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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.

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 fluororphore 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 μmol E m^{-2} 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 μg 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 × g, 10 min) and the supernatant removed. Cell pellets were then washed in 500 μl PBS buffer, centrifuged (4000 × 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× 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 ddH_20 and then transferred to a reaction tube. This was followed by two further washes using 10 μl of ddH_20 to ensure all cells had been transferred. For all samples, extraction was carried out using