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Occurrence of four species of algae in the marine water of Hong Kong

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ABSTRACT

Harmful algal blooms (HABs) have broken out frequently throughout the world in recent decades; they are caused by the rapid multiplication of algal cells in near-coastal waters polluted with nitrogen and phosphorus and greatly affect the quality of marine water and human health. Over the past several decades, climate change and increasing environmental degradation have provided favourable growth conditions for certain phytoplankton species. Therefore, it is essential to rapidly identify and enumerate harmful marine algae to control these species. In this study, quantitative PCR (qPCR) was used to detect four representative species of HABs that are widespread in the marine water of Hong Kong, namely, *Alexandrium catenella*, *Pseudo-nitzschia* spp., *Karenia mikimotoi* and *Heterosigma akashiwo*. We applied qPCR with the dye SYBR Green to detect *Alexandrium* spp. and *Pseudo-nitzschia* spp. and used TaqMan probe for the enumeration of *Karenia mikimotoi* and *Heterosigma akashiwo*. The total genomic DNA of these algae from Hong Kong marine water was extracted successfully using the CTAB method, and for each kind of alga, we constructed a ten-fold series of recombinant plasmid solutions containing certain gene fragments of 18S rDNA and ITS1-5.8S-ITS2 as standard samples. Ten-fold dilutions of the DNA of known numbers of the extracted algal cells were also used to create an additional standard curve. In this way, the relationship between the cell number and the related plasmid copy number was established. The qPCR assay displayed high sensitivity in monitoring marine water samples in which the low concentrations of harmful algae were not detected accurately by traditional methods. The results showed that the cell numbers of the four species were all in low abundance. For *Alexandrium catenella*, the cell abundances at 12 sites ranged from 3.8×10^2 to 4.3×10^3 cells L^{-1}, while *H. akashiwo*, *K. mikimotoi* and *Pseudo-nitzschia* ranged from 1.1×10^2 to 1.3×10^3, from 23 to 6.5×10^2 and from 45 to 3.3×10^3 cells L^{-1}, respectively. The concentrations of these algae were much lower than those observed during outbreaks of HABs in Hong Kong. These results may be useful for local aquaculture development and may provide effective suggestions and a theoretical basis for HAB monitoring and management.

1. Introduction

Harmful algal blooms (HABs), which pose a major threat to aquatic environments and human health (Anderson, 2009; Medlin, 2013), result from the rapid propagation of algal cells under certain marine conditions promoted by increasing water pollution and climate change (Handå et al., 2014). In recent years, HAB outbreaks have occurred frequently around the world, including the seas around the United States (Anderson et al., 2008), Japan (Imai et al., 2006) and Australia (Hallegraeff, 1993), and have resulted in severe economic losses (Bertrand et al., 2013) and ecological damage (Pettersson and Pozdnyakov, 2013). Hong Kong is one of the worst HAB-affected areas in the world, with a high diversity of harmful and toxic algal species. Among over 4000 kinds of microalgae, approximately 330 aquatic organisms worldwide are known to cause red tides, of which >260 kinds of microalgae can result in HABs and >70 can produce biotoxins (Law and Lee, 2013). In Hong Kong, 76 species have been recorded to cause red tides. Of the 76 local species that cause red tides, 19 are recognised as HABs, some of which produce toxins that are harmful to marine organisms (Law and Lee, 2013). Some algal species that cause red tides produce toxins that accumulate in the shellfish that feed on these algae, resulting in shellfish poisoning in human consumers. Common forms of shellfish poisoning include paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP) and amnesic shellfish poisoning (ASP).

Dinoflagellates and diatoms (Bacillariophyceae) are the two most common groups represented in the phytoplankton assemblage in Hong Kong, and many of the known red tide-causative species belong to these two groups. In this study, we detected four kinds of microalgae, all of which are abundant and have caused HABs in Hong Kong. The dinoflagellates of the genus *Alexandrium* are notorious for their production of toxins leading to PSP, which can cause neurological disorders and even death (Anderson, 1997). *Alexandrium* spp. are widely

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distributed in cold temperate coastal waters and have caused HABs in Australia, Japan, the East China Sea and the South China Sea, including Hong Kong. *Karenia mikimotoi* is another well-known dinoflagellate that is globally distributed and can produce and release hemolytic toxins (Zaccaroni and Scaravelli, 2008). *K. mikimotoi* is a common species that causes red tides in Hong Kong which is known to be associated with fish kills. *Pseudo-nitzschia* is a genus of diatom species that is famous for the production of domoic acid, which can cause ASP and threaten both humans and other organisms (Bates et al., 1989). This genus is often observed in the waters of Hong Kong. The last of the four major flagellated algae which were studied herein are *Heterosigma akashiwo* (Raphidophyceae), not belong to the two groups we mentioned above. However, this species has received international attention for causing massive fish kills. Research suggests that *H. akashiwo* may be responsible for these fish kills via gill damage caused by the production of neurotoxins (Hallegraeff, 1993). This species is also a common cause of red tides in Hong Kong.

The traditional methods to identify and enumerate microalgae include light microscopy (LM) and electron microscopy (EM) (Booth, 1993). Although these classical methods are still used today, they have several disadvantages, being time-consuming, laborious and dependent upon taxonomic knowledge and experience. In addition, morphologically similar species can induce mistakes in identification by the operator. Quantitative real-time PCR (qPCR or qRT-PCR) is an extremely powerful and sensitive method for the quantitative detection of microorganisms that has been widely applied in recent decades. Several studies have demonstrated the potential of this method for quantitative analysis of microorganisms. Galluzzi et al. (2004) applied a qPCR assay to rapidly detect the numbers of *Alexandrium minutum* in both fixative-preserved environmental samples and cultures. Murray et al. (2011) developed a qRT-PCR assay to detect and quantify a domain of the gene sxtA, encoding a unique enzyme (sxtA4) putatively involved in the saxitoxin (STX) pathway in marine dinoflagellates. The gene copy number of sxtA4 has been shown to be relatively constant among *Alexandrium catenella* strains, allowing the detection and quantification of blooms of *A. catenella* that lead to STX uptake in oysters. Yuan et al. (2012) used the ITS (including the 5.8S rDNA) region of *K. mikimotoi* to construct plasmids as standard samples for qPCR. The study used two standard curves to establish the linear relationship between the cell numbers and the correlated plasmid copy numbers. Tests using field and laboratory samples then confirmed the effectiveness of the method for the detection of *K. mikimotoi*. Fitzpatrick et al. (2010) developed a qPCR approach for the quantification of the genus *Pseudo-nitzschia* on the East Coast of the United States. They used SYBR Green as a fluorescent dye and added a lysis protocol for cell rupture to improve the DNA yields. Genus-specific primers were designed based on 18S rDNA sequences. However, no species-specific designs were possible, indicating the low level of 18S variability among *Pseudo-nitzschia spp*. Coyne et al. (2005) also used 18S rDNA sequences to generate a standard curve, using plasmid and DNA extraction from cultures with known concentrations of the target species, for the quantification of *Heterosigma akashiwo* in field water based on the TaqMan probe method.

Hong Kong has a long history of HABs. As early as the beginning of the 1970s, formal reports appeared of HABs in Hong Kong. As one of the areas with the most frequent occurrence of HABs, Hong Kong has attracted increasing research attention focused on the study of harmful algae. Lam and Ho (1989) proposed that the increased urbanisation of Hong Kong was the principal reason for the increasing frequency of HABs and related fish-killing events. HABs occur with a higher probability in regions characterised by greater human activity. Yan et al. (2010) counted the frequency of red tide occurrence across the coastal areas of Hong Kong and the Pearl River Estuary. Tolo Harbour was found to have been involved in >70% of the total HAB events, followed by the regions of Mirs Bay and Port Shelter. These three areas are all located to the northeast of Hong Kong. Few studies have reported the algal levels of the central areas and west coast of Hong Kong, though these areas are more affected by human activity and more vulnerable to pollution. HAB appears to be seasonably variable: according to the records, 70% of the HAB outbreaks in Hong Kong occurred during the period from December to May. Although many studies have focused on qualitative and quantitative research into harmful algae, no studies have applied qPCR to environmental samples or summarised the existing methods for the simultaneous detection of several common species in Hong Kong. Therefore, we sampled 12 sites in the central areas and on the west coast of Hong Kong in December, reviewed previous research, and applied the qPCR method to enumerate four common species simultaneously. Our objective was to construct a qPCR method for the rapid and accurate enumeration of representative red tide species in Hong Kong, which would provide a theoretical foundation for HAB monitoring and management, as well as aquaculture development.

2. Materials and methods

2.1. Algal cultures

All of the algal strains used in this study were kindly provided by the South China Sea Institute of Oceanology. The cultures were maintained in f/2 medium at 23 °C ± 1 °C under a light intensity of 60 to 100 μmol photons m^{-2} s^{-1} on a 12-h/12-h dark/light cycle. The numbers of microalgae were counted using an optical microscope at a magnification of 200× (Olympus). Cultures were harvested over 10 days for DNA extraction and the estimation of gene copy numbers.

2.2. Sampling

To examine the numbers of various microalgae in the natural marine water of Hong Kong, we chose 12 sampling sites from the southern (S1–S6) and western coasts (W1–W6), which were all near point sources and hotspots. These sites were all associated with high incidences of red tide and vulnerability to pollution. The sites are shown in Fig. 1. Surface seawater samples (depth 1 to 2 m) were collected by a submersible pump (6 m^3 h^{-1}) at each sampling station. Temperature, salinity and pH were measured simultaneously during the sampling. Each water sample was passed through a 200-μm plankton net to exclude large plankton, and 500-mL samples of the filtered water at every site were extracted into LDPE bottles with a volume of 1 L and kept at 4 °C until arrival in the laboratory.

2.3. DNA preparation

All water samples from the field environment were filtered onto 0.22-μm nitrocellulose filters (Millipore). The filters were submersed into 1 mL of CTAB buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA, 2% [w/v] cetyltrimethylammonium bromide [CTAB], 1% [v/v] β-mercaptoethanol) amended with 20 ng/μL exogenous plasmid DNA (pGEM, Promaga) using for a reference standard (Coyne et al., 2005), then preheated the extraction solution to 55 °C, and the suspended cells were incubated at 55 °C for 1 h with gentle, inverted shaking (20 to 30 times every 10 min). The suspension was then cooled at 4 °C for 3 min, and 1 mL of a mixture of chloroform/isoamyl alcohol (24:1, *v*/v) was added to the suspension and mixed thoroughly by gentle inversion (20 to 30 times) until an emulsion was formed. The samples were centrifuged at 11,000*g* for 10 min at 4 °C. The supernatant (approximately 700 mL) was transferred into a new 2-mL micro-tube, mixed with precooled ethanol (2 times the volume of the suspension) and sodium acetate solution (10% of the suspension volume) and stored at −20 °C for 3 to 4 h. Finally, the DNA precipitate in the micro-tube was rinsed with cold 70% alcohol, dried at room temperature, dissolved in 20 μL of TE buffer and stored at −20 °C for use.

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