Development of a PNA Probe for Fluorescence In Situ Hybridization Detection of *Prorocentrum donghaiense*

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Abstract

*Prorocentrum donghaiense* is a common but dominant harmful algal bloom (HAB) species, which is widely distributed along the China Sea coast. Development of methods for rapid and precise identification and quantification is prerequisite for early-stage warning and monitoring of blooms due to *P. donghaiense*. In this study, sequences representing the partial large subunit rDNA (D1–D2), small subunit rDNA and internal transcribed spacer region (ITS-1, 5.8S rDNA and ITS-2) of *P. donghaiense* were firstly obtained, and then seven candidate DNA probes were designed for performing fluorescence in situ hybridization (FISH) tests on *P. donghaiense*. Based on the fluorescent intensity of *P. donghaiense* cells labeled by the DNA probes, the probe DP0443A displayed the best hybridization performance. Therefore, a PNA probe (PP0443A) analogous to DP0443A was used in the further study. The cells labeled with the PNA probe displayed more intensive green fluorescence than that labeled with its DNA analog. The PNA probe was used to hybridize with thirteen microalgae belonging to five families, i.e., Dinophyceae, Prymnesiophyceae, Raphidophyceae, Chlorophyceae and Bacillariophyceae, and showed no visible cross-reaction. Finally, FISH with the probes PP0443A and DP0443A and light microscopy (LM) analysis aiming at enumerating *P. donghaiense* cells were performed on the field samples. Statistical comparisons of the cell densities (cells/L) of *P. donghaiense* in the natural samples determined by FISH and LM were performed using one-way ANOVA and Duncan’s multiple comparisons of the means. The *P. donghaiense* cell densities determined by LM and the PNA probe are remarkably higher than that (p=0.05) that determined by the DNA probe, while no significant difference is observed between LM and the PNA probe. All results suggest that the PNA probe is more sensitive that its DNA analog, and therefore is promising for the monitoring of harmful algal blooms of *P. donghaiense* in the future.

Introduction

The occurrence of harmful algal blooms (HABs) reportedly has been increasing on a global scale, which is associated with a series of economic and environmental problems [1]. To warn of the occurrence of HABs and avoid the loss due to them, strict monitoring of the causative algae is necessary. Therefore, precise detection methods should be developed to facilitate the identification and quantification of harmful algae.

*Prorocentrum donghaiense*, which belongs to Dinophyta, Dinophyceae, Prorocentrophyceae and Prorocentrales, is a common *Prorocentrum* species widely distributed along the China coast. Meanwhile, this species has always been one of the most dominant HABs species in the East China Sea since 2000 [2,3]. It has also been reported that blooms of the same species have occurred in Japan, South Korea and Turkey. In China several major blooms of over 1000 km² have occurred in the last decade causing significant local concern [4]. Considering its negative impact on the marine ecosystem, aquaculture and public health, it is essential for precise identification and quantification in the phytoplankton research and to provide important data for water quality assessment and early warning of the hazards of *P. donghaiense* to fisheries and aquaculture.

Unfortunately, correct identification and enumeration of *P. donghaiense* is not trivial. The cells are smallish, with a length of 16–22 μm and width of 9.5–14 μm, and are fragile and cell morphology often changes under different water conditions [3]. This species has not been recognized for a long time until it was first reported and established by Lu and Goebel [5] in 2001. Even after the establishment of *P. donghaiense*, it has also been confused with another related species *P. dentatum* [3,6,7]. Specially, the taxonomy of *P. donghaiense* has been very recently discussed in Percopo et al [8]. This paper has commented the similarity of *P. donghaiense* and *P. maximum*, indicating a potential synonymy of the two species, which is however still not resolved due to the lack of taxonomical information on *P. maximum*. One clear implication is...
that much experience is required to identify and enumerate *P. donghaiense* by light and electron microscopy using morphological characters known to be present in both cultured and wild samples. Things become more complicated when *P. donghaiense* is only a minor component of planktonic assemblages, or when trying to distinguish between morphologically similar species or strains, such as *P. dentatum, P. minimum* and *P. micans*. Moreover, the traditional methods relying on microscopical examination is laborious, tedious and time-consuming, especially when large numbers of samples are to be analyzed. For the above reasons it is necessary to develop a simple, rapid, and effective identification and quantification method for this species.

In previous studies, biochemical, immunological and molecular techniques have been introduced to facilitate identification and enumeration of phytoplankton [8]. Among these, molecular methods are the most favored, because they aim for nucleic acid in cells, which is relatively invariable compared with other target molecules. Lots of techniques, including fluorescence in situ hybridization (FISH) [8,9], real-time PCR [10,11], sandwich hybridization assay (SHA) [12,13], loop-mediated isothermal amplification [14], nucleic-merization-assy/sandwich hybridization (NPA-SH) [15] and nucleic acid sequence-based amplification (NASBA) [16] have been reported. However, few efforts were made on *P. donghaiense*. Polyclonal antibodies targeting cell surface antigens of *P. donghaiense* were firstly developed by Wang et al. [17]. Despite that this method could distinguish *P. donghaiense* from other unrelated species, the antiserum against *P. donghaiense* showed weak cross-reactions with the closely related species. Another problem is that the detection reliability needs to be further tested, since the cell surface tends to change with water conditions. Moreover, the serum preparation is comparatively complicated and troublesome. Recently, Chen et al. [2] established an assay for *P. donghaiense* with NPA-SH. However, this method requires the quantitative extraction of high quality RNA, which is more difficult for *Prorocentrum* with hard thecae than for fragile and naked species (e.g. *Heterosigma akashiwo*) [18,19]. Specially, uniform extraction of RNA from a diverse range of organisms is necessary for environmental monitoring. These suggest that NPA-SH may be not promising.

**Results and Discussion**

**Probes design**

The final aim of this study is to develop a PNA probe for FISH detection of *P. donghaiense*. Screening an optimal probe among few candidate probes is crucial for this. Direct PNA probe screening must be costly, since the current price of a PNA probe is more than 10 times higher than that of its DNA analog. Therefore, we obtained the optimal probe of best hybridization performance by testing a few DNA probes, and then used its PNA analog for the further study.

So far, the probes targeting rRNA have been widely used for FISH detection of several harmful algae [12,20], with less work done to develop rDNA-targeted probes [8,32]. In this study, a wide range of probes were screened from the LSU D1–D2, SSU rDNA, and ITS sequences, among which both the LSU D1–D2 and SSU were used for rRNA targeting probes, while the ITS for rDNA targeting probes design. BLAST search and alignment analysis showed that different stains of *P. donghaiense* have identical nucleic acid sequences of LSU D1–D2, SSU rDNA and ITS (data not shown), implying that they are conservative and competent for probe design for different stains of the species. However, they display comparatively different variability within *Prorocentrum*. Among them, LSU D1–D2 shows higher variable degree, whereas SSU rDNA and ITS are relatively conservative to be difficult to search for specific regions.

Remarkably, the conservation of the ITS sequence of *P. donghaiense* is out of expectation, since more findings demonstrate that many species usually have more variable ITS than their LSU and SSU [33,34] due to the less evolutionary pressure and relatively rapid divergence rates [35]. Finally, a total of 9 DNA probes, including 4 targeting LSU rRNA (DP0387A22, DP0602A23, DP0512A19 and DP0443A19), 1 targeting SSU rRNA (DP1704A25), 2 targeting ITS rDNA (DP0159A25 and DP0498A21), and 2 control probes (DU0512A18 and DU0499S18) [36–38], were introduced for further probes screening, as shown in Table 1.

**Probes screening**

The results of FISH using all the DNA probes are summarized in Table 2 and Fig. 1. *P. donghaiense* could not be labeled by the probes targeting both SSU rRNA (DP1704A25) and ITS rDNA (DP0159A25, DP0498A21). The complex second structure of rRNA may preclude its hybridization with DP1704A25, since rRNA expression in cells is often thought to be at a high level. Except for certain species [8], rDNA is generally thought to be unsuitable for probe targeting, because the cells labeled with rDNA targeting probe tend to display weak fluorescence [32], which disturb their differentiation from other species in natural samples [39]. Things seem to get worse for *P. donghaiense*, since the cells marked by both DP0159A25 and DP0498A21 did not display...
any visible fluorescence under epifluorescence microscopy. The possible reason for this is that the copies of ITS rDNA within genomic DNA of *P. donghaiense* are at least less than *A. catenella* [32], *A. tamarens* [32] and *H. akashiwo* [8]. Therefore, *P. donghaiense* cells could not provide enough binding molecules for the rDNA targeting probe, and the hybridized cells with less fluorescein labeled probe naturally give out weak and even invisible fluorescence, as shown in this study.

The effect of the secondary structure of the LSU rRNA on the accessibility of probes to the target sites has been shown in previous studies [40–42]. Again, our findings reconfirm this. The four rRNA-targeted probes with even slight alternation in the 5′ portion of the sequence displayed different performance (Table 2 and Fig. 1). Among them, only DP0443A labeled *P. donghaiense* cells with fluorescent intensity equivalent to the positive control probe (DU0512A18), while *P. donghaiense* cells marked by DP0443A did not show any fluorescence. The cells labeled with DP0602A and DU0512A displayed more or less intensive fluorescence compared with the positive control probe labeling cells, respectively. The further quantification analyses of fluorescent intensity of cells labeled with different probes were shown in Fig. 2. Apparently, the fluorescent intensity of DP0443A labeling cells were significantly more intensive (p<0.05) than that of the cells marked by other LSU rDNA-targeted probes.

Based on these findings, DP0443A could be considered as the best among these designed DNA probes. Consequently, we synthesized a PNA probe (PP0443A) with the same nucleotide sequence as DP0443A and utilized it to hybridize with *P. donghaiense*. As expected, the PNA probe PP0443A labeled *P. donghaiense* cells with more intensive fluorescence than the positive control and DP0443A (Fig. 1). Moreover, the difference in fluorescent intensity between them was significant (p<0.05) (Fig. 2). Thus, we gain the ideal PNA probe for FISH detection of *P. donghaiense*.

### Specificity of the PNA probe

The specificity of the PNA probe (PP0443A) should be considered as a critical point for FISH detection. To achieve this, the probes were firstly designed based on the multiple sequence alignment involving the LSU D1–D2 sequences of *P. donghaiense* and all other *Prorocentrum* available in Genbank. Next, BLAST searches were performed on the designed probes, confirming that the sequences of probes could exclusively match with *P. donghaiense*. Finally, cross-reactivity of the screened probe against other microalgae was tested. The positive (DU0512A) and negative (DU0499S18) control treatments were included to define a range of labeling intensities possible for any given sample and thereby provided a reference from which to assess the reactivity of specific probe.

The FISH trials served as an intermediate step to determine whether a candidate probe could access its target sequence. Therefore, no attempt was made to optimize the whole cell hybridization conditions and the list of species used in the trials was also limited. The results of hybridization with all test species using the PNA probe and control probes are shown in Table 2. The positive probe could react with all test species, repeatedly giving bright and uniform labeling intensity for all species examined. Contrarily, the negative probe could not label any species, and the cells treated by negative probe appeared uniformly dark. In contrast, PP0443A reacted exclusively with *P. donghaiense*. Based on these, the specific PNA probe may be speculated to be useful for molecular identification of the target species in natural samples containing many different microalgae.

### Table 2. Sensitivity of probes to *Prorocentrum donghaiense* determined by the FISH assays a.

| Probes       | DU0512A | DU0499S | DP0587A | DP0602A | DU0512A | DP0443A | DP1704A | DP0159A | DP0498A | PP0443A |
|--------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| **Sensitivity** | ++++    | –       | –       | ++      | +       | +++     | –       | –       | –       | +++     |

*aCells with signal intensity similar to the positive control were scored as "+++"; signal intensity equivalent to the negative control was scored as "–"; signal intensities clearly above the negative but below the positive control were scored as "++" or "+", depending on the brightness relative to the positive and negative probes; signal intensity above the positive control was scored as "++++".

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Application of DNA and PNA probes to detect *P. donghaiense* in natural samples

Both the DNA (DP0443A) and PNA (PP0443A) probes were used to analyze twelve natural samples from different stations located in the East China Sea. The representative micrographs of FISH analysis are shown in Fig. 3. Some dying or dead target cells, deduced from their blurry contours with weaker color compared with the surrounding living cells under light microscope (LM), were observed to be included in the field samples (Fig. 3 C). Both the DNA and PNA probes could enter the algal cells easily and bound strongly with the target species, rendering the target cells green (Fig. 3 A, B). However, the PNA labeled cells were expected to give stronger fluorescence on average than the DNA probe labeled cells (Fig. 3 A, B). The reason for this is that the PNA probe has much stronger binding capability [26,27] and higher hybridization efficiency [29] than its DNA analog. This also explains why the dying or dead cells could well be stained by the PNA probe, but scarcely stained by the DNA probe (Fig. 3 B, C). Moreover, the hybridizations with both probes are specific, since only the *P. donghaiense* cells were labeled in the field samples, without non-specific binding to other algal species (Fig. 3).

All the natural samples were used for direct enumeration by LM and indirect enumeration after FISH treatments with both the DNA and PNA probes. The results showed that the *P. donghaiense* cell densities determined by LM and the PNA probe were remarkably higher than that determined by the DNA probe (p<0.05) (Fig. 4). No significant difference was observed between the cell densities determined by LM and PNA probe (Fig. 4). Whether the dying or dead cells were stained or not due to the sensitivity may be one of the most possible reasons for the difference in cell densities between the DNA and PNA probes. Obviously, the PNA probe is more competent for target cell enumeration than the DNA probe. These also indicate that the PNA probe and the hybridization protocol are effective for the detection of *P. donghaiense* in the field samples.
Many factors are speculated to influence efficiency and detection sensitivity of a molecular probe, such as sample treatment methods, autofluorescence of chlorophyll, and physiological station of target cells. Firstly, several necessary steps are usually taken to deal with the samples prior to observing fluorescent labeled target cells under the epifluorescence microscope. Lots of target cells are likely to be lost in the sample treatment steps, such as repeated centrifugation, pipetting, and washing in the earlier studies [32,39]. This is specifically not fit for the natural samples in which the target species is a minor component. However, the subsequent filtration methods for the capture of target cells in the field samples [9,20], as being adopted in this study, have already overcome this problem, avoiding the loss of even single cell. Secondly, red autofluorescence from abundant chlorophylls in algal cells could interfere with observation of the green fluorescence of target cells, which would possibly result in an underestimation of target cells. Therefore, an additional decolorization is likely a prerequisite prior to FISH analysis. This is sometimes true for the cells fixed by paraformaldehyde, which need a further ethanol or acetone treatment to reduce autofluorescence [43,44]. However, the cells treated with the more widely used saline ethanol fixative are often competent for direct FISH analysis, without additional decolorization, because ethanol in the fixative could well destruct the chlorophylls. Some harmful algae, such as P. micans and Karenia spp. are exceptional (data not shown). When performing FISH analysis on them, the further methanol treatment to remove intensive red autofluorescence is necessary. Fortunately, the autofluorescence of P. donghaiense cells fixed by ethanol-based fixative was entirely decomposed which should lead to reduced fluorescent intensity of labeled cells [20]. However, the more sensitive PNA probe will work well despite of less rRNA content. Therefore, it could be inferred that the PNA probe should be more suitable than its DNA analog for FISH analysis of field samples preserved for a long time.

In summary, the hybridization protocol adopted in this study is competent, and the PNA probe is more sensitive that its DNA analog, and therefore is promising for the monitoring of P. donghaiense in the natural samples in the future.

Materials and Methods

Algal cultures

Clonal P. donghaiense and other microalgae employed in this study were shown in Table 3. All the cultures were established by pipeting single cells or chains of cells, sequentially through droplets of sterile seawater. Cultures were grown at 20–22°C in Guillard’s f/2 medium [46] on a 12:12-h light:dark cycle with light provided by cool white fluorescent tubes at a photon flux density of 50–100 μmol m⁻² s⁻¹. Silicate (110 μM) was added to the f/2 medium to support the growth of Skeletonema (used for probe cross-reactivity testing). All cultures were maintained in 250 ml flasks containing 100 ml f/2 (+Si) medium.

Dna extraction, PCR amplification, cloning and sequencing

Total genomic DNA was isolated according to the protocol described previously by Chen et al. [8]. The LSU D1–D2, SSU and ITS sequences were specifically amplified by PCR with the universal primer pairs, D1 (5′-ACCCGCTGAATTTAAGCATA-3′)/D2 (5′-CCTTGGTGCCGTTCTTCAGAGA-3′) [47], 65S1N (5′-TCCTGGCCATATGCATATGC-3′)/16S2N (5′-TGATCTCTGTCGTGTGGAG-3′) [48], and TW81 (5′-GGGATCCGTTCGTTGCCAGTGACCCCTCAG-3′)/AR82 (5′-GGGATCCATATGCTTTTAACTTACGCG-3′) [49,50] using a DNA Thermal Cycler (Takara, Japan).

Table 3. List of species investigated in this study.

| Species                          | Geographic origin                  |
|---------------------------------|------------------------------------|
| Prorocentrum donghaiense         | East China Sea, West Pacific Ocean |
| Prorocentrum minimum             | East China Sea, West Pacific Ocean |
| Prorocentrum micans              | East China Sea, Zhejiang, China    |
| Prorocentrum dentatum            | Daya Bay, Guangdong, China         |
| Alexandrium tamarense            | East China Sea, West Pacific Ocean |
| Karenia sp1                      | Wenzhou, East China Sea, West Pacific Ocean |
| Karenia sp2                      | Hangzhou, East China Sea, West Pacific Ocean |
| Gymnodinium sp.                  | Jiaozhou Bay, Yellow Sea, West Pacific Ocean |
| Phaeocystis globosa              | Daya Bay, Guangdong, China         |
| Heterosigma akashiwo             | Jiaozhou Bay, Yellow Sea, West Pacific Ocean |
| Platy-monas cordiformis          | Bohai Sea Bay, West Pacific Ocean  |
| Skeletonema tropicum             | Qingdao Fishery, Yellow Sea, West Pacific Ocean |
| Skeletonema dohrnii              | Jiaozhou Bay, Yellow Sea, West Pacific Ocean |
| Skeletonema costatum             | Xiamen, Taiwan Strait, West Pacific Ocean |

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Dalian, China), respectively. The amplification conditions were as follows: denaturing at 94°C for 4 min, followed by 29 cycles of 94°C 1 min, 50°C 50 s, 72°C 50 s, and a final extension at 72°C for 7 min. Amplification products were purified and recycled using TIANquick Midi Purification Kit (TIANGEN Biotech Co., Ltd., Beijing, China) according to the manufacturer’s instructions. Purified PCR products were ligated with pMD 18-T Vector (Sangon Biotech Co., Ltd., Shanghai, China) and transformed into competent Escherichia coli DH-5α (Sangon Biotech Co., Ltd., Shanghai, China). The positive colonies containing the objective DNA fragments were identified by colony PCR and then sequenced using Vector primer M13 as sequencing primer. Sequencing was performed in Sangon (Shanghai) Biotech Co., Ltd. The obtained sequences were submitted to GenBank, acquiring the accession numbers of DQ336340 (LSU D1–D2), AY465116 (ITS), and DQ336054 (SSU).

**DNA alignment and probe design**

The obtained LSU D1–D2, SSU and ITS sequences were used for BLAST search, respectively, and the corresponding sequences of all *P. donghaiense* strains and *Prorocentrum* spp. deposited in GenBank were downloaded. All sequences of *Prorocentrum* used in this study were shown in Table 4. Three independent alignments containing the LSU D1–D2, SSU and ITS sequences, respectively, were conducted using computer software BioEdit for visually searching for specific regions for *P. donghaiense*. Oligonucleotide probes targeting the SSU, ITS and LSU were designed with the help of Premier Primer 6.0, respectively. The candidate probes were then refined with the aid of Oligo 6.0, excluding unsuitable probes mainly according to the potential problems associated with secondary structure and homomer/dimer formation. The probes were screened with BLAST to examine their specificity against a wide range of organisms. Both the DNA (Invitrogen Biotechnology Co., Ltd., Shanghai, China) and PNA (Paide Biotechnology, Chengdu, China) probes were synthesized commercially with fluorescein isothionate (FITC) attached to the 5’ end. The probes received in a lyophilized form were dissolved in 0.1 M Tris-HCl (pH 7.5) to a final concentration of 100 μM, and aliquots were stored at −20°C in the dark. The probes are named following a changed nomenclature firstly outlined by Wheeler Alm et al. [51]. Using the probe ‘DNA-Pdon-0587-A-22’ as an example, the first three letters stand for the kind of the probe. The second four-letter code is for the species targeted. The next number is the 5’ position of the probe relative to either *Escherichia coli* or target organism (*P. donghaiense*). The next letter is for whether the probe is identical to the DNA sense (S) or antisense (A) strand. The last number is the length of the probe. All probes used in this study are listed in Table 1. In the rest of the table, figures and text, the probe name is shortened for brevity: for example, DNA-Pdon-0587-A-22 becomes DP0587A.

**Fluorescence in situ hybridization tests for optimal probe**

Comparative study on the hybridization performance of candidate probes was performed to screen the best probe. Approximately 10 ml of mid-exponential culture was pipetted gently into a 50 ml centrifuge tube containing 30 ml of saline ethanol fixative [1.25 ml deH2O, 3.75 ml 20×SET buffer (3.00 M NaCl, 20 mM EDTA, 0.40 M Tris HCl, pH 7.8) and 25 ml of 95% ethanol] [37]. The mixture was left to stand at room temperature for 5 min before gently mixing by inversion, allowed to stand for an additional hour, and then centrifuged at 6000 g for 2 min at 4°C. The supernatant was removed, and the fixed cells were washed twice in 5×SET hybridization buffer by centrifugation at 6000 g for 2 min at 4°C. About 1–1.5 ml of 5×SET hybridization buffer was added to re-suspend the precipitated cells. The pelleted cells were aliquoted to 1.5 ml Eppendorf tubes. After centrifugation at 6000 g for 2 min at 4°C, as much supernatant as possible was removed for each tube. Then, 200 μl of 5×SET hybridization buffers containing probes were added. For probes targeting nuclear ITS DNA, cells were incubated at 97°C for 3 min to denature genomic DNA and incubated on ice for 5 min prior to hybridization. The reaction tubes were incubated for 1 h at 45°C. After hybridization, the labeled cells were washed twice with 1×SET for 3 min at 50°C. The labeled cells were at once mounted on glass microscope slides with SlowFade Light antifade.

| Species                  | GenBank accession number (LSU) | GenBank accession number (ITS) | GenBank accession number (SSU) |
|--------------------------|-------------------------------|-------------------------------|-------------------------------|
| Prorocentrum donghaiense | DQ336340, EU586259, AY863007, AY833516, AY822610 | DQ336340, AY465116 | DQ336054, AY803743, AJ841810, AY551272 |
| Prorocentrum minimum     | EU780639                      | DQ662403                      | AY803741, AY803740 |
| Prorocentrum micans      | EU780638                      | EU927531                      | AY803739                   |
| Prorocentrum dentatum    | FJ823581                      | FJ823581                      | DQ336057, AY803742 |
| Prorocentrum balticum    | AF042816                      | EU927547                      |                               |
| Prorocentrum rostratum   | EU244471                      | EU244471                      |                               |
| Prorocentrum rhathymum   | EU165279                      | EU244466                      | EU287487                   |
| Prorocentrum triestinum  | AF042815                      | EU927551                      | DQ004734                   |
| Prorocentrum mexicanum   | DQ336183                      | AY886763                      | EU287485                   |
| Prorocentrum lima         |                               |                               | FJ823582                   |
| Prorocentrum cassubicum   |                               |                               | EU244475                   |
| Prorocentrum compressum   |                               |                               | EU927558                   |
| Prorocentrum gracile      |                               |                               | AY443019                   |
| Prorocentrum tsawwassenense |                            |                               | EF657885                   |

Table 4. List of *Prorocentrum* introduced into alignment for design of probes, with GenBank accession numbers of their LSU rDNA, ITS, and SSU rDNA sequences.
solution (Molecular Probes Inc., Eugene, OR, USA) for epifluorescence microscopic observation or stored at 4 or -20°C in the dark for future analysis.

Image capture and quantification of fluorescent intensity of labeled cells

Both image capture and quantification of fluorescent intensity of labeled cells were carried out as described in Miller and Scholin [20]. Microscopic observations of cells were performed at 522 nm under an epifluorescence microscope (Nikon Eclipse E800, Tokyo, Japan) when stimulated with 494 nm wavelength and fluorescent micrographs of cells were taken with Nikon digital camera equipped with the microscope. For comparative study, the configuration of the microscope remained constant throughout all trials, and all images were captured using a manual exposure setting of 3-s integration with all other camera parameters at default settings. Images were analyzed using computer program Scion Image. The freehand selection tool was used to manually determine the pixel density of cells by defining labeled cells being analyzed. Twenty randomly selected cells were examined from each treatment and pixel density was averaged to provide a quantitative estimate of cell fluorescence intensity. The final cell fluorescence intensity was represented by the value of 255 subtracted by the mean pixel density of 20 cells.

Cross reactivity test

The PNA analog (PP0443A) to the DNA probe (DP0443A) of the best hybridization performance was used to hybrdize with thirteen microalgae cultured in our laboratory, including common HAB causative species, such as *P. minimum*, *P. micans*, *P. dentatum*, *Karenia* spp., *H. akashiwo*, *A. tamarense*, *Phaeocystis globosa* and *Skeletonema* spp. (Table 3), following the already described FISH procedure for *P. donghaiense*.

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**FISH and light microscopy (LM) analysis of field samples**

Natural samples were collected from East China Sea, where the cell density of *P. donghaiense* bloom is commonly at 10⁶ cells/L [3]. The improved protocol for the filed material was summarized as follows. Briefly, 1.5 ml field sample was fixed for 30 min with 5.5 ml of saline ethanol solution, filtered using Whatman 25 mm diameter 0.2 μm pore size Nuclepore filter, and then rinsed twice with 1 ml of hybridization buffer (5×SET). Wrapped filters could be stored at 4°C for at least 4 weeks or processed immediately. Next, the filter was placed on a glass slide and 500 μl of probe (10 μM) [PP0443A or DP0443A] dissolved in 5×SET was added. The filter was hybridized in the dark for 1 h at 45°C, washed twice for 3 min at 30°C with 1 ml of pre-warmed washing buffer (1×SET) to remove excess probe. The labeled cells were examined and counted under an epifluorescence microscope. Also, the natural samples were used for direct enumeration by LM with haemacytometer. The morphological characteristics used to distinguish *P. donghaiense* from other taxa were as being described in Lu et al. [3,7] and Lu and Goebel [3].

**Statistical analysis**

Statistical analysis of fluorescent signal intensity of labeled cells was carried out using the software SPSS 13. One-way ANOVA and Duncan’s multiple comparisons of the means were done to compare the data obtained.

**Author Contributions**

Conceived and designed the experiments: GC CZ BZ. Performed the experiments: GC. Analyzed the data: GW ZX PY. Contributed reagents/materials/analysis tools: BZ GW DL. Wrote the paper: GC.
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