In Situ Instrumentation

BY JOHN PAUL, CHRIS SCHOLIN, GER VAN DEN ENGH, AND MARY JANE PERRY

NEEDS AND CHALLENGES

Ocean-observing systems are changing the way ocean science is accomplished. No longer is ocean science limited to observations made by ships, whose scheduling and expense often constrain research to short forays that result in data streams limited in space and time. Such observations have been described as being "frozen in the invisible present," offering thin slices of the ocean record that often miss processes that function on multiple spatial (e.g., boundary current, eddy, gyre, ocean basin) and temporal (e.g., monthly, seasonal, annual, decadal) scales. The key to autonomous observations of microbes in the ocean is continuing development of sensing technologies in the laboratory, transitioning sensors from the bench to the field, and integrating sensor suites into observing platforms appropriate to the spatial and temporal dimensions of specific processes and phenomena.

With regard to platforms, the last several decades have witnessed an impressive evolution of in-water platforms that extend the temporal and spatial reach of ships. Bottom-tethered coastal and deep-sea moorings provide time-series data at single locations (i.e., OASIS: http://www.mbari.org/oasis) and as integrated observing networks (i.e., GoMOOS: http://www.gomoos.org). Enhanced battery life and new technologies that locally produce energy are enabling longer mooring deployments and additional instrumentation. More recently, the development of shore-powered, cabled observatories with high bandwidth is freeing researchers from constraints of power limitation and enabling rapid two-way communication with sensors and other devices (i.e., Martha's Vineyard Cabled Observatory: http://www.whoi.edu/mvco/description/description2.html; Venus: http://www.venus.uvic.ca; LEO-15: http://marine.rutgers.edu/cool/LEO/LEO15.html; and others in planning). Mobile platforms such as profiling floats, drifters, autonomous underwater vehicles (AUVs), and gliders allow questions to be addressed on a range of spatial scales; mobile platforms either follow water masses in a Lagrangian mode or operate in a survey mode (Rudnick and Perry, 2003). Distributed networks of diverse and complementary ocean-observing systems offer the possibility of integrated, continuous, real-time observing of oceanic phenomena over large areas without the limitations imposed by shipborne observations (Figure 1).

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Despite the successes of moorings, gliders, and other observational platforms in routinely making long-term autonomous measurements of physical or meteorological data, biological sensing systems—particularly those capable of microbiological measurements—are in their infancy. With a few notable exceptions, most autonomous biological sensing systems are optically based and

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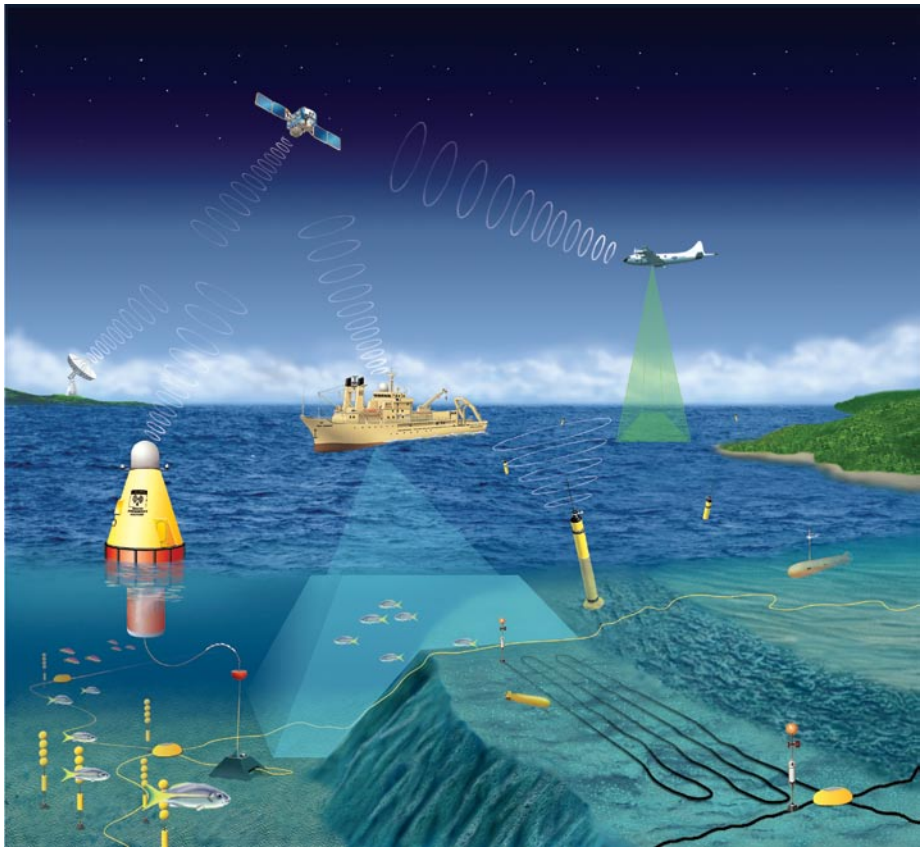

Figure 1. Vision of the components of an ocean-observing system, including cabled observatories, autonomous underwater vehicles, gliders, buoys, moorings, satellites, and a traditional observing platform (research vessel). *Image courtesy of Harris Maritime Communications*

typically focus on bulk optical measurements. In contrast, laboratory-based technologies include rapidly evolving, highly capable molecular techniques for taxonomic and functional analysis and optical methods for analysis of single cells. The challenge for observatories is to transition technology capable of microbiological measurements into the ocean.

There are six major considerations in the development and deployment of this nascent technology:

1. What is the concentration or frequency of occurrence of the target organisms? Certain targets may always be present at a relatively high concentration (i.e., bacteria) while others may only occur episodically (i.e., harmful algae), and yet others (human pathogens) may be so dilute as to require sample sizes in the hundreds of liters.
2. What is required for sample preparation prior to analysis? Certain detection technologies require nucleic acid extraction and purification, while others require staining or probe hybridization to nearly intact cells. Simple sample preparation is certainly better than a lengthy series of extraction and purification steps.
3. How complex is the detection assay? If simple staining or hybridization is required, results will be available in a relatively short period of time (therefore enabling a higher data-collection frequency), whereas amplification may take one to several hours.
4. Is it desirable to archive samples for examination and verification after instrument retrieval? Archiving requires some preservation as well as storage capacity of the system.
5. What are the design criteria for sensors in terms of size and power consumption? Size and power budget will limit the type of platform on which a particular sensor can be deployed (i.e., cabled observatory versus glider).
6. How long can the sensing system (sensor and platform) operate between service visits? Biofouling, stability of reagents, and sample capacity are among the factors that will determine frequency of sampling and length of deployment. Ultimately, a desirable goal is service frequencies of months (even better, years).

APPROACHES

Optical Techniques

Optical methods have long been used to study autotrophic phytoplankton, either at the community level or as individual cells. Chlorophyll *a* fluorescence is widely used to assess phytoplankton abundance (Lorenzen, 1966), and a wide variety of small, power-stingy sensors exist. Variable fluorescence (Fv/Fm), based on saturation kinetics of Photosystem II, is used to determine key photosynthetic parameters for computation of phytoplankton primary productivity (Kolber and Falkowski, 1993). The current generation of variable fluorometers has typically been used in ship-based profiling or

flow-through modes, but newer instruments are smaller with lower power consumption, making them more compatible with autonomous deployment. In-water spectrometers measure either a complete visible absorption spectrum or a limited number of wavelengths and have been used for months on moorings and days on mobile platforms. Absorption spectra are used in assessing physiology (photoadaption) (Roesler and Zaneveld, 1994) and species composition (Robbins et al., 2006).

In contrast to bulk optical properties, flow cytometers identify and count individual particles that stream past an array of light detectors. These instruments were originally intended for biomedical studies, but are now successfully used in the analysis of marine microbes (Chisholm et al., 1988; Olson et al., 1989; Binder et al., 1996; Shalapyonok et al., 1998). The use of flow cytometry is still largely restricted to the laboratory, but special instruments that can be deployed in the field are becoming available (Olson et al., 2003; Dubelaar et al., 1989; http://www.cytobuoy.com). Rapid advances in the technology, especially the use of solid-state lasers, will make it possible to deploy grids of flow-cytometry detectors at permanent observation sites. Real-time, on-site plankton detectors will allow biological oceanographers to remotely observe the dynamics and spatial distribution of algae blooms and the proliferation associated microbes.

Molecular Biological Techniques

Although optical methods are highly evolved and used routinely in ocean science, they do not allow for distinction of many microbial groups, nor do they provide an indication of the genomic capacity (e.g., Culley et al., 2006; DeLong and Karl, 2005). Molecular biological techniques do offer a range of methods for addressing genetic capability and/or phylogeny, complementing information gleaned using optics. The application of molecular analytical techniques in the environmental sciences has historically required the return of samples to a laboratory. Thus, an integrated view of the presence and activities of a natural community of microbes often emerges long after samples were collected. Application of molecular analytical techniques in a remote, in situ context is clearly feasible, but from the perspective of instrumentation development and ocean observing systems, it is in its infancy.

> The challenge for observatories is to transition technology capable of microbiological measurements into the ocean.

In the laboratory, different steps associated with sample processing—sample collection, extraction, analysis—are generally accomplished using distinct instrumentation for each process. Some companies offer complete systems that automate sample preparation and analysis (e.g., Cepheid), and simple field portable systems have also been devised for detecting microbes (e.g., Bavykin et al., 2001: Casper et al., in press). As far as we are aware, however, only the autonomous microbial genosensor (AMG) and environmental sample processor (ESP), discussed in greater detail later, have been advanced as single systems that make it possible to conduct cell-free, molecular analyses remotely beneath the ocean surface.

Methods that rely on nucleic acid amplification (e.g., Suzuki et al., 2000; Casper et al., 2004) offer the most sensitive assays for detecting low levels of target sequences, and they are commonly applied in environmental sciences. Reaction mixtures are produced by adding a suite of reagents supplied in liquid or dehydrated form, the resulting cocktail is subjected to an appropriate thermal profile, and the reaction is often complete in less than one hour. At least one company, Cepheid, offers a complete laboratory system (GeneXpert®) for processing water samples and applying quantitative polymerase chain reaction (PCR), in many ways resembling the core functionality of AMG.

Less developed are methods that allow for direct analysis of target molecules without a requirement for amplification. This can be achieved by retaining target molecules on a solid support, or reacting probes with target molecules in solution (e.g., Ellison and Burton, 2005; Anthony et al., 2005; Ahn et al., 2006).

Probe arrays offer a means of detecting a large number of target sequences in

a single sample simultaneously. Current methods generally favor extensive sample-preparation procedures to obtain labeled and amplified material that is suitable for analysis, but direct detection of target sequences is also possible (e.g., Marcelino et al., 2006; Hashsham et al., 2004; Small et al., 2001). Using such methods in an autonomous system deployed in the ocean poses significant, though not insurmountable, challenges. For example, STMicroelectronics offers the In-Check® platform, a microfluidic chip that combines PCR amplification and probe array detection functions. Integrated devices like this system could find application for deploying "conventional probe array chemistries" in an ocean setting.

CASE STUDIES

Cytometry

Sallie Chisholm, Rob Olson, Zachary Johnson, Charles Yentsch, and Daniel Vaulot have made significant contributions in establishing criteria for the identification of microbes by flow cytometry and have conducted extensive field studies that describe the temporal and geographical distribution of, most notably, the cyanobacteria (Chisholm et al., 1988; Legendre and Yentsch, 1989; Vaulot et al., 1995; Mann and Chisholm, 2000; Johnson et al., 2006).

Typical measurements of marine samples determine the forward scatter and side scatter and the fluorescence from chlorophyll and phycoerythrin (a reddish pigment found mainly in cyanobacteria and red algae). Chisholm and Vaulot and their collaborators have shown that these parameters are useful in measuring primary producers (*Prochlorococcus* and *Synechococcus* at different locations) (Vaulot et al., 1995). Li (1994) and Worden et al. (2004) use these parameters to quantitate picoeukaryotic grazers of the cyanobacteria.

Among the group of optical parameters that remains to be explored, only the use of scatter polarization has been reported. Olson et al. (1989) observed that differences in polarization of forward scatter can be used to distinguish among coccolithophores, diatoms, and other microbes. Scatter depolarization is a promising parameter to determine the degree of calcification of coccolithophores, and it may be useful in determining the productivity and carbon fixation of this ecologically important group of microorganisms (Iglesias-Rodriguez et al., 2002, 2006).

Recent engineering efforts by author van den Engh and Tim Petersen, now at Cytopeia, Seattle, have led to greatly improved detectors for polarized scatter measurement. This new generation of detectors can register particles as small as 100 nm and determine scatter- and fluorescence-depolarization with great precision. When combined with photomultipliers with a high current capacity, the dynamic range can be adjusted to cover six or even eight decades of signal intensity.

Flow cytometers are complex instruments, and their fragile character is an obstacle for use in the field. Historically, flow cytometers used finicky, power-hungry lasers. This situation is rapidly changing. In recent years, a wide range of solid-state lasers has become available. At this moment, solid-state lasers offer a wide choice of wavelengths and light intensities between 355 nm and 700 nm. The availability of adequate light sources no longer is an obstacle to field applications.

Current flow cytometers require a particle-free carrier fluid to transport particles through the measurement area. Prolonged operation at a remote location requires a constant supply of clean sheath fluid. The two systems that have been built for use at sea recycle the carrier fluid and remove particles by filtration as new sample is injected into the core of the fluid stream. The mechanism that Rob Olson and Heidi Sosik (Olson et al., 2003) developed for their system is remarkably robust and has operated for months at the test site.

A plankton detector that does not require a sheath fluid is being developed (Jarred Swalwell, School of Oceanography, University of Washington, *pers. comm.*, 2006). The detection system of this instrument determines the position of the particles in front of the detector (Position Sensitive Detector, PSD). Only particles that follow a trajectory through the optical optimum are accepted for analysis. The PSD has been shown to perform accurate measurements on unfiltered seawater flowing though a simple fluidic system. Developments like this will lead to simpler designs with increased reliability and longevity in the field.

Optical Phytoplankton Discriminator

The Optical Phytoplankton Discriminator (OPD) (Figure 2) is a highly adaptive phytoplankton-sensing module developed by Mote Marine Laboratory, Sarasota, Florida, under the direction of Gary Kirkpatrick (Robbins et al., 2006). The instrument is designed to discrimi-

nate the Gulf of Mexico red tide organism *Karenia brevis* from other phytoplankton based upon optical properties. The heart of the module is a liquid waveguide capillary cell (LWCC) attached to a fiber-optic spectrometer, illuminated by a fiber-optic tungsten/deuterium light source. The operational sequence of this instrument is to first draw a sample into the LWCC, take a spectral reading, and then draw in a reference solution from an onboard reservoir to take a reference spectrum. Finally, the LWCC pulls in a filtered (cell-free) sample of the ambient water to get the spectral properties of the dissolved components of the sample in question. Pigment absorbance peaks are transformed using fourth derivative analysis and compared to values obtained with a reference *K. brevis* culture. A similarity index is computed that ranges from 0 to 1, a value of 1 being most similar to *K. brevis*.

The OPD can be deployed on stationary moorings or mobile platforms such as the BSOP (Bottom Stationed Ocean Profiler; http://cot.marine.usf.edu/Bsop/Bsop.htm) and autonomous underwater vehicles such as gliders and REMUS (Remote Environmental Monitoring UnitS) (Robbins et al., 2006). A distinct advantage of the OPD is the minimal sample preparation time that enables it to process multiple samples quickly, as required for AUV deployment.

Figure 3 shows data obtained from the deployment of the OPD on an AUV off the coast of southwestern Florida in January 2005. The proportion of the phytoplankton attributed to *K. brevis* is reported in conjunction with salinity (reported as density). These data show that *K. brevis* is more abundant in the western portion of the transect (left side of figure).

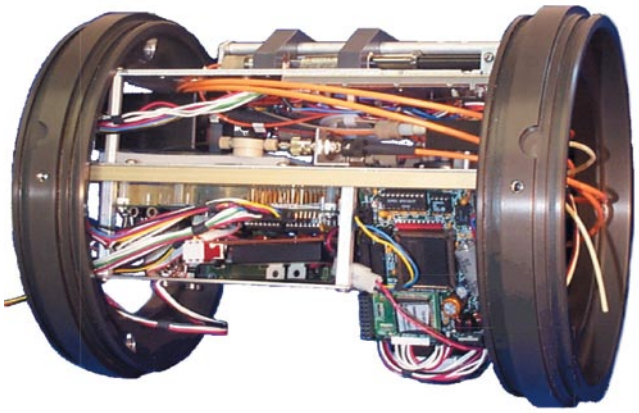

Figure 2. Optical Phytoplankton Discriminator (aka Brevebuster). *From http://coolgate.mote.org/socool/breve-def.html*

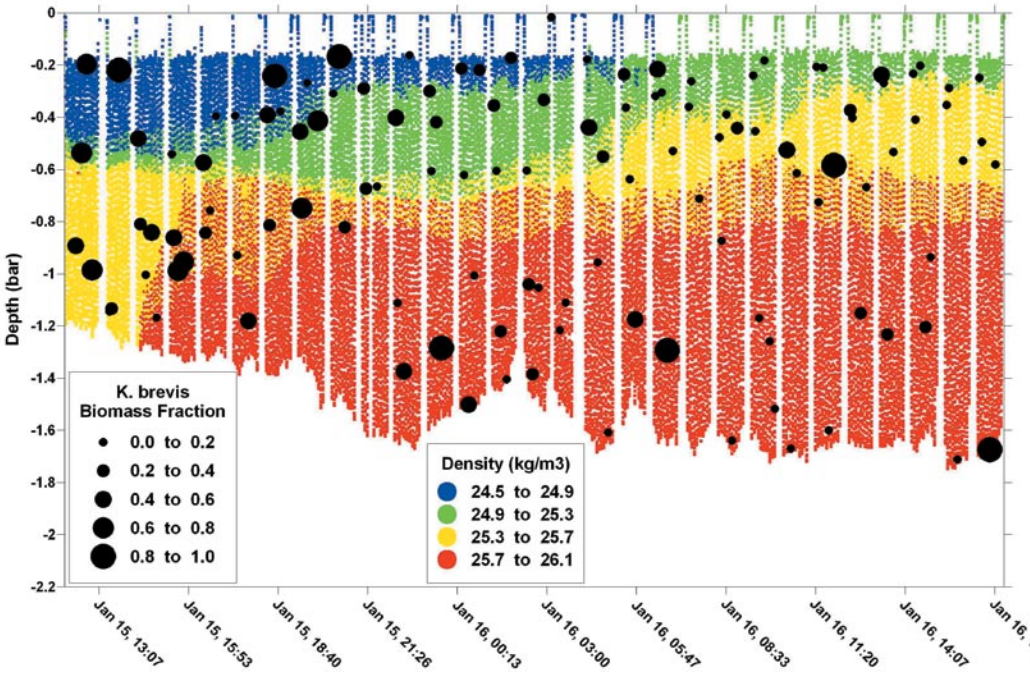

Figure 3. Cross section of water density and *Karenia brevis* chlorophyll biomass fraction obtained from a BreveBuster-equipped glider on January 15–16, 2004. From the beginning of the plot to approximately 2130 hrs on January 15, the glider was moving west-southwest across the shelf. It then turned and proceeded southeast, parallel to the coast, until it was recovered. Due to the sampling scheme of the BreveBuster, the vertical positions of the biomass fraction values are rough approximations. These positions could vary by approximately 50% of the bottom depth. Although it is not possible to give the depth of the *K. brevis* observations precisely, the horizontal distribution shows a higher biomass fraction at the northern (left side) extent of the survey. Note that the density values are individual measurements, not contours. *Gary Kirkpatrick, Mote Marine Laboratory*

The Environmental Sample Processor

The ESP is an electromechanical/fluidic system that collects discrete water samples from the ocean subsurface, concentrates microorganisms (particulates), and permits exchange of various reagents in a timed sequence (http://www.mbari.org/microbial/esp). The instrument executes user-defined macros that specify a sequence of steps for accomplishing high-level tasks, such as collecting a sample and generating a lysate, developing a probe array, collecting and archiving a sample, or flushing the system. Sample manipulations are carried out in reaction chambers, called pucks, which are loaded into and removed from various stations using robotic mechanisms. Pucks clamped in a process position can be exposed to seawater or reagents that are accessed through various valve manifolds using a syringe pump (Scholin et al., in press; Babin et al., 2005). Central to the current functioning of the ESP are custom rRNA-targeted DNA probe arrays that are applied using a sandwich hybridization technique (e.g., Greenfield et al., 2006) (Figure 4).

Following sample collection, cells are homogenized using detergent and heat, and the resulting crude homogenate is applied to a probe array printed on reinforced nitrocellulose. Direct capture of the target molecule ensues, followed by hybridization of a signal probe and chemiluminescent reporting. An image of the array is captured using a CCD camera and transmitted to shore for interpretation. The ESP supports a variety of environmental contextual sensors. For example, data from a CTD/fluorometer/transmissometer are also uploaded periodically to provide a context for viewing results of the probe array assays. The entire automated process, from collecting a live sample to broadcasting an imaged DNA or protein probe array takes about two hours and occurs subsurface. Reagents employed in these assays are stable for extended periods (none used in the ESP require refrigeration), and the chemical reactions themselves are amenable to microfluidic scaling. The ESP also has the capability to archive samples for various laboratory analyses, including fluorescent in situ hybridization (FISH), nucleic acid analyses, and algal toxin detection.

First-generation prototypes of the ESP have been deployed in Monterey Bay and the Gulf of Maine. Development of a second generation ESP, or 2G ESP, was recently completed (Figure 5). The 2G ESP was successfully deployed in Monterey Bay in 2006. To date, the ESP

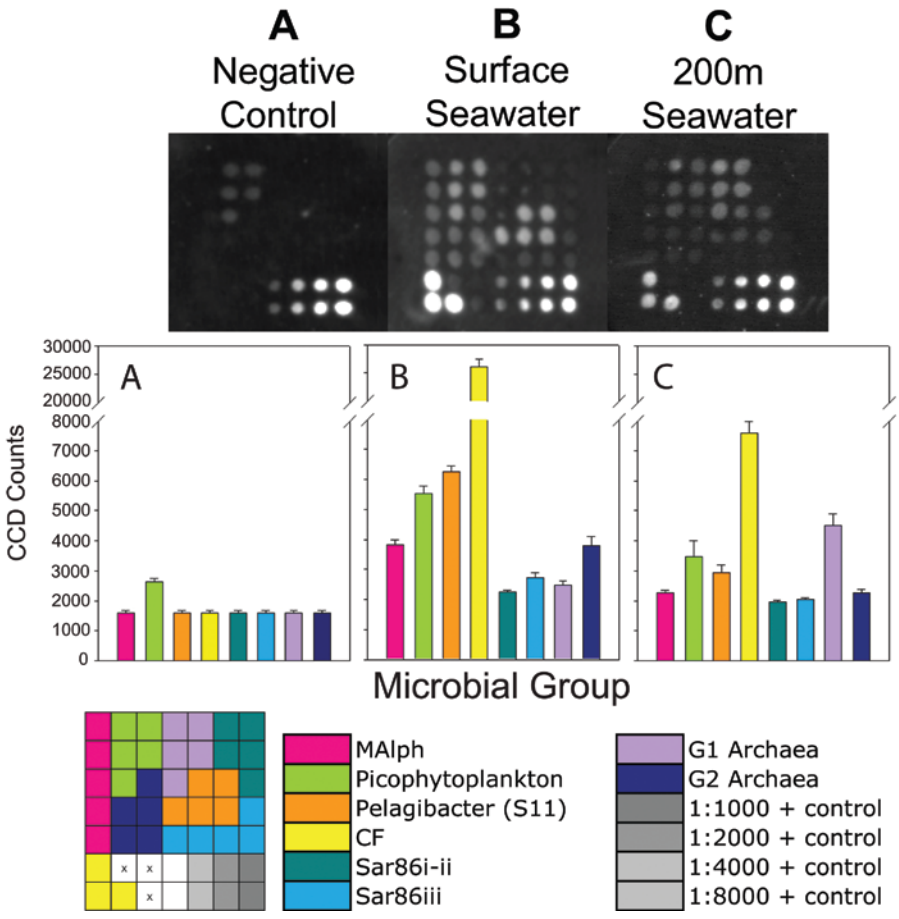

Figure 4. These are 16s rRNA-targeted DNA probe arrays printed with probes for marine microbial groups developed using the ESP supplied with different samples. The bottom panel shows the pattern of probes and an abbreviation of the group targeted. The top panel shows the actual arrays exposed, left to right, to a lysis buffer only, a sample collected near the surface, and a sample collected at 200 m. The arrays demonstrate change in the microbial community as a function of depth, quantified as mean pixel intensity in the middle panel. The actual size of the arrays shown are ~ 15 mm^2. *Figure courtesy of Christina Preston, Monterey Bay Aquarium Research Institute, 2006. After Greenfield et al. (2006)*

Figure 5. The second-generation Environmental Sample Processor (2G ESP) being tested in a seawater tank ahead of deployment in Monterey Bay. The instrument is moored subsurface and an electromechanical cable provides for communications between a remote station and the ESP's surface buoy. An integral conductivity-temperature-depth (CTD) package is visible at left. The ESP operates on 12-volt rechargeable batteries (at bottom, above the anchor). *Photo credit: Todd Walsh, Monterey Bay Aquarium Research Institute*

has automated application of three different classes of DNA probe arrays in single field deployments lasting 20 days, targeting detection of marine planktonic organisms ranging from heterotrophic and photosynthetic bacteria, archaea, and harmful algae to small invertebrates found in the upper ocean (Christina Preston, Monterey Bay Aquarium Research Institute, *pers. comm.*, 2006; Goffredi et al., 2006; Greenfield et al., 2006; Babin et al., 2005). A competitive ELISA (Enzyme-Linked ImmunoSorbent Assay) for the algal biotoxin domoic acid, a neurotoxic amino acid, was also fielded in concert with the probe arrays (Gregory Doucette, NOAA/National Ocean Service, *pers. comm.*, 2006; http://www.mbari.org/microbial/esp/esp_technology.htm). This is the first record of sensing in situ both a harmful algal species and the toxin it produces (an amino acid metabolite) using molecular probe assays.

Autonomous Microbial Genosensor

The AMG (Figure 6) is a microbiological sensing buoy under development by the University of South Florida's College of Marine Science (http://www.marine.usf.edu/systems/?q=amg). The AMG is the first microbiological detection buoy to be designed using nucleic acid sequence-based amplification (NASBA). NASBA is an RNA-based amplification technology that starts with RNA, converts the target RNA into a cDNA by the action of reverse transcriptase, and synthesizes cDNA by the action of T7 RNA polymerase (Compton, 1991; http://www.marine.usf.edu/microbiology/nasba.shtml).

Although the AMG can be tailored to many different microbial targets, the initial configuration is for detection of the Gulf of Mexico red tide organism *Karenia brevis*. The target for amplification is the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*) mRNA. Because mRNA has a relatively rapid turnover time, only transcriptionally active (i.e., viable) cells are targeted. The functional design of the AMG includes a syringe pump for sampling, a series of fluidic valves that direct the sample onto custom-made filtration/extraction columns, a rotating wheel that houses the columns, and motorized injectors that vertically move the columns in and out of a waste stream collection device or into reaction tubes. The purified RNA is injected into reaction tubes in a second rotating wheel that traverses into the reaction module. The NASBA reaction module includes a blue LED excitation lamp, a photomultiplier, and an infrared heater and thermistor. Amplification is measured as an increase in fluorescence versus time, with the potential to provide quantitative data on *K. brevis* abundance. Currently, the AMG is designed to transmit data through a WiFi connection and is battery powered for complete autonomous operation, but it could be just as easily connected to a cabled network system for data transmission and power.

Sustained investment in the development of small, robust, in situ instrumentation is essential to bring to fruition the testing of ideas and models discussed in this special issue.

Figure 6. The Autonomous Microbial Genosensor (left) and pressure vessel (right).

Figure 7. Deployment of the Autonomous Microbial Genosensor in Bayboro Harbor, St. Petersburg, Florida.

CONCLUSIONS AND FUTURE DIRECTIONS

Optical and molecular technologies are the bases for measuring microbes in the ocean, and specialized instruments for in situ applications are improving rapidly. Bulk optical methods provide the framework for assessing temporal and spatial distributions of autotrophic microbes as well as certain key species; in the near future, most optical sensors should be integrated into all types of autonomous platforms. Flow cytometers enumerate and analyze individual cells, and the full limits of this technology have not yet been explored. New electronics, algorithms, and functional stains will yield improved methods for identifying and counting marine microbes as well as providing insight into their roles in ocean ecosystems. As plankton cytometers become more robust and protocols standardized, they will be routinely deployed on moorings, cabled observatories, and ships of opportunity. As devices for concentrating cells from seawater and extracting nucleic acids become smaller and easier to reconfigure for different applications, the ability to sense a diversity of microbes will become widespread. In the long term, instruments designed for in situ use will likely benefit from an ability to apply multiple molecular analytical techniques to a single sample. Novel technologies under development that combine microfluidics with array amplification (Microfluidic digital PCR) hold promise for characterizing the genetic capabilities of single cells (Ottesen et al., 2006).

No doubt much work remains to define the assays that will be deployed in situ and the concomitant, upstream sample collection and processing requirements. Putting all the pieces together from a systems point of view remains a ripe area for future investigation. Sustained investment in the development of small, robust, in situ instrumentation is essential to bring to fruition the testing of ideas and models discussed in this special issue.

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