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Development and field assessment of a quantitative PCR for the detection and enumeration of the noxious bloom-former *Anabaena planktonica*

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Abstract

Anabaena planktonica is a harmful, bloom-forming freshwater cyanobacterium, which has arrived recently in New Zealand. In the short time since its incursion (<10 yr), *A. planktonica* has spread rapidly throughout lakes in the North Island. To date, the identification and enumeration of *A. planktonica* has been undertaken using light microscopy. There is an urgent demand for a highly sensitive and specific quantitative detection method that can be combined with a high sample processing capability in order to increase sampling frequency. In this study, we sequenced 36 cyanobacterial 16S rRNA genes (partial), complete intergenic transcribed spacers (ITS), and 23S rRNA genes (partial) of fresh-water cyanobacteria found in New Zealand. The sequences were used to develop an *A. planktonica* specific TaqMan QPCR assay targeting the long ITS1-L and the 5´ terminus of the 23S rRNA gene. The QPCR method was linear (R^2 = 0.999) over seven orders of magnitude with a lower end sensitivity of approximately five *A. planktonica* cells in the presence of exogenous DNA. The quantitative PCR (QPCR) method was used to assess the spatial distribution and seasonal population dynamics of *A. planktonica* from the Lower Karori Reservoir (Wellington, New Zealand) over a five-month period. The QPCR results were compared directly to microscopic cell counts and found to correlate significantly (95% confidence level) under both bloom and non-bloom conditions. The current QPCR assay will be an invaluable tool for routine monitoring programs and in research investigating environmental factors that regulate the population dynamics and the blooming of *A. planktonica*.

The growing number of anthropogenic activities associated with urban, agricultural, and industrial land development has resulted in the eutrophication of numerous fresh-water ecosystems worldwide. The excess of nutrients, in particular phosphorus and nitrogen, can increase the growth rate of phytoplankton resulting in the formation of dense populations (i.e., algae blooms). Algae blooms, in particular cyanobacterial blooms, have been shown to pose environmental and social problems (Vargas-Montero and Freer 2004; Haande et al. 2007; Stone and Bress 2007; Zhang et al. 2007). For example, blooms reduce water quality and the recreational value of aquatic ecosystems (Rahman at al. 2005; Smith and Lester 2006) and numerous cyanobacterial species produce potent toxins, which pose serious health risks to both human and animals (Codd et al. 2005).

A. planktonica is a bloom-forming, noxious cyanobacterial species that has arrived recently in New Zealand and has quickly become the dominant phytoplankton in numerous North Island lakes and rivers (Ryan et al. 2003; Wood et al. 2004). *A. planktonica* first was detected in New Zealand in 2000, however there is evidence that the organism has been present since the late 1990s but misidentified (Wood et al. 2004). During the past 5 yr, *A. planktonica* has spread throughout the North Island (Wood 2005) and, once present in a lake ecosystem, has been shown to dominate the phytoplankton community rapidly (Ryan et al. 2003). *A. planktonica* blooms have affected both the recreational use of waterbodies and drinking water supplies. Recently, *A. planktonica* was implicated in the production of saxitoxins and taste and odor

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compounds in the water supply of more than 960,000 households (Kouzminov et al. 2007). *A. planktonica* appears to have a number of adaptive traits that may account for its dominance and spread throughout the North Island. It is highly buoyant, can "overwinter" in a planktonic vegetative state and moderate populations are maintained even when a water column is well mixed with light availability and temperatures being low (<8°C) (Hicks et al. 2007). To date, little is known about the invasive nature and environmental requirements of *A. planktonica.* Thus, the ability to rapidly and accurately identify and enumerate this species at low (≤10 cells mL^{-1}) and high (≥15,000 cells mL^{-1}) cell concentrations is essential to aid in bloom prediction and management.

Blooms arise because of a complex interaction of chemical, physical, and biological variables including lake stratification and circulation, lake shape and depth, nutrient input, light supply, lake catchment use, and zooplankton densities (Oliver and Ganf 2000). Recently, computer models have been developed, which assist in understanding the complex processes that control lake dynamics (e.g., Hamilton and Schladow 1997; Schladow and Hamilton 1997). Such models can be used in early warning of imminent bloom development and also can help to investigate the responses of cyanobacterial populations to different management strategies. However, these models require validation with chemical, physical, and biological data from the waterbody being investigated. Development of in situ technologies and reporting equipment, e.g., telemetry, has allowed collection of data on numerous chemical and physical variables at frequent intervals. However, the technology to analyze biological data (e.g., differentiation and enumeration of phytoplankton) has not advanced as rapidly and commonly relies on traditional microscopic methods, which are laborious and often cannot differentiate to species level.

Detection of microbial species using molecular techniques allows specificity and sensitivity combined with speed and high sample processing capability (e.g., Coyne et al. 2001). For instance, the ability of quantitative PCR (QPCR) to determine cell abundance by measuring the input copy number of a specific DNA target sequence has resulted in the recognition and acceptance of this technique as a tool for environmental monitoring of a range of cyanobacteria and micro-algae taxa (Kurmayer and Kutzenberger 2003; Popels et al. 2003; Vaitomaa et al. 2003; Coyne et al 2005*a*; Rinta-Kanto et al. 2005).

In this study, a QPCR assay targeting the hyper-variable regions of long intergenic transcribed spacer (ITS1-L) and the 5´-terminus of the 23S rRNA gene was developed in order to identify and enumerate *A. planktonica* rapidly and accurately in environmental water samples. The QPCR has been optimized and validated for data analysis using the comparative C_T method for relative quantification (Livak and Schmittgen 2001). An internal reference standard QPCR was incorporated into the data analysis by adding a known concentration of exogenous plasmid DNA to each sample (Coyne et al. 2005*b*). Unlike absolute quantification, the relative quantification approach has been demonstrated to compensate for methodological bias and sample-to-sample variation in extraction and amplification efficiencies and thus provides more accurate and realistic quantification results (Lebuhn et al. 2004; Coyne et al. 2005*b*). The QPCR for *A. planktonica* was applied to environmental water samples from the Lower Karori Reservoir in Wellington, New Zealand over a five-month period to assess the spatial distribution and seasonal population dynamics during bloom and non-bloom conditions and to compare cell abundances by QPCR to microscopic cell counts. This method will improve the ability to predict and understand the factors contributing to the dominance of *A. planktonica*, and will be valuable in understanding the physiological requirements of this invasive organism.

Materials and procedures

Isolation, culturing, and cell harvesting—Single filaments were isolated by micro-pipetting from lake water samples and were transferred to 24-well plates containing 500 μL MLA medium (Bolch and Blackburn, 1996) per well. The samples were incubated under the following conditions; 100 μEin × m^{-2} × s^{-1}; 12: 12 hour light : dark at 18 ± 1°C (Contherm, BioSyn 6000CP). Successfully isolated strains were maintained in 50 mL plastic bottles (Biolab) under the same conditions as described. Cultures were harvested by centrifugation for 10 min at 16.1*g* with a benchtop centrifuge (Eppendorf 5415R) and the pellet stored at –20°C until processed.

Collection of environmental samples—Twenty-eight phytoplankton samples (500 mL) were collected from the water column of the Lower Karori Reservoir (41°17′S, 174°45′E) between 22 May 2006 and 13 September 2006. Samples were taken from the reservoir surface and three different depths (5, 10, and 15 m) using a Van Dorn sampler. A 40 mL aliquot of each sample was filtered onto GF/C glass microfiber filter (47 mm ∅, Whatman, England) using low-pressure vacuum filtration. Filters were folded once enclosing the filtered cells and stored separately in a sterile plastic bags at –20°C until processed. A 100 mL aliquot of each sample was preserved with Lugol's iodine (10% [w/v] potassium iodide, 5% [w/v] iodine, 10% [v/v] acetic acid) for microscopic cell enumeration.

Microscopic cell enumeration—Microscopic enumeration of environmental samples was carried out using an inverted microscope (CKX41, Olympus) and Utermöhl settling chambers (Utermöhl 1958). After mixing, a sub-sample of 10 mL was pipetted into each Utermöhl chambers and allowed to settle for at least 4 h. *A. planktonica* cells in one central transect were counted at ×400 magnification. If fewer than 150 cells were observed, then transect counts were undertaken at ×200 magnification or cells on the whole chamber floor were counted. Each sample was counted in triplicate. The counting error of this method is approximately ±20% (Hötzel and Croome 1999).

DNA extraction—DNA from environmental samples was extracted using a modified protocol of Kurmayer et al. (2003).

Table 1. Cyanobacteria strains used in this study and analysis of the strains by TaqMan *A. planktonica* QPCR.

Organisms	Culture ID	Location within New Zealand	QPCR
Anabaena planktonica	CYN01	Lower Karori Reservoir	+
Cylindrospermopsis raciborskii	CYN03	Lake Waahi	–
Microcystis sp.	CYN04	Lake Rotorua	–
Microcystis sp.	CYN06	Lake Hakanoa	–
Microcystis sp.	CYN07	Lake Hakanoa	–
Microcystis sp.	CYN09	Lake Horowhenua	–
Anabaena circinalis	CYN13	Lake Rotorua	–
Aphanizomenon issatschenkoi	CYN16	Lake Hakanoa	–
Microcystis panniformis	CYN17	Lake Rotoehu	–
Anabaena planktonica	CYN19	Helensville Dam	+
Anabaena planktonica	CYN20	Helensville Dam	+
Microcystis sp.	CYN21	Lake Pauri	–
Anabaena planktonica	CYN23	Lake Hakanoa	+
Microcystis wesenbergii	CYN26	Lake Hakanoa	–
Microcystis sp.	CYN27	Lake Waahi	–
Anabaena planktonica	CYN28	Lower Karori Reservoir	+
Anabaena sp.	CYN29	Lake Rotoehu	–
Anabaena circinalis	CYN30	Lake Rotoiti	–
Anabaena circinalis	CYN31	Lake Rotoiti	–
Anabaena circinalis	CYN32	Ohau Channel	–
Anabaena circinalis	CYN33	Ohau Channel	–
Microcystis wesenbergii	CYN34	Lake Ngaroto	–
Anabaena planktonica	CYN36	Lake Kainui	+
Anabaena planktonica	CYN37	Lake Kainui	+
Phormidium sp.	CYN38	Red Hills Tarn	–
Anabaena lemmermannii	CYN40	Lake Henly	–
Nondularia sp.	CYN43	Lake Forsyth	–
Phormidium sp.	CYN47	Ashley River	–
Phormidium sp.	CYN48	Ashley River	–

Briefly, filters were cut into pieces (approximately 16 mm^2) with a sterile scalpel and subjected to osmotic shock treatment in 15 mL Falcon tubes (3 mL of 25% [w/v] saccharose, 50 mM Tris-HCl, 100 mM EDTA, pH 8) for 2 h on ice with each sample containing an aliquot of 14 ng of exogenous pGEM plasmid (pGEM-32 Vector, Promega) as an internal extraction control (Coyne et al. 2005*b*). Cells were lysed with lysozyme (5 mg mL^{-1} at 37°C for 1 h) and then incubated with proteinase K (50 μg mL^{-1}) and sodium dodecyl sulfate (2% final sample concentration) at 60°C for 1 h. DNA was extracted with phenol: chloroform (1:1) followed by two extractions using chloroform:isoamyl alcohol (24:1). Centrifugation was performed with a Jouan centrifuge (Jouan CR 4-11,) at 5000*g*. DNA was precipitated with 0.1 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol at –80°C for 1 h. Aliquots of the samples then were transferred into 1.5 mL plastic tubes and DNA collected at 2°C for 45 min at 16.1*g* with a benchtop centrifuge (Eppendorf 5415R). DNA pellets were washed twice with ice chilled 80% ethanol, air dried, and re-suspended in 20 μL of 0.1 × TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). An extraction negative control sample treated in the same way as described but with no added pGEM plasmid and microfilter was included in each experiment.

DNA from laboratory cultures (Table 1) was extracted from pellets using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions.

Determination of 16S rRNA gene and ITS sequences—The 16S rRNA gene and ITS was amplified using primer 338F 5´-ACT CCT ACG GGA GGC AGC-3´ (Muyzer et al. 1993) and a modification of primer 23S30R 5´-CHT CGC CTC TGT GTG CCW AGG T-3´ (Taton et al. 2003). PCR was performed in 25 μL reaction volumes containing approximately 20 ng of DNA, 600 nM of each primer, 0.2 mM dNTPs (Roche Diagnostics), 1 × Taq PCR buffer (Roche Diagnostics), 0.75 U of Taq (Roche Diagnostics), 4 mM $MgCl_2$ (Roche Diagnostics) and 0.8 μg non-acetylated bovine serum albumin (BSA) (Sigma). Thermal cycling conditions were: 94°C for 2 min followed by 19 cycles of 94°C for 45 s; 65°C for 30 s; 72°C for 2 min with annealing temperature being gradually decreased by 0.5°C per cycle, followed by 14 cycles of 94°C for 45 s, 55°C for 30 s, 72°C for 2 min. Final extension was at 72°C for 7 min. PCR reactions were run on a PTC-200 Peltier Thermal Cycler (MJ Research).

The PCR products between 1500 and 3000 bp were excised from a 1.5% agarose gel under ultraviolet (UV) light subsequent to electrophoresis. The excised bands were wrapped with parafilm and frozen at –20°C. A firm and constant pressure was applied to the frozen and wrapped bands using fingers of both hands and the exudate was collected into 1.5 mL Eppendorf tubes. The DNA samples were extracted with phenol, chloroform, and isoamyl alcohol and isopropanol precipitated as described above. Cloning of the PCR products was performed with a TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. Plasmids were extracted following the protocol described in Kotchoni et al. (2003) and bi-directional sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing employed primers T3, T7 (TOPO TA cloning kit, Invitrogen) and 1392R (5´-ACG GGC GGT GTG TRC-3´) (Lane et al. 1985), respectively.

Primers and probe design—The target position for the forward primer and hybridization probe was visually deduced using multiple sequence alignment (ClustalW; Thompson et al. 1994) of 36 novel cyanobacterial 16S rDNA-ITS sequences and sequences of closely related species from GenBank (http://www.ncbi.nlm.nih.gov/). The reverse primer employed a modification of the universal cyanobacterial primer 23S30R (5´-CHT CGC CTC TGT GTG CCW AGG T-3´, Taton et al. 2003). Primers were synthesized by Invitrogen. The TaqMan probe was synthesized with a 6-FAM reporter dye and at the 5´-end a Black Hole Quencher 1 at the 3´-end (Integrated DNA Technology).

Evaluation of QPCR specificity—The specificity of the *A. planktonica* assay was verified by QPCR analysis using our

cyanobacterial culture collection listed in Table 1. Genomic DNA was extracted from the cultures as described above and >5 ng of DNA used for QPCR analysis. A C_T value of 45 (maximum number of PCR cycles) indicated that the tested species was not detected.

Assay sensitivity—The sensitivity of the QPCR was evaluated with genomic DNA extracted from an *A. planktonica* culture (CYN01). Cell enumeration was carried out at ×200 magnification (BX51, Olympus) in a Sedgwick-Rafter chamber according to McAlice (1971). For that purpose, an aliquot of 10 mL was fixed with Lugol's iodine solution and 1 mL transferred into the chamber, allowed to settle for 30 min and ten transverses counted for each sample. Ten mL of the culture were harvested on a GF/C filter and the DNA extracted as described above. Ten-fold serial dilutions were prepared from the extracted DNA and analyzed in triplicate reactions by QPCR analysis. Linear regression was used to assess the sensitivity range of the QPCR assay by plotting the C_T-values over the log transformed cell equivalents.

Quantitative PCR—QPCR for the target and the internal pGEM reference standard was performed in triplicate for each sample in two separate reactions. The primers and probe concentration were optimized on a Rotor Gene 6000 using genomic DNA of *A. planktonica* (CYN01). The optimized assay consisted of a 12.5 mL reaction containing 6 mM $MgCl_2$, 0.2 mM dNTPs (Roche Diagnostics), 1 × PCR reaction buffer, 0.5 units of Platinum *Taq* DNA polymerase (Invitrogen), 450 nM forward primer, 150 nM reverse primer, 200 nM probe, 0.8 µg non-acetylated BSA (Sigma), and 1 µL of DNA template. PCR cycling used the following conditions: 95°C for 2 min and 45 cycles of 95°C for 7 s and 60°C for 40 s.

QPCR amplifying the internal reference standard (pGEM-32, Promega) used 300 nM forward primer M13f (5´-CCC AGT CAC GAC GTT GTA AAA CG-3´), 900 nM reverse primer pGEMr (5´-TGT GTG GAA TTG TGA GCG GA-3´), and 200 nM TaqMan probe (5´-CAC TAT AGA ATA CTC AAG CTT GCA TGC CTG CA-3´) (Coyne et al. 2005*b*). The pGEM TaqMan probe was synthesized with a Cy5-reporter dye at the 5´-end and a Black Hole Quencher 2 at the 3´-end (Integrated DNA Technology). QPCR cycling conditions were as described above.

Data analysis and validation of the comparative C_T method—Data analysis for determination of cell abundance was performed using the comparative C_T method according to Coyne et al. (2005*b*). The comparative C_T method requires that amplification efficiencies ($E = 10^{[-1/slope]}$) of the target gene and pGEM reference standard PCR are near equal (Livak and Schmittgen 2001). Validation of the comparative C_T method for the *A. planktonica* and pGEM assay was performed by QPCR on serial dilutions of filter-extracted DNA from *A. planktonica* (CYN01) in the presence of pGEM reference standard. QPCR amplification of serial DNA dilutions was carried out in triplicate for both assays in separate reactions. The amplification efficiency of both PCR assays was determined from the slope of the regression lines for mean C_T values plotted over the log relative DNA concentrations. Delta C_T ($C_{T[target]}$–$C_{T[pGEM]}$) was calculated for each dilution and plotted over the log relative DNA concentration used for QPCR.

Assessment

Determination of 16S rRNA gene and ITS sequences—Thirty-six 16S rRNA gene (partial; >1100 bp), complete ribosomal ITS and partial 23S rRNA gene sequences (>40 bp) of cyanobacterial cultures and environmental clone libraries were retrieved by sequencing. The sequences of forward and reverse strand were aligned and sequence discrepancies resolved using the electropherograms. Both sequences were assembled into a consensus sequence and compared with sequences in the NCBI GenBank DNA database. The sequences were deposited in the NCBI nucleotide sequence database under the accession numbers listed in Table 2.

Primer and probe development—An *A. planktonica* specific forward primer and hybridization probe was designed from multiple sequence alignments using the 36 new cyanobacterial sequences and those obtained from GenBank. The priming site chosen for forward primer (5´-GGT ATA TTC CTT TTG AAT TTT GCC TTT TGA-3´) is located within the hyper-variable large stem-loop V2 region of the long ITS1-L between the $tRNA^{Ils}$ and $tRNA^{Ala}$ genes (Iteman et al. 2000). This sequence was unique to *A. planktonica* and its specificity also was confirmed by an in silico analysis using NCBI blast for short sequences (http://www.ncbi.nlm.nih.gov/BLAST). The sequence of the hybridization probe (5´-ACA GAC ATG AGA GTG TTT CGT G-3´) targets the variable stem-loop V3 region between the D4 and D5 domain of the ITS1. The primers amplified a 313 bp region of the ITS1-L of *A. planktonica*. The ribosomal operon is present in multiple copies in the genome of prokaryotes and the number of copies can vary between species and strains (Klappenbach et al. 2001). Inconsistent ribosomal operon numbers between strains of *A. planktonica* therefore can induce errors in the determination of cell numbers. On the other hand, multiple quantification markers in the genome increase the sensitivity of the QPCR assay and the ability to detect low cell numbers.

Assay specificity—The specificity of the assay was validated experimentally using QPCR with genomic DNA of the cyanobacterial species listed in Table 1. The QPCR assay showed no cross-reactivity when DNA from non-target species was used as template.

Assay sensitivity—Validation of the QPCR with DNA extracts of known numbers of *A. planktonica* cells demonstrated a high degree of sensitivity and accuracy for the current method. The QPCR had a linear ($R^2 = 0.999$) dynamic range of detection over seven orders of magnitude with a lower limit of detection of five *A. planktonica* cell equivalents, which equates to approximately 25 fg of template DNA for the current assay assuming an average DNA content of 5 fg per bacterial cell (Bakken and Olsen 1989; Fig. 1).

Table 2. NCBI accession numbers of the novel cyanobacterial 16S rRNA gene (partial), intergenic transcribed spacer (complete), and 23S rRNA gene (partial) sequences.

Genera/species	Clone ID	Location within New Zealand	Accession Number
Anabaena planktonica	1–3	Lower Karori Reservoir	EF638707
Anabaena planktonica	1–9	Lower Karori Reservoir	EF638708
Anabaena planktonica	19–1	Helensville Dam	EF638709
Anabaena planktonica	23–10	Lake Hakanoa	EF638710
Anabaena planktonica	H6	Lake Hakanoa	EF638711
Anabaena planktonica	4J–20	Lake Rotoiti	EF638712
Anabaena planktonica	2D–16	Lake Rotoiti	EF638713
Anabaena circinalis	13–3	Lake Rotorua	EF634471
Anabaena circinalis	13–4	Lake Rotorua	EF634472
Anabaena circinalis	33–1	Ohau Channel	EF634473
Anabaena circinalis	33–10	Ohau Channel	EF634474
Anabaena lemmermannii	29–2	Lake Rotoehu	EF634475
Anabaena sp.	Okare–25	Lake Okare	EF634476
Anabaena compacta	4J–13	Lake Rotoiti	EF634477
Anabaena compacta	4J–14	Lake Rotoiti	EF634478
Anabaena compacta	4J–8	Lake Rotoiti	EF634479
Microcystis sp.	6–1	Lake Hakanoa	EF634465
Microcystis sp.	10–7	Lake Horowhenua	EF634466
Microcystis sp.	17–1	Lake Rotoehu	EF634467
Microcystis sp.	21–2	Lake Pauri	EF634468
Microcystis sp.	34–5	Lake Ngaroto	EF634469
Microcystis sp.	26–6	Lake Hakanoa	EF634470
Cylindrospermopsis raciborskii	3–1	Lake Waahi	EF638714
Aphanizomenon issatschenkoi	16–1	Lake Hakanoa	EF638715
Aphanizomenon gracile	SC8–3	Lake Waahi	EF687685
Aphanizomenon sp.	SC4–4	Barnes Dam	EF638716
Nodularia spumigena	Forsyth–N6	Lake Forsyth	EF638717
Synechococcus sp.	19–3	Helensville Dam	EF638719
Synechococcus sp.	2D–18	Lake Rotoiti	EF638718
Synechococcus sp.	Kanui–2	Lake Kanui	EF638720
Synechocystis sp.	2D–15	Lake Rotoiti	EF638721
Coelosphaerium sp.	SC1–1	Okawa Bay	EF638722
Coelosphaerium sp.	SC1–5	Okawa Bay	EF638723
Phormidium autumnale	Hut	Hut River	EF222209
Phormidium murrayi	SC 7–1	Red Hills Tarn	EF638724
Planktothricoides raciborskii	SC 5–2	Lake Rotoehu	EF638725

Validation of the comparative C_T method—The amplification efficiencies for both assays calculated from the slope of the regression lines were ≥1.98 (Fig. 2), indicating near optimal amplification rates, i.e., there is a doubling in the number of target copy sequences during each PCR cycle. Figure 3 demonstrates that the slope of the regression line for ΔC_T ($C_{T[target]}$–$C_{T[pGEM]}$) over the log relative DNA concentrations of template was –0.0368 and within the range of ±0.1, validating the comparative C_T method to enumerate *A. planktonica* in environmental samples.

Cell enumeration of environmental samples—The QPCR method developed in this study was tested for its applicability to quantitatively detect *A. planktonica* in environmental water samples. We monitored the spatial distribution of *A. planktonica* in the water column from the Lower Karori Reservoir over a 5-month period and compared the cell numbers calculated by QPCR to microscopic cell counts. According to microscopy, *A. planktonica* was present in all samples from all depths (i.e., 0, 5, 10, and 15 m depths) with cell numbers varying over five orders of magnitude (Table 3). *A. planktonica* was distributed evenly within the water column, independent of cell abundance or sampling date. The average cell abundance within the water column was highest in May 2006–June 2006 with 12,600 cells mL^{-1}. From the beginning to the end of July 2006, the average cell abundance of *A. planktonica* decreased gradually from 4,695 to 293 cells mL^{-1} and was lowest in August

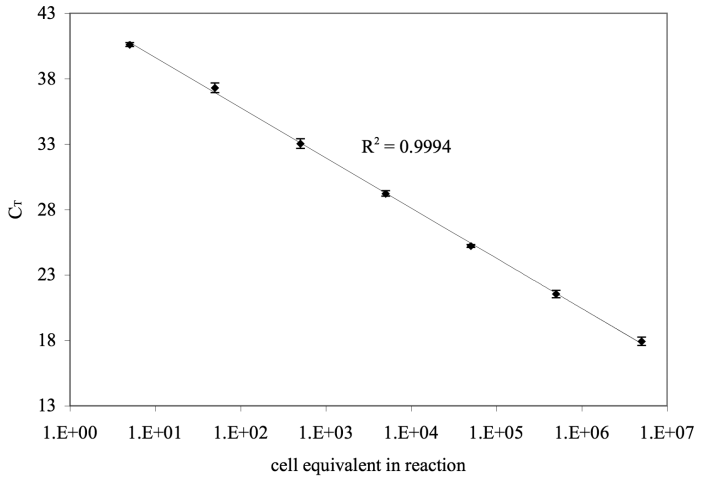

Fig. 1. Sensitivity of quantitative PCR for *A. planktonica*. Dilutions were prepared from genomic DNA and mean values of C_T plotted over the log transformed cell equivalents. Error bars represent standard deviations for three replicates.

2006–September 2006 with 27 cells mL^{-1}. With the surface sample from 03 July 2006 as a calibrator, cell concentrations for environmental samples were determined by QPCR using the comparative C_T method (Table 3). In all but one of the samples, *A. planktonica* was detected with QPCR. Although microscopy identified *A. planktonica* in the 15 m-depth sample from 31 July 2006, we obtained no PCR signal for either the target- or the pGEM assay, indicating that a substantial amount of co-extracted PCR inhibitors was preventing amplification. It is possible that this sample was contaminated with lake sediment during sampling due to the close proximity of sampling depth and lake bottom. The calculated cell concentrations in the Lower Karori Reservoir were highest between May 2006 and June 2006 with on average 9,640 cells mL^{-1}. In July 2006, the month of bloom subsidence, the average cell

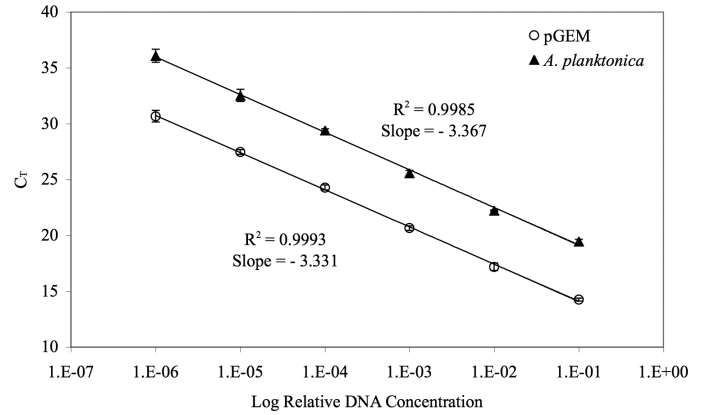

Fig. 2. Quantitative PCR plot of target and internal reference standard QPCR. DNA template employed ten-fold serial diluted filter-extracted *A. planktonica* cells in the presence of pGEM reference standard DNA. Error bars represent standard deviations for three replicates.

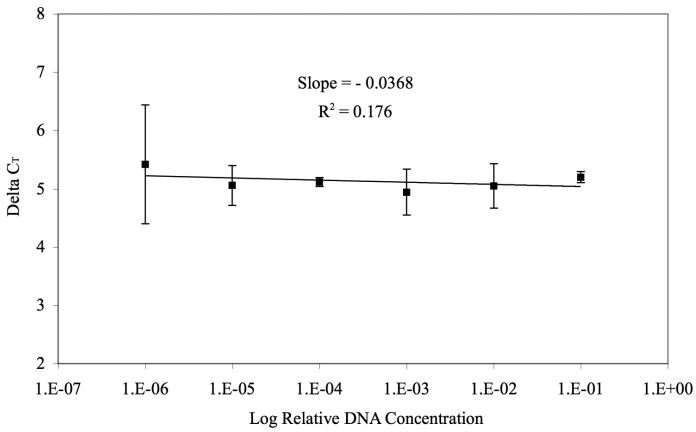

Fig. 3. Validation of the comparative C_T method by calculating ΔC_T ($C_{T,[target]}$–$C_{T,\ [pGEM]}$) for each serial dilution of filter-extracted *A. planktonica* DNA in the presence of the internal reference standard pGEM. Error bars represent standard deviations for three replicates.

concentration within the water column declined from 4,927 to 470 cells mL^{-1} and was lowest in August and September 2006 with on average 46 cells mL^{-1}.

Cell numbers determined by QPCR correlated significantly with the microscopically determined cell numbers. The accuracy of the QPCR results was within the 95% confidence level of the microscopically determined cell concentrations. The error between both methodologies was somewhat higher for the 10 m-depth sample from 3 July 2006 and two of the lower count samples collected on 13 September 2006, i.e., surface- and 15 m-depth sample. The reason for this discrepancy is, at least in part, due to inherent errors in both methodologies. For example, microscopic cell count can vary markedly depending on the subjective decision of the operator undertaking the counting. We experienced up to two-fold variation in estimated cell numbers between operators. Inaccuracies in cell count also can be accentuated when morphologically similar phytoplankton taxa are present due to misidentification of the target organisms. Another possible source of error can occur during pipetting of *A. planktonica*, which is a filamentous organism with cells occurring in long chains of up to approximately 100 cells per filament. Cell concentration in a subsample will vary greatly depending on the number of filaments transferred. This error is likely to be compounded particularly at low cell densities.

QPCR on the other hand can be problematic as the method equates the input copy number of target sequences to cell abundance. The ratio of the copy number of target sequences to actual cell number is not constant and varies depending on the growth phase of the cell population (Ludwig and Schleifer 2000). For instance, the cellular DNA content of a single cell doubles just prior to cell division and a population in the exponential growth phase will produce an earlier C_T-value than a corresponding population in the stationary growth phase. Thus, the comparative C_T method may under- or

Table 3. Comparison of estimated cell numbers to QPCR calculated cell numbers for environmental water from the Lower Karori Reservoir. Filtration of environmental samples used 40 mL.

Sampling date	Sample depth	Cell count [mL^{-1}] (± 95% CL)	Average $C_{T,\ target}$ (± SD)	Average $C_{T,\ pGEM}$ (± SD)	Average $\Delta\Delta C_T$ (± SE)	Average $E^{-\Delta\Delta CT}$ (± SD)	Calculated cells [mL^{-1}] (± SD)	Calculated cells relativeto microscopy
22 May 2006	0 m	1.78×10^4 (6995)	14.49 (0.071)	19.00 (0.164)	–1.303 (0.131)	2.35 (0.199)	1.07×10^4 (905)	0.60
	5 m	1.12×10^4 (2298)	14.56 (0.119)	18.97 (0.085)	–1.200 (0.199)	2.21 (0.292)	1.00×10^4 (1324)	0.89
	10 m	1.57×10^4 (6502)	14.49 (0.047)	18.88 (0.l00)	–1.170 (0.099)	2.15 (0.136)	9.77×10^3 (619)	0.62
	15 m	9.76×10^3 (NA)	14.88 (0.172)	19.04 (0.067)	–0.947 (0.180)	1.87 (0.219)	8.47×10^3 (996)	0.87
06 Jun 2006	0 m	1.14×10^4 (5730)	14.37 (0.136)	18.87 (0.173)	–1.290 (0.161)	2.33 (0.238)	1.06×10^4 (1082)	0.93
	5 m	1.42×10^4 (3214)	15.27 (0.202)	20.13 (0.182)	–1.650 (0.285)	2.98 (0.554)	1.35×10^4 (2514)	0.95
	10 m	1.22×10^4 (NA)	15.01 (0.086)	19.49 (0.375)	–1.273 (0.425)	2.36 (0.649)	1.07×10^4 (2946)	0.88
	15 m	1.13×10^4 (13421)	16.07 (0.189)	19.46 (0.278)	–0.180 (0.428)	1.15 (0.311)	5.23×10^3 (1413)	0.46
19 Jun 2006	0 m	1.01×10^4 (1449)	14.72 (0.035)	18.78 (0.121)	–0.874 (0.122)	2.09 (0.136)	9.48×10^3 (615)	0.93
	5 m	1.28×10^4 (1518)	14.81 (0.197)	19.36 (0.071)	–1.337 (0.130)	2.40 (0.199)	1.09×10^4 (904)	0.85
	10 m	1.13×10^4 (1825)	14.67 (0.081)	19.09 (0.279)	–1.203 (0.357)	2.24 (0.488)	1.01×10^4 (2216)	0.89
	15 m	1.38×10^4 (2635)	15.39 (0.227)	19.58 (0.488)	–0.973 (0.650)	2.01 (0.837)	9.10×10^3 (3800)	0.66
03 Jul 2006	0 m [(a)]	4.54×10^3 (1121)	15.44 (0.101)	18.65 (0.115)	0.000 (0.176)	1.00 (0.112)	4.55×10^3 (507)	1.00
	5 m	6.37×10^3 (2918)	16.30 (0.064)	19.00 (0.090)	0.517 (0.152)	0.72 (0.072)	3.25×10^3 (326)	0.51
	10 m	4.48×10^3 (1682)	14.88 (0.040)	18.77 (0.110)	–0.677 (0.130)	1.56 (0.133)	7.08×10^3 (602)	1.58
	15 m	3.39×10^3 (1536)	15.32 (0.180)	18.59 (0.223)	–0.063 (0.396)	1.06 (0.252)	4.83×10^3 (1146)	1.43
31 Jul 2006	0 m	2.58×10^2 (254)	21.20 (0.059)	21.06 (0.076)	3.347 (0.038)	0.11 (0.003)	5.08×10^2 (13)	1.97
	5 m	3.43×10^2 (252)	20.47 (0.121)	20.41 (0.170)	3.280 (0.050)	0.12 (0.004)	5.31×10^2 (17)	1.55
	10 m	3.34×10^2 (97)	20.71 (0.075)	20.08 (0.194)	3.840 (0.246)	0.08 (0.012)	3.71×10^2 (57)	1.11
	15 m	2.38×10^2 (183)	NA	NA	NA	NA	NA	NA
21 Aug 2006	0 m	1.40×10^1 (6)	23.36 (0.262)	18.54 (0.300)	8.033 (0.235)	0.0053 (0.0008)	2.40×10^1 (4)	1.70
	5 m	8.00×10^0 (4)	25.52 (0.081)	18.26 (1.131)	10.467 (1.185)	0.0013 (0.0010)	6.00×10^0 (4)	0.77
	10 m	4.1×10^1 (55)	24.51 (0.125)	19.13 (0.257)	8.590 (0.296)	0.0037 (0.0007)	1.70×10^1 (3)	0.40
	15 m	1.9×10^1 (20)	29.07 (0.255)	20.50 (1.056)	11.783 (1.223)	0.0006 (0.0004)	2.54×10^0 (2)	0.14
13 Sep 2006	0 m	3.90×10^1 (29)	21.13 (0.388)	18.92 (0.441)	5.430 (0.276)	0.03 (0.005)	1.31×10^2 (24)	3.37
	5 m	5.90×10^1 (55)	22.14 (0.125)	18.70 (0.155)	6.653 (0.252)	0.01 (0.002)	5.90×10^1 (10)	1.00
	10 m	3.00×10^1 (3)	23.01 (0.140)	19.13 (0.365)	7.093 (0.505)	0.01 (0.003)	4.50×10^1 (16)	1.53
	15 m	9.00×10^0 (15)	22.03 (0.074)	19.10 (0.436)	6.140 (0.509)	0.02 (0.006)	8.40×10^1 (25)	9.75

*(a), Calibrator sample; NA, Not available; SD, standard deviation; SE, standard error.

overestimate the actual cell number of an unknown treatment sample if the cell population used to establish the calibrator sample was in a different physiological growth phase. In addition, the presence of free DNA from lysed cells could increase the apparent number of target sequences over the actual cell number and thus contribute to an increasing error in cell enumeration between QPCR and microscopy.

Discussion

The frequency and severity of cyanobacterial blooms is increasing globally and there is a corresponding intensified effort to develop multi-tiered approaches to assist with bloom mitigation, management, and risk assessment (Vargas-Montero and Freer 2004; Haande et al. 2007; Stone and Bress 2007; Zhang et al. 2007). Quantification and accurate identification of problematic species is paramount in any monitoring or research program aimed at predicting or understanding bloom formation. The present microscopy-based methods used for the identification and enumeration of cyanobacterial species require taxonomic expertise and sample processing is both slow and limited by the number of experienced personnel available. PCR is a genetic tool combining high specificity and sensitivity with high sample processing capability and rapid turnaround times.

To date most cyanobacterial specific PCRs have focused primarily on hepatotoxic producing species targeting the microcystin synthetase gene (*mcy*) cluster within microcystin producing genera (Foulds et al. 2002; Kurmayer et al. 2003; Kurmayer and Kutzenberger 2003; Vaitomaa et al. 2003; Rinta-Kanto et al. 2005) or the nodularin synthetase gene (*ndaF*) within *Nodularia* species (Koskenniemi et al. 2007). The intergenic spacer region within the phycocyanin operon (Kurmayer and Kutzenberger 2003) and the evolutionary conserved ribosomal 16S rRNA genes also have been used to detect and enu-

merate *Microcystis* species by QPCR (Rinta-Kanto et al. 2005). While these target sequences have been demonstrated to be useful in detecting cyanobacterial genera, they do not provide sufficient taxonomical resolution to discriminate at species level or beyond. The ribosomal ITS between the 16S rRNA and 23S rRNA genes provides the needed variability in both nucleotide sequence and length and has proven to be useful for detection to species level and beyond for a variety of pro and eukaryotes including cyanobacteria and diatoms (e.g., Jensen et al. 1993; Zechman et al. 1994; Nagpal et al. 1998; Janse et al 2003; Gugger et al. 2005; Créach et al. 2006).

Anabaena spp. are among the most cosmopolitan bloom formers (Paerl et al. 2001). With the exception of one QPCR assay that targets the *mcy*E gene specifically from this genera (Vaitomaa et al. 2003) we are unaware of published QPCR assays focusing on species within this genus. Our objective was to develop a QPCR assay that could detect and enumerate the invasive cyanobacteria *A. planktonica* rapidly and accurately during both bloom and non-bloom conditions. The QPCR assay developed in this study proved to be highly specific for *A. planktonica*, and sensitive over a wide dynamic range with a demonstrated detection limit of approximately five *A. planktonica* cells. The level of sensitivity theoretically can be improved by increasing the volume of sample filtered making the QPCR assay suitable for even extreme low-level cell detection.

QPCR data typically are analyzed absolutely and/or relatively. Absolute quantification employs an internal or external calibration curve to determine the input number of DNA target sequences (expressed in cell equivalents) in an unknown sample. Relative quantification, on the other hand, determines the input number of target sequences (expressed in cell equivalents) in an unknown sample relative to the input number of target sequence from a known number of target organisms in a calibrator sample. This is done after normalizing for the total DNA extraction recoveries from both samples by comparing these values to the DNA quantities of an internal reference standard sequence (measured by a second QPCR) that was added in equal amounts prior to DNA extraction (Livak and Schmittgen 2001). The incorporation of internal controls (either as plasmids or laboratory cultures) in relative quantification also assists in assessing differences in amplification efficiencies. For example, in environmental samples co-purified compounds often can inhibit PCR producing increased C_T values or false negative results. Although absolute quantification usually is considered to be more accurate (Yuan et al. 2006), it does not incorporate controls to verify the accuracy of the results. For example, in the current study, the C_T-values for the internal pGEM reference standard in the environmental samples varied by up to 2.8 C_T-units, highlighting the importance of compensating for methodological bias and thus improving sample-to-sample comparisons. Relative quantification has been recommended for environmental surveys (Brinkman et al. 2003; Lebuhn et al. 2004; Coyne et al. 2005*b*) and was applied in this study to compare cells concentration for *A. planktonica* calculated by QPCR to microscopic cell counts in water samples from the Lower Karori Reservoir.

There was a significant correlation between QPCR and microscopic cell counting for *A. planktonica* under both bloom and non-bloom conditions. In general, cell densities determined with QPCR were within the 95% confidence level of their microscopic counts. The error in *A. planktonica* counts was higher in three samples (out of 28), in particular for two samples containing low cell densities. We postulate that the reasons for this are likely due to the methodological limitations of QPCR and microscopy.

Given the excellent specificity and ability to detect *A. planktonica* over a wide range of cell concentrations, the QPCR assay developed in this study offers numerous benefits over traditional microscopic methods. We envisage that the method will provide scientists and lake managers with a molecular tool allowing for the rapid and accurate identification and enumeration of *A. planktonica* from multiple sampling sites and different time points during bloom initiation. The method has the potential for high-throughput and, in combination with physical and chemical data, this information will be crucial in identifying environmental factors contributing to the dominance of *A. planktonica* in lakes across New Zealand and elsewhere. The knowledge will be valuable for early warning of *A. planktonica* bloom occurrence and in the development of strategies for lake preservation and restoration.

Comments and recommendations

For the purpose of this study, we used our most robust and reliable protocol for DNA extraction. We felt this method would provide the highest quality DNA with the least interference to the development and evaluation of our QPCR assay. However, this extraction method is time-consuming and employs corrosive chemicals that are not amenable to a high-throughput sample processing. We currently are evaluating several commercially available DNA extraction kits in order to develop the method to a higher throughput capacity.

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