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Development and evaluation of a DNA microarray assay for the simultaneous detection of nine harmful algal species in ship ballast and seaport waters*

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Abstract Rapid, high-throughput and reliable methods are urgently required to accurately detect and monitor harmful algae, which are responsible for algal blooms, such as red and green tides. In this study, we successfully developed a multiplex PCR-based DNA microarray method capable of detecting nine harmful algal species simultaneously, namely *Alexandrium tamarense*, *Gyrodinium instriatum*, *Heterosigma akashiwo*, *Karenia mikimotoi*, *Prorocentrum donghaiense*, *Prorocentrum minimum*, *Ulva compressa*, *Ulva ohnoi* and *Ulva prolifera*. This method achieved a limit of detection (LOD) of 0.5 ng of genomic DNA (orders of magnitude of the deci-nanogram range) in the tested algae cultures. Altogether, 230 field samples from ship ballast waters and seaport waters were used to evaluate the DNA microarray. The clinical sensitivity and specificity of the DNA microarray assay in detecting field samples were 96.4% and 90.9%, respectively, relative to conventional morphological methods. This indicated that this high-throughput, automatic, and specific method is well suited for the detection of algae in water samples.

Keyword: ballast waters; DNA microarray; harmful algae; limit of detection; multiplex PCR; seaport waters

1 INTRODUCTION

The proliferation of algae in marine environments can generate harmful algal blooms (HAB), i.e., red and green tides. The former, manifesting as a discoloration of the waters, are mostly caused by certain species of microalgae, such as dinoflagellates (Handy et al., 2008; Anderson et al., 2012). By contrast, the formation of masses of certain macroalgae (such as *Ulva* spp.) is responsible for green tides (Yabe et al., 2009; Duan et al., 2012; Liu et al., 2013a). These algal species are considered highly detrimental for two main reasons: they produce toxins that can kill fish and invertebrates or affect human health (Hallegraeff, 2010; Anderson et al., 2012), and they generate high biomass blooms, which disrupt the marine ecosystem (Smetacek and Zingone, 2013; Lai and Yin, 2014). Blooms caused by these harmful algae in marine, brackish, and continental waters are becoming more frequent worldwide, posing a serious threat to fisheries, aquaculture, human health and tourism (Anderson et al., 2012; Smetacek and Zingone, 2013). These issues are of particular concern in China; a green tide occurred off the Yellow Sea coast near Qingdao in 2008, spanning an area of

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2 500 km^2, and was responsible for direct economic losses of appropriately CNY 1.3 billion (State Oceanic Administration People's Republic of China, 2009). A red tide off the Huangqi coast in Fujian Province lasted for 13 days in 2012 and led to direct economic losses of more than CNY 1 billion (State Oceanic Administration People's Republic of China, 2013). In addition, with the development of international business, the continuous increase in international shipping, and other anthropological factors, species of harmful algae have greatly expanded their geographical range. For example, these algae can be transported in ship ballast waters (Doblin et al., 2002; McCollin et al., 2007; Anderson et al., 2012). Therefore, all countries with coastlines or continental waters require the detection and monitoring of harmful algae.

Traditionally, algae are identified by morphological observation using light or electron microscopy (Culverhouse et al., 1996). This method provides direct visual confirmation of the target algae and is applicable for the monitoring of total phytoplankton or algal biomass. However, it is time-consuming and laborious, and requires professional taxonomic expertise to discriminate certain algal species from total phytoplankton (Scorzetti et al., 2009). Furthermore, samples with unidentified species have to be cultivated for 20 or more days before morphological identification, and this lag often makes an outbreak of harmful algal blooms inevitable. In addition, misidentification is common because of morphological similarities among related algal species (Hayden et al., 2003). For macroalgae, significant complexity in morphology (such as similarities among different algal species, and polytropic morphological characters presented by a single algal species), is frequently encountered as a result of environmental changes (e.g., light intensity, temperature, salinity and nutrient levels), which greatly hinders the identification of these algae (Reddy et al., 1992; Hayden et al., 2003). As the frequency and duration of both red and green tides have increased globally, new techniques are urgently required to detect harmful algae rapidly. Molecular methods based on nucleic acid testing are preferred for their ability to identify species in field samples without a requirement for culturing. A variety of PCR-dependent methods have been developed and have proved reliable for the identification of single species of harmful algae, particularly microalgae (Coyne et al., 2005; Handy et al., 2008; Yuan et al., 2012; Antonella and Luca, 2013). However, PCR with specific primers can be used to identify only one species at a time, and parallel assays are necessary to analyze multiple algal species present in a given field sample; this makes such methods somewhat laborious and time-consuming. A variant of PCR, termed multiplex PCR, allows for the rapid detection of multiple microorganisms in a single reaction and has been described in the detection of HAB species (Handy et al., 2006; Genovesi et al., 2011; Baker et al., 2013).

As a more efficient method, DNA microarray technology presents a significant advantage in high-throughput nucleic acid testing, because of the compatibility of numerous probes that can be simultaneously detected on a small carrier, such as a glass wafer. Other important advantages of microarray-based detecting microorganisms include its high-throughput ability, high sensitivity and specificity, and its amenability for automation and miniaturization (Severgnini et al., 2011; Shi et al., 2012). Recently, several PCR-based DNA microarrays have been successfully developed to detect harmful microalgae (Ahn et al., 2006; Ki and Han, 2006; Galluzzi et al., 2011; McCoy et al., 2013). In addition, the concept of an "array" was introduced in the Environmental Sampling Processor (ESP) at the Monterey Bay Aquarium Research Institute (MBARI), an instrument that autonomously collected water samples, concentrated particles from the water, and achieved detection by probes and data transmission (Roman et al., 2007; Greenfield et al., 2008; Scholin et al., 2009). The ESP uses custom DNA probe arrays for nucleotide sequences indicative of specific species and accomplishes detection following the principles of the sandwich hybridization assay (SHA). These developed microarray methods mostly focused on the identification of microalgal species responsible for red tides, such as dinoflagellates. However, no microarray methods have been developed for monitoring macroalgae, like *Ulva* spp.

Ballast water is regarded as a prominent human-mediated mechanism involved in the introduction of phytoplankton species into new areas, including a variable proportion of HAB species, e.g. *Alexandrium catenella*, *Gymnodinium catenatum*, *Pfiesteria piscicida*, *P. shumwayae*, and *Heterosigma akashiwo* (Hallegraeff and Fraga, 1998; Doblin et al., 2002; Lilly et al., 2002; Butrón et al., 2011). In China, Ningbo and Shanghai are the two most important ports in the Changjiang (Yangtze) River Delta. Biological invasions from ship ballast waters, particularly invasive species of algae responsible for red or green

Table 1 Algal species analyzed in the DNA microarray study

Phylum	Algae	Isolate	Reference
Dinophyta	*Alexandrium lusitanicum*	NMBjah043	Wang et al., 2010
	Alexandrium minutum	A	Zhou et al., 2007
	Alexandrium tamarense	NMBjah048	Wang et al., 2010
	Gyrodinium instriatum	NMBjah046	Wang et al., 2010
	Karenia mikimotoi	NMBjah052	Wang et al., 2010
	Karlodinium micrum	NMBjah047-1	Wang et al., 2010
	Prorocentrum donghaiense	NMBjah045	Zhou et al., 2007
	Prorocentrum micans	NMBjah041	Wang et al., 2010
	Prorocentrum minimum	NMBjah049	Wang et al., 2010
	Scrippsiella trochoidea	NMBjah044	Wang et al., 2010
Bacillariophyta	*Chaetoceros debilis*	-	Tian et al., 2013
	Phaeodactylum tricornutum	NMBguh001	Wang et al., 2010
	Skeletonema costatum	-	Tian et al., 2013
	Thalassiosira pseudonana	NMBguh005	Wang et al., 2010
Xanthophyta	*Heterosigma akashiwo*	H1	Zhou et al., 2007
	Heterosigma akashiwo	H2	Zhou et al., 2007
Haptophyta	*Isochrysis galbana*	NMBjih021-4	Wang et al., 2010
	Pavlova viridis	NMBjih024	Wang et al., 2010
Chlorophyta	*Chlorella pyrenoidosa*	NMBluh015-1	Wang et al., 2010
	Ulva compressa	SDF10	Duan et al., 2012
	Ulva compressa	SDF12	Duan et al., 2012
	Ulva compressa	SDF0813	Duan et al., 2012
	Ulva flexuosa	XS4	Sun et al., 2011
	Ulva flexuosa	55	Sun et al., 2011
	Ulva ohnoi	FJ4	Unpublished
	Ulva pertusa	SDF30	Duan et al., 2012
	Ulva pertusa	SDF30	Duan et al., 2012
	Ulva prolifera	SDF2	Duan et al., 2012
	Ulva prolifera	SDF25	Duan et al., 2012
	Ulva prolifera	XS-1-1	Sun et al., 2011
	Ulva prolifera	XS5	Sun et al., 2011

tides, are considered a severe environmental concern at these ports (Yang et al., 2011; Dai et al., 2012; Zhou et al., 2012). To reduce the risk of introducing invasive species via ballast water, an effective detection method should be developed that is adapted for port quarantine. In this study, we describe a rapid, specific and sensitive microarray method for the simultaneous detection of nine harmful algal species, *Alexandrium tamarense*, *Gyrodinium instriatum*, *Heterosigma akashiwo*, *Karenia mikimotoi*, *Prorocentrum donghaiense*, *Prorocentrum minimum*, *Ulva compressa*, *Ulva ohnoi*, and *Ulva prolifera*. The suitability of this method was evaluated by screening both ship ballast waters (BW) and port waters (PW), in comparison with conventional morphological methods and DNA sequencing. Our established microarray method for simultaneous detection of algal species will be useful for investigating these species, and the probes designed here may be further adopted for developing high-throughput detection of algal species in ballast waters.

2 MATERIAL AND METHOD

2.1 Isolates of algae

Thirty-one algal isolates were used in the study (Table 1). Among them, 19 were microalgal species,

while the remaining 12 were macroalgal species. Nine algal species (Section 1) were chosen as target organisms for microarray design. The remaining algal species were used in the specificity assay for the DNA microarray.

2.2 Culture conditions of algae

The microalgal isolates used in the present study were cultured in our laboratory following previously reported methods (Zhou et al., 2006, 2007; Wang et al., 2010; Tian et al., 2013). Briefly, all the tested microalgal isolates, except the Bacillariophyta, were cultured in sterile seawater enriched with fresh nutrients (the NML3# medium): 100 mg/L KNO_3, 10 mg/L KH_2PO_4, 0.25 mg/L $MnSO_4 \cdot H_2O$, 2.50 mg/L $FeSO_4 \cdot 7H_2O$, 10 mg/L Na_2EDTA, 6 μg/L vitamin B_1, and 0.05 μg/L vitamin B_{12}. The Bacillariophyta isolates were cultured in a similar medium with the addition of 20 mg/L Na_2SiO_3. The cultures were maintained at a salinity of 25–30, at 20–25°C (15–20°C for Dinophyta) under irradiance of 80–110 μmol photons/($m^2 \cdot s$) with a light regime of 12 h:12 h light:dark cycle. Morphological characterization was performed under an inverted microscope (Axiovert 40 CFL, Zeiss, Germany) at 200× or 400× magnifications, using the Utermöhl method (Edler and Elbrächter, 2010). The macroalgal isolates were cultured in a Climacell incubator (MMM Medcenter Einrichtungen GmbH, Germany) at a salinity of 25, at 20°C under 80–100 μmol photons/($m^2 \cdot s$) with a light regime of 12 h:12 h light:dark cycle (Duan et al., 2012). GeO_2 was added at 5 mg/L to suppress the growth of diatoms (Shea and Chopin, 2007). Morphological characterization was performed following previously described criteria (Hiraoka and Enomoto, 1998; Loughnane et al., 2008).

2.3 Field sample collection and treatment

Two types of marine water samples were obtained: ship BW and PW. A total of 150 BW samples were taken from the vessels boarding at five wharfs in Ningbo port (Suanshan, Beilun, Daxie, Meishan, and Chuanshan), China, between January 2011 and June 2012 (Fig.1). Before sample collection, BW in the ballast water tanks (BWT) of each vessel was agitated for 10 min using circulation apparatus. Approximately 5 L of BW was then sampled from 3–5 sites depending on the BWT counts and the gross register of each vessel. A total of 80 PW samples were collected, of which 50 were sampled from 12 coastal sites near Qingdao port, China, over four time frames, namely July 2008, July 2009, August 2010 and July 2011; these time frames correspond to the times of year when green tides typically occur in this region (Fig.1). The remaining 30 PWs were collected from the five aforementioned wharfs at Ningbo port from July 2010 to August 2013 (Fig.1).

An aliquot of each water sample (250 mL) was fixed using 0.5 mL acidic Lugol's solution (10 g/mL potassium iodide, 5 g/mL iodine, 10 g/mL concentrated acetic acid, sealed and stored in darkness) (Edler and Elbrächter, 2010) and stored in the dark at 4°C for the settling and subsequent detection of microalgae. Morphological identification of microalgae was performed as described previously (Edler and Elbrächter, 2010). To detect macroalgal species, another aliquot of each sample was cultured following the protocol of Duan et al. (2012). Morphological identification of macroalgal species was performed as described previously (Hiraoka and Enomoto, 1998; Loughnane et al., 2008).

2.4 Genomic DNA extraction

Genomic DNA was extracted from each algal isolate using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The purified DNA standards were quantified using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Serial dilutions of genomic DNA were used for the measurement of the limits of detection (LOD) of the DNA microarray. To extract genomic DNA, each 250-mL sample was filtered through a 150-μm mesh. Genomic DNA was extracted from the filtrates using the DNeasy Plant Mini Kit and stored at -20°C until use.

2.5 Conventional PCR assay combined with DNA sequencing

Genomic DNA extracted from algal isolates or field samples was used as a template for PCR amplification. The ITS1-5.8S-ITS2 region and the genes encoding the large subunit of Ribulose,-1,5-bisphosphate carboxylase (*rbcL*) were used as the target genes for macroalgal species, while 18S and 28S rDNA genes were used for microalgal species. For macroalgal species, the primers 18S-1505 (5'-TCTTTGAAACCGTATCGTGA-3') (Hayden et al., 2003) and ENT-26S (5'-GCTTATTGATATGCT-TAAGTTCAGCGGGT-3') (Blomster et al., 1998) were used to amplify the ITS1-5.8S-ITS2 region, producing an amplicon of 870 bp, while the primers rbcL-SHF1 (5'-CCGTTTAACTTATTACACGCC-3') and rbcL-SHR4 (5'-TTACATCACCACCTTCAGAT-

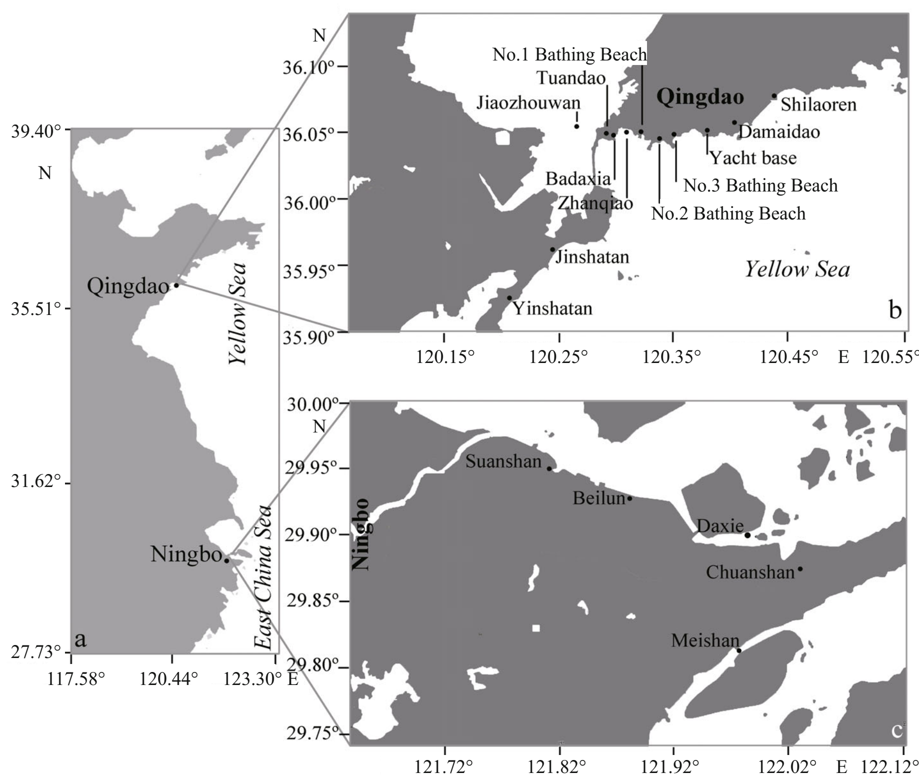


Fig.1 Map of the Yellow Sea and the East China Sea, China (a) showing the sampling sites of ship ballast waters (BW) (c) and seaport waters (PW) (b and c) in the present study

GC-3') (Heesch et al., 2009) were used to amplify the *rbcL* gene, producing an amplicon of 1 200 bp. For microalgal species, the primers 1F (5'-AACCTGGT-TGATCCTGCCAGT-3') and 1528R (5'-TGATCCTT-CTGCAGGTTCACCTAC-3') (Diercks et al., 2009) were used to amplify the 18S rDNA gene, producing an amplicon of 1 800 bp, while primers D1R (5'-ACCGCTGAATTTAAGCATA-3') and D2C (5'-CCTTGGTCCGTGTTTCA AGA-3') (Diaz et al., 2010) were used to amplify the 28S rDNA gene, producing an amplicon of 670 bp. Each PCR reaction (20 μL) contained 500 nmol/L of each primer, 200 μmol/L deoxynucleoside triphosphate, 2.5 U LA Taq DNA polymerase (TaKaRa, Dalian, China), 1×PCR buffer, 2.5 mmol/L $MgCl_2$, and 2 μL genomic DNA template. PCR reactions were performed in an Eppendorf Mastercycler Pro S thermal cycler (Eppendorf AG, Hamburg, Germany) with a previously described 3-step program (Hayden et al., 2003; Diercks et al., 2009; Heesch et al., 2009; Diaz et al., 2010). All PCR reactions were terminated with an elongation step at 72°C for 10 min, verified by agarose gel electrophoresis, and purified using a Minelute PCR purification kit (Qiagen, Hilden, Germany). The purified PCR products were then cloned into vector pMD19-T (Simple) (TaKaRa, Dalian, China), and positive clones were sequenced on an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.6 Oligonucleotide probe design and microarray construction

Forty-two probes (40 or 59 bases in length, Table 2) to detect the nine aforementioned algal species were designed, based on multiple sequence alignment analysis. The specificity of each probe was evaluated at both the genus and species levels by performing nucleotide BLAST searches against a nucleotide database (nr/nt) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Among them, seven probes with a short sequence (20 bp each) were added at the 5' end using a spacer with 20 poly (T) oligonucleotides, for the purpose of covalent immobilization on the aldehyde-coated glass surface (Table 2). Additionally, a set of control probes (HEX, NC, and PC) was used (Table 2). The HEX probe, which was used to monitor probe spotting and fixing on the microarray, was a hexachloro-6-carboxylfluorescein (Hex)-labeled oligomarker (50 nt) (Shi et al., 2012). The NC probe, which was used for quality control of nonspecific hybridization, comprised

Table 2 Probes used in the study on DNA microarray analysis of harmful algal species

Name	Abbr.	Species	Isolate	Target gene	Sequence (5' to 3')
HEX	HEX		-		GTCACATGCGATGGATCGAGCTCCTTTATCATCGTTCCCACCTTAATGCA
PC	PC		-		TTTTTTTTTTTTTTTTTTTTCTCATGCCCATGCCGATGC
NC	NC		-		TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
Ata-18S-1	Ata1	*Alexandrium tamarense*	NMBjah048	18S	agattggaggttgttacttgtatgacttgttcagcaccctatgcgacatcaaagtgttt
Ata-18S-2	Ata2	*Alexandrium tamarense*	NMBjah048	18S	agtatttggcacagcctgagcatttatcttgaaagtacaactgcacttggctgtgtggt
Gin-18S-1	Gin1	*Gyrodinium instriatum*	NMBjah046	18S	ACCGCAGCAGCGTTTGGTTCCTGAATGCTGCCGTGGAAAG
Gin-18S-2	Gin2	*Gyrodinium instriatum*	NMBjah046	18S	AGTTTGTTACCTTGACTGGAATGGTTGGGCAATCTTCTGA
Gin-18S-3	Gin3	*Gyrodinium instriatum*	NMBjah046	18S	CGTCCTAGGCATCATCTTGGAGAGCGTCGCTGCAATTGAT
Gin-18S-4	Gin4	*Gyrodinium instriatum*	NMBjah046	18S	GCGTTTGGTTCCTGAATGCTGCCGTGGAAAGCTTCGTGAA
Gin-18S-5	Gin5	*Gyrodinium instriatum*	NMBjah046	18S	GTAGACTTTAAATCACTTTGCAAGTATCGAGTGCAGGGCA
Gin-18S-6	Gin6	*Gyrodinium instriatum*	NMBjah046	18S	TGCATGGAATGGTTGGGCAATCTTCTGAAACTGCATCGTG
Gin-28S-1	Gin7	*Gyrodinium instriatum*	NMBjah046	28S	ATCTGGTTGCGCGTGGTCGCTTCTTGCTTTGGGTGTCAGC
Gin-28S-2	Gin8	*Gyrodinium instriatum*	NMBjah046	28S	CGGTAGTGCAAACACGGCTGCAGGCGTCGATCTGGTTGCG
Gin-28S-3	Gin9	*Gyrodinium instriatum*	NMBjah046	28S	CTCAAGTGAGGCCCTGCTCCGGTAGTGCAAACACGGCTGC
Gin-28S-4	Gin10	*Gyrodinium instriatum*	NMBjah046	28S	TAGCCTGGAGATGCGTCGCCAACAGGGGCGTAGGTGCGAA
Gin-28S-5	Gin11	*Gyrodinium instriatum*	NMBjah046	28S	TTGCTTTGGGTGTCAGCGTCAGCTCAACTCTGAGGACAAC
Hak-18S-1	Hak1	*Heterosigma akashiwo*	H1	18S	tcggcggacgggatgtatttattagatggaaaccaataccgagttttctcggtttttgt
Hak-18S-2	Hak2	*Heterosigma akashiwo*	H1	18S	aaggggtctgcacctgtattgtcctttgccatccttcaggaaggcgcttcttgtattaa
Hak-18S-3	Hak3	*Heterosigma akashiwo*	H1	18S	tggaataatgagatagggccttggtggttttctattttgttggtttgcacgccaaggta
Hak-18S-4	Hak4	*Heterosigma akashiwo*	H1	18S	atcgtcctttgccatccttcaggaaggcgcttcttgtattaacttacgggttgcgaact
Hak-18S-5	Hak5	*Heterosigma akashiwo*	H1	18S	tcccaactgcttcggcggacgggatgtatttattagatggaaaccaataccgagttttc
Hak-18S-6	Hak6	*Heterosigma akashiwo*	H1	18S	gaacgagacccccgcctgctaaatagtgtcggtaatgcttctgcattgccgtttctact
Hak-18S-7	Hak7	*Heterosigma akashiwo*	H1	18S	gcttctgcattgccgtttctacttcttagagggactttcggtgactaaccgaaggaagt
Hak-18S-8	Hak8	*Heterosigma akashiwo*	H1	18S	cctaccgattgaatgattcggtgaaaatcttggactttggcatggaactttattgtgac
Hak-18S-9	Hak9	*Heterosigma akashiwo*	H1	18S	ggtttttgtggtgaatcatagtaactgtgcgaatcgacttcggtcgatggttcattcaa
Hak-18S-10	Hak10	*Heterosigma akashiwo*	H1	18S	accaataccgagttttctcggtttttgtggtgaatcatagtaactgtgcgaatcgactt
Hak-28S-1	Hak11	*Heterosigma akashiwo*	H1	28S	AGGGAAGCGATTGAAGTCAGTGTTGCTCCTGGTCTTTTTG
Hak-28S-2	Hak12	*Heterosigma akashiwo*	H1	28S	GGAGCGTTTCAACATGCGTTCTGTTCCTCGGGAAATGTTC
Hak-28S-3	Hak13	*Heterosigma akashiwo*	H1	28S	AGTGTGCTGGAACCTTCGGGGAAACGCACTGTTCTTGTCG
Hak-28S-4	Hak14	*Heterosigma akashiwo*	H1	28S	TGGTTAGGACGGAGGACCTTTGCTCCTTTGACTGCGCGTT
Hak-28S-5	Hak15	*Heterosigma akashiwo*	H1	28S	CCTCTCTCGGGTATGCTGGTGTCTACTGCTTGCCGTTTTC
Kmi-28S-1	Kmi1	*Karenia mikimotoi*	NMBjah052	28S	ttttttttttttttttttttgcttgcctgcgatcttctg
Pdo-18S-1	Pdo1	*Prorocentrum donghaiense*	NMBjah045	18S	ttttttttttttttttttttagcttatgaccttgcctgga
Pdo-18S-2	Pdo2	*Prorocentrum donghaiense*	NMBjah045	18S	GACTGCTGCGGTGTCAAGTTCCTGGACCTGCTTGCGGAAA
Pdo-18S-3	Pdo3	*Prorocentrum donghaiense*	NMBjah045	18S	CGAGCTTATGACCTTGGCTGGAAAGGTTGGTTAATCTTTT
Pdo-18S-4	Pdo4	*Prorocentrum donghaiense*	NMBjah045	18S	CGGACTGCTGCGGTGTCCAGTTCCTGGACCTGCTTGCGGA
Pmi-28S-1	Pmi1	*Prorocentrum minimum*	NMBjah049	28S	GGTGCATGACGCGCTTGATCGTCTCTTGCCTTGTGTGTCA
Pmi-28S-2	Pmi2	*Prorocentrum minimum*	NMBjah049	28S	GGTGGATGACGCGCTTGATCGTCTCTTGCCTAGTGTGCCA
Uco-18S-1	Uco1	*Ulva compressa*	SDF10	ITS1-2[a]	GGTCCTTGCGGCCGGGCTGGCTGAAGTACAGAGGTTCGTG
Uco-18S-2	Uco2	*Ulva compressa*	SDF10	ITS1-2[a]	TCGGTGCCGTGTGCTATGAGCCACGAAGGATACTAACTTT
Uco-18S-3	Uco3	*Ulva compressa*	SDF10	ITS1-2[a]	ttttttttttttttttttttGGCTCATAGCACACGGCACC
Uoh-18S-1	Uoh1	*Ulva ohnoi*	FJ4	ITS1-2[a]	ttttttttttttttttttttGGGCAGAGGGTTTGCTGGGT
Uoh-rbcL-1	Uoh2	*Ulva ohnoi*	FJ4	rbcL	ttttttttttttttttttttTCATACACAGCACGTCCGTA
Upr-rbcL-1	Upr1	*Ulva prolifera*	XS-1-1	rbcL	ttttttttttttttttttttATGCGTCGTCACCGAAGATT
Upr-rbcL-2	Upr2	*Ulva prolifera*	XS-1-1	rbcL	ttttttttttttttttttttCACATGTACCCGCAGTTGCA

[a] ITS1-2 represents ITS1-5.8S-ITS2.

40 poly (T) oligonucleotides. The PC probe, which could hybridize with a fluorescently labeled complimentary sequence (PCc, 5'-GCATCGGCATGG-GCATGAG-3', labeled with HEX at the 5′-end), was used for quality control of specific hybridization (Shi et al., 2012). All the probes were commercially synthesized by Invitrogen Co., Shanghai, China.

All the probes were dissolved in 50% dimethyl sulfoxide (DMSO) at a final concentration of 1 μg/μL and printed onto aldehyde group-modified OPAminoSlide™ glass slides (CapitalBio Corp., Beijing, China). Each probe was spotted in quadruplicate to eliminate irregular data arising from physical defects on the glass slides. In each array, the probe controls (HEX, PC, and NC) were spotted as shown in Fig.3. After probe spotting, the slides were baked for 1 h at 80°C in an oven. The processed slides were then rinsed, stored at room temperature in the dark, and scanned at 532 nm to assess and confirm spotting quality.

2.7 Microarray hybridization and fluorescent detection

The 5′ end of the forward primer (primer 18S-1505, rbcL-SHF1, 1F and D1R) of each primer pair described in Section 2.5 was labeled with carboxytetramethylrhodamine (TAMRA), which was incorporated into the PCR amplicons for subsequent fluorescence detection. The PCR reactions and the purification of PCR amplicons were carried out as described in Section 2.5.

Before hybridization, the processed slides were washed with 0.2% (w/v) SDS to remove non-covalently bound nucleic acids. Seven microliters of labeled PCR amplicons and 0.2 μL of PCc were mixed with 15 μL of hybridization solution containing 3× SSC, 25% (v/v) formamide, 0.2% (w/v) SDS, and 5× Denhardt's solution. The slides were hybridized by incubation in the mix at 42°C for 2 h. They were then washed with solution II (0.2×SSC) for 4 min followed by washing with solution I (2×SSC and 0.2% SDS) for 4 min, and scanned by a LuxScan™ 10 K microarray scanner at an excitation wavelength of 532 nm (CapitalBio Corp., Beijing, China). The fluorescence intensities were quantified using SpotData™ Pro 3.0 (CapitalBio Corp.) and calculated for all true-positive spots by subtracting the background values of the median signal intensities of the negative control. A positive spot was defined as having a signal intensity of more than 1 000.

2.8 LOD of microarray assay

The LODs of the microarray assay were roughly measured using a 10-fold dilution series of genomic DNA of targeted algal isolates. Genomic DNA amounts of 50, 5, 0.5, and 0.05 ng were used as the template for labeling PCR amplification followed by microarray hybridization, respectively. All experiments were performed in triplicate. Finally, LODs were determined based on the relationship between target DNA concentrations and hybridization signals.

2.9 Parallel detection of multiple algal species

Genomic DNA with known LOD concentrations for four algae, namely *G. instriatum*, *P. minimum*, *U. compressa*, and *U. ohnoi*, were used as templates to evaluate the reproducibility of the DNA microarray. The labeled PCR amplicons targeting the aforementioned seven genes, i.e., 18S and 28S of *G. instriatum*, 28S of *P. minimum*, 18S and *rbcL* of *U. compressa*, and 18S and *rbcL* of *U. ohnoi*, were purified. Fifty nanograms of genomic DNA for each sample was mixed with 0.2 μL of PCc and hybridized with the microarray, as described in Section 2.7. All experiments were performed in triplicate.

To test the performance of simultaneous amplification using multiple pairs of primers, a multiplex PCR assay was carried out with a genomic DNA mix as the template. Genomic DNA samples of each of the four algal species were mixed as described, at their respective LOD concentrations; 350 ng of purified PCR products were then hybridized with the microarray as described in Section 2.7. All experiments were performed in triplicate.

2.10 Clinical sensitivity and specificity

150 BW and 80 PW samples were collected as described in Section 2.3 to evaluate the applicability of the DNA microarray technology. The clinical sensitivity and specificity of the DNA microarray were calculated using conventional morphological methods as the control. With the clinical sensitivity and specificity of conventional morphological methods arbitrarily set at 100%, these parameters of the DNA microarray method and DNA sequencing were calculated using the formula: Sensitivity=true positives/(true positives+false negatives)×100%; Specificity=true negatives/(true negatives+false positives)×100%. In addition, 95% confidence intervals (95% CI) for both clinical sensitivity and specificity were calculated.

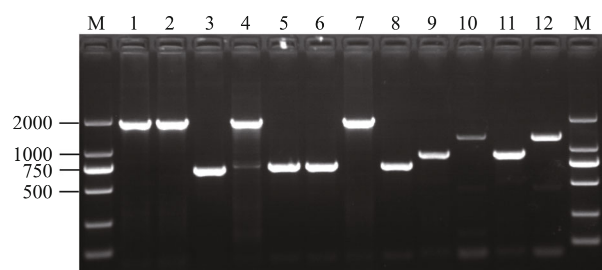


Fig.2 Purified PCR products after amplification of target genes from harmful algal species using different primers

Lane 1: *Alexandrium tamarense* (Primers: 1F/1528R); Lane 2: *Gyrodinium instriatum* (Primers: 1F/1528R); Lane 3: *Gyrodinium instriatum* (Primers: D1R/D2C) Lane 4: *Heterosigma akashiwo* (Primers: 1F/1528R); Lane 5: *Heterosigma akashiwo* (Primers: D1R/D2C); Lane 6: *Karenia mikimotoi* (Primers: D1R/D2C); Lane 7: *Prorocentrum donghaiense* (Primers: 1F/1528R); Lane 8: *Prorocentrum minimum* (Primers: D1R/D2C); Lane 9: *Ulva compressa* (Primers: 18S-1505/ENT-26S); Lane 10: *Ulva prolifera* (Primers: rbcL-SHF1/rbcL-SHR4); Lane 11: *Ulva ohnoi* (Primers: 18S-1505/ENT-26S); Lane 12: *Ulva ohnoi* (Primers: rbcL-SHF1/rbcL-SHR4); M: DL 2000 DNA Marker (TaKaRa Co., Dalian, China).

3 RESULT

3.1 PCR amplification

For microalgal species, the target regions of both 18S and 28S rDNA genes were successfully amplified using universal primers 1F/1528R and D1R/D2C, respectively (Fig.2). For macroalgal species, the target regions of both ITS1-5.8S-ITS2 and the *rbcL* gene were specifically amplified using the universal primers 18S-1505/ENT-26S and rbcL-SHF1/rbcL-SHR4, respectively (Fig.2).

3.2 Probe design and screening

Sequence alignments of the target genes (i.e., both 18S and 28S rDNA genes for microalgal species and both ITS1-5.8S-ITS2 and *rbcL* genes for macroalgal species) were performed before probe design to maximize the hybridization specificity of the consensus sequences of the targeted species. The specificity of each probe was confirmed in silico using a BLAST search. Forty-two probes were screened out to differentiate nine algal species, comprising six microalgal species and three macroalgal species (Table 2).

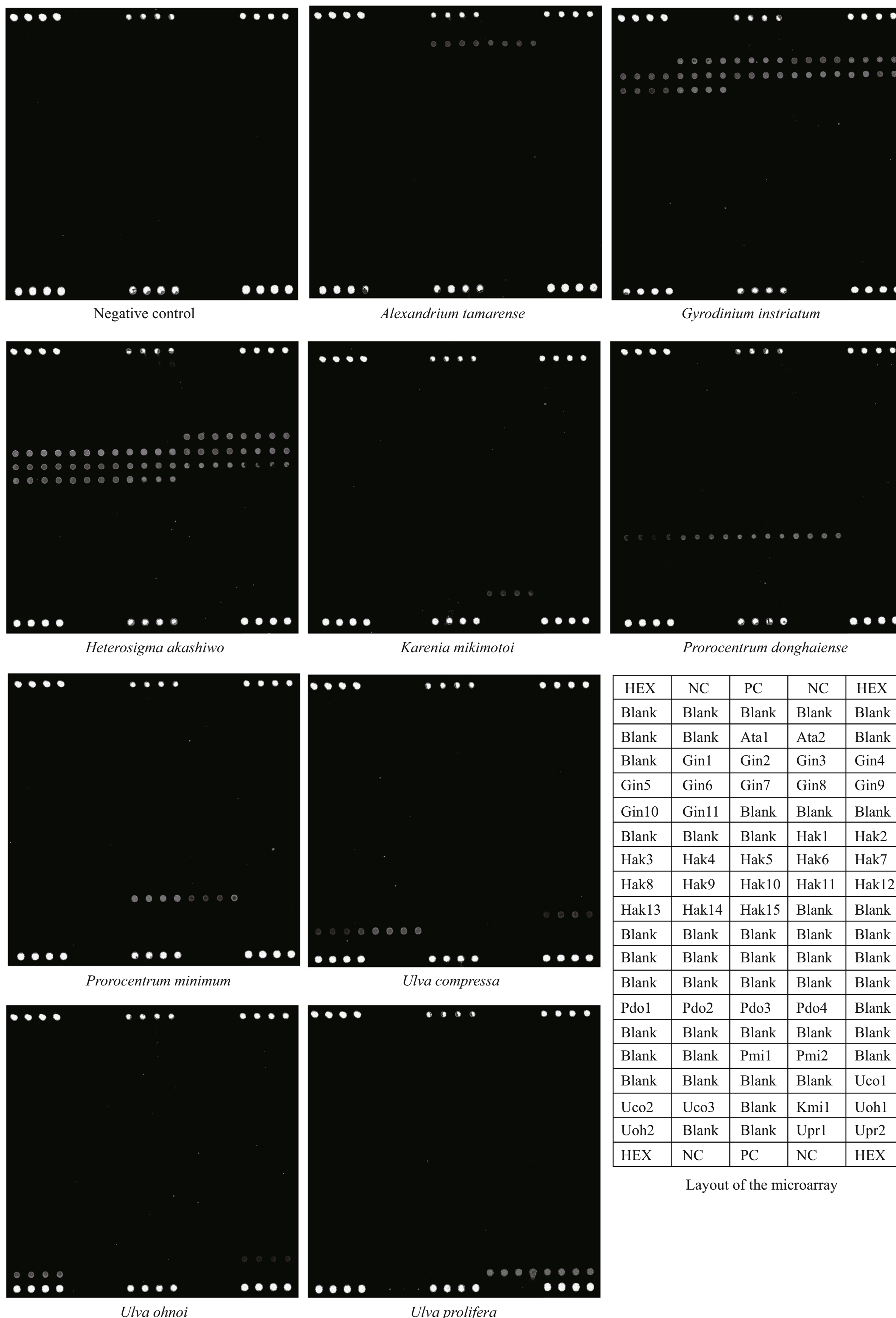
The probes were then printed on the microarray and tested for their actual specificity (Fig.3). Positive spots were easily differentiated from negative spots, and a clear, specific pattern of reactivity was observed for each tested species. As expected, the labeled PCR amplicons presented species-specific hybridization results in the microarray assay (Fig.3). The three quality control probes (HEX, NC, and PC) exhibited good reliability and stability (Fig.3). On the other hand, the PCR amplicons of the genomic DNA of other algal species used in the assays, i.e., *Alexandrium lusitanicum*, *Alexandrium minutum*, *Chaetoceros debilis*, *Chlorella pyrenoidosa*, *Isochrysis galbana*, *Karlodinium micrum*, *Pavlova viridis*, *Phaeodactylum tricornutum*, *Prorocentrum micans*, *Scrippsiella trochoidea*, *Skeletonema costatum*, *Thalassiosira pseudonana*, *Ulva flexuosa*, and *Ulva pertusa*, showed negative signals when hybridized with this microarray (data not shown).

3.3 Evaluation of the LOD of the DNA microarray

To measure the LOD of the DNA microarray assay for algal species, serial 10-fold dilutions of *G. instriatum* genomic DNA were used as the templates in PCR reactions targeting the 18S and 28S rDNA. When using a gDNA template concentration ≥ 0.5 ng, PCR amplicons could hybridize successfully with all probes of *G. instriatum* and generate signal intensities higher than 1 000, indicating positive results (Fig.4). When using 0.05 ng of gDNA template, the hybridization signal intensities of PCR amplicons with six probes (Gin-18S-1, Gin-18S-2, Gin-28S-2, Gin-28S-3, Gin-28S-4, and Gin-28S-5) were <1 000 (Fig.4), suggesting negative results. Similar detection limits for other tested algae were obtained. The LOD of our DNA microarray was defined as 0.5 ng (in orders of magnitude in the deci-nanogram range) gDNA of each tested algal species.

3.4 Parallel detection of multiple algae

Four singleplex PCR reactions were performed using 0.5 ng of gDNA from each of the four algae and their corresponding primer pairs. After amplification, the singleplex PCR products were pooled. Simultaneously, one multiplex PCR assay was carried out using 2 ng of mixed gDNA (0.5 ng of gDNA from each of the four previously indicated algal species) and four primer pairs. The mixture of the singleplex PCR products and the direct product of multiplex PCR were used in the subsequent hybridizations. Compared with hybridization with the former, the signal intensities of the latter were always somewhat weaker; however, robustly positive signals were still observed (fluorescence intensity >1 000) (Fig.5). Compared with the singleplex PCR using their corresponding



HEX	NC	PC	NC	HEX
Blank	Blank	Blank	Blank	Blank
Blank	Blank	Ata1	Ata2	Blank
Blank	Gin1	Gin2	Gin3	Gin4
Gin5	Gin6	Gin7	Gin8	Gin9
Gin10	Gin11	Blank	Blank	Blank
Blank	Blank	Blank	Hak1	Hak2
Hak3	Hak4	Hak5	Hak6	Hak7
Hak8	Hak9	Hak10	Hak11	Hak12
Hak13	Hak14	Hak15	Blank	Blank
Blank	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank
Pdo1	Pdo2	Pdo3	Pdo4	Blank
Blank	Blank	Blank	Blank	Blank
Blank	Blank	Pmi1	Pmi2	Blank
Blank	Blank	Blank	Blank	Uco1
Uco2	Uco3	Blank	Kmi1	Uoh1
Uoh2	Blank	Blank	Upr1	Upr2
HEX	NC	PC	NC	HEX

Fig.3 Specificity testing results of the DNA microarray assay for nine harmful algal species

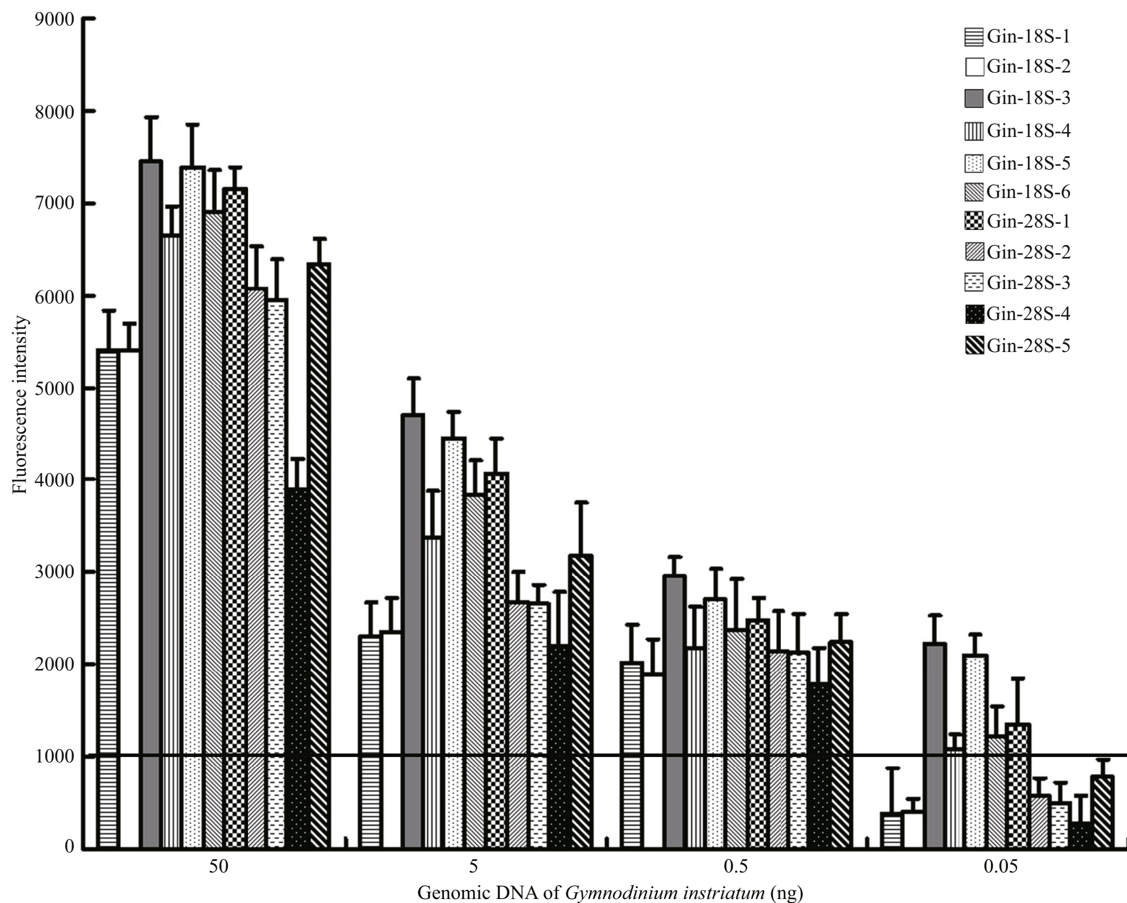


Fig.4 Evaluation of limits of detection (LOD) of the DNA microarray assay using genomic DNA of *Gyrodinium instriatum* as a template

The solid line indicates the threshold value of positive signal intensity (>1 000).

primers, effective amplification was achieved for targeted nucleotide sequences of other tested algae using the multiplex PCR with the pooled four pairs of primers. Therefore, the multiplex PCR method was used in the subsequent detection of field samples.

3.5 Clinical sensitivity and specificity of microarray assay

The algal species collected from the 150 BW and 80 PW samples were first identified by morphological observation and DNA sequencing. Next, these results were used to evaluate the clinical sensitivity and specificity of the newly developed DNA microarray method (Table 3). With respect to the nine species of algae evaluated in this study, all samples tested negative for *A. tamarense*, *G. instriatum*, *K. mikimotoi*, *P. donghaiense*, *P. minimum*, and *U. ohnoi*. Fifty-five of the 230 samples were confirmed to be positive for four species, i.e., *H. akashiwo* (1), *U. compressa* (7), *Ulva ohnoi* (3) *and U. prolifera* (44), by morphological observation (Table 3). The remaining 175 samples were found to be negative for all nine species. The microarray assay and DNA sequencing methods detected 53 and 49 positives, respectively, among the 55 positive samples indicated (Table 4). Among the 175 samples that tested negative by morphological observations, 159 and 166 samples were indicated to be negative by microarray assay and DNA sequencing, respectively. The clinical sensitivity and specificity of the DNA microarray, relative to morphological methods, were 96.4% and 90.9%, respectively; DNA sequencing displayed 89.1% clinical sensitivity and 94.9% clinical specificity, respectively (Table 4).

4 DISCUSSION

The prevalence of marine environment degradation caused by harmful algal species has increased worldwide, particularly in China, where coastal waters are subject to annual outbreaks of both red tide (caused by microalgae) and green tide (caused by macroalgae, such as *Ulva*. spp) (Keesing et al., 2011; Liu et al., 2013a, b). Some algae may be transported

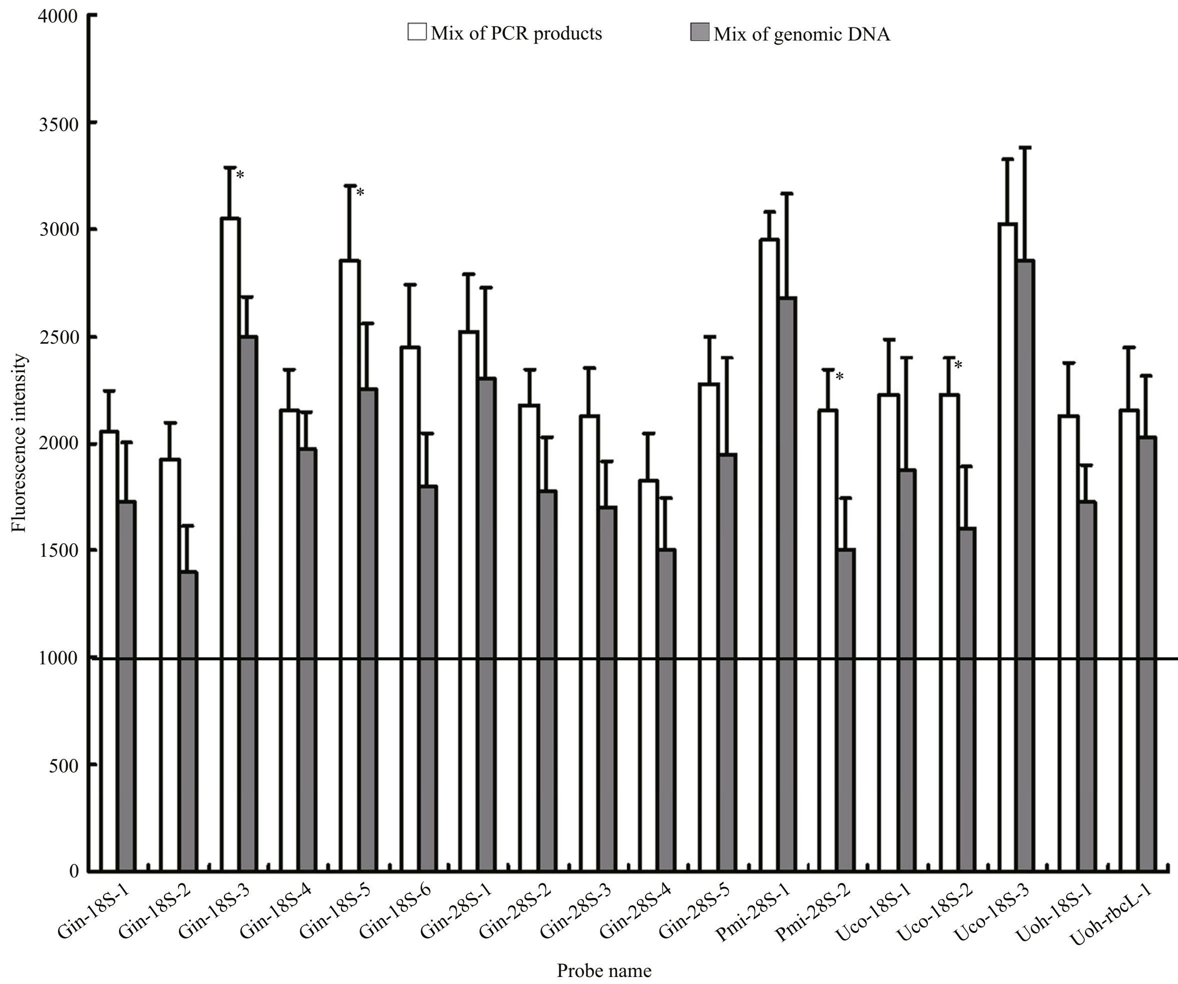


Fig.5 The signal intensities of hybridization

0.5 ng of genomic DNA of each tested algal species, i.e., *Gyrodinium instriatum*, *Prorocentrum minimum*, *Ulva compressa*, and *Ulva ohnoi* was used as the template. 50 ng of each labeled PCR product plus 0.2 μL PCc was mixed and hybridized using a DNA microarray (indicated by empty boxes). The signal intensities of hybridization with the product of the multiplex PCR assay were determined (shown by solid boxes). The solid line indicates the threshold value of positive signal intensity (>1 000). The statistical test was performed using one-way ANOVA analysis of variance followed by the Bonferroni method of multiple comparisons using SPSS Version 16.0 (SPSS Inc., Chicago, IL, USA). Asterisks indicate significant differences relative to the identical probe hybridized with the two amplification products ($P<0.05$).

Table 3 Analysis of water samples using the DNA microarray method, compared with conventional morphological methods and DNA sequencing

Algae	Morphological methods		DNA microarray		DNA sequencing	
	NOPS[a]	Origin	NOPS[a]	Origin	NOPS[a]	Origin
Heterosigma akashiwo	1	BW in Ningbo port	1	BW in Ningbo port	1	BW in Ningbo port
Ulva compressa	7	4 from PW near Qingdao port3 from PW inside Ningbo port	7	4 from PW near Qingdao port3 from PW inside Ningbo port	7	4 from PW near Qingdao port3 from PW inside Ningbo port
Ulva ohnoi	3	3 from PW near Qingdao port	3	3 from PW near Qingdao port	3	3 from PW near Qingdao port
Ulva prolifera	44	32 from PW near Qingdao port11 from PW inside Ningbo port1 from BW inside Ningbo port	58	38 from PW near Qingdao port17 from PW inside Ningbo port3 from BW inside Ningbo port	47	34 from PW near Qingdao port10 from PW inside Ningbo port3 from BW inside Ningbo port
Total	55		69		58	

[a] number of positive samples.

Table 4 Clinical sensitivity (true-positive rate) and specificity (true negative rate) of DNA microarray and DNA sequencing, relative to conventional morphological methods as the standard (100%)

Morphological methods (n)	DNA microarray		DNA sequencing	
	Positive	Negative	Positive	Negative
Positive (55)	53	2	49	6
Negative (175)	16	159	9	166
Clinical sensitivity	96.4% (95% CI: 87.7%–99.0%)		89.1% (95% CI: 78.2%–94.9%)	
Clinical specificity	90.9% (95% CI: 85.7%–94.3%)		94.9% (95% CI: 90.5%–97.3%)	

in ship BW or sediment into a country's contiguous waters (McCollin et al., 2007; Butrón et al., 2011); therefore, it is necessary to develop a rapid and high-throughput detection technique to establish exit and entry inspections and quarantine procedures. To that end, we developed a DNA microarray for the simultaneous detection of nine species of environmentally harmful algae. The species *U. prolifera*, which is suspected to be the dominant species responsible for green tide in China (Duan et al., 2012; Huo et al., 2013), was frequently detected in PW samples, while both *U. prolifera* and *H. akashiwo* were detected at low frequencies in BW samples. This suggested that ship ballast waters could be important sources of invasive species of harmful algae.

Selection of an appropriate target gene is critical for the parallel detection of multiple biological species. Ribosomal DNA sequences, such as 18S, ITS1-5.8S-ITS2, and 28S, are common options for algal species for several reasons (De la Herrán et al., 2000; Mayta et al., 2000; King et al., 2008). First, these genes contain both highly conserved regions and highly variable regions, and this facilitates the design of universal primers for different algal species and enables discrimination at the genus, species and even strain levels. Second, ribosomal genes are repeated in multiple copies and are present in a tandem pattern in the algal genome (Duan et al., 2012; Zhang et al., 2014), providing a greater number of target molecules to be bound by oligonucleotide probes. In the present study, both the ribosomal genes (i.e., 18S, ITS1-5.8S-ITS2, and 28S) and the *rbcL* gene were selected to design microarray probes. After screening, 42 probes were confirmed to be species-specific, with respect to the nine species of interest.

The LOD is one of the key benchmarks to determine the applicability of DNA microarray assays (Diaz et al., 2010; Galluzzi et al., 2011; Shi et al., 2012; Kegel et al., 2013). It is dependent mostly on the efficiency of PCR amplification and probe hybridization. Ahn et al. (2006) developed a DNA fiber-optic microarray, based on microspheres, with a LOD of approximately five cells for three algal species responsible for red tide, namely *Alexandrium fundyense*, *Alexandrium ostenfeldii*, and *Pseudo-nitzschia australis*. Recently, Galluzzi et al. (2011) developed an oligonucleotide microarray targeting the ITS1-5.8S-ITS2 rDNA region to detect nine harmful dinoflagellate species; the reported LOD was 2 ng of the PCR product. More recently, McCoy et al. (2013) used a universal microarray, MIDTAL, for the detection of harmful microalgal species, with a LOD of 5 ng of algal RNA. A preliminary application of this test in Arcachon Bay (France) generated results that compared favorably with traditional cell-count procedures for most genera, except *Prorocentrum* (Kegel et al. 2013). In our study, the DNA microarray displayed a rough LOD of orders of magnitude of deci-nanogram genomic DNA. Assuming that these algae possess a certain minimum cell DNA amount, similar to that reported for *Karenia brevis* (approx. 50 pg/cell) (Yoon et al., 2005), the LOD of our microarray can be extrapolated to represent approximately 10 cells, which is comparable to the LODs reported in previous studies (Ahn et al., 2006; Galluzzi et al., 2011; McCoy et al., 2013).

Multiplex PCR promises the simultaneous detection of more than one target sequence by incorporation of multiple pairs of primers in a single reaction vessel (Markoulatos et al., 2002). However, interaction and/or interference among multiple primers significantly decreases the assay's LOD, compared with each of the corresponding singleplex assays (Operario and Houpt, 2011). The dramatic inequality relationships among algal templates (i.e., one target is in excess of the other), as well as large amplicons, were probably responsible for the reduction in the efficiency and sensitivity of multiplex PCR (Handy et al., 2006). This strategy has been applied in DNA microarray assays to simultaneously detect more than one target (Susana et al., 2011; Bierbaum et al., 2012). In the present study, we evaluated the suitability of using our multiplex PCR

amplification in a microarray assay. Although signal intensities of the multiplex PCR-microarray hybridization were slightly weaker than that of the singleplex PCR-microarray hybridization (Fig.5), our method still reliably detected the harmful algae.

Not all test results match the actual status of the tested targets. Therefore, the concepts of clinical sensitivity and specificity are used to gauge the ability of a test to identify positives or negatives accurately (Fawcett, 2006). Both indexes are often used to compare the reliability of new detection techniques and well-established methods (Zhu et al., 2007; Xi et al., 2012; Surabattula et al., 2013; Zhou et al., 2014). For algal species identification, morphological observation is still the predominant strategy, because of the complexity of algal taxonomy (Culverhouse et al., 1996). It is inevitable that new techniques will initially diverge from conventional morphological methods, with respect to accuracy and precision (Zhu et al., 2007; Xi et al., 2012; Zhou et al., 2014). For example, a LyssaChip was developed to successfully discriminate seven major lyssavirus species and presented 100% sensitivity and 93.94% specificity compared with a fluorescent antibody test (FAT) (Xi et al., 2012). In this study, our microarray assay displayed 96.4% clinical sensitivity and 90.9% clinical specificity (Table 4), which is comparable to that reported in other studies (Zhu et al., 2007; Xi et al., 2012; Zhou et al., 2014). This indicated that our microarray assay is practical and reliable for preliminary screening of harmful algal species in field samples.

Although some works on multiple detection of HAB species, such as multiplex PCR (Handy et al., 2006) and DNA microarray (Ahn et al., 2006; Ki and Han, 2006; Galluzzi et al., 2011; McCoy et al., 2013), have been reported, few studies dealt with detection methods aimed at macroalgae for green tides. BW is a well-known vector for the transmission of microalgae species, particularly cyst-forming HAB species (Hamer et al., 2001). Resting cysts of HAB species are relatively resistant to adverse environmental conditions, such as the ballasting process and long-term darkness in ballast tanks, which favors ship-assisted dispersal (Hamer et al., 2001). The conditions in ballast tanks could induce spore and propagule formation of microscopic stages; therefore, BW is also a potential vector for the invasive macroalgal species (Flagella et al., 2007; Kolwalkar et al., 2007). Thus, the DNA microarray method can be integrated within larger observatory networks and applied not only in governments' procedures for routine monitoring of HAB species or investigators interested in ecological maintenance against HAB species, but also port quarantine of BW. This method provides a time- and labor-saving alternative for preliminary screening of harmful algal species in field samples.

5 CONCLUSION

We established a multiplex PCR-based microarray method for the simultaneous detection of nine species of environmentally harmful algae. This method presented acceptable clinical sensitivity and specificity; thus, it can be used to provide a convenient platform for the routine monitoring of harmful algal species causing red and green tides. Further studies are necessary to generate complementary probes that can increase the total number of detectable species of harmful algae.

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