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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**A B S T R A C T**

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 10^6 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: Haloogens 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.

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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; Gilbert 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; Bajaras et al., 2003; Montejo 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 (Boonyapitwat, 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 AcSM01 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 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 microscopy. Furthermore, both cultured and environmental samples of *Alexandrium* species often carried 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 taxaspecific 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. (Vanderver et al., 2012), *Ostreopsis* cf. *ovata* (Perini et al., 2011; Hariganea et al., 2013), the diatom *Pseudo-nitzschia* spp. (Fitzpatrick et al., 2010) and harmful raphodophytes, *Chattonella subalsa* 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 (SHELB, 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.p.r.) 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, dSTX, 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
Fig. 1. Fluorescent micrographs of *Alexandrium tamiiyavanichii* AcSM01 from Samariang, Sarawak. (A) Ventral view showing the first apical plate, 1’. Inset: sulcal plate (s.a.) with precingular part (p.pr.); (B) Antapical view showing the longer than wide posterior sulcal plate (s.p.). Bars = 10 µm.

(Minimum Information for Publication of Quantitative Real-time PCR Experiments; Bustin et al., 2009). The MIQE checklist for the assay is listed in Supplementary material Table S2.

The nucleotide sequence of the ITS region from *Alexandrium tamiiyavanichii*, AcSM01 (GenBank accession: KP063148) was multiple-aligned with sequences from other *Alexandrium* species using Clustal X (Thompson et al., 1997), and subsequently edited by BioEdit Sequence Alignment Editor 7.0.9.0 (Hall, 1999). A total of 68 taxa from 18 *Alexandrium* species (*A. affine*, *A. andersoni*, *A. australiense*, *A. catenella*, *A. fraterculus*, *A. fundyense*, *A. insuetum*, *A. lusitanicum*, *A. margalefi*, *A. minutum*, *A. ostenfeldii*, *A. pacificum*, *A. peruvianum*, *A. pseudogonyaulax*, *A. tamutum*, *A. tamarense*, *A. taylori* and *A. tamiiyavanichii*) were retrieved from the GenBank nucleotide database (NCBI) (Supplementary material Table S3) and used in the multiple alignments.

Potential species-specific primer sites were selected for testing within the second internal transcribed spacer (ITS2) that would yield amplicons of <150 base pairs (bp) and that contained one or more unique sites where the hydrolysis probe could bind. These unique primers and probe sites were further analyzed in silico using IDT OligoAnalyzer 3.1 (http://www.idtdna.com/calc/oligoanalyzer/) to determine the GC content, melting temperature (T_m) and possible dimer formation. Primer sites with T_m in the range of 58–60 °C and percentage of GC content between 45 and 65% were selected. Probe sequence was further evaluated by Primer Express 3.0.1 (Applied Biosystems, Tokyo, Japan) to determine if they met criteria typical for probes to work well. The T_m of the probe sites was 8–10 °C above that of the primer pair. The specificity of the primers and probe was then evaluated in silico using Primer-BLAST (Ye et al., 2012) to determine potential cross-reactivity with non-target species. The final primer pair and probe selected for use in this study are listed in Table 1. Taqman® hydrolysis probe, Tamia-probe, was synthesized with 6-FAM (6-carboxy fluorescein) reporter dye at the 5’ end, and the quencher, minor groove binder (MGB), at the 3’ end (Applied Biosystems, CA, USA).

### 2.3. qPCR assay and specificity

The qPCR assay was performed on an Applied Biosystems® 7500 Fast Real-time PCR System ver. 2.0.6 (Applied Biosystems). The assay was run using a Taqman® Fast Advanced Master Mix (Applied Biosystems) in a total reaction volume of 20 µl. The optimized qPCR mixtures contained 200 nM of probe; 300 nM of each forward and reversed primer, and incorporated 2 µl of DNA template in each reaction. The qPCR cycling condition consisted of a holding stage at 50 °C for 2 min and 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The assay was run in triplicate for all samples; and with no template control (NTC), exogenous control and positive control. The threshold cycle (C_t) was determined from the exponential phase of all amplification plots using the default settings.

Specificity of the qPCR assay and species cross-reactivity were evaluated by conventional PCR and qPCR assays using gDNAs from non-targeted *Alexandrium* species as well as other phytoplanktonic species. The conventional PCR was performed in a 25 µl reaction mixture containing 1× PCR buffer (Fermentas, St. Leon-Rot, Germany), 2 mM MgCl₂ (Fermentas), 0.2 mM of deoxynucleoside triphosphate (Fermentas), 1 µM of each primer, 1U of Taq polymerase (Fermentas) and 100–200 ng of gDNA template. The gene amplification was performed using an Arktik™ Thermal Cycler (Thermo Scientific, Vantaa, Finland), with the following cycling conditions: 95 °C for 4 min, 35 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 1.5 min, followed by a final extension of 72 °C for 7 min. Cross-reactivity with the exogenous control, pGEM-3Z plasmid was also tested.

### 2.4. Calibration curve construction

Two calibration curves were constructed in this study: (1) synthetic gene fragment-based (hereafter referred to as gene-based) and (2) cell-based calibration curves. The first was constructed from 10-fold serial dilutions of the synthetic gene fragment spanning the ITS2 rDNA sequence flanked by the qPCR primers of the target species, while cell-based calibration curves were constructed from gDNAs extracted from 10-fold serial dilutions of known cell concentrations. The former was used to determine total extractable gene copies per cell in the samples; the latter was used to determine the linear range of cell densities that can be detected by the qPCR assay.

### Table 1

<table>
<thead>
<tr>
<th>Primer and probe sequences for the qPCR assay used in this study.</th>
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<tbody>
<tr>
<td>DNA target</td>
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<tr>
<td>---------------------</td>
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<tr>
<td><em>A. tamiiyavanichii</em></td>
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<tr>
<td>ITS2 rDNA</td>
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[Table 1](#) Primer and probe sequences for the qPCR assay used in this study.
2.4.1. Construction of the gene-based calibration curves

A synthetic DNA, gBlocks® gene fragment containing the assay target sequence of * Alexandrium tamiaiyanichii* ITS2 rDNA, was synthesized (Integrated DNA Technologies, IA, USA) and used in the construction of the gene-based calibration curves. The synthetic gene fragment flanked the *A. tamiaiyanichii* species-specific primer pair with extra 10 bp long at both ends. The lyophilized gBlocks® gene fragment was re-suspended in 200 μl TE buffer (Tris 1 M, EDTA 0.5 M, pH 8) to obtain a stock concentration of 1 ng μl⁻¹. The copy number of gene fragment in 1 ng μl⁻¹ was then calculated as

\[
\text{amplicon concentration (ng μl}^{-1}) \times 6.022E23 \left(\text{molecule m}^{-1}\right) \times 1 \text{ target sequence per molecule}
\]

for 1 bp of double stranded DNA × 1E9 ng g⁻¹

Dilutions of stock solution to 1 × 10⁸ gene copies, followed by 10-fold serial dilutions of the gene fragment (1 × 10³ to 1 × 10⁸ gene copies), were performed in triplicate qPCR runs.

To account for the loss of DNA during gDNA extraction and the effect of PCR inhibition by the environmental sample matrix, a set of treatments was performed as follows. A separate set of serially diluted gene fragments was subjected to the DNA extraction procedure using 2 μl aliquots from each serial dilution by the same commercial DNA extraction kit, DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) that was applied to the environmental samples. They were subsequently spiked with 2 μl of SHIVA environmental gDNA that was confirmed to be absent of *Alexandrium tamiaiyanichii* cells (by microscopy and post-amplification melting-curve analysis based on SYBR Green I-based qPCR). The samples were then amplified using the qPCR assay as described above. With the gene fragments having undergone the same DNA extraction procedure as was used for the environmental samples, the constructed calibration curves are assumed to have similar and consistent extraction efficiencies as for the environmental samples.

The calibration curves were constructed by plotting triplicate Cq values against the log-transformed copy numbers using GraphPad Prism v. 5.03 (GraphPad Inc., USA). A linear regression was performed onto the calibration curve to determine the R² value and slope. Amplification efficiency (AE) was calculated as AE = [10⁻¹¹/slope] − 1) × 100%. Calibration curves were constructed for the serial dilutions of gene fragments with and without undergoing gDNA extraction, respectively. The AEs calculated from both experiments were then compared.

2.4.2. Cell-based calibration curve construction and pGEM-3Z spike control

Cells grown in multiple tubes to the early to mid-exponential phase were mixed and harvested, and a series of cell concentration was made in 1 ml of total sample volume ranging from 1 to 10⁶ cells ml⁻¹ (1, 5, 10, 10², 10³, 10⁴ cells ml⁻¹; with three analytical replicates); these were used to construct the cell-based calibration curves. Samples with cell concentrations of 1–10 cells ml⁻¹ were prepared by single-cell isolation using the micropipetting technique. Samples with cell concentrations of >10² cells ml⁻¹ were obtained by 10-fold serial dilutions of a known cell concentration determined by microscopic cell counts. Cell concentrations in each dilution were further confirmed using a Sedgewick–Rafter counting slide under an Olympus IX51 microscope.

An exogenous DNA, pGEM-3Z (Promega, Tokyo, Japan) was used to determine the efficiency of DNA extraction and amplification inhibition of the qPCR assay (Coyne et al., 2005). The plasmid was linearized by the restriction enzyme, Ndel (Thermo Fisher Scientific Inc., Vilnius, Lithuania), and amplified using the primer pair M13F and pGEM R (Coyne et al., 2005). The concentrations of pGEM-3Z amplicons were checked using a UVmini-1240 spectro-photometer ( Shimadzu, Tokyo, Japan). Copy number of pGEM-3Z was calculated based on the above equation. A 10-fold serial dilution ranging from 1 × 10² to 1 × 10⁷ copies of pGEM-3Z was performed.

A set of linearized pGEM-3Z dilutions, and a random volume of preserved SHIVA environmental samples (500 μl) that had been tested for the absence of the target cells, were spiked into each of the cell dilution samples. The spiked mixtures were then extracted using DNeasy® Plant Mini Kit (Qiagen). The qPCR reactions were performed in separate runs using the primer-probe sets of *A. tamiaiyanichii* (Table 1) and pGEM-3Z (Coyne et al., 2005), respectively. Calibration curves were constructed for *A. tamiaiyanichii* and pGEM-3Z by plotting triplicate Cq values against the respective log-transformed concentration and gene copy number. Linear regression was performed on the calibration curves and AEs were determined.

2.5. Efficiency assessment of DNA extraction and qPCR amplification by pGEM-3Z spike control

The protocol used in this study followed Hariganey et al. (2013). In brief, samples containing target cells (samples 1 and 2) were prepared. Sample 1 was spiked with 1 × 10⁵ copies of pGEM-3Z prior to gDNA extraction, while the same copies of pGEM-3Z were spiked into sample 2, after gDNA extraction. The qPCR assay was performed using pGEM-3Z primers and probe set. The number of copies of pGEM-3Z, in sample 1 (a) and 2 (b), were determined from Cq values on the pGEM-3Z calibration curve. The extraction efficiency (or correction factor) was calculated as follows:

\[
\text{Extraction efficiency } = \frac{a}{b}
\]

Taking the extraction efficiency into consideration, the quantification data (x) was corrected based on the correction factor:

\[
\text{Corrected rRNA gene copy numbers } = \frac{(b)}{a}
\]

2.6. Determination of extractable gene copies per cell

To determine the extractable mean ITS2 copies per cell of *Alexandrium tamiaiyanichii*, qPCR was performed on 10 independent samples ranging from 5 to 9800 cells ml⁻¹. Cell densities of the samples were determined via microscopic cell count. Copy number was defined as the slope of simple linear regression of ITS2 copies detected by qPCR based on the gene-based calibration curve vs. cell densities determined microscopically. The slope of the linear regression was then defined as the extractable gene copy number per cell.

2.7. Assay validation of the comparative Cq method

To examine the deviation of the potential cell density calculated based on the qPCR assay, a set of treatments was performed with random-spiked cell samples. Actual cell densities were determined microscopically by triplicate cell counts using a Sedgewick–Rafter counting slide under an Olympus IX51 microscope. The random-spiked samples of *A. tamiaiyanichii* were then premixed with environmental samples and known copies of linearized pGEM-3Z. Genomic DNAs were extracted using the above commercial kit and the qPCR assay was then performed as above. The potential cell density was calculated using the comparative Cq method based on the gene-based calibration curve. Microscopic
cell counts were then compared with the cell numbers estimated by the qPCR assay.

Since gDNAs were extracted in the presence of pGEM-3Z, the extraction efficiency and PCR inhibition of both the target and pGEM-3Z are equal and consistent (Lebuhn et al., 2004; Coyne et al., 2005). Using an exogenous control and the spiked environmental samples with a known number of target cells allows the use of the comparative $C_q$ method for quantification, where $C_q$ values for the unknown samples were used to quantify target gene copies from the calibration curve. To validate the comparative $C_q$ method, AEs of the target and exogenous control must be near equal (Livak and Schmittgen, 2001; Coyne et al., 2005).

2.8. Field application of the qPCR assay

Samples were collected from 71 stations during a scientific cruise of the RV Sonne, for the EU SHIVA project (http://shiva.iup.uni-heidelberg.de/index.html), between 15 and 29 November, 2011. The sampling route covered the coastline of Malaysian Borneo up to Manila, Philippines (Fig. 2). GPS locations of each station are listed in Supplementary material Table S4. At each station, 150ℓ surface (5–6 m) seawater samples were collected from the vessel’s moon pool with a pumping system; samples were also collected from discrete depths (5–66 m depth) at selected stations (Supplementary material Table S5). Samples were passed through a 10-μm mesh plankton net, and cells retained in the cod end of the net were filtered onto 0.2-μm pore-size Whatman™ nylon membranes (Thermo Fisher Scientific, MA, USA). Cells, together with the nylon membrane filter, were preserved in 30 ml of modified saline ethanol (Miller and Scholin, 2000). Samples were kept at −20 °C.

Total environmental gDNAs were extracted from 1 ml aliquots using the DNeasy™ Plant Mini Kit. Each environmental sample incorporated known copies of the pGEM-3Z exogenous control as described above. The qPCR assay was performed on pGEM-3Z to assess amplification efficiency. A positive control using the known copies of synthetic gene fragments was included in each qPCR run to assess if $C_q$ was delayed. The SHIVA environmental gDNA samples were then assayed by qPCR as described above. The cell abundance for each station was then calculated taking into account the preserved volume (30 ml) and total sample volume (150 l). Potential cell densities (cells l⁻¹) in the environmental samples were determined. To assess PCR inhibition, random SHIVA environmental gDNAs were selected and diluted 1:10 prior to the qPCR assay. Cell densities calculated from undiluted and diluted gDNAs were then compared.

Physico-chemical data were obtained underway, along cruise transect of the RV Sonne (Cheah et al., 2013). In brief, underway sea surface temperature and salinity were measured by a shipboard thermosalinometer, while the vertical profiles were measured using a SBE 911plus CTD (Sea-Bird Electronics, Washington, USA). Macronutrients were analyzed photometrically using a 120 QuAA-tro auto-analyser (SEAL Analytical, UK). Cell densities of A.

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Fig. 2. Map showing the sampling route by the RV Sonne cruise from November 15–21, 2001 and station locations (black circles) listed in Supplementary material Table S2. Square indicates the location, Samariang, Sarawak Borneo, where plankton samples were collected for culture establishment.
**3. Results**

**3.1. Assay specificity and sensitivity**

The species-specific primers amplified a 149 bp fragment of the ITS2 rDNA of *Alexandrium tamiyavanichii* (Table 1). The primers alone contained up to 17 bp mismatches with other species in the genus. Primer-BLAST demonstrated that the two primer sequences hit all ITS sequences of *A. tamiyavanichii* in GenBank (Accession: AB436948, AB436947 [Japan], AF145224, KP063148 [Malaysia], Fj668713, Fj668712, Fj668711 [Brazil]), with 100% identity to the Malaysian and Japanese isolates, but 99% identity to the Brazilian isolate; one mismatch was observed in the primer sites when comparing to the Brazilian *A. tamiyavanichii* sequence.

Primer specificity was further confirmed by a cross-reactivity test on other species of microalgae, including *Alexandrium* species. While the non-target species showed no amplification in both conventional PCR and the qPCR assay (Supplementary material Fig. S1), cultured and environmental samples that showed positive amplification were further verified by DNA sequencing on the resulting amplicons, and the Blastn search revealed the sequences of *A. tamiyavanichii*. Cross-reactivity of the exogenous DNA, pGEM-3Z, using the *A. tamiyavanichii* primer-probe set, showed no amplification, confirming no cross-reactivity between *A. tamiyavanichii* and pGEM-3Z. Furthermore, the primer-probe set targeting pGEM-3Z could easily amplify the gDNAs of dinoflagellate cultures and environmental samples showed no amplification. The qPCR run on NTCs showed no amplification throughout the study.

The gene-based calibration curve had a linear detection ranging over seven-orders of magnitude ($R^2 = 1.00$, $AE = 94\%$; Fig. 3A), with uniformly separated amplification plots (Supplementary material Fig. S2). The gene-based calibration curve showed here represents the average $C_q$ values for the gene fragments that were used for constructing the calibration curve and those that were used as positive controls. The slopes of the calibration curves constructed from the serially diluted gene fragments that underwent gDNA extraction were nearly identical to those of gene fragments that did not undergo gDNA extraction (slopes of $-3.41$ and $-3.48$, respectively; Fig. 3), indicating that the DNA extraction efficiency was consistent over a wide range of gene copies and that the amplification inhibition was negligible, although a slight deviation was observed at lower gene copy numbers (Fig. 3B). The qPCR assay was able to detect as low as $10^2$ gene copies.

The cell-based calibration curve was linear over four-orders of magnitude (5–$10^4$ cells ml$^{-1}$, $R^2 = 1.00$; $AE = 91.7\%$; Fig. 4). The amplification plots were uniformly separated (Supplementary material Fig. S2). At a cell density of $10^6$ cells ml$^{-1}$, the $C_q$ values were similar to those at $10^4$ cells ml$^{-1}$. As such, the upper limit of cell quantification of *A. tamiyavanichii* by this qPCR assay is $10^6$ cells ml$^{-1}$. The spiked exogenous DNA, pGEM-3Z, extracted together with the cell dilution samples, showed a linear range of detection ($R^2 = 1.00$), and exhibited a high $AE$ of 96.7% (Fig. 4). $AE$s of both cell-based and pGEM-3Z calibration curves were comparable (Fig. 4), indicating that the extraction and amplification efficiencies were consistent over a wide range of cell concentrations.

Evaluation of the assay sensitivity using a lower range of cell dilutions (1, 5 and 10 cells ml$^{-1}$) showed that our qPCR assay was able to detect a signal from a single cell (1 cell ml$^{-1}$) but the $C_q$ was often inconsistent and fell beyond the total reaction cycles of 40. On the other hand, samples containing 5 cells ml$^{-1}$ were consistently amplified and yielding a $C_q$ that was within the linear dynamic range ($27.41 \pm 0.32$). Therefore, the lower LOD of this assay was 5 cells ml$^{-1}$. The mean extractable ITS2 copy numbers per *A. tamiyavanichii* cell was $527,835 \pm 7617$ (slope $\pm SD$) ($R^2 = 1.00$, $P < 0.0001$, Fig. 5). Cell densities estimated using this copy number had a good fit to those determined microscopically (Fig. 6).

We evaluated the feasibility of gene- and cell-based calibration curves to quantify *Alexandrium tamiyavanichii* cells by comparing the CV of the two calibration curves. Our results showed that the slopes of the both the gene- and cell-based calibration curves ($-3.41$ and $-3.54$, respectively) had a CV of 0.01, indicating consistent $AE$s in both calibration curves. Thus, both calibration curves could be used to determine the corresponding cell concentrations at the same $C_q$ values. This is because both the gene fragment and cell dilutions samples had undergone the same gDNA extraction procedure.

To assess the extraction and amplification efficiency, the qPCR results of the pGEM-3Z exogenous control in samples before and after gDNA extractions showed $C_q$ delayed by three units, with the correction factor calculated as 0.08. No differences were observed in data analyzed with or without considering the correction factor. Moreover, the extraction efficiencies among samples were equivalent and consistent since a similar gDNA extraction procedure was applied throughout the study. Hence, quantification in this study was performed without considering the correction factor.

![Fig. 3](image-url) Calibration curves of the cycle threshold ($C_q$) generated using 10-fold serial dilutions of *Alexandrium tamiyavanichii* ITS2 gBlock$^{\text{TM}}$ gene fragment, without undergoing gDNA extraction (A) and with gDNA extraction (B) procedures. Error bars denote the standard deviation from triplicate amplifications. $AE$, amplification efficiency.
3.2. Assay accuracy

Accuracy of the assay was tested with samples containing known target cell densities based on microscopic cell counts. Note that gDNA was extracted from cultured cells and that spiked environmental samples were extracted using the procedure specified in the QIAGEN DNeasy Plant Mini Kit. The potential cell densities calculated based on the gene-based and cell-based calibration curves were strongly correlated with the actual *Alexandrium tamiyavanichii* cell densities obtained by microscopic counts ($R^2 = 1.00, P < 0.0001$), but with a slight deviation at higher cell density (Fig. 6). The results showed that the potential cell densities estimated from either gene-based or cell-based calibration curves were not significantly different ($P > 0.05$). The *A. tamiyavanichii* cell densities in the environmental samples from the southeastern SCS-SS determined by the qPCR assay also showed a strong correlation between those calculated from the gene-based and cell-based calibration curves ($R^2 = 1.00, P < 0.0001$; Fig. 7).

3.3. Spatial distribution of *A. tamiyavanichii* in the southeastern SCS-SS

To evaluate the inhibition of amplification, cell densities of *Alexandrium tamiyavanichii* in the environmental samples collected along the southeastern SCS-SS were estimated from $C_q$ values derived from undiluted and 10-fold diluted environmental gDNAs. Our results showed no significant difference in the cell densities obtained, suggesting negligible qPCR inhibition. All environmental samples were analyzed without diluting the gDNAs, as diluted environmental gDNAs in samples with low cell densities might give negative amplifications, leading to false negative results. On the other hand, our qPCR results of the pGEM-3Z exogenous control in the environmental samples showed no significant delays in $C_q$, implying insignificant PCR inhibition. In addition, no significant deviation in $C_q$ was observed for the gene fragment positive controls.

Cells of *Alexandrium tamiyavanichii* were detected in the surface water samples of 21 stations, with cell densities ranging from 1 to 150 cells l$^{-1}$; other stations showed extremely low abundances (<1 cell l$^{-1}$) or were below the detection limit (Fig. 8A). Stations...
along the offshores of Borneo were generally characterized by constant sea surface temperatures (SST; 29.0–29.5 °C), with the exception of stations offshore of northern Borneo, where SSTs declined to 28.5 °C (Fig. 8B). Surface salinities ranged between 31.0 and 32.5 (Fig. 8C). *A. tamiyavanichii* was present at higher abundances (Fig. 8) at stations in southern Borneo, with the highest cell densities found at stations near the coastal areas of Kuching (UW29; 152 ± 6 cells l⁻¹), Bintulu (UW39; 43 ± 2 cells l⁻¹), and offshore of Miri (UW46; 56 ± 19 cells l⁻¹). Notably, *A. tamiyavanichii* was not detected at stations offshore of northern Borneo nor at coastal stations (Kota Kinabalu, UW60–63; Sandakan, UW80–81).

Moderate *Alexandrium tamiyavanichii* cell abundances were again detected at stations near the southern Palawan Island, Sulu Sea (UW71–76), with cell densities of 10–20 cells l⁻¹ (Fig. 8A, Supplementary material Table S4). SSTs in this deeper basin were similar to those in southern Borneo (29.0–29.5 °C), but surface salinities were slightly higher (salinity of >32.5). At all stations, surface nutrient concentrations were low but homogeneous, with NO₃ + NO₂ < 1 μmol l⁻¹ and PO₄ < 0.1 μmol l⁻¹ (data not shown).

The vertical distribution of *Alexandrium tamiyavanichii* offshore of Malaysian Borneo is shown in Fig. 9A. The southern offshore is characterized by shallow waters of the southern Sunda Shelf (<100 m depth), while the northern offshore is adjacent to a sedimentary basin, the Brunei basin (>100 m depth). Environmental parameters of the water mass where samples were collected, ranged from 26.2 to 29.6 °C (Fig. 9B), salinity of 31.9–33.5 (Fig. 9C), 0.003–9.932 μmol l⁻¹ NO₂ (Fig. 9D) and 0.001–0.709 μmol l⁻¹ PO₄ (Fig. 9E).

Cells were generally detected at most stations above the pycnocline, ranging from >1 to 70 cells l⁻¹, except for stations UW34 and UW42 where no cells were detected (Fig. 9A). A patchy distribution of *Alexandrium tamiyavanichii* was observed offshore of Miri (UW46), with a surface cell density of ~60 cells l⁻¹ and a cell maximum of 73 ± 1 cells l⁻¹ at a 30 m depth; but no cells were detected deeper (66 m). At stations with low cell densities (<15 cells l⁻¹), cells generally appeared at depths between 20 and 30 m (Fig. 8A).

4. Discussion

4.1. Development of *A. tamiyavanichii* qPCR assay

A species-specific hydrolysis probe-based qPCR assay was successfully developed for the highly toxic *Alexandrium tamiyavanichii* occurring in the South China Sea (SCS) region. The assay targeted the ITS2 rDNA region because of its higher sequence divergences in the rDNA region when compared with other species (e.g., Leaw et al., 2005; John et al., 2014; Kremp et al., 2014; Wang et al., 2014). This provides numerous unique primer and probe sites that can be used to discriminate species in the genus.
Furthermore, the high copy numbers of rDNA per cell enhanced the sensitivity of the qPCR assay to detect extremely low cell numbers or even down to a single cell (Bowers et al., 2000; Dyhrman et al., 2006; Park et al., 2012, 2014; this study).

The specificity of the short primer regions selected for the *Alexandrium tamiyavanichii* qPCR assay was confirmed by demonstrating no amplification on other *Alexandrium* species found in the SCS region (Supplementary material Fig. S1). Screening of the primer sequences against GenBank, however, revealed that the assay may have to be modified for application in detecting the Atlantic population of *A. tamiyavanichii*. As in Menezes et al. (2010), the LSU rDNA phylogenetic tree inferred that the Asian Pacific and Atlantic populations of *A. tamiyavanichii* are separated, and that the primer sequences used in this study vary by one base pair. Further
sequencing of the Atlantic clones will be needed to determine if primers with a single polymorphic site will be sufficient to allow the current *A. tamiyavanichii* assay to be applied globally. The available data, however, indicate that the qPCR assay developed here can be widely applied in the Pacific for accurate enumeration of *A. tamiyavanichii* in environmental samples as has been previously accomplished for many other harmful species (e.g., Galluzzi et al., 2004; Coyne et al., 2005; Hosoi-Tanabe and Sako, 2005; Dyhrman et al., 2006; Galluzzi et al., 2010; Perini et al., 2011; Howard et al., 2012; Vandersea et al., 2012; Hariganeya et al., 2013; Park et al., 2014).

In this study, we employed two calibration curve strategies: one using a synthetic, sequence-verified gene fragment spanning the *Alexandrium tamiyavanichii* ITS2 target gene sequence (gene-based), and another using gDNAs extracted from samples with known cell densities (cell-based). The synthetic gene fragment is convenient and could serve as an alternative calibrator for qPCR absolute quantification other than plasmid DNA constructs and purified target PCR products. The cell-based calibration curve, on the other hand, provides a reliable dynamic range of cells that can be quantified by the qPCR assay. Cell quantification by either gene or cell-based curves was consistent for the spiked and environmental samples, suggesting that the assay is robust to variations that occur in different cell sizes, growth phases, sexes and populations (Galluzzi et al., 2010).

The extractable gene copy numbers per cell determined in this study were comparable to those reported for other dinoflagellate species (*Alexandrium* [Galluzzi et al., 2010]; *Cambriodiscus* [Vandersea et al., 2012]; *Ostreopsis* [Hariganeya et al., 2013]). It is important to note that the data presented here do not provide an absolute gene copy number per cell for *Alexandrium tamiyavanichii*. This is because, an empirical relationship was used to convert the total extractable gene copies in a sample to cell estimates, provided that the samples were extracted and the qPCR was carried out following the exact procedures described in this study. We applied the same gDNA extraction method throughout this study. Thus, it is assumed that the extraction efficiency and the losses of DNA were normalized among treatments, resulting in comparable gene copy and cell density estimates.

### 4.2. Distribution and abundance of *A. tamiyavanichii* in the SCS

The SCS is the largest semi-enclosed sea in the world, and has a long history of HABs since the 1970s, involving cases of human illness and deaths (Azanza and Taylor, 2001; Usup et al., 2012). Intensive coastal aquaculture and human activity have enriched the coastal waters of SCS countries with nutrients, explicitly increasing the risk of HABs. The major threat associated with HABs in this region is PSP. The species *Pyrodinium bahamense* remains the major toxin producer; likewise, several toxic species of *Alexandrium* have been subsequently discovered in the region. Among them, *A. tamiyavanichii* is known to be the most commonly distributed toxic *Alexandrium* species in the Asian Pacific region; the species was first recorded from the Gulf of Thailand in the 1980s (Kodama et al., 1988). In this study, we document that the distribution and abundance of *A. tamiyavanichii* covers a broader area of the south-eastern SCS and SS, and is a continuing occurrence in the region. Its distribution and abundance reported here is in good agreement with the data collected during the SEAFDEC cruise in the SCS during 1996–1997 (Boonyapiwat, 1999b). It is interesting to note that, despite the time gap between the two studies, the data support the endemism of the species in these waters. The distribution data suggest that the species was unlikely introduced via anthropogenic activity or human dispersal (e.g., ballast water), but is rather endemic in the region.

Environmental factors play a significant role in the growth of *Alexandrium*, subsequently affecting its distribution and abundance (Gilbert et al., 2005a; Anderson et al., 2008). The optimum nutrient, salinity, temperature and light conditions for the growth of *Alexandrium tamiyavanichii* have been well studied in the laboratory (Ogata et al., 1990; Lim et al., 2004, 2006), but how environmental variability in these factors affects natural *A. tamiyavanichii* populations is not well understood. A comparison of the *A. tamiyavanichii* distribution with the environmental data reported in this study provide a fundamental overview of how these factors influence the relative abundances of this species.

The distribution data showed higher abundances of *Alexandrium tamiyavanichii* in the coastal area of Sarawak Borneo, with the highest cell densities found at the station closest to Kuching Bay (station UW29). This region is characterized by extensive nutrient inputs, with ambient NO<sub>3</sub> and PO<sub>4</sub> concentrations 17- and 5-fold higher compared to other stations (9–10 μmol l<sup>−1</sup> and <0.6 μmol l<sup>−1</sup>, respectively). Kuching Bay receives nutrient loading from river runoff; for example, river discharges from an adjacent river (Sibu Laut River) contained NO<sub>3</sub>–N, the major dissolved inorganic nitrogen, as high as 30 μmol l<sup>−1</sup> (Soo et al., 2014). High NO<sub>3</sub> levels in Kuching Bay may promote the patchy distribution of *A. tamiyavanichii*. It is presumed that the optimum temperature and light in the region enhanced and stimulated the cell size and nutrient uptake of *A. tamiyavanichii*. In laboratory cultures of *A. tamiyavanichii*, the nitrate uptake rate is greater at optimum growth temperature (Lim et al., 2006). Likewise, nutrients other than nitrate may contribute to the patchy distribution in Kuching Bay. Urea and ammonium are other nitrogen sources that simulate phytoplankton growth in coastal waters (Gilbert et al., 2001; Burkholder et al., 2006) and they have been shown to promote shifts in phytoplankton composition (Berg et al., 2003; Gilbert et al., 2005b). While nutrients may influence the distribution of *A. tamiyavanichii*, the surface nutrient concentrations of other stations along the Borneo coast, the SCS and the SS are depleted (NO<sub>3</sub> < 0.6 μmol l<sup>−1</sup> and PO<sub>4</sub> < 0.12 μmol l<sup>−1</sup>), and are likely limiting phytoplankton production. This is supported by observation of high cyanobacteria abundances in the region (Cheah et al., 2013). On the contrary, the vertical distribution of *A. tamiyavanichii* along the coast of Malaysia Borneo could not be explained by favorable nutrient levels, as both nitrate and phosphate were depleted throughout the depths sampled (Fig. 9). Its patchy distribution was thus more likely explained by the salinity regime (discussed below).

Salinities in the southern Borneo were lower (<32.5) compared to the stations off northern Borneo and in the Sulu Sea (>33.5); this may partially account for the higher cell concentrations of *Alexandrium tamiyavanichii* in the southern Borneo. In the laboratory, *A. tamiyavanichii* was reported as a stenohaline species, showing a weak tolerance at both extremes of the optimal salinity range. It grew at salinities of 20–30, and optimally at 25 (Lim and Ogata, 2005). The lower salinity ranges in the southern Borneo are likely due to the freshwater runoff from several rivers along the Sarawak coast (Batang Salak, Batang Sadong, Batang Laporan, Batang Saribas, Batang Rajang and Batang Betawai). Studies showed that blooms of toxic dinoflagellates were often associated with freshwater runoff and enhanced rainfall (Hallegraeff et al., 1995; Weise et al., 2002). Furthermore, our observation on the vertical profile showed a subsurface-layer aggregation of *A. tamiyavanichii* cells where salinity stratifications (salinity < 32.5) are observed horizontally. The layer somehow developed at nutrient depleted depths that did not promote growth. Phytoplankton species have often adapted strategies to grow in sub-optimal conditions by forming layers (Gentien et al., 2005). It has been predicted that a patch of *A. tamiyavanichii* cells could be transported out from the nearshore in river plumes, for example the Baram River plume.
and that this may be the reason for offshore salinity stratifications. The growth of such populations may be reduced or increased over time, depending on other promoting factors such as nutrients. This could not be elucidated further as no temporal data was collected from this study.

The vertical distribution of *Alexandrium tamarensis* showed cells as deep as 60 m, which is above the euphotic zone; below that, no cells were observed. Nearly all species of *Alexandrium* demonstrate a high light-adaptation strategy (Smayda, 2008), and laboratory experiments have shown that photo inhibition was not detected, even at 800 μmol photons m⁻² s⁻¹ (*Alexandrium catenella*, Carignan et al., 2002; Laabir et al., 2011). In the laboratory, optimum growth of *A. tamarensis* was observed at 100 – 140 μmol photons m⁻² s⁻¹ (Lim and Ogata, 2005; Lim et al., 2006). Given the mean PAR of 700 μmol photons m⁻² s⁻¹ in the mixed layer (15–50 m) of the region (Cheah et al., 2013), the distribution was consistent with the light conditions in the water column. While irradiances along the cruise transect might be optimum for growth, *A. tamarensis* cells were not detected at most of the stations. Likewise, the consistent high sea surface temperatures are growth optimum. Although these two factors play significant roles in phytoplankton growth, they are unlikely the important ones influencing the occurrence and distribution of *A. tamarensis* observed in this study. As in most tropical regions, sea surface temperatures and light at the surface and mixed layer are almost constant, conditions that usually favor the growth of tropical phytoplankton. Thus other environmental factors (salinity, upwelling etc.) explain to the abundance and distribution of this species in the region.

It is noteworthy that *Alexandrium tamarensis* cells were observed mainly in the neritic zone (<200 m). We did not detect any cells at stations along the Sabah Trough (northern Borneo), nor at offshore stations in the Sulu Sea, which are located at the deep Sunda Basin (depths >200 m). This is in agreement with other toxic *Alexandrium* species, such as *A. fundyense*, where blooms often occur in shallow shelf waters (e.g., Wyatt and Jenkinson, 1997; Mcgillicuddy et al., 2014). Notably, blooms of *A. tamarensis* are also almost absent in the region, especially in southern Borneo. This could be partly explained by the high flushing rates due to the semi-diurnal tidal cycle. The growth response of dinoflagellates in natural environments is complicated and multifaceted. Future investigation on the adaptive strategies of this species would provide a better insight into its distribution patterns in the region.

4.3. The qPCR assay in monitoring

In the context of harmful microalgal monitoring, the qPCR assay developed in this study is proven to be successful in quantitatively detecting *Alexandrium tamarensis* in the environmental samples. The assay is able to detect cells at extremely low abundances (down to 5 cells ml⁻¹), and could thus be used to detect cells especially during the bloom initiation stage. In conclusion, the specificity and sensitivity of this assay provide a reliable molecular platform for the early detection of this toxic *Alexandrium* species, as well as for identifying potential hot spots of PSP, which makes it an asset for HABs monitoring.

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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.jhal.2015.10.002.

References


