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Simultaneous enumeration of multiple raphidophyte species by quantitative real-time PCR: capabilities and limitations

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Abstract

Quantitative real-time PCR (QPCR) is a powerful method for species-specific detection and enumeration of harmful algal bloom (HAB) algae, but can be quite time-consuming and expensive for multiple species detection when only a single species is analyzed in each reaction. Rapid enumeration methods would be valuable for the investigation of multiple HAB events such as the mixed blooms of harmful raphidophyte species (*Chattonella* cf *verruculosa*, *C. subsalsa*, and *Heterosigma akashiwo*) that co-occur in the Delaware Inland Bays (DIB), USA. This technology uses multiple species-specific probes that fluoresce at different wavelengths and allows for the simultaneous detection and enumeration of more than one species, limited only by the detection capabilities of the instrument. The approach can cut analysis time and cost at least in half, depending on the number of species that can be accurately detected and enumerated in the mixture. Here, we compared multiprobing—or using a single primer set with species-specific probes—to multiplexing, in which species-specific primers and probes are used, targeting the 18S rDNA of 3 raphidophyte species and an internal standard. Although both methods were effective for multispecies detection compared to simplex results, multiplexing was more accurate than multiprobing for this particular group of species. We investigated the accuracy and sensitivity of multiplex/multiprobe QPCR using plasmids, genomic DNA from cultures, or DNA extracted from environmental samples as template. Smaller amplicon size and sequence heterogeneity between amplicons increased the accuracy of the results. We found that environmental samples of raphidophytes can be successfully multiplexed or multiprobed with only minimal losses in sensitivity. In addition, we explored probe reporter dyes and quenchers for their compatibility, robustness, and reproducibility in multitemplate detection, and we compared and evaluated the capabilities and limitations of this method for detecting and enumerating multiple phytoplankton species simultaneously.

Introduction

Quantitative real-time polymerase chain reaction (QPCR) is a powerful tool for identification and enumeration of microbial species in environmental samples (Cullen et al. 2002; Gray et al. 2003; Popels et al. 2003; Coyne et al. 2005; Handy et al. 2005). QPCR measures the change in product concentration during each PCR cycle using real-time detection (Heid et al. 1996). Fluorescent, template-specific probes are used, commonly labeled with a fluorescent reporter dye at the 5′ end and a quencher molecule at the 3′ end. Fluorescence is generated by cleavage of the reporter dye from the oligonucleotide probe, releasing it from the quencher. The increase in product concentration is measured as an increase in fluorescence emission (R_n). The threshold cycle number (Ct) for each reaction is the cycle number at which the fluorescence emission changes significantly over baseline levels (Walburger et al. 2001).

Quantification of microbial species can be accomplished by the standard curve method (e.g., Cullen et al. 2002) in which dilutions of plasmids or culture DNA with a known concentration of the target template are used as standards, and the concentrations of the target species in unknown samples are determined by linear regression analysis. The standards must be included for each sample set, occupying much of the 96-well plate during a QPCR run (Popels et al. 2003). Alternatively, the comparative Ct method ($2^{-\Delta\Delta Ct}$ method) uses a single sample as a calibrator (Livak and Schmittgen 2001). For enumeration of phytoplankton species in natural water samples, the calibrator can consist of DNA extracted from an envi-

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ronmental water sample with a known number of cells of the target species (determined by microscopic cell counts) (Coyne et al. 2005; Handy et al. 2005). This sample is then used to enumerate the species of interest in other samples.

For improved accuracy, an internal reference standard may also be used to normalize for DNA extraction and amplification efficiencies (Coyne et al. 2005). As a result, the target species and the internal standard concentrations must be determined independently with specific primer/probe sets for each. Because greater accuracy would be obtained by measuring both target and internal standard within the same reaction, it would be an advantage to be able to quantify multiple targets in the sample tube.

Multiprobing/multiplexing with QPCR—Simplexing is defined as the use of a single set of primers and a single fluorescent probe in QPCR. Multiplexing makes use of multiple template-specific primers and fluorescent probes, allowing for the enumeration of more than one species in a single reaction. Multiprobing, on the other hand, uses multiple template-specific fluorescent probes, but with a single primer set, which can be advantageous because competition between primer pairs is eliminated (Yang et al. 2002). By using multiple primer/probe sets, the costs of QPCR can be reduced by half, if not more, depending on the number of templates that can be accurately detected and enumerated. In addition, multiplexing/multiprobing saves time over simplexing (Vet et al. 1999).

Multitarget detection is possible through the use of probes (reporters) that fluoresce at different wavelengths. A limitation for multiplexing and multiprobing is the potential for overlapping spectra between the fluorophores, which reduces resolution. Nonfluorescent quenchers, such as black hole quenchers (BHQs) (Biosearch Technologies), have recently been developed that can be combined with reporter dyes, thus increasing the spectra available for multiprobe QPCR. BHQs are designed specifically for reporters that fluoresce within a range of wavelengths. They can employ fluorescence resonance energy transfer (FRET) (Clegg 1992) more efficiently and therefore quench more effectively (Parkhurst and Parkhurst, 1995a,b; Tyagi et al. 1998). In addition, a nonfluorescent quencher allows the instrument to measure reporter fluorescence (spectral resolution) more effectively since the fluorescence of the quencher does not have to be resolved by the instrument (ABI Chemistry Guide 2003). To our knowledge, however, a comparison between fluorescent and nonfluorescent quenchers for use in multiplex assays has not been described.

Multiplexing has had limited but increasing use by the biomedical community (Walburger et al. 2001; Yang et al. 2002; Richtsteiger et al. 2003; Ng et al. 2005). These studies often obtain results that are comparable to simplexing, but a specific protocol for development of these assays is lacking. Klein et al. (2000), for example, used multiplexing for the estimation of transduction efficiency in preclinical trials, normalizing the results to a marker gene. They found that multiplexing gave results similar to that of other non-PCR-based methods and was favored because of its increased accuracy over simplexing (referred to as monoplexing in Klein et al. 2000). The steps involved in assay development, however, were not described. Vet et al. (1999) also demonstrated that up to 4 targets of pathogenic retroviruses can be identified using multiplexing. However, the protocol for developing a multiplex assay was not discussed, nor was the difference in reaction efficiencies for simplexing and multiplexing evaluated.

Multiplex and multiprobe assays must be carefully evaluated (Klein 2002) for each new system. Optimization of primers and probes is an important first step for any QPCR assay (ABI Chemistry Guide 2003). For multiplex/multiprobe assays, however, cross reactivity between primers and probes as well as amplification products must also be evaluated. Competition for reagents may lead to decreased sensitivity and amplification efficiencies, especially with large differences in template concentrations (Wawrik et al. 2002). Each of these will also have an impact on the accuracy of QPCR multiplex/multiprobe assays and must be rigorously evaluated before application to environmental samples.

Although there has been progress in the use of QPCR for harmful algae research (e.g., Bowers et al. 2000; Gray et al. 2003; Popels et al. 2003; Galluzzi et al. 2004; Handy et al. 2005), few studies have used multiplexing/multiprobing for investigations of phytoplankton in environmental samples. Wawrik et al. (2002) used multiplexing to determine the expression level of rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase) in diatom and pelagophyte species. In this case, however, the collective gene expression was monitored and species-specific expression was not determined.

Harmful algae belonging to the class Raphidophyceae are widespread and have been linked to mass mortalities of fish in many areas of the world (Odebrecht and Abreu 1995; Munday and Hallegraeff 1998), including Delaware's Inland Bays (DIB) (Bourdelais et al. 2002). Using species-specific primers and probes, raphidophyte species from the DIB have been accurately enumerated by QPCR in simplex reactions (Coyne et al. 2005; Handy et al. 2005). This method is preferred to traditional microscopic cell counts because it reduces subjectivity of person-to-person cell count analyses and is more accurate for counts of low biomass samples. In addition, long-term storage of fixed raphidophyte samples is problematic (Throndsen 1997; Handy et al. 2005).

It has been noted that 2 or more of the 4 species of raphidophytes within the DIB often form mixed blooms (Whereat 2003; Handy et al. 2005). Using multiplex or multiprobe technology, multiple species could be simultaneously detected and enumerated within a single reaction. We used the DIB raphidophyte populations as a model system to develop multiplex and multiprobe assays of harmful algal species. QPCR assays targeting the 18S rDNA in simplex, multiplex, and multiprobe analyses were compared using plasmid and culture DNA of DIB isolates, with and without background DNA, over a range of dilutions. The differences in sensitivity, range of detection, and

Table 1. Primers used for this study.

DNA target	Primer (5′-3′)	Citation
C. subsalsa 18S rDNA	Cs 1350F CTAAATAGTGTGGGTAATGCTTAC	Coyne et al 2005
	Cs 1705R GGCAAGTCACAATAAAGTTCCAA	
H. akashiwo 18S rDNA	Ha 1350F CTAAATAGTGTCGGTAATGCTTCT	Coyne et al 2005
	Ha 1705R GGCAAGTCACAATAAAGTTCCAT	
C. subsalsa 18S rDNA and *H. akashiwo* 18S rDNA	*Raph 1350F GCTAAATAGTGTSGGTAATGCTT	This publication
	*Raph 1705R GGCAAGTCACAATAAAGTTCCA	
C. subsalsa 18S rDNA, *H. akashiwo* 18S rDNA, and *C.* cf. *verruculosa* 18S rDNA	**Raph 1519F TTAGATGTCCTGGGCTGCAC	This publication
	**Raph 1610R ATCTATCCCTATCACGATGCAC	
C. cf. *verruculosa* 18S rDNA	Cv 1561F ATGCATACAGCGAGTCTAGA	This publication
	Cv 1780R TCACTCCGAAAAGTGTCAAC	
pGEM internal standard	pGEMR TGTGTGGAATTGTGAGCGGA	Coyne et al 2005
	M13F CCCAGTCACGACGTTGTAAAACG	

*Raphidophyte primer set 1
**Raphidophyte primer set 2

reaction efficiencies were investigated for each method. We also compared fluorescent and nonfluorescent quencher molecules.

Simplex, multiplex, and multiprobe assay results were then compared for DNA extracted from environmental samples from the DIB. Reaction efficiencies with respect to target DNA concentrations within complex mixtures are discussed, as well as the application of these methods for environmental monitoring programs. In addition, the protocol presented here for development of QPCR methods for single-tube detection of multiple species would be valuable for anyone wishing to investigate these and other algae, and may be used as a primer for researchers developing their own assays for multispecies detection.

Materials and procedures

Plasmid development—Plasmids for *Chattonella subsalsa* and *Heterosigma akashiwo* were prepared as described in Coyne et al. (2005). To develop a plasmid from the currently unculturable *C.* cf *verruculosa*, an environmental water sample of a unialgal *C.* cf *verruculosa* bloom was extracted as described below. The 18S rDNA was amplified using Euk A/Euk B primers (Medlin et al. 1988), cloned into pCR4 TOPO plasmid vector (Invitrogen, Carlsbad, CA, USA) and bidirectionally sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The sequence was verified by comparison to the 18S rDNA sequence for *C.* cf *verruculosa* (GenBank acc. no. AY788946).

Extraction of field samples—Field samples were collected by prefiltering (250 μm Nitex) to remove large zooplankton and detritus and filtering under gentle vacuum (~85 kPa) onto a 3-μm polycarbonate filter. The filters were submersed in CTAB buffer (100 mM Tris-HCL pH 8, 1.4 M NaCl, 2% wt/vol cetyltrimethylammonium bromide [CTAB], 0.4% vol/vol β-mercaptoethanol, 1% wt/vol polyvinylpyrollidone, 20 mM EDTA) (Dempster et al. 2003) amended with 20 ng/mL pGEM plasmid (Promega, Madison, WI, USA) as an internal standard (Coyne et al. 2005). The samples were stored at –80°C until extraction as described in Coyne et al. (2001), and DNA concentrations were determined by spectrophotometry.

Development of quantitative PCR primers and probes—Species-specific primers and probes for *C. subsalsa*, *H. akashiwo*, and the internal standard pGEM are described in Coyne et al. (2005). Two sets of general raphidophyte primers and species-specific primers for *C.* cf *verruculosa* were designed by aligning 18S rDNA sequences of Delaware Inland Bay raphidophyte strains and 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). Raphidophyte general primer set 1 (Raph1350F/1705R) was designed to amplify 377 bp of the 18S rDNA gene of *C. subsalsa* and *H. akashiwo*. Raphidophyte general primer set 2 (Raph1519F/1610R) amplified 113 bp of the 18S rDNA gene of DIB raphidophyte strains of *C. subsalsa, C.* cf *verruculosa*, *H. akashiwo*, and *Fibrocapsa japonica* in addition to other Raphidophyte species such as *Haramonas dimorpha*, and some diatom species. The *C.* cf *verruculosa*-specific primers amplified 239 bp of the 18S rDNA gene. All primer sequences are listed in Table 1.

Taqman probes were designed with Primer Express software (Applied Biosystems). Probes were synthesized with one of the following reporter dyes: 6-FAM (6-carboxyfluorescein), HEX (6—carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein), CY5 (CY Dye), or Texas red at the 5′ end and either TAMRA (6-carboxytetramethylrhodamine), Black Hole Quencher (BHQ), or Iowa Black Hole Quencher (IBHQ) (Integrated DNA Technologies) at the 3′ end. All probe sequences are listed in Table 2.

Primers and probes were optimized for the ABI Prism 7500 Sequence Detection System (Applied Biosystems) using cloned plasmids that contained the 18S rDNA sequences of *C.* cf *verruculosa*, *C. subsalsa*, *H. akashiwo*, or the internal standard pGEM. For species-specific *C. subsalsa* and *H. akashiwo* detection, optimized conditions were the same as in Coyne et al. (2005), with the exception of the HEX-labeled *H. akashiwo* probe, which had an optimized concentration of 0.075 μM (final concentration).

Table 2. Probes and reporter dyes used for this study.

Probe Sequence	Target	Citation
HEX-CAACGAGTAACGACCTTTGCCGGAA-IBFQ	*H. akashiwo*	Coyne et al 2005
FAM-CAACGAGTACTTTCCTTGGCCGGAA-BHQ1	*C. subsalsa*	Coyne et al 2005
CY5-CAAGAGTACCCAGGCCTCTCGACC-IBRQ	*C.* cf. *verruculosa*	This publication
FAM-CACTATAGAATACTCAAGCTTGCATGCCTGCA-BHQ1	pGEM internal standard	Coyne et al 2005

In addition, primer set 1 (Raph 1350F/1705R) had an optimized concentration of 1.2 μM for each primer. For *C.* cf *verruculosa*, the optimized conditions for 25-μL reactions were 12.5 μL Taqman Universal Master Mix (Applied Biosystems), 0.3 μM of each *C.* cf *verruculosa* primer (Cv 1561F/1780R), 0.225 μM of the CY5-labeled Taqman probe (Cv), and 2.5 μL diluted template.

The Taqman Universal Master Mix contains Taq polymerase, dNTPs, buffer, magnesium, uracil-*N*-glycosylase, and the passive reference dye, ROX. The cycling parameters for all reactions were 2 min at 50°C for activation of the uracil-*N*-glycosylase, 10 min at 95°C for activation of the polymerase, and 50 cycles of 15 s at 95°C and 1 min at 60°C. The concentrations of the individual primers and probes when multiplexing or multiprobing were the same as above, but were combined in a single reaction.

Brilliant Multiplex QPCR Master Mix (Stratagene) was also evaluated for multiplexing and multiprobing by substituting the mix for the 12.5 μL of the Taqman Universal Master Mix. The Brilliant mix contains SureStart Taq DNA polymerase (a modified version of Taq2000 DNA polymerase with hot-start capability), dNTPs, buffer, and magnesium. For the Brilliant Multiplex QPCR Master Mix, 0.45 mM of an internal reference dye (ROX) was added separately.

Primer and probe specificity—All plasmids were evaluated for target specificity by PCR with *C. subsalsa*, *H. akashiwo*, and *C.* cf *verruculosa* species-specific primer sets and with the raphidophyte primer set 1 (Raph 1350F/1705R). The 20-μL PCRs contained 0.2 mM dNTPs, 0.5 μM of each of the forward and reverse primers, 10 μg BSA, 2.5 mM $MgCl_2$, 1× Taq polymerase buffer (Sigma Chemical, St. Louis, IL, USA), and 0.5 units JumpStart Taq Polymerase (Sigma). Cycle parameters are the same as in Coyne et al. (2005). In addition, all primer/probe combinations were evaluated for specificity by QPCR amplification of DNA extracted from *C. subsalsa* (CCMP 217 and 2191), *C. marina* (CCMP 2049), *C. antiqua* (CCMP 2050), and several isolates of *H. akashiwo* (CCMP 2393, 1912, 302, 1596, and 1870).

Multiplex and multiprobe QPCR with plasmids—The accuracy and sensitivity of simplex reactions were compared to multiplex and multiprobe reactions by combining species-specific or raphidophyte general primers and species-specific probes with equal concentrations of plasmid templates as well as with a range of dilutions of plasmids. Mixtures of plasmid templates were also prepared in which the concentration of one template DNA was constant (0.01 ng/μL final concentration) with 10-fold dilutions of the second template. Simplex (one target) and multiplex or multiprobe (more than one target) reactions were directly compared within the same QPCR run.

The M13F primer site is present on both the pGEM internal standard template and on the species-specific plasmids. For this reason, the 18S rDNA inserts of the species-specific plasmids were first reamplified and the resulting PCR products used as template when pGEM was one of the targets used for multiplexing. The inserts were amplified from 5 μL of 0.1 ng/μL plasmid with 0.5 μM EUK A/B primers (Medlin et al. 1988) in a 50-μL reaction with the following parameters: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 2.5 min, and a final extension of 72°C for 5 min. Resulting PCR products were cleaned up with GenElute PCR cleanup kit (Sigma-Aldrich). The products were diluted in LoTE (3mM Tris-HCl pH 7.5, 0.2 mM EDTA) to 0.2 ng/μL and used as template with pGEM plasmids in multiplex reactions.

Multiplex and multiprobe QPCR with pure culture DNA—Genomic DNA extracted from cultures of DIB species *C. subsalsa* (CCMP 2191) and *H. akashiwo* (CCMP 2393) was used as template in multiplex/multiprobe assays with *C. subsalsa*– and *H. akashiwo*–specific primers or raphidophyte primer set 1. DNA template concentrations for one species were held constant at 2.5 ng/μL (final concentration) while varying the other by 10-fold dilutions for a final concentration of 2.5, 0.25, 0.025, or 0.0025 ng/μL. The mixtures of genomic DNA were diluted in LoTE or with 25 ng/μL sheared herring sperm (SHS) DNA (Invitrogen) to provide a similar concentration of background DNA as in environmental samples.

Multiplex and multiprobe QPCR with environmental samples—Fifteen environmental water samples from the DIB containing both *H. akashiwo* and *C. subsalsa* (verified by microscopy) were used to compare the calculated cell abundances obtained by multiprobing to simplexing in environmental samples. In addition, 6 environmental samples from the summer of 2005 were used to compare results of simplexing, multiprobing, and multiplexing of *C.* cf *verruculosa* and *C. subsalsa* within the same QPCR run. Six other environmental samples were also used to compare simplexing and multiplexing of *C.* cf *verruculosa* and the internal standard pGEM. DNA extracted from environmental samples was diluted 1:200 in LoTE for final concentrations of 4 to 13 ng/μL. Dilutions are necessary for DIB samples to reduce concentrations of DNA or substances such as humic acids which commonly cause inhibition of PCR. DNA extracted from environmental samples also contained the internal

standard pGEM, which was used to correct for variability due to extraction and amplification efficiencies (Coyne et al. 2005). Target cells were enumerated using the comparative Ct method in which a calibrator sample was included to calculate cell numbers. Calibrator samples consisted of DNA extracted from field samples during raphidophyte blooms. Accurate cell counts of the target raphidophyte species in the calibrator samples provided a comparative basis for calculating cell abundances in unknown field samples (Coyne et al. 2005; Handy et al. 2005; Demir et al. 2005) according to the following equations:

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

where

$$\Delta\Delta Ct = (\Delta Ct_{\text{unknown}} - \Delta Ct_{\text{calibrator}}) \quad (2)$$

and

$$\Delta Ct = Ct_{\text{target}} - Ct_{\text{pGEM}} \quad (3)$$

Cell abundances calculated by QPCR for each method (simplex, multiplex, and multiprobe) were then compared to each other. For multiplexing and multiprobing, unless otherwise noted, the Brilliant Multiplex QPCR Master Mix was used.

Comparative Ct method validations—Validation of the comparative Ct method for *H. akashiwo* and *C. subsalsa* has been reported previously (Coyne et al. 2005). The amplification efficiencies of target and reference standard of the calibrator used for *C.* cf *verruculosa* were examined with DNA extracted from environmental water sample during a *C.* cf *verruculosa* bloom. The water sample (BC3T17) was collected on 31 August 2004 from the Delaware Inland Bays and validated for use as a calibrator sample as in Coyne et al. (2005).

Efficiency estimates—Amplification efficiencies were calculated using 10-fold dilutions of the DNA (either plasmid or genomic), using the following equation:

$$E = 10^{(-1/\text{slope})} - 1.$$

Assessment

Secondary structures—All primers and probes were evaluated for secondary structure using the Operon oligo toolkit (http://www.operon.com/oligos/toolkit.php). The ideal sequence affinity is less than 6 base pair complements. When comparing primer and probe combinations that would be used together, the following had a greater than 6-bp complement: Cv probe to itself (8-bp complement), pGEM probe to itself (8-bp complement), and primer Cv1780F to itself (6-bp complement). This does not mean that the primers and probes listed above are unusable but that they may reduce amplification efficiencies in QPCR reactions (Peters et al. 2004). The other combinations of primers and probes used in multiplexing assays described here have fewer than 6 bp complements and should not form primer-dimers.

Probe design—**Choice of quencher.** We compared robustness (measured as the change in fluorescence) and reproducibility of probes synthesized with a FAM reporter and either a nonfluorescent (BHQ; Biosearch Technologies) or fluorescent (TAMRA) quencher. Both probes were purchased from the same company (Integrated DNA Technologies). Using *C. subsalsa* plasmid (0.01 ng/μL) as template in triplicate reactions, the BHQ probe outperformed the TAMRA probe in both reproducibility and robustness (data not shown). In addition, the lower Ct value when using BHQ indicates a greater sensitivity of detection.

Choosing reporter dyes. The ABI 7500 is capable of detecting a range of fluorophores, including FAM (6-carboxyfluorescein, emission spectrum 554 nm), HEX (6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein, emission spectrum 555 nm), TXRD (Texas red, emission spectrum 583 nm), and Cy 5 (CY Dye, emission spectrum 668 nm), which are spectrally distinct enough from each other to use in multiplexed reactions (Boeckman et al. 2003). TXRD was initially used as a reporter dye for the internal standard pGEM but was found to be incompatible with other reporter dyes in multiplex reactions. Each time the TXRD-labeled pGEM probe was combined with any of the other probes, it caused them to fluoresce, even if the target DNA for the other probes was not present during the reaction (data not shown). Because the passive reference dye (ROX) and TXRD have similar emission wavelengths, it is possible that fluorescence from TXRD interfered with ROX detection. A FAM-labeled pGEM probe was then synthesized and found to be compatible with HEX- and CY5-labeled species-specific probes.

Primer/probe specificity—All species-specific primer sets amplified only the species for which they were designed, and the raphidophyte primer set 1 amplified only *C. subsalsa* and *H. akashiwo*. Raphidophyte primer set 2 amplified all DIB raphidophytes. Primers for the internal standard, pGEM, did not amplify PCR products generated by the other plasmids or environmental samples (Coyne et al. 2005). Specificity of the *H. akashiwo*– and *C. subsalsa*–specific primers and probes was demonstrated previously (Coyne et al. 2005).

The probes were also evaluated for specificity against all plasmids used, to be sure they were only targeting 1 species (Table 3). Fluorescence generated in QPCR reactions with species-specific probes occurred only in the presence of the DNA template they were designed for. Positive PCR amplification resulted using the general raphidophyte primer set 2 for each of the *C.* and *H.* isolates (data not shown). QPCR amplification with primer set 2 and species-specific probes, however, resulted in no fluorescence when the *H. akashiwo* probe was used in reactions with DNA extracted from *Chattonella* isolates, nor when the *C. subsalsa* probe was used with DNA extracted from *H. akashiwo*, *C. antiqua*, and *C. marina* isolates (data not shown).

Multiplex validation of 3 targets using plasmids—After optimizing each primer/probe set, accuracy and sensitivity of multiplex reactions were evaluated for *C. subsalsa*, *H. akashiwo*, and *C.* cf *verruculosa.* Initially, equal concentrations of each plasmid were combined (0.01 ng/μL final concentration of each tem-

Table 3. Specificity of primer and probe combinations.

Target 0.1 ng/μL plasmid	Primers used	Probes Ha	Cs	Cv
C. cf. *verruculosa* multiplex	Raph primer set 1			
	Cv 1561F/1780R	–	–	+
C. subsalsa multiplex	Raph primer set 1			
	Cv 1561F/1780R	–	+	–
H. akashiwo multiplex	Raph primer set 1			
	Cv 1561F/1780R	+	–	–
H. akashiwo simplex	Raph primer set 1	+	–	–
C. subsalsa simplex	Raph primer set 1	–	+	–
C. cf. *verruculosa* simplex	Raph primer set 1	–	–	–
C. cf. *verruculosa* simplex	Cv 1561F/Cv 1780R	–	–	+
H. akashiwo simplex	Cv 1561F/ Cv 1780R	–	–	–
C. subsalsa simplex	Cv 1561F/ Cv 1780R	–	–	–

Cv, *C.* cf. *verruculosa*; Cs, *C. subsalsa*; Ha, *H. akashiwo*.

plate). Raphidophyte primer set 1 and Cv primers were used in a multiplex reaction with species-specific probes. Results were compared to simplex reactions using the same primer/probe combinations in separate reactions containing only 1 template. At equal template concentrations, the Ct values obtained in simplex and multiplex reactions were not significantly different, with *P* values of 0.75, 0.39, and 0.33 for *H. akashiwo*, *C. subsalsa,* and *C.* cf *verruculosa*, respectively (Student *t* test: 2-sample, equal variance, unpaired). In addition, the robustness of the reactions (measured by the overall change in fluorescence) for the simplex and multiplexed reactions was not different for *C.* cf *verruculosa* (data not shown) and only marginally different (difference in $\Delta Rn < 5$) for the 2 other species.

Although this result is encouraging, it is not realistic for environmental monitoring since species are rarely in equal abundance. We found that when concentrations of one or more of the target plasmids decreased, the fluorescence was shifted to a higher Ct value or lost altogether compared with the same concentration of template in the simplex reaction. This result could be caused by competition for reagents or difficulties in amplifying one template when others are present in great excess (Wawrik et al. 2002). It would appear that with the technology currently available, accurately quantifying cell abundances for these 3 targets over a range of dilutions may be extremely difficult if not impossible.

Multiplex/multiprobe validations with C. subsalsa and H. akashiwo plasmids—A series of dilution experiments were then conducted to determine the efficiencies of simplex, multiplex (using species-specific primers), and multiprobe (using raphidophyte primer set 1) reactions for *C. subsalsa* and *H. akashiwo*. To accomplish this, the concentration of one template was held constant and the other was varied by a series of 10-fold dilutions (i.e., *C. subsalsa* at 0.01 ng/μL in combination with 10-fold dilutions of *H. akashiwo*). By using plasmids instead of genomic DNA, the copy number of the initial template DNA can be normalized between target species. Multiplex and multiprobe reactions produced similar results. When the dilutions resulted in a 100-fold difference in DNA concentration of the 2 targets, the efficiency of the reaction would diminish (data not shown). Results were the same even if total starting concentration of DNA (for both species) was reduced (data not shown). Amplification efficiencies ($10^{(-1/slope)} - 1$) for *H. akashiwo* were always better than for *C. subsalsa* when they were multiplexed/multiprobed (64% vs. 39% efficiencies as an example).

It has been noted by Becker et al. (2000) that probes which have asymmetric distributions of AT- and GC- rich domains might have a tendency toward a higher degree of base pair mismatching. They recommend that a probe should not target a sequence with an AT-rich section. This phenomenon may cause the *C. subsalsa* (Cs) probe to be less efficient than the *H. akashiwo* (Ha) probe. These two probes are similar in sequence except for a 4-bp segment near the middle of the probe where the Cs probe sequence is CTTT and the Ha probe sequence is ACGA. Although the calculated Tm is the same for both probes, it is possible that the difference in sequence may result in slightly lower probe-target duplex stability for the Cs probe than the Ha probe.

The amplicon size and sequence could also account for the reduced sensitivity and amplification efficiency for the template at lower concentrations. The relatively large length of the amplicons (377 bp) may result in reduced Taq enzyme processivity. For multiplex/multiprobe reactions, this reduction in processivity would be exacerbated for templates at lower concentrations. In addition, the sequences of the amplicons are 97% identical. As a result, the amplicon of the template in higher concentration may be annealing to the template in lower concentration, competing with primers during the annealing step. This would decrease reaction efficiency whether in multiplex or multiprobe conditions.

Raphidophyte primer set 2 was designed to amplify a shorter segment to see if this would increase the efficiency of the multiprobing reactions (data not shown). Whereas the amplification efficiencies for simplex reaction increased with reduction of amplicon size (96% to 100%), the results of multiprobing reactions with primer set 2 were slightly worse than with primer set 1, with amplification efficiencies decreasing from 64% to 60% (data not shown). We concluded that the nearly identical sequences of the *H. akashiwo* and *C. subsalsa* amplicons have a greater impact on amplification efficiency in multiplex/multiprobe reactions than amplicon size.

Multiplex validation with heterogeneous plasmid targets—The primers used for *C.* cf *verruculosa* yield shorter amplicons than the ones used for *C. subsalsa* and *H. akashiwo* (239 bp vs. 377 bp). Because *C.* cf *verruculosa* is phylogenetically distinct from *C. subsalsa* (Bowers et al. 2004), the 18S rDNA sequences for these 2 species are more heterogeneous than *C. subsalsa* and *H. akashiwo.* To test our hypothesis that formation of amplicon heteroduplexes is inhibiting multiplex/multiprobing reactions between *H. akashiwo* and *C. subsalsa*, we compared

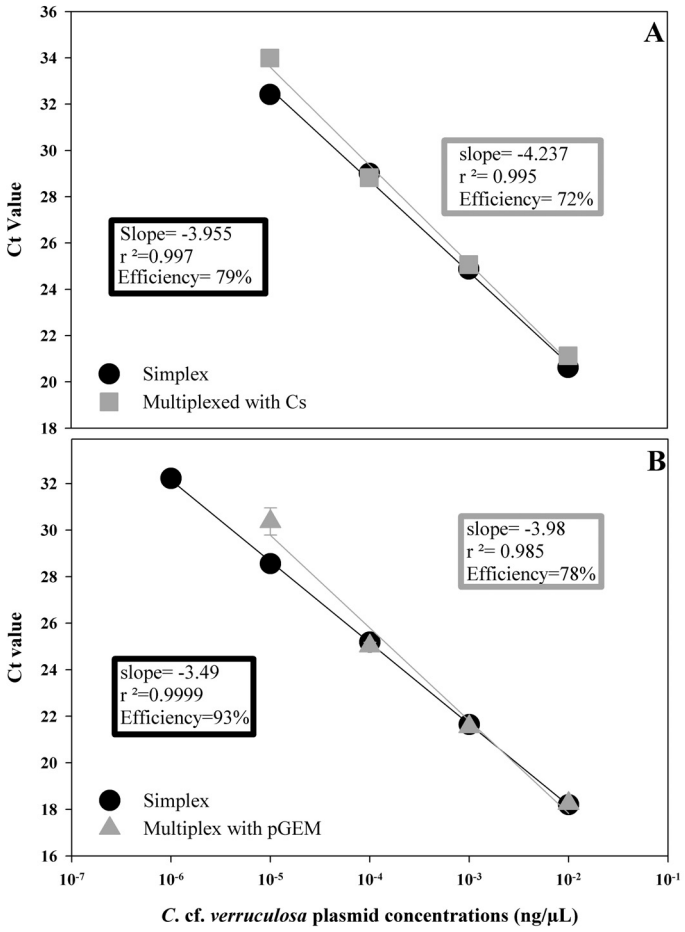

Fig. 1. Comparison of simplexed *C.* cf *verruculosa* using Cv1561F/1708R primers (●) to multiplexed reaction with *C. subsalsa* using raphidophyte prime set 1 (■) (A) or with pGEM internal standard (▲) (B). Error bars are the standard deviation of the Ct value; if not visible, error bars were smaller than symbols.

those results to multiplex reactions of *C.* cf *verruculosa* with *C. subsalsa*. A dilution series of *C.* cf *verruculosa* and *C. subsalsa* plasmids was prepared as described for *C. subsalsa* and *H. akashiwo* (Figure 1A). The amplified insert of *C.* cf *verruculosa* was also multiplexed with the pGEM internal standard plasmid (Figure 1B). The simplexed and multiplexed reactions resulted in nearly identical Ct values over 3 orders of magnitude for the *C.* cf *verruculosa* plasmid (Figure 1A,B). When 10-fold dilutions of *C. subsalsa* were combined with a constant concentration of *C.* cf *verruculosa*, however, the result was the same as with *H. akashiwo*, in which the Ct values were shifted to higher values with a > 100-fold difference in concentration. Here, the results for *C.* cf *verruculosa* suggest that shorter amplicons allow multiplex reactions to proceed with greater efficiency. It is not always possible to design primers that produce short amplicons and are species-specific, but our results demonstrate that these characteristics improve the accuracy of multiplex/multiprobe reactions. In addition, multiplex reactions for species that are genetically distant from each other yield more accurate results.

Validation of multiplexing/multiprobing with environmental samples—Calibrator samples were prepared from environmental samples collected during blooms of *C. subsalsa*, *C.* cf *verruculosa*, and *H. akashiwo*. Each species was enumerated by microscopic cell counts. DNA extracted from these field samples was then used to calculate abundances by QPCR for water samples [see Handy et al. (2005) and Coyne et al. (2005) for review]. It is more appropriate to compare simplexing and multiplexing using calculated cell counts rather than Ct values, because small changes in the Ct value will correspond to exponentially greater differences in calculated cell numbers. A comparison of results indicates the accuracy of the calibrator samples in calculating cell abundances for unknown samples (i.e., whether results using a multiplexed calibrator would be the same as a simplexed calibrator). Three different groups of environmental samples were examined.

Fifteen samples collected from DIB during the summer of 2004 were used to directly compare cell counts calculated by multiprobing vs. simplexing of *C. subsalsa* and *H. akashiwo* (Figures 2 and 3A,B). For phytoplankton populations containing these 2 species of Raphidophytes in the DIB, *C. subsalsa* tends to be the dominant one (K.J.C. , unpublished data). Species-specific primers were used for the simplexing, whereas raphidophyte primer set 1 was used for multiprobing with species-specific probes. In this case, Universal Master Mix was used. The results are presented in both a bar graph (Figure 2) to depict calculated cell counts for both species within each sample, and as a linear regression (Figure 3A,B) of simplex vs. multiprobing results for each species. Figures 2 and 3A,B show that

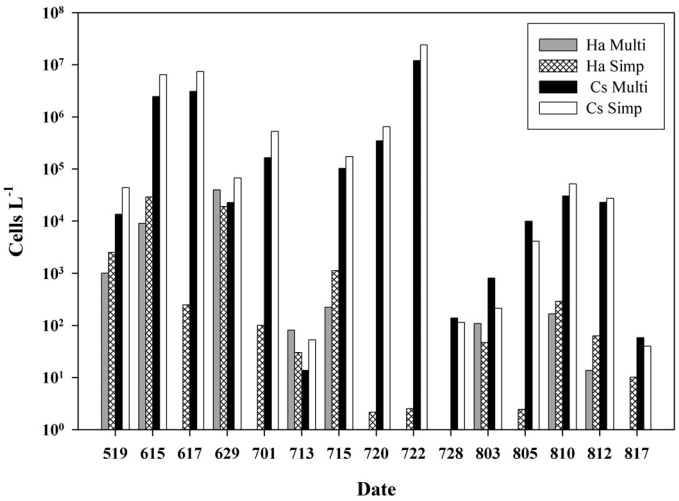

Fig. 2. Comparison of cell abundances determined by simplex (simp) and multiprobe (multi) QPCR analysis of *C. subsalsa* (Cs) and *H. akashiwo* (Ha) in environmental water samples. The black and white bars indicate values obtained for Cs by multiprobe and simplex reactions, respectively, and the light gray and hatched bars indicate the values obtained for Ha by multiprobe and simplex reactions, respectively. Raphidophyte primer set 1 was used for the multiprobe reactions.

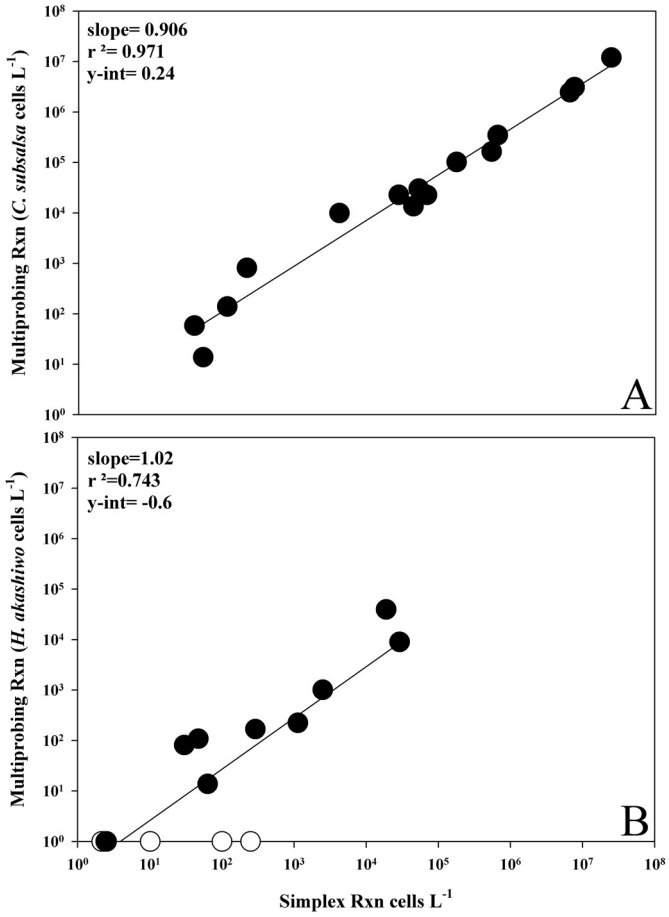

Fig. 3. Linear regression analysis of cell abundances determined by simplex vs. multiprobe reactions for environmental samples containing both *C. subsalsa* (A) and *H. akashiwo* (B). Error bars are the standard deviation of the Ct value; if not visible, error bars were smaller than symbols. ○, samples in which *C. subsalsa* was 1000-fold more abundant than *H. akashiwo*.

QPCR-calculated cell counts for both the simplex and multiprobing reactions were strikingly similar for both species. The only times that the multiprobing resulted in a loss of signal, compared to the simplex reactions, were when the more dominant species (*C. subsalsa* in all of these cases) was 1000-fold greater than the lesser species (*H. akashiwo*; samples 617, 701, 720, 722, and 805) and/or when the less abundant species was present at 10 cells L^{-1} or less (*H. akashiwo*; samples 720, 722, 805, and 817; Figure 2). In each of these cases, *H. akashiwo* was present at concentrations less than 250 cells L^{-1} (calculated by the simplex QPCR results). This level of sensitivity is vastly better than the sensitivity of microscopic cell counts (about 10,000 cells L^{-1}) commonly used by monitoring programs.

Linear regression analysis of the multiprobe versus simplex results shows how closely results from the two methods agree with one another (correlation coefficient), whether there is a trend for one method to overestimate cell abundances (y-intercept) and if there is a density-dependent error in estimating cell abundances (slope). The correlation coefficient for simplex versus multiprobe of *C. subsalsa* (r^2 = 0.96) demonstrates an excellent agreement in calculated cell abundances between this group of samples. The y-intercept was essentially 0 (0.24 cells L^{-1}), and the slope of the line (0.906) indicates that cell densities calculated by multiprobing QPCR will be approximately 10% lower than simplexing (Figure 3A).

Figure 3B illustrates the differences between simplex and multiprobing results for *H. akashiwo*. The y-intercept of –0.6 with a slope of 1.02 suggests that, overall, multiprobe results are comparable to those obtained by simplexing over the range of cell concentrations tested. The correlation coefficient (r^2 = 0.751), however, suggests less agreement between these two methods compared to the results for *C. subsalsa*. This is not surprising, as lower template concentrations tend to lead to greater variability in QPCR results (Peirson et al. 2003; S.M.H., K. Portune, D.A.H., and K.J.C. , unpublished data). Comparing the abundance of *H. akashiwo* (2 to 29,000 cells L^{-1} by simplex results) to *C. subsalsa* (40 to 24,000,000 cells L^{-1} by simplex results), one would expect greater error in combined QPCR results for *H. akashiwo*. By removing the points representing times when cell abundances of *C. subsalsa* were 1000-fold greater than *H. akashiwo* (Figure 3B, ○), the correlation coefficient increases to 0.889 (data not shown).

In spite of the weak agreement between simplex and multiprobing results, amplification of *H. akashiwo* in multiprobe reactions occurred even when it was much less abundant than *C. subsalsa* within the same field sample. These results are surprising compared to those obtained by using plasmid templates in multiprobe reactions. The differences in results between using plasmid and environmental DNA as template may be a result of differences in gene copy number. Currently the copy number of the 18S rRNA gene in these harmful algal species is unknown. It is possible that *H. akashiwo* has a much higher gene copy number than *C. subsalsa*, so that even low numbers of cells are detectable in environmental samples, but not when plasmids of equal copy number are used for multiprobing. Alternatively, the primer specificity may increase with the complexity of the DNA from environmental samples (discussed below).

Six environmental water samples from the DIB containing *C. subsalsa* and *C.* cf *verruculosa* were also examined. DNA extracted from these samples was simplexed, multiplexed, and multiprobed for both *C. subsalsa* and *C.* cf *verruculosa* (Figure 4A-D). For the simplex and multiplex reactions, species-specific primers and probes were used. Raphidophyte primer set 2 was used for the multiprobe reactions. Cell numbers were calculated with the use of calibrator samples. When the calculated cell abundances were compared by linear regression (multiplex or multiprobe vs. simplex), the results were striking (Figure 4A-D). For both species, r^2 values suggest that simplex results agree more closely with results from multiplex reactions than with multiprobing (0.999 vs. 0.867 for *C. subsalsa*, and 0.963 vs. 0.890 for *C.* cf *verruculosa*). For both species, y-intercepts were close to 0, but the slopes of the lines

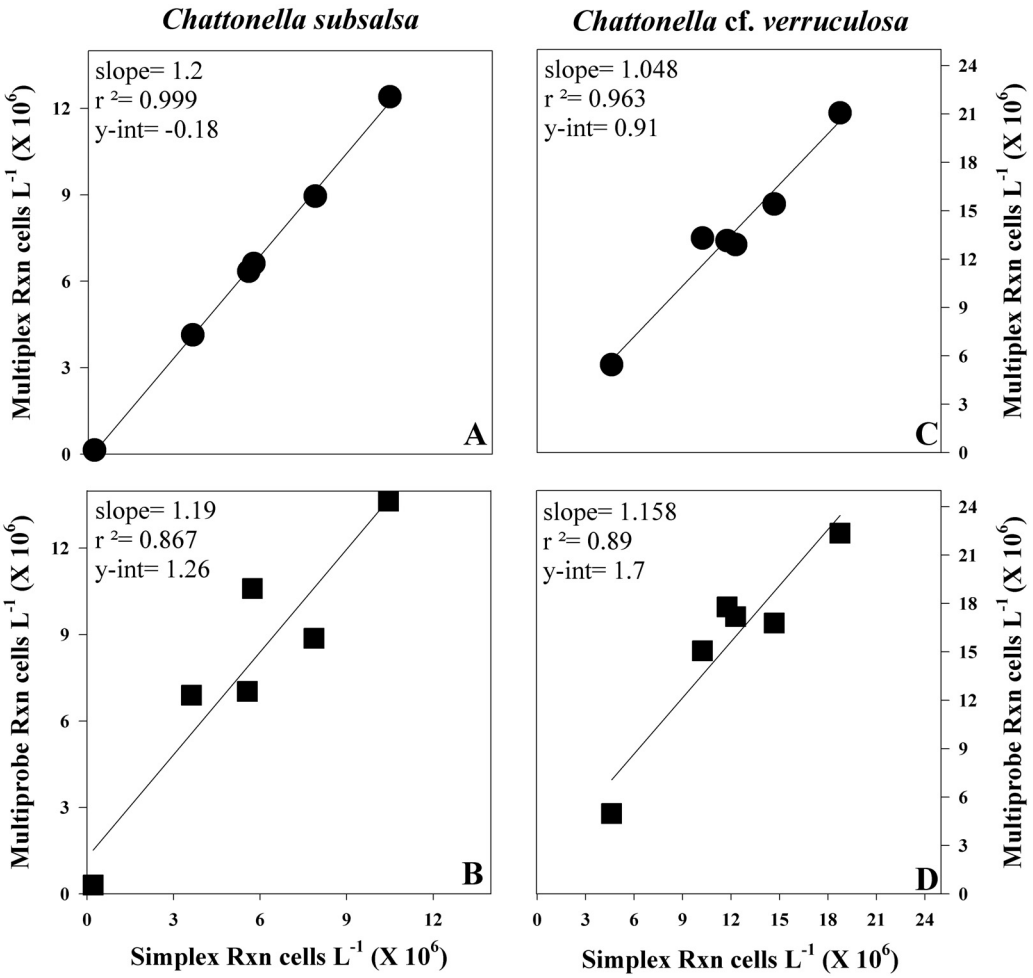

Fig. 4. Comparison of *C. subsalsa* cell abundance in simplex versus multiplex (A) or multiprobe (B) reactions and of *C.* cf *verruculosa* in simplex versus multiplex (C) and multiprobe (D) reactions for environmental samples containing both *C. subsalsa* and *C.* cf *verruculosa*. ●, multiplex reactions; ■, multiprobe reactions.

for each graph indicate some overestimation of cell abundances in multiplex/multiprobe reactions compared to simplex QPCR results.

A final group of 6 environmental water samples was examined to compare simplexing and multiplexing for *C.* cf *verruculosa* and the internal standard pGEM (Figure 5). The slope of the direct comparison of the two methods was 1.02, with $r^2 = 0.96$. These results demonstrate that multiplexing the internal standard and the harmful species *C.* cf *verruculosa* can be achieved in field samples with little to no loss of sensitivity or accuracy.

Multiplex/multiprobe with culture DNA—We wanted to determine whether the favorable multiplex/multiprobe results with DNA from environmental samples were generated because of inherent differences between genomic and plasmid DNA. Genomic DNA was extracted from cultures of *C. subsalsa* (CCMP 2191) and *H. akashiwo* (CCMP 2393). A comparison was done with a starting concentration of 2.5 ng μL^{-1} of genomic DNA for each of the targets. Ten-fold dilutions were then made with either LoTE for simplex reactions or a constant concentration of the other target for multiplex and multiprobe reactions. For the simplex and multiplex reactions, the species-specific primers were used. For the multiprobing reactions, raphidophyte primer set 1 was used. Results using the genomic DNA were similar to the plasmid DNA in that when differences in template concentrations were a 100-fold or greater, the Ct values increased (data not shown). As an example, when *C. subsalsa* genomic DNA was diluted, amplification efficiencies decreased from 82% for simplex to 24% for multiprobe reactions. These data suggest that the greater correlation between simplex and multiplex/multiprobe reactions for *C. subsalsa* and *H. akashiwo* observed in environmental samples, when compared to plasmids, does not seem to be a result of the copy number.

Multiplex/multiprobe of plasmids and genomic DNA with added SHS—We hypothesized that the higher level of DNA complexity found in the environmental samples had a positive effect on the accuracy of the results for multiprobe reactions. To test this, we compared simplex to multiplex/multiprobe results for

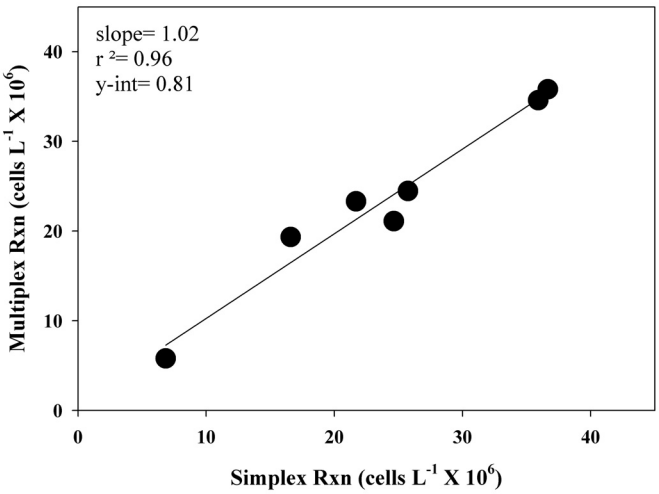

Fig. 5. Comparison of *C.* cf *verruculosa* cell abundances in environmental water samples for simplex vs. multiplex reactions with pGEM.

H. akashiwo and *C. subsalsa* using either plasmid or genomic DNA mixed with an environmentally relevant background level of nontarget DNA (25 ng/μL of SHS DNA). *H. akashiwo* and *C. subsalsa* were chosen because they were difficult to multiprobe with the plasmid DNA alone yet produced more accurate results in the environmental samples. In both simplex and multiprobe reactions, the addition of SHS made no difference in results when using either genomic DNA or plasmid template (data not shown). This would imply that the background level of genomic DNA in environmental samples is not a factor in the improvement we see for the multiplex/multiprobe reactions. It is possible, though, that the increased complexity of DNA extracted from environmental samples may have a positive effect on these reactions.

Discussion

Here, we designed and evaluated QPCR assays for single tube detection of multiple raphidophyte species in the DIB using both multiplexing and multiprobing. We found that 3 DIB raphidophyte species can be accurately detected and enumerated when the templates are at equal concentrations. With plasmid DNA template, however, a reduction in amplification efficiency and sensitivity in multiplex/multiprobe QPCR assays occurs when template concentrations are not equal. This could be a consequence of amplicon size or homology. Large amplicons reduce the efficiency of the reaction (Suzuki and Giovannoni 1996; Vet et al. 1999) owing to reduced enzyme processivity (Kainz 2000) and potential misincorporation of nucleotides during the extension step (Cheng et al 1994). In addition, amplicons from closely related species, such as *H. akashiwo* and *C. subsalsa*, may form heteroduplexes, outcompeting primers during the annealing step. This explanation for reduced amplification efficiency was further supported when raphidophyte primer set 2 did not alleviate the problem, even though the amplicons were shorter and could be more efficiently amplified. It is recommended then, that targets chosen for multiplexing or multiprobing should have primers designed for short amplicons and have heterogeneous sequences.

The problems encountered when using plasmid or genomic DNA as template were not observed for environmental samples. We found that raphidophytes in environmental water samples were accurately detected and enumerated in multiprobing QPCR reactions when cell abundances for the two species are within 3 orders of magnitude. In fact, multiprobing of the environmental samples examined worked very well, with sensitivities much better than the limits of microscopy (the lower limit for these environmental samples was 30 cells L^{-1} for *H. akashiwo*, compared with 10,000 cells L^{-1} with microscopy). Accuracy is only greatly affected when one target is in excess of the other by a factor of 1000 cells or more. During these instances, however, the lesser species was present at very low cell concentrations (< 250 cells L^{-1}). In all environmental samples tested, *C. subsalsa* was always at a higher abundance than *H. akashiwo*, so the amplification efficiencies could not be determined for multiprobing reactions in which *H. akashiwo* is dominant.

Results obtained using DNA extracted from environmental samples demonstrated greater amplification efficiency for multiplexing and multiprobing compared to plasmid templates. This result was surprising, as DNA from environmental samples is typically more difficult to amplify (Bostrom et al. 2004) than DNA from lab cultures or plasmids due to inhibition. Humic acids, for example, may coprecipitate with DNA from field samples and are known to decrease PCR efficiency (Tebbe and Vahjen 1993). Conversely, results of this investigation indicate similar or increased amplification efficiency for all targets investigated in environmental samples. Reasons for this may be the increased level of nontarget DNA in environmental samples such that primer specificity increases with the complexity of the DNA. However, amplification of plasmid and genomic DNA in the presence of sheared herring sperm DNA did not improve multiplex/multiprobe results.

When enumerating cell abundances by QPCR, normalizing differences in extraction and amplification efficiencies to an internal control will provide more accurate results (Coyne et al. 2005). Typically, two separate reactions are required to determine first the Ct values of the target species, and then the Ct values of the internal standard (pGEM). A source of error could be introduced because these assays are performed in different wells (often at different times), and with a different DNA aliquot for each assay. Any variation in pipetting would also affect the results. However, because pGEM has no homology to the raphidophyte DNA targets, our results suggest that the two can be multiplexed from the same DNA aliquot.

Although there have be a few publications on enumerating raphidophyte species by QPCR in simplex reactions (Bowers et al. 2004; Coyne et al. 2005; Handy et al. 2005), there have been no reports of multiplexing of these species or of any harmful algal

species. Using existing methods, we advanced these assays one step further. In addition, we have outlined a protocol and identified limitations of this technique that may be of use to others interested in developing multiplex or multiprobe QPCR assays. This methodology is appropriate for monitoring agencies interested in using QPCR to determine cell abundances for multiple harmful species, or for any investigator interested in efficiently enumerating multiple algal species in a mixed community at a lower cost. The method has been shown to be robust and sensitive for environmental samples targeting harmful DIB raphidophytes as well as the internal standard pGEM. This technology offers a time- and money-saving alternative to simplexing, without losing the sensitivity and specificity of QPCR.

Comments and recommendations

Expenses—Currently the master mix is the most expensive part of QPCR, at approximately $3.70 per sample if the Stratagene Brilliant Multiplex master mix is used. Mixes can be prepared in the lab, but for simplicity and consistency we decided to use a manufactured master mix that had been optimized for multiplexing. Work is also being done in our laboratory to validate the use of smaller reaction volumes (K. Portune and K.J.C. , unpublished data).

Probe design—For the purpose of these experiments, only linear hydrolysis probes were investigated, and only with 4 reporter dyes (FAM, HEX, TXRD, and CY5). There are many different types of probes [see Wong and Medrano (2005) for review] and reporter dyes (for a list see http://www.idtdna.com/Catalog/DNAProbes/Page1.aspx). We found that probes synthesized with CY5 and FAM as the reporter molecule are spectrally well resolved and work well together.

Development of multiplexing and multiprobing techniques—Although multiplexing and multiprobing are powerful tools that could reduce costs and increase accuracy for molecular investigations of specific groups of harmful algae, the development and validation process can be difficult and expensive. The probes and primers must be optimized for each successive target, and, as demonstrated here, the results can be unpredictable. Researchers must use caution when interpreting results that show a large difference in template quantity or very low template concentrations for environmental samples. These assays work well outside the noted constraints. Newer QPCR instruments and probe advancement will no doubt make these methods easier and more efficient in the future (Klein 2002). However, it is our intention here to critically evaluate the capabilities and limitations of this technique with equipment and reagents in common use today so that the protocol outlined in this article may be directly relevant to other investigations of raphidophytes or for those attempting to design multiplexing or multiprobing protocols for other species.

References

Applied Biosystems International. 2003. Sequence Detection Systems Applied Biosystems 7900HT Real Time PCR System and 7300/7500 Real Time PCR System and 7700 Sequence Detection System; GeneAmpR 5700 Sequence Detection System Chemistry Guide, Applied Biosystems, Foster City, CA, USA

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. Envir. Microbiol. 66:4945-4953.

Boeckman, F., K. Hamby, and L. Tan. 2003. The icycler detection system for multiplex real-time PCR assays. Bio-Rad Tech Bull. 2620. Hercules, CA, USA.

Bourdelais, A.J., C. R. Tomas, J. Naar, J. Kubanek, and D. G. Baden. 2002. New fish-killing alga in coastal Delaware produces neurotoxins. Envir. Health Persp. 110:465-470.

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, 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. Envir. Microbiol. 66:4641-4648.

Bowers, H., T. Tengs, S. Goto, C. Thomas, C. Ono, S. Yoshimatsu, and D. Oldach. 2004. Development of real-time PCR assays for the detection of *Chattonella* species in culture and environmental samples. *In*: Steidinger, K., J.H. Landsberg, C. Tomas, G. A. Vargo, Eds. Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, p 231-233.

Cheng, S., C. Fockler, W. M. Barnes, and R. Higuchi. 1994. Effective amplification of long targets from cloned inserts and human genomic DNA. Proc. Nat. Acad. Sci. U. S. A. 91: 5695-5699.

Clegg, R. M. 1992. Fluorescence resonance energy-transfer and nucleic-acids. Methods Enzymol. 211:353-388.

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.

———, and others. 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.

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.

Demir, E., K. J. Coyne, M. A. Doblin, and D. A. Hutchins. 2005. Microzooplankton grazing on *Heterosigma akashiwo* in the Delaware Inland Bays, an application of QRT-PCR technique. *In*: 3rd Symposium on Harmful Algae in the U.S., Monterey, CA.

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.

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, A., 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.

Handy, S. M., and others. 2005. Evaluating vertical migration behavior of harmful raphidophytes in the Delaware Inland Bays utilizing quantitative real-time PCR. Aquat. Microb. Ecol. 40:121-132.

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

Kainz, P. 2000. The PCR plateau phase: towards an understanding of its limitations. Biochim. Biophys. Acta 1494:23-27.

Klein, D., B. Bugl, W. H. Gunzburg, and B. Salmons. 2000. Accurate estimation of transduction efficiency necessitates a multiplex real-time PCR. Gene Ther. 7:458-463.

———. 2002. Quantification using real-time PCR technology: applications and limitations. Trends Mol. Med. 8:257-260.

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.

Munday, B. L., and G. M. Hallegraeff. 1998. Mass mortality of captive southern bluefin tuna (*Thunnus maccoyii*) in April/May 1996 in Boston Bay, South Australia: a complex diagnostic problem. Fish Pathol. 33:343-350.

Ng, C. T., C. A. Gilchrist, A. Lane, S. Roy, R. Haque, and E. R. Houpt. 2005. Multiplex real-time PCR assay using scorpion probes and DNA capture for genotype-specific detection of *Giardia lamblia* on fecal samples. J. Clin. Microbiol. 43:1256-1260.

Odebrecht, C. , and P. C. Abreu. 1995. Raphidophycean in Southern Brazil. Harmful Algal News 12/13:4.

Parkhurst, K. M., and L. J. Parkhurst. 1995a. Donor-acceptor distance distributions in a double-labeled fluorescent oligonucleotide both as a single-strand and in duplexes. Biochemistry 34:293-300.

———, and L. J. Parkhurst. 1995b. Kinetic-studies by fluorescence resonance energy-transfer employing a double-labeled oligonucleotide: hybridization to the oligonucleotide complement and to single-stranded-DNA. Biochemistry 34:285-292.

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. Nucleic Acids Res. 31:73.

Peters, I. R., C. R. Helps, E. J. Hall, and M. J. Day. 2004. Real-time RT-PCR: considerations for efficient and sensitive assay design. J. Immunol. Methods 286:203-217.

Popels, L., and others. 2003. The use of quantitative polymerase chain reaction for the detection and enumeration of the harmful alga *Aureococcus anaphagefferens* in environmental samples along the United States East Coast. Limnol. Oceanogr. Methods 1:92-102.

Richtsteiger, R., C. Henke-Gendo, M. Schmidtke, G. Harste, and A. Heim. 2003. Quantitative multiplex real-time PCR for the sensitive detection of interferon β gene induction and viral suppression of interferon β expression. Cytokine 24:190-200.

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

Suzuki, M., and S. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62:625-630.

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. Envir. 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. *In*: C. R. Tomas, Ed. Identifying Marine Phytoplankton. Academic Press, Harcourt Brace, p. 591-729.

Tyagi S, D.P. Bratu, and F.R. Kramer. 1998. Multicolor molecular beacons for allele discrimination. Nature Biotechnol. 16:49-53.

Vet, J.A.M., and others. 1999. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc. Nat. Acad. Sci. U. S. A. 96:6394-6399.

Walburger, D.K., I. A. Afonina, and R. Wydro. 2001. An improved real time PCR method for simultaneous detection of C282Y and H63D mutations in the HFE gene associated with hereditary hemochromatosis. Mutation Res. Genomics 432:69-78.

Wawrik, B., J. H. Paul, and F. R. Tabita. 2002. Real-time PCR quantification of rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase) mRNA in diatoms and pelagophytes. Appl. Environ. Microbiol. 68:3771-3779.

Whereat, E. D. 2003. Phytoplankton monitoring report. Volunteer Phytoplankton Monitoring Program. University of Delaware, Lewes, DE, USA.

Wong, M.L., and J. F. Medrano. 2005. Real-time PCR for mRNA quantification. Biotechniques 39:75-85.

Yang, S., and others. 2002. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. J. Clin. Microbiol. 40:3449-34.

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