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REVIEW

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In Situ Phytoplankton Analysis: There's Plenty of Room at the Bottom

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Approximately 70% of the earth is covered with water, within which the most dominant organism is phytoplankton. Not only is phytoplankton at the base of the marine food web, but it also fixes excess carbon dioxide and regulates sulfur on a global scale.[1] Changes in phytoplankton populations have been linked to toxicity to humans and marine life, pollution, and global climate change.[2] Routine monitoring of both fresh and salt water ecosystems has been taking place for many years, with consortia set up explicitly for this purpose. Consensus indicates that five categories of information are useful for early warning systems.[3,4] These are (1) local nutrient concentrations, especially nitrogen and phosphorus, (2) overall chlorophyll concentration, (3) mean phytoplankton density, (4) phytoplankton community analysis, including size distributions, chlorophyll distributions, and density of toxic species, and (5) physical variables, such as water temperature, salinity, pH, and turbidity. Some of this information can be obtained over very large areas and long time intervals by remote monitoring with satellites, aircraft, or balloon. Satellites such as SeaWiFS and MODIS have yielded daily coverage with algorithms developed that focus on harmful algal blooms (HABs) and red tide detection[5,6] as well as chlorophyll concentration.[7] However, current satellites can only independently identify the culprit of blooms that have very distinct optical properties, such as *Trichodesmium*, *Coccolithophores*,[8] or *Karenia brevis*.[9] Most HABs cannot be remotely tracked until the organism has first been identified at the ocean surface.

Many methods have been adapted for the laboratory analysis of phytoplankton including visual microscopy, pigment analysis by high-performance liquid chromatography (HPLC),[10,11] immunochemical and fluorescent staining,[1,12−14] radiolabeling, genetic analysis, spectrophotometry, light scattering, and fluorometry. Traditional methods of study require expensive laboratory settings and time-consuming analysis: samples are obtained and cultured or preserved for later study. However, these methods have a serious bias: many forms of phytoplankton do not preserve well or at all. For example, most picoplankton and many soft bodied dinoflagellates lyse upon preservation. More recently, there has been a trend to move increasingly complex analysis techniques to the site of collection or to perform these analyses in a submersible instrument, many of which are available commercially.[15−17] Monitoring stations, moored platforms, and other fixed installations at the surface can obtain data at regular intervals, although networks are required to obtain even moderate spatial resolution. Discrete bottle samples, especially those taken during cruises on ocean vessels, and towed instruments can cover a reasonable spatial area, but their use is labor intensive and temporal resolution can be difficult to achieve.

In contrast, instruments mounted in autonomous underwater vehicles (AUVs) can provide an adequate level of both spatial and temporal resolution. Although not strictly correct, we include moored vertical profilers[18] in this category; autonomously run instruments in both types of housings can monitor local conditions before, during, and after HABs without prior warning that the bloom will occur. Unlike remote monitoring, submersible instrumentation can usually penetrate below one optical depth, is not affected by cloud cover, and may be sensitive to concentrations of particles lower than the detection limit of current satellites.[8] Data collected by remote monitoring and by instruments mounted in AUVs is complementary. A combined approach can both provide warning of HABs, pollution, or climate change and can generally improve our understanding of marine ecology (Figure 1).

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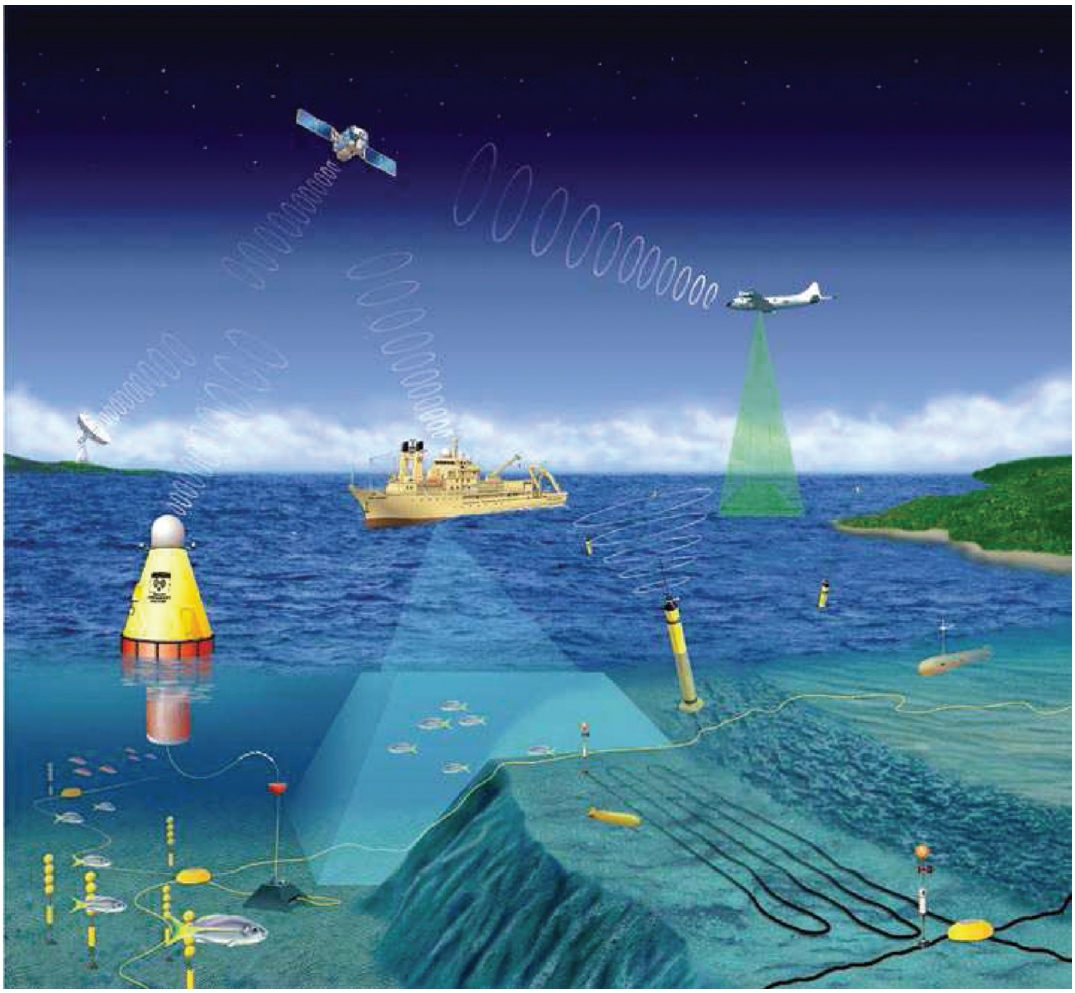

Figure 1. Complementary systems for understanding phytoplankton ecology. The components include cabled observatories, autonomous underwater vehicles, gliders, buoys, moorings, satellites, and a research ship. Reprinted with permission from ref 85. Copyright 2007 The Oceanography Society.

The focus of this review is phytoplankton measurement techniques that have been tested *in situ* (i.e., underwater) or that have potential for transition to autonomous *in situ* operation, particularly in AUVs. Mounting an instrument inside an AUV can be a difficult proposition. Payloads tend to be very small due to geometric constraints, and available power is usually severely restricted. In addition, deployment times can be limited due to maintenance requirements as well as biofouling of the instrument from continuous exposure to the marine environment. At present, virtually all biological data collected by AUV-mounted instruments is restricted to simple observations such as light scatter, imaging, spectroscopy, fluorometry, and advanced laser fluorescence techniques. While true species-level identification is beyond the scope of these techniques (except for imaging), it is possible to obtain information about phytoplankton functional groups, to follow the most abundant members of the ecosystem, or to monitor for blooms or a targeted species.

Not all particles in the marine environment are phytoplankton; many are detritus or heterotrophic species. Understanding how to distinguish phytoplankton from other substances and how to classify phytoplankton in a meaningful way is important to scientists and engineers who wish to develop new methods or instrumentation for *in situ* use. In order to appreciate the information that can be obtained by submersible systems, we focus on two groups of measurement and classification techniques: those that evaluate and classify phytoplankton by morphology and those that characterize and classify phytoplankton by pigment color.

IDENTIFICATION AND CLASSIFICATION OF PHYTOPLANKTON BASED ON MORPHOLOGY

Some theories of marine ecology have suggested that size (and to a limited extent, shape and volume) is a basic unit for the trophic classification of phytoplankton, if not the most important feature.[14] Phytoplankton is traditionally classified into three size groups: picoplankton (0.2−2 μm), nanoplankton (2−20 μm), and microplankton (20−200 μm). Historically, a research vessel may deploy a phytoplankton recorder which collects phytoplankton on a continuously moving filter,[19] although discrete bottle samples are also commonly analyzed. Phytoplankton morphology is subsequently determined through visual microscopy,[20] although this technique is most effective only for nanoplankton and microplankton (Figure 2). Smaller phytoplankton tend to have less intrinsic fluorescence and are difficult to study by standard phase contrast, differential interference contrast, or fluorescence microscopies. While the smaller phytoplankton generally appear in higher population densities (10^2−10^6/mL), larger

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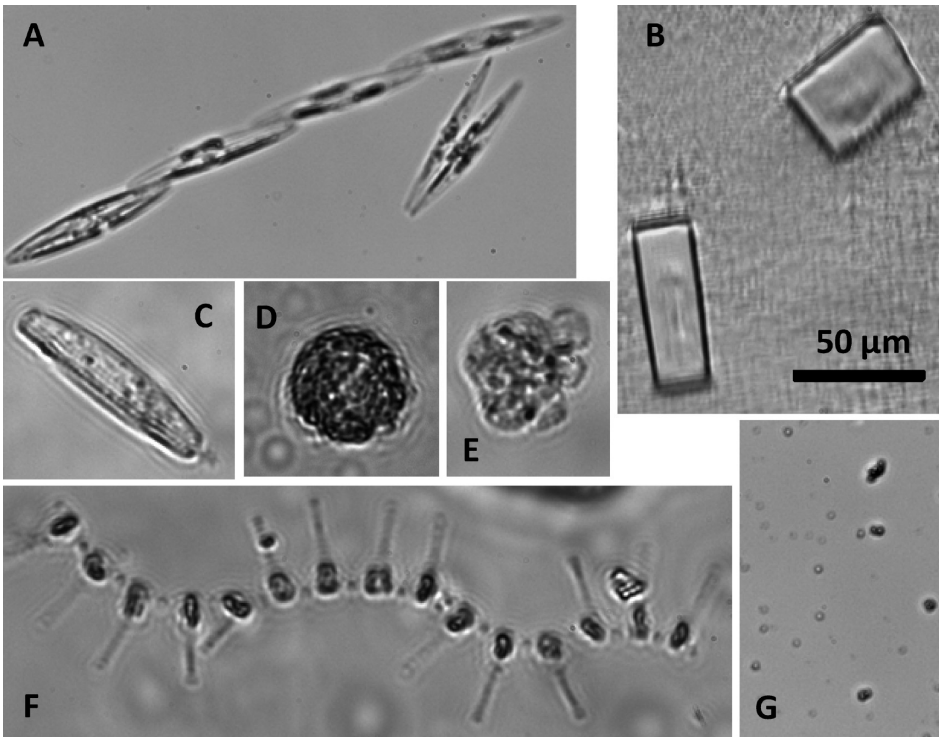

Figure 2. Micrographs of six distinct phytoplankton species showing variations in shape and sizes ranging from ~50 to 0.5 μm: (A) *Pseudonitzschia*, (B) *Thalassiosira pseudonana*, (C) *Nitzschia curvilineata*, (D) *Alexandrium*, (E) *Karenia brevis*, (F) *Asterionellopsis glacialis*, (G) *Synechococcus* sp.

cells are correspondingly more rare, thus requiring cell concentration prior to imaging.[21,22]

Microscopy is cumbersome and time-consuming and is especially difficult to perform on a moving ship. Furthermore, because the use of phytoplankton to understand environmental changes is often based on dominant taxa at the genus level or higher,[23] taxonomic detail at the species level may not be required for monitoring. There are exceptions. With the advent of scanning electron microscopy (SEM), it has become possible to obtain even more detailed visual information about phytoplankton species. Much of phytoplankton ultrastructure that is used for species identification can only be observed with SEM techniques. This is especially true for diatoms. While unnecessary in the vast majority of studies, it is sometimes difficult (if not impossible) to determine if a particular genus is harmful algae without definitive species identification, as provided by SEM. For example, there are many species in the genus *Pseudonitzia* but only a few produce toxins. In this rare case, species identification is critical for detection of HABs.

Light Scattering. In the absence of microscopy, phytoplankton size distributions are analyzed *in situ* using a number of methods, the most common two being imaging instruments and light scattering.[24] Impedance measurements, to a lesser degree, have also been used to estimate volume. Light scattering at a near-forward angle has been correlated with cell volume, although many workers point out that it is also a function of the refractive index of the particle with respect to that of the medium, which changes with species, nutrients, and salinity.[25] Furthermore, many plankton form colonies and chains; automated instruments may be unable to distinguish these larger clusters from individual cells. For larger particles, the intensity of forward scattered light varies erratically with size, and nonspherical particles can be difficult to size using forward scattering. Mie theory describes a framework from which calculations can be made, but the equations are complex in all cases except spherical.[26] There are also limits in most light scattering instrumentation caused by obstruction and obscuration of cells. Light scattering at larger angles is considered "side scattering", which is less sensitive to particle size but more sensitive to shape and ultrastructure (i.e., granularity). Backscattering is sometimes also used when the geometry is advantageous, but the measurements for individual cells are highly affected by pigment concentration.[27,28] Time-of-flight measurements in instruments with flow chambers are used to measure particle lengths, from which volume can be inferred assuming they are spherical.[29] By far, the most accurate sizing is obtained using imaging instruments. While manual visual microscopy allows the greatest accuracy because the operator can account for detritus, buried cells, and clusters, it is also time intensive and therefore provides a small number of data points, giving statistical uncertainty. Newer automated video imaging methods are much less labor intensive, but they may overestimate size distributions due to overlapping objects. Obviously, there is a trade-off in community sizing methods. In addition to size, phytoplankton shape can also be important. Shape information is often expressed in terms of sphericity or an aspect ratio whereby radials are created and compared. More modern approaches have also been used. For example, shape information can be inferred by rapid signal processing of laser pulses such as happens with the Cytosense,[30] a commercially available flow cytometer specialized for oceanographic applications.

A number of commercial light scattering instruments, turbidity meters, transmissometers, and particle sizers exist for *in situ* use, as reviewed by Moore et al.[15] Imaging instruments add another degree of complexity, providing additional shape, colony, and sometimes fluorescence information. Imaging instruments are

complementary to light scattering measurements: they are best suited for microplankton and larger cells and are currently being pushed into the nanoplankton range. Time-of-flight measurements also fall into this size range. Light scattering measurements, on the other hand, are well suited for characterizing picoplankton because most scattering theories require the object being measured to be smaller than the diameter of the interrogation beam.[26] While the main purpose of light scattering instruments is to obtain information about the size of phytoplankton and detritus, it can also provide valuable information about the transmission of sunlight through the ocean as a function of depth due to the correlation of phytoplankton size and light availability.

Imaging. Imaging has been performed for the *in situ* study of phytoplankton since approximately 1980.[16] As technology has moved forward, imaging instruments have been developed that can take pictures of moving cells, can autofocus, and can digitize images with high resolution using modern CCD elements. While video instruments have the potential to provide a powerful amount of information, the vast quantity of images they produce make them labor intensive to use. In an effort to reduce the workload, automated image analysis software is beginning to emerge. However, it is still in its infancy; we expect much work to be done in this field over the next decade.[16,31,32]

A great many submersible video imaging instruments have been used *in situ*.[24] In addition, a few such instruments are commercially available. The FlowCAM and ZooSCAN are examples of commercially available imaging systems. The submersible FlowCAM[33] uses a microscope objective with both a CCD element and fluorescence detectors to obtain images and fluorescence data for particles moving through a flow cell. The instrument can be run in two modes: fluorescence triggered detection and autotriggered mode, where the instrument takes pictures on a regular interval. One thing that makes the instrument unique is its very large depth of field: FlowCAM uses a custom-made element to introduce a controlled amount of spherical aberration into the field of view. This has the effect of degrading the image slightly but increasing the depth of focus. The amount of spherical aberration added optimizes the particle sizing accuracy over a nearly ± 500 μm focal range within the flow. The instrument can perform automated image analyses to determine object sizes and distributions or the user can manually identify and size objects based on the raw images. The FlowCAM also provides information on shape and some fluorescence parameters, and in many respects it behaves like a flow cytometer, although it does not use a core-in-sheath flow. The ZooSCAN[34] uses the flatbed scanner model to obtain images and morphology information about phytoplankton ranging in size from 200 μm to several centimeters. In addition, it has dedicated image processing software. To the best of our knowledge, ZooSCAN has not been incorporated into an AUV, although it has been used on many cruises to examine discrete bottle samples.

Holography. One emerging technique with exciting potential for *in situ* morphological studies is holography (Figure 3). A hologram is an image of the diffraction pattern resulting from the interference between light scattered from particles and the undisturbed part of a coherent and collimated beam (or reference beam). An in-line digital holographic microscopy system is composed of a laser source (providing coherent and collimated light) illuminating a sample volume of particles and a digital camera recording the magnified image of the light field on the other side of the sample volume. Holographic microscopy is advantageous over conventional light microscopy in that the focal plane (or sample volume) is 2−3 orders of magnitude greater. Holography can provide nonintrusive, nondestructive, high-resolution 3-D imaging of objects in their natural environment at a resolution and sample volume size that no other instrument can currently achieve. Because of the ability to optically section the hologram into individual image planes during reconstruction, it allows the extraction of all individual particle characteristics (at the same lateral resolution), their 3-D spatial distribution, and motion (in short pulsed serial holograms).

Obtaining *in situ* information about the unperturbed size and shape of every particle/organism in a sample volume has application to a broad spectrum of science disciplines.[35,36] For example, processes of aggregation, particle interaction, and disaggregation from local shear or turbulence as well as sedimentation could be directly visualized and quantified with *in situ* holography, as a system could both characterize the particles and determine 3-D velocity fields by recording short pulse exposures of particle displacements in flow fields.[37−39] *In situ* holography can also be used to determine the vertical distributions of various organisms and other nonliving particles, along with nearest neighbor distance between all organisms and particles. Malkiel et al.[40] used *in situ* holography to show evidence of harpacticoid copepods clustering around detritus particles, as well as examining copepod-generated feeding flows.[41] There are numerous biological applications that could employ *in situ* holography, including quantifying predator−prey behavioral interactions, micro- and fine-scale biophysical interactions, and spatial distributions, behaviors, and identification of organisms. Sheng et al.[42] used holographic microscopy to reveal prey-induced modification of swimming behaviors in heterotrophic dinoflagellates.

Holographic microscopy systems could be adapted to deployment on a variety of moorings or ocean observing platforms. These types of systems could be used to quantify the temporal evolution of the concentration, size, shape, orientation, and spatial distribution of particles or plankton species as well as assess the impacts of mixing events on particle size structure (particle disruption/aggregation). Real time visualization of *in situ* holographic images could be also be a major tool for adaptive sampling, where structures, organisms, or particles of interest would be visualized by the system and used to guide the location of *in situ* sampling.

■ CHARACTERIZATION AND CLASSIFICATION OF PHYTOPLANKTON BASED ON PIGMENT COLOR

The concept of phytoplankton functional groups is a middle ground stance between the simplistic test for chlorophyll (which indicates the presence of phytoplankton) and complete taxonomic identification at the genus-species level. Functional groups subdivide phytoplankton based on similar physiological traits or adaptations, such as a high affinity for phosphorus or carbon dioxide, skeletal silicon, or a good light antenna. The presence of a dominant functional group in an environment might indicate certain limited nutrients, or the sudden growth of one functional group over another might reflect changing environmental conditions.

Reynold's original functional group description currently includes 31 different groups.[43] However, most of the variables required to make such classifications are not easily measurable by ecologists interested in remote monitoring for HABs. Instead, a classification scheme following the same spirit but using more easily measurable variables is commonly used. Unlike the

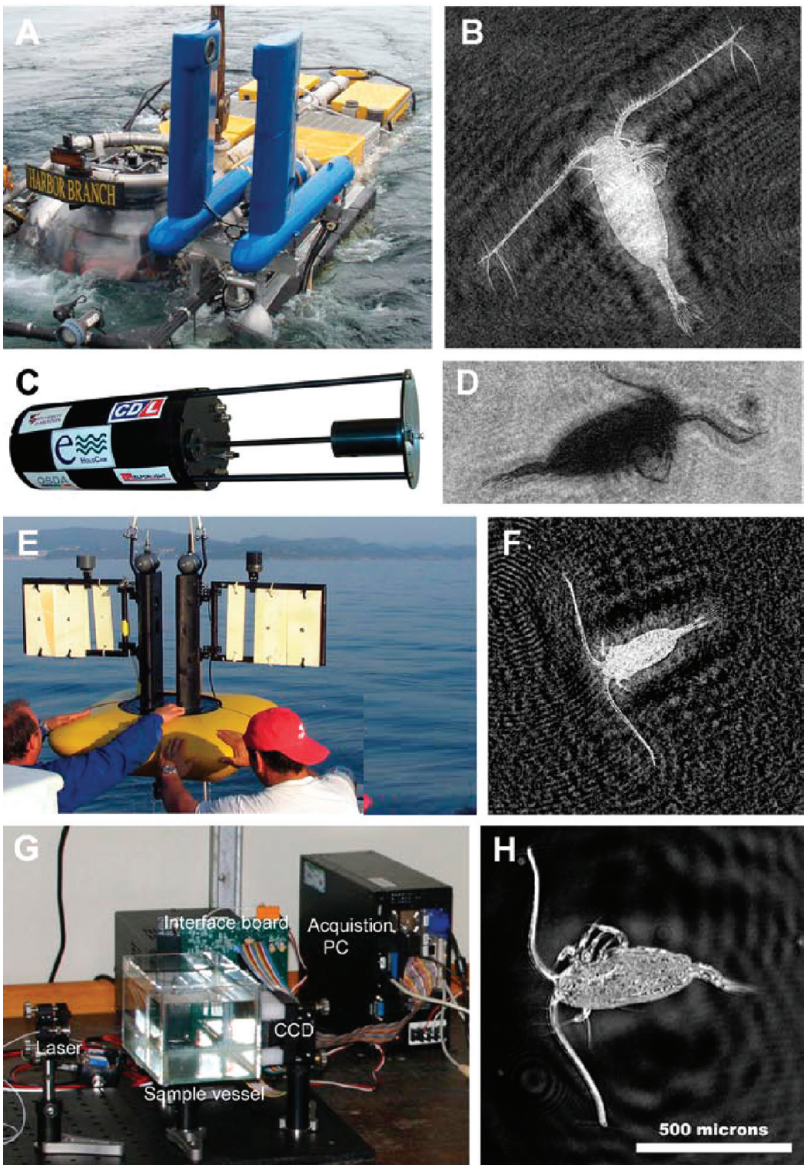

Figure 3. *In situ* holographic imaging systems. Holographic systems offer a means of imaging plankton over a broad range of sizes while preserving their spatial interrelationships. Examples of systems that are operational or under development along with example plankton images include (A,B) the Holocamera. Image: J. Katz, Johns Hopkins University. (C,D) The Holocam. Image: CDL Ltd., Aberdeen and University of Aberdeen. (E,F) The Digital Holosubmersible. Image: E. Malkiel, The Johns Hopkins University. (G,H) The DHI: Digital Holographic Instrument. Images: N. Loomis, MIT, C. Davis, WHOI.[16]

terrestrial environment, competition for sunlight is one of the limiting factors in phytoplankton growth.[44] Because of this evolutionary pressure, different types of phytoplankton have developed unique combinations and amounts of accessory pigments, allowing them to harvest complementary portions of the light spectrum to varying degrees and thus coexist. This differentiation allows phytoplankton families to be characterized by the types and ratios of accessory pigments present. In fact, it is the presence of these accessory pigments that are used in remote sensing algorithms for discrimination of broad groups of phytoplankton by satellite (e.g., dinoflagellates, cyanobacteria).

Phytoplankton Pigments. The primary pigment in phytoplankton, chlorophyll, has absorption maxima in the blue and red parts of the spectrum. However, it is the large number of accessory pigments in phytoplankton that supplement chlorophyll in order to harvest light energy across the visible spectrum that are of interest for classification of phytoplankton into functional groups. From a very simplistic point of view, energy is transferred from these pigments into chlorophyll reaction centers using electron transport. Other substances found in the marine environment are also known to fluoresce, including detritus and colored dissolved organic matter (CDOM),[45] which is operationally defined as organic matter that passes through a filter with a specified size cutoff, usually 0.22 μm. Chlorophyll fluorescence has traditionally been a good indicator of the presence of phytoplankton. However, detritus contains degradation products of chlorophyll and therefore emission spectra can be similar. Particle aggregates, which are often dominated by living phytoplankton that have become tangled up together, can also bias pigment measurements.

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While chlorophyll and other pigments are present in varying amounts in different species of phytoplankton, the amounts found in a population of cells of the same species may also have a high degree of variation. Normalizing the chlorophyll content (measured e.g. by fluorescence) to cell size, mass, or volume will reduce the coefficient of variation. However, more problematic for species identification is the variability of pigment production due to the levels of light exposure or nutrients. Phytoplankton grown in a dark environment have been shown to produce significantly larger amounts of pigment per unit volume than phytoplankton grown in a bright environment.[1,29] Upwelling, turbulence, and currents can mix phytoplankton into different ocean depths, which adds variability to fluorescence data sets. Furthermore, fluorescence measurements suffer from the "package effect", meaning that the pigments involved in photosynthesis are contained within discrete packages (chloroplasts) instead of uniformly distributed throughout the phytoplankton. This causes absorbance values to be smaller than a corresponding uniform solution and introduces errors in the quantification of pigments.[44]

Such effects can be moderated by considering ratios rather than absolute measurements. Various researchers have tried to classify plankton according to ratios such as the chlorophyll accessory pigment ratio or the ratio of pigment to cell volume. These attempts have met with mixed success. The greatest success has been group differentiation exploiting specific accessory pigments such as phycocyanin for bluegreen cyanobacteria or peridinin in dinoflagellates

Spectrophotometry. Spectroscopic absorbance plots can be obtained through hyperspectral measurements in both remote monitoring applications as well as *in situ* instrumentation. The principle is to illuminate a water sample with broadband radiation (the sun, in the case of satellite based measurements) and observe the amount of light it absorbs as a function of wavelength. *In situ* measurements also subtract background due to the medium itself as well as the detritus and dissolved organic substances present in the medium. Because phytoplankton contain different accessory pigments in differing amounts, the resulting absorption spectrum is unique to a particular phytoplankton functional group. Unfortunately, the changes in raw absorption plots are typically small, making them insufficient for the differentiation of phytoplankton. Instead, workers typically compute the fourth derivative of absorption data,[46,47] where accessory pigments are more easily identified.

Various parameters and tests have been developed for the comparison of absorbance spectra, most notably the similarity index[46] and the partial least-squares regression method.[48] The commercial Optical Phytoplankton Discriminator or "Brevebuster" uses this approach to identify HABs composed of *Karenia brevis*.[49] This submersible instrument includes a long flow channel illuminated with a fiber-coupled tungsten/deuterium light source and monitored with a fiber optic spectrometer. Results are compared to calibration data and used to create and report a similarity index to *K. brevis*. Of course, the detection algorithm itself is not unique to *K. brevis*; by introducing other calibration standards, an entire family of instruments could be developed with sensitivities to single or even multiple species of interest.

Fluorometry. Compared to spectrophotometry and HPLC analysis,[10,11] fluorometry provides less detailed information about both the relative and absolute amounts of pigments in phytoplankton. However, it is a fast technique requiring little or no sample preparation and is easily incorporated into *in situ* methods. Commercial instruments utilizing fluorometry continue to emerge to provide information difficult to obtain in other ways.[15] Two complementary methods of phytoplankton fluorescence spectral analysis include fluorescence emission (FEM) and fluorescence excitation (FEX). FEM provides information about pigment fluorescence excited at a fixed wavelength, while FEX relies on measuring the dependence of pigment fluorescence intensity on the excitation wavelength and provides information about the spectral absorption of accessory pigments. Rather than measuring the absorption of light at various wavelengths, however, the FEX method measures the emission of chlorophyll A, typically at 680 nm. An early example of this technique is the chlorophyll accessory pigment ratio, which is a comparison of FEX efficiencies at two different excitation wavelengths, typically around 450 and 530 nm.[25]

The field of fluorometry in marine ecology is moving toward more complex methods in order to obtain additional information from phytoplankton. New techniques include the measurement and interpretation of time-resolved peak information. The idea behind these "pump-and-probe" methods is to saturate the photochemical performance within the photosystem II reaction with an intense pulse of light and then measure the size and shape of the chlorophyll A peak. Maximum quantum efficiency, Fv/Fm, is calculated as the ratio of the total amount of variable fluorescence (Fv) and the maximum fluorescence yield (Fm).[50] In natural phytoplankton populations, Fv/Fm measured in the dark with the photosystem II single turnover protocol may vary in a range of 0.65 for a nutrient enriched environment to nearly zero for nutrient poor or environmentally stressed conditions. In this way, it is possible to obtain information not only about the phytoplankton itself but also the environmental conditions in which it lives. The FAST family of commercial fluorometers is an example of the *in situ* application of pump-and-probe techniques.[51]

A second example of an instrument that uses pump-and-probe methods is the advanced laser fluorometer (ALF).[52] The ALF has incorporated multiple excitation laser wavelengths, broadband hyperspectral detectors, spectral deconvolution algorithms and Raman peak normalization to assess the major water constituents. Although the current ALF instrument has not yet been developed for *in situ* use, it has been used for the shipboard analysis of discrete bottle samples.

A different approach has been taken by Hill et al.,[53] who developed a specialized set of optical filters called multivariate optical elements (MOEs) for phytoplankton identification. The approach uses a broadband lamp as an excitation source but with custom excitation interference filters designed around experimentally measured absorption profiles of specific phytoplankton species over a relatively narrow range of wavelengths. The process begins by studying the single-cell fluorescence spectra of a number of individual phytoplankton species through the use of a fluorometer equipped with an optical trap.[54] Typically, up to 100 individual cells from each particular culture are scanned in order to obtain meaningful average spectra over the natural range of variability within each culture. A principle component analysis is performed on the spectra to help identify the best linear discriminants, which are realized by fabricating custom MOEs to precisely control the spectra of excitation light to which the phytoplankton will be exposed, typically by specifying the exposure intensity over the range of 570−610 nm.[55] By incorporation of sets of two or more of these excitation filters and observing the intensity of chlorophyll fluorescence at 680 nm in a

Figure 4. The left photo depicts a CytoSense benchtop scanning flow cytometer which is transformed into a CytoBuoy for moored operation. The right photo shows researchers lowering the FlowCytobot onto the WHOI research vessel Mytilus for deployment at the Martha's Vineyard Coastal Observatory (Photo courtesy Tom Kleindinst).[87,88]

custom flow-through instrument, discrimination of several kinds of phytoplankton has been demonstrated. Currently, this instrument is being further developed for *in situ* studies in the open ocean.

■ ANALYSIS OF INDIVIDUAL PHYTOPLANKTON CELLS AND COLONIES

Video imaging and holography are capable of providing *in situ* information for individual phytoplankton cells, clusters, and colonies. However, until automated image analysis techniques are greatly improved, the interpretation of data collected by those methods is extremely labor intensive. In contrast, two techniques have emerged that can classify individual members of a bulk population of phytoplankton *in situ* without the requirement of visual identification.

Flow Cytometry. The advantage of using flow cytometry for the study of marine ecology is the ability to perform analysis on single particles, rather than measuring a population average. A typical flow cytometer combines light scattering measurements with fluorometry, but some flow cytometers also include imaging or sorting/cell capture capabilities.[56] Occasionally, other advanced measurements are performed, such as polarization scattering measurements, time-of-flight measurements, or pulse shape analysis.[57] Several excellent reviews of flow cytometry exist.[58,59]

In flow cytometry, cells are pushed through a nozzle and into a sheath fluid at a high velocity, so that they pass through the interrogation region in single-file. Statistics can be quickly obtained on a large number of cells. Flow cytometry requires that the particles to be interrogated are smaller than the nozzle diameter (for a typical nozzle, particles should be <30−50 μm) and is most efficient when the particle concentrations are very high (on the order of 10^4−10^7 objects/mL) due to the very low sample volumetric flow rate (typically 10−50 μL/min). This makes most commercially available flow cytometers perfectly suited for the study of picoplankton. In contrast, the use of traditional flow cytometry on larger and rarer species like nanoplankton and microplankton is difficult and has not found widespread acceptance. Furthermore, working with phytoplankton introduces a number of other issues: seawater can result in instrument biofouling, and nonspherical phytoplankton may take a number of orientations in flow, causing a loss of resolution in the data. The very nature of creating core-in-sheath flow introduces shear forces, which can break apart fragile colonial phytoplankton and aggregates, introducing a bias in the measurement. Finally, because natural samples of phytoplankton come in such a wide variety of sizes and shapes, it can be difficult to distinguish between single objects and clusters, or to recognize clumps of cells that include detritus or other inclusions. This can lead to sampling biases. For the interested reader, Rutten and co-workers thoroughly compare the use of flow cytometry to traditional microscopy techniques in phytoplankton monitoring.[4]

Despite its difficulties, the number of studies using flow cytometry over the last few decades has increased rapidly, and the technique has been responsible for a stunning discovery: a new species of phytoplankton, *Prochlorococcus*, was detected using flow cytometry.[60] Interestingly, *Prochlorococcus* is regarded as both the smallest photosynthetic organism as well as among the most abundant. This made the discovery even more remarkable; *Prochlorococcus* had completely eluded previous detection by visual microscopy (or any other method). The autofluorescence intensity of this species is especially dim, and exposures to light long enough to detect it under a microscope were sufficient to either completely photobleach or even lyse the cells.

Flow cytometers are in use onboard ships, and submersible versions have been developed. Perhaps the biggest advantage of performing cytometry for phytoplankton analysis is that it removes the need to preserve the sample and provides data on a rapid time frame. In the case of submersible autonomous instruments, data may be obtained over a wide swath of space and time. Two of the challenges in flow cytometry instrumentation that have emerged are (1) the development of instruments suitable to study an expanded range of phytoplankton sizes and (2) the development of autonomous instruments for *in situ* (underwater) operation over extended periods of time, especially on AUVs. To address these challenges, one must either modify an existing commercial instrument[61] or else custom-build a cytometer[62,63] (Figure 4). Most researchers seem to prefer the former method, although one commercial line of flow cytometers is now available that is specialized for oceanographic studies.[30]

The first family of flow cytometers based on a custom device is called the Optical Plankton Analyzer (OPA), first introduced in 1989.[62,63] The stated goal of this instrument was to analyze

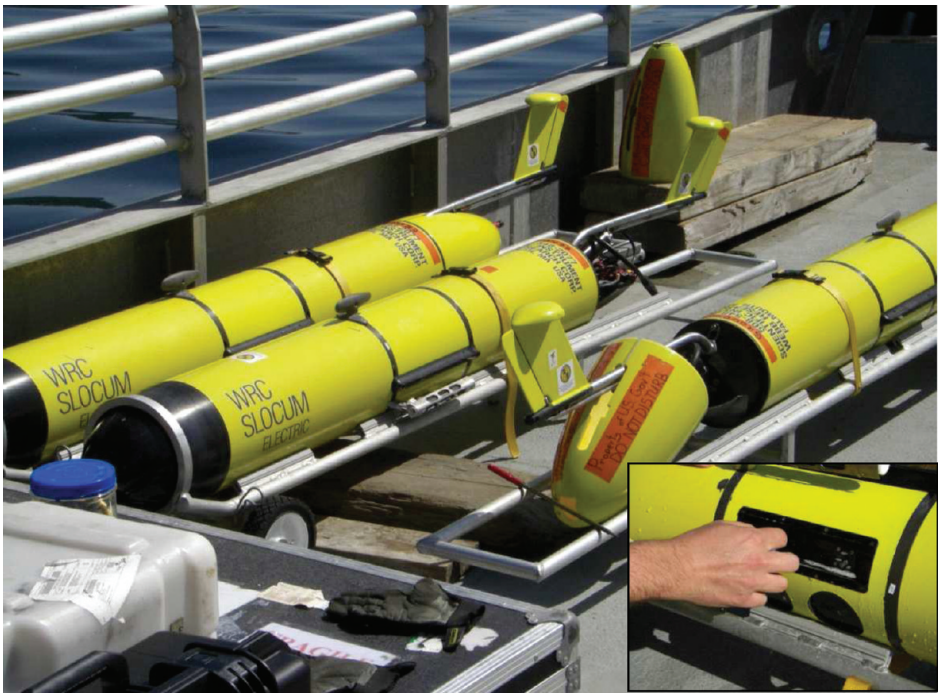

Figure 5. Example of an AUV for deployment of miniaturized analytical instruments. The Slocum Glider (Teledyne Webb Research, Inc.) has been deployed with instruments for measurements of bulk scatter and fluorescence. The NRL Microflow Cytometer is being redesigned to fit into the limited space of the instrument compartment (inset).[89]

phytoplankton over a wide variety of sizes, from approximately 1 to 1000 μm swept diameters; thus, the authors developed a new flow cell with relatively large-diameter constriction points and very high flow rates. They subsequently optimized the optics around the new geometry. Various improvements have resulted in an entire line of instruments with different capabilities, some of which are now commercially available. These include the European Optical Plankton Analyzer (EurOPA)[64] and the CytoSense family of cytometers.[65] In order to become rugged enough for extended field use, the CytoSense has incorporated an interesting set of technologies including a holographic diffraction grating to replace traditional dichroic mirrors and a multipixel hybrid photodiode to replace the traditional photomultiplier tube. Additional features that have been used in this instrument family include diffracted scattered light analysis, imaging in flow, and peak shape analysis routines.

A second notable family of instruments, the Flow CytoBot,[66,67] was developed starting in 2003 at Woods Hole Oceanographic Institute (WHOI) based on the goals of collecting *in situ* data autonomously over very long periods of time, and later, adding image identification in 2007.[68,69] These instruments differ from the OPA family in that their submersible and automated versions are semipermanent installations, with cables for power supply and data transmission. In contrast, the OPA family (CytoBuoy and CytoSub) use batteries and, in the case of CytoBuoy, radio communications. However, the Flow Cytobot family of instruments is designed to obtain samples regularly over very long-term operation, measured in years as opposed to weeks. A commercial version is currently under development with a much smaller footprint and lower power requirements. The flow Cytobot has been used on both short- and long-time cruises, and in a recent study it was responsible for identifying the first harmful *Dinophysis* bloom in the Gulf of Mexico.[70] Though the authors were looking for *Karenia brevis*, not *Dinophysis*, they were able to not only identify the species but also provide early warning shortly before an annual oyster festival that attracts up to 30 000 people. Because *Dinophysis* is known to cause diarrhetic shellfish poisoning, the oysters were recalled in time to prevent disaster.

While both cytometers described above are routinely used for *in situ* studies, to our knowledge neither has (yet) been mounted inside of an AUV. This type of project presents special challenges. Extended deployment is required, typically weeks, during which the instrument must run autonomously. The overall volume of the housing tends to be very small, and the amount of power available to the instrument is severely constrained. For example, mounting a cytometer in the science bay of a Slocum Glider (Figure 5) would require an instrument with a volume of no more than a few liters, irregular shape (the glider has support members going right through the center of the bay), and operation on approximately 20 W of power. Onboard thresholding, processing, and storage of data must take place within the instrument, as radio communication in real time is not always reliable or feasible. Fragile components must be made sufficiently rugged; instruments must be made insensitive to shock, vibration, humidity, and movement, sometimes during the course of a measurement. Finally, instrument drift issues must be addressed, including those due to the (sometimes very significant) differences between the temperature of the water in which the instrument operates and the laboratory in which it was assembled. The regular use of calibration standards, monitoring, and controlling fluctuations in the light sources, fine-tuning or otherwise adjusting the optical components, and keeping the flow cell clean due to natural biofouling issues are viable strategies that have been employed.

Several laboratories are trying to facilitate the deployment of flow cytometers on AUVs by developing miniature devices based

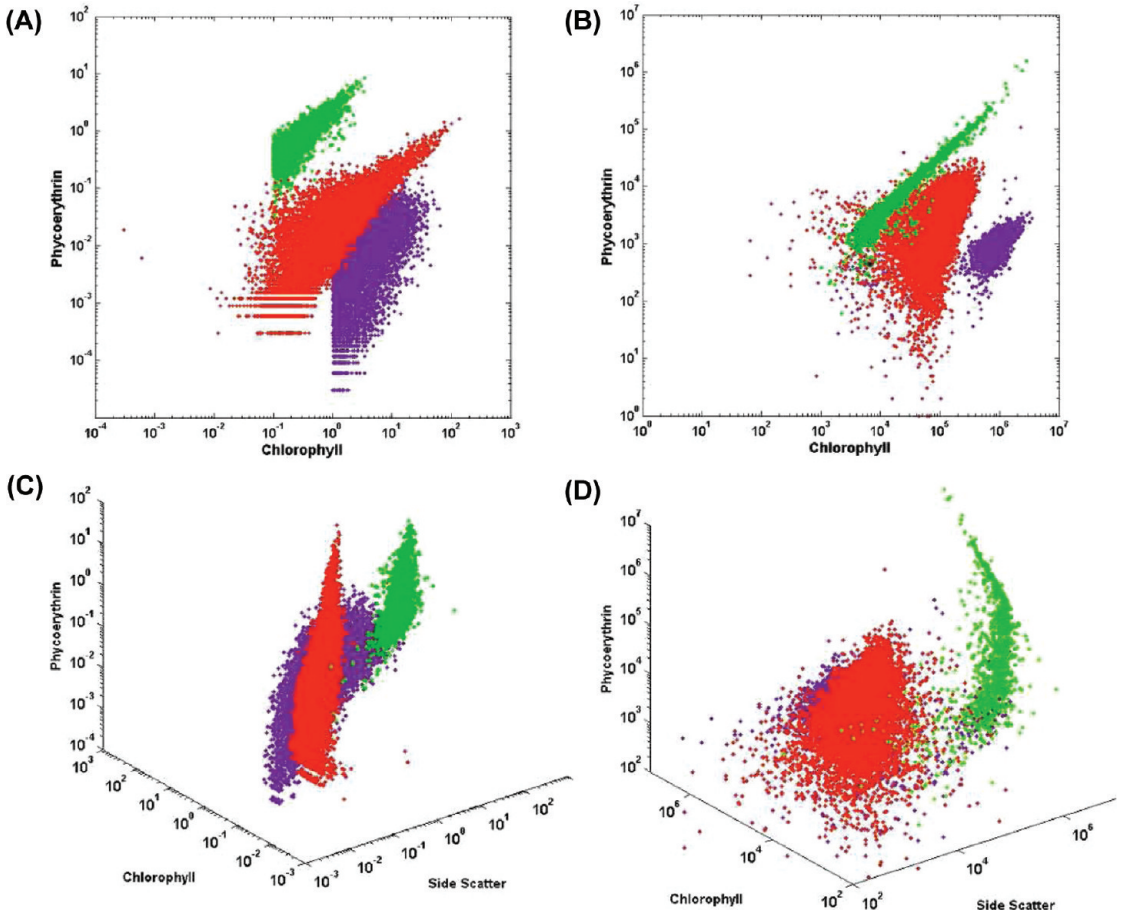

Figure 6. Microflow cytometry for discriminating phytoplankton populations. Figures in the left column show data obtained using the NRL Microflow Cytometer while scatter plots in the right column were obtained using the commercial Accuri flow cytometer. Phycoerythrin (*y*-axis) intensities plotted vs chlorophyll fluorescence (*x*-axis) for each individual cell are shown in parts A and B. Parts C and D depict 3D scatter plots of chlorophyll fluorescence, phycoerythrin fluorescence, and side scatter. *Synechococcus sp.* (0.5−1.0 μm in diameter) is represented by red dots, *Nitzschia d.* (16−80 μm in length and 2−5 μm in width) by green dots, and *Thalassiosira p.* (4−29 μm in width) by purple dots. Reprinted from ref 75, Copyright 2011.

on microfluidic chip technology. The first generation of these microflow cytometers has been tested in the laboratory for the ability to analyze phytoplankton or its products. A briefcase-sized system with two-color fluorescence capability was used to measure reduced esterase activity in a single species of microplankton passing through a 28 μm × 21 μm microfluidic channel as an indicator of Cu^{2+} toxicity.[71] The NRL Microflow Cytometer includes a microfluidic chip with integrated optical fibers to excite cells with two excitation wavelengths and collect both 90° light scatter and up to three colors of fluorescence.[72,73] Because the sample is sheathed on all four sides, channel dimensions can be maintained at 390 μm × 130 μm while the sample stream is focused to approximately 40 μm in diameter. The ability to discriminate different populations of phytoplankton was demonstrated using two different excitation schemes (488 or 532/635 nm); cells ranging in size from 0.5 μm (*Synecococcoccus*) to 80 μm were analyzed[74,75] (Figure 6). The same microflow cytometer was also fabricated using hard plastic instead of soft polydimethyl methacrylate (PDMS)[76] and modified for sheath fluid recycling.[77] Investigators at the University of Southhampton have taken a different approach to a microflow cytometer, combining optical analysis of intrinsic fluorescence excited at 532 or 633 nm with impedance measurements for size determination.[78] The channel was 11 μm × 20 μm in PDMS. Three different species of phytoplankton, 0.5−7.0 μm in size, could be distinguished by fluorescence, but the impedance measurements failed to size particles less than 2 μm (*Synecococcus*). In order to deploy microflow cytometers such as these on submersible platforms, especially autonomous vehicles, the space and power required by the optics must be reduced, the channel dimensions configured to resist clogging and accommodate the maximum particle size range, and fluidics materials selected for operation at subsurface pressures.

Molecular Identification and Genomics. Possibly the most rapidly developing area of phytoplankton analysis involves molecular recognition using antibodies or oligonucleotide probes. Staining of concentrated phytoplankton with antibodies or oligonucleotide probes may then be used to identify phytoplankton species in whole cell assays using either microscopy[21] or flow cytometry. Alternatively, the rRNA molecules in the cytoplasm or the nuclear DNA can be extracted and amplified using a polymerase for identification. It is important to remember that antibody and oligonucleotide probes are created and validated using laboratory samples of phytoplankon and are usually limited to species that have been cultured. Over the past few years, metagenomic analyses of complex populations of uncultured cells has begun to reveal a much greater diversity of species.[79,80] The focus of these analyses is to understand the function of complex populations at the molecular level and to monitor ecological and evolutionary processes.

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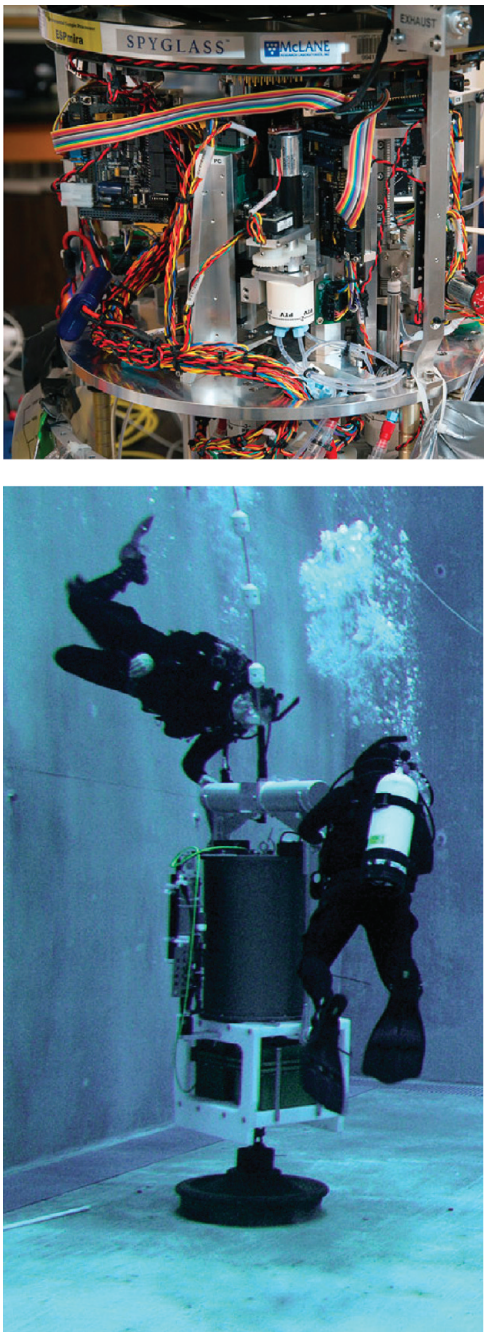

Figure 7. Environmental Sample processor (ESP) for *in situ* genetic analysis. The ESP was first developed at the Monterey Bay Aquarium Research Institute, which deployed it on piers and buoys at sea. The photo (Todd Walsh, Copyright 2006 MBARI) depicts initial tank testing. A new version (upper panel) is currently being constructed at the Woods Hole Oceanographic Institution in collaboration with McLane Research Laboratories for deployment in 2011. (Photo by Tom Kleindinst). A history of this project is available.[90]

Two instruments have been developed for the automated *in situ* use of molecular probes (Figure 7). The Environmental Sample Processor (ESP)[81] collects discrete water samples *in situ* and automates the application of rRNA targeted DNA probes for near real-time detection of target sequences. It uses "pucks", which are custom-designed reaction chambers that are stored in a rotating carousel, moved to separate processing stations as needed, and sealed with a clamp to connect to fluidic manifolds. The ESP has demonstrated the detection of *Pseudonitzchia australis*[82] both through an automated sandwich hybridization assay as well as archival and storage of samples on a filter for a later fluorescence *in situ* hybridization assay performed in a laboratory. In addition, the ESP can perform competitive enzyme-linked immunosorbent assays for shellfish toxins such as domoic acid.[83] A second instrument, the Autonomous Microbial Genosensor (AMG)[84] is still under development at the University of South Florida. This instrument is designed to perform several autonomous assays underwater over a period of 3 days using real-time nucleic acid sequence based amplification with *K. brevis* as a specific target.[85]

FUTURE DIRECTIONS

Understanding changes in phytoplankton populations can provide real-time information about changes in climate or ocean currents, marine diversity, and pollution. Observations from space reveal the geographic scope of widespread distributions and are useful for evaluating long-term trends, but they do not provide either depth-related information or population analyses. Cell analyses provide information essential to interpret the large-scale data and evaluate events at different depths. Laboratory analyses not only look at the function of individual cell types if they can be cultured or assess their morphology by microscopy but, via modern genomic approaches, have begun to provide information on functions of multispecies populations and to identify new species refractory to culture.[86] *In situ* analyses, especially imaging and flow cytometry, can provide species identification and a means to monitor changes in functional phytoplankton populations, respectively. Moving these analyses to AUVs will provide real-time data at the single-cell level over a range of depths and geographic distributions. The continued miniaturization of optics, electronics, and fluidic systems should expedite this process. Although it is not quite what Richard Feynman meant, in the field of underwater phytoplankton analysis it is still true that "there's plenty of room at the bottom."

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BIOGRAPHIES

Dr. Jeffrey Erickson received his Ph.D. (2004) in Chemical and Biomolecular Engineering from the Johns Hopkins University. He accepted a postdoctoral position and later became a federal employee at NRL in Washington, DC. His current research focus is on the development, integration, and testing of biosensor systems, especially those involving microfluidics or nanotechnology.

Dr. Nastaran Hashemi received her Ph.D. (2008) from Virginia Tech in Mechanical Engineering, with studies focused on nonlinear dynamics in atomic force microscopy. For the last 3 years, she has been working as a postdoctoral fellow at NRL with a focus on the application of microfluidic flow cytometry for the identification of phytoplankton. Dr. Hashemi has joined the faculty at Iowa State University.

Dr. James M. Sullivan received his M.S. (1994) and Ph.D. (2000) from the University of Rhode Island, Graduate School of Oceanography (URI-GSO) with concentrated studies on phytoplankton physiology, optics, and biophysics. He accepted a research faculty position at URI-GSO and has recently started

working as a research scientist for WET Laboratories Inc. His current research focus is on ocean optics and sources of optical backscattering, understanding the biological and physical mechanisms that control the spatial-temporal distribution of optical fine-structure and phytoplankton populations in coastal oceans, and oceanographic instrument development (including *in situ* holographic devices and autonomous sampling platforms).

Dr. Alan Weidemann got his Ph.D. in biology from the University of Rochester, NY, in 1985 investigating optical properties associated with phytoplankton blooms in Irondequoit Bay. He then went on to NORDA/NRL where he has spent the last 25 years studying ocean optical properties and characterizing the key absorption and scattering components of the ocean environment. His research deals with identifying the critical factors, such as thin layers of phytoplankton or optical turbulence that degrade image quality for Navy underwater and airborne electro-optical systems.

Dr. Frances Ligler is the Navy's Senior Scientist for Biosensors and Biomaterials and a member and past chair of the Bioengineering Section of the National Academy of Engineering. She earned a B.S. from Furman University and both a D.Phil. and a D.Sc. from Oxford University. Currently working in the fields of biosensors and microfluidics, she has also performed research in biochemistry, immunology, and proteomics. She has over 350 full-length publications and patents, which have led to 11 commercial biosensor products and have been cited over 7000 times.

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