonstrates that the comparative Ct method with an exogenous DNA reference standard provides both accurate and reproducible quantification of HAB species in environmental samples.

Quantitative real-time PCR (QPCR) is an extremely powerful and sensitive method for quantitative detection of microorganisms. In contrast to end-point analysis by conventional PCR, real-time detection by QPCR measures the change in product concentration as an increase in fluorescence (ΔRn) during each PCR cycle (Heid et al. 1996). The fractional cycle number (Ct) is calculated for each reaction at a point where the fluorescence signal crosses a certain threshold. Several studies have demonstrated the potential of the methodology for the quantitative analysis of microorganisms (Cullen et al. 2002; Fontaine and Guillot 2002; Phister and Mills 2003; Skovhus 2004; Suzuki et al. 2000; Vaitomaa et al. 2003) including harmful algal bloom (HAB) species (Bowers et al. 2000; Galluzzi et al. 2004; Gray et al. 2003; Popels et al. 2003; Saito et al. 2002) in environmental samples. The basic approach used in these studies is to generate a standard curve using plasmids with the target DNA sequence or DNA extracted from cultures with known concentrations of the target species. While this process is relatively straightforward, the application of QPCR to environmental studies sometimes produces variable results (Cullen et al. 2002; Kolb et al. 2003; Vaitomaa et al. 2003). There are two main reasons for this. First, the amplification efficiencies of plasmids or laboratory cultures used for generation of a standard curve may not accurately represent the amplification efficiencies of DNA extracted from environmental samples (*see* e.g., Becker et al. 2000). Second, QPCR methods often fail to incorporate controls to assess the accuracy of the results. DNA extracted from environmental samples, in particular, can vary

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significantly in quantity and quality, affecting the outcome of quantitative PCR by several-fold (Bostrom et al. 2004). Coprecipitation of compounds that inhibit PCR also confounds molecular analyses of environmental samples (Stults et al. 2001; Tebbe and Vahjen 1993; Wilson 1997) by producing false negative results.

The relative QPCR approach, in which the target gene is normalized to a reference standard, provides a more accurate assessment of cell abundances. Methods have been described, for example, in which cells are spiked into samples (Brinkman et al. 2003; Lebuhn et al. 2004), reducing error due to inherent differences in extraction or amplification efficiencies. These methods rely on the assumption that the lysing efficiency of spiked cells is the same as target cells for every sample. In addition, samples are spiked independently, potentially introducing another source of error. Other methods incorporate reference standards that are sequence-specific and preclude the QPCR quantification of multiple and diverse targets from the same field sample. Widada et al. (2002), for example, spiked cells containing an artificial construct competitor DNA directly into the extraction buffer for use in competitive QPCR. Analysis of multiple species, however, requires that a separate construct be prepared and calibrated for each species under investigation.

Here, we describe the development of relative QPCR assays for quantification of two Raphidophyte species, *Chattonella subsalsa* and *Heterosigma akashiwo*. These organisms have gained recognition as fish-killing phytoplankton, causing massive mortalities of fish and resulting in millions of dollars in damage to the aquaculture industry (Black et al. 1991; Horner 1999; Yang et al. 1995). In addition, brevetoxin-like compounds produced by Raphidophytes (Haque and Onoue 2002; Khan et al. 1996*a*, 1996*b*, 1997) pose a threat to higher trophic levels (including wildlife and humans) since they can potentially be concentrated during food web transfer (Ishida et al. 2004; Plakas et al. 2004; Stommel and Watters 2004; Woofter et al. 2005). Conventional microscopic methods for identifying and estimating the abundance of Raphidophyte species in complex environmental samples are time-consuming and often lack the sensitivity required for background level detection. Some species, such as *Heterosigma akashiwo*, can also be pleomorphic, making them difficult to identify in complex mixtures. Further, Raphidophytes are very fragile, and cell counts of environmental samples prepared with standard phytoplankton fixation methods may be unreliable (Throndsen 1997).

Our objectives were to develop QPCR methods for rapid, sensitive, and accurate identification and enumeration of Raphidophytes in environmental water samples. To eliminate errors due to extraction and amplification efficiencies, a known concentration of exogenous plasmid DNA was introduced into the extraction buffer as a reference standard. We determined the sensitivity of the assay and range of detection for each of the target genes. Intra-sample variability (precision) was evaluated for environmental samples with both high and low abundances of the target species. Finally, we evaluated the accuracy of the comparative Ct method (Livak and Schmittgen 2001) using an environmentally relevant calibrator sample for QPCR quantification of *Chattonella subsalsa* and *Heterosigma akashiwo* in several field samples. The calibrator consisted of DNA extracted from field samples during blooms of *H. akashiwo* and *C. subsalsa*. Accurate cell counts of each target species in the calibrator samples provided a comparative basis for calculating cell abundances in unknown field samples. The approach described here may be applied to development of QPCR assays of other microbial species in complex environmental samples.

Materials and procedures

Determination of 18S rDNA sequences—Delaware Inland Bays (DIB) isolates *Chattonella subsalsa* (CCMP 2191) and *Heterosigma akashiwo* (CCMP 2393) were cultured at 24°C in f/2 growth medium (Guillard 1975). Cells were collected by centrifugation and lysed in 0.7 mL CTAB buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA, 2% [w/v] cetyltrimethylammonium bromide [CTAB], 0.4% [v/v] β-mercaptoethanol, 1% [w/v] polyvinylpyrollidone; [Dempster et al. 1999]). DNA was extracted as in Coyne et al. (2001). The region spanning the 18S through ITS2 region of the rDNA gene was amplified by PCR in a 20-µL reaction volume containing 0.2 mM dNTPs, 0.5 µM Euk A (5′ AACCTGGTTGATCCTGCCAGT 3′) (Medlin et al. 1988), 0.5 µM Raph ITS R (5′ YGCCAGGTGCGTTCGAA 3′), 2.5 mM $MgCl_2$, 1X Taq polymerase buffer (Sigma Chem. Co.), and 0.5 units Jump-Start Taq Polymerase (Sigma Chem. Co.). The reaction consisted of 35 cycles of 30 s at 94°C, 30 s at 56°C, and 2.5 min at 72°C, followed by a 5-min extension at 72°C. PCR products were cloned into pCR4 TOPO plasmid vector (Invitrogen) and bi-directionally sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem).

Quantitative real-time PCR primers and probes—Primer and probe sites for *Chattonella subsalsa* and *Heterosigma akashiwo* were identified by aligning the 18S rDNA sequences of the DIB isolates to sequences of closely related species in GenBank (www.ncbi.nlm.nih.gov) using Clustal (Thompson et al. 1994) in the Genetic Data Environment (Smith et al. 1994). Each primer pair was designed to amplify approximately 350 bp of the 18S rDNA gene (Table 1).

Taqman probes were designed using Primer Express software (Applied Biosystems). The probes were synthesized with a 6-FAM (6-carboxyfluorescein) reporter dye at the 5′ end and a TAMRA (6-carboxytetramethylrhodamine) quencher molecule at the 3′ end. Primer and probe concentrations were optimized for quantitative real-time PCR on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using cloned plasmids containing the 18S rDNA sequence for *Chattonella subsalsa* and *Heterosigma akashiwo* as template. Optimized reaction conditions for each target species consisted of a 25-µL

Table 1. Primer and probe sequences for QPCR of *Chattonella subsalsa*, *Heterosigma akashiwo*, and pGEM plasmid

DNA Target	Primer	Sequence (5′-3′)
Chattonella subsalsa 18S rDNA	Cs 1350F*	CTAAATAGTGTGGGTAATGCTTAC
	Cs 1705R*	GGCAAGTCACAATAAAGTTCCAA
	Raph Probe	CAACGAGTACTTTCCTTGGCCGGAA
Heterosigma akashiwo 18S rDNA	Hs 1350F	CTAAATAGTGTCGGTAATGCTTCT
	Hs 1705R	GGCAAGTCACAATAAAGTTCCAT
	Hs Probe	CAACGAGTAACGACCTTTGCCGGAA
pGEM plasmid DNA	M13F	CCCAGTCACGACGTTGTAAAACG
	pGEM R	TGTGTGGAATTGTGAGCGGA
	pGEM Probe	CACTATAGAATACTCAAGCTTGCATGCCTGCA

*F designates forward primer, R designates reverse primer.

reaction containing 12.5 µL of Taqman Universal Master Mix (Applied Biosystems), 0.9 µM each primer, 0.2 µM Taqman probe, and 2.5 µL diluted template. The Universal Master Mix includes Taq polymerase, dNTPs, buffer, magnesium, and uracil-N-glycosylase. Cycling parameters were 2 min at 50°C for activation of the uracil-N-glycosylase, 10 min at 95°C for activation of the polymerase, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C.

An exogenous DNA, pGEM plasmid (pGEM-3Z Vector; Promega), was added to the CTAB buffer stock solution at 20 µg L^{-1} concentration for extraction of environmental samples. Primers and probe were designed to target specific sequences within the pGEM plasmid (Table 1) and were optimized for QPCR as described above.

Evaluation of primer and probe specificity—Primer specificity was initially evaluated by amplification of environmental water samples. PCR products of water samples that were positive for *Chattonella subsalsa* and *Heterosigma akashiwo* (independently verified by microscopy) were cloned and sequenced as described above to confirm the specificity of primers and probes for the target species. Primers and probes for *Chattonella subsalsa* and *Heterosigma akashiwo* were further evaluated using DNA extracted from cultures of *C. subsalsa* (CCMP 217), *Chattonella* sp. (CCMP 218), *C. harima* (UTEX 2424), *C. japonica* (UTEX 2162), *C. marina* (CCMP 2049), *C. antiqua* (CCMP 2050), several isolates of *H. akashiwo* (CCMP 1912, 302, 1596, and 1870), and *Fibrocapsa japonica* (Delaware Inland Bays isolate, University of Delaware Phytoplankton Culture Collection). A cloned plasmid of the 18S rDNA gene from *Chattonella* cf *verruculosa* was also tested for cross-reactivity (cultures of *C.* cf *verruculosa* were not available). DNA was evaluated by amplification with eukaryotic primers, Euk29F (5′ GTCTCAAAGATTAAGCCATGC 3′) and Euk 517R (5′ GGACCAGACTTGCCCTC 3′), in a positive PCR control reaction. DNA from each species was then used as template in QPCR reactions with *H. akashiwo* and *C. subsalsa* primers and probes (Table 1) to assess specificity.

Probe sensitivity—The sensitivity of the QPCR protocol was determined by amplification of known amounts of plasmids containing the 18S rDNA gene for each of the target species. Ten-fold dilutions of plasmids were made in LoTE [3 mM Tris-HCl (pH 7.5), 0.2 mM EDTA] to achieve a concentration range of 0 to 100 million copies of *Chattonella subsalsa* 18S rDNA and 0 to 140 million copies of *Heterosigma akashiwo* 18S rDNA per microliter. Plasmid dilutions were then used as template in duplicate QPCR reactions under the optimized conditions, resulting in a range of 0 to 250 million copies of *C. subsalsa* 18S rDNA and 0 to 350 million copies of *H. akashiwo* 18S rDNA per reaction. Reactions were allowed to proceed for 50 cycles using optimized conditions described above.

Intra-sample variability—To assess intra-sample variability, we processed triplicate sub-samples of an environmental water sample (designated 062904A, B, and C) collected 29 June 2004 from Loop Canal, Bethany (site IR62) in Indian River Bay, Delaware, U.S.A. Both *Chattonella subsalsa* and *Heterosigma akashiwo* were identified in this water sample by microscopy, where *C. subsalsa* cell abundances were estimated to be approximately 1000-fold higher than *H. akashiwo* cell abundances. The water samples were filtered through a 250-µm Nitex filter to remove debris and macrozooplankton and then onto a 47 mm, 3 µm pore size polycarbonate filter (Osmonics). The 3 µm filter was submersed in CTAB buffer amended with 20 µg L^{-1} of pGEM plasmid (Promega) and heated at 50°C for 15 min. Samples were then frozen at –80°C until extraction. DNA was extracted as described in Coyne et al. (2001) and stored at –80°C until analysis. DNA concentrations were determined spectrophotometrically and further diluted 1:50 in LoTE buffer for QPCR amplification of *H. akashiwo* and 1:200 for QPCR amplification of *C. subsalsa*. Diluted DNA was used as template in separate reactions for *C. subsalsa*, *H. akashiwo*, and the pGEM reference standard under optimized conditions described above.

Validation of the comparative Ct method—We evaluated the amplification efficiencies of target and reference standard using DNA extracted from environmental water samples during blooms of *Heterosigma akashiwo* and *Chattonella subsalsa*. A water sample was collected during a bloom of *C. subsalsa* (sample BC3T11) on 31 Aug 2004 from a canal in Holly Terrace Acres (site IR32) in Indian River Bay. A second water sample containing *H. akashiwo* at concentrations > 10^7cells L^{-1}

(sample 101104) was collected from the east end of Russell Canal, Little Assawoman Bay, Delaware, on 11 Oct 2004. Samples (0.05-0.10 L volumes) were filtered through a 250-µm Nitex filter and then onto 47 mm, 3 µm polycarbonate filters and extracted as described above. DNA was diluted 1:200 (sample BC3T11) or 1:50 (sample 101104) in LoTE buffer. Tenfold dilutions of the diluted DNA were then used as template in QPCR reactions using species-specific primers under optimized conditions described above. Co-extracted pGEM plasmid DNA was also amplified for each of the 10-fold dilutions in separate reactions using pGEM-specific primers and probe.

Calibration using environmental water samples—The accuracy of environmental water samples for use as calibrators was evaluated by comparison of calculated QPCR results to cell counts derived by microscopy. A calibrator sample (sample BC3T0) was collected from site IR32 on 30 Aug 2004 during a bloom of *Chattonella subsalsa*. Triplicate 1-mL aliquots were fixed briefly with a 0.009% (final concentration) glutaraldehyde solution for cell counts of *C. subsalsa*. Immediately after the samples were preserved, eight fields of view for each replicate were counted using a Speirs-Levy counting chamber (Hausser Scientific) at × 10 magnification using light microscopy. A second (test) sample (sample BC3T17) was collected from surface waters at the same location on 31 Aug 2004. A 1-mL aliquot was fixed with 0.009% (final concentration) glutaraldehyde solution and counted as described above. Water samples (0.05-0.1 L) were processed and DNA extracted as described above. DNA was diluted 1:200 in LoTE buffer for QPCR analysis. Ct values were obtained for each sample in duplicate QPCR reactions with *C. subsalsa* primers and probes under optimized conditions described above. Co-extracted pGEM plasmid DNA was also amplified for the diluted DNA samples in separate reactions using pGEM-specific primers and probe.

To determine the accuracy of *Heterosigma akashiwo* QPCR analysis, DNA was extracted from three sub-samples (a calibrator sample and two test samples) of *H. akashiwo* bloom sample 101104 (described above). A 1-mL aliquot of each sub-sample was fixed briefly with a 0.009% (final concentration) glutaraldehyde solution and eight fields of view were counted using a Speirs-Levy counting chamber at ×40 magnification using light microscopy. DNA from each sub-sample was diluted 1:50 and used as template in triplicate QPCR reactions with *H. akashiwo* primers and probes. Co-extracted pGEM plasmid DNA was also amplified for the diluted DNA samples in separate reactions using pGEM-specific primers and probe.

Nine water samples with estimated cell abundances ranging from 10^5 to 10^7 cells L^{-1} of *Chattonella subsalsa* and six water samples with abundances ranging from 10^4 to 10^7 cells L^{-1} of *Heterosigma akashiwo* were collected and DNA extracted as described above. Estimated cell abundances were determined by microscopic cell counts of 2 to 3 replicates of 40 µL of unfixed (live) water samples as described in Andersen and Throndsen (2003). Extracted DNA was diluted 1:50 or 1:200 in LoTE to achieve concentrations of about 5 to 10 ng μL^{-1} and analyzed by QPCR for *C. subsalsa* or *H. akashiwo* and pGEM as described above.

Table 2. Primer and probe specificity

Identification	Source†	PCR Euk	QPCR Ha*	QPCR Cs*
Chattonella subsalsa	CCMP 2191‡	+	–	+
Chattonella subsalsa	CCMP217	+	–	+
Chattonella harima	UTEX 2424	+	–	–
Chattonella marina	CCMP 2049	+	–	–
Chattonella antiqua	CCMP 2050	+	–	–
Chattonella sp.	CCMP 218	+	–	–
Chattonella japonica	UTEX 2162	+	–	–
Chattonella cf *verucullosa*	Plasmid	+	–	–
Heterosigma akashiwo	CCMP 2393‡	+	+	–
Heterosigma akashiwo	CCMP 1912	+	+	–
Heterosigma akashiwo	CCMP 302	+	+	–
Heterosigma akashiwo	CCMP 1596	+	+	–
Heterosigma akashiwo	CCMP 1870	+	+	–
Fibrocapsa japonica	UD	+	–	–

*Ha, *Heterosigma akashiwo* primers and probes; Cs, *Chattonella subsalsa* primers and probes.
†CCMP, Provasoli Guillard National Center for Culture of Marine Phytoplankton; UTEX, University of Texas at Austin, Culture Collection of Algae; UD, University of Delaware College of Marine Studies, Phytoplankton Culture Collection; Plasmid of 18S rDNA gene sequence (cultures are unavailable).
‡Isolates of *C. subsalsa* and *H. akashiwo* from the Delaware Inland Bays.

Assessment

Primer and probe development—Cloned 18S rDNA sequences for the DIB isolates of *Chattonella subsalsa* and *Heterosigma akashiwo* matched 18S rDNA sequences in GenBank (Accession numbers AY788938 and AY788936.1, respectively). To test for specificity and cross-reactivity, DNA extracted from geographical isolates of *C. subsalsa* and *H. akashiwo* as well as several closely related species were amplified with primers and probes described here (Table 1). The QPCR amplification demonstrated no cross-reactivity (no increase in fluorescence) when DNA from non-target species was used as template (Table 2).

The specificity of primers for target species in field samples from the Delaware Inland Bays was confirmed by sequence analysis of positive PCR reactions. DNA extracted from several water samples (without addition the pGEM reference standard) was also amplified with primers designed to target the pGEM plasmid. These reactions were negative, demonstrating the lack of sequences complementary to the pGEM primers in complex environmental samples (data not shown).

Probe sensitivity—A distinct advantage in using QPCR is the extreme sensitivity and large range of detection. Although standard protocols suggest a limit of 40 PCR cycles, we found that linear detection of the target DNA under conditions described here could be maintained for at least 50 cycles, increasing the sensitivity of the assay. We determined the sensitivity of the reac-

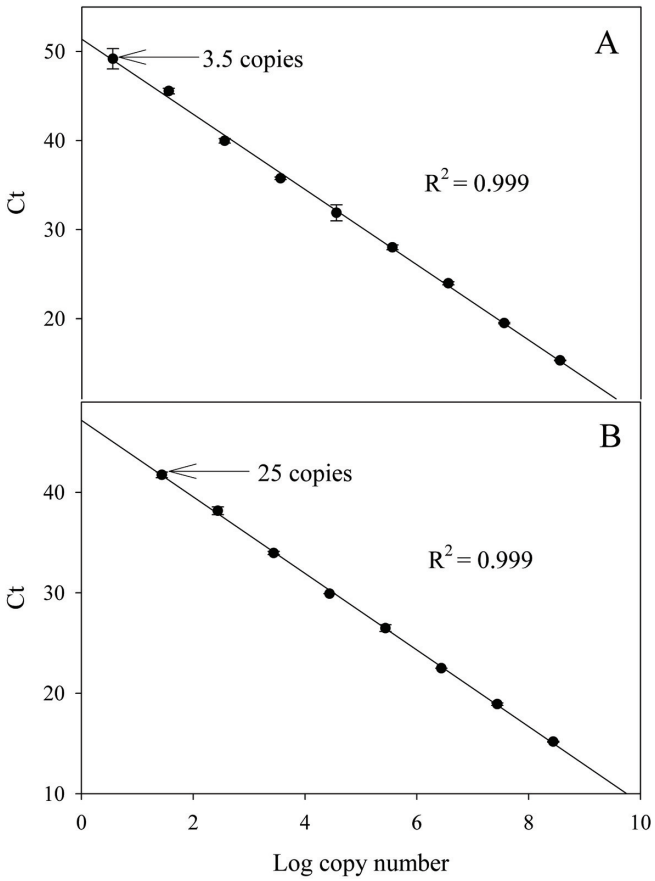

Fig. 1. Sensitivity of QPCR for *Heterosigma akashiwo* (A) and *Chattonella subsalsa* (B). Dilutions were generated from plasmid template containing the 18S rDNA sequence for each species. Error bars represent standard deviation of triplicate reactions.

tion for each template using known copy number plasmids with inserts containing the target 18S rDNA gene. The lowest level of detection for the *C. subsalsa* primer-probe set was 25 copies with a linear (R^2 = 0.999) range of detection over 7 orders of magnitude (Fig. 1B). The detection limit for *H. akashiwo* 18S rDNA was 3.5 copies with a linear (R^2 = 0.999) range of detection over 8 orders of magnitude (Fig. 1A).

Validation of the comparative Ct method—Cell densities in environmental water samples may be determined using the comparative Ct method in which unknowns are compared to a calibrator sample, containing a known abundance of the target organism (as determined by microscopy). For this method to be valid, the amplification efficiencies of the target and the reference standard must be approximately equal (Livak and Schmittgen 2001). To validate the comparative Ct method for *Heterosigma akashiwo* and *Chattonella subsalsa*, we prepared a dilution series of environmental DNA from natural bloom samples extracted in lysis buffer amended with the pGEM reference standard. The Ct values for target and reference DNAs were obtained by QPCR and the reaction efficiencies for each species were calculated (Table 3) from the slopes of regression lines for Ct versus relative dilutions (Fig. 2). The slopes of regression lines for ΔCt versus dilution of DNA from the *C. subsalsa* and *H. akashiwo* bloom samples were within the range of –0.1 to +0.1 (Fig. 3) for both target species, confirming the validity of the comparative Ct method for relative quantification for these species.

Table 3. Amplification efficiencies calculated from the slope of regression line for QPCR analysis of *Heterosigma akashiwo* and *Chattonella subsalsa*

Template	Slope of Ct versus 10-fold dilutions	Amplification efficiency [E = $10^{(-1/slope)}$]*
H. akashiwo Sample 101104	–3.909	1.80
C. subsalsa Sample BC3T11	–3.3195	2.00

*E = 2.0 represents 100% efficiency.

We found that the inclusion of the plasmid DNA in the extraction buffer provides a reference standard that is both

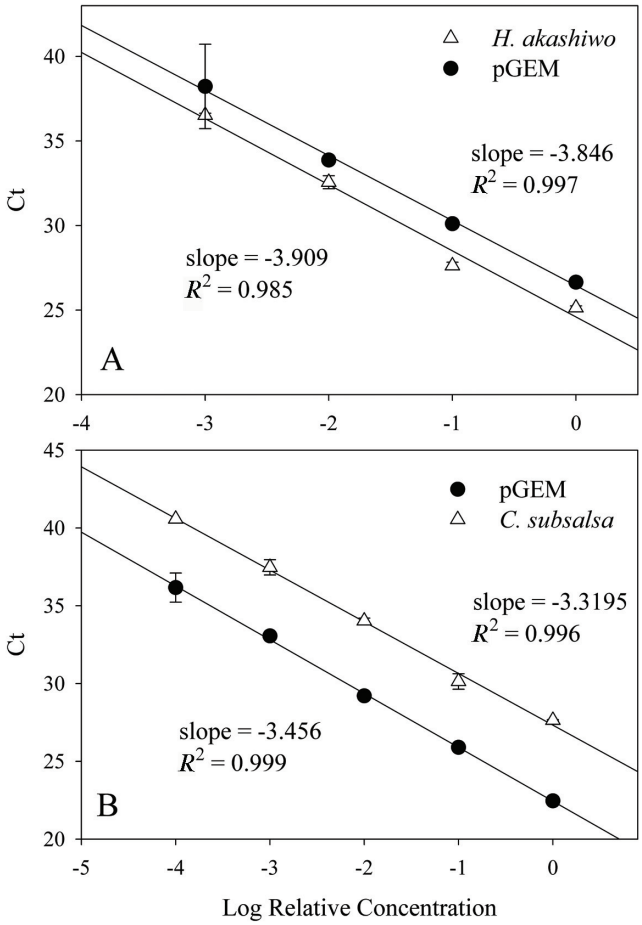

Fig. 2. Ct values for 10-fold dilutions of DNA extracted from natural bloom samples of *Heterosigma akashiwo* (A) and *Chattonella subsalsa* (B) in the presence of pGEM reference standard DNA. Error bars represent standard deviations of triplicate reactions.

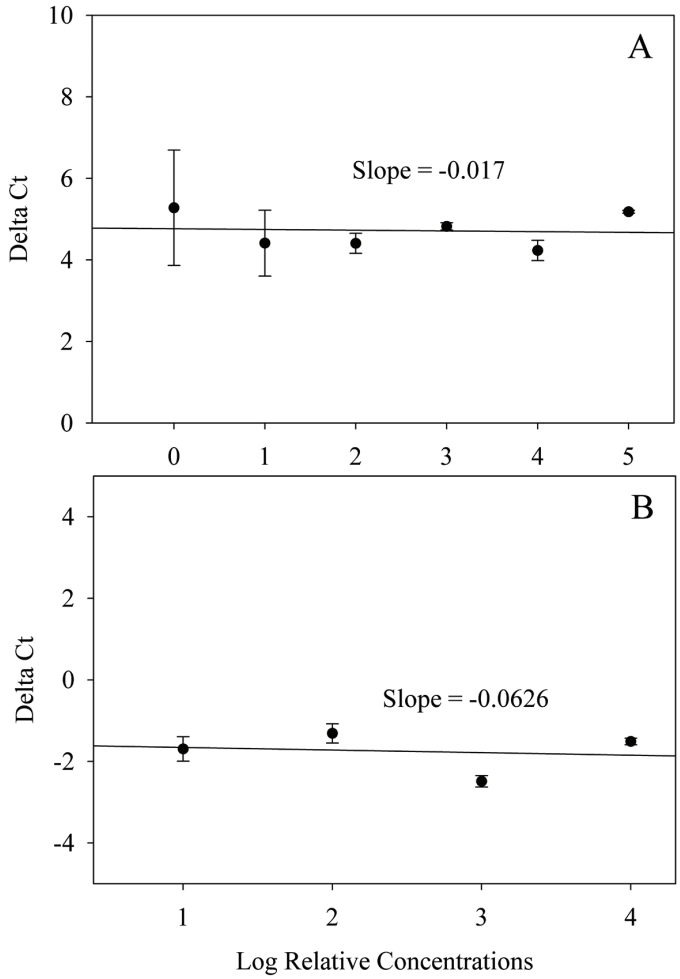

Fig. 3. Calculated ΔCt values ($Ct_{(Target)} - Ct_{(pGEM)}$) for 10-fold dilutions of DNA extracted from natural bloom samples of *Chattonella subsalsa* (A) and *Heterosigma akashiwo* (B) in the presence of pGEM reference standard DNA. Error bars represent standard error of triplicate reactions.

practical and robust. The stock solution could be stored at room temperature for at least 6 months without degradation of the plasmid DNA so that all samples processed during a sampling season included a constant concentration of the reference standard.

Precision—Intensive sampling strategies often do not permit the processing of more than one sample for each site at each time point. We examined intra-sample variability by filtering and extracting DNA from three sub-samples of water collected from a bloom of *Chattonella subsalsa*. Microscopic observation of the live sample indicated that *Heterosigma akashiwo* was also present at abundances that were approximately 1000 times lower than the abundance of *C. subsalsa*. The results for each sample were normalized to 1-L extraction volume for comparison (Table 4). The variability in cell abundance can be calculated from the standard deviation of ΔCt values (normalized to 1 L vol) for the triplicate samples. If E is the amplification efficiency (Table 3) and SD is the standard deviation among replicate ΔCt values normalized to 1L, the % variability may be calculated by

$$\% \text{ variability} = 100 \times (E^{SD} - 1) \quad (1)$$

These values represent less than 3% variability in cell abundance from the mean for replicate samples of *H. akashiwo* and less than 27% variability among replicate samples for *C. subsalsa*.

The improvement in QPCR results by the addition of a reference standard is illustrated in Fig. 4 for the data generated for *H. akashiwo* in the replicate water samples. While small differences in extraction and amplification efficiencies or dilution of samples resulted in Ct values that varied from the ideal (represented by the line on the graph, Fig. 4A), normalization of Ct values for the amplification of *H. akashiwo* 18S rDNA to Ct values for the pGEM reference resulted in a higher correlation coefficient and less intra-sample variability (Fig. 4B).

Accuracy—The accuracy of calculated cell abundances by QPCR using the comparative Ct method was first evaluated by comparing the results to cell counts derived by microscopy for

Table 4. Variability between triplicate water samples processed from the same site (062904)

	Volume filtered	Avg Ct_{target} (± SD)	Avg Ct_{pGEM} (± SD)	ΔCt (± SE)	ΔCt Normalized to 1 L (± SD)
H. akashiwo					
A	0.039 L	40.37 (0.565)	22.18 (0.141)	18.19 (0.336)	13.48
B	0.061 L	40.06 (0.221)	22.63 (0.035)	17.44 (0.129)	13.41
C	0.034 L	40.95 (0.139)	22.48 (0.040)	18.37 (0.083)	13.49
Avg					13.46 (0.044)
C. subsalsa					
A	0.039 L	26.71 (0.323)	22.24 (0.121)	4.47 (0.199)	–0.21
B	0.061 L	26.70 (0.665)	22.93 (0.161)	3.77 (0.395)	–0.26
C	0.034 L	26.28 (0.354)	22.23 (0.193)	4.05 (0.253)	–0.83
Avg					–0.43 (0.344)

ΔCt normalized to 1-L volume was calculated by ΔCt-$\log_2$(1/vol filtered). SD, standard deviation; SE, standard error.

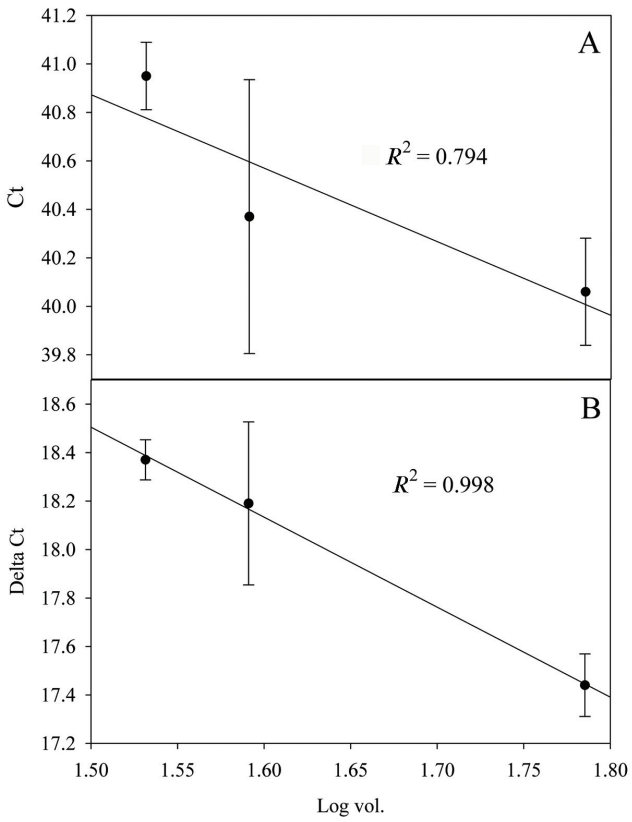

Fig. 4. Comparison of $Ct_{(H.\ akashiwo)}$ values (A) to $\Delta Ct_{(H.\ akashiwo\ -\ pGEM)}$ values (B) for different volumes of replicate water samples processed from sample 062904. Error bars represent standard deviation (A) and standard error (B) of duplicate reactions.

natural bloom samples. Cell counts of *Heterosigma akashiwo* were obtained after fixing triplicate sub-samples of water (bloom sample 101104) in very low concentrations of glutaraldehyde solution. Using one sub-sample as a calibrator sample, the *H. akashiwo* cell abundances for the other two sub-samples were calculated using the comparative Ct method (Table 5) according to the following equation:

$$\text{Cells L}^{-1} = E^{-\Delta\Delta Ct} \times [\text{cells L}^{-1}_{(calibrator)}] \times [\text{Vol filtered}_{(calibrator)}/\text{Vol filtered}_{(unknown)}] \quad (2)$$

where

$$\Delta\Delta Ct = (\Delta Ct_{(unknown)} - \Delta Ct_{(calibrator)}) \quad (3)$$

and

$$\Delta Ct = Ct_{(target)} - Ct_{(pGEM)} \quad (4)$$

Calculated cell abundances were 0.84 and 0.77 times the values obtained by microscopic cell counts for samples 1 and 2, respectively. These results are within the 95% confidence level for cell counts.

For *Chattonella subsalsa*, cell counts were determined for two water samples (samples BC3T0 and BC3T17) collected during a bloom. With sample BC3T0 as a calibrator sample, the cell abundance for sample BC3T17 was determined by QPCR using the comparative Ct (Eq. 2) method (Table 5). The

Table 5. Accuracy of comparative Ct method evaluated for triplicate sub-samples of *Heterosigma akashiwo* and two separate samples collected from a bloom of *Chattonella subsalsa*

	Volume filtered	Cell counts ($\times 10^7$ L^{-1}) (95% CL)*	Avg Ct_{target} (± SD)*	Avg Ct_{pGEM} (± SD)*	ΔCt (± SE)*	$E^{-\Delta\Delta Ct}$ (range)†	Calculated cells L^{-1} ($\times 10^7$) (range)†	Calculated relative to microscopic cell counts‡
H. akashiwo								
Sample 101104 (Calibrator)	0.100 L	1.544 (1.235-1.851)	26.71 (0.354)	28.19 (0.099)	–1.48 (0.260)			
Replicate 1	0.100 L	1.325 (1.039-1.611)	27.39 (0.127)	28.33 (0.325)	–0.940 (0.247)	0.728 (0.630-0.842)	1.124 (0.972-1.300)	0.84
Replicate 2	0.100 L	1.437 (0.833-2.042)	27.74 (0.276)	28.64 (0.396)	–0.905 (0.341)	0.713 (0.584-0.872)	1.101 (0.901-1.345)	0.77
C. subsalsa								
Sample BC3T0 (Calibrator)	0.050 L	1.084 (0.962-1.205)	31.60 (0.311)	24.26 (0.064)	7.35 (0.224)			
Sample BC3T17	0.054 L	1.763 (NA)*	28.21 (0.148)	22.56 (0.007)	5.65 (0.105)	3.24 (3.01-3.48)	3.250 (3.021-3.500)	1.84

*CL, Confidence limits; SD, standard deviation; SE, standard error; NA, not available.
†Asymmetric range of values calculated from average ΔCt ± SE.
‡Calculated cells L^{-1} divided by cell counts.

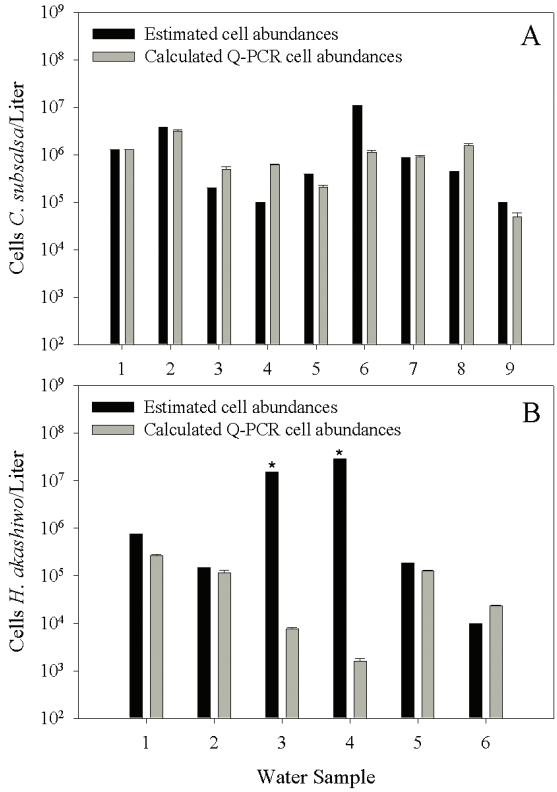

Fig. 5. Comparison of estimated cell abundances (black bars) to QPCR calculated cell abundances (gray bars) for environmental water samples with *Chattonella subsalsa* (A) and *Heterosigma akashiwo* (B). Asterisks indicate samples (3 and 4, panel B) that were misidentified as *H. akashiwo* during microscopic cell counts. Error bars represent standard error for duplicate reactions.

calculated cell abundance of BC3T17 by QPCR was 1.84 times greater than microscopic cell counts. Although this sample was from the same location as the calibrator sample, both cell counts and sample processing were done on different days and by different people. Inter-personnel differences and day-to-day variability in laboratory technique may have affected the results, suggesting that these protocols may also need to be standardized for better accuracy. Alternatively, the difference may be due to increased DNA content for the test sample (BC3T17). The calibrator and test samples were collected 17 h apart and may represent samples that are in different phases of the cell cycle.

We then compared QPCR results to estimated cell counts for several unfixed (live) environmental water samples shown in Fig. 5. The error in live estimated cell counts can be quite high due to cell motility and the small volume examined. A comparison of estimated cell counts and calculated cell counts based on QPCR show surprisingly good correlation, however, with differences within an order of magnitude for 13 of 15 field samples. Two of the samples (3 and 4, Fig. 5B) contained high abundances (>10^7) of cells that were identified by microscopy as *Heterosigma akashiwo*. 18S rDNA sequence analysis of these water samples, however, indicates the presence of a phylogenetically distinct species (manuscript in preparation), suggesting that cells of this species may have been mistaken for *H. akashiwo* in microscopic cell counts.

Discussion

Potentially toxic Raphidophyte species in the Delaware Inland Bays often form mixed blooms and can achieve concentrations exceeding 10^8 cells L^{-1} (http://www.ocean.udel.edu/mas/DIBCMP/reports.html). Raphidophyte cells are particularly fragile and pleomorphic, so that conventional phytoplankton fixation and microscopic techniques for obtaining accurate cell counts can be unreliable. Detection of microbial species using molecular methods provides a level of sensitivity and accuracy that is not possible with conventional microscopic (e.g., Coyne et al. 2001) or immunological techniques (Popels et al. 2003). Problems associated with QPCR analysis of field samples, however, have precluded extensive application of this technology to ecological studies. Our objectives were to develop quantitative real-time PCR assays that can rapidly and accurately identify and enumerate cell abundances of two Raphidophyte species, *Chattonella subsalsa* and *Heterosigma akashiwo*, in field samples during both bloom and nonbloom conditions. In this study, we determined the sensitivity of the assay and range of detection for the target gene in each species. We also evaluated the precision and accuracy of the comparative Ct method using environmentally relevant calibrator samples for enumeration of *C. subsalsa* and *H. akashiwo* in field samples.

The method proved to be extremely sensitive over a wide dynamic range with a demonstrated detection limit of about 4 copies of the *Heterosigma akashiwo* 18S rDNA gene and 25 copies of the *Chattonella subsalsa* 18S rDNA gene. Although the ribosomal copy numbers for these species are not known, the rDNA operon is tandemly repeated up to thousands of times in other protists (Galluzzi et al. 2004; Guay et al. 1992; Le Blancq et al. 1997; Saito et al. 2002), suggesting that detection of fewer than 10 cells of *H. akashiwo* and *C. subsalsa* may be possible. This level of sensitivity is probably not necessary for environmental monitoring (as such low abundances pose minimal environmental risk), but instead serves to demonstrate the potential of the method.

Two obstacles in obtaining accurate and reproducible results for PCR analysis of environmental samples are variability in extraction efficiencies and the presence of inhibitory compounds. Cells of target organisms (Lebuhn et al. 2004), closely related species (Brinkman et al. 2003) or cells containing target DNA (Okano et al. 2004), or competitor DNA constructs (Widada et al. 2002) have been employed as reference standards in quantitative PCR methods to minimize these errors. These whole cell standards are used to normalize differences in lysis efficiency and may be essential to validate extraction of species that are difficult to lyse. The addition of cultured cells to samples or extraction buffer, however, could

potentially introduce inhibitory compounds, and assumes that lysis of the target cells is identical to the introduced cells. There is also a reasonable probability that primers and probes targeting the introduced standard cells may detect naturally occurring species present in environmental samples and result in decreased Ct values for the standard.

Mumy and Findlay (2004) found that the recovery of DNA from cells containing an exogenous plasmid was similar to addition of the plasmid itself directly to samples prior to extraction. Bostrom et al. (2004) also described the addition of an exogenous DNA plasmid to the extraction buffer to measure extraction efficiencies of different methods. Neither of these publications, however, described the application of plasmid DNA as a reference standard in the development of quantitative real-time PCR assays. In the protocol described here, we included pGEM plasmid (Promega) as an exogenous reference standard in the lysis extraction buffer for extraction of all environmental samples. Since the exogenous DNA is extracted in the presence of the target, PCR inhibitors affect the amplification of both the target and the reference standard equally (Lebuhn et al. 2004; Widada et al. 2002). In addition, reference and target DNA undergo the same treatment so that errors due to downstream manipulations, such as preparing dilutions, are reduced.

Another challenge to obtaining accurate and realistic results with QPCR is in the design of standards for the target species. Absolute quantification methods by QPCR often use plasmid dilutions (Fontaine and Guillot 2002; Galluzzi et al. 2004) or laboratory cultures (Bowers et al. 2000; Phister and Mills 2003; Popels et al. 2003; Saito et al. 2002) to generate a standard curve. Although these standards are relatively easy to prepare, they may not always be appropriate for comparison to natural populations of target cells. With the method described here, cell abundances were calculated relative to a calibrator sample, consisting of natural environmental bloom samples for each target species. The use of natural environmental samples as opposed to purified plasmids for calculation of amplification efficiencies more accurately represents efficiencies of other environmental samples (*see* Peirson et al. 2003). We evaluated the accuracy of the comparative Ct method ($E^{-\Delta\Delta Ct}$) by comparing cell densities of test samples calculated by QPCR to microscopic cell counts. QPCR results were within the 95% confidence level for cell counts for replicate samples of the *Heterosigma* bloom sample, but approximately 1.84 times higher than the value determined by microscopy for *Chattonella* bloom sample BC3T17. Small differences in Ct values may represent large differences in calculated results. Under optimal conditions (efficiency = 2.0), a 2-fold increase in initial template concentration results in a decrease of only 1 Ct unit. The difference between QPCR results and microscopic cell counts for *C. subsalsa* sample BC3T17 (Table 5), for example, represents a difference in Ct value of less than 0.88 units.

Other potential sources of error in the comparative Ct method using an exogenous DNA reference standard are due to pipetting of the lysis buffer, volume measurements of water samples, and cell counts for calibrator samples. The lysis buffer described here is somewhat viscous, so that precise pipetting will be important to achieve a constant concentration of reference plasmid DNA in the extracted sample. If cell concentrations are reported per unit volume, inaccurate volume measurements of field samples will also affect the results. Finally, errors in microscopic cell counts of the calibrator sample will affect results of calculated cell densities by QPCR. Obtaining accurate cell counts of calibrator samples for Raphidophytes can be difficult because these species do not retain their shape and often burst under standard fixation protocols (Throndsen 1997). We found that by fixing samples in very low concentrations of glutaraldehyde solution and observing them immediately under the microscope, we were able to obtain accurate cell counts for environmental bloom samples of *Chattonella subsalsa* and *Heterosigma akashiwo* for use as calibrators.

The application of the comparative Ct method to other species will need to be validated as described in the Materials and Procedures section. For the method to be valid, the absolute value of the slope of the regression line must be less than 0.1 (*see* Assessment). If the pGEM reference standard is not compatible with the target species, a dilution series of standards must be run for both the reference standard and the calibrator sample.

An unexpected result of our investigation highlights the increased accuracy in species identification when using molecular methods. Microscopic examination of two of the samples (3 and 4, Fig. 5B) chosen for validation of the QPCR assay identified cells resembling *Heterosigma akashiwo* at very high densities (> 10^7 cells L^{-1}). Our calculated QPCR results, however, were several orders of magnitude less than the estimated cell counts for *H. akashiwo*. Subsequent sequence analysis confirmed that the bloom species in these samples is phylogenetically distinct (manuscript in preparation) and represents a case of mistaken identity.

In conclusion, we have described modifications to the QPCR method that improve both precision and accuracy of quantitative detection of microbial species in environmental water samples. The addition of the exogenous DNA plasmid, pGEM, as a reference standard permits analysis of multiple species in the same sample and is applicable to any microbial species of interest. In addition, the reference standard may be used to identify false negative results due to PCR inhibition. The QPCR assays described here are currently being used to investigate factors that affect Raphidophyte population dynamics in the natural environment (Handy et al. in press). We have found that the sensitivity of this method permits identification and quantification of *Chattonella subsalsa* and *Heterosigma akashiwo* in field samples where cell abundances are below the level of detection (and enumeration) by routine microscopy. The ability to rapidly and accurately quantify these species over a large range of cell concentrations will allow us to identify environmental variables that impact bloom initiation and progression.

References

Andersen, P., and J. Throndsen. 2003. Estimating cell numbers, p 99-129. *In* G. M. Hallegraeff, D. A. Anderson, and A. D. Cembella [eds.], Manual on harmful marine microalgae. Unesco Publishing Co.

Becker, S., P. Boger, R. Oehlmann, and A. Ernst. 2000. PCR bias in ecological analysis: A case study for quantitative Taq nuclease assays in analyses of microbial communities. Appl. Environ. Microbiol. 66:4945-4953.

Black, E. A., J. N. C. Whyte, J. W. Bagshaw, and N. G. Ginther. 1991. The effects of *Heterosigma akashiwo* on juvenile *Oncorhynchus tshawytscha* and its implications for fish culture. J. Appl. Ichthyol. 7:168-175.

Bostrom, K. H., K. Simu, A. Hagstrom, and L. Riemann. 2004. Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. Limnol. Oceanogr. Methods 2:365-373.

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

Brinkman, N. E., R. A. Haugland, L. J. Wymer, M. Byappanahalli, R. L. Whitman, and S. J. Vesper. 2003. Evaluation of a rapid, quantitative real-time PCR method for enumeration of pathogenic *Candida* cells in water. Appl. Environ. Microbiol. 69:1775-1782.

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

Cullen, D. W., A. K. Lees, I. K. Toth, and J. M. Duncan. 2002. Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. Plant Pathol. 51:281-292.

Dempster, E. L., K. V. Pryor, D. Francis, J. E. Young, and H. J. Rogers. 1999. Rapid DNA extraction from ferns for PCR-based analyses. Biotechniques 27:66-68.

Fontaine, M., and E. Guillot. 2002. Development of a TaqMan quantitative PCR assay specific for *Cryptosporidium parvum*. Fems Microbiol. Lett. 214:13-17.

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

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

Guay, J. M., A. Huot, S. Gagnon, A. Tremblay, and R. C. Levesque. 1992. Physical and genetic mapping of cloned ribosomal DNA from *Toxoplasma-gondii*—primary and secondary structure of the 5s gene. Gene 114:165-171.

Guillard, R. R. L. 1975. Culturing of phytoplankton for feeding marine invertebrates, p 29-60. *In* W. L. Smith and M. H. Chanley [eds.], Culturing marine invertebrate animals. Plenum Publishing Co.

Handy, S. M., and others. In press. Evaluating vertical migration behavior of harmful raphidophytes in the Delaware Inland Bays utilizing quantitative real-time PCR. Aquatic Microbial Ecology.

Haque, S. M., and Y. Onoue. 2002. Variation in toxin compositions of two harmful raphidophytes, *Chattonella antiqua* and *Chattonella marina*, at different salinities. Environ. Toxicol. 17:113-118.

Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. Gen. Res. 6:986-994.

Horner, R. A. 1999. Harmful algal blooms in the USA: An update, p. 10. *In* J. L. Martin and K. Haya [eds.], Proceedings of the Sixth Canadian Workshop on Harmful Marine Algae. No. 2261, Can. Tech. Rep. Fish. Aquat. Sci.

Ishida, H., A. Nozawa, H. Nukaya, and K. Tsuji. 2004. Comparative concentrations of brevetoxins PbTx-2, PbTx-3, BTX-B1 and BTX-B5 in cockle, *Austrovenus stutchburyi*, greenshell mussel, *Perna canaliculus*, and Pacific oyster, *Crassostrea gigas*, involved neurotoxic shellfish poisoning in New Zealand. Toxicon 43:779-789.

Khan, S., O. Arakawa, and Y. Onoue. 1996*a*. Growth characteristics of a neurotoxin-producing chloromonad *Fibrocapsa japonica* (Raphidophyceae). J. World Aquacult. Soc. 27: 247-253.

———. 1996*b*. A toxicological study of the marine phytoflagellate, *Chattonella antiqua* (Raphidophyceae). Phycologia 35:239-244.

———. 1997. Neurotoxins in a toxic red tide of *Heterosigma akashiwo* (Raphidophyceae) in Kagoshima Bay, Japan. Aquacult. Res. 28:9-14.

Kolb, S., C. Knief, S. Stubner, and R. Conrad. 2003. Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. Appl. Environ. Microbiol. 69:2423-2429.

Le Blancq, S. M., N. V. Khramtsov, F. Zamani, S. J. Upton, and T. W. Wu. 1997. Ribosomal RNA gene organization in *Cryptosporidium parvum*. Mol. Biochem. Parasitol. 90:463-478.

Lebuhn, M., M. Effenberger, G. Garces, A. Gronauer, and P. A. Wilderer. 2004. Evaluating real-time PCR for the quantification of distinct pathogens and indicator organisms in environmental samples. Water Sci. Technol. 50:263-270.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402-408.

Medlin, L., H. J. Elwood, S. Stickel, and M. L. Sogen. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71:491-499.

Mumy, K. L., and R. H. Findlay. 2004. Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR. J. Microbiol. Methods 57:259-268.

Okano, Y., and others 2004. Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Appl. Environ. Microbiol. 70: 1008-1016.

Peirson, S. N., J. N. Butler, and R. G. Foster. 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucl. Acids Res. 31:e73.

Phister, T. G., and D. A. Mills. 2003. Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine. Appl. Environ. Microbiol. 69:7430-7434.

Plakas, S. M., and others. 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon 44:677-685.

Popels, L. C., and others. 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., T. Drgon, J. Robledo, D. Krupatkina, and G. Vasta. 2002. Characterization of the rRNA locus of *Pfiesteria piscicida* and development of standard and quantitative PCR-based detection. Appl. Environ. Microbiol. 68:5394-5407.

Skovhus, T. L., N. B. Ramsing, C. Holmstrom, S. Kjelleberg, and I. Dahllof. 2004. Real-time quantitative PCR for assessment of abundance of Pseudoalteromonas species in marine samples. Appl. Environ. Microbiol. 70:2373-2382.

Smith, S. W., R. Overbeek, C. R. Woese, W. Gilbert, and P. M. Gillevet. 1994. The genetic data environment. An expandable GUI for multiple sequence analysis. Comput. Appl. Biosci. 10:671-675.

Stommel, E. W., and M. R. Watters. 2004. Marine neurotoxins: Ingestible toxins. Curr. Treat. Options Neurol. 6:105-114.

Stults, J. R., O. Snoeyenbos-West, B. Methe, D. R. Lovley, and D. P. Chandler. 2001. Application of the 5′ fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. Appl. Environ. Microbiol. 67:2781-2789.

Suzuki, M. T., L. T. Taylor, and E. F. Delong. 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. Appl. Environ. Microbiol. 66:4605-4614.

Tebbe, C. C., and W. Vahjen. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl. Environ. Microbiol. 59:2657-2665.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22:4673-4680.

Throndsen, J. 1997. The planktonic marine flagellates, p 591–729. *In* C. R. Tomas [ed.], Identifying marine phytoplankton. Academic Press, Harcourt Brace.

Vaitomaa, J., and others 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Appl. Environ. Microbiol. 69:7289-7297.

Widada, J., H. Nojiri, and T. Omori. 2002. Recent developments in molecular techniques for identification and monitoring of xenobiotic-degrading bacteria and their catabolic genes in bioremediation. Appl. Microbiol. Biotechnol. 60: 45-59.

Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63:3741-3751.

Woofter, R. T., K. Brendtro, and J. S. Ramsdell. 2005. Uptake and elimination of brevetoxin in blood of striped mullet (*Mugil cephalus*) after aqueous exposure to *Karenia brevis*. Environ. Health Perspectives 113:11-16.

Yang, C. Z., L. J. Albright, and A. N. Yousif. 1995. Oxygen radical-mediated effects of the toxic phytoplankter *Heterosigma carterae* on juvenile rainbow trout *Oncorhynchus mykiss*. Dis. Aquat. Org. 23:101-108.

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