Rapid Identification and Quantification of *Aureococcus anophagefferens* by qPCR Method (Taqman) in the Qinhuangdao Coastal Area: A Region for Recurrent Brown Tide Breakout in China

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Abstract Since 2009, *Aureococcus anophagefferens* has caused brown tide to occur recurrently in Qinhuangdao coastal area, China. Because the algal cells of *A. anophagefferens* are so tiny ($\sim 3\ \mu\text{m}$) that it is very hard to identify exactly under a microscope for natural water samples, it is very urgent to develop a method for efficient and continuous monitoring. Here specific primers and Taqman probe are designed to develop a real-time quantitative PCR (qPCR) method for identification and quantification continually. The algal community and cell abundance of *A. anophagefferens* in the study area (E $119^{\circ}20'–119^{\circ}50'$ and N $39^{\circ}30'–39^{\circ}50'$) from April to October in 2013 are detected by pyrosequencing, and are used to validate the specification and precision of qPCR method for natural samples. Both pyrosequencing and qPCR shows that the targeted cells are present only in May, June and July, and the cell abundance are July > June > May. Although there are various algal species including dinoflagellata, diatom, Cryptomonadales, Chrysophyceae and Chlorophyta living in the natural seawater simultaneously, no disturbance happens to qPCR method. This qPCR method could detect as few as 10

targeted cells, indicating it is able to detect the algal cells at pre-bloom levels. Therefore, qPCR with Taqman probe provides a powerful and sensitive method to monitor the brown tide continually in Qinhuangdao coastal area, China. The results provide a necessary technology support for forecasting the brown tide initiation, in China.

Keywords Harmful algal bloom (HAM) · Brown tide bloom · *Aureococcus anophagefferens* · Pyrosequencing · Real-time quantitative PCR (qPCR)

Introduction

Liaodong Bay is located in the northeast of the Bohai Sea, China, and is semi-enclosed. Qinhuangdao coastal area locates at the south-western corner of Liaodong Bay, and this region is a shallow embayment. Since 2009, large-scale recurrent harmful algal blooms (HAB) have occurred in this region and caused destructive effects on the local shellfish mariculture industry [1]. The causative species of this kind of bloom had been identified as *A. anophagefferens* using clone libraries of 18S rDNA sequence [1], thus China become the third country all over the world that has occurred *Aureococcus* blooms except for USA and South Africa [1, 2]. Qinhuangdao coastal region has attracted widely attention by the related researchers rapidly. The detection of the dominant algal species' succession process within the algal assemblages in the concerned region may be of specific importance for eutrophication mechanism and algal bloom breakout as algal cells affiliated to different species favor the different environment conditions. Furthermore, the rapid detection and early-warning method for *A. anophagefferens* should be formulated as soon as possible.

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For traditional methods of algal species identification, the algal cells are observed under a microscope, and the algal population densities (cells ml⁻¹) are estimated using the sedimentation technique. However, the algal cells of *A. anophagefferens* are so tiny (~3 µm) and no specific morphological characteristics that it is very hard to identify them under a microscope. Alternatively, molecular techniques which do not depend on the morphological characteristics of algal cells have therefore been used to monitor algal populations in a variety of environmental samples. The next generation sequencing technology of pyrosequencing allows a more complete view of the grain communities' overall composition, and ensure that even a small proportion of the population is detected [3]. In previous studies, pyrosequencing have been used to monitor the microbial communities successfully in various environments [4–7]. Meanwhile, real-time quantitative PCR (qPCR) is developed to quantify microbial population based on specific genes, and qPCR has become an effective and powerful tool for the detection of harmful algal bloom species [8–10].

All the monitoring is performed from April to October that belong to a breakout sensitive period of brown tide bloom over 1 year. The results monitored by pyrosequencing are used to validate the specification and precision of qPCR method for detecting the targeted cells in the field.

Materials and Methods

Study Area, Samples Collection and DNA Extraction

Five stations are selected in the Qinhuangdao coastal area where brown tide has broken out recurrently (Supplementary Fig. 1). The field sampling is carried out in the early days from April to October, 2013. A global positioning system (GPS) is used for precise positioning of each station. The water temperature (T), pH and dissolved oxygen (DO) at the surface water (<0.5 m depth) is detected using the Multi-parameter Water Quality Meter (YSI, USA) in situ. Surface water (0–20 cm) are collected for determine chemical oxygen demand (COD_{Mn}), total dissolved nitrogen (TDN), nitrate nitrogen (NO₃-N), nitrite nitrogen (NO₂-N), ammonia nitrogen (NH₄-N), total dissolved phosphorus (TDP) and phosphate phosphorus (PO₃-P) using standard methods. For chlorophyll a (Chla) analysis, a known volume of water is filtered into GF/F filter, and the filter is kept refrigerated and analyzed spectrophotometrically in acetone extracts. Another surface water samples are collected in clean sterile sampling bottle. The water samples are prefiltered (250 µm) to remove the large

zooplankton and detritus, and filtered using 0.45 µm within 12 h. The filters are stored in -20 °C for DNA extraction. The PowerMax Soil DNA Isolation Kits (MO BIO Laboratories Inc., Carlsbad, CA, USA) is used for DNA extraction according to the manufacturer's instruction. All DNA samples are purified using AxyPrep™ DNA Gel Extraction Kit. The DNA extract of 5 station samples in each month are mixed evenly, respectively. And the mixed DNA extract as the representative sample of each month are used for the following analysis.

Primer and Probe Development

qPCR are performed to quantify the *A. anophagefferens* in triplicate using the ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). In order to improve the monitoring sensitivity, a new primer set (Aap-18S-F, Aap-18S-R) and a probe (Aap-probe) are designed based on our available sequences in the clone libraries, which specifically targeted 167-bp 18S fragments of *A. anophagefferens*. The sequences are as following:

Aap-18S-F: 5'-AAAGCTCGTAGTTGGATTCTCTGG-3'

Aap-18S-R: 5'-GTGTTCAACGCAGGCTTACG-3'

Aap-probe: 5'-CTTGCGATGGTCTATCCT-3'

The qPCR is performed in 10-µl volume mixture. The reaction mixture contains 5 µl of KAPA probe fast qPCR Master Mix (2×) Universal, 0.25 µM of each primer (Aap-18S-F, Aap-18S-R), 0.25 µM Aap-probe, 1 µl of the purified DNA template, 0.2 µl of ROX adjust dye, and 3.15 µl ddH₂O. The qPCR is started with 3 min pre-incubation at 95 °C and followed by an amplification of 40 cycles of 3 s at 95 °C and 20 s at 60 °C, and melting curves of 15 s at 95 °C, 15 s at 60 °C and 15 s at 95 °C. Agarose gel electrophoresis and melting-curve analysis are used to confirm the specificities of qPCR primer/probe.

qPCR Standard Curves

The plasmids containing specific 18S fragments of *A. anophagefferens* are used as quantitative standards. The specific gene fragment amplified from the pure algal cells of *A. anophagefferens* (CCMP1784) are ligated into the pMD18-T vector and cloned into JM109 competent cells (TAKARA BIO INC, Shiga, Japan). The method of obtaining plasmid DNA is described in detail in Wang et al. [7]. The positive plasmids with targeted sequence are sent to Beijing Liuhe Tong Trade Co., LTD for sequencing. The sequence is 168 bp, and 100 % homogenous with 18S rRNA of *A. anophagefferens* with accession no. JQ420084.1, JQ420083.1, JQ420082.1, JQ420078.1, JQ420077.1, HQ710572.1, AF117778.1, AF117776.1, AF117779.1, AF119119.1, AF117777.1 and AF118443. Therefore,

these plasmids with 100 % homogenous with 18S rRNA of *A. anophagefferens* can be used to develop the standard curve. The sequence of plasmid is submitted to the GenBank with the Accession no. KT585673. The concentration of *A. anophagefferens* recombinant plasmid solution is measured by the determination of optical density at 260 nm. A series of plasmid standard samples are prepared using ten times series dilution of plasmid DNA with 3.79×10^3 to 3.79×10^9 gene copies μl^{-1} , and each gradient are repeated three times.

In addition, DNA standards with known *A. anophagefferens* cells from the purified cultured algal strains are extracted, and amplified with the universal primers for eukaryote (SSU-F: 5'-ACC TGG TTG ATC CTG CCA GT-3' and SSU-R: 5'-TCA CCT ACG GAA ACC TTGT-3'). The PCR conditions are as follows: 95 °C for 2 min; 35 cycles of denaturation (94 °C; 20 s), annealing (54 °C; 30 s), and extension (72 °C; 2 min); followed by the final elongation (72 °C; 12 min). Then the PCR product is sent to Beijing Liuhe Tong Trade Co., LTD for sequencing. The sequence is 1717 bp, and highly homogenous with 18S rRNA of *A. anophagefferens* with accession no. HQ710572.1 (100 %), JQ420078.1 (99 %), AF117778.1 (99 %), AF117776.1 (99 %), AF119119.1 (99 %), AF117777.1 (99 %), AF118443.1 (99 %), AF117779.1 (99 %), JQ420083.1 (99 %), JQ420077.1 (99 %), JQ420082.1 (99 %), JQ420084.1 (99 %), JQ420080.1 (99 %), JQ420079.1 (99 %) and JQ420081.1 (99 %). Thus the DNA solution is confirmed as the DNA standard of *A. anophagefferens* and is able to be used to develop the standard curve. The sequence of DNA standard is submitted to the GenBank with the Accession no. KT585674. A series of cell standard samples (from 3.0×10^1 to 3.0×10^6 cells) is prepared by ten times series dilution of the crude DNA solution. The DNA standards are used as quantitative standards.

Thus two standard curves are obtained (Fig. 1): threshold cycle (C_t) versus the denary logarithms of the recombinant plasmid copy numbers ($\lg C_{\text{plasmid}}$); C_t versus the denary logarithms of cell numbers ($\lg N_{\text{cell}}$). Finally, the qPCR standard curve of plasmids versus algal cell numbers is developed. Each standard sample concentration is carried out in triplicate.

PCR and Pyrosequencing

DNA-amplification is performed with the universal 16S rRNA gene primers for eukaryote (Euk1F: 5'-GGA GGG CAA GTC TGG T-3' and 5'-ARC GGC CAT GCA CCA CC-3'). The PCR mixture (final volume, 20 μl) contains 4 μl fivefold reaction buffer (TransStart™ FastPfu Buffer, TransGen Biotech), 10 ng sample DNA, 0.4 μl for each primer (5 μM), 0.4 μl Pfu polymerase (TransStart™

FastPfu DNA Polymerase, TransGen Biotech), and 2 μl dNTPs (2.5 mM), finally added ddH₂O to 20 μl . For each sample, three independent PCRs are run using a MG96 + Thermal Cycler (LongGene Scientific Instruments Co., Ltd). The PCR conditions are as follows: 95 °C for 2 min, and 30 PCR cycles of 95 °C for 0.5 min and 55 °C for 0.5 min, then extension of 72 °C for 1 min, followed by the final elongation (72 °C; 10 min). A quantitative analysis of DNA is made by TBS-380 Mini-Fluorometer (Promega Corporation, CA, USA).

The pyrosequencing and data analysis are performed according to Zheng et al. [11]. The DNA distance matrices are calculated by the DNADIST program in PHYLIP. The matrices are used to define the number of operational taxonomic units (OTUs). OTUs that reached the level of 97 % similarity are used to analyze based coverage estimator (ace), richness (chao) and Shannon diversity by the MOTHUR program [12]. The 16S rRNA gene sequences derived from pyrosequencing are deposited in the NCBI Short Read Archive under accession number SRP051296.

Validation of qPCR Method

The algal cells of *A. anophagefferens* are collected at the exponential phase from the purified cultured algal strains. The algal cell density is 3.2×10^6 cells l^{-1} . Then the cell samples with 3.2×10^6 , 3.2×10^3 and 3.2×10^2 cells are obtained by filtering known volumes of algal culture. The DNA is extracted from these sample filters and purified following the method as described in “[Study Area, Samples Collection and DNA Extraction](#)” section. The DNA extracted from the algal cells of *Skeletonema costatum*, *Phaeodactylum tricornutum*, *Chlorella* sp., and *Prorocentrum micans* are used as the negative control. The qPCR is performed according to the method in “[Primer and Probe Development](#)” section. In addition, the DNA solution from the field samples in “[Study Area, Samples Collection and DNA Extraction](#)” section are mixed with qPCR reagents, and the cell abundance of *A. anophagefferens* in the study area is determined by using the qPCR method as described above. Thus validation of qPCR method is performed using laboratory samples and natural seawater.

Statistics Analysis

Each qPCR run includes the standard curve, established by serially diluted plasmid containing the target sequence or DNA standards of the target algal cells, and a no-template control. And each sample is performed in triplicate.

Student's *t* test is applied in comparing the following cases. For the validation of qPCR method, cell abundances calculated by qPCR are compared with the results by traditional microscopy techniques.

Fig. 1 The qPCR amplification graph (a) and standard curve (b) of recombinant plasmids. $C_t = 42.341 - 3.241 \times \lg C_{\text{plasmid}}$ ($R^2 = 0.999$; Eff% = 103.474); the qPCR amplification graph (c) and standard

curve (d) of *A. anophagefferens* cells. $C_t = 38.631 - 3.200 \times \lg N_{\text{cell}}$ ($R^2 = 0.998$; Eff% = 105.334)

Results

The Environmental Conditions in the Study Region

The environmental conditions including T, pH, DO, COD_{Mn} , nutrients and Chla are determined at the sampling time, and the trophic level index (TLI) is calculated. Each environmental factor has certain fluctuation with the changing month. The T, pH, DO and COD_{Mn} range from 9.0–28.5 °C, 6.69–8.56, 4.46–8.76 mg l^{-1} and 1.16–3.44 mg l^{-1} , respectively. The concentration (mg l^{-1}) of TDN, $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ range from 1.006–2.677, 0.116–0.660, 0.089–0.264, 0.015–0.164, respectively. The concentration (mg l^{-1}) of TDP and $\text{PO}_3\text{-P}$ range from 0.015–0.048 and 0.009–0.026, respectively. The Chla varies between 1.978 and 23.215 $\mu\text{g l}^{-1}$, and the highest concentration occurs in August. The TLI varies between 37.99 and 57.91, indicating a mesotrophic state at the sampling time.

Specificity of Primer, Probe and Detection of Laboratory Samples

The results are showed in Supplementary Fig. 2, and it confirms that the primer and probe are specific to *A. anophagefferens*.

The comparison of cell abundance quantified by microscope and qPCR for laboratory examples is showed in Table 1. There are no significant differences in cell abundance between microscope and qPCR method ($p > 0.05$), suggesting good potential of qPCR in quantification for *A. anophagefferens* with high specificity.

Algal Monitoring in the Field

The number of sequences after filtering and OTUs, richness (chao1, ace) and Shannon index of the algal communities by pyrosequencing analysis in the studied area is showed in Supplementary Table 1. Total 56,047 high quality partial

Table 1 Comparison of microscope and qPCR for detection of *A. anophagefferens* with laboratory examples

Cell number by microscope (cells)	C_t value	Cell number calculated by qPCR (cells)	t test
3.2×10^6	18.0562	2.69×10^6	$p > 0.05$
	18.0033	2.79×10^6	
	17.7759	3.29×10^6	
	Mean: 17.9451 ± 0.1489	Mean: $(2.92 \pm 0.32) \times 10^6$	
3.2×10^3	26.9956	4.33×10^3	$p > 0.05$
	27.0688	4.10×10^3	
	27.1635	3.83×10^3	
	Mean: 27.0760 ± 0.0842	Mean: $(4.09 \pm 0.25) \times 10^3$	
3.2×10^2	30.6672	3.08×10^2	$p > 0.05$
	30.6425	3.14×10^2	
	30.8653	2.67×10^2	
	Mean: 30.7250 ± 0.1221	Mean: $(2.96 \pm 0.26) \times 10^2$	

16S rDNA sequences and 1469 OTUs (at the 97 % similarity level, corresponding to taxonomically effective species) are obtained from the seven samples. For each sample, 145–263 OTUs are detected at 3 % sequences divergence. And the richness, diversity of the algal communities is calculated. The results show that the highest richness appears in September (ace = 280, chao = 283), while the lowest one (ace = 156, chao = 153) appears in April. The highest algal diversity (Shannon = 4.31) is found in September, while the lowest one is in April (Shannon = 3.39).

The composition at phylum level in the different months by pyrosequencing is showed in Supplementary Fig. 3. Diatomea are the first predominated phylum in April, July, August, September and October, accounting for 33.39, 46.49, 67.38, 40.01 and 77.03 %, respectively. Protalveolata is the first predominant phylum in May with 39.61 %, while Dinoflagellata become the first predominant phylum in June with 48.15 %. Dinoflagellata are the second predominant phylum in April, May, July, August and September, accounting for 24.45, 28.94, 21.14, 17.18 and 28.15 %, respectively. Chlorophyta is the second predominant phylum in June with 24.59 %, while Protalveolata become the second predominant phylum in October with 9.70 %.

The composition at species level by pyrosequencing is showed in Supplementary Fig. 4. A lot of unclassified and uncultured species are detected. There are significant differences in the algal community compositions and their relative abundance in the different month. In April, the first dominant species is *Thalassiosira nordenskioldii* with 17.74 %, followed by *Noctiluca scintillans* with 4.29 %; In May, the first dominant species is *Guinardia solstherfothii* with 8.09 %, followed by *Noctiluca scintillans* with 6.45 %; In June, the first dominant species is *Micromonas pusilla* with 11.31 %, followed by *Noctiluca scintillans* with

2.19 %; In July, the first dominant species is *Pseudo-nitzschia multiseriis* with 6.08 %, followed by *Noctiluca scintillans* with 2.85 %; In August, the first dominant species is *Peridinium quinquecorne* endosymbiont with 14.76 %, followed by *Gonyaulax polygramma* with 8.24 %; In September, the first dominant species is *G. polygramma* with 6.07 %, followed by *P. quinquecorne* endosymbiont with 4.89 %; In October, the first dominant species is *Eucampia antarctica* with 15.85 %, followed by *Thalassiosira rotula* with 15.62 %.

The targeted algal cells of *A. anophagefferens* is detected by pyrosequencing in May (5 OTUs), June (6 OTUs) and July (78 OTUs), accounting for 0.05, 0.12 and 1.05 %, respectively. The cell abundance is as following in declining order: July > June > May. In contrast, no targeted algal cells are detected in April, August, September and October.

The monitoring result of *A. anophagefferens* using qPCR is showed in Table 2. The amplification is detected only in May, June and July, and the C_t values are 31.1997 ± 0.1212 , 26.5253 ± 0.0435 and 22.8394 ± 0.1375 , respectively. The cell abundance (cells l^{-1}) in May, June and July are $(4.92 \pm 0.42) \times 10^2$, $(1.36 \pm 0.42) \times 10^4$ and $(1.88 \pm 0.18) \times 10^5$, respectively. All of the DNA samples from April, August, September and October have no signal amplification, indicating that no *A. anophagefferens* are present in these period.

Discussions

A great advantage of qPCR comparing with microscopic methods to evaluate cell abundance include the following: (1) it is not necessary to be done on fresh samples; (2) for the tiny algal cells hard to distinguish under a microscope, it is easy to enumerate as same as other algal cells; (3) it is

Table 2 Result comparison by pyrosequencing and qPCR for detection of *A. anophagefferens*

	Cell abundance by pyrosequencing	C _t value	Cell abundance (cells l ⁻¹)
Apr	–	No amplification	–
May	5 OTUs (0.05 %)	31.0875	5.32 × 10 ²
		31.3283	4.48 × 10 ²
		31.1834	4.97 × 10 ²
		Mean: 31.1997 ± 0.1212	Mean: (4.92 ± 0.42) × 10 ²
June	6 OTUs (0.12 %)	26.4951	1.39 × 10 ⁴
		26.5751	1.31 × 10 ⁴
		26.5057	1.38 × 10 ⁴
		Mean: 26.5253 ± 0.0435	Mean: (1.36 ± 0.42) × 10 ⁴
July	5 OTUs (1.05 %)	22.8256	1.89 × 10 ⁵
		22.7093	2.05 × 10 ⁵
		22.9832	1.69 × 10 ⁵
		Mean: 22.8394 ± 0.1375	Mean: (1.88 ± 0.18) × 10 ⁵
Aug	–	No amplification	–
Sep	–	No amplification	–
Oct	–	No amplification	–

labor-saving and time-saving, especially for a lot of field samples; (4) it is specific and sensitive. This method is already developed to monitor a lot of HAB algal cells and used in the field successfully [10, 13, 14]. Here we design a set of specific primers and a Taqman probe, and develop a real-time PCR method to identify and quantify *A. anophagefferens*. The high quality sequences obtained from pyrosequencing can ensure that microorganisms with tiny percentage in the population are detected. The results used by qPCR and pyrosequencing for monitoring the targeted algal cells in the field are consistent (Table 2). It indicates pyrosequencing technique achieve the main objective of this study that confirm the applicability of qPCR method in quantification of targeted algal cells from the field samples. Thus qPCR method (Taqman probe) is able to be used to identify and quantify the *A. anophagefferens* in complex environmental samples.

If there is no any algal cell in the ecosystem, there are no chances for bloom occurrence. Therefore, monitoring continually the algal cells even in low abundance would aid in forecasting the potential outbreak of algal bloom. In present study, the qPCR with Taqman probe is able to detect as few as 10¹ cells of *A. anophagefferens*, and it can also identify brown tide when even the cell density reached 10⁶ cells (Fig. 1). If in the field where the densities are much lower or too higher, we can also detect them by changing the collecting volume of natural seawater. Therefore, the qPCR with Taqman probe can be used to monitor continually the initiation, development and disappear of brown tide in the field. Furthermore, the early warning of brown tide breakout can be given according to the development of cell abundance.

However, it is very hard to identify the algal cells of *A. anophagefferens* under a microscopy for the complex seawater because the targeted cells are too small and hard to distinguish from the other particles with the similar size. Other limitations of light microscopy, such as the inexperience to morphology and the decomposition of preserved cells, would make the results false. These results suggest that light microscopy is not an appropriate selection to identify and quantify the algal cells of *A. anophagefferens* in complex natural seawater, while qPCR with Taqman probe is a powerful and sensitive method for monitoring brown tide.

Conclusions

The primers and probe of *A. anophagefferens* are designed for monitoring the brown tide using qPCR in the coastal area along Qinhuangdao city, China. The results of validation by pyrosequencing suggest that the primers and probe developed has species-specific, and the precision meet the need of monitoring brown tide that the algal cells at pre-bloom levels can be detected. Thus the qPCR method could be successfully used to monitor the cell abundance of *A. anophagefferens* in natural seawater. Therefore, this study provides a necessary technology support for forecasting the brown tide initiation, in China.

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