J Appl Phycol (2016) 28:1683–1693
DOI 10.1007/s10811-015-0682-6

Development of a quantitative PCR for detection and quantification of *Prorocentrum donghaiense*

Chun Yun Zhang[1,2] · **Guo Fu Chen**[1,2] · **Jin Zhou**[3] · **Yuan Yuan Wang**[1] · **Dou Ding Lu**[4]

Received: 24 May 2015 / Revised and accepted: 30 July 2015 / Published online: 13 August 2015

Abstract *Prorocentrum donghaiense* is a dinoflagellate with a high frequency of bloom formation in the East China Sea. These blooms harm coastal ecosystems, marine fisheries, aquatic environments, and public health. Therefore, new and rapid methods that accurately process and specifically detect this alga are crucial to facilitate long-term monitoring or to provide timely warnings of *P. donghaiense* blooms. We report the development of a quantitative real-time PCR (qPCR) method to identify and detect *P. donghaiense*. The partial large subunit (LSU) rDNA D1–D2 was cloned and sequenced to design specific amplification primers. The specificity of the primers was tested using regular PCR and fluorescent PCR against a wide range of microalgae widely distributed along the Chinese coast. The qPCR detection protocol was based on two standard curves. Both curves were constructed from standard samples of tenfold serially diluted solutions of the recombinant plasmid containing the LSU D1–D2 fragment and crude DNA extracts with a known number of target cells. A quantitative relationship between the cell numbers and their corresponding plasmid copy numbers was established; this relationship can be used to determine the target cell number of unknown samples in combination with a standard curve that was generated from tenfold-diluted plasmid solutions and the determined C_t value of target DNA. The effectiveness of the developed protocol was tested with a series of simulated and field samples. The developed qPCR had a detection sensitivity of up to 3.45 cells. The performance of qPCR was not affected by nontarget DNA. The detection test with a series of samples fixed for 40 days showed that qPCR is competent for long-term monitoring programs that require the quantitative analysis of fixative-preserved samples. qPCR can identify the target cells in the field samples within 3–4 h. No significant differences in the quantitative results of the target cells were observed between qPCR and light microscopy. Overall, the established qPCR method is specific, sensitive, rapid, accurate, and promising for the field detection of *P. donghaiense* in natural samples.

Keywords *Prorocentrum donghaiense* · LSU rDNA · Quantitative PCR · Detection · Dinoflagellate

✉ Guo Fu Chen
chenguofu@hitwh.edu.cn

[1] School of Marine Science and Technology, Harbin Institute of Technology (Weihai), Weihai 264209, People's Republic of China

[2] Key Laboratory of Marine Ecology and Environmental Science, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, People's Republic of China

[3] Division of Ocean Science and Technology, Graduate School at Shenzhen, Tsinghua University, Shenzhen, Guangdong 518055, People's Republic of China

[4] The Second Institute of Oceanography, SOA, Hangzhou 310012, People's Republic of China

Introduction

Harmful algal blooms (HABs) have increased in frequency, number, and span worldwide over the past few decades. The China Sea has frequently suffered from devastating HABs since the 1990s. In particular, large-scale dinoflagellate blooms have began to occur in the East China Sea area adjacent to the Changjiang River estuary during late spring and early summer since the beginning of the twenty-first century. The causative organism of these blooms in the East China Sea was identified as a new species named *Prorocentrum donghaiense* (Lu and Goebel 2001; Lu et al. 2003, 2005).

Prorocentrum donghaiense has caused approximately 120 algal blooms in the East China Sea from 2000 to 2006 (Chen et al. 2013a, b). The Ministry of Land and Resources of the People's Republic of China has recorded 16 *P. donghaiense* blooms in the East Sea in 2013 alone, accounting for almost 50 % of the total number of blooms in China for that year (http://www.coi.gov.cn/gongbao/nrhuanjing/nr2013/201403/t20140325_30705.html). Although it does not produce toxins, *P. donghaiense* is still harmful because its blooms usually reach a high biomass with a wide spatial distribution that lasts for a long period (Lu et al. 2003). *P. donghaiense* blooms negatively affect the aquatic environment, public health, and marine fisheries economy (Li et al. 2009). The direct loss of marine fisheries caused by *P. donghaiense* amounts to more than several million dollars per year (Tang et al. 2006a, b; Long et al. 2008).

The development of a monitoring program that provides adequate early warnings of possibly imminent blooms is essential to mitigate economic losses caused by *P. donghaiense*. Current monitoring programs of HAB occurrence detect and identify target species via light microscopy (LM) analysis based on morphological criteria. However, the delineation of *P. donghaiense* is problematic. *P. donghaiense* cells are known for their small size, 18.9–21.6 μm length and 9.6–13 μm width; the morphology of these cells often varies under different physiological (Lu et al. 2003) and natural conditions (Cai et al. 2006). Controversies on the taxonomy of *P. donghaiense* lasted long before it was finally established as a new species (Lu and Goebel 2001; Lu et al. 2003; Qi and Wang 2003). However, *P. donghaiense* is still confused with other analogous species, including *Prorocentrum dentatum* (Lu et al. 2003, 2005; Qi and Wang 2003) and *Prorocentrum maximum* (Percopo et al. 2011). These studies indicate that the identification of *P. donghaiense* by morphological methods requires taxonomic knowledge and skills.

With the rapid development of molecular biology and related techniques, an increasing number of promising alternatives to the traditional method of microscopic analysis have been applied to identify and quantify environmental microorganisms. These methods usually employ ribosomal RNA operons, including small subunit rDNA, large subunit (LSU) rDNA, and internal transcribed spacers, to differentiate harmful algae at the species level. Protocols to detect harmful algae include fluorescence in situ hybridization (FISH) (Scholin et al. 1996; Chen et al. 2013a, b), sandwich hybridization assay (Diercks et al. 2008; Mikulski et al. 2008; Zhen et al. 2009), DNA arrays (Ki and Han 2006; Smith et al. 2012), and quantitative real-time PCR (qPCR) (Popels et al. 2003; Yuan et al. 2012a, b). qPCR is promising for the field monitoring of environmental microorganisms. This technique offers all the advantages of conventional PCR, such as high sensitivity and specificity, and allows for the quantification of PCR products.

qPCR was originally developed to quantify gene expression. However, recent studies have employed this method to detect environmental microorganisms. The first application of qPCR to detect harmful algae dates back to 2000 (Bowers et al. 2000). This technique has become increasingly popular in the monitoring or the microalgal population dynamic studies of harmful algae (Penna and Galluzzi 2013) because of its high sensitivity, rapidity, and high throughput. In the current study, we developed a qPCR assay that targets the LSU D1–D2 region to detect *P. donghaiense*. The LSU D1–D2 region was PCR amplified, sequenced, and used to design specific primers. The specificity of the primers was tested against other microalgal species. Two standard curves were constructed with tenfold-diluted solutions of plasmids containing the target sequence and tenfold-diluted crude DNA extracts with a known number of target cells. A mathematical relationship between the cell numbers and their corresponding plasmid copy numbers was determined from these standard curves. Consequently, a series of tenfold-diluted plasmid solutions was used in qPCR to construct a standard curve for the quantification of the target cells. qPCR was performed in parallel with samples containing unknown cell numbers. The performance of the developed qPCR assay was appraised by testing with fixative-preserved cultures, as well as spiked and environmental samples.

Materials and methods

Prorocentrum donghaiense strain MABT-11 and all other microalgae were obtained from commercial sources or from private isolations (Table 1). All cultures were maintained in sterile-filtered f/2 or f/2+Si seawater medium (Guillard 1975) at a salinity of 36 psu. All cultures were grown at 20–22 °C under a 12:12-h light/dark cycle with a light intensity of 50–100 μmol photons m^{-2} s^{-1}. The cultures were manually stirred daily, with the nutrient medium replaced on a biweekly or monthly basis.

Sequencing of the LSU D1–D2 of *P. donghaiense*

The genomic DNA of the *P. donghaiense* strain MABT-11 was extracted from algal cultures during the log phase. Target cells were harvested through centrifugation, rinsed with double-distilled water, resuspended in cetyltrimethylammonium bromide (CTAB) buffer (2 % CTAB, 1.4 M NaCl, 0.02 M EDTA, 0.1 M Tris-Cl, and 0.2 % β-mercaptoethanol), and incubated for 30–35 min at 60 °C. Total nucleic acids were sequentially extracted with equal volumes of phenol, phenol/chloroform/isoamyl alcohol (25:24:1, *v*/*v*/*v*), and chloroform/isoamyl alcohol (24:1, *v*/*v*). Nucleic acids were concentrated with two

Table 1 List of strains and PCR results using the specially designed primers for *Prorocentrum donghaiense*

Species	Taxonomy	Geographic origin	Strain	Specificity test[a]
Prorocentrum donghaiense	Dinophyceae	Zhejiang, East China Sea	MABT-11	+
Prorocentrum micans	Dinophyceae	East China Sea	CCMA-56	−
Prorocentrum triestinum	Dinophyceae	East China Sea	LAMB100721	−
Prorocentrum lima	Dinophyceae	Daya Bay, East China Sea	CCMA-62	−
Prorocentrum minimum	Dinophyceae	Daya Bay, East China Sea	CCMA-15	−
Amphidinium carterae	Dinophyceae	USA	CCMA-26	−
Gymnodinium sanguineum	Dinophyceae	Xiamen Bay, East China Sea	CCMA-132	−
Takayama pulchellum	Dinophyceae	Xiamen Bay, East China Sea	CCMA-25	−
Alexandrium minutum	Dinophyceae	Hong Kong, East China Sea	CCMA-17	−
Alexandrium tamarense	Dinophyceae	East China Sea	MABT-12	−
Scrippsiella trochoidea	Dinophyceae	South China Sea	CCMA-47	−
Karenia mikimotoi	Dinophyceae	Wenzhou, East China Sea	MABT-5	−
Karlodinium veneficum	Dinophyceae	Longboat Key near Sarasota, FL, USA	LAMB090611	−
Nitzschia closterium	Bacillariophyceae	Weihai Bay, Yellow Sea	MABT-9	−
Skeletonema costatum	Bacillariophyceae	Shenzhen Bay, East China Sea	MABT-7	−
Heterosigma akashiwo	Raphidophyceae	South China Sea	MABT-10	−
Prymnesium parvum	Prymnesiophyceae	Bohai Sea	CCMA-74	−
Tetraselmis chuii	Chlorophyceae	Weihai Bay, Yellow Sea	MABT-13	−

[a] The positive and negative PCR/qPCR results are represented by "+" and "−", respectively

volumes of ethanol, and a 1/10 volume of sodium acetate (3 M, pH 5.2) was added to accelerate the precipitation. The precipitates were rinsed twice with two volumes of 70 % ethanol. Finally, DNA was dissolved in TE buffer (10 mM Tris-Cl, pH 7.6; 0.05 mM EDTA) and then stored at 4 °C or frozen at −16 °C. Genomic DNA was used as a template for the PCR amplification of the D1–D2 region of the 28S rDNA gene with universal primers D1 and D2 (Table 3) (Scholin et al. 1996). The PCR products were purified using the Sanprep Type DNA Gel Extraction Kit (Sangon Biotech., China). The purified PCR products were ligated to the pMD 18-T vector (TaKaRa, China) and then transformed into competent *Escherichia coli*. The positive clones were screened via colony PCR and then sequenced at the Beijing Nuosai Genome Research Center Co., Ltd. (Beijing, China). The obtained sequences were submitted to GenBank (accession no. KF032444).

Primer design and specificity verification

The obtained target sequence and related sequences from other *Prorocentrum* species in GenBank (Table 2) were used to perform multiple sequence alignment with CLUSTALW as implemented in the BioEdit program (Hall 1999). Variable regions were identified to manually design species-specific primers (Table 3). Primer quality was assessed by Oligo 6.0, and primer specificity was further confirmed by Basic Local Alignment Search Tool (BLAST) search. The primers were synthesized at the Beijing Nuosai Genome Research Center Co., Ltd. The specificity of the designed primers was experimentally verified with a series of microalgal species that are widely distributed along the Chinese coast (Table 1). The genomic DNA of all test microalgae was extracted as described in the section "Sequencing of the LSU D1–D2 of *P. donghaiense*." The specificity of the primers was tested using conventional PCR and qPCR with the extracted genomic DNA as a template. The conventional PCR was performed in a 25-μL reaction containing 2.5 μL of 10× PCR buffer, 1.5 μL of $MgCl_2$ (1.5 mM), 1 μL of dNTP (0.4 mM), 1 μL of each primer (0.4 mM), 19.3 μL of PCR-grade water, 0.2 μL of *Taq* polymerase (1 U; Sangon Biotech Co., Ltd., Shanghai, China), and 1 μL of genomic DNA (5–10 ng). The PCR protocol was 94 °C for 5 min, followed by 29 cycles of 94 °C for 45 s, 50 °C for 30 s, and 72 °C for 50 s, with a final extension step at 72 °C for 7 min.

qPCR

qPCR assays were performed with an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, USA). Reactions were conducted in 96-well plates with a final volume of 20 μL per well, which contained 10 μL of Premix Ex Taq, 0.4 μL each of the forward (Q-Pd-f) and reverse (Q-Pd-r) primers (10 μM), 0.4 μL of ROX Reference Dye II, 2 μL of DNA template, and 5.8 μL of ddH_2O. The following quantification cycling protocol was used: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. At the end of each run, a dissociation step (95 °C for 15 s, 60 °C for 1 min, 95 °C for 1 min, and 60 °C for 15 s) was added to generate a melting

Table 2 List of *Prorocentrum* introduced into alignment analysis, with GenBank accession numbers of their partial LSU rDNA sequences

Species	GenBank accession number (LSU)	References
Prorocentrum arabianum	EF566752	
Prorocentrum arenarium	EF566747	
Prorocentrum balticum	EU927548	
Prorocentrum belizeanum	DQ238042	Faust et al. 2008
Prorocentrum cassubicum	EU927557	
Prorocentrum concavum	EF566751	
Prorocentrum consutum	FJ842378	Chomerat et al. 2010
Prorocentrum dentatum	AY863006	
Prorocentrum donghaiense	AY822610	
Prorocentrum emarginatum	DQ336192	Murray et al. 2007
Prorocentrum faustiae	EF566744	
Prorocentrum foveolatum	AY259173	
Prorocentrum fukuyoi	EU196416	
Prorocentrum gracile	EF517251	
Prorocentrum gracile	AY259165	
Prorocentrum hoffmannianum	DQ336185	Murray et al. 2007
Prorocentrum levis	FJ489619	
Prorocentrum lima	DQ336186	Murray et al. 2007
Prorocentrum mexicanum	DQ336183	Murray et al. 2007
Prorocentrum micans	AY032654	
Prorocentrum minimum	EU927535	
Prorocentrum rhathymum	EF566745	
Prorocentrum sculptile	EF566749	
Prorocentrum sigmoides	EF566746	

curve thermal profile and confirm the amplification of single PCR products with the expected melting profile. Experiments were performed in triplicate for each standard curve point and with duplicates for each lysate sample. Each PCR run included the standard curve, which was established by serially diluted plasmids containing the target sequence, and a template-free control.

Construction of standard curves using plasmid DNA and crude DNA extracts from target cells

The recombinant plasmid containing the target sequence was extracted with a TaKaRa MiniBEST Plasmid Purification Kit (Ver. 4.0; Takara Bio Inc., Japan). After assessing the purity and quality through 2 % agarose gel electrophoresis, the double-stranded plasmid was digested with *Bam*HI to produce single-stranded products. The enzyme-digested plasmid was purified with the Sanprep Type DNA Gel Extraction Kit (Sangon Biotech., China). The concentration and purity of the purified plasmid were determined with a NuDrop microspectrophotometer (NAS99, ACTGene, USA). The copy number concentration of plasmid (NCP; copies μL^{-1}) was calculated as follows: $NCP=(PC\times10^{-9}\times6.02\times10^{23})/(N\times660)$, where PC represents the concentration of plasmid (ng μL^{-1}) and N represents the base number of recombinant plasmids. According to the NCP, a series of tenfold-diluted plasmid solutions was prepared with deionized water and respectively used as templates for qPCR to establish a plasmid standard curve.

Table 3 Summary of primers used in this study

Primer name	Primer sequence (5′–3′)	Amplification type	Reference
D1	ACCCGCTGAATTTAAGCATA	PCR	Scholin et al. 1994
D2	CCTTGGTCCGTCTTTCAAGA	PCR	Scholin et al. 1994
Q-pd-f	ATCGTCTCCTGCCTTGTGTG	PCR/qPCR	This study
N-pdl-r	GTCCGCAAATGAGTTCTGCC	PCR/qPCR	This study

Two methods of preparing DNA templates for qPCR were compared using 3.45×10^6 cells that were collected from the algal cultures. One method is that described in the section "Sequencing of the LSU D1–D2 of *P. donghaiense*." The other method involves resuspending algal pellets in deionized water and then subjecting the algal cell solution to ultrasonic treatment. Each method for DNA template preparation was performed thrice. The tenfold-diluted DNA templates prepared from known cell numbers with deionized water were used for qPCR to construct a standard curve for the target cells. Simultaneously, qPCR was run in a microplate to construct standard curves for the plasmid and the target cells.

Anti-interference experiment of qPCR

Common microalgae *Skeletonema costatum* and *Prorocentrum triestinum* were used to test whether or not nontarget cells interfere with the quantification of the target cells. In brief, 10 mL of *P. donghaiense* cultures of known cell concentration was analyzed via LM and then mixed with equal volumes of *S. costatum* and *P. triestinum* cultures. Algal cells were collected by centrifugation, washed, resuspended in 1 mL deionized water, and then subjected to ultrasonic treatment for crude DNA extract preparation. The crude DNA extract was used for qPCR to quantify the target cells with the standard curves, which were constructed by simultaneous qPCR with tenfold dilutions of the plasmid containing the target sequence. The quantitative results of LM and qPCR were compared to demonstrate whether or not the nontarget cells affect the quantification of the target cells in the field samples.

qPCR test with target species fixed for different time intervals

After determining the cell density of each algal culture, 14 replicates of the algal cultures were pipetted into 50-mL centrifuge tubes; each replicate contained 10^6 cells of the target microalgae. All of the algal cultures were classified into two groups (I and II). Seven replicates of the algal solutions in group I were fixed with 2 % Lugol's solution for 0, 1, 3, 5, 10, 20, and 40 days. By contrast, no fixative was added to the algal cultures in group II. Each replicate of the algal culture was used to collect target cells via centrifugation and prepare a crude DNA extract for subsequent qPCR as previously described.

qPCR test with simulated and environmental samples

The capacity of qPCR to detect *P. donghaiense* from environmental water samples spiked with a known number of organisms was assessed. The results of qPCR showed that environmental water samples from Weihai Bay (Weihai, Shandong province) tested negative for the presence of *P. donghaiense*. In brief, algal cultures were mixed with aliquots of field seawater at ratios of 1:1, 1:3, and 1:19 to prepare final target cell concentrations of 3.15×10^5, 1.58×10^5, and 3.15×10^4 cells mL^{-1}. The field samples were collected from different sites of East China Sea during a spring cruise in April 2014. The morphological criteria described by Lu et al. (2005) were used to identify *P. donghaiense* cells in the natural samples. The target cells were identified using a plankton-counting chamber under a light microscope. The simulated and natural samples were vacuum filtered onto 5-μm cellulose membranes (Millipore). The membranes were cut into small pieces with autoclaved scissors and then placed in a 1.5-mL microtube with 1 mL of deionized water. After vigorous shaking and ultrasonic treatment, the supernatants were used to perform qPCR.

Statistical analysis

A two-tailed Student's *t* test was used to compare the cell densities between LM and qPCR. All statistical calculations were performed using SPSS 13.0 (SPSS, Chicago, IL, USA). Statistical significance was considered at $P<0.05$.

Results

Specificity verification of specific primers for *P. donghaiense*

DNA extraction and PCR amplification, including regular PCR and qPCR, were performed on the cultures of *P. donghaiense* and other control organisms to verify experimentally the specificity of the designed specific primers. These test microalgae belonged to five classes, namely, Dinophyceae (13 species), Bacillariophyceae (2 species), Raphidophyceae (1 species), Prymnesiophyceae (1 species), and Chlorophyceae (1 species). The specificity test results are summarized in Table 1, and the electrophoresis results of regular PCR and qPCR amplification are shown in Fig. 1. All DNA templates from the test microalgae were PCR amplified with the universal primers, and ca. 650-bp PCR fragments were generated; this result indicates that all of the DNA templates work well (Fig. 1a). By contrast, PCR with the specific primers exclusively amplified *P. donghaiense* (Fig. 1b) and yielded 111-bp PCR fragments. Furthermore, qPCR showed that the fluorescent signal denoting successful amplification with the specific primers was only detectable in the reactions containing DNA from *P. donghaiense* (Fig. 1c). That is, this fluorescent signal was not detectable via qPCR in the reactions containing DNA templates from nontarget species and in the negative control without a DNA template (Fig. 1c). These results indicate that the designed primer pair is specific for *P. donghaiense* and thus is suitable for further detection experiments of the field samples.

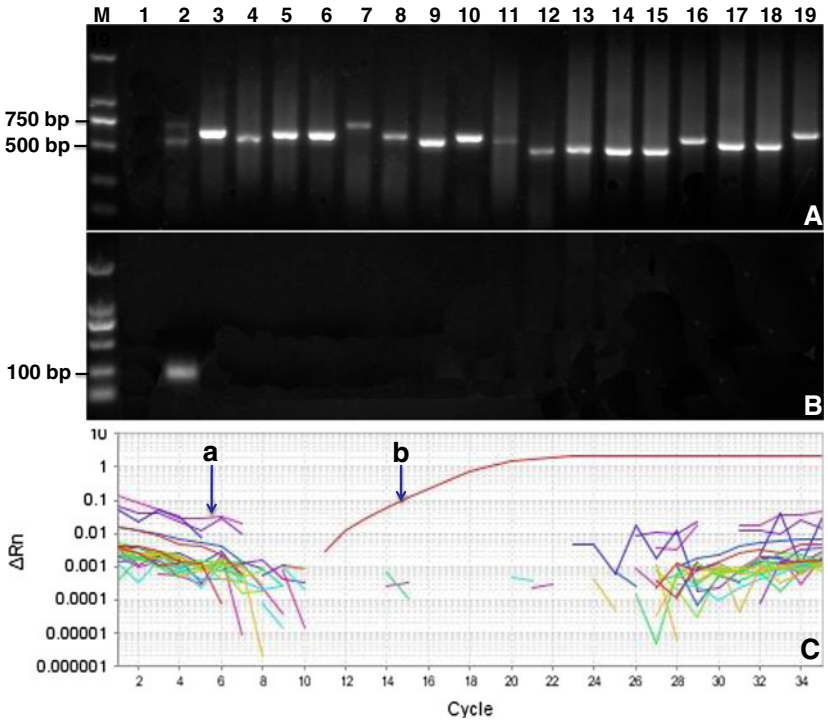

Fig. 1 Specificity test of the specific primers for *P. donghaiense*. **a** Regular PCR with the universal primers (D1/D2). **b** Regular PCR with the specific primers (Q-Pd-f/Q-Pd-r). **c** qPCR with specific primers. *M* DL 2000 or DL 500 DNA markers, *L1* blank control, *L2–L27* are the respective test algae in Table 1 shown from top to bottom; *a* and *b* show the amplification curves of nontarget species and *P. donghaiense*, respectively

Standard curves

Both standard curves with tenfold-diluted recombinant plasmids and crude DNA extracts that represent different cell densities of *P. donghaiense* were established by performing qPCR in the same run. The establishment of these curves was based on the linear relationship between the C_t and the common logarithms for logarithms to base 10 of plasmid copy numbers and cell numbers. The regression equation between the recombinant plasmid and C_t (RE-P) is $y=-3.3407x+39.401$ ($R^2=0.999$), where x and y represent the denary logarithm of plasmid copy numbers ($\lg N_{plasmid}$) and the C_t value (Fig. 2a), respectively. The efficiency of the reaction (E) was calculated to be 99.22 % by using the formula $E=10^{(1/m)}-1$, where m is the slope of the standard curve. Similarly, the regression equation between the *P. donghaiense* cells and C_t is $y=3.2653x+29.907$ ($R^2=0.999$), where x and y represent the basic logarithm of *P. donghaiense* cells ($\lg N_{cell}$) and the C_t value (Fig. 2b), respectively. The efficiency of the reaction was 102.41 %. On the basis of these results, a regression curve representing the mathematical relationship between the cell numbers and the correlated plasmid copy numbers was finally constructed. The corresponding regression equation is $y=0.9774x+2.8418$ ($R^2=0.999$), where x and y represent the basic logarithm of *P. donghaiense* cells ($\lg N_{cell}$) and corresponding plasmid copy numbers (Fig. 3), respectively.

Comparison of DNA template preparation methods

To assess the high sensitivity and efficiency of the established qPCR in detecting field samples, equal cell amounts of *P. donghaiense* (3450 cells) were used to obtain pure DNA via CTAB extraction and crude DNA extract via ultrasonic treatment. Both extracts were used as templates for qPCR. The corresponding plasmid numbers calculated using the RE-P and the C_t value generated by qPCR with the two DNA templates are summarized in Table 4. The estimated corresponding plasmid numbers per cell were 6.53±0.23 for regular DNA extraction (CTAB extraction) and 628.07±38.23 for crude DNA extraction. Therefore, crude DNA extraction was approximately 100-fold more sensitive than regular DNA extraction ($P<0.01$). Additional qPCR with tenfold dilutions (10^0–10^{-5}) of the crude DNA extract (standard curve construction) demonstrated that the C_t value varied from 18.24±0.09 (3450 cells) to 31.20±0.41 (3.45 cells; data not shown). However, no amplification signals were detected with the crude DNA extract representing less than 3.45 cells. Therefore, the developed qPCR method has a limit of 3.45 cells.

Effects of background DNA on qPCR

The performance of the developed qPCR assay was assessed in the presence of background DNA by adding *P. triestinum* or *S. costatum* to the *P. donghaiense* cultures. The results of qPCR with 1 μL of the crude DNA extract for 666 cells are summarized in Table 5. The cell numbers determined by qPCR were estimated to be 509.43±105.99 and 760.61±168.56 for the samples containing *P. triestinum* and *S. costatum*, respectively. The cell numbers calculated by qPCR were not equal to the actual cell numbers determined

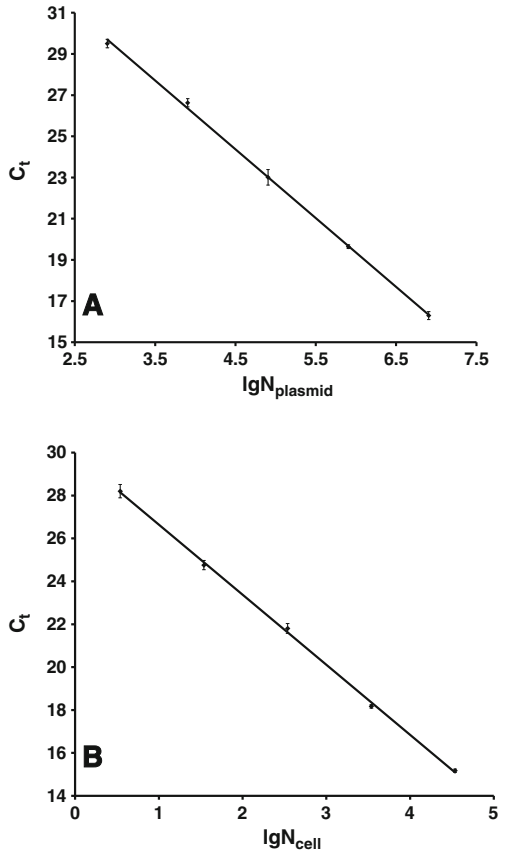

Fig. 2 Standard curves constructed with specific primers for the tenfold-diluted plasmid containing the LSU D1–D2 region of *P. donghaiense* (**a**) and the tenfold-diluted *P. donghaiense* cells (**b**). Values of lg $N_{plasmid}$ and lg N_{cell} represent the common logarithms for logarithms to base 10 of plasmid copy number and cell number, respectively. Values are means± standard deviation n=3

by LM, but no significant differences (P>0.05) were detected between these values. These data indicate that nontarget cells exert no effect on the qPCR detection of target cells.

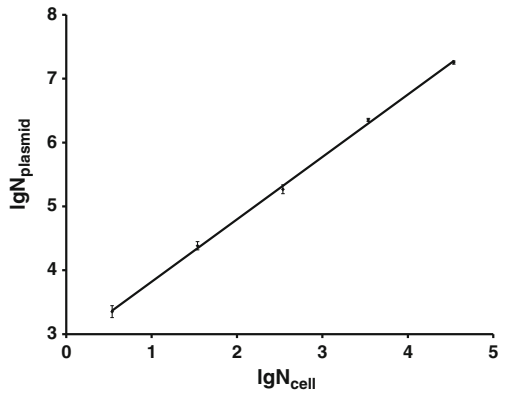

Fig. 3 Regression curve generated from the number of plasmids containing the LSU D1–D2 region and the number of *P. donghaiense* cells. Values of lg $N_{plasmid}$ and lg N_{cell} represent the common logarithms for logarithms to base 10 of plasmid copy number and cell number, respectively. Values are means±standard deviation n=3

Detection stability

The capability of qPCR to recover and detect *P. donghaiense* DNA over a span of 40 days from fixative (Lugol's solution)-preserved and unpreserved algal cultures was assessed with a known number of organisms (Fig. 4). The C_t value remarkably changed from 26.5±0.79 (day 0) to 35.7±0.86 (day 20) in the unpreserved algal cultures. No amplification was detected in the algal cultures that were fixed for more than 20 days. By contrast, amplification signals were detected in the cultures fixed for 40 days. Moreover, the long-term stability was apparent despite the approximate shift of three cycles from a C_t value of 26.5±0.89 to 29.3±0.74.

qPCR assay of simulated and field samples

The applicability of the developed qPCR was tested using simulated and field samples by collecting target cells with a filter membrane instead of centrifugation. For simulation, field seawater was spiked with a known cell number of *P. donghaiense* cultures at various ratios to prepare samples with different target cell densities. The results of qPCR quantification are summarized in Table 6. Despite the tenfold change in cell numbers (from 315.0 cells to 31.5 cells) of the simulated samples, the target cell numbers determined by LM were highly comparable with those determined by qPCR without any significant differences (P>0.05). For further practical application, eight field surface seawater samples (samples 1 to 8) for testing were randomly obtained from the East China Sea (120° 52′ 30″ E, 24° 32′ 28″ N). The cell density of *P. donghaiense* varied from 3.5×10^3 to 6.5×10^4 cell L^{-1} as calculated by LM enumeration. LM and qPCR produced different quantification results, but the difference was not significant for most samples (P>0.05; Fig. 5). Only one sample (sample 8) displayed a significant difference (P<0.05) in the cell densities quantified by LM and qPCR. Therefore, the quantitative results of qPCR are generally comparable with those of LM. In addition, the detection of qPCR is rapid and can be completed within 3–4 h. In conclusion, qPCR is an effective tool for the quick detection of *P. donghaiense* and is particularly promising for the field monitoring of *P. donghaiense*.

Discussion

Considering the serious harm of *P. donghaiense* to marine economy and ecological environment, researchers have focused on searching for novel techniques to identify and quantify this species. Previous studies developed several molecular methods to detect *P. donghaiense*; these methods include FISH (Zhang et al. 2005; Chen et al. 2011), immunofluorescence assay (IA) (Wang et al. 2007), lectin probe-based assay

Table 4 Method comparison of DNA template preparation for qPCR (values are means±standard deviation, $n=3$)

Method for DNA template preparation	Cell number (cells) determined by LM	C_t	Corresponding plasmid number (copy)	Corresponding plasmid number per cell	Randfold P value
DNA extraction	3450	24.86±0.05	$(22.51\pm0.80)\times10^3$	6.53±0.23	1.13E−04
Crude DNA extract	3450	18.24±0.09	$(2.17\pm0.13)\times10^6$	628.07±38.23	

(LPBA) (Huang et al. 2008), sandwich hybridization integrated with nuclease protection assay (NPA-SH) (Zhen et al. 2009), and loop-mediated isothermal amplification (LAMP) (Chen et al. 2013a, b). However, the current methods display obvious disadvantages, including poor sensitivity (FISH, IA, LPBA, and NPA-SH), inaccurate quantification due to unstable RNA (NPA-SH), and nonquantitative results (LAMP). In addition, SYBR Green I real-time PCR yields unspecific results. Thus, Yuan et al. (2012a, b) established a real-time PCR method (Taqman) that targets internal transcribed spacers to quantify *P. donghaiense* cells. However, this PCR method requires a specific Taqman probe, rendering it less simple and less economical compared with SYBR Green I qPCR. Considering its specificity, sensitivity, precise quantification, economy, and rapidity, a qPCR assay with SYBR Green I was developed in this study to target the LSU and quantify *P. donghaiense* cells.

Specificity is an important factor to be considered in molecular detection. The detection specificity of qPCR is mainly dependent on specific primers. Three sequential steps were adopted to guarantee primer specificity. First, all the representative LSU sequences of *Prorocentrum* species available in GenBank were downloaded for alignment to search for variable regions, from which specific primers were designed. Second, the designed primers were used in a BLAST search to confirm their specificity for *P. donghaiense*. Finally, the designed primers were tested with several microalgae in our laboratory. As expected, the results demonstrated that the primers were specific. However, this specificity is "temporary." One reason is that only a limited number of *Prorocentrum* have their related sequences deposited in GenBank. Another reason is that we were unable to perform the current specificity test with all the algal species of the genus *Prorocentrum*. Only four *Prorocentrum* species, namely, *Prorocentrum micans*, *P. triestinum*, *Prorocentrum lima*, and *Prorocentrum minimum*, were used because of the unavailability of other species. *P. maximum* and *P. dentatum* are both potentially synonymous to *P. donghaiense*; hence, the possible cross-reactivity with these species cannot be excluded. The specific primers were tested against several microalgae from five taxonomic classes that represent widespread species in the East China Sea. These microalgae possibly coexist with *P. donghaiense*, which may result in nonspecific detection. No cross-reactions occurred with these microalgae, proving that the primers used for qPCR are suitable for detecting samples from the East China Sea.

Two main types of quantitative strategies that rely on standard curves are employed in the current qPCR protocols to quantify algal cells. One method depends on cellular standard curves generated by using serial dilutions of DNA extracts from clonal cultures (Shi et al. 2010; Andrée et al. 2011) or crude lysates of target cell serial dilutions spiked into natural and filtered seawater or culture media (Fitzpatrick et al. 2010; Garneau et al. 2011) to determine the cell number in the field sample. The other method involves calculating the number of target genes per cell from a standard curve generated from the serial dilutions of plasmids containing the cloned target sequence (generally ribosomal RNA genes) combined with another curve generated from unknown target cells (Créach et al. 2006; Cary et al. 2014). Given their simple and convenient extraction and their stability in storage, recombinant plasmids are suitable for standard samples. However, degradation may occur after frequent freezing and thawing of genomic DNA, especially crude DNA extracts (Bowers et al. 2000). This phenomenon results in unreliable calculations for the target sequences. Another advantage of using plasmids is that the measured optimal density can be easily used to calculate the plasmid concentration even after long-term storage and frequent freezing and thawing. In the present study, two series of standard samples were used in parallel for qPCR to establish a mathematical relationship between the plasmid copy number and the cell number. The cell number in the unknown samples could then be easily determined from the plasmid standard samples. Therefore, the proposed quantification method is clearly simple. The effectiveness of this quantification strategy was also validated by the subsequent tests.

Table 5 Effects of nontarget species on the detection of target species by qPCR (values are means±standard deviation, and $n=3$)

Nontarget species	Target cell number (cells) determined by LM	C_t	Target cell number (cells) determined by qPCR	Randfold P value
Prorocentrum triestinum	666	21.04±0.31	509.43±105.99	0.08
Skeletonema costatum	666	20.46±0.30	760.61±168.56	0.52

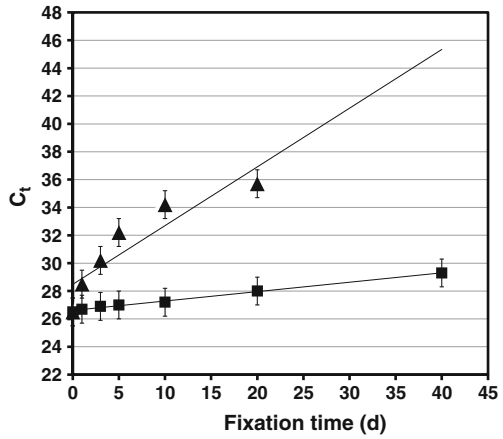

Fig. 4 qPCR detection of *P. donghaiense* in fixative (Lugol's acid solution)-preserved cultures for 45 days. DNA from all time points was assayed in the same PCR run. *Filled triangles* represent unpreserved samples and *filled squares* represent fixative-fixed samples. Values are means±standard deviation $n=3$

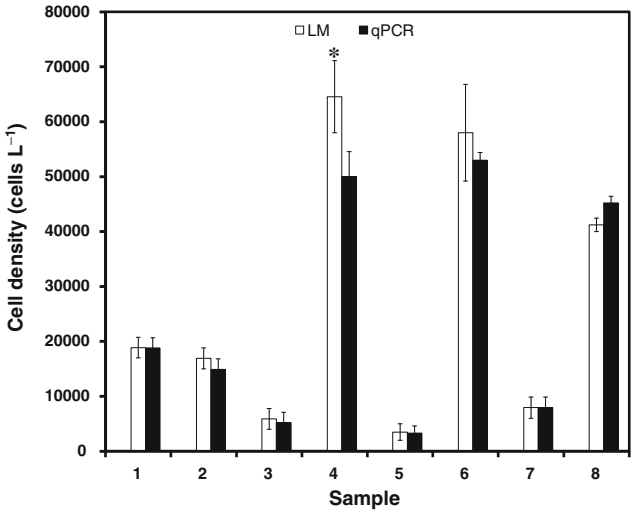

Fig. 5 Detection of field samples through LM and qPCR. Values are means±standard deviation. *Error bars* represent standard deviation, $n=3$. *Asterisk* indicates a statistically significant difference ($p<0.05$) between LM and qPCR methods

DNA extraction influences the detection sensitivity of molecular methods such as RQT-PCR. Methods that have been employed to isolate genomic DNA use phenol (Hosoi-Tanabe and Sako 2005; Yuan et al. 2012a, b), phenol/CTAB (Kamikawa et al. 2007), CTAB (Lin et al. 2006; Murray et al. 2011), and commercial kits (Casper et al. 2004; Erdner et al. 2010). Highly pure genomic DNA can be obtained from marine algae with these methods, but the DNA yield and extraction reproducibility may be poor. The unstable extraction efficiency would lead to over- or underestimation of cell numbers. Therefore, crude lysates of cultured and natural samples may be ideal alternatives for qPCR quantification. In the present study, two preparation methods for DNA templates were compared. Crude DNA extraction was proven more efficient than the CTAB method; thus, the former method may produce more sensitive results than the latter. Further tests for sensitivity showed that qPCR has a detection limit of 3.45 cells. In addition, crude DNA extraction is simpler, faster, and cheaper than the CTAB method.

The biological composition of field samples is diverse and changes with the physical, chemical, and biological conditions of seawater. A favorable molecular method should be adaptable for variable field samples. Given this point, the practicality of the established qPCR was evaluated with DNA mixtures of target and nontarget DNA. Algal mixtures containing the target and nontarget cells were simulated to test whether or not nontarget cells interfere with the detection of target cells by qPCR. *S. costatum* and *P. triestinum* were selected as nontarget species in the present study because *Skeletonema* species are likely to occur in nearly all samples from the coast because of its cosmopolitan nature (Zingone et al. 2005) and *P. triestinum* is a coexisting *Prorocentrum* species in the East China Sea (Wang et al. 2012). The current results showed that the detection performance of qPCR was not affected by nontarget species, indicating that this method may be competent for natural samples that contain different components other than the target cells.

Most samples for environmental monitoring and ecological investigation usually need to be sent to the laboratory for qPCR quantification. Therefore, much time is often spent on the shipping and treatment of samples before cell quantification is completed. Consequently, an experiment was designed to explore the effects of sample preservation time on the detection performance of qPCR. Reasonableness suggests that DNA is relatively stable. However, trace DNA degradation may be detected by the highly sensitive qPCR, as confirmed by Bowers et al. (2000) and the current study. Nonetheless, the present results indicate that fixative (Lugol's acid solution)

Table 6 Verification of qPCR by using simulated field samples (values are means±standard deviation, $n=3$)

Volume ratios of field samples to algal cultures	Target cell number (cells) determined by LM	C_t	Target cell number (cells) determined by qPCR	Randfold *P* value
10 mL:10 mL	315.0	21.70±0.07	317.80±15.69	0.75
15 mL:5 mL	157.5	22.42±0.19	193.47±26.48	0.06
19 mL:1 mL	31.5	22.76±0.22	38.05±5.92	0.08

preservation can greatly prevent the destruction of nucleic acid molecules. Moreover, the effects of fixation time on the detection performance may be ignored because the subtle change in C_t did not cause an order of magnitude change in the cell change (data not shown), which is important for environmental monitoring. Therefore, the developed qPCR is competent for fixative-preserved samples. However, a competent alternative fixative to Lugol's acid solution is required to increase the accuracy of the qPCR detection of samples preserved for a long time. This concern should also be considered in future studies.

The ultimate objective of this study was to apply qPCR to environmental monitoring and ecological investigation. The applicability of the developed method for quantifying algal cells should be confirmed using simulated and field samples before its formal use. For this purpose, simulated samples representing a tenfold change in the target cell density were used for qPCR. These conditions indicate that the probable density of the target cells in the natural samples is greatly variable. Results showed that qPCR can accurately quantify the target cells, even in natural samples with different target cell numbers. Furthermore, natural samples randomly collected from the East China Sea were analyzed via qPCR. The cell density determined by LM was generally comparable with that determined by qPCR. In addition, the detection procedure would save time if cells are collected by filtration and the crude DNA extract is used as the amplification template. These adjustments allow a large number of samples to be analyzed. However, at least two more tests should be performed to assess the established qPCR method before its practical use. First, more field samples should be used to test the effectiveness of the method. Second, different strains of *P. donghaiense* should be used to assess the accuracy of the method because the possible variability of the rDNA copy number among strains could impair the accuracy of quantitative analysis.

In summary, we developed a qPCR assay to detect *P. donghaiense*. This protocol employed a specific primer pair designed from the LSU D1–D2 sequence of *P. donghaiense.* The specificity of the primers was confirmed using several microalgae distributed along the Chinese coast. Two standard curves were constructed with the tenfold-diluted plasmid solution and crude DNA extract from known cell numbers of target species. These standard curves were used to establish a mathematic relationship between plasmid number and cell number. The tenfold-diluted plasmid was conveniently used to perform qPCR in parallel to quantify the target cells in the samples. The performance of the developed qPCR was unaffected by background DNA. The detection effect is relatively stable and suitable to quantify fixative (Lugol's acid solution)-preserved samples. The developed qPCR can be applied to analyze field samples, thereby providing a convenient tool for various critically important environmental monitoring initiatives, such as long-term monitoring programs or timely warnings of *P. donghaiense* blooms.

Acknowledgments This work was supported by the National Scientific Foundation of China (41476086, 41106082, 41176141); the S & T Project of Shenzhen Science and Technology Innovation Committee (JSKF201505293000320); the Open Fund of Key Laboratory of Marine Ecology and Environmental Science, Institute of Oceanology, Chinese Academy of Sciences (KLMEES201303); and the Basic Research of Harbin Institute of Technology Outstanding Talents Cultivation Plan of Class III.

References

Andrée KB, Fernandez-Tejedor M, Elandaloussi LM, Quijano-Scheggia S, Sampedro N, Garcés E, Camp J, Diogene J (2011) Quantitative PCR coupled with melt curve analysis for detection of selected *Pseudo-nitzschia* spp. (Bacillariophyceae) from the northwestern Mediterranean Sea. Appl Environ Microbiol 77:1651–1659

Bowers HA, Tengs T, Glasgow HB, Burkholder JM, Rublee PA, Oldach DW (2000) Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl Environ Microbiol 66:4641–4648

Cai Q, Li R, Zhen Y, Mi T, Yu Z (2006) Detection of two *Prorocentrum* species using sandwich hybridization integrated with nuclease protection assay. Harmful Algae 5:300–309

Cary SC, Coyne KJ, Rueckert A, Wood SA, Kelly S, Gemmill CEC, Vieglais C, Hicks BJ (2014) Development and validation of a quantitative PCR assay for the early detection and monitoring of the invasive diatom *Didymosphenia geminata*. Harmful Algae 36: 63–70

Casper ET, Paul JH, Smith MC, Gray M (2004) Detection and quantification of the red tide dinoflagellate *Karenia brevis* by real-time nucleic acid sequence-based amplification. Appl Environ Microbiol 70:4727–4732

Chen GF, Zhang CY, Zhang BY, Wang GC, Lu DD, Xu Z, Yan PS (2011) Development of a PNA probe for fluorescence in situ hybridization detection of *Prorocentrum donghaiense*. PLoS One 6:e25527

Chen GF, Liu Y, Zhang CY, Ma CS, Zhang BY, Wang GC, Xu Z, Lu DD (2013a) Development of rRNA-targeted probes for detection of *Prorocentrum micans* (Dinophyceae) using whole cell in situ hybridization. J Appl Phycol 25:1077–1089

Chen GF, Ma CS, Zhang CY, Zhou J, Wang YY, Wang GC, Zhang BY, Xu Z, Lu DD (2013b) A rapid and sensitive method for field detection of *Prorocentrum donghaiense* using reverse transcription-coupled loop-mediated isothermal amplification. Harmful Algae 29:31–39

Chomerat N, Sellos DY, Zentz F, Nezan E (2010) Morphology and molecular phylogeny of *Prorocentrum consutum* sp. nov. (Dinophyceae), a new benthic dinoflagellate from South Brittany (northwestern France). J Phycol 46:183–194

Créach V, Ernst A, Sabbe K, Vanelslander B, Vyverman W, Stal LJ (2006) Using quantitative PCR to determine the distribution of a semicryptic benthic diatom, *Navicula phyllepta* (Bacillariophyceae). J Phycol 42:1142–1154

Diercks S, Medlin LK, Metfies K (2008) Colorimetric detection of the toxic dinoflagellate *Alexandrium minutum* using sandwich hybridization in a microtiter plate assay. Harmful Algae 7:137–145

Erdner DL, Percy L, Keafer B, Lewis J, Anderson DM (2010) A quantitative real-time PCR assay for the identification and enumeration of *Alexandrium* cysts in marine sediments. Deep-Sea Res II 57:279–287

Faust MA, Vandersea MW, Kibler SR, Tester PA, Litaker RW (2008) *Prorocentrum levis*, a new benthic species (Dinophyceae) from a mangrove island, Twin Cays, Belize. J Phycol 44:232–240

Fitzpatrick E, Caron D, Schnetzer A (2010) Development and environmental application of a genus-specific quantitative PCR approach for *Pseudo-nitzschia* species. Mar Biol 157:1161–1169

Garneau M, Schnetzer A, Countway PD, Jones AC, Seubert EL, Caron DA (2011) Examination of the seasonal dynamics of the toxic dinoflagellate *Alexandrium catenella* at Redondo Beach, California, by quantitative PCR. Appl Environ Microbiol 77:7669–7680

Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Press, New York, pp 29–60

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hosoi-Tanabe S, Sako Y (2005) Species-specific detection and quantification of toxic marine dinoflagellates *Alexandrium tamarense* and *A. catenella* by real-time PCR assay. Mar Biotechnol 7:506–514

Huang H, Wu MG, Lei HL, Lai HY, Hou JJ (2008) Quantitative detection of harmful algae species using lectin probes. J Fish Sci China 15: 279–292

Kamikawa R, Nagai S, Hosoi-Tanabe S, Itakura S, Yamaguchi M, Uchida Y, Baba T, Sako Y (2007) Application of real-time PCR assay for detection and quantification of *Alexandrium tamarense* and *Alexandrium catenella* cysts from marine sediments. Harmful Algae 6:413–420

Ki JS, Han MS (2006) A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridisation of consensus PCR products of LSU rDNA D2 domain. Biosens Bioelectron 21:1812–1821

Li J, Glibert PM, Zhou MJ, Lu SH, Lu DD (2009) Relationships between nitrogen and phosphorus forms and ratios and the development of dinoflagellate blooms in the East China Sea. Mar Ecol Prog Ser 383:11–26

Lin S, Zhang H, Dubois A (2006) Low abundance distribution of *Pfiesteria piscicida* in pacific and western Atlantic as detected by mtDNA-18S rDNA real-time polymerase chain reaction. J Plankton Res 28:667–681

Long H, Zhou Y, Yu J, Hu YF, Fu GJ (2008) Analyses on harmful algal blooms in Zhejiang coastal waters from 2001 to 2007. Mar Environ Sci 27(suppl 1–4):11–18

Lu DD, Goebel J (2001) Five red tide species in genus *Prorocentrum* including the description of *Prorocentrum donghaiense* Lu sp. nov. from the East China Sea. Chin J Oceanogr Limnol 19:337–344

Lu DD, Qi YZ, Goebel J, Zou JZ, Gao YH (2003) Redescription of *Prorocentrum donghaiense* Lu and comparison with relevant *Prorocentrum* species. Chin J Appl Ecol 14:1060–1064

Lu DD, Goebel J, Qi YZ, Zou JZ, Han XT, Gao YH, Li RX (2005) Morphological and genetic study of *Prorocentrum donghaiense* Lu from the East China Sea, and comparison with some related *Prorocentrum* species. Harmful Algae 4:493–505

Mikulski CM, Park YT, Jones KL, Lee CK, Limb WA, Lee Y, Scholin CA, Doucette GJ (2008) Development and field application of rRNA-targeted probes for the detection of *Cochlodinium polykrikoides* Margalef in Korean coastal waters using whole cell and sandwich hybridization formats. Harmful Algae 7:347–359

Murray SA, Nagahama Y, Fukuyo Y (2007) Phylogenetic study of benthic, spine-bearing prorocentroids, including *Prorocentrum fukuyoi* sp. nov. Phycol Res 55:91–102

Murray SA, Wiese M, Stüken A, Brett S, Kellmann R, Hallegraeff G, Neilan BA (2011) STXA-based quantitative molecular assay to identify saxitoxin-producing harmful algal blooms in marine waters. Appl Environ Microbiol 77:7050–7057

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 20:6851–6862

Percopo I, Siano R, Cerino F, Sarno D, Zingone A (2011) Phytoplankton diversity during the spring bloom in the northwestern Mediterranean Sea. Bot Mar 54:243–267

Popels LC, Cary SC, Hutchins DA, Forbes R, Pustizzi F, Gobler CJ, Coyne KJ (2003) The use of quantitative polymerase chain reaction for the detection and enumeration of the harmful alga *Aureococcus anophagefferens* in environmental samples along the United States East Coast. Limnol Oceanogr Methods 1:92–102

Qi YZ, Wang Y (2003) What the *Prorocentrum* species should be?—a review on identification of *Prorocentrum* species from the East China Sea. Chin J Appl Ecol 14:1188–1190

Scholin CA, Herzog M, Sogin M, Anderson DM (1994) Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae): sequence analysis of a fragment of the LSU rRNA gene. J Phycol 30:999–1011

Scholin CA, Buck KR, Britschgi T, Cangelosi G, Chavez FP (1996) Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. Phycologia 35:190–197

Shi YH, Zhang FY, Ma LB (2010) Development of a real-time PCR assay for rapid detection and quantification of *Heterocapsa circularisquama*. J Fish Sci China 17:267–273

Smith MW, Maier MA, Suciu D, Peterson TD, Bradstreet T, Nakayama J, Simon HM (2012) High resolution microarray assay for rapid taxonomic assessment of *Pseudo-nitzschia* spp. (Bacillariophyceae) in the field. Harmful Algae 19:169–180

Tang DL, Di BP, Wei GF, Ni IH, Oh IS, Wang SF (2006a) Spatial, seasonal and species variations of harmful algal blooms in the South Yellow Sea and East China Sea. Hydrobiologia 568:245–253

Tang JL, Mao HY, Guo MR (2006b) Analysis on the red tide of *Prorocentrum dentatum* in the north coastal water of Zhejiang. Mar Environ Sci 25:63–66

Wang DZ, Huang XG, Chan LL, Hong HS (2007) Development of an immunofluorescence technique for detecting *Prorocentrum donghaiense* Lu. J Appl Phycol 19:325–332

Wang HX, Lu DD, He PX, Xia P, Dai XF (2012) Morphological characteristic and ITS sequence analysis of *Prorocentrum triestinum* isolated from the East China Sea. Acta Oceanol Sin 34:155–162

Yuan J, Mi TZ, Zhen Y, Yu ZG (2012a) Development of a rapid detection and quantification method of *Karenia mikimotoi* by real-time quantitative PCR. Harmful Algae 17:83–91

Yuan J, Mi TZ, Zhen Y, Yu ZG (2012b) Development of a real-time PCR method (Taqman) for rapid identification and quantification of *Prorocentrum donghaiense*. J Ocean Univ China 11:366–374

Zhang BY, Wang GC, Qi YZ, Zou JZ, Tseng CK (2005) Identification of two species of dinoflagellate using fluorescence in situ hybridization. Chin High Technol Lett 15:101–105

Zhen Y, Mi TZ, Yu ZG (2009) Detection of several harmful algal species by sandwich hybridization integrated with a nuclease protection assay. Harmful Algae 8:651–657

Zingone A, Percopo I, Sims PA, Sarno D (2005) Diversity in the genus *Skeletonema* (Bacillariophyceae) I. A reexamination of the type material of *S. costatum* with the description of *S. grevillei* sp. nov. J Phycol 41:140–150