Harmful Algae 47 (2015) 86–96

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Development and evaluation of a reverse dot blot assay for the simultaneous detection of common toxic microalgae along the Chinese coast

Chun Yun Zhang [a,b], Guo Fu Chen [a,b,*], Pan Pan Cai [a], Yuan Yuan Wang [a], Chang Lu Guo [a]

[a] College of Oceanology, Harbin Institute of Technology (Weihai), Weihai 264209, PR China
[b] Key Laboratory of Marine Ecology and Environmental Science, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, PR China

ARTICLE INFO

Article history:
Received 24 December 2014
Received in revised form 8 June 2015
Accepted 8 June 2015
Available online 24 June 2015

Keywords:
Toxic microalgae
Detection
LSU rDNA
Multiplex PCR
Reverse dot blot assay

ABSTRACT

Toxic microalgae currently pose a great threat to human health, ecosystem, fishery, tourism, and aquaculture along the Chinese coast. The detection of toxic microalgae by routinely monitoring natural waters is necessary to provide timely mitigation. Therefore, an effective, simultaneous detection protocol should be established for the simple, rapid, and accurate identification of causative algae. This study developed and evaluated a reverse dot blot assay (RDBA) combined with a low-density membrane-based DNA array for the rapid and simultaneous detection of toxic microalgae that are commonly distributed along the Chinese coast. The large subunit rDNA D1–D2 regions of the target species were first sequenced to design taxonomic probes. Probe specificity was validated by performing a cross-reactivity test with dot blot hybridization. The tailed probes were immobilized onto a nylon membrane to prepare a low-density DNA array for RDBA. The established detection procedure involved DNA extraction, biotin (Bio)-labeling of objective sequences by multiple polymerase chain reaction (M-PCR), RDBA, coloration, and judgment of hybridization by the naked eye. Bio-labeled primer-based labeling proved to be an economical and effective method to prepare Bio-labeled PCR products for RDBA. The detection limits of RDBA using the M-PCR-labeling products from DNA templates prepared by different methods were also compared, and a kit-based DNA extraction method displayed the lowest detection limit of 0.5 cells. Simulation results showed that RDBA can recover all target species and was not affected by background DNA. RDBA was proven effective, specific, and sensitive for the simultaneous detection of toxic microalgae in the field samples. Therefore, this method may be used in the field monitoring of natural samples.

1. Introduction

Microalgae determine the primary productivity and influence the trophic networks in marine economic systems; however, the incidence of algal blooms has increased in coastal waters worldwide during the last years. The Chinese coast has been suffering from harmful algal blooms (HABs) for several years (Zhou et al., 2001). The latest public report from the National Bureau of Oceanography revealed that 73 blooms occurred in the China Sea, which caused a great economic loss of more than $ 300 million (http://www.soa.gov.cn/zwgk/hygb/zghyzhgb/zhgb/201303/t20130306_24229.html). An increasing number of microalgal species have been discovered to form blooms along the Chinese coast; in particular, 29 species of toxic phytoplankton have been recorded (Zhang et al., 2012; Dai et al., 2014). For example, *Aureococcus anophagefferens* and *Karlodinium veneficum* have been recently identified as bloom-forming species in China (Zhang et al., 2012; Dai et al., 2014). The most common causative species of large-scale blooms in the East and South China Sea include the toxic species *Karenia mikimotoi*, which often causes serious poisoning events. Toxic microalgae negatively affect marine ecological environment, fishery economy, and aquatic food safety and health. Thus, the routine monitoring of toxic microalgae in natural waters is crucial to reduce economic losses and ensure public health safety.

Total phytoplankton biomass is relatively easy to monitor by estimating the concentration of chlorophyll or measuring the cell

* Corresponding author at: School of Marine Science and Technology, Harbin Institute of Technology at Weihai, Wenhua West Road, 2#, Weihai, Shandong Province, PR China. Tel.: +86 631 5687059; fax: +86 631 5687059.
E-mail address: chenguofu@hitwh.edu.cn (G.F. Chen).

http://dx.doi.org/10.1016/j.hal.2015.06.003
1568-9883/

density using microscopy. The identification and quantification of individual species, however, remain difficult because of the high morphological variability under different water conditions and the high morphological similarity among related species. In fact, distinguishing species by morphological characteristics requires cells to be examined by light, epifluorescence, and even electron microscopy. These microscopic methods are time consuming, laborious, costly, and complex. In addition, examining water samples that contain morphologically related species possibly results in misidentification. Therefore, morphological examination is not suitable for the routine monitoring of coastal areas that involve large-scale sample analysis. Molecular methods have recently been developed as valid alternatives to the traditional microcopy-based methods for the rapid and sensitive identification of harmful algae. The current methods, which generally involve using ribosomal operons, such as 18S, 28S, 5.8S, ITS1, and ITS2 sequences as targets, can be categorized into two main groups. The first group is based on nucleic acid amplification. Methods belonging to this group include restriction fragment length polymorphism (Chen et al., 1999; Persich et al., 2006), random amplification polymorphism (Han et al., 2004; Martínez et al., 2006), heteroduplex mobility assay (Oldach et al., 2000), denaturing gradient gel electrophoresis (Wang et al., 2005), loop-mediated isothermal amplification (Chen et al., 2013a,b), and real-time PCR (Penna and Galluzzi, 2013). The second group is based on nucleic acid probe hybridization. Methods belonging to this group include fluorescence *in situ* hybridization (Scholin et al., 1996; Chen et al., 2011; Chen et al., 2013a,b), sandwich hybridization (Scholin et al., 1996; Mikulski et al., 2008), and nuclease protection assay integrated with sandwich hybridization (Zhen et al., 2011; Zhu et al., 2013).

Existing molecular protocols facilitate the specific and rapid detection of harmful algae in water samples (Blair et al., 2009); however, only one species at a time can be identified from water samples. Toxic microalgae are diverse, and more than 70 of the approximately 4000 established microalgae worldwide reportedly produce toxins (Zhou et al., 2001). At least 29 toxic microalgae have been found along the Chinese coast (Zhou et al., 2001; Dai et al., 2014), and diverse toxic organisms are usually found in the same field sample. Therefore, the simultaneous detection of potential toxin-producing microalgae in samples collected from areas with high incidence rates of algal blooms is required. Using methods for single species is time consuming, costly, and delays the detection of potentially imminent HABs. Therefore, other methods for the simultaneous detection of several microalgae are required.

DNA microarray is a state-of-the-art technology in molecular biology that enables the detection of target DNA/RNA sequences in bulk samples. This technology was originally developed for gene expression analysis (Sherlock, 2000; Takahashi et al., 2011). Recent studies have coupled DNA microarray with taxonomic probes, such as PhyloChip to detect pathogenic microorganisms (Sun et al., 2014). The application of DNA microarray to identify marine organisms is relatively new and innovative. Ye et al. (2001) and Peplies et al. (2003) first applied DNA array to investigate the biodiversity of marine environmental microorganisms. Research on using DNA array to detect harmful algae is still in its infancy. The current DNA array technologies can be classified into four categories depending on the carriers used for taxonomic probes: glass slide-based DNA array (Ki and Han, 2006; Galluzzi et al., 2011), fiber-optic microarray (Ahn et al., 2006), bead array (Scorzetti et al., 2009), and electrochemical sensor DNA array (Orozco and Medlin, 2011). These DNA array technologies have two obvious drawbacks. One is that they use specific materials as probe carriers, including tailor-made glass slide, fiber-optic, electrode, and bead. Another is that special instruments, such as fluorescence scanners, flow cytometers, and optical or electrical signal detectors, are required to recognize hybridization signals. Hence, the practical applications of DNA array technologies are limited by their relatively high cost and skill requirements. Glass slide-based microarrays that target rRNA have been recently established to detect toxic algae and quantify target cells (Dittami et al., 2013; Kegel et al., 2013); however, high cost and skill requirements again make them not the best choice for analyzing field samples.

In the present study, a cheap and simple multiple polymerase chain reaction (M-PCR)/reverse dot blot assay (RDBA) was developed for the simultaneous detection of six common toxic microalgae distributed along the Chinese coast. The assay generally includes membrane-based DNA array preparation, M-PCR-labeling, hybridization, and hybridization signal recognition. The detailed parameters for DNA array preparation and hybridization were optimized. The protocol was also evaluated by measuring the detection limit with different methods for DNA template preparation and by assessing the detection stability with DNA mixtures that contain different ratios of target DNA to nontarget DNA. The applicability of the developed RDBA was further validated on simulated and field samples.

2. Materials and methods

2.1. Microalgal strain

The algal strains used in this study were obtained from commercial sources or from private isolations (Table 1). All cultures were maintained in f/2 ± Si medium (Guillard, 1975) at pH 8.2 and 20 °C to 22 °C. Light was provided by cool-white fluorescent bulbs (photon flux of approximately 100 $\mu E\ m^{-2}\ s^{-1}$ provided by cool-white) on a 12 h light–12 h dark cycle.

Table 1
List of microalgal species used in this study.

Species	Taxonomy	Geographic origin
Heterosigma akashiwo	Raphidophyceae	South China Sea
Amphidinium carterae	Dinophyceae	East China Sea
Karlodinium veneficum	Dinophyceae	East China Sea
Karenia mikimotoi	Dinophyceae	Wenzhou, East China Sea
Alexandrium tamarense	Dinophyceae	East China Sea
Prorocentrum lima	Dinophyceae	Hongkong, East China Sea
Chaetoceros debilis	Bacillariophyceae	Weihai Bay, Yellow Sea
Prorocentrum donghaiense	Dinophyceae	Zhejiang, East China Sea
Prorocentrum minimum	Dinophyceae	East China Sea
Nitzschia closterium	Dinophyceae	Weihai Bay, Yellow Sea
Skeletonema tropicum	Bacillariophyceae	Weihai Bay, Yellow Sea
Phaeocystis globosa	Prymnesiophyte	South China Sea
Nitzschia angularis	Bacillariophyceae	Weihai Bay, Yellow Sea
Thalassiosira pseudonana	Bacillariophyceae	East China Sea
Pseudo-nitzschia pungens	Bacillariophyceae	Zhujiang Estuary, East China Sea
Symbiodinium sp.	Dinophyceae	Hangzhou, East China Sea
Phaeodactylum tricornutum	Bacillariophyceae	East China Sea
Prorocentrum triestinum	Dinophyceae	East China Sea
Chaetoceros curvisetus	Bacillariophyceae	Weihai Bay, Yellow Sea
Alexandrium minutum	Dinophyceae	Hongkong, East China Sea
Gymnodinium sp.	Dinophyceae	Yellow Sea
Karenia sp.2	Dinophyceae	Wenzhou, East China Sea
Chlorella sp.	Chlorophyta	Bohai Sea
Karenia brevis	Dinophyceae	Hongkong, East China Sea
Dierateria zhanjiangensis	Prymnesiophyte	Xiamen, East China Sea
Prymnesium parvum	Prymnesiaceae	South China Sea
Nannochloropsis oculata	Eustigmatophyceae	South China Sea