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Fiber-Optic Microarray for Simultaneous Detection of Multiple Harmful Algal Bloom Species

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Harmful algal blooms (HABs) are a serious threat to coastal resources, causing a variety of impacts on public health, regional economies, and ecosystems. Plankton analysis is a valuable component of many HAB monitoring and research programs, but the diversity of plankton poses a problem in discriminating toxic from nontoxic species using conventional detection methods. Here we describe a sensitive and specific sandwich hybridization assay that combines fiber-optic microarrays with oligonucleotide probes to detect and enumerate the HAB species *Alexandrium fundyense*, *Alexandrium ostenfeldii*, and *Pseudo-nitzschia australis*. Microarrays were prepared by loading oligonucleotide probe-coupled microspheres (diameter, 3 μm) onto the distal ends of chemically etched imaging fiber bundles. Hybridization of target rRNA from HAB cells to immobilized probes on the microspheres was visualized using Cy3-labeled secondary probes in a sandwich-type assay format. We applied these microarrays to the detection and enumeration of HAB cells in both cultured and field samples. Our study demonstrated a detection limit of approximately 5 cells for all three target organisms within 45 min, without a separate amplification step, in both sample types. We also developed a multiplexed microarray to detect the three HAB species simultaneously, which successfully detected the target organisms, alone and in combination, without cross-reactivity. Our study suggests that fiber-optic microarrays can be used for rapid and sensitive detection and potential enumeration of HAB species in the environment.

Harmful algal blooms (HABs) result from the proliferation of certain types of phytoplankton species. In some cases, accumulation of these organisms can cause a discoloration of the seawater, giving rise to the name "red tides" (3). HABs pose a serious threat to public health because many HAB species produce potent toxins, which are responsible for a variety of shellfish poisoning syndromes. Consumption of HAB-contaminated shellfish has been linked to mortalities of wild and farmed fish, seabirds, and mammals (13, 24) and can result in illness or death in humans (4, 31). In addition, HABs adversely affect the coastal economy, causing economic loss due to restrictions on seafood industries and reduced tourism (22, 37). The economic loss caused by HAB is estimated to be at least $49 million each year in the United States alone.

As the frequency of HAB occurrences has increased worldwide (22), new techniques have been developed to monitor seawater for the presence of HAB species. Traditionally, phytoplankton are detected and enumerated by direct observation using light or electron microscopy of live or preserved seawater samples. Although this method can provide direct visual confirmation of target organisms, it is both time-consuming and requires expertise in phytoplankton taxonomy because of the difficulty in identifying morphologically similar species or strains (20, 36). The latter problem frequently occurs in the study of HAB species, because toxic and nontoxic phytoplankton species can coexist in the collected sample, and in most samples, the HAB species of interest is often a minor component of the mixed plankton community (5). Alternatively, the identification and enumeration of HAB species have been facilitated by the development of molecular methods for HAB cell detection.

Antibody-based detection methods for HAB species have been developed that target specific molecules on the cell surface (26, 30, 33, 40). Detection of a wide range of HAB species using immunological methods has been reported (9, 39, 41). Many immunological detection methods suffer from poor quantification, primarily due to cross-reactivity problems and to cell loss during sample processing (6, 7).

More commonly, oligonucleotide probes have been employed to identify HAB species using short, synthetic DNA that selectively binds to sequences specific to a target organism. DNA-based methods for HAB analysis are particularly focused on the detection of rRNA. Ribosomal genes have several significant advantages as detection targets for HAB species. First, they contain regions ranging from highly conserved to highly variable, which enables discrimination of cells at various levels, from broad phylogenic groups to species and even strains (27, 36). Second, ribosomal genes are present in high copy numbers, providing abundant target molecules to which oligonucleotide probes can bind (28, 38). The use of oligonucleotide probes targeting rRNA in HAB species has been approached in several different ways, including whole-cell hybridization (1, 29, 32, 36), sandwich hybridization (34, 35), and PCR-based methods (11, 19, 20, 21).

Fiber-optic arrays have been successfully employed as platforms for various applications ranging from diagnostics to artificial noses (10, 15, 43). In particular, microsphere-based fiber-optic arrays provide many advantages over other array-based methods: higher sensor-packing density, smaller assay

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TABLE 1. Oligonucleotide probe sequences for HAB target rRNAs

Probe type and name	Target organism	Length (bp)	Sequence (5′→3′)
Capture probes			
NA1S	*A. fundyense*	21	GCAAGTGCAACACTCCCACCA
AO2	*A. ostenfeldii*	21	GTGGACGCAACAATCTCACCA
auD1S	*P. australis*	23	AAATGACTCACTCCACCAGGCGG
Signal probes			
AlexS	*A. fundyense*, *A. ostenfeldii*	24	TTCAAAGTCCTTTTCATATTTCCC
PseudS	*P. australis*	33	CTCTTTAACTCTCTTTTCAAAGTTCTTTGCATC

sample volumes, increased array reusability, flexible array design, and reduced false positives and false negatives (16). Previous work has demonstrated that the microsphere-based fiber-optic array can detect as few as 600 target DNA molecules and is sensitive enough to discriminate a single-base mismatch from a perfect match (15, 17).

In this paper, we describe a simple, specific, and sensitive method for simultaneous detection of multiple HAB species using microsphere-based DNA fiber-optic microarrays. Three HAB species were chosen as target organisms for this study: *Alexandrium fundyense*, *Alexandrium ostenfeldii*, and *Pseudo-nitzschia australis*, all of which are associated with toxic blooms in the Gulf of Maine (*A. fundyense* and *A. ostenfeldii*) or on the West Coast of the United States (*P. australis*). A sandwich hybridization methodology was employed in which target rRNA in the sample was hybridized to capture probes immobilized on the microspheres, followed by a second hybridization with Cy3-labeled signal probes. The resulting fluorescent signals from the hybridization were observed using a charge-coupled device (CCD) camera.

(A preliminary report of this work was presented previously by Anderson et al. [8].)

MATERIALS AND METHODS

Materials. Optical fiber bundles of with diameters of 500 μm and 1 mm were obtained from Galileo Electro-optics Corp., Sturbridge, MA (the company no longer exists; a large spool of fiber was purchased before the company went out of business) and Illumina Inc. (San Diego, CA), respectively. Amine-modified poly(methylstyrene)-divinylbenzene microspheres (diameter, 3.1 μm) were purchased from Bangs Laboratories, Inc. (Carmel, IN). Europium(III) thenoyltrifluoroacetonate · $3H_2O$ (Eu-dye) and cyanuric chloride were obtained from Acros Organics (Morris Plains, NJ). Glutaraldehyde (25% aqueous solution) was purchased from Polysciences (Warrington, PA). Acetonitrile was purchased from Aldrich Chemical Company (Milwaukee, WI). Ammonium fluoride, hydrofluoric acid, tetrahydrofuran (THF), methanol, Tween 20, dimethyl sulfoxide, succinic anhydride, formamide, and polyethyleneimine (PEI) were purchased from Sigma Chemical Co. (St. Louis, MO). All oligonucleotide probes used in this study were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Sterile water used to resuspend oligonucleotide probes was purchased from Abbott Laboratories (North Chicago, IL). Cell lysis buffer was obtained from Orca Research Inc. (Bothell, WA). Buffers used in this study were diluted from concentrated stock solutions of 100× Tris-EDTA (TE) buffer, (pH 8.0; Sigma Chemical Co., St. Louis, MO), or 10× phosphate-buffered saline (PBS) (pH 7.4; Fluka, Seelze, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. All reagents were used without further purification.

Microsphere encoding. Internal encoding of microspheres was performed as reported previously (2). Aliquots (50 μl) of 3.1-μm-diameter amine-modified poly(methylstyrene)-divinylbenzene microspheres were washed three times each with 150 μl PBS (1×; pH 7.4) and then 150 μl THF. A 150-μl aliquot of Eu-dye in THF (0.025 M, 0.1 M, or 0.5 M) was added to the microspheres, and the mixture was shaken for 2 h at room temperature. To avoid clumping of microspheres, the mixture was vortexed every 15 min with shaking. Encoded microspheres were recovered after centrifugation (9,000 × *g*, 5 min) and washed thoroughly with methanol and 1× PBS (pH 7.4). The encoded microspheres were stored in 0.01% Tween 20 in PBS at 4°C until use.

Oligonucleotide probes. Previously designed DNA oligonucleotide probes were used for detection of *Alexandrium fundyense* and *Pseudo-nitzschia australis* (7, 34). The capture probe for *Alexandrium ostenfeldii* (AO2) was designed for this study based on sequence and probe information reported previously (23). Probe sequences are listed in Table 1. The probes were stored at a concentration of 100 μM in DNase- and RNase-free water.

Coupling of oligonucleotide probes to the microsphere surface. Oligonucleotide probes were activated as previously described (2). A 250-μl aliquot (25 nmol) of DNA (100 μM) was activated by the addition of 40 μl of 50 mM cyanuric chloride in acetonitrile. The mixture was shaken for 2 h at room temperature, and the activated DNA probes were separated from the unreacted cyanuric chloride by Amicon centrifugal filters of with a 3,000-molecular-weight cutoff (Millipore, Bedford, MA). DNA probes were recovered in 200 μl of 100 mM sodium borate buffer (SBB; pH 8.6).

An aliquot of each encoded microsphere solution (10 μl) was suspended in 200 μl of 8% (vol/vol) glutaraldehyde in 1× PBS with 0.01% (vol/vol) Tween 20 (PBST; pH 7.4), and shaken for 1 h at room temperature. The mixture was vortexed every 15 min to avoid aggregation of microspheres. After three washes with PBST, the microspheres were suspended in 200 μl of 5% (vol/vol) PEI in PBS. The mixture was shaken for 1 h at room temperature, and the microspheres were rinsed with PBST followed by SBB. An aliquot (100 μl) of cyanuric chloride-activated DNA was added to the PEI-functionalized microspheres, and the mixture was shaken overnight at room temperature. The microspheres were then rinsed with SBB three times. To prevent nonspecific binding, the remaining free amine groups on the microspheres were capped with 100 μl of succinic anhydride (0.1 M in 90% dimethyl sulfoxide, 10% SBB) by shaking for 1 h at room temperature. The microspheres were washed three times with SBB and three times with TE buffer containing 0.1% NaCl and 0.1% sodium dodecyl sulfate (SDS) and were then stored at 4°C prior to use.

Microarray formation. Optical fiber bundles with diameters of 500 μm and 1 mm, containing about 6,000 and 50,000 individual 3-μm-diameter optical fibers, respectively, were used in this study. The 1-mm-diameter optical fiber bundles were obtained from Illumina Inc. (San Diego, CA) already polished and etched. Optical fiber bundles of 500 μm diameter were polished on a fiber polisher followed by chemical etching to form microwells as described previously (2, 14). A DNA microarray was prepared by pipetting an aliquot (0.5 μl) of each microsphere suspension for a single-probe-type array, or a mixture of the different probe-functionalized microsphere solutions for a multiplexed array, onto the distal end of the etched fiber bundle containing the microwells. The loaded microspheres spontaneously and randomly distribute into individual microwells by capillary forces during solvent evaporation. After solvent evaporation, the distal end of the fiber bundle was wiped with an antistatic swab to remove excess microspheres. Registration for each probe type position on the randomized array was based on the "optical bar code," the encoding dyes contained within each microsphere, as previously described (18, 42).

Imaging system. The customized imaging system used in this study has been described previously (14, 17). Fluorescent signals were captured by a CCD camera (Orca ER; Hamamatsu Photonics, Trenton, NJ) and analyzed using IPLab software (Scanalytics, Fairfax, VA).

Cultures. *A. fundyense* (strain GTCA28), *A. ostenfeldii* (strains HT-240D2 and HT-120D6), and *P. australis* (strain 1BA) were grown in modified f/2 medium as previously described (5). At the mid-exponential phase of growth, cell counts were taken, and dilutions of the cultures were made with fresh f/2 medium to

yield a range of cell densities from 5 to 5,000 cells/ml. After dilution, aliquots of each diluted culture were filtered onto 25-mm, 0.65-μm-pore-size Durapore filters (Millipore Corp., Bedford, MA), which were placed in 2.0-ml cryovials and frozen in liquid nitrogen. Frozen samples were then stored at −80°C until use.

Sample preparation. The filtered cells in the cryovial were lysed by adding 500 μl of lysis buffer and vortexing to wet the filter completely. The vial was then heated for 5 min at 85°C and cooled in ice for 1 min. The resulting cell lysate was syringe-filtered through a 0.45-μm Durapore Millex-HV filter (Millipore Corp., Bedford, MA) into a fresh tube and was used as a target sample in the sandwich hybridization described below. When necessary, the filtered lysate was treated with RNase-free DNase by incubating the lysate samples with RQ1 DNase (Promega, Madison, WI) at 37°C for 5 min according to the manufacturer's instructions.

To prepare simulated field samples, 10 liters of coastal seawater (Vineyard Sound, MA) were filtered through 20-μm Nitex nylon mesh to concentrate the natural algal cells. The collected cells were washed into a tube with 10 ml of seawater, and this seawater concentrate was used for preparation of target HAB cells to provide different levels of background matrix. One milliliter of seawater concentrate prepared in this manner could provide background organisms corresponding to 1 liter of raw seawater. Note: to prepare multiplexed samples containing *P. australis* cells, rRNA from *P. australis* had to be prepared separately and mixed with other rRNA samples, instead of being directly prepared from a filtered cell mixture, since *P. australis* was collected and shipped separately from *A. fundyense* and *A. ostenfeldii*.

Microarray hybridization. Sandwich hybridization was performed by exposing the microarray first to 200 μl of target sample lysates, during which the target rRNA sequences from HAB species bound to their complementary capture probes immobilized on the microspheres. After incubation for 30 min, the array was washed twice with prewarmed TE containing 2% SDS (40°C). Following the wash steps, three fluorescence images were acquired with a 150-ms camera exposure time. The average signal intensity from individual bead types (~100 beads per microarray) was calculated from each image, and the average value from these three images was used as the background signal. A second hybridization was then performed by exposing the array to 200 μl of Cy3-labeled signal probes (1 μM) for 15 min and washing with prewarmed TE containing 2% SDS (40°C). After a wash, three fluorescence images were taken, and their average value was calculated as the hybridization signal as explained above. In this study, a positive hybridization signal was defined as any net signal (hybridization signal − background) greater than 3 times the standard deviation of the background intensity. After the measurement, the arrays were washed with dehybridization buffer containing 90% formamide solution in 1× PBS heated to 85°C until the fluorescence intensity returned to background levels, to prepare the arrays for reuse. All probe hybridizations were performed at room temperature.

RESULTS

Development of DNA microarray. Three species of algae, *Alexandrium fundyense*, *Alexandrium ostenfeldii*, and *Pseudonitzschia australis*, were selected for this study, since all three are known to be toxin-producing organisms that can co-occur in the Gulf of Maine (*A. fundyense*, *A. ostenfeldii*) or on the West Coast of the U.S. (*P. australis*). For each algal species, a specific capture probe was used (Table 1). While *A. fundyense* and *A. ostenfeldii* shared the same signal probe, AlexS, *P. australis* had its own signal probe, PseudS. Three types of DNA probe-functionalized microspheres, containing either NA1S, AO2, or auD1S capture probes, were prepared as described above for *A. fundyense*, *A. ostenfeldii*, and *P. australis*, respectively.

The performance of each capture probe was first tested using microarrays in which only a single probe sequence was present on all the microspheres in the array. Each of these arrays was exposed to Cy3-labeled synthetic targets with sequences complementary to the capture probe. Hybridization was performed at room temperature using 100 μl of synthetic target solution at two different concentrations, 1 nM and 1 pM, with hybridization times of 10 min and 30 min, respectively. Net signal intensities for each probe type were obtained by subtracting the averaged background signal intensity from the averaged hybridization signal intensity (Table 2). All probes were able to detect 1 pM target solution within 30 min, demonstrating the capacity of the microsphere-based DNA array for sensitive detection. The arrays were further tested with lower target concentrations; while all probe types could detect 100 fM target solution within 30 min, only NA1S gave a positive signal to 10 fM after 30 min of exposure (data not shown). The arrays were regenerated between the different target concentrations by dehybridizing with 90% formamide solution (85°C).

TABLE 2. Average net hybridization signals obtained with synthetic targets hybridized to single bead arrays[a]

Probe	Net hybridization signal ± SD for the following target concn/hybridization time:	
	1 nM/10 min	1 pM/30 min
NA1S	623 ± 73	376 ± 46
AO2	556 ± 43	272 ± 24
auD1S	656 ± 81	185 ± 25

[a] The standard deviation of the background was 21, and the threshold limit for a positive signal was calculated to be 63 (3 × 21).

Optimization of sandwich hybridization. In a sandwich hybridization assay, two separate hybridization steps are performed: a primary hybridization between the capture probe and the target rRNA and a secondary hybridization between the target rRNA and the signal probe. We optimized each hybridization step by varying the hybridization time from 5 to 60 min and from 1 to 30 min for capture and signal probes, respectively. For this study, we used a NA1S single-probe array, containing approximately 60 replicate microspheres, as a representative microarray for *A. fundyense* detection.

To optimize the primary capture probe hybridization time, target samples containing rRNA from 5 and 500 cells of *A. fundyense* were tested. First, the primary hybridization times were varied from 5 to 60 min while a fixed 10 min was employed as the secondary hybridization time for the signal probe (Fig. 1A). As expected, signal intensity increased with longer hybridization times, and samples with more cells reached a signal plateau in less time; this plateau was observed after less than 20 min with 500 cells, while it took 30 min to reach a plateau with 5 cells. From this result, 30 min was selected as an optimal time for the primary hybridization between capture probes and target rRNA. We then optimized the secondary hybridization time, using a rRNA sample prepared from 50 cells of *A. fundyense*, by varying the secondary hybridization time from 1 to 30 min. As shown in Fig. 1B, signal intensity increased with increased hybridization time, reaching a plateau after 15 min. Based on these results, 30- and 15-min hybridization times were used in the remainder of this study for capture and signal probes, respectively.

Detection limits of fiber-optic microarrays. Detection limits for each DNA capture probe were determined using three single-probe-type microarrays, containing either the NA1S, AO2, or auD1S probe, with 5 to 5,000 cultured cells of *A. fundyense*, *A. ostenfeldii*, and *P. australis*, respectively (Table 3). All three microarrays could detect 5 target cells, the lowest number of cells tested in this study, and the signal intensity

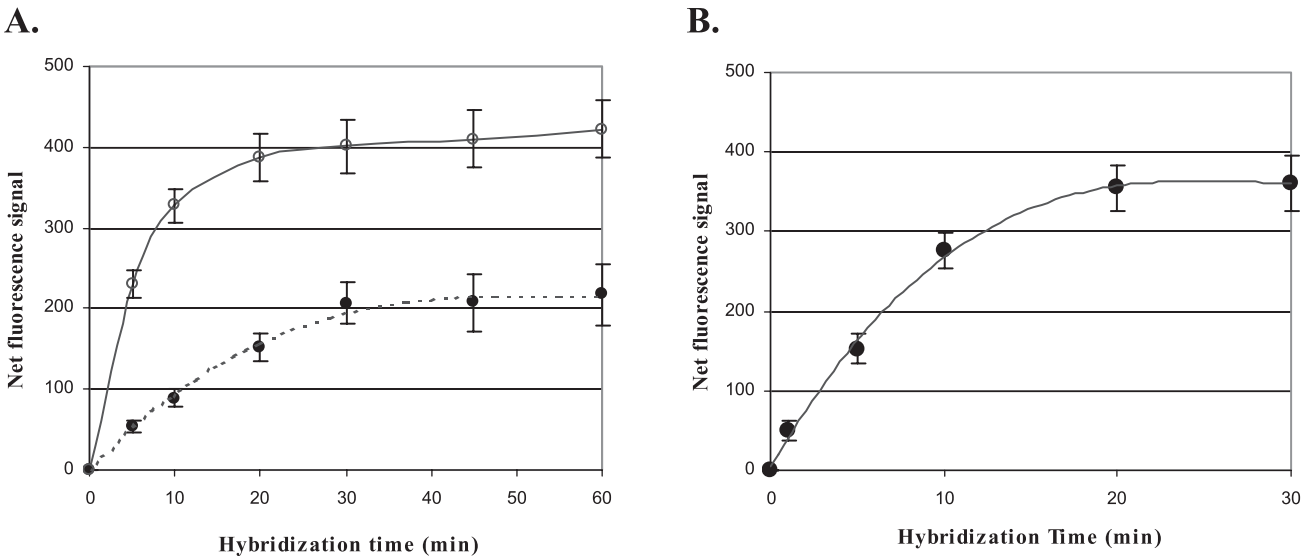

FIG. 1. Optimizations of sandwich hybridization for the capture probe (NA1S) (A) and signal probe (AlexS) (B) using *A. fundyense* as a target cell. Error bars, standard deviations from three measurements. (A) Hybridization time with the capture probe was optimized with two different numbers of cells: 500 cells and 5 cells of *A. fundyense*. The solid curve represents the polynomial fit for 500 cells, and the dashed curve represents that for 5 cells, with R^2 values of 0.996 and 0.994, respectively. (B) Hybridization time with the signal probe was optimized with 50 cells of *A. fundyense*. The solid curve represents the polynomial fit, with an R^2 of 0.998. Each point represents the average of triplicate measurements.

increased with higher target cell numbers for all three capture probes.

The detection limit was also determined for rRNA by using a representative microarray composed of only the NA1S probe microspheres. The approximate amount of rRNA in the target *A. fundyense* cells was calculated from a measurement of total cellular RNA, which indicated that *A. fundyense* contains approximately 34 pg of total cellular RNA (D. M. Anderson, unpublished data). Since 75 to 80% of total cellular RNA is rRNA (12, 44), one *A. fundyense* cell is calculated to contain 25.5 pg of rRNA. Assuming that the rRNA pool comprises equimolar amounts of 28S, 18S, 5.8S, and 5S rRNA, at 3,400 nucleotides (nt), 1,800 nt, 160 nt, and 120 nt, respectively (5,480 nt total), and that the average nucleotide weight is 5.4×10^{-22} g, we calculate the amount of the target 28S rRNA to be approximately 8.6×10^6 molecules/cell. Serially diluted rRNA samples were prepared and hybridized to the *A. fundyense* NA1S single-probe microarray, yielding a detection limit of approximately 4×10^4 rRNA molecules (Table 4). The dose-response curve shown in Fig. 2 exhibits a dynamic range of 4 orders of magnitude, between 4×10^6 and 4×10^{10} molecules, which will be useful for target HAB enumeration.

To study the effect of DNA present in the sample, *A. fundyense* lysate samples of 5 to 5,000 cells were prepared either with or without DNase treatment as described above. Even with DNase treatment, the single-probe-type microarray containing the NA1S probe could detect 5 cells with signal intensity decreased by 10% compared to the signal without DNase treatment. With higher target cell numbers (50 to 5,000 cells), the signal intensities decreased as much as 7% from that without DNase treatment (data not shown).

Detection of *A. fundyense* in seawater concentrate. To study the effect of co-occurring plankton and detritus on the detection of target cells in field samples, seawater concentrate samples were prepared as described above to provide simulated background matrices. Various numbers of *A. fundyense* cells

TABLE 3. Averaged net fluorescence signal intensity from three single-probe-type arrays with varying numbers of target cells[a]

No. of target cells	Net hybridization signal ± SD for the following probe type/target organism:		
	NA1S/*A. fundyense*	AO2/*A. ostenfeldii*	AuD1S/*P. australis*
5	189 ± 17	73 ± 14	65 ± 15
50	280 ± 31	120 ± 18	140 ± 25
500	410 ± 56	258 ± 38	223 ± 32
5,000	502 ± 64	399 ± 65	346 ± 66

[a] The standard deviation of the background was 17, and the threshold limit for a positive signal was calculated to be 51 (3 × 17).

TABLE 4. Detection limits of NA1S probe obtained from single-probe-type microarray

No. of rRNA molecules[a]	Net hybridization signal ± SD
4×10^3	(23 ± 4)[b]
4×10^4	32 ± 5
4×10^5	55 ± 7
4×10^6	106 ± 11
4×10^7	189 ± 17
4×10^8	280 ± 19
4×10^9	410 ± 35
4×10^{10}	502 ± 57

[a] The amount of rRNA is estimated to be 8.6×10^6 molecules/cell in *A. fundyense*.

[b] Signals in parentheses are considered nondetectable according to the definition of a positive signal as greater than 3 times the standard deviation of the background. The standard deviation of the background was 10, and the threshold limit for a positive signal was calculated to be 30 (3 × 10).

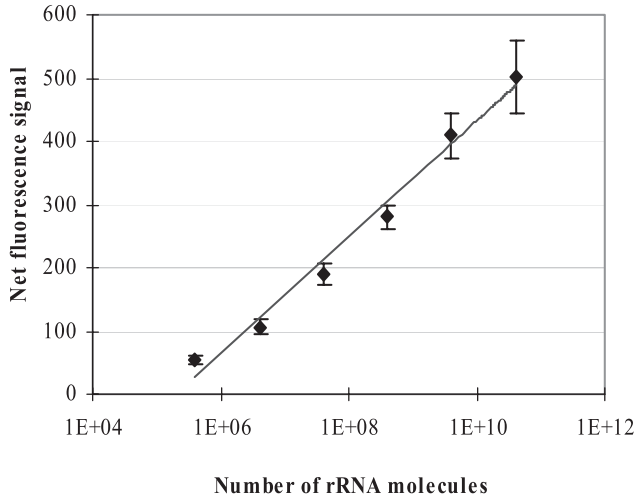

FIG. 2. Dynamic range of the dose-response curve from the DNA microarray. The NA1S single-probe microarray was used as a representative array, and serially diluted rRNA samples from *A. fundyense* were used as the target. The solid line represents the linear fit, with an R^2 of 0.984. Each point represents the average of triplicate measurements. Error bars, standard deviations from three measurements.

were mixed with 1 ml seawater concentrate (equivalent to 1 liter of seawater) and tested using a NA1S single-probe-type microarray. As shown in Fig. 3(A), even in the presence of other co-occurring wild plankton cells and detritus, the microarray was able to detect rRNA from as few as 5 cells, the same detection limit achieved with the pure culture of *A. fundyense*.

In addition, different volumes of seawater concentrate spiked with 1,000 cells of *A. fundyense* were tested: 0.1 ml, 0.25 ml, 0.5 ml, and 1.0 ml of seawater concentrate, corresponding to 0.1 liter, 0.25 liter, 0.5 liter, and 1.0 liter of raw seawater, respectively. As expected, due to mass transport limitations, signal intensities decreased slightly with increasing seawater volume (Fig. 3B). With a 10-fold increase in seawater volume from 0.1 liter to 1.0 liter, the signal intensity decreased by 10%.

Multiplexed detection of HAB organisms. To study the specificity of the probes on the microarray, all three HAB targets were detected using a multiplexed DNA microarray. The multiplexed microarray was prepared to contain all three capture probes, NA1S, AO2, and auD1S, and the microarray responses to different concentrations of synthetic targets (100 fM, 10 pM, and 10 nM) were examined. The multiplexed microarray was able to detect 100 fM of each sequence within 30 min of hybridization. Furthermore, the tested probes gave positive signals only with their complementary target sequences, and no positive hybridization signals were observed from any of the noncomplementary targets (data not shown).

The multiplexed microarray was then tested against single-target samples containing rRNA from one of four strains of target organisms (5,000 cells) *A. fundyense* (strain GTCA28), *A. ostenfeldii* (strains HT-240D2 and HT-120D6), and *P. australis* (strain 1BA). Positive signals were observed only from sandwich hybridization between probes and their corresponding target organisms (Fig. 4A). All signal intensities from nontarget algal cells were lower than the threshold for a positive signal. The AO2 probe gave positive signals with both strains of *A. ostenfeldii* (HT-240D2 and HT-120D6), and no cross-reactivity between NA1S and AO2 probes was observed. The multiplexed microarray was further tested with lower target cell numbers: 5, 50, and 500 cells. The multiplexed array could successfully detect 5 target cells of all four strains tested without any cross-reactivity between probes, and the signal intensity increased with higher target cell numbers for all three capture probes (data not shown).

Additionally, the multiplexed microarray was examined with a mixture of target cells containing 5,000 cells of each target

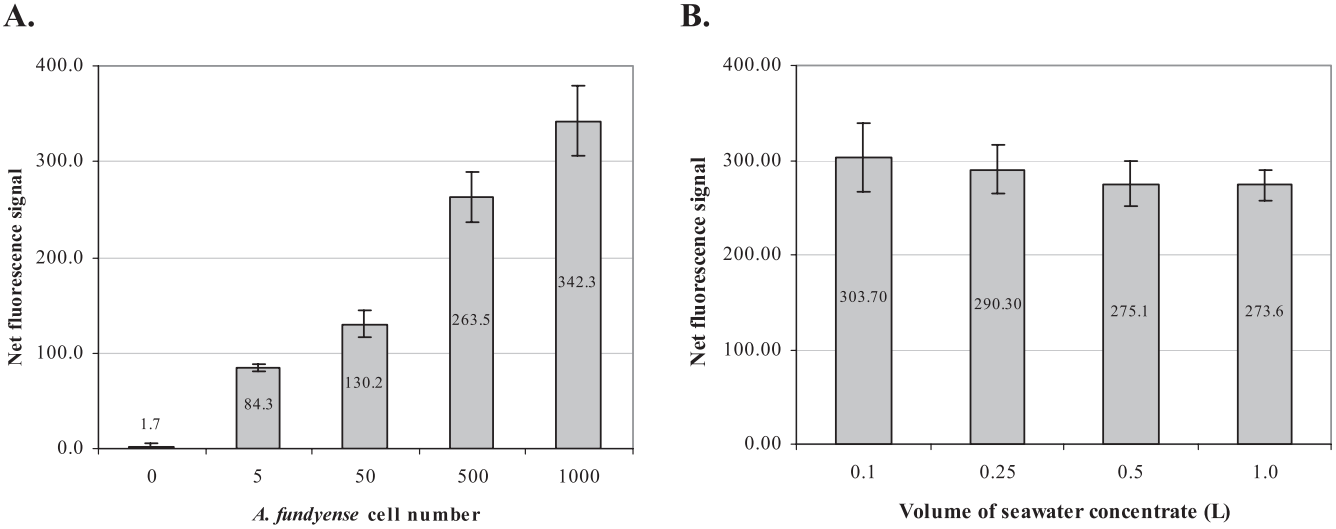

FIG. 3. Effect of natural plankton cells and detritus present in seawater. (A) Signal intensities from target samples obtained using various numbers of *A. fundyense* cells spiked into 1 ml seawater concentrate, which is the equivalent of 1 liter of raw seawater. (B) Signal intensities obtained using various volumes of seawater concentrate spiked with 1,000 *A. fundyense* cells. The single bead-type array with the NA1S probe was used for the signal measurement. The standard deviation of the background was 15, and the threshold for a positive signal was calculated to be 45 (3×15). Each data point represents the average of triplicate measurements. Error bars, standard deviations from three measurements.

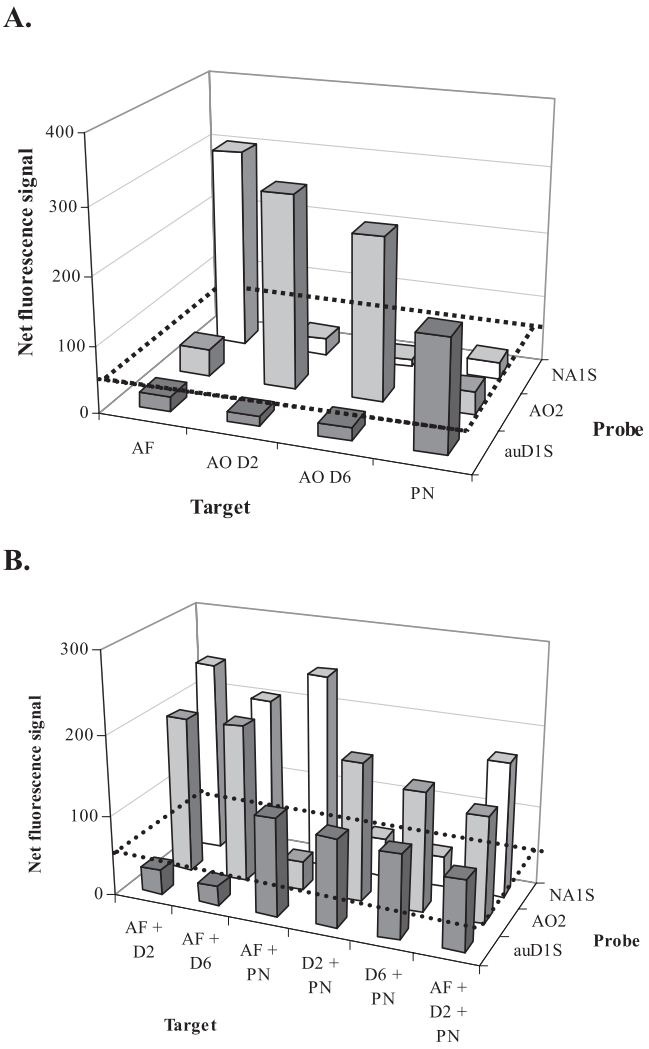

FIG. 4. Simultaneous detection of single (A) or multiple (B) HAB species using a multiplexed microarray containing three probe types: NA1S, AO2, and auD1S. The standard deviation of the background was 17, and the threshold limit for a positive signal was calculated to be 51 (3×17). The positive threshold is shown as dashed lines. Each point represents the average of triplicate measurements. AF, *A. fundyense* GTCA 28; AO D2 (A) or D2 (B), *A. ostenfeldii* HT-240D2; AO D6 (A) or D6 (B), *A. ostenfeldii* HT-120D6; PN, *P. australis* 1BA.

species. The algal cultures were mixed and collected on a 0.65-μm-pore-size membrane filter, and the rRNA lysates were prepared from the filtered cells as described above. All sandwich hybridizations were performed with hybridization times of 30 min and 15 min for capture and signal probes, respectively. Hybridization signals were observed only from probes matching the target organisms in the sample (Fig. 4B).

DISCUSSION

The fiber-optic DNA microarray reported here has many advantages over other detection methods. The high density of DNA probe molecules attached to each bead's surface can provide a low detection limit with a short analysis time. The presence of replicate DNA probe microspheres on each array serves to increase the signal-to-noise ratio as well as to minimize false-positive and false-negative signals. The fiber-optic microarray is easy to fabricate and enables direct monitoring of hybridization in the target solution. Additional probe sequences can be added to the array by simply including additional microsphere types in the bead mixture. Furthermore, the microarrays can be reused after dehybridization of targets. These advantages make a fiber-optic microarray a promising alternative to conventional detection methods.

In this study, we developed a microsphere-based fiber-optic microarray for the detection of HAB cells by using a sandwich hybridization protocol. The methodology utilizes two probes (capture probe and signal probe) designed to specifically target the rRNA sequences of the HAB organisms *A. fundyense*, *A. ostenfeldii*, and *P. australis*. Since rRNA molecules are present in large numbers in a cell, constituting as much as 75 to 80% of total cellular RNA, rRNA can provide a sufficient number of target molecules to generate signals without any amplification step, which is required for methods targeting chromosomal DNA (19, 25). Another advantage of using rRNA as a target is that its sequence contains both highly conserved and highly variable regions. This characteristic makes it possible to design species- or even strain-specific probe sequences. The performance of the capture probes was confirmed using Cy3-labeled synthetic oligonucleotide targets whose sequences were complementary to the capture probe on single-probe microarrays.

The hybridization times for the primary hybridization between the capture probe and target DNA and the secondary hybridization between the captured target DNA and signal probe were optimized to 30 min and 15 min, respectively. These results suggest that the capture probes were completely hybridized to rRNA molecules after a short incubation time (30 min). It is notable that a much shorter incubation time (15 min) is required to obtain saturation of the target rRNA sequence by the signal probes. This result could arise from two steps in our procedure (or a combination of both). First, the washing step prior to the secondary hybridization of the signal probe removes most nontarget DNA and RNA as well as other impurities that can interfere with hybridization between the target sequence and signal probe. Since the target rRNA sample is prepared by simple cell lysis and filtration, there are probably large amounts of nontarget DNA and RNA molecules as well as other low-molecular-weight impurities in the lysates. Primary hybridization between the target rRNA and capture probe may be hindered by the presence of these nontarget molecules in the solution, requiring longer incubation time for probe saturation. The washing step prior to secondary hybridization, however, eliminates any of these nontarget molecules, removing any interference and facilitating the binding of the signal probe to its target rRNA sequences. Second, the high concentration of signal probe (1 μM) used for hybridization facilitates a rapid reaction. A previous study with synthetic target showed that probes were saturated and reached the signal plateau within 5 min in the presence of high concentrations of targets (≥100 nM) (18). Although we optimized the hybridization time for capture and signal probes at 30 min and 15 min, respectively, we observed that the signal intensities obtained with rRNA from HAB-spiked seawater samples (containing wild algal cells) were lower than the signals from pure cultures. This result suggests that more than 45 min of total

hybridization time (30 min and 15 min) might be needed to obtain complete hybridization for environmental samples containing background algal cells in addition to the target organisms. Detection of environmental samples might also be improved by replacing the simple sample preparation steps of cell lysis and filtration with more specific RNA purification protocols, which would further remove many of the impurities present in seawater. On the other hand, this procedure would add another step and additional costs and is therefore to be avoided if at all possible.

The detection limits for the three different probe types were determined with serially diluted rRNA samples, from 5 to 5,000 cells per membrane filter, using three single-probe microarrays. All three microarrays could detect 5 cells, with statistically higher signal intensity than background, in both pure cultures and samples with a natural seawater background matrix. Even with DNase treatment of the samples, 5 cells could be detected with only a slight decrease in signal intensity ($\leq$10%). This result suggests that the signal is produced by specific hybridization between probes and rRNA targets, with a minimal effect of DNA in the sample. A dose-response curve was obtained for the NA1S single-probe-type microarray as a representative microarray. The detection limit determined from the dose-response curve for cultured *A. fundyense* was approximately 4×10^4 rRNA molecules, corresponding to 5×10^{-3} of the amount of rRNA contained in a single cell. This detection limit is much lower than previously reported detection limits of 0.2 to 0.6 HAB cells using PCR-based methods (11, 19). This low detection limit is likely due to high copy numbers of rRNA existing in the target cell (estimated to be 8×10^6 molecules/cell in *A. fundyense*), compared to 2 to 100 copies of ribosomal DNA, employed as a target in other studies. Since these results were obtained without any separate amplification step, the fiber-optic microarray developed in this study provides sensitive detection with minimal assay time. In addition, the fact that 5 cells could be detected even in the presence of co-occurring algae/plankton cells suggests the potential for application of this technology for direct detection of environmental samples. In the dose-response curve, the dynamic range was between 4×10^6 and 4×10^{10} molecules. Even though the detection limit itself is much lower, this range could be used for target HAB cell enumeration because of its linearity.

The specificity of the probes was studied using a multiplexed microarray containing all three probes: NA1S, AO, and auD1S, for *A. fundyense*, *A. ostenfeldii*, and *P. australis*, respectively. The performance of the multiplexed microarray was first tested with synthetic targets that had sequences complementary to each probe type. Positive signals were observed only from hybridization between probes and their complementary targets. The detection limits of all three probes in a multiplexed array were 100 fM with a 30-min hybridization time, which was comparable to detection limits obtained from single-probe-type microarrays of 10 fM for NA1S and 100 fM for AO2 and auD1S probes. With the same multiplexed microarray, four strains of the target organisms *A. fundyense* (strain GTCA28), *A. ostenfeldii* (strains HT-240D2 and HT-120D6), and *P. australis* (strain 1BA) were tested, either as single organisms or as mixed samples. Each probe produced positive signals only when the specific target organism was present, and no cross-reactivity of probes was observed, supporting the feasibility of simultaneously detecting three target HAB organisms using a single microarray system. In mixed samples, the signal intensities were lower than those obtained when only a single organism was present. This decrease in signal intensity could be explained by competition for the probes between target sequences and nontarget sequences as a result of the high numbers of other organisms present in the sample.

The specificity of the microarray described in this study is likely due to the use of two specific probes (capture and signal) employed in the sandwich hybridization format. In sandwich hybridization, the signal can be produced only when both capture and signal probes bind to their target sequences. This dual-probe system can circumvent the false-positive signals occurring in other detection assay formats that rely on a single probe. Another reason for the high specificity could be the large number of replicates of each probe type (approximately 100 microspheres per probe type), which can reduce the frequency of both false-positive and false-negative signals.

The multiplexed array described in this paper employed three probe types for the simultaneous detection of multiple HAB species. We demonstrated the ability to detect target HAB species either from pure cultures or from spiked seawater samples containing natural algae and other plankton. The bead-based microarray can be expanded to more probes simply by adding additional probe microspheres to the existing array, without affecting the performance of the preexisting probes. Presently, we are developing additional HAB probes in hopes of expanding this microarray to a large number of HAB species. We are also continuing to test the microarray with natural seawater samples containing the target species. With its sensitivity and specificity, the microarray system presented in this study provides great potential for the rapid detection of HAB species in environmental samples, representing an improvement in speed and specificity over a number of current screening methods. In addition, the simplicity of the microarray format and its ease of reuse mean that this approach is highly amenable to automation for direct shipboard detection of HABs or for deployment on remote, moored instruments capable of detecting HAB species in an early warning system.

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