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Application of environmental sample processor (ESP) methodology for quantifying *Pseudo-nitzschia australis* using ribosomal RNA-targeted probes in sandwich and fluorescent in situ hybridization formats

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Abstract

In this contribution, we assess the application of methodology used in a novel in situ remote sampling platform, the environmental sample processor (ESP), for the identification and enumeration of a harmful algal species, the domoic acid–producing diatom *Pseudo-nitzschia australis*, using 2 molecular assays: a sandwich hybridization assay (SHA) and fluorescent in situ hybridization (FISH). Both the SHA and FISH assays were initially designed as laboratory-based methods that are now emulated in the ESP. Response of the SHA to a range of concentrations of *P. australis* using the laboratory (96-well plate) and ESP (DNA probe array) formats showed that the two were highly correlated. Enumeration of cells filtered and archived for FISH using a manifold designed for laboratory applications agreed well with counts of cells filtered and archived in the ESP at ≥2.5 × 10^4 cells L^{-1}. At lower concentrations, it becomes statistically unlikely to derive a reliable abundance estimate, suggesting that FISH is better suited for qualitative analyses unless the target organism is very abundant. We also assessed the suitability of an oligonucleotide as synthetic target sequence to act as a SHA reagent quality control and internal standard for the plate assay. This was successful, but the probe and/or associated reagents were stable for only ~60 days. Our results show that the ESP is capable of detecting *P. australis* in near real-time and also supports whole-cell archival samples, making it a potentially useful tool for future research and monitoring initiatives.

Introduction

A recent shift from using traditional light microscopy to molecular approaches for identifying and quantifying marine harmful algal bloom (HAB) species has been driven by the need to expedite sample processing for both research and monitoring purposes (Anderson 1995; Scholin et al. 2003). In addition, light microscopy does not always afford sufficient resolution to discern species. For example, distinguishing between species of pennate diatoms within the genus *Pseudo-nitzschia* H. Peragallo requires electron microscopy (e.g., Hasle 1965, 1994; Smith et al. 1990; Lundholm et al. 2006). Some species of *Pseudo-nitzschia* produce the neurotoxin domoic acid (DA) that can be harmful to both humans and wildlife, so accurate and rapid identifications are desirable (Fryxell and Hasle 2003). A variety of molecular probe assays applied in cell-free and whole-cell formats aid in the detection and quantification of *Pseudo-nitzschia* species (e.g., Scholin et al. 2003), as well as a wide range of naturally occurring microorganisms. Such approaches include fluorescent in situ hybridization (FISH) (e.g., Lim et al. 1993; Groben et al. 2004), fluorescently labeled antibodies and lectins (e.g., Vrieling et al. 1994; Sako et al. 1996; Rhodes 1998; Peperzak et al. 2001), sandwich hybridization (e.g., Scholin et al. 1996; Tyrrell et al. 2002), probe arrays (e.g., Loy et al. 2002; Metfies and Medlin 2004; Ellison and Burton 2005), and a variety of nucleic acid amplification methods that target specific signature sequences (e.g., Bowers et al. 2000; Penna and Magnani 2000; Bolch 2001; Litaker and Tester 2002; Casper et al. 2004).

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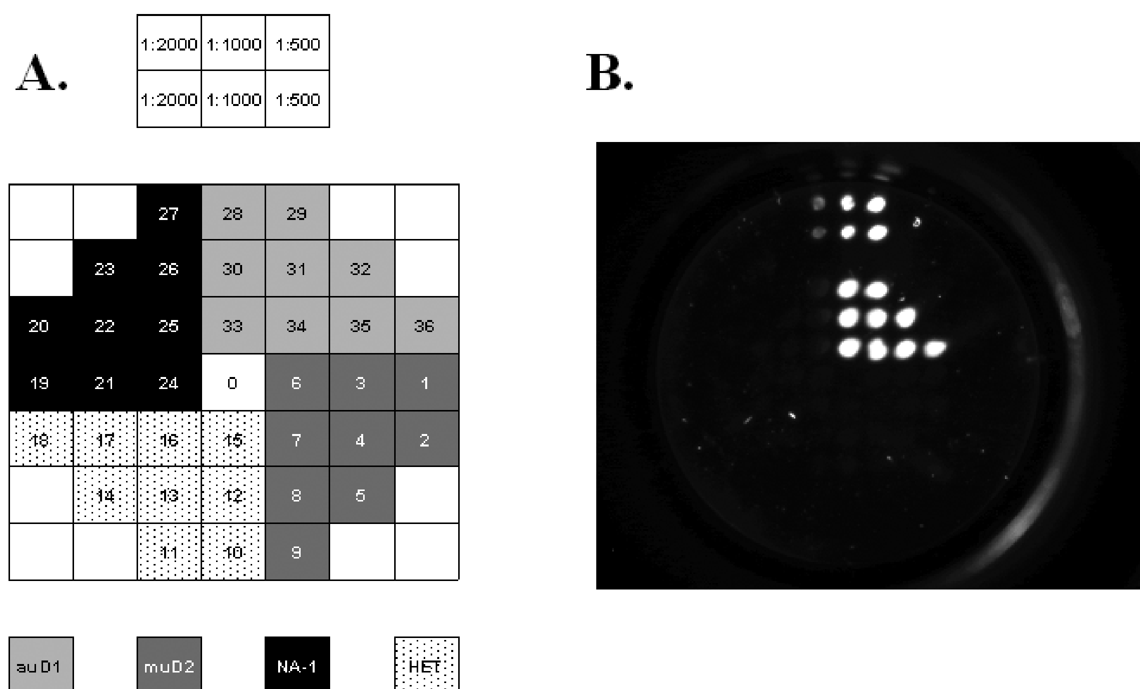

Fig. 1. Example of a 25-mm-diameter ESP array printed with a variety of probes for HAB species. (A) Map showing location of capture probes. auD1, *Pseudo-nitzschia australis*; muD2, *Pseudo-nitzschia multiseries*; NA-1, *Alexandrium catenella*; Het1, *Heterosigma akashiwo* (Scholin et al. 1999; Tyrrell et al. 2001). The top 6 squares are a dilution series of control probes (independent of sample) that verify assay chemistry and orient the image. (B) Imaged array developed using the 1G ESP, 1.6×10^4 *P. australis* cells mL^{-1} lysate, and a 30-s camera exposure with 2-by-2 binning.

Most of these techniques, however, require access to shore-based laboratories for sample processing and analysis, which can be both time- and labor-intensive. Moreover, laboratory processing constrains the number of samples that can be acquired for a given area and period, especially when a ship is needed to reach sampling sites. Consequently, there has been a recent surge in the development of instruments that enable in situ species identification (Sieracki et al. 1998; Babin et al. 2005; See et al. 2005).

The system discussed in this contribution is the environmental sample processor, or ESP (Scholin et al. 1998, 2001). The ESP offers a number of options for deploying molecular probe technology remotely, including the direct detection of a target species or group of species using DNA probes. We assess the quality of ESP-generated data by comparing instrument results to values derived from 2 established laboratory molecular assays, a sandwich hybridization assay (SHA) and FISH. We have 3 specific objectives: first, to compare sensitivity and dynamic range of the SHA for *P. australis* using the 96-well plate and ESP array formats (Scholin et al. 1999 and in press, respectively); second, to evaluate the ESP's ability to archive samples for FISH relative to archival samples using a standardized filter-based method (Miller and Scholin 1996, 1998); and third, to establish a positive control for the SHA that is independent of target cell material. The motivations for the latter are to provide a reference for reagent-quality control procedures and ultimately facilitate future remote instrument calibration.

The ESP is an electromechanical/fluidic system that collects discrete water samples from the ocean subsurface, concentrates microorganisms (particulates), and automates application of ribosomal RNA (rRNA)-targeted DNA probe arrays (Figure 1) that detect target sequences in near real-time. The ESP also archives samples for nucleic acid analyses, microscopy, and other procedures after the instrument is recovered from deployment (Goffredi et al. 2005). The ESP employs "pucks" (Figure 2) that are used for executing different sample collection and processing protocols. Pucks are custom-designed reaction chambers that support a wide variety of filters or chemically adsorptive media depending on protocol requirements. Pucks are highly configurable but conform to a common outer shape and overall size. Pucks are stored in a rotating carousel and are manipulated to processing station positions by elevator and shuttle mechanisms (Roman et al. 2005). The same mechanisms can also move pucks to an imaging station where a CCD camera records results of DNA probe arrays. Once pucks are in a processing position, they are sealed in a clamp, thus providing fluidic connections to the sample port and reagent valve manifolds. Syringe pumps draw in seawater samples and dispense the required reagents. Modular valving supports the use of many custom-defined reagents. Additional valves allow a syringe pump to pull reagents "top-to-bottom" or to push reagents from "bottom-to-top" across the filter or adsorptive medium supported in a puck. Processing stations are equipped with heaters so that reagents can be trapped within a puck for

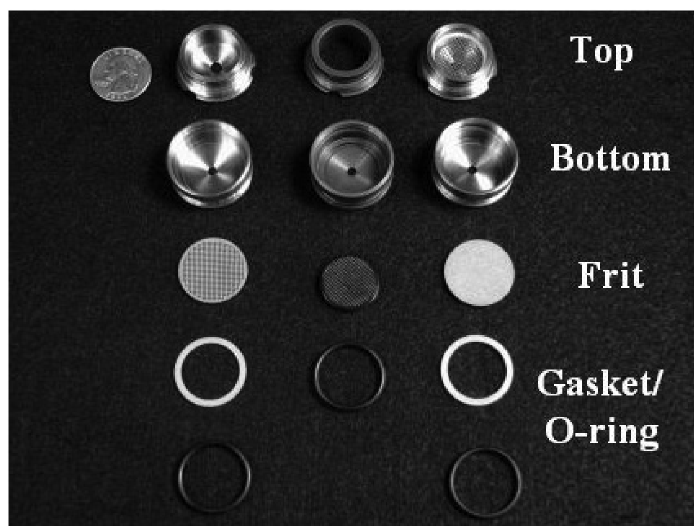

Fig. 2. Custom-fabricated pucks for the 2G ESP shown L to R as sample collection and filtration, array processing, and FISH archival. Quarter shown for size.

an extended period and held at a specific temperature. Material eluted from a puck may be pulled into the syringe pump and used in a subsequent protocol requiring a different solid-phase medium (e.g., cell lysate applied to a DNA probe array). Processed or spent pucks are unloaded into an empty carousel tube using the elevator and shuttle described above.

The first-generation (1G) ESP underwent field trials during which all of the above-described mechanical functions and molecular assays were successfully implemented for deployments lasting up to 20 days (Goffredi et al. 2005; Scholin et al. in press) (C. Preston, unpublished observations). Those field trials, however, were primarily qualitative and verified basic ESP design concepts. Based on that work, a second-generation (2G) ESP was built, and field trials using that instrument began in March 2006. In concert with the 2006 field trials, we present here studies that quantitatively compare ESP performance to the laboratory-based methods that the instrument emulates. Specifically, we focus on the complete SHA procedure and the sample archival step associated with FISH using a cultured isolate of *P. australis* collected from Monterey Bay, California, USSA.

Methods and Procedures

SHA and FISH assays for P. australis—Detailed descriptions of the SHA and FISH assays for *P. australis* are given elsewhere (e.g., Scholin et al. 1996, 1999, Miller and Scholin 2000, with modification as detailed below). Both assays employ large-subunit rRNA-targeted oligonucleotide probes designed against cultured cells collected from Monterey Bay, California. Both methods use filtration to initially collect and concentrate cells, but the subsequent treatment of samples differs substantially. In the SHA, a crude homogenate is prepared by disrupting cells with a chaotrope and heat. That homogenate is filtered to remove particulates and analyzed directly (i.e., without further purification) using a semiautomated 96-well plate format. The SHA employs 2 probes, capture and signal, to detect rRNA sequences indicative of *P. australis*. The result is a colorimetric reaction wherein intensity can be used to estimate cell abundance in the original sample using a standard curve derived from either cultured material or natural samples. In contrast, material collected for FISH is first treated with a saline ethanol preservative that maintains cell integrity. Samples are then treated with a fluorescent-labeled probe, and cells that retain the probe are enumerated using epifluorescence microscopy.

Preparation, processing, and image analysis of ESP arrays—Use of arrays in the ESP entails 3 phases: cutting and printing of arrays, the SHA, and image analysis. To cut arrays, we used an Osborne no. 149 arch punch and hammer to section 24-mm-diameter discs of Predator membrane (Pall Corp., East Hills, NY, USA) placed on a polypropylene board covered with a lint-free TX TechniCloth II (Upper Saddle River, NJ, USA). Cut membranes were loosely inserted into an open-faced array puck (Figure 2) and stored at room temperature until needed.

Probe inks, capture and control, were then prepared to print on the cut membrane. Capture probe (species-specific and control) ink consisted of an oligonucleotide with biotin at its 5′ end attached to the probe via three C9 spacers (100 ng μL^{-1} in 1× Tris-EDTA, pH 7.4 [Sigma, St. Louis, MO, USA]), recombinant streptavidin (1 mg mL^{-1} distilled water; Pierce, Rockford, IL, USA), and 20× sodium saline phosphate-EDTA buffer (SSPE) (Sigma) added in a ratio of 5:1:1.2, respectively, and in that order, with finger-vortexing and microcentrifugation (2 to 3 s) between each addition. The sequence of the *P. australis* probe is given in Scholin et al. (1999). The control probe (5′-GGGAAATATGAAAAGGACTTTGAA-3′) is the complement of the SHA signal probe that detects *Alexandrium* sp. (AlexS-alt; Anderson et al. 2005). It was prepared as above, then diluted 1:500, 1:1000, and 1:2000 in 1× Tris-EDTA, nutravidin (1 mg mL^{-1} distilled water; Pierce), and 20× SSPE mixed at a 5:1:1.2 ratio, respectively. Control probes return a positive reaction independent of sample to verify that assay chemistry is working properly and also to orient the array image (Figure 1). All solutions were RNase and DNase free; probe and RSA concentrations were measured using a NanoDrop ND-1000 Full-spectrum UV/Vis Spectrophotometer (NanoDrop, Rockland, DE, USA). Fifty microliters of probe ink was dispensed into individual wells of a 384-well polypropylene plate (Nunc, Rochester, NY, USA). Separate 384-well plates were used for capture and control inks. Each probe had a corresponding wash column containing 75 µL/well of distilled water (Sigma).

Probe inks were printed using a custom-built arrayer composed of a motorized X, Y, Z translation table (Danaher Precision Systems, Salem, NH, USA) fitted with Galil motion controllers (Galil Motion Control, Rocklin, CA, USA) and custom user interface software (Microsoft Visual Basic; Microsoft, Redmond, WA, USA) that allowed precise positioning of a microarray flat TI W72.9 diameter by DELRIN w/0.600 mm needle (Point Technologies, Boulder, CO, USA). The array

printer operated in a fashion similar to other commercially available units by dipping the needle into the probe ink, and then moving the needle to the appropriate spot (e.g., Figure 1a) and touching the array support. This action was repeated 4 times; each touch deposited ~25 nL probe ink. The "four spot touch" created replicate spots for up to 8 arrays per printing event. Before spotting the next capture probe, the needle was dipped through the corresponding column of wash wells. After printing the capture probes, control probes were printed from highest to lowest dilution. All printing steps were carried out at room temperature in a humidified chamber. Printed arrays were baked at 70°C for 20 min and stored in a desiccator at room temperature until needed. Conventional nitrocellulose membranes can also be printed in this fashion. The arrays appear to be stable for several months under these conditions, but were typically used within weeks of printing.

The second phase of ESP array processing, the SHA, involves 5 steps: sample collection, sample lysis, lysate filtration, hybridization, and imaging. Both the 96-well plate assay and the ESP employ the same steps and use many of the same reagents, but assay execution and signal transduction vary. To collect samples, log-phase culture was concentrated on 25-mm 0.45-μm pore size hydrophilic Durapore filters (Millipore, Bedford, MA, USA), membranes were rolled and placed in cryovials (Nalge, Rochester, NY, USA) sample side facing in, and capped vials were stored in liquid nitrogen until needed. Details of the SHA plate assay are found elsewhere (Tyrrell et al. 2001; Goffredi et al. 2005). In the ESP, printed arrays were removed from the puck used for printing and reloaded into a clean array puck on top of a 5-μm pore size hydrophilic Durapore backing filter (Millipore), leaving a 0.009-inch gap above the array, and the puck was placed into the instrument's rotating carousel. The ESP can collect samples, generate lysate, and filter samples automatically, but for experiments presented here, sample lysing and filtration were manual (e.g., Anderson et al. 2005; Goffredi et al. 2005). The filtered lysate was split between the 96-well plate assay and the ESP. For the ESP, an aliquot of lysate was drawn into the ESP's processing syringe. The ESP then loaded the puck to the SHA processing position and heated the puck to 25–30°C. The 1G and 2G ESPs add sample and reagents in the same sequence, but the 1G used 2 mL of each whereas the 2G uses 1 mL each. Lysate and processing solutions (see Goffredi et al. 2005, except where noted) were applied automatically as follows: lysate, 20 min; 1× wash, 2 min; signal probe cocktail in 2 M guanidinium thiocyanate (GuSCN) signal buffer, 10 min; 2× wash, 2 min each; anti-Dig HRP (Pierce; Rockford, IL, USA) diluted 1:1500 in stabilized diluent blocker (1 mL) (Pierce), 5 min; 4× wash, 2 min each; HRP substrate (Pierce SuperSignal West Femto Maximum Sensitivity Substrate: Stable Peroxidase Buffer and Luminol/Enhancer Solution mixed 1:1 before delivery to the array), 10 s. Afterward, the puck was immediately positioned beneath the camera and the image was captured using a CCD camera (1G ESP: Santa Barbara Instruments model ST-8EI [Santa Barbara, CA, USA] with a Fujinon [Japan] HF35A-2M1 lens; 2G ESP: Starlight Xpress model SXV-H9 [England] with a Fujinon model HF16HA-1B lens).

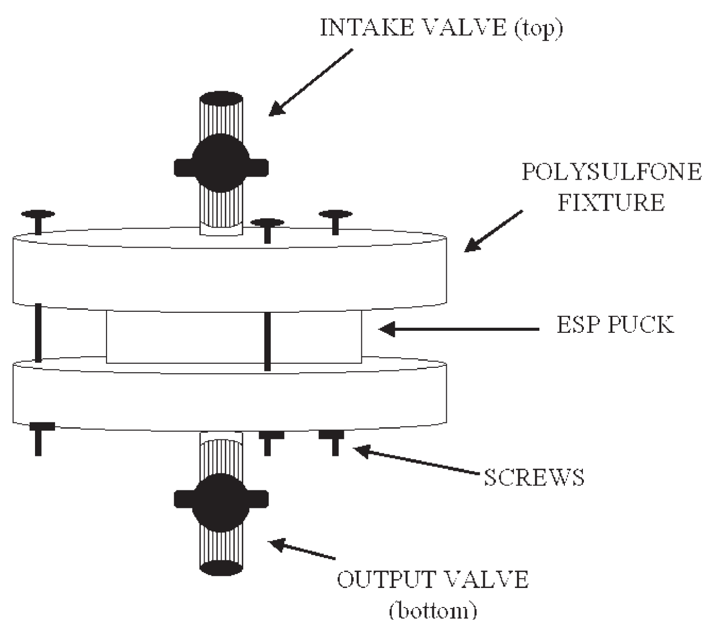

Fig. 3. Fixture assembly for holding pucks and mimicking ESP processing protocols. The fixture assembly consists of two 50 mm diameter by 12 mm height polysulfone cylinders that anchor and seal the puck while samples and processing solutions are injected. Each cylinder is configured with a 5/16-inch hex Natural Kynar PVDF fitting and a Discofix 1-way stopcock. The fixture can be used for any puck (Figure 2) and all of the protocols used by the ESP, but in the case of work presented here was used for whole-cell archival samples only.

The final phase of ESP array processing, image analysis, was done using V++ Precision Digital Imaging System, v. 4.0 (Digital Optics, Auckland, NZ). The grand mean (± SE) spot intensity per DNA probe was determined for each image by measuring a 10-by-10 pixel area per spot then recording average (± SD) pixel intensity. An array spot with a grand mean intensity significantly higher than background (array region where no probe spotting occurred) indicated a positive reaction for that probe. Background intensity was determined as above by taking the grand mean of 3 randomly selected unspotted regions of the imaged array.

Manifold vs. ESP FISH filtration and archival—Sample preparation for FISH entails filtration, archival, and hybridization. Both benchtop manifold and ESP procedures use identical chemistries, but sample filtration and archival differ. The manifold designed for laboratory use is described elsewhere (Miller and Scholin 1998, 2000). ESP FISH sample archival involved a custom-made puck and 20-μm pore size sintered frit, 25 mm diameter, equipped with a 1.2 μm Isopore membrane (Millipore) loaded on top of a 0.45-μm pore size Metricell backing filter (Pall Corp., Ann Arbor, MI, USA) (Figure 2). To process multiple samples simultaneously and facilitate experiments reported here, we used a test fixture (Figure 3) that enables us to mimic instrument sampling and reagent delivery, thereby

permitting laboratory experimentation. Counts of cells archived using the fixture assembly were not statistically different (*t* test, $\alpha = 0.05$) from counts using instrument-archived samples (data not shown).

The puck fixture assembly was used to mimic ESP sample filtration and archival process as follows: samples were drawn through Teflon tubing at 5 mmHg vacuum, the intake and outtake were removed, and 5- and 10-mL syringes were attached to the top and bottom valves, respectively. Samples were preserved by adding 2 mL modified saline ethanol solution (Miller and Scholin 2000) to the 5-mL syringe, the bottom valve was closed, and the top valve was closed once the plunger reached 1 mL. In this fashion, the entire volume of fixative entered the puck where it was retained under pressure and incubated for 20 min at ambient temperature. Fixative was removed by opening the bottom valve, pulling the 10-mL syringe plunger to 5 mL, and opening the top valve while pulling the bottom plunger to 10 mL. The bottom valve was closed and syringes were removed, emptied, and replaced for 2 more incubations. Afterward, the fixture assembly was left at room temperature, valves closed, for ~24 h. Samples can be stored in pucks for ~1 month without loss of signal in a FISH assay, so we do not believe that our archival procedure compromised cellular integrity during our study (see also Miller and Scholin 2000).

To hybridize samples archived in ESP pucks, the Isopore filter was removed from the fixture assembly and sectioned with a razor into 4 equivalent-sized pies. Sectioning was done because field samples would generally receive multiple probe treatments (e.g., universal positive control, universal negative control [no probe], and one or more species probes; e.g., Miller and Scholin 1998). Quartering filters for sample analysis is preferable to taking discrete samples for each probe treatment because (1) there are limited pucks and reagents that the ESP can support and (2) the instrument requires ~ 1.5 h to sample and archive for FISH, making it difficult to relate samples temporally and potentially within the same water mass. Consequently, it was important to assess intrafilter cell distribution variability for a single sample (see below). Each quartered filter was placed sample side up on a 25-mm-diameter circle of Whatman 3MM CHR paper (Maidstone, England), saturated with 500 µL hybridization buffer (Miller and Scholin 2000) for 10 min, then moved to a second 25-mm Whatman paper situated in an inverted 50-mL disposable polypropylene centrifuge tube cap (e.g., Corning, Corning, NY, USA). The corresponding centrifuge tube contained 1 sheet of lint-free paper soaked with 1 mL hybridization buffer to humidify the sample during hybridization. Fluorescein-labeled auD1 probe (Miller and Scholin 1998) at 500 µL and 4.8 ng mL^{-1} hybridization buffer was applied to the sample in the dark, the centrifuge tube was sealed atop the cap, and the assembly was placed top-down in an oven at 45°C for 75 min. After labeling, quartered filters were placed on a third 25-mm circle of Whatman paper saturated with 500 µL 5× SET (Scholin et al. 1996) preheated to hybridization temperature for 5 min in the dark. Filters were mounted on a microscope slide with 20 µL SlowFade Light (Molecular Probes, Eugene, OR, USA), and all labeled cells were enumerated using a Zeiss Axiophot 2 epifluorescent microscope.

Preparation of SHA positive control and processing reagents—A positive control for the SHA that is independent of cellular material will allow us to interrogate assay chemistry and better correlate results obtained using the 96-well plate assay with those derived from the ESP. We thus assessed the application of a "linker," a synthetic oligonucleotide complementary to both capture and signal probes (see also Goffredi et al. 2005), that theoretically should produce consistent absorbance values, thereby enabling assay comparisons. To prepare the linker, a stock (1 ng μL^{-1} in 1× Tris-EDTA) of NA1S/ALXS alt L, (5′-CCATGAGGGAAATATGAAAAGGACAAATGGTGGGAGT-GTTGCACTTGC-3′), henceforth NA1-L (the complement of capture and signal probes for the North American ribotype of the *A. tamarense/catenella* species complex; Anderson et al. 2005), was made then stored at 4°C. The stock was diluted to 5 and 25 pg mL^{-1} in 0.2 µm-filtered 3 M GuSCN lysis buffer, and both concentrations were stored at room temperature and 4°C. Absorbance at 450 nm (A_{450}) was determined by the 96-well plate SHA (after Goffredi et al. 2005) using a Spectromax plate reader (Molecular Devices, Sunnyvale, CA) that has a dynamic range of 0.0 to 4.0. Each assay used the probes NA1 (capture) and Alex-alt (signal) as described by Anderson et al. (2005). A_{450} was compared between stored NA1-L and probe prepared fresh within 1 h of running the assay at 0 h, 2 days, 7 days, and monthly for 5 months. The same batch of processing solutions and prongs were used throughout the study.

Phytoplankton culturing and enumeration—*Pseudo-nitzschia australis* (MBARI clone CB4) was grown under a 14:10 light: dark cycle at 15°C in 0.2 µm–filtered f/2 media (Guillard and Ryther 1962) using water collected from Monterey Bay at 200 m depth near mooring M1 (36.75 latitude, –122.03 longitude, 34‰). *P. australis* concentrations were determined by sampling three 1.8-mL aliquots of log-phase culture, staining aliquots with 1 drop of Lugol's iodine solution, removing triplicate 5-µL subsamples from each aliquot, and placing each 5-µL subsample under a separate 18-by-18-mm cover slide for a total of 9 discrete counts. The entire slide was enumerated using a light microscope.

Standard curves for 96-well plate and ESP array SHA—To generate a SHA standard curve using the 96-well plate assay, frozen sample filters ($n = 5$) were thawed briefly to room temperature, lysed, and analyzed as per Goffredi et al. (2005), except that the homogenate was clarified using a 0.2-µm hydrophilic Durapore filter, and we used probes for *P. australis* (auD1 capture, PSDS signal at 150 ng mL^{-1} in 2 M GuSCN signal buffer; Scholin et al. 1999). Lysates were prepared and serially diluted in lysis buffer to give samples with ~6.4×10^4, 3.2×10^4, 1.6×10^4, 8×10^3, 4×10^3, 2×10^3, and 0 cells mL^{-1} lysate. A_{450} exhibited a linear response until ~2.5×10^4 cells

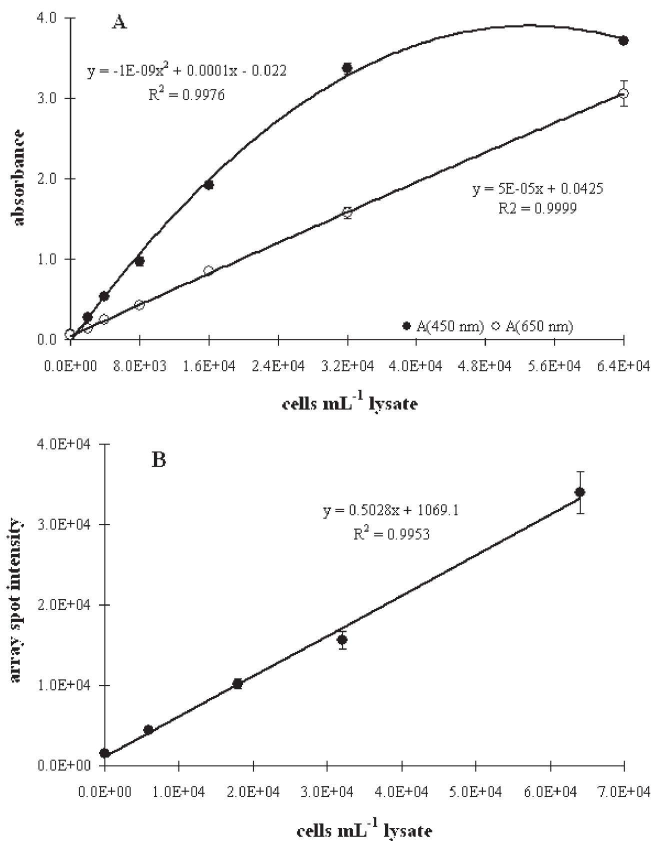

Fig. 4. SHA standard curves for *P. australis* using 96-well benchtop assay (n = 5 replicate wells ± SE) (A) and ESP array spot intensity (n = 12 replicate array spots ± SE) (B).

mL^{-1} lysate, and absorbance was linear for all concentrations measured at 650 nm (Figure 4a). A_{450} for 0 cells mL^{-1} lysate (lysis buffer only) was 0.073 ± 0.001 SD, which was within the typical range of 0.060 to 0.080 based on previous experiments.

To generate standard curves using the ESP, sample filters (n = 3 per array) were homogenized as before to generate lysates with a target concentration of 6.4 × 10^4, 3.2 × 10^4, 1.6 × 10^4, 8 × 10^3, or 0 cells mL^{-1} lysate. Each lysate was split such that an aliquot was applied to the ESP and a matched aliquot was analyzed using the 96-well plate SHA. The results of the plate assay were used to estimate actual cell concentrations applied to the ESP arrays. This was done because replicate samples have a range of cells retained on the filter, and we used 3 filters for the ESP runs as opposed to the 5 filters used for constructing standard curves with the plate assay (5 replicate sample filters yield a more accurate number of cells than 3).

Assessment

Relationship between benchtop SHA absorbance and array spot intensity—A major objective of this study was to compare SHA standard curves between the 96-well plate assay and ESP arrays given a range of range of cell concentrations biased toward lower densities associated with the onset of a harmful bloom (6.4 × 10^4 to 2 × 10^3 cells mL^{-1} lysate). This range was chosen based on the ESP filtering a 400-mL sample and homogenizing material retained in 2 mL lysis buffer, corresponding to target cell concentrations ranging from ~1 × 10^4 to 3.2 × 10^5 cells L^{-1}. For Monterey Bay, CA, we believe that DA production by *P. australis* generally does not pose a health or ecological concern with densities < 2.5 × 10^4 cells L^{-1}. Globally, alert levels for *Pseudo-nitzschia* spp. used for monitoring purposes vary from 1 × 10^3 to 2 × 10^5 cells L^{-1} (Todd 2003).

Results showed that cell concentration and grand mean ESP array spot intensity were highly correlated (Figure 4b). These are the first data showing that ESP arrays yield results that are directly comparable to the 96-well plate assay. Many options to improve the detection limit are available and are being explored at this time, but are not considered here.

Results of the SHA reflect the concentration of targeted rRNA molecules. Values obtained for a given number of cells may vary depending on organism age or nutritional status (e.g., Anderson et al. 1999, Miller et al. 2004, Smith et al. 1992). The extent to which rRNA content is sensitive to environmental fluctuations in natural populations of *P. australis* is not known. We view the SHA assay as measuring whether a particular species is present within some abundance range knowing sample and lysis volumes, but results should not be interpreted as providing a precise cell density estimate per se in the absence of deriving standard curves using natural samples (see also Scholin et al. 1999, 2000).

Manifold vs. ESP FISH filtration and archival—To determine if the ESP sample filtration and archival for FISH produces cell counts comparable to laboratory manifold filtration and fixation, *P. australis* cells were collected and preserved using both approaches. The samples were then treated with the *P. australis*–specific probe auD1 (Scholin et al. 1996, Miller and Scholin 1998). Since ESP filters were to be quartered, sample volumes were 10 mL per filtration manifold tube (n = 3) and 40 mL per ESP filter (n = 3, n = 12). Miller and Scholin (1998) found ~ 20% SE for enumeration of 2.5 × 10^4 cells L^{-1} target *P. australis* diluted in whole seawater and ~10% to 35% SE for 1 × 10^4 cells L^{-1} target *P. australis* with a filtration manifold (derived from Tables 4, 5 in Miller and Scholin 1998), so we used target cell concentrations of 9 × 10^4, 2.5 × 10^4, 1 × 10^4, and 1 × 10^3 cells L^{-1} 0.2-μm filtered FSW.

The area of the quartered 25-mm ESP filter was 87.4 mm^2, or 1.2 times the area of the 13-mm filter exposed to the sample using the manifold. When accounting for this difference in filter area, cell abundance estimates using the manifold were excellent predictors of cell abundances using the ESP method, particularly for high cell densities (Figure 5a).

Despite the strong correlation of cell counts between approaches, some error terms were high. For example, whereas interfilter variability was not significant for any ESP treatment (ANOVA, single-factor, $P > 0.05$), indicating sample collection was highly repeatable, standard errors tended to be greater for the quarter ESP filters than for the manifold

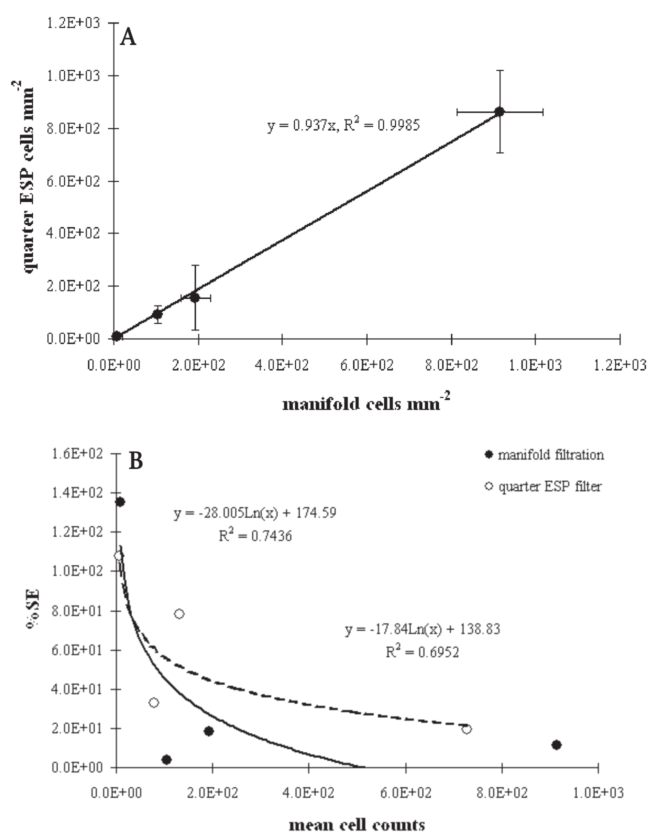

Fig. 5. Whole-cell archive and hybridization comparing mean filtration manifold (n = 3 ± SE) and ESP (n = 12 ± SE) approaches for abundance estimates (A) and 20% error LLD (B). Solid regression line represents manifold; dashed represents quarter ESP filter counts.

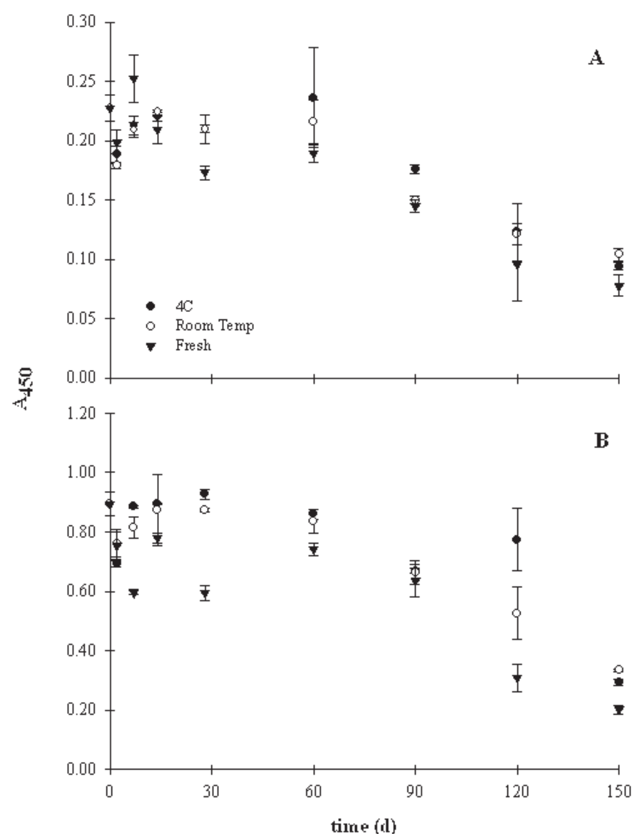

Fig. 6. SHA absorbance values for NA1-L over a 5-month period, stored at 4°C and room temperature (~23°C) and freshly prepared, at 5 pg mL^{-1} (A) and 25 pg mL^{-1} (B).

approach, indicating substantial intrafilter variability for the ESP method (Figure 5a). Intrafilter variability resulting from uneven distribution of cells was particularly apparent in our study because *P. australis* is a chain-forming species. We often observed chains consisting of 10 to 15 cells, occasionally up to 20 cells. Our %SE for the filtration manifold at 1 × 10^4 cells L^{-1} was 3.6, but %SE was 18.2 for 2.5 × 10^4 cells L^{-1}, suggesting that a conservative estimate for a 20% lower limit of detection (LLD) of *P. australis* counts using a filtration manifold is ~2.5 × 10^4 cells L^{-1} (Figure 5b). These results are similar to Miller and Scholin (1998). The LLD for the *entire* ESP filter (i.e., sum of all 4 quarters) was ~1.2 × 10^4 cells L^{-1} (data not shown), but that dropped significantly to ~7 × 10^4 cells L^{-1} per *quartered* filter due to intrafilter variability. The ESP method is therefore unlikely to produce statistically reliable abundance estimates unless cells occur in very high abundances or one enumerates the entire filter.

Considering that DA poisoning appears to be associated with moderate cell densities (Todd 2003), we believe that FISH is an acceptable quantitative accompaniment to the SHA. At < 2.5 × 10^4 cells L^{-1}, however, FISH is unlikely to produce statistically reliable data as the methods were applied here and is better suited for qualitative analysis.

Potential of NA1-L as a SHA control—At the start of the study, A_{450} for the low (5 pg mL^{-1}) concentration of NA1-L was 0.227 (± 0.011 SD) (Figure 6a) and 0.894 (± 0.040) for the high (25 pg mL^{-1}) concentration (Figure 6b). A_{450} ranged between 0.593 (± 0.024) and 0.779 (± 0.016) for freshly made linker during the first 60 days. NA1-L stored at room temperature at both 5 and 25 pg mL^{-1} yielded relatively constant SHA absorbance values for 60 days, but then declined. Similar results were found for NA1-L kept at 4°C, but a white precipitate formed within 48 h, indicating that refrigeration is not a preferred storage medium. When these results are presented as ratios of fresh to stored NA1-L (data not shown), trends generally followed the absorbance values for freshly prepared probe. The exception, 5 pg mL^{-1} at 4°C, followed trends in absorbance values for freshly prepared probe until day 60, when ratios remained ~0.8 for the duration of the study. These results suggest that one possible explanation for the overall decline after 60 days could be that one or more of the SHA reagents and/or prongs degraded. Another possibility could be an apparent degradation of the linker stock itself, since at the end of the 5-month study the concentration had decreased by ~20%. Although this does not explain the entire signal decline, it could be a contributing factor.

We believe that oligonucleotide linkers that have complementary sequences to capture and signal probes are suitable

SHA positive controls with a few caveats. First, for NA1-L, we recommend the higher concentration (25 pg mL^{-1}). Second, although it is possible to make the linker fresh for each assay, this is time- and reagent-consuming and also prone to dilution errors. We therefore suggest preparing a linker stock in lysis buffer and storing that at room temperature for a maximum duration of 60 days. Third, to the best of our knowledge, all SHA reagents used in the 96-well plate format should be stable for at least 6 months, but our results suggest that may be an overestimate. It is not yet clear what component(s) are responsible for the signal decline observed. For that reason, it is essential to maintain vigorous quality control protocols when the highest-fidelity data are required and be mindful of dates when batches of reagents were prepared. Despite these constraints, NA1-L has proved to be valuable tool for assessing the quality of the 96-well plate SHA chemistry. We are now in the process of assessing how we could employ a linker within the ESP to periodically check reagent quality over extended deployments, as well as how to standardize pre- and postdeployment checks to ensure that results throughout a mission are directly comparable.

Discussion

We have shown that the ESP can successfully archive, detect, and provide a means for estimating the abundance of *Pseudo-nitzschia australis* in near real-time. The strong correlation between ESP array spot intensity and the 96-well plate values demonstrates that ESP-derived data are both comparable to and verifiable by established laboratory assays. These findings show that the ESP offers tremendous potential for detecting HABs and potentially other organisms remotely in situ. The application of the SHA, a cell homogenate approach, for developing arrays may provide a more comprehensive notion of phytoplankton community assemblages than methods requiring intact cells (archival for FISH, preservation of whole water for light microscopy, etc.) because one can detect organisms that are too delicate to withstand most sampling and fixing approaches (e.g., raphidophytes, ciliates, some unarmored dinoflagellates). Furthermore, array results can be viewed in near real-time from a remote location. Despite these advantages, one should remain cognizant that arrays as used here reflect the concentration of rRNA. The extent to which rRNA content changes over the course of a *P. australis* bloom and/or in response to environmental fluctuations, for example, is not yet fully understood. Consequently, array data should be interpreted as providing approximate, not precise, cell density estimates.

Our comparisons between ESP and manifold sample archival for FISH elucidated the influence of cell density and sample volume on the statistical reliability of abundance estimates. Our whole-cell method used native (i.e., no preconcentration) seawater. The sample volume is restricted such that the filter is covered with a fine layer of material so as to not obscure target cells. The LLD of 2.5×10^4 cells L^{-1} *P. australis* using the laboratory manifold and a 10-mL sample makes FISH a reasonable method for monitoring (e.g., Rhodes et al. 1998). The nearly 3-fold higher LLD for quartered filters archived using the ESP, however, suggests that unless a very dense bloom of *P. australis* occurs, samples processed for FISH represent a qualitative rather than quantitative interpretation of cell abundances. This does not preclude using ESP-archived samples for alternate downstream analysis (e.g., gene libraries, PCR) when abundances are below the LLD, but instead suggests that cell enumeration using FISH is best saved for blooms unless entire filters are dedicated for single-species analysis.

The issue of statistical reliability using FISH becomes particularly acute when one considers HAB species that pose human health and ecological threats at very low cell densities. For example, "red tide" dinoflagellates of the genus *Alexandrium* Halim include toxic isolates that can cause paralytic shellfish poisoning at ~10^2 cells L^{-1} (Prakash et al. 1971), which is several orders of magnitude below the LLD for *P. australis*. Unlike *Pseudo-nitzschia* sp., cells of *Alexandrium* sp. are spherical and occur singly or form short chains (2-4 cells) during exponential growth. Whereas the whole cell LLD will probably differ between phytoplankton species according to cell morphology, chain-forming behavior, and how samples are handled upstream (e.g., Anderson et al. 2005), it is unlikely to vary by orders of magnitude. Moreover, the small sample volume required to easily view field samples constrains the extent to which sample volume can be increased. Given the widespread application of FISH for enumerating field samples, caution for interpreting results in a quantitative sense is highly advisable.

There is considerable potential to use the ESP to detect other HAB phytoplankton and consequently enhance in situ monitoring. We have shown that the molecular assays used within the instrument are verifiable by laboratory-based methods, thereby enabling truthing of ESP data. Additionally, because the ESP is moored, data represent temporal variability of a particular location. This could be advantageous for some HAB initiatives that previously have had to rely on shipboard collections. The detailed, long-term, and near real-time data sets that can be acquired with the ESP could therefore allow for rapid identification of a potentially toxic event while simultaneously advancing our understanding of bloom dynamics and associated oceanographic processes. Great care should be taken, however, to continually ground truth field and laboratory data with established approaches and quality control procedures to ensure proper interpretation of results.

Future Directions

This contribution focused on applying ESP technology to the detection and enumeration of *P. australis* and potentially other HAB species, but it is important to realize that the ESP is a highly configurable platform. Sampling and processing protocols can be tailored to user specifications. For example, assay chemistry can be modified, new probes can be developed, and sampling/processing procedures (sample volume, archiving, filtration, lysis, etc.) can be amended. Numerous other applications and instrument configurations are possible depending on

what is appropriate for a particular initiative. In that regard, there is considerable potential to explore new detection modules and applications of the instrument. Our hope is that this flexibility will facilitate transfer of the ESP technology to other research groups. To that end, we are constructing 4 additional instruments to aid with technology transfer, as well as exploring modifications for deep ocean (~4 km) sampling. Although we cannot guarantee a completion date for the additional ESPs due to the engineering and testing time required for each instrument, we are making considerable progress toward assembling and testing them during 2006–2007. In the long term, it is our hope that research groups will either acquire/modify their own instrument for their specific research purposes through a third-party manufacturer or perhaps make arrangements for use of one of the existing prototypes.

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