CYTOMETRY
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Monitoring Microbial Communities in the Marine Environment

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A healthy environment on land depends on a healthy ocean. The condition of the ocean is largely determined by the activities of microscopic plankton cells that harvest the energy that supports the food chain. Microbes turn CO_2, H_2O, and nutrients into organic compounds. They dissolve, transform, and deposit large amounts of minerals. They control the composition of the atmosphere and influence climate on a global scale. If microbes change their activities and cycles, life on land will experience the consequences.

The oceans are increasingly affected by human activity. The planet-warming effect of green-house gases is generally recognized, but the long-term effect of society's waste may be even more significant. Nearly indestructible (and indigestible) man-made materials are accumulating at a rapid pace (1). It is estimated that the amount of plastic increases by 275 million metric tons per year (2). A significant portion of this material ends up at sea. In the near future, plastic debris is expected to be more abundant than fish (3). Chemical waste, fertilizer, and other industrial compounds leach into our rivers and are flushed into the oceans. Pesticides, drugs, hormone-mimicking substances that affect various life forms, are being detected at biological effect levels (4). The influence of individual factors may be uncertain, but there is no question that at least some of these changes, if not most, will have long-lasting effects. To make sensible decisions, we need to understand what these effects may be. We need to understand how they might affect the balance of life in the oceans. We need to monitor the state of the microbial communities in the ocean to detect shifts in the equilibrium in a timely manner.

Our understanding of the ways in which life forms may fare in a changing milieu is fragmentary at best. Most analyses of the marine environment rely on bulk observations such as: integrated chlorophyll fluorescence, uptake rates and metabolism of radiolabeled compounds, the meta-genome at a certain place and time. Among the technologies that may be used to measure marine microbial parameters, flow cytometry holds a unique position. Instead of measuring overall activity, flow cytometry may be used to identify, count, and study the individual constituents of a microbial network. By building a picture of a community from the base up, flow cytometry may provide a unique perspective on the composition and interactions within microbial systems.

The void in our knowledge of marine microbes is illustrated by recent discoveries about the distribution of marine cyanobacteria throughout the oceans. Two representatives of this group, *Synechococcus* and *Prochlorococcus,* can be identified by flow cytometry. *Synechococcus*, first described in 1979 (5,6), is detectable as micron-sized particles with a characteristic orange fluorescence in marine waters all over the planet. The even smaller, *Prochlorococcus*, which has a very dim chlorophyll fluorescence, was only discovered in the late 1980's (7). Before the discovery of *Prochlorococcus*, the productivity of the oligotrophic tropical oceans, which cover more than half of our planet, was considered of secondary importance. It was not until investigators took flow cytometers to sea and observed large populations of these tiny, fluorescent particles over large areas and across considerable depths, that the significance of *Prochlorococcus* became evident (8–10). It is estimated that there are 3×10^{27} *Prochlorococcus* cells in the oceans (11). The

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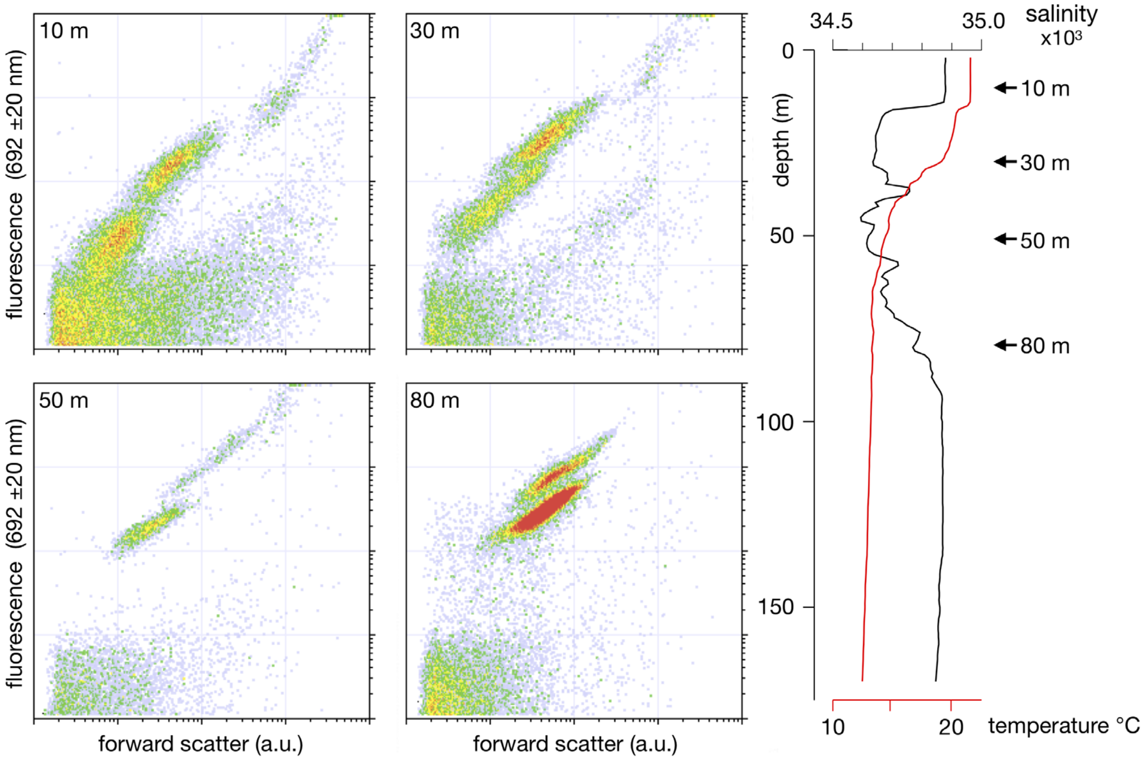

Figure 1. Four plankton samples from different depths over the Atacama Trench (Atacamex, Universidad de Concepción, February, 2018). The panels display chlorophyll fluorescence (692 ± 20 nm) versus low-angle forward scatter; all excited by 488 nm. The traces show the salinity and temperature gradients.

photosynthetic activity of *Prochlorococcus* alone is thought to exceed that of all plants on land and this species may produce, as much as 1 of every 3 oxygen molecules we breath (12,13). Moreover, *Prochlorococcus* turns over at a rapid pace. While maintaining constant population numbers, the cells appear to divide on a daily basis, implying that every day half of the biomass contained in *Prochlorococcus* is recycled or enters into the food chain. Because of their predominance, the dynamics of cyanobacteria are a crucial factor in the quality of our environment. Small changes in turnover can be expected to cause large shifts in the cycles that control the steady state of our environment and atmosphere.

The billions of years of evolution that produced *Prochlorococcus* and *Synechococcus* have led to a large genetic heterogeneity. DNA-sequence analysis reveals thousands of genetic variants differing in genome size, gene composition, and variance in gene expression (14,15). Although the genetic heterogeneity is suspected to be linked to ecotype specialization (16), most hypotheses about the role of genetic variants in the environment and the food web remain to be tested. Little is known about the environmental factors that drive genetic adaptation. The effect of anthropogenic changes in the environment on *Prochlorococcus* and *Synechococcus* and their ecotypes cannot be predicted. An indication of ecotype specialization may be gleaned from the distribution of cyanobacteria in water layers at different locations. At eutrophic sites, where both *Prochlorococcus* and *Synechococcus* are abundant, *Synechococcus* dominates in the surface layers and *Prochlorococcus* is found in the deeper, darker waters. In the well-lit oligotrophic ocean, *Prochorococcus* is numerically dominant at all depths and can be found down to a depth of 150 m or more, whereas *Synechococcus* is present at low numbers in shallower waters (17,18). Comprehensive studies of the distribution of the various genotypes in different marine environments will give clues about the interplay of microbial ecotypes and the physical and chemical environment.

The discovery of *Prochlorococcus and Synechococcus*, long overlooked in microscopic studies and now considered to be among the most important primary producers, illustrates the value of flow cytometry in describing environmental communities. Flow cytometry registers particles with a refractive index that differs from that of the surrounding medium. Because DNA has a high refractive index, living cells, including most viruses and many non-living detrital particles, can be detected by light scatter, provided the instruments are sensitive enough. Many organisms contain characteristic chromophores that can be made to fluoresce (19). In addition, there exists a range of fluorescent reagents that can be used to quantitatively test for specific biochemical components or physiological functions. Thus multi-color, multi-angle-scatter measurements (FCM phenotypes) may provide detailed, quantitative information about every particle in a given volume of ocean water.

Although rich in detail, the accurate optical signatures (flow phenotypes or FCM phenotypes) generated by multiparameter flow cytometers are of limited value without a

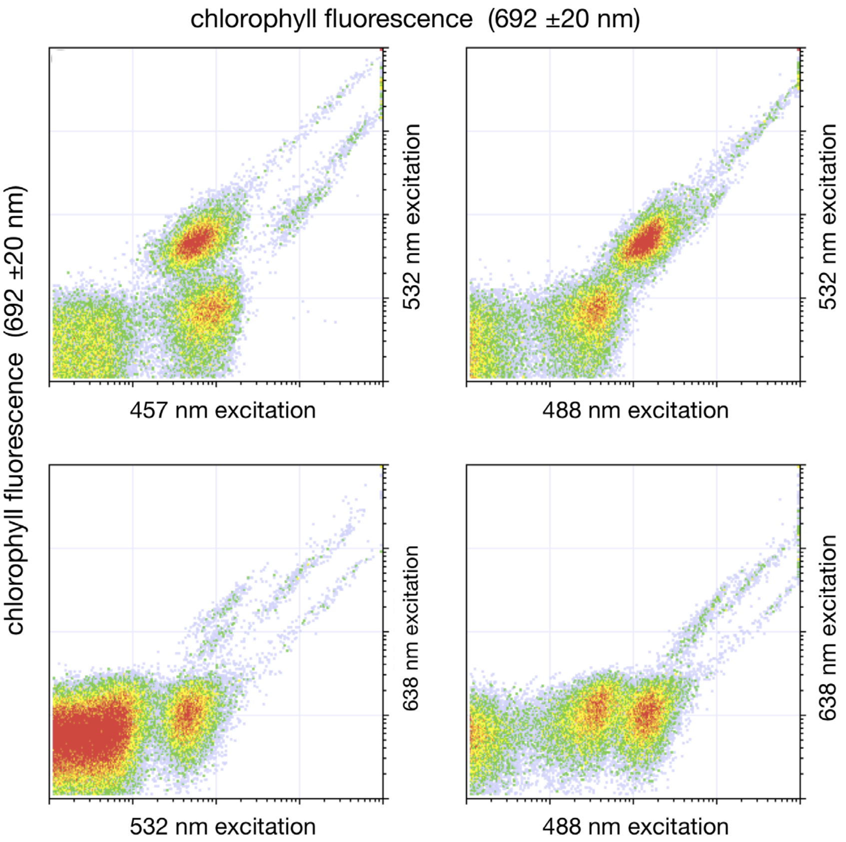

Figure 2. Four different representations of a plankton sample collected at a depth of 20 m over the Atacama Trench on January 29, 2018. All axes display chlorophyll fluorescence (692 ± 20 nm). Depending on the excitation wavelength (457, 488, 532, or 638 nm), the panels show different arrangements of clusters indicating that chlorophyll fluorescence is the result of light absorbed and transferred by accessory pigments.

proper functional or morphological interpretation. The plankton world is mostly known through microscopic images, metabolic activity, and increasingly, by DNA sequence and transcription products. The quantitative but abstract optical measurements of flow cytometry are not easily correlated with these established biological properties. The challenge is to combine environmental parameters and biological components with the optical signature of individual particles into a single, coherent model. For flow ctyometry to become relevant for environmental studies, the field first needs to develop the necessary rules and tools that pertain to plankton analysis.

Few flow cytometers have been optimized for plankton analysis. The majority of current instruments and software has been developed in hematological research. The promise and shortcomings of biomedical research instruments for plankton analysis may be illustrated by two examples.

The first example considers plankton flow phenotypes of samples from different depths of the Pacific Ocean over the Atacama Trench (Atacamex expedition, Universidad de Concepción, February 2018). The water layers at this site are brought in by currents that originate at different areas of the Southern Hemisphere creating different physical environments along the depth profile. The panels of Figure 1 display the red fluorescence (emission 692 nm, presumably chlorophyll) against the low-angle light scatter of particles that are illuminated with a 488-nm laser beam. The character of the dot plots varies along the depth gradient. The variation in population clusters reflects differences in the microbial communities that thrive at different temperature, salinity, and nutrient conditions. What are those populations? How can we interpret the measurements? The common interpretation of such dot plots, established and validated in hematological research, is that scatter is proportional to particle size (more is bigger) and that the 692 nm fluorescence is proportional to chlorophyll content. This simple linear interpretation, that may be valid for stained lymphocytes, however does not apply to plankton samples.

Blood cells are several times larger than the wavelength of light. The majority of plankton particles is about the wavelength of light or smaller. Wavelength-sized particles interact with light in fundamentally different ways than particles that are several times larger (20). Scatter by very small particles is subject to angle-dependent constructive and destructive interference. Consequently, very small changes in a particles' shape or the angle of observation can cause large jumps in signal intensity. The scatter of large, spherical particles, like lymphocytes, can be expected to increase smoothly and monotonically with their cross sectional area. One cannot expect a similar continuous scatter-size relationship for the much smaller and often non-spherical microbial particles.

The relationship between fluorescence and chromophore content of the phototrophs is equally uncertain. Photosynthetic organisms must utilize light over a broad spectral range and for this purpose have evolved complex systems in which multiple chromophores cooperatively channel energy into the molecular pathways that convert photons into chemical energy. The chlorophyll fluorescence that is observed reflects the transfer of energy within the photosynthetic antennas rather than the excitation/emission cycle of a single molecule (21). Figure 2 demonstrates that in microbes, chlorophyll fluorescence cannot simply be proportional to chlorophyll content. All axes in the four panels display chlorophyll fluorescence intensity at 692 nm. The difference between the panels is that the fluorescence is induced with different colors of excitation light. If chlorophyll fluorescence is a simple proportional metric of chlorophyll content, all axes should be correlated and the plots should present straight lines. Instead, the panels show well-separated clusters whose relative position varies with the excitation wavelengths (457, 488, 535, and 645 nm). Fluorescence measured in traditional flow cytometry applications (carefully designed to yield a proportional reagent to analyte relationship) can be considered to represent dye content. In the case of sub-micrometer photosynthetic organisms, we need more sophisticated models and methods to arrive at appropriate size and pigment estimates. Because of lack of a suitable theory, flow cytometry should be considered a descriptive tool that shows that plankton populations are different at different sites. But such results lack the quantitative and analytical quality that allows one to interpret the observed differences in a meaningful way. The empirically observed differences cannot be translated in terms that make sense from an environmental taxonomic perspective.

The interpretation of flow cytometry results is further complicated as living organisms can modulate their phenotype (i.e., pigment content) to match environmental conditions. Clusters in a dot plot collected at one depth may not correspond to clusters with the same coordinates in other samples. An example of the extreme plasticity of *Prochlorococcus* is given in Figure 3 (19). In the oligotrophic ocean north of the Hawaiian Islands, *Prochlorococcus* is the numerically dominant photosynthetic organism (98% of fluorescent particles). Within the upper 100 m, the distribution of *Prochlorococcus* DNA ecotypes is relatively constant (23). Yet, chlorophyll fluorescence and low-angle scatter measurements show a steady increase as one descends down the water column. *Prochlorococcus* fluorescence intensity at a depth of 100 m is about 80-fold brighter than that at the surface (Fig. 3). Layers separated by as little as 5 m, may show significant differences in fluorescence. A similar relationship is observed for *Synechococcus* (19). The variability of the two species is so extreme that the fluorescence of the *Prochlorococcus* phenotype at 100 m is the same as that of *Synechococcus* around 50 m. Obviously, the properties in flow phenotypes do not express fixed characteristic properties that can be used to identify a microbe, but the characteristics are subject to major modifications by environmental acclimation.

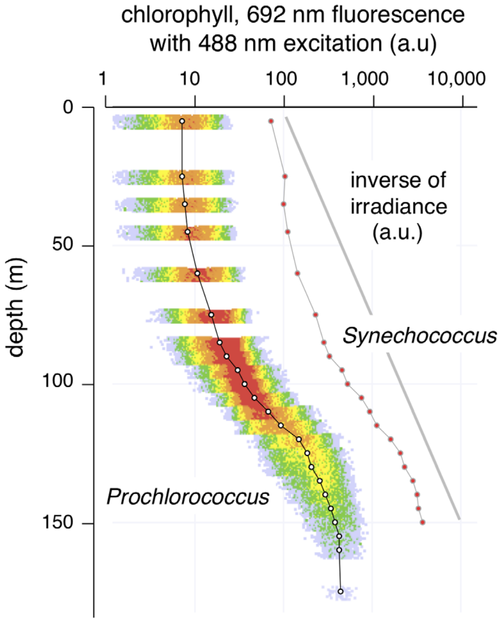

Figure 3. Chlorophyll fluorescence (measured at 692 ± 20 nm) of *Prochlorococcus* and *Synechococcus* at different depths at Station Aloha (6 nautical mile radius around 22° 45′ N, 158° 00′ W (22). The excitation wavelength is 488 nm. The open circles represent the mean fluorescence of *Prochlorococcus.* The red dots represent *Synechococcus.* The mean fluorescence of both species increases monotonically with the inverse of the local light intensity (solid gray line) resulting in a 50-fold difference over a range of 125 m. This suggests that both species acclimate in a similar manner to ambient light. If chlorophyll content is inversely related to light intensity, the photon collection rate, and therefore primary production, can be expected to be independent of depth.

The state of the marine environment is crucial for the well-being of our planet. Many aspects of the interactions within microbial communities and between microbes and their environment are unknown. Flow cytometry is well suited to reveal and clarify such relationships. The technology is in principle capable of providing a detailed index of the identity and activities of all members of a microbial community. Flow cytometry produces results in real time and therefore is also well-suited to configure monitoring stations at crucial locations. However, for flow technology to become relevant and practical for plankton studies, current methodology and concepts developed in biomedical research, need to be reconsidered, and must be made suitable for the analysis of plankton cells and other very small particles (0.2–2.0 µm). Instrumentation and methodology should be adapted for particles that contain multiple interacting chromophores. Current flow cytometry paradigms assume linearity, smooth transitions, and chromatically isolated pigments. For flow cytometry to become an analytical tool with which the composition of microbial assemblages from different sites and times can be compared in a meaningful way, the theory of light scatter by wavelength-sized particles needs to be implemented in flow methodologies. Ideally, instruments suitable for plankton analysis perform multi-color scatter measurements with precise angular and polarization information (24). Such instruments should be equipped with spectral analyzers for collecting both excitation- and emission-spectra of the fluorescence (25). Software should integrate samples from transects or time series into datasets that correlate the composition and behavior of microbial populations at a given time and location with local physical and environmental parameters. Such software should allow normalized comparison of samples gathered in different experiments. The instruments should be combined with efficient single cell sorting procedures that allow reliable single-cell genome analysis, either by PCR or whole genome or RNA-expression sequencing, to validate the identity of clustered populations. For flow cytometry to become applied on a broader scale in environmental sciences a new class of instruments needs to become available. Standardized analytical procedures must become established and generally accepted. Only if the flow community works toward establishing appropriate tools and rules that are relevant for plankton analysis, flow cytometry will develop its full promise and potential for monitoring the state our oceans and the extent to which anthropogenic activities are affecting the quality of the marine environment.

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