

Development of a multiplex real-time qPCR assay for simultaneous enumeration of up to four marine toxic bloom-forming microalgal species



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ABSTRACT

Harmful algal blooms (HAB) pose serious economic and health risks worldwide. Current methods of identification require high levels of taxonomic skill and can be highly time-consuming thus limiting sample throughput. So, new rapid and reliable methods for detection and enumeration of HAB species are required. Here we describe a high-throughput, multiplex-qPCR (M-qPCR) method using hydrolysis probe technology for the simultaneous detection of four HAB species commonly found in many coastal areas worldwide: *Alexandrium tamarense*, *Karenia mikimotoi*, *Karlodinium veneficum* and *Prymnesium parvum*. Primers and probes were species-specific and highly efficient when tested in simplex. Species were then added in succession and the assay conditions adjusted until all four species could be quantitatively evaluated simultaneously. Enumeration accuracy of the M-qPCR assay as a monitoring tool was evaluated using spiked natural environmental samples from Danish coastal waters. Comparison of estimates of cell abundances obtained by the M-qPCR technique with those obtained by light microscopy (Sedgwick Rafter technique) showed no statistically significant difference across a range of concentrations. We were also able to identify and enumerate target cells that would be below the detection limit of light microscopy making this a suitable method for early bloom detection or for low biomass species. With the development of molecular probes for a greater number of algal species M-qPCR will be of great benefit to phytoplankton monitoring programmes and the aquaculture industry worldwide.

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1. Introduction

Over the last few decades there has been an increase in the incidences and problems worldwide associated with harmful algal bloom (HAB) species (Anderson, 1994). Many countries are now being challenged by a large number of toxic or harmful species and their associated effects (Anderson et al., 2002). Early detection is critical due to the array of serious health effects and economic problems associated with HAB (Gowen et al., 2012). Within Europe (EU) marine environmental policy means that EU member states are required under a number of directives to monitor the aquatic environment. The EU Shellfish Hygiene Directive (91/492/EEC) looks for the presence of phycotoxins within shellfish flesh, as well as the causative phytoplankton in water samples. Many operators and resource managers use the published regulatory results to plan their harvesting and develop effective strategies for the

management of HAB in order to minimise any potential risks (Eckford-Soper et al., 2013; Main et al., 2014). The health and economic problems associated with HAB species have resulted in a rapidly advancing monitoring effort that is occurring alongside the development, testing and deployment of new fast and reliable detection methods.

The traditional approach for detecting, identifying and enumerating phytoplankton is by direct observation by light microscopy on preserved material using the Utermöl technique (LeGresley and McDermott, 2010; Utermöl, 1958). This technique can be time consuming and requires a high level of expertise which will often limit sample throughput, thus making it difficult to obtain data in real time (Karlson et al., 2010; Medlin, 2013). Furthermore, use of light microscopy for monitoring HAB species is extremely difficult for species which have a variable morphology, or when they only make up the background component of the phytoplankton community (Main et al., 2014). Fixative induced changes in cell morphology can also be problematic; Lugol's often distorts naked dinoflagellates e.g. *Karlodinium* and *Karenia* making identification next to impossible.

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The increasing number of nucleotide sequences in Genbank in combination with the development of new tools has enabled the use of molecular methods as an early warning detection system. This allows (close to real-time) prediction of the composition of the phytoplankton community before it becomes problematic (Al-Tebrineh et al., 2012; Anderson et al., 2012; Bertozzini et al., 2005). Some of the molecular methods include: fluorescent in situ hybridisation (FISH) (Touzet et al., 2010), fluorescent in situ hybridisation-flow cytometry (FISH-FC) (Eckford-Soper et al., 2013), enzyme-linked immunosorbent assay (ELISA), microarray for the detection of toxic algae (MIDTAL) (Medlin, 2013) and real-time qPCR (Penna and Galluzzi, 2013).

The invention of PCR and qPCR technologies has vastly improved the analysis of nucleic acids from both quantitative and throughput perspectives. qPCR is commonly preferred over traditional microscopic cell counts as it reduces person to person variation, time and ultimately cost. qPCR uses either non-specific inter-calculating fluorescent dyes (SYBR) or species-specific fluorescent probe technology (Taqman) (Schmittgen et al., 2000). qPCR using Taqman hydrolysis probe technology, (henceforth referred to as hydrolysis probes) previously has been used for the detection and quantification of a number of HAB species belonging to: dinoflagellates, diatoms, haptophytes, dictyochophytes and raphidophytes (e.g. Dittami et al., 2013; Handy et al., 2008; Park et al., 2009; Touzet et al., 2010).

Previously, most qPCR methods described have been limited to a single target species. These 'simplex' (S-qPCR) assays utilise a single primer pair and probe that targets just one individual species or genus (Al-Tebrineh et al., 2012). Multiplex-qPCR (M-qPCR) has many advantages over S-qPCR as it allows for the simultaneous amplification of more than one target sequence in a single reaction, not only conserving valuable samples but also increasing sample throughput making it more time and cost effective (Zhong et al., 2011). Many commercial real-time thermocyclers can detect up to four different coloured targets at one time. This is only possible due to the development of hydrolysis probe assays where each target has a specific primer pair as well as a specific probe labelled with a unique fluorescent dye or fluorophore that fluoresce at different wavelengths. The qPCR reader uses the signal from each dye to separately quantify the amount of each target (Handy et al., 2006).

The main goal of multiplexing is to accurately quantify the amount of each target present without interference or competition from non-target DNA or inhibiting chemical compounds i.e. DNA polymerase, dNTPs, buffer and $MgCl_2$. Each assay can inhibit the others through interactions between primers, probes, targets or amplicons. A critical concern in multiplexing reactions is the competition for reagents among the different amplicons, therefore we must optimise the reaction conditions for every assay combination in the multiplex reaction. To achieve a high efficiency (E) either requires the reduction in primer concentrations and/or increasing the concentration of the other components. Often we do not know the concentrations of each target or if the targets are present at the same concentrations. A qPCR assay will be more efficient with a more abundant target, which will use up additional dNTPs leaving fewer for the other targets. This problem can be overcome by making each reaction primer limited, so the primers of the more abundant organism are used up rapidly causing it to plateau quickly leaving plentiful dNTPs for the less abundant targets. When the concentration of the target organisms is unknown, such as in environmental samples, the reaction should also be primer limited (Handy et al., 2006). To primer limit an assay we must determine the optimal primer concentrations by finding the lowest concentration that does not cause an increase in C_q value. This is calculated by running a serial dilution of primer concentrations.

Here we developed a multiplex assay for four HAB species commonly found in coastal waters including the dinoflagellates: *Alexandrium tamarense*, *Karenia mikimotoi* and *Karlodinium veneficum* and a haptophyte *Prymnesium parvum*. Firstly we optimised S-qPCR reaction conditions for each species before adding one target at a time and further optimising conditions until all four could be detected simultaneously in a single reaction. We then compared M-qPCR results for each target with their corresponding S-qPCR reactions over a range of concentrations. To validate its potential use on field populations we tested the accuracy and sensitivity of the assay by using artificially spiked field samples with known concentrations of the four different species. We propose that this approach can be used for developing multiplex assays for additional HAB species.

2. Materials and methods

2.1. Cultures

The following non-axenic microalgal cultures were used: *Alexandrium tamarense* (CCAP 1119/28) isolated from Shetland, Scotland and obtained from the Culture Collection of Algae and Protozoa, Oban, Scotland. Also, *Karenia mikimotoi* (SCCAP K-0260) isolated from Oslofjorden, Norway, *Karlodinium veneficum* (SCCAP K-1661) isolated from Nordhavn, Denmark and the haptophyte *Prymnesium parvum* (SCCAP K-0081) isolated from Flade Sø, The latter three cultures were all obtained from the Scandinavian Culture Collection of Algae and Protozoa, Copenhagen, Denmark. The following: *A. tamarense*, *K. mikimotoi* and *K. veneficum* were grown in L1 media and *P. parvum* in TL10 media at 15 °C under a light intensity of 110 $\mu\text{mol E m}^{-2} \text{s}^{-1}$ and a 16:8 h light:dark cycle.

2.2. Serial dilutions

To determine the performance of the qPCR assays 10-fold serial dilutions of known cell concentrations from cultured material were prepared for the standard curves in triplicates. Concentrations ranged from 10^1 – 10^5 cells for *P. parvum* and *K. veneficum* and 10^1 – 10^4 cells for *A. tamarense* and *K. mikimotoi*. Cells were removed aseptically from exponentially growing cultures and fixed in acidified Lugol's (660 $\mu\text{g I}_2$) before being diluted. The 10^0 and 10^1 samples were obtained by single cell isolations. All samples except for the 10^0 and 10^1 samples were pelleted by centrifugation ($4000 \times g$, 10 min) and the supernatant removed. Cell pellets were then washed in 500 μl PBS buffer, centrifuged ($4000 \times g$, 10 min) and the supernatant removed. Finally cell pellets were stored at -20°C until extraction of total genomic DNA.

2.3. Cell counts

A 1.0 ml aliquot from each dilution was removed and counted using a Sedgewick–Rafter counting chamber (LeGresley and McDermott, 2010) at 100 \times magnification by microscope (Olympus CH-2 CHK-BI45). This cell number was used to estimate the total number of cells within the serial dilutions.

2.4. DNA extraction

For the 10^0 and 10^1 samples DNA was extracted using a freeze-thaw protocol (-80°C for 10 min and room temperature for 10 min) followed by mechanical disruption using bead beating. For all other samples the cell pellets were re-suspended in 10 μl of ddH₂O and then transferred to a reaction tube. This was followed by two further washes using 10 μl of ddH₂O to ensure all cells had been transferred. For all samples, extraction was carried out using

the Powerplant Pro DNA isolation kit (MO BIO, USA) according to manufacturers recommendations with one exception; an aliquot (4 μ l) of Internal DNA extraction control (Primer Design, UK) was added to the lysis buffer prior to extraction. The control was purified along with the sample DNA and used as a positive control of the extraction process. A separate primer and probe provided by Primer Design, UK was used to detect the control DNA. This procedure allowed us to calculate loss (%) from absolute qPCR values generated for the control before and after extraction. Total cell numbers were then reduced by the same percentage for each individual sample. Total elution volume of DNA was 50 μ l. Samples were then stored at -20°C until analysis.

2.5. qPCR

The species-specific qPCR primers and probe sets used in this study for *A. tamarense* and *K. mikimotoi* were developed by Toebe et al. (2013) and Yuan et al. (2012), respectively. Primers and probes for *K. veneficum* and *P. parvum* were designed and developed by Eckford-Soper and Daugbjerg (2015).

Each species was amplified using the primer and probe sequences, modifications and concentrations that are described in Eckford-Soper and Daugbjerg (2015). S-qPCR and M-qPCR conditions were optimised by performing both S-qPCR and M-qPCR assays individually and in tandem. Firstly temperature was adjusted for the CFX96 Touch Real-time PCR detection system (Biorad) and the lowest optimum temperature of 60°C was used. The assay was made primer limited by running a serial dilution of primer concentrations until the lowest concentration that did not cause the C_q value to increase was found. The optimised S-qPCR conditions with 20 μ l reactions were: 4 μ l of HOT FIREPol[®] Probe qPCR Mix Plus (no ROX) ($1 \times$ final concentration) (Solis BioDyne), 2 μ l of template, 0.5 μ l of each appropriate primer, 1 μ l of probe and made up to 20 μ l with ddH₂O. The target species were then added in sequentially and successively optimised conditions for each M-qPCR assay by running a serial dilution of each parameter (MgCl₂, DNA polymerase and dNTPs) to achieve optimal M-qPCR conditions. M-qPCR reagent concentrations are listed in Table 1. Optimisation continued until similar C_q values were achieved for both simplex and multiplex reactions. C_q values varying by more than one cycle between simplex and multiplex reactions were deemed unacceptable. The cycling parameters for all reactions were: 15 min at 95°C for activation of the polymerase, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C .

2.6. Standard curves

Standard curves were obtained by amplifying extracted DNA from the 10-fold serial dilutions of known cell concentrations in

triplicate. For the M-qPCR assay target templates were added in equal concentrations to each individual reaction. qPCR reactions were carried out with a negative control containing ddH₂O only.

2.7. M-qPCR validations using unequal DNA concentrations

We conducted a serial dilution experiment to assess whether the accuracy of the assay is affected when one target is in excess. DNA concentrations of the other templates were kept constant at either 10^4 cells for *A. tamarense* and *K. mikimotoi* or 10^5 cells for *K. veneficum* and *P. parvum*. The species of interest was varied by a tenfold dilution ranging from 10^1 – 10^5 total cell numbers for *P. parvum* and *K. veneficum* and 10^0 – 10^4 total cell numbers for *A. tamarense* and *K. mikimotoi* in triplicate.

The primers and probes sets were then tested against both target and non-target species, which are found to co-occur in European coastal waters. These included other strains and HAB species including the dinoflagellates *Alexandrium tamarense*, group III ribotype *Alexandrium pseudogonyaulax*, *Alexandrium ostenfeldii*, *Alexandrium minutum*, the dictyochophytes *Pseudochattonella farcimen*, *Pseudochattonella verruculosa* and *Dictyocha speculum* and the haptophytes *Prymnesium polylepis* and other *Prymnesium parvum* strains.

2.8. Spiked environmental samples

Seawater samples (1 l) were collected by a ship deployed Niskin bottle from Danish coastal waters (Øresund) in August 2014. Samples were collected between 10 m and 15 m. Samples were collected from three sites: site 1, $56^{\circ} 03.097\text{ N}$, $12^{\circ} 38.969\text{ E}$, site 2, $56^{\circ} 03, 522\text{ N}$, $12^{\circ} 39.662\text{ E}$ and site 3, $56^{\circ} 03.125\text{ N}$, $12^{\circ} 38.446\text{ E}$. In the laboratory the samples were screened through a 100 μ m filter to remove larger zooplankton. Aliquots (200 ml) in triplicate were removed and spiked with known concentrations of all four species in triplicate. As none of our target species were observed at the time of collection samples were spiked with a wide range of cell concentrations. Spiked concentrations were as follows: 5^1 – 1.5^4 total cell number for *A. tamarense* and *K. mikimotoi* and 1^3 – 1.2^5 total cell number for *P. parvum* and *K. veneficum*. Samples were then centrifuged ($5000 \times g$, 10 min), and treated as stated in Section 2.2. The resultant cell pellet was stored at -20°C before DNA extraction. Cell concentrations were calculated by measuring the C_q values and comparing them to the standard curve.

2.9. Statistics

Statistical procedures were carried out using Minitab statistical software. A Student's *t*-test was used on log-transformed data to test the differences between the C_q values. A linear regression analysis was used to assess the differences between the S-qPCR and M-qPCR standard curves. $P < 0.05$ was considered significant and variability was measured by standard error of the mean (S.E.M.).

3. Results

3.1. Optimisation

Temperature and primer and probe concentrations were all optimised separately by running a serial dilution or temperature gradient to obtain optimal conditions for the S-qPCR assay. The optimal annealing temperature for all reactions was 60°C . Species were then added in one at a time and the constituents (MgCl₂, DNA polymerase and dNTPs) of each assay were adjusted individually. Primer and probe concentrations are listed in Table 1 and the M-qPCR assay was based on these conditions. Under these conditions

Table 1
MgCl₂, dNTPs, and DNA polymerase concentrations required for a duplexing, triplexing and quadruplexing assay.

	Concentration
Duplex	
MgCl ₂	6.5 mM
dNTPs	300 μ M
Taq Pol	0.09 U/ μ l
Triplex	
MgCl ₂	7.5 mM
dNTPs	500 μ M
Taq Pol	0.115 U/ μ l
Quadruplex	
MgCl ₂	8.5 mM
dNTPs	600 μ M
Taq Pol	0.115 U/ μ l

each M-qPCR assay (duplex, triplex and quadraplex) achieved comparable results to the S-qPCR assay ($P > 0.05$).

3.2. Specificity and sensitivity

Each primer and probe set was tested against other species and strains. Only the target species were detected in the respective channel with no cross-species reactivity being observed. The fluorescence spectra of the different fluorophores used in the M-qPCR assay were distinct with no spectral overlap in the quantification range. The M-qPCR assay was able to identify each species accurately with a detection level that was comparable to the S-qPCR.

The reproducible limit of detection for cell numbers was 10^0 for *A. tamarensis* and *K. mikimotoi* and 10^1 for *P. parvum* and *K. veneficum* for both the M-qPCR and S-qPCR assays. An internal extraction control was used to assess extraction efficiency of the DNA extraction kit. Percentage loss varied between 8 and 11% and did not vary between species ($P > 0.05$).

3.3. S-qPCR and M-qPCR comparison using standard curves

The R^2 values of all the standard curves were over 0.99. The S-qPCR and M-qPCR reactions resulted in almost identical C_q values over the five orders of magnitude ($P > 0.05$), with the exception of the highest *A. tamarensis* concentration where the C_q value was slightly higher (Fig. 1). To estimate the natural variation and to determine the error between the methods the coefficient of variation (CV) was determined for the replicates for each dilution. The coefficients of variation were low for both methods varying between 0.001 and 0.012% with no difference between the assays. To assess whether the specificity had been altered a linear regression analysis was carried out using Minitab on the standard curves generated by each assay. The results showed that the sensitivity had not been altered ($P > 0.05$).

For all species amplification efficiencies M-qPCR were either similar or higher compared to the S-qPCR assay. Efficiency increased from 84.1% to 87.9% for *A. tamarensis* (Fig. 1a) for M-qPCR and S-qPCR, respectively, while it was similar for *K. mikimotoi* at 90.4% and 91% (Fig. 1b) and *K. veneficum* at 80.2% and 80.1% (Fig. 1c). The efficiency also increased from 83.4% to 89.9% for *P. parvum* (Fig. 1d).

3.4. Multiplex validations

A series of dilution experiments were conducted to determine the efficiencies of the M-qPCR assay when the concentrations of the templates are not equal. One template was held constant while the others were varied tenfold dilution (10^0 – 10^5). The M-qPCR assay provided similar results even with a 10^4 – 10^5 -fold dilution difference in DNA concentration between targets ($P > 0.05$) for all species (Fig. 2). Amplification efficiencies between the S-qPCR and MqPCR assays were similar for *K. mikimotoi* and *K. veneficum* but higher under M-qPCR conditions for *A. tamarensis* and *P. parvum* increasing from 87% to 100.6% and 85.6% to 93.5%, respectively.

3.5. Spiked environmental samples

Fig. 3 compares the spiked cell concentrations determined by light microscopy and by qPCR in natural seawater samples. Over the range of cell concentrations M-qPCR and Sedgwick Rafter counts closely matched. A linear regression analysis carried out in Minitab showed that the two methods did not differ from each other ($P > 0.05$).

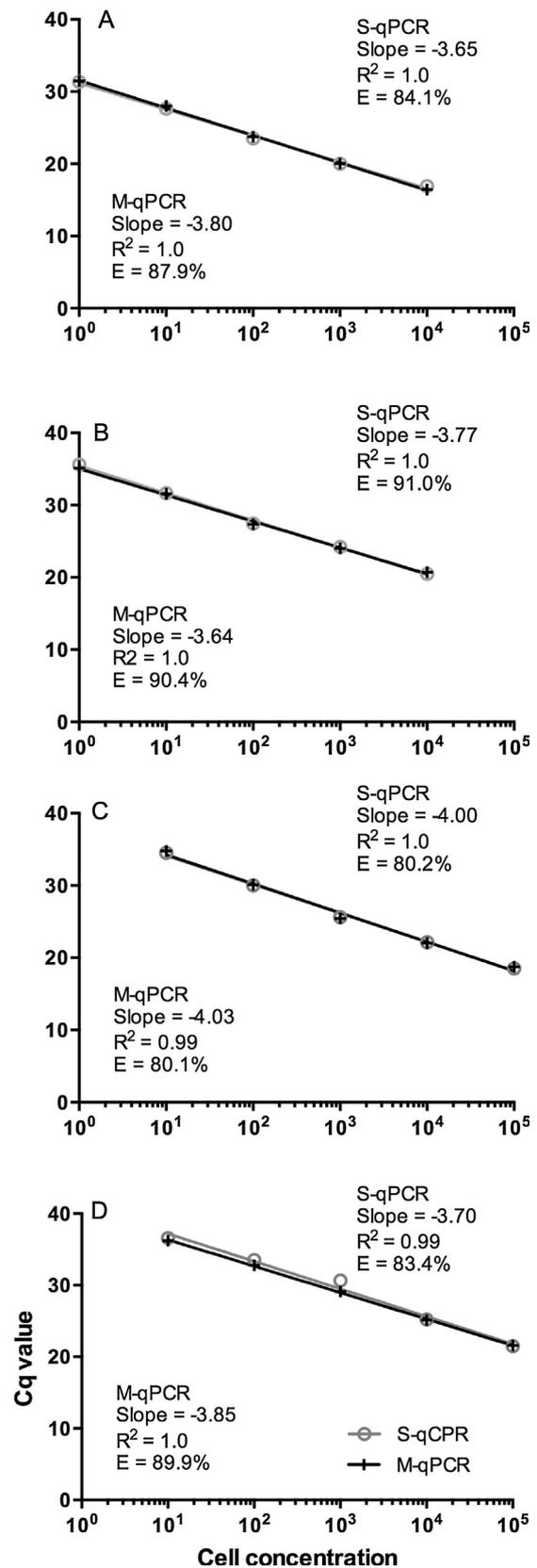


Fig. 1. Comparison of simplex (S-qPCR) and multiplex (M-qPCR) reactions for (a) *A. tamarensis*, (b) *K. mikimotoi*, (c) *K. veneficum* and (d) *P. parvum*. All results are means of triplicate samples. Error bars represent the SEM of the C_q value.

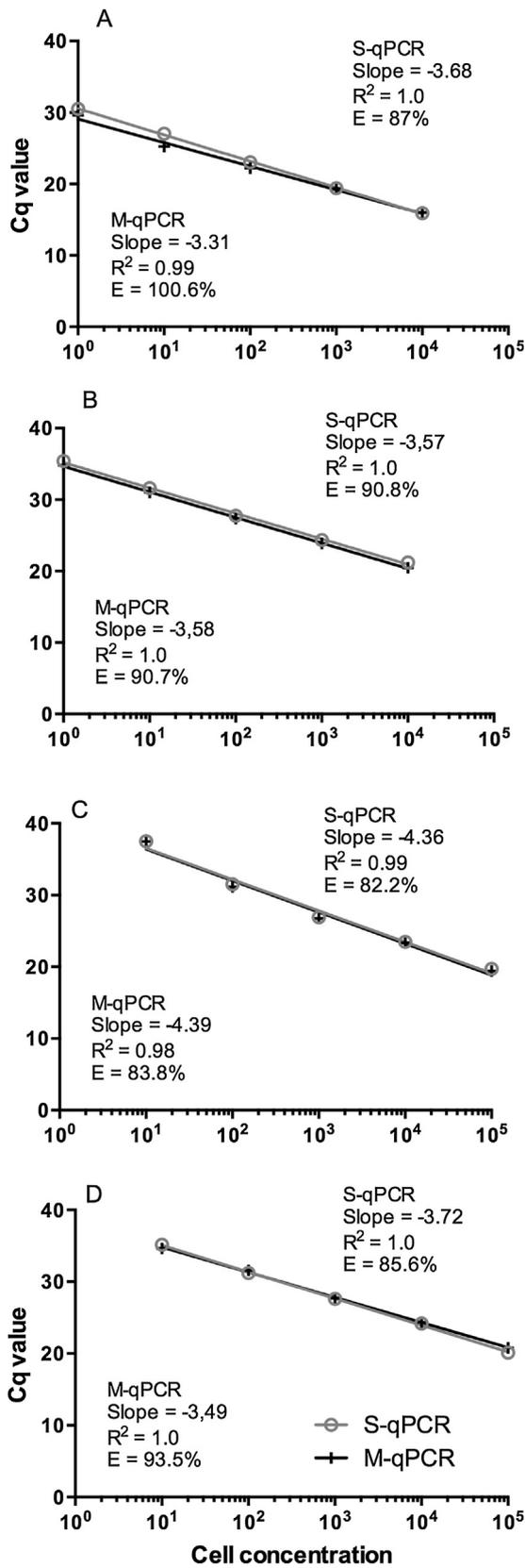


Fig. 2. Comparison of simplex (S-qPCR) and multiplex (M-qPCR) reactions for (a) *A. tamarense*, (b) *K. mikimotoi*, (c) *K. veneficum* and (d) *P. parvum* when DNA concentrations were unequal. DNA concentrations of the other templates were kept constant at either 10⁴ cells for *A. tamarense* and *K. mikimotoi* or 10⁵ cells for *K. veneficum* and *P. parvum* while the others were varied by a tenfold serial dilution series ranging from 10⁰ to 10⁵. All results are means of triplicate samples. Error bars represent the SEM of the C_q value.

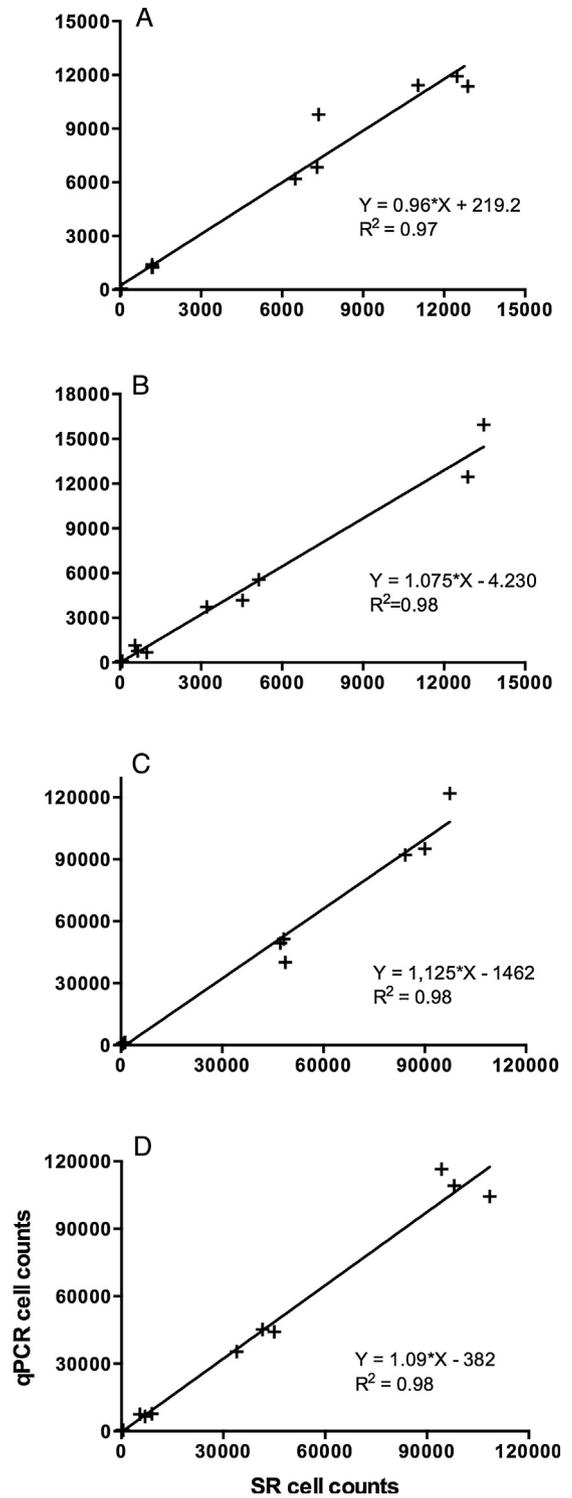


Fig. 3. Comparison of M-qPCR and microscope derived (SR) concentrations of (a) *A. tamarense*, (b) *K. mikimotoi*, (c) *K. veneficum* and (d) *P. parvum* from natural seawater spiked samples.

4. Discussion

Currently researchers and monitoring agencies are looking for new techniques to identify and enumerate harmful algae, as it is often difficult to distinguish between species or populations of interest using light microscopy alone. Rapid evaluation of the distribution and concentration of toxic HAB species is crucial, especially in regions where multi-species bloom populations are known to occur (Al-Tebrineh et al., 2012). qPCR is becoming progressively popular for the identification and quantification of microalgae as the combination of species-specific primers and probes are able to decidedly increase precision, sensitivity and sample throughput. Nevertheless, careful design as well as extensive optimisation of assay conditions and concentrations is still required (Al-Tebrineh et al., 2012). In this study we evaluated both S-qPCR and M-qPCR assays and were able to modify and optimise each protocol for accurately detecting and enumerating up to four marine HAB species simultaneously.

Combining primers and probes for multiple assays change the thermodynamic efficiencies and the chemical kinetics of the reaction. Therefore M-qPCR often requires large amounts of troubleshooting and optimisation (Zhong et al., 2011). During testing fluorescence was only generated in the qPCR reactions when the species of interest was present, demonstrating 100% specificity for their targets with no observed cross reactivity. When the group I *A. tamarensis* primer was tested against a group III ribotype no cross reactivity was observed. Being able to distinguish between toxin and non-toxin producing species is of great importance as it could prevent unnecessary fishery closures in areas where these ribotypes are known to co-occur e.g. Scotland (Bresnan et al., 2008).

Due to competition between primers, we used relatively high concentrations of DNA polymerase compared to the S-qPCR assay (Table 1). Al-Tebrineh et al. (2012) also described a M-qPCR assay that required a high DNA polymerase concentration due to the presence of PCR products amassing in the later cycles inhibiting the DNA polymerase. Overall, the DNA polymerase concentration is related to the number of primer pairs and the amount of amplification of the targets in the assay (Al-Tebrineh et al., 2012).

After optimisation, the efficiency and sensitivity of the simplex and multiplex reactions were evaluated. Results for the multiplex reactions were comparable with the simplex reactions. The C_q values obtained in the simplex and multiplex reactions were not significantly different with the exception of the highest *A. tamarensis* concentration where the C_q value was slightly higher. This value was within 0.4 of a cycle and well within the natural variation of the assay (Karlen et al., 2007).

When comparing the two separate assays the efficiency should not vary more than 5% (Ogino, 2001). The efficiencies of the M-qPCR assay were comparable if not greater than the S-qPCR assay. Commonly multiplexing provides a reduction in the overall efficiency of the assay as seen in Handy et al. (2006), rather than an increase. For the S-qPCR assay a standard commercially purchased qPCR master mix for use with hydrolysis probes was used. Hence, we must assume that the master mix purchased may not have had the optimal concentrations of certain reagents. So, making a custom made mastermix in the laboratory could result in an overall increase in reaction efficiencies.

The detection limits of the four species was analysed by examining DNA extracted from a tenfold serial dilution of pure cultures. Here we observed sensitivities, which are better than the limits set by light microscopy (Penna et al., 2007) with both the S-qPCR and M-qPCR assays being able to detect 1–10 cells of each species with clear signals in any given sample. Light microscopists can encounter a number of different problems due to the large range of cell numbers present in the environment. Many species

such as *A. tamarensis* rarely exceed 10^4 cells l^{-1} and are only found as the background component of the overall phytoplankton community. Rapid identification of some of the smaller species e.g. when using light microscopy *K. veneficum* is difficult as it superficially resembles other small dinoflagellates such as *Pfiesteria shumwaye* which co-occurs in Scandinavian waters (Jakobsen et al., 2002). Even for a highly trained taxonomist, identification down to genus level can be a highly skilled task (Eckford-Soper et al., 2013). The Utermöhl method can enumerate cells with a concentration as low as 20 cells l^{-1} (when using a 50 ml sedimentation chamber) but with reduced precision and reproducibility. In contrast, molecular methods such as qPCR can have a standard deviation that is not affected by target cell concentrations (Godhe et al., 2007). In the past qPCR has been able to accurately quantify cells at concentrations as low as 5 cells per reaction for *K. mikimotoi* (Yuan et al., 2012) and 0.5 cells per reaction *K. veneficum* (Park et al., 2009), 2 cells per reaction for *P. parvum* (Galluzzi et al., 2008) and 1 cell per reaction for *A. tamarensis* (Collins et al., 2009). These detection limits are similar to those found in the present study. This makes qPCR a strong technique for the early detection of harmful algal blooms and allows fish farmers to react quickly to preserve their stocks.

To determine whether the accuracy of the M-qPCR assay was affected when one target is in excess we conducted a series of dilution experiments. While DNA concentrations for the other species were kept constant one species was varied by using tenfold serial dilutions (10^0 – 10^5 cells). C_q values remained similar even when there was a 10^4 – 10^5 -fold difference in DNA concentrations, suggesting that this method is able to identify low-density background species even when they are being swamped by high-density bloom species.

Light microscopic examination of the three spiked natural samples revealed a phytoplankton community with a low species diversity dominated by a few centric diatoms. In all samples *Dactyliosolen fragilissimus* and *Coscinodiscus* sp. were present but *D. fragilissimus* dominated at sites 1 and 2 whereas *Coscinodiscus* spp. was dominant at site 3. Sites 1 and 2 also contained dinoflagellates (*Ceratium tripos*, *Ceratium longipes*, *Protoperidinium* spp. and unidentified thecate and naked dinoflagellates), the silicoflagellate *Dictyocha speculum*, cryptophytes, prasinophytes and unidentified nanoflagellates. The species diversity of site 3 differed slightly as this sample contained a few species not observed at sites 1 and 2 (*Chaetoceros* sp., *Dinophysis acuminata*). Ciliates (e.g. *Mesodinium rubrum*) were also present in all the samples. When the M-qPCR method was tested on the spiked field samples we did not experience the problems associated with unidentified contaminants that have affected other field studies (Toebe et al., 2013). To robustly test this method it should be tested against traditional light microscopy on environmental samples containing natural assemblages of the species of interest. When tested on environmental samples the assay could be complicated by the potential variation in the copy number if the gene of interest changes, either throughout the growth cycle or between different strains. This has been highlighted in *Alexandrium* (Erdner et al., 2010). One way of overcoming this potential problem could be to create the standard curve using a number of strains to generate an average range of the overall copy number (Erdner et al., 2010).

The assay method developed by individually optimising $MgCl_2$, DNA polymerase and dNTP concentrations was also tested and it worked on other strains and species combinations including: *Alexandrium pseudogonyaulax*, *Alexandrium ostenfeldii*, *Alexandrium minutum*, *Pseudochattonella verruculosa*, *Pseudochattonella farcimen* and *Dictyocha speculum*. The *Prymnesium parvum* primer set was tested on 14 different strains with a global distribution (unpublished results).

M-qPCR allows for a large sample throughput and can also quantify samples quickly with a 96 well plate taking 1.5–2 h to complete. A 96 well plate allows for 26 triplicate samples plus the standard curve to be analysed simultaneously. Ideally, a number of water samples could arrive in the morning. These are then processed, their DNA is extracted and analysed by M-qPCR with results by late afternoon. This is in contrast to light microscopy, which can require a 24-h settling time prior to analysis (Eckford-Soper et al., 2013) with each sample taking upwards of 30 min (depending on cell numbers) to count. So, the use of the M-qPCR assay with the development of molecular probes for a greater number of algal species will be of great benefit to phytoplankton monitoring programmes worldwide.

5. Conclusions

We designed and tested a M-qPCR assay that allowed us to simultaneously detect and enumerate up to four coastal HAB species. Due to the thorough optimisation process the assay was highly specific, sensitive and reliable. Based on the results it was revealed that there is great potential for laboratory and field use, especially for the swift evaluation of complex bloom communities. Furthermore this method allows for high throughput, making it rapid and more cost effective compared to traditional light microscopy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.hal.2015.06.009](https://doi.org/10.1016/j.hal.2015.06.009).

References

- Al-Tebrineh, J., Pearson, L.A., Y.S.N.B., 2012. A multiplex qPCR targeting hepato- and neurotoxic cyanobacteria of global significance. *Harmful Algae* 25, 19–25.
- Anderson, D.M., 1994. Red tides. *Sci. Am.* 271, 62–68.
- Anderson, D.M., Cembella, A.D., Hallegraeff, G.M., 2012. Progress in understanding harmful algal blooms: paradigm shifts and new technologies for research, monitoring, and management. *Ann. Rev. Mar. Sci.* 4, 143–176, <http://dx.doi.org/10.1146/annurev-marine-120308-081121>.
- Anderson, D.M., Glibert, P.M., Burkenholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition and consequences. *Estuaries* 704–726.
- Bertozzini, E., Penna, A., Pierboni, E., Bruce, I., Magnani, M., 2005. Development of new procedures for the isolation of phytoplankton DNA from fixed samples. *J. Appl. Phycol.* 17, 223–229.
- Bresnan, E., Turrel, E.A., Fraser, S., 2008. Monitoring PSP toxicity and *Alexandrium* hotspots in Scottish waters.
- Collins, C., Graham, J., Brown, L., Bresnan, E., 2009. Evaluation of quantitative real-time TaqMan PCRs for detection and quantification of *Alexandrium* species in Scottish waters 1–13.
- Dittami, S.M., Riisberg, I., Edvardsen, B., 2013. Molecular probes for the detection and identification of ichthyotoxic marine microalgae of the genus *Pseudochattonella* (Dictyochophyceae, Ochrophyta). *Environ. Sci. Pollut. Res. Int.* 20, 6824–6837, <http://dx.doi.org/10.1007/s11356-012-1402-2>.
- Eckford-Soper, L.K., Daugbjerg, N., 2015. Examination of six commonly used laboratory fixatives in HAB monitoring programs for their use in quantitative PCR based on Taqman probe technology. *Harmful Algae* 42, 52–59, <http://dx.doi.org/10.1016/j.hal.2014.12.007>.
- Eckford-Soper, L.K., Davidson, K., Bresnan, E., 2013. Identification and quantification of toxic and nontoxic strains of the harmful dinoflagellate *Alexandrium tamarense* using fluorescence in situ hybridization and flow cytometry. *Limnol. Oceanogr. Methods* 11, 540–548, <http://dx.doi.org/10.4319/lom.2013.11.540>.
- Erdner, D.L., Percy, L., Keafer, B., Lewis, J., Anderson, D.M., 2010. A quantitative real-time PCR assay for the identification and enumeration of *Alexandrium* cysts in marine sediments. *Deep. Sea. Res. Part 2. Top. Stud. Oceanogr.* 57, 279–287, <http://dx.doi.org/10.1016/j.dsr2.2009.09.006>.
- Galluzzi, L., Bertozzini, E., Penna, A., Perini, F., Pigalarga, A., Graneli, E., Magnani, M., 2008. Detection and quantification of *Prymnesium parvum* (Haptophyceae) by real-time PCR. *Lett. Appl. Microbiol.* 46, 261–266, <http://dx.doi.org/10.1111/j.1472-765X.2007.02294.x>.
- Godhe, A., Cusack, C., Pedersen, J., Andersen, P., Anderson, D.M., Bresnan, E., Cembella, A., Dahl, E., Diercks, S., Elbrächter, M., Edler, L., Galluzzi, L., Gescher, C., Gladstone, M., Karlson, B., Kulis, D., LeGresley, M., Lindahl, O., Marin, R., McDermott, G., Medlin, L.K., Naustvoll, L.-J., Penna, A., Töbe, K., 2007. Intercalibration of classical and molecular techniques for identification of *Alexandrium fundyense* (Dinophyceae) and estimation of cell densities. *Harmful Algae* 6, 56–72, <http://dx.doi.org/10.1016/j.hal.2006.06.002>.
- Gowen, R., Tett, P., Bresnan, E., Davidson, K., McKinney, A., Harrison, P.J., Milligan, S., Mills, D., Silke, J., Crooks, A.-M., 2012. Anthropogenic nutrient enrichment and blooms of harmful phytoplankton. In: Gibson, R.N., Atkinson, R.J.A., Gordon, J.D.M., Hughes, R.N. (Eds.), *Oceanography and Marine Biology an Annual Review*. Taylor & Francis, pp. 65–126.
- Handy, S.M., Demir, E., Hutchins, D.A., Portune, K.J., Whereat, E.B., Hare, C.E., Rose, J.M., Warner, M., Farestad, M., Cary, S.C., Coyne, K.J., 2008. Using quantitative real-time PCR to study competition and community dynamics among Delaware Inland Bays harmful algae in field and laboratory studies. *Harmful Algae* 7, 599–613, <http://dx.doi.org/10.1016/j.hal.2007.12.018>.
- Handy, S.M., Hutchins, D.A., Cary, S.C., Coyne, K.J., 2006. Simultaneous enumeration of multiple raphidophyte species by quantitative real-time PCR: capabilities and limitations. *Limnol. Oceanogr. Methods* 4, 193–204, <http://dx.doi.org/10.4319/lom.2006.4.193>.
- Jakobsen, K.S., Tengs, T., Vatne, A., Bowers, H.A., Oldach, D.W., Burkholder, J.M., Glasgow, H.B., Rublee, P.A., Klaveness, D., 2002. Discovery of the toxic dinoflagellate *Pfiesteria* in northern European waters. *Proc. Biol. Sci.* 269, 211–214, <http://dx.doi.org/10.1098/rspb.2001.1852>.
- Karlen, Y., McNair, A., Perseguers, S., Mazza, C., Mermod, N., 2007. Statistical significance of quantitative PCR. *BMC Bioinform.* 8, 131, <http://dx.doi.org/10.1186/1471-2105-8-131>.
- Karlson, B., Cusack, C., Bresnan, E., 2010. *Intergovernmental Oceanographic Commission of UNESCO. IOC Manuals and Guides no. 55*, pp. 1–114.
- LeGresley, M., McDermott, G., 2010. Counting chamber methods for quantitative phytoplankton analysis – haemocytometer. In: Karlson, B., Cusack, C., Bresnan, E. (Eds.), *Palmer-Maloney Cell and Sedewick-Rafter Cell*. UNESCO (IOC Manuals and Guides), Paris, pp. 25–30.
- Main, C.R., Doll, C., Bianco, C., Greenfield, D.I., Coyne, K.J., 2014. Effects of growth phase, diel cycle and macronutrient stress on the quantification of *Heterosigma akashiwo* using qPCR and SHA. *Harmful Algae* 37, 92–99, <http://dx.doi.org/10.1016/j.hal.2014.05.014>.
- Medlin, L.K., 2013. Molecular tools for monitoring harmful algal blooms. *Environ. Sci. Pollut. Res.* 20, 6683–6685.
- Ogino, S., 2001. A method to compensate for different amplification efficiencies. *J. Mol. Diagn.* 3, 189–190.
- Park, T.-G., Park, Y.-T., Lee, Y., 2009. Development of a SYTO9 based real-time PCR probe for detection and quantification of toxic dinoflagellate *Karlodinium veneficum* (Dinophyceae) in environmental samples. *Phycologia* 48, 32–43, <http://dx.doi.org/10.2216/08-52.1.Conventional>.
- Penna, A., Bertozzini, E., Battocchi, C., Galluzzi, L., Giacobbe, M.G., Vila, M., Garces, E., Lugliè, A., Magnani, M., 2007. Monitoring of HAB species in the Mediterranean Sea through molecular methods. *J. Plankton Res.* 29, 19–38, <http://dx.doi.org/10.1093/plankt/fbl053>.
- Penna, A., Galluzzi, L., 2013. The quantitative real-time PCR applications in the monitoring of marine harmful algal bloom (HAB) species. *Environ. Sci. Pollut. Res. Int.* 20, 6851–6862, <http://dx.doi.org/10.1007/s11356-012-1377-z>.
- Schmittgen, T.D., Zakrajsek, B.A., Mills, A.G., Gorn, V., Singer, M.J., Reed, M.W., 2000. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal. Biochem.* 285, 194–204, <http://dx.doi.org/10.1006/abio.2000.4753>.
- Toebe, K., Alpermann, T.J., Tillmann, U., Krock, B., Cembella, A., John, U., 2013. Molecular discrimination of toxic and non-toxic *Alexandrium* species (Dinophyta) in natural phytoplankton assemblages from the Scottish coast of the North Sea. *Eur. J. Phycol.*, <http://dx.doi.org/10.1080/09670262.2012.752870>, <<http://dx.doi.org/10.1080/09670262.2012.752870>>, hdl:10013/epic.41143.
- Touzet, N., Davidson, K., Pete, R., Flanagan, K., McCoy, G.R., Amzil, Z., Maher, M., Chapelle, A., Raine, R., 2010. Co-occurrence of the West European (Gr.III) and North American (Gr.I) Ribotypes of *Alexandrium tamarense* (Dinophyceae) in Shetland, Scotland. *Protist* 161, 370–384.
- Utermöhl, H., 1958. Zur Vervollkommnung der quantitativen Phytoplankton-Methodik. *Mitt. Int. Ver. Theor. Angew. Limnol.* 1–38.
- Yuan, J., Mi, T., Zhen, Y., Yu, Z., 2012. Development of a rapid detection and quantification method of *Karenia mikimotoi* by real-time quantitative PCR. *Harmful Algae* 17, 83–91, <http://dx.doi.org/10.1016/j.hal.2012.03.004>.
- Zhong, Q., Bhattacharya, S., Kotsopoulos, S., Olson, J., Taly, V., Griffiths, A.D., Link, D.R., Larson, J.W., 2011. Multiplex digital PCR: breaking the one target per color barrier of quantitative PCR. *Lab Chip* 11, 2167–2174, <http://dx.doi.org/10.1039/c1lc20126c>.