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REMOTE DETECTION OF MARINE MICROBES, SMALL INVERTEBRATES, HARMFUL ALGAE, AND BIOTOXINS USING THE ENVIRONMENTAL SAMPLE PROCESSOR (ESP)

Divers inspecting the subsurface instrument package during pre-deployment testing.

ABSTRACT. The advent of ocean observatories is creating unique opportunities for deploying novel sensor systems. We are exploring that potential through the development and application of the Environmental Sample Processor (ESP). ESP is an electromechanical/fluidic system designed to collect discrete water samples, concentrate microorganisms, and automate application of molecular probe technologies. Development and application of ESP grew from extensive partnerships galvanized by the National Oceanographic Partnership Program. Near-real-time observations are currently achieved using low-density DNA probe and protein arrays. Filter-based sandwich hybridization methodology enables direct detection of ribosomal RNA sequences diagnostic for groups of bacteria and archaea, as well as a variety of invertebrates and harmful algal species. An antibody-based technique is used for detecting domoic acid, an algal biotoxin. To date, ESP has been deployed in ocean waters from the near surface to 1000 m. Shallow-water deployments demonstrated application of all four types of assays in single deployments lasting up to 30 days and provided the first remote detection of such phylogenetically diverse organisms and metabolites on one platform. Deep-water applications focused on detection of invertebrates associated with whale falls, using remotely operated vehicle-based operations lasting several days. Current work emphasizes incorporating a four-channel, real-time polymerase chain reaction module, extending operations to 4000-m water depth, and increasing deployment duration.

INTRODUCTION

Application of molecular analytical techniques for identifying marine microbes, specific genes, and gene products currently demands collecting discrete samples, often in liter quantities, and transporting samples to a laboratory for processing. These requirements typically result in delays ranging from many hours to days between collection of material and its analysis. Establishing new sample collection and processing paradigms is an essential step towards overcoming this impediment (e.g., Goodwin and Litaker, 2008; Palmer et al., 2008; Mariella, 2008). We have approached this problem through development of the Environmental Sample Processor (ESP), a field-deployable system that combines sample collection with molecular analytical functionality. We refer to this type of device as an "ecogenomic sensor" in keeping with the vision cast by NEPTUNE (2008) and Scholin (2009).

The initial drivers behind ESP development were aimed at understanding and monitoring harmful algal blooms (HABs), but a diverse set of applications has emerged since. Laboratory and field trials have demonstrated near-real-time detection of bacterioplankton, invertebrates, HAB species, and the algal biotoxin domoic acid (DA; for reviews on DA, see Landsberg et al., 2005; Ramsdell, 2007). All of these applications use a common set of sample collection and processing protocols. This uniform methodology makes it possible to detect a variety of targets using a single instrument system (e.g., Goffredi et al., 2006; Greenfield et al., 2006, 2008; Paul et al., 2007; Jones et al., 2008; Doucette et al., in press).

ESP development would not have been possible without support from the National Oceanographic Partnership Program (NOPP) through funds allocated by the National Science Foundation (NSF). Preliminary investigations had proven that remote application of molecular probe technology was feasible, but much work remained to bring the ESP prototype to operational status (Scholin et al., 2008). Building on that foundation, NOPP support allowed us to delve into the design further and to construct the current, "second generation" ESP. Work on the new instrument began in 2004 with field trials commencing two years later. Our original aim was to moor the instrument in ocean surface waters and carry out time-series measurements. Design of the system architecture also took into account the possibility of future deep-sea deployments on platforms such as remotely operated vehicles (ROVs) and benthic observatories. Though such developments were beyond the scope of the original NOPP project, support from the Keck Foundation and NASA's Astrobiology Program provided an opportunity to explore those ideas further. Now, four years after the NOPP project began, a small fleet of ESPs has been constructed, instruments have been fielded repeatedly in coastal waters in a networked configuration, and operations at 1000-m water depth have been achieved. All of these deployments took place in Monterey Bay, California. Work to extend deployment duration, geographic coverage, depth rating, and analytical capacity is ongoing. Here, we present examples of field trials conducted during 2007–2008 that highlight ESP's capabilities, emphasizing some of the many collaborations that

grew from our involvement with NOPP. Current developments and plans for future deployments are outlined, along with a discussion of the prospects for transferring ESP technology to the wider oceanographic research and resource management communities.

THE ESP SYSTEM

ESP consists of three major components: the core sample processor ("core ESP"; Figure 1a–c), sampling modules, and analytical modules (Scholin et al., 2006). The "core ESP" performs DNA and protein array analyses on target molecules extracted from particulate matter concentrated by filtration. The core ESP is designed to handle small- to moderate-sized samples (milliliters to several liters) at depths to 50 m. At depths greater than 50 m, the core ESP requires an external "sampling module" to introduce de-pressurized seawater into the ~ 1 atm core. Finally, "analytical modules" are stand-alone molecular detection systems that can be added to or removed from the core ESP to impart different suites of analytical functions downstream of common sample-processing operations. These three components are controlled using the Ruby scripting language running on Linux. Ruby scripts control high-level tasks, such as collecting a sample and generating a homogenate, developing a probe array, collecting and archiving material, or flushing the system (Roman et al., 2007).

Within the core ESP, the "puck" is a surrogate for the laboratory bench (Figure 1b,c). Pucks can contain a variety of filters, or chemically reactive media, depending on the specific application required. Pucks are stored in a rotating carousel and manipulated to collection, processing, or imaging stations by robotic mechanisms (ESP Works, 2008). By specifying a sequence of loading pucks, collecting a sample, and adding reagents in a timed and temperature-defined sequence, ESP can function as an autonomous "lab in a can" to detect in situ a variety of organisms and substances. In addition, the instrument is bundled with chemical and physical sensors so that samples obtained can be referenced against prevailing environmental conditions.

Real-time detection chemistries currently rely on DNA probe and protein arrays to detect target molecules (ESP Technology, 2008). DNA probes are printed onto a nitrocellulose filter in an ordered array (e.g., Greenfield et al., 2006, 2008). These "molecular spots" act to immobilize and concentrate target ribosomal RNA (rRNA) sequences contained in a crude sample homogenate produced onboard the ESP. Captured rRNA molecules are "sandwiched" via a secondary hybridization of one or more signal probes (thus the term sandwich hybridization assay, or SHA). The location and concentration of those signal probes on the array is revealed by a chemiluminescent reaction, in turn denoting the presence and abundance of target sequences. A CCD camera records an image of the array, which is then broadcast via radio telemetry to a remote

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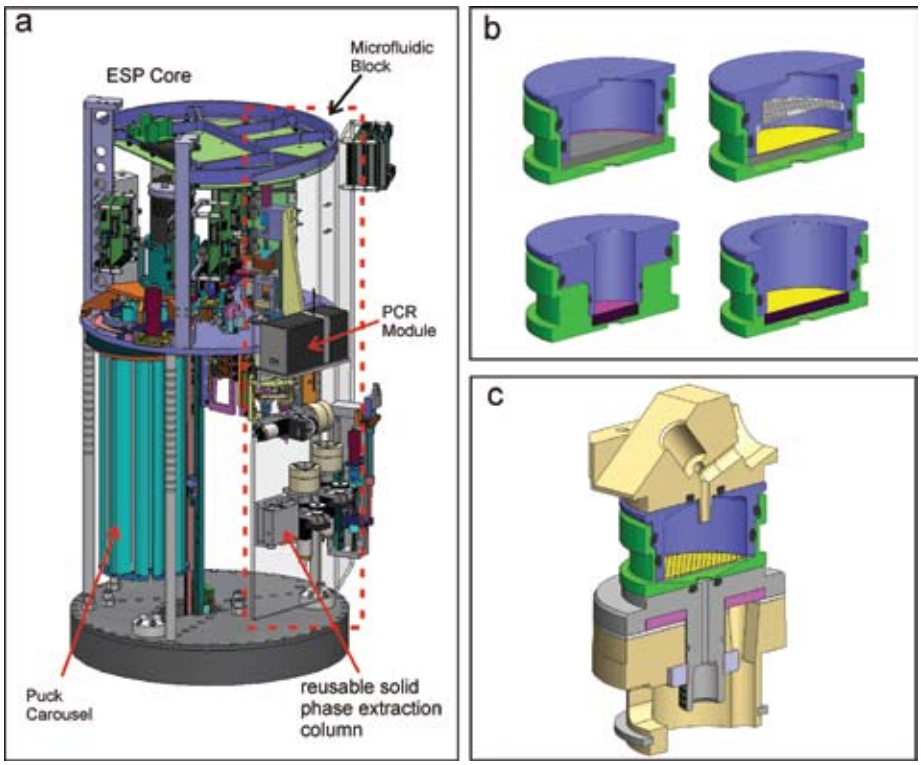

Figure 1. SolidWorks® models showing: (a) the core ESP with a microfluidic block (MFB) and polymerase chain reaction module attached on the right-hand side, (b) different types of pucks used for processing samples, and (c) the sample-collection station clamp in the closed position holding a sample puck. Puck assemblies are specially designed for different operations. In (b), the pucks shown are designed for collecting and homogenizing large-volume samples (top left), archiving material for microscopy (top right), and developing probe arrays printed on 12-mm or 25-mm membranes (bottom left and right, respectively). All pucks conform to the same overall size and shape so that robotic systems used to move and utilize the pucks can be standardized against a constant form factor. ESP with MFB is ~ 0.5-m diameter and ~ 1-m tall. A puck is ~ 30-mm diameter x 17-mm tall. ESP is protected under US Patent No 6187530.

location for interpretation. An increase in the abundance of target organisms is reflected by a proportional increase in light emission from corresponding probes on the array.

The protein arrays use a competitive Enzyme-Linked ImmunoSorbent Assay (cELISA) technique for detecting target substances such as DA (Doucette et al., in press). Like DNA probe arrays, ordered arrays are created by depositing onto a nitrocellulose filter specific protein molecules that retain and/or compete with other substances found in a sample extract. In the case of the DA cELISA assay, an extract produced by the core ESP is combined with a DA-specific antibody and exposed to an array spotted with a DA-protein conjugate. The toxin in solution competes with the immobilized toxin-protein conjugate for the DA antibody in solution. The amount and location of antibody binding to the DA-protein conjugate spots on the array is revealed by a second antibody via chemiluminescence. Like the DNA probe arrays, results of the reaction are visualized using a CCD camera; however, conversely, the signal intensity is inversely proportional to the toxin concentration in the sample extract. For both the SHA and the cELISA, the automated process from collection of a live sample to broadcast of an imaged array takes about three to four hours.

DETECTION OF BACTERIOPLANKTON

A major advantage of the assays conducted by ESP is that the detection of unique rRNA sequences is possible without requiring nucleic acid purification or amplification. In addition, liquid reagents used in the process are stable for extended periods at temperatures from 2–25°C (e.g., Jones et al., 2008; Greenfield et al., 2008; Haywood et al., 2009). Recently, Preston et al. (2009) demonstrated that the same approach is suitable for detecting marine bacterioplankton remotely (e.g., Figure 2). These experiments are among the first of their kind and were limited to well-known microbial groups found in the ocean. Opportunities for extending this capability to detect a wider range of organisms and expression of functional genes are under active investigation. It is not yet clear if the SHA methodology will suffice when there is a need to detect low copy number targets. For example, quantification of messenger RNAs or functional genes that may occur as a very small fraction of total biomass (but that are also critical to biogeochemical cycling and other microbial processes) will likely demand higher-fidelity analytical methodologies such as those that use nucleic acid purification and amplification.

DEEP-WATER OPERATIONS

The development of deep-sea observatories is creating unique opportunities for studying microorganisms in situ (e.g., NEPTUNE, 2008; ORION, 2008). Among the desired technological advances are instruments to assess temporal changes in microbial populations and dynamics of specific genes indicative of geomicrobial metabolic processes. With that in mind, we considered how ESP might be adapted for use in such programs. Early work using ESP for detecting bacterioplankton and invertebrates provided a test case for deploying ESP in deep waters (e.g., Paul et al., 2007; Jones et al., 2008; see also Huber et al., 2007). In particular, whale carcasses in Monterey Bay are known to be "biological hotspots" akin to gas seeps and hydrothermal vents (Goffredi et al., 2008). At such sites, there is a clear distinction in biomass and species diversity relative to background seawater. With such sites in mind, we modified the instrument for use to 1000-m water depth. A Deep-Water Sampling Module (DWSM) was devised and attached external to ESP. DWSM allowed for acquisition of a ~ 2-liter sample and for depressurizing the sample to ~ 1 atm by expanding the volume of the collection chamber (Scholin et al., 2006). Once

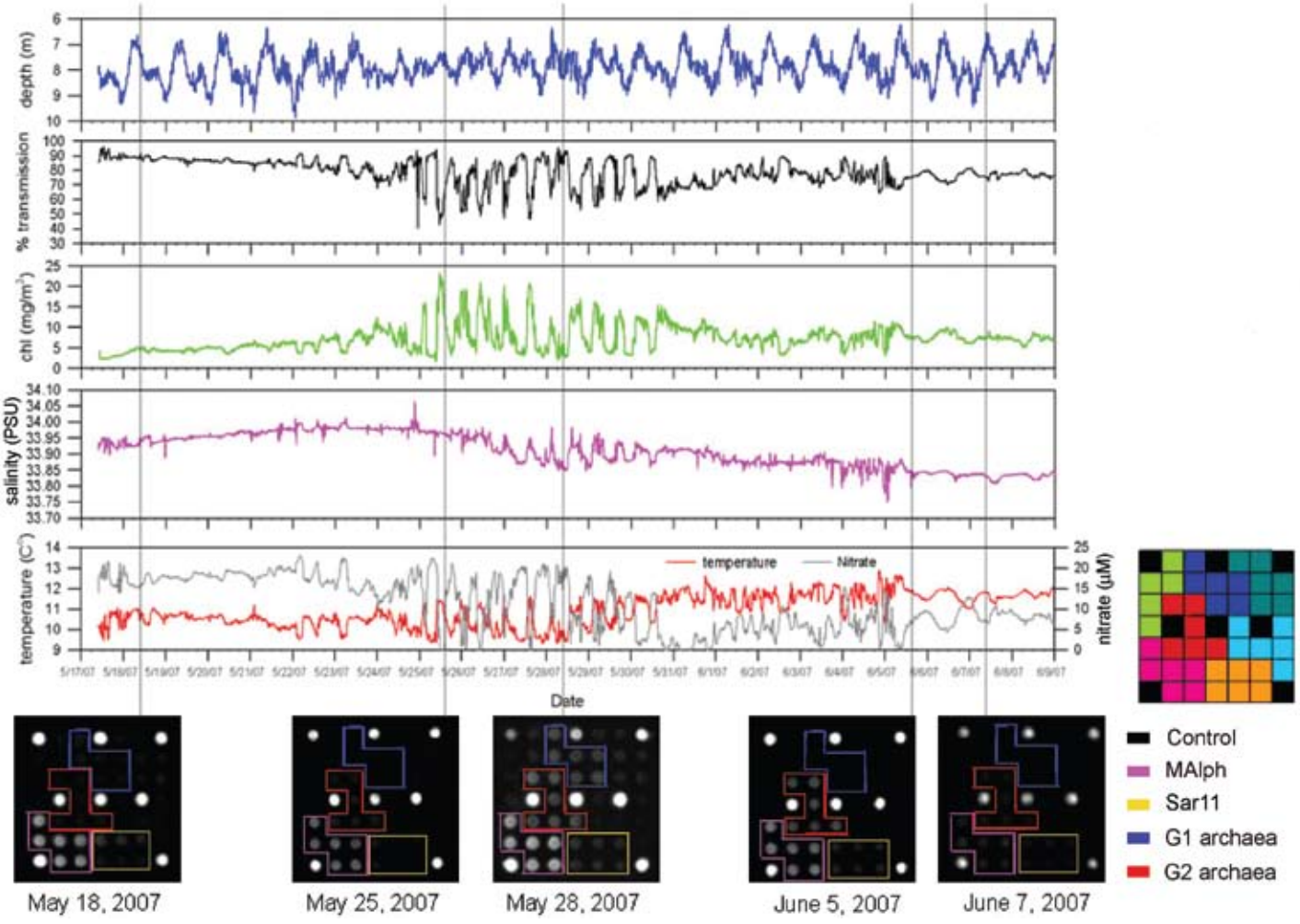

Figure 2. Detection of marine bacterioplankton using ESP moored in Monterey Bay, California, May–June 2007. The top five graphs show physical and chemical data collected by contextual sensors on the ESP mooring during the deployment. The instrument is held subsurface on a taut, compliant assembly (Scholin et al., 2008) so the tidal cycle is evident in the depth panel. The bottom panel shows arrays developed and imaged by ESP. Colored boxes indicate the different bacterioplankton groups detected; sample date and volume for each are shown underneath. Actual size of the arrays is ~ 15 mm x 15 mm. Alterations in the rRNA pool are accompanied by changing chemical and physical oceanographic conditions (after Preston et al., 2009). During this same deployment, assays targeting groups of harmful algae and invertebrates were also run on the same instrument (e.g., Greenfield et al., 2008).

depressurized, the sample was passed through a filter puck located in the core ESP, followed by development of probe arrays as described above. This system is referred to as the deep-sea ESP, or D-ESP (Figure 3).

The D-ESP prototype was mounted beneath ROV *Ventana* and fitted with a sampling wand that allowed a mechanical arm to precisely position the sample intake at ~ 2–3 m from D-ESP itself (Figure 3c). A pump was used to pull fluid continuously through the wand, past the DWSM intake. When the wand was at the desired location, DWSM was activated and then followed by ESP sample processing. Those deployments proved that capturing a volume of raw seawater at depth and interrogating it using conventional ESP SHA probe array techniques was feasible (Figure 3a,b). We also succeeded in returning preserved samples to the laboratory for microscopic analysis and nucleic acid extraction (recent work of author Jones; after Jones et al., 2008; Scholin et al., 2008). Construction of a 4000-m-rated DWSM and ESP is now underway. The new DWSM allows for capturing and depressurizing up to 10 liters of water prior to its delivery to the core ESP. The extent to which depressurizing a sample affects the perception of rRNA profiles

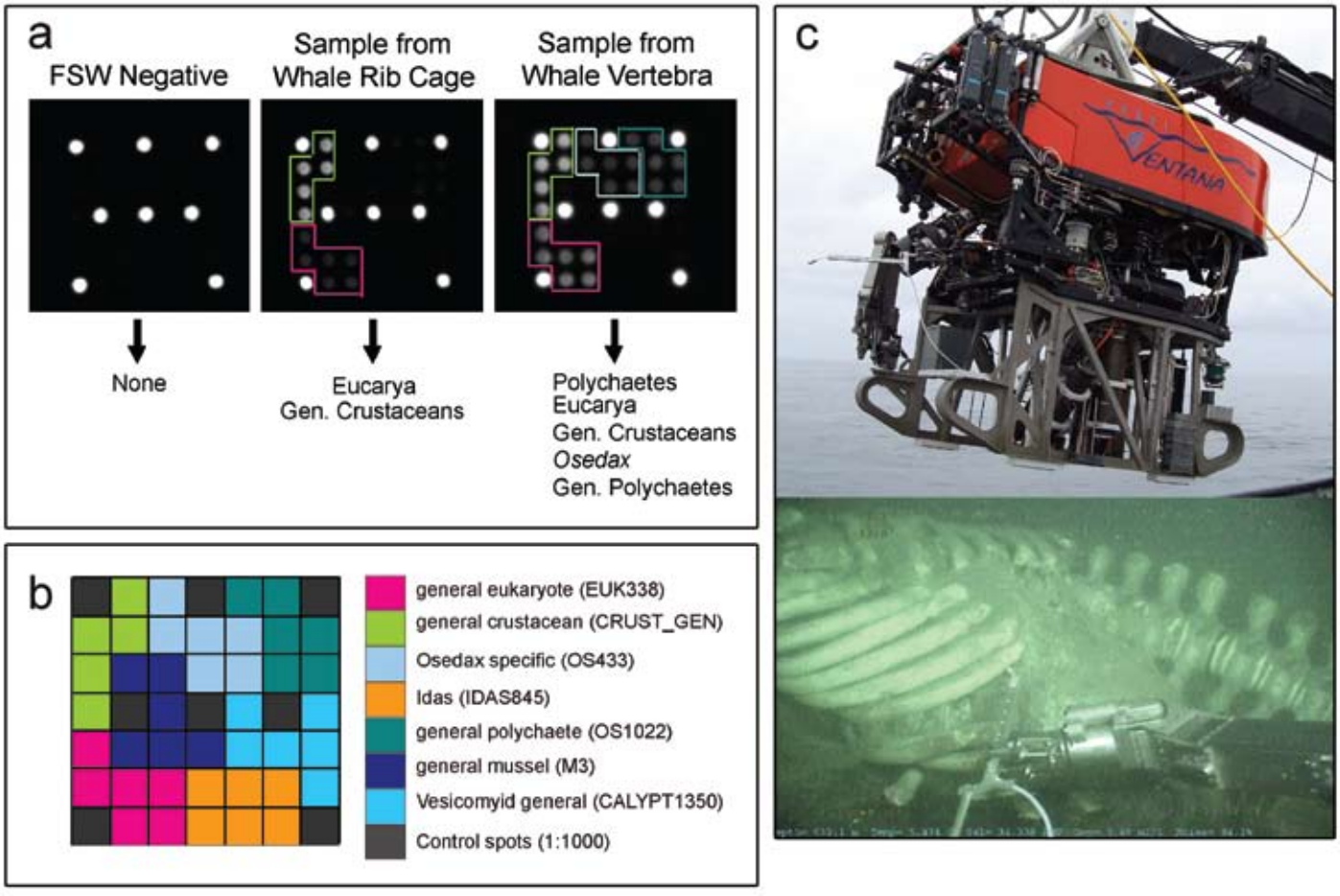

Figure 3. Application of ESP for detection of invertebrates associated with a whale fall at 633-m depth in Monterey Bay, California, in August 2007. (a) From left to right are arrays from pre-deployment negative control (filtered seawater [FSW]) and two arrays from material collected from different portions of the carcass. Different sets of probes reacted positively depending on sample source. Actual size of the arrays is ~ 15 mm x 15 mm. (b) Array key showing locations of probes for different invertebrate rRNA sequences, including universal probe for Eucarya. Colored boxes surrounding probe spots on arrays (a) correspond to invertebrate species detected. (c) The top picture shows deployment of ROV *Ventana* with the D-ESP mounted below; the sampling wand is held in a robotic arm. The bottom picture is a video frame grab showing the sampling wand extended during sampling of the rib cage.

is not yet fully understood. However, current work sponsored by NASA aims to modify the core ESP so as to allow for collection of samples at ambient pressure. Thus, in the near future, we expect to be able to address the "depressurization effect" empirically.

HARMFUL ALGAL BLOOM RESEARCH

The identification and quantification of aquatic microorganisms that are a public health concern is a priority (e.g., GEOHAB, 2008; Ramsdell et al., 2008). For HAB research and monitoring, identification of causative species and associated toxins is critical to understanding bloom dynamics and toxicity, as well as mitigating impacts associated with harmful blooms. This idea was central to the NOPP-supported ESP development, solidifying the partnership between teams at the Monterey Bay Aquarium Research Institute (MBARI) and NOAA's Marine Biotoxins Program (Charleston, SC). Implicit in the design of ESP was the recognition that collection and processing of samples for organism and toxin detection share many similarities. For example, both SHA and cELISA require sample concentration, chemical extraction, a timed sequence of reactions at specific temperatures, and use of a CCD camera to image probe arrays. In addition to providing presence/absence information, these assays can also provide a quantitative assessment of their target concentrations in seawater. Recent work shows that signal intensities correlate well with the number of cells collected (Greenfield et al., 2006, 2008), and very closely with the amount of toxin found in the particulate phase of a sample (Doucette et al., in press). Thus, it should be possible to use ESP to remotely assess changes in both the abundance and toxicity of particular HAB species.

Tandem deployment of the HAB SHA and DA cELISA began in 2006. Early trials resulted in the first-ever autonomous, subsurface detection of an HAB species and associated phycotoxin (Greenfield et al., 2008; Doucette et al., in press). Since then, a number of refinements to both assays have been implemented and the quantitative nature of the assays' chemistries further explored. Field trials conducted in 2008 in Monterey Bay are illustrative of progress made. Figure 4 shows a small subset of the data obtained. Such observations confirm that ESP is capable of detecting DA-producing species and particulate DA at levels well below that of public health concern. At the same time, these observations also reveal how low levels of toxin may enter the food web under "nonbloom" conditions, raising the issue of how chronic delivery of DA may affect higher trophic levels (e.g., Lefebvre et al., 2002; Kvitek et al., 2008; Ramsdell and Zabka, 2008).

THE QUEST FOR HIGH-DENSITY PROBE ARRAYS

Detecting a suite of pathogens, HAB species, and toxins in order to protect public health (Sandifer et al., 2007), as well as numerous applications of environmental genomic data, all require the simultaneous detection of tens to hundreds, if not thousands, of unique molecules in a single sample. Probe arrays offer one means of accomplishing this objective (e.g., Ellison and Burton, 2005; Ahn et al., 2006; Rich et al., 2007; Frias-Lopez et al., 2008). Generating probe arrays for ESP was originally achieved using a contact pin printer to deposit "probe ink" on filter membranes (e.g., Figures 2 and 3; Greenfield et al., 2006, 2008). That method allowed us to create arrays consisting of ~ 50–60 features within ~ 15 mm x 15 mm space, a very coarse array by today's standards. Using a positive displacement "ink jet" (piezo) type printer, we are now able to deposit DNA and protein probes in much higher densities. For example, printing ~ 800 probe features on 12 mm x 12 mm flow-through membranes is possible (e.g., Figure 5). Such densities are still quite moderate compared to what can be accomplished using glass slides or microbead arrays, but they nonetheless open up unique research opportunities when considering they can be used autonomously in situ.

MICROFLUIDIC BLOCK

Homogenizing cells and parsing small, concentrated aliquots of that material to various analytical chemistries appears to offer the best solution for meeting a serialized "collect and analyze" functionality, yet still allowing for variable sample analysis methodologies. While future ESP use will likely employ techniques like SHA and cELISA, we also expect other applications to require more extensive sample preparation and higher-fidelity analytical operations. To that end, the core ESP was designed to handle large volumes of natural samples and then render material collected for analysis on a millifluidic or high-end microfluidic scale. Although this method is appropriate for some assay chemistries, many others demand microfluidic-scale processing. To meet that need, we have designed a separate

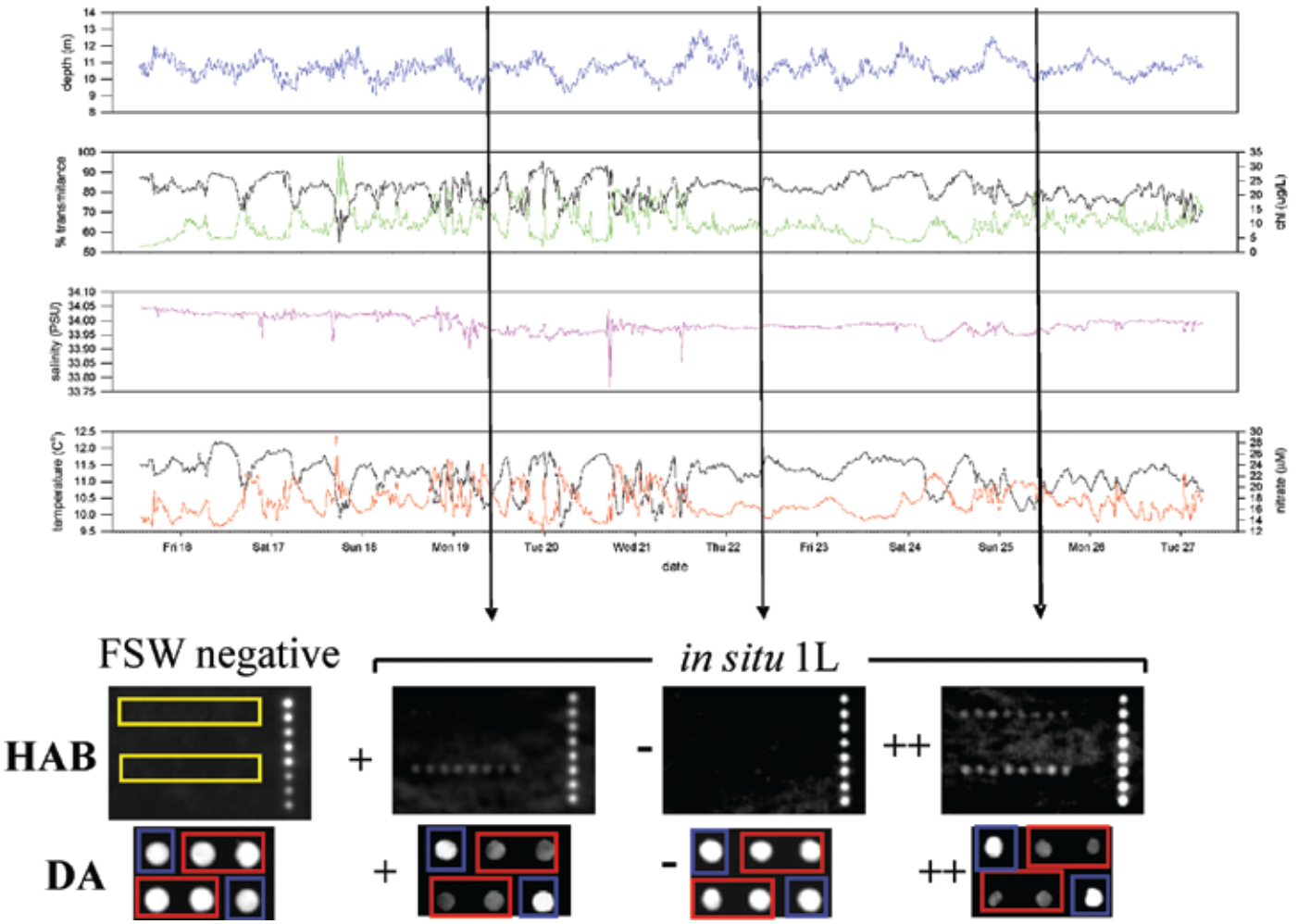

Figure 4. Use of ESP to detect *Pseudo-nitzschia* spp. (P-n) and domoic acid (DA) in Monterey Bay, California, in May 2008. As in Figure 2, sensors mounted to ESP provide the environmental context in which to view results of DNA and protein arrays. Only small sections of the arrays are shown. Harmful algal bloom (HAB) arrays emphasize probes for *P. multiseries* (top row) and *P. multiseries/pseudodelicatissima* (bottom row) printed horizontally, with positive control probes printed vertically. DA arrays show section with two control (top left, bottom right) and four DA spots. The negative controls show resulting arrays when ESP is provided filtered seawater (FSW). Arrays for both HAB species and DA obtained during the deployment are based on 1-liter sample volume. Low numbers (~ 5–10 x 10^3 cells l^{-1}) of P-n species appear at the mooring site, then disappear during a strong upwelling event, and finally reappear as upwelling relaxes and a population of toxin-producing cells is re-established. The DA pattern follows that of the P-n cells, with toxin initially detectable (~ 10 ng DA l^{-1}), disappearing, and then present at higher concentration (~ 60 ng DA l^{-1}) upon relaxation of upwelling.

fluid-handling system that can be added to the existing core ESP. This work was carried out in collaboration with researchers at Lawrence Livermore National Laboratory (LLNL) with support from NASA and the Gordon and Betty Moore Foundation. We refer to this device as the "microfluidic block," or MFB (Figure 1). MFB is an interface for distributing samples and reagents from ESP to one or more analytical modules. The analytical module currently under development consists of a reusable solid phase extraction column for purifying nucleic acids and a four-channel, real-time polymerase chain reaction (PCR) module (after Belgrader et al., 2003). The fluidics support reagents for a variety of PCR master mixes, primer/probe combinations, and control templates. Ultimately, we aim to use MFB to arrange multiple functions in an analytical cascade (e.g., employ probe arrays and/or capillary electrophoresis downstream of PCR).

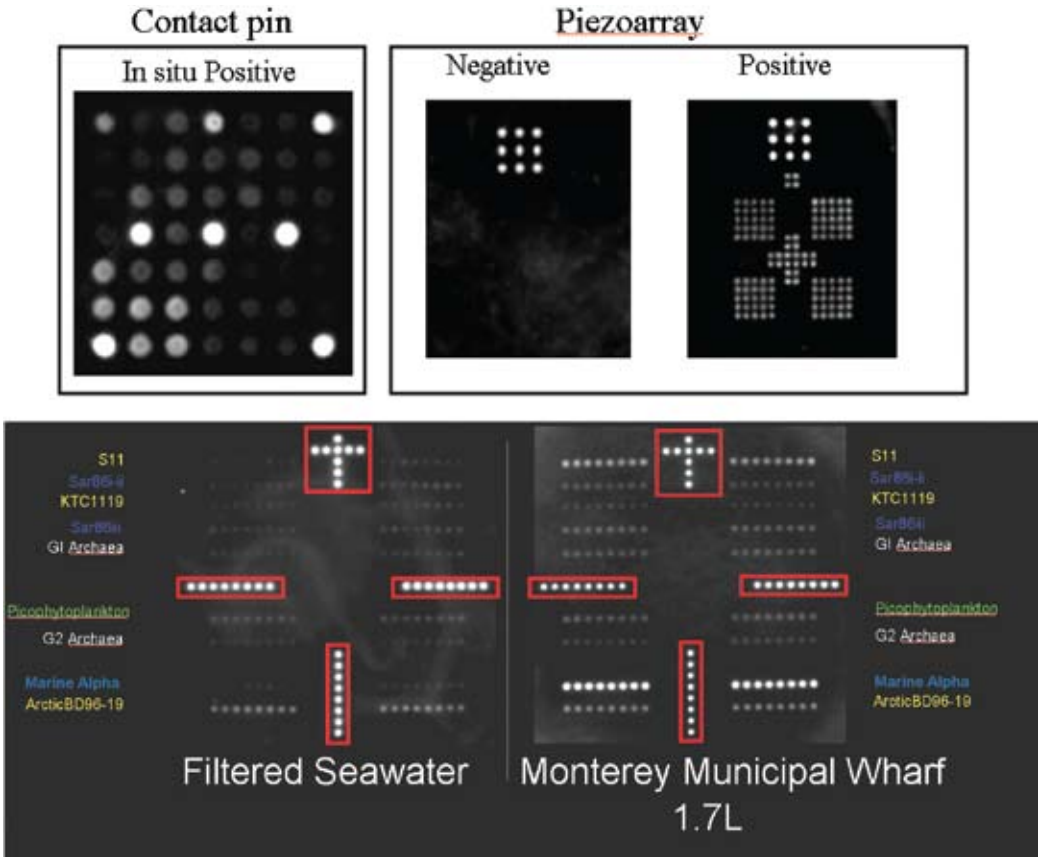

Figure 5. Examples showing refinement of probe-array printing techniques from contact pin to "inkjet" (piezo) technology. The upper panel illustrates how much smaller and more compact the probe features are using the piezoarray. The bottom panel shows rRNA-targeted probe arrays for bacterioplankton (as in Figure 2) printed using the piezo technique. Red boxes show positive controls; the presence of various microbial groups is indicated by light emission from probe spots for a 1.7 l native seawater sample versus that obtained for filtered seawater (negative control). All images shown are roughly equivalent in scale.

LOOKING TOWARD THE FUTURE

The ESP is now at a stage where it is feasible to transfer technology to groups with needs for a remote, in-water sensing capability and for integration with larger-scale observatory operations. Indeed, these were the primary objectives set forth under the NOPP-sponsored development. To that end, we are working to construct copies of the core ESP as well as MFBs with support from NOPP, NSF, and the Moore Foundation. Our goal is to transfer those devices to a limited number of labs in the United States and elsewhere for method development and testing. With that network in place, we hope to bridge the gap among regulatory agencies, academicians, and environmental research interests, and thoroughly evaluate how this kind of technology may be used for research and monitoring purposes. This work will be greatly facilitated by furthering academic, government, and industry partnerships initiated under the NOPP program. While challenges remain, basic in-water operations underlying the ecogenomic sensor concept have been attained and the vision cast by NEPTUNE (2008) and others is within reach.

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