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Spatial distribution of toxic *Alexandrium tamiyavanichii* (Dinophyceae) in the southeastern South China Sea-Sulu Sea: A molecular-based assessment using real-time quantitative PCR (qPCR) assay

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ABSTRACT

In this study, a quantitative real-time PCR (qPCR) assay targeting the second internal transcribed spacer (ITS2) of the nuclear-encoded ribosomal RNA gene (rDNA) was developed for *Alexandrium tamiyavanichii*, a harmful tropical marine dinoflagellate. This species is of concern because it produces toxins that cause paralytic shellfish poisoning (PSP). The qPCR assay employed hydrolysis probe technology and showed high specificity, with a detection limit of 10^2 gene copies (less than one cell equivalent). Using this assay, the spatial distribution of *A. tamiyavanichii* was assessed, for the first time, in the southeastern South China Sea and the Sulu Sea. Plankton samples were collected from 71 stations during a scientific cruise from the *Research Vessel Sonne* as part of the joint EU project on Stratosphere ozone: Halogens in a Varying Atmosphere (SHIVA), conducted in November 2011. The highest cell densities were detected offshore of Kuching, southern Borneo (150 cells l^{-1}) and exceeded the threshold level of 20–40 cells l^{-1} where the bioaccumulation of PSP toxins by shellfish is of concern. The distribution of *A. tamiyavanichii* was patchy horizontally with the highest cell concentrations found mainly offshore of southern Borneo, and a heterogeneous vertical distribution was observed above the pycnocline. The *A. tamiyavanichii* qPCR assay proved its applicability, specificity and sensitivity, and provides an alternative implementation tool for harmful microalgae monitoring programs.

1. Introduction

The South China Sea (SCS), located in the equatorial Western Pacific Ocean, one of the world's busiest shipping transition points, is not only ecologically important with its tremendous marine biodiversity and diverse ecosystems, but is also rich in its fisheries resources. The utilization of those resources has expanded rapidly over the past decade, as the Southeast Asian countries surrounding the SCS have experienced the greatest annual increase in harvests of cultured and wild-caught fish worldwide between 2008 and 2012 (SEAFDEC, 2014). The regional investment in fish aquaculture has been particularly intensive and now accounts for a majority of global production (i.e., 53.3% in 2012; SEAFDEC, 2014). The rigorous fish cultivation operation in Southeast Asia, however, has been accompanied by significant ecosystem deterioration (Chua et al., 1989), including coastal eutrophication. The increased number of fish farms compared to a decade ago has contributed to high nutrient levels in the coastal waters. Among the many adverse effects from high nutrient inputs is the promotion of harmful algal blooms (HABs) (Smayda, 1990; Hallegraeff, 1993; Gilbert et al., 2005a; Glibert et al., 2010; Anderson et al., 2008). The blooms produce adverse shifts in phytoplankton species composition, toxins that kill or impair many species and excess biomass that promotes development of hypoxic conditions (Sunda et al., 2006).

Over the past four decades, the region investigated in this study has been particularly susceptible to dinoflagellate blooms that produce paralytic shellfish toxins (PSTs) associated with paralytic shellfish poisoning (PSP) (reviewed in Usup et al., 2012). Until 1970, PSP was confined only to the temperate waters of Europe, North America and Japan (Dale and Yentsch, 1978), but was subsequently dispersed throughout the Southern Hemisphere (Hallegraeff, 1993), including the SCS (Usup et al., 2012). The main source of PSTs in the Southeast Asian waters is the toxic marine dinoflagellate, *Pyrodinium bahamense* var. *compressum*. In

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Malaysia, blooms of this species are prominent along the coast of Sabah, Malaysian Borneo (Lim et al., 2012; Usup et al., 2012). Several *Alexandrium* species are similarly able to produce PSTs. Incidents of PSP related to these *Alexandrium* species were reported in 1991 and 2001 from locations along the Peninsular Malaysia (Usup et al., 2002; Lim et al., 2004). These intoxications led to an intensive investigation of the *Alexandrium* species diversity in this region. Five species of *Alexandrium* were initially identified (*A. affine*, *A. leei*, *A. minutum*, *A. tamarense*, *A. tamiyavanichii*); *A. minutum* and *A. tamiyavanichii* were found to be highly toxic (Usup et al., 2002; Lim et al., 2006, 2007; Hii et al., 2012). Two weakly toxic species, *A. peruvianum* and *A. taylori*, were subsequently discovered off the coast of Sarawak, Malaysian Borneo (Lim and Ogata, 2005; Lim et al., 2005). Another PSP toxin-producing dinoflagellate species, *Gymnodinium catenatum* is also present (Adam et al., 2011).

This study focused on characterizing the distribution of *Alexandrium tamiyavanichii*. Moreover, no information is available on the spatial variability and abundance of *A. tamiyavanichii* in the SCS and adjacent Sulu Sea (SS). This species is widely distributed in tropical and temperate waters and has been reported in the Gulf of Thailand (Kodama et al., 1988), the Philippines (Furio and Gonzales, 2002; Bajarias et al., 2003; Montojo et al., 2003), Malaysia (Usup et al., 2002; Lim et al., 2006, 2007), Japan (Ogata et al., 1990; Nagai et al., 2003; Kim and Sako, 2005; Beppu et al., 2008; Oh et al., 2009), and northeastern Brazil (Menezes et al., 2010). It has also been observed offshore of east Peninsular Malaysia and Sarawak, Malaysian Borneo (Boonyapiwat, 1999a,b). Relatively little is known about the larger scale biogeography of this species in the SCS and SS. This is of concern because human intoxication could occur even at very low cell densities due to high intracellular toxin concentrations. Work with the Malaysian *A. tamiyavanichii* strain AcMS01 isolated from Sebatu (Strait of Malacca) showed that cellular PSTs content can exceed 180 fmol PSTs cell^{-1} (Lim et al., 2006). Similarly, specific toxicities of up to 100×10^{-6} MU cell^{-1} were reported from Japanese isolates (Beppu et al., 2008). Hence, the presence of toxic *Alexandrium* spp. in the water column of as low as 20–40 cells l^{-1} 1 is enough to pose a trigger warning (e.g., Food Standard Agency, UK; Sea Fisheries Protection Authority).

Cell counts alone, however, do not accurately assess the actual risk of PSP because toxic species frequently co-occur with morphologically similar non-toxic *Alexandrium* species such as *A. leei* and *A. affine* (Usup et al., 2002). Conventional taxonomic discrimination of *Alexandrium* species is based on detailed microscopic observation of the thecal plate characteristics (e.g., Balech, 1995; Lim et al., 2007), which can be examined by staining the thecal plates with iodine solution (Imamura and Fukuyo, 1987) or fluorescein calcoflour white (Fritz and Triemer, 1985) and examining the cells under light or epi-fluorescence microscopes; nevertheless, the morphological characters are so subtle that it is notoriously difficult and challenging to differentiate and enumerate species using microscope. Furthermore, both cultured and environmental samples of *Alexandrium* species often possessed a degree of morphological plasticity. This has been demonstrated for numerous species, including *A. tamiyavanichii* (Lim et al., 2007), *A. ostenfeldii – A. peruvianum* (Kremp et al., 2014) and the *A. tamarense* complex (Anderson et al., 1994; John et al., 2014). This inability to readily distinguish *Alexandrium* species using conventional methods has driven the need to develop a rapid, sensitive and effective tool to detect and quantify *A. tamiyavanichii* in the region.

Molecular-based species-specific assays have been widely explored as alternatives to laborious traditional microscopic techniques. These assays include molecular probes coupled with fluorescence in situ hybridization (e.g., Sako et al., 2004; Kim and Sako, 2005), the multiplex PCR assay (Nagai, 2011), and taxa-specific quantitative, real-time PCR (qPCR). The latter method is the most extensively used because of its specificity, sensitivity and high throughput in detecting and quantifying the nucleic acids found in target cells. The method has been used to monitor various harmful dinoflagellate species, including *Alexandrium minutum* (Galluzzi et al., 2004), *Alexandrium tamarense* and *Alexandrium catenella* (Hosoi-Tanabe and Sako, 2005), *Alexandrium fundyense* (Dyhrman et al., 2006), *A. catenella* and *Alexandrium taylori* (Galluzzi et al., 2010), *Cochlodinium polykrikoides* (Howard et al., 2012; Park et al., 2014), *Gambierdiscus* spp. (Vandersea et al., 2012), *Ostreopsis* cf. *ovata* (Perini et al., 2011; Hariganeya et al., 2013), the diatom *Pseudo-nitzschia* spp. (Fitzpatrick et al., 2010) and harmful raphydophytes, *Chattonella subsalsa* and *Heterosigma akashiwo* (Coyne et al., 2005; Park et al., 2012).

To date, there is no qPCR assay developed for the toxic *Alexandrium tamiyavanichii*. Here, we describe a hydrolysis probe-based real-time quantitative PCR (qPCR) assay for the quantification of *A. tamiyavanichii* cells. This high-precision molecular method was then used to investigate the spatial dynamics of this toxic dinoflagellate in the southeastern SCS, including the waters offshore of Malaysian Borneo, and in the SS. The study involved the development of a *A. tamiyavanichii* species-specific qPCR assay, and validation of the practical application using environmental samples. This study provides the first insight into the spatial variability and vertical distribution of this toxic species in the region.

2. Materials and methods

2.1. Algal cultures

Clonal cultures of dinoflagellates used in this study (Supplementary material Table S1) were grown in test tubes containing 25 ml of sterile ES-DK medium (Kokinos and Anderson, 1995) with a pH of 7.8–8.0 and a salinity of 30. Cultures were maintained at 25 ± 0.5 °C under a 12:12-h light:dark photoperiod in a temperature-light controlled growth chamber (SHEL LAB, Oregon, USA), illuminated by cool-white fluorescent bulbs with light intensity of 100 μmol photons m^{-2} s^{-1}.

A strain of *Alexandrium tamiyavanichii* (AcSM01) was established from Samariang, Sarawak, Malaysian Borneo (1.6092°N, 110.3244°E) and used in this study. The species identity was morphologically confirmed by microscopic observation in conjunction with the DNA amplifications and sequencing of the large subunit (LSU) ribosomal DNA and the internal transcribed spacer (ITS) (Leaw et al., 2005, 2010). Microscopic species identification was performed by calcoflour white staining on the thecal plates of dinoflagellates (Usup et al., 2002; Lim et al., 2007), and subsequently observed under an Olympus IX51 inverted research microscope (Olympus, Tokyo, Japan), equipped with a mercury lamp and a UV filter set, at 400–1000× magnification. The distinctive morphological features of *A. tamiyavanichii* are readily observed in the strain AcSM01 (Fig. 1). These features included an oblique posterior margin of the first apical plate (1′), a triangular to trapezoid-shaped of precingular part (p.pr.) of the anterior sulcal plate (s.a.) (Fig. 1A), and a longer than wide posterior sulcal plate (s.p.) (Fig. 1B). The strain forms long chains of up to 30 cells. The strain is confirmed toxic, with GTX1–5, STX, dcSTX, neoSTX, and traces of C1–C2 detected (Law et al., 2015).

2.2. Alexandrium tamiyavanichii *species-specific primer-probe design*

The *Alexandrium tamiyavanichii* species-specific qPCR assay developed in this study conforms to the guidelines of MIQE