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Marine phytoplankton distributions measured using shipboard flow cytometry

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Abstract—Flow cytometry is an alternative to traditional methods of studying the distribution and abundance of phytoplankton that allows rapid, objective analysis of light scattering and fluorescence of individual cells in a natural water sample. We report here the first flow cytometric analysis of phytoplankton communities carried out at sea. Light scatter and autofluorescence of individual cells were measured in water samples from the Gulf Stream. These flow cytometric 'signatures' allowed us to discriminate between different groups of phytoplankton, enumerate them, and detect changes in average pigment fluorescence resulting from changes in population structure. The results indicate that flow cytometric techniques can be used on board ship with minimal modification of available equipment. They also show promise for automated analyses of plankton and particles in the sea.

INTRODUCTION

THE distribution and abundance of marine phytoplankton is studied traditionally by techniques involving microscopic examination of water samples or cell harvesting by filtration, which sacrifices information about the distribution of properties among the cells. Flow cytometry is an alternative to these methods; it allows rapid and quantitative analyses of optical properties of individual particles in a fluid (MELAMED *et al.*, 1979; VISSER and VAN DEN ENGH, 1982; YENTSCH *et al.*, 1983a). This is accomplished by hydrodynamically focusing a sample stream in the center of a fluid sheath so that sample particles are carried in single file through an intense beam of light with a spot size small enough to illuminate only one particle at a time. Quantitative analysis of light scattering and fluorescence properties can then provide information as to the size and composition of each individual particle. Physical sorting of particles according to predetermined optical criteria can also be carried out.

The utility of flow cytometry for studying phytoplankton recently has been recognized in laboratory studies using cultured organisms (YENTSCH *et al.*, 1983b; TRASK *et al.*, 1982; PAAU *et al.*, 1978, 1979; OLSON *et al.*, 1983), but the obvious extension of these studies to natural populations has been forestalled by anticipated problems with instrument portability, shipboard performance, and dilute cell concentrations. Commercially available flow cytometers with cell sorting capability use a laser light source which must be precisely

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focused on the sample stream. In a first evaluation, laser alignment would appear to be quite susceptible to vibration and/or motion effects on board a ship. Moreover, optimum sample concentrations for flow cytometry (on the order of 10^6 particles per ml) are higher than those of most phytoplankton populations occurring *in situ*.

To evaluate the feasibility of shipboard operation, and to begin to investigate methods of sample preparation, we carried out analyses using a Coulter EPICS V flow cytometer/cell sorter aboard the R.V. *Iselin* in the Gulf Stream during April of 1984. We focused our study on the unicellular cyanobacterium *Synechococcus* because its relatively high concentrations (up to 10^5 ml^{-1}) and distinctive fluorescent pigment (phycoerythrin) make this organism a near-ideal subject for flow cytometry.

METHODS

Water samples for flow cytometric analysis were collected using 5-l Niskin bottles from a depth profile in the Gulf Stream (38°24.8'N, 67°49.3'W). All samples were prefiltered through 53-μm Nitex mesh immediately before analysis to eliminate large particles. Samples were analyzed unconcentrated, and after 300-fold concentration by gentle filtration (vacuum < 150 mm Hg) onto 1-μm Nuclepore filters and resuspension. Laser emission of 450 mW at wavelength 488 nm was used as the light source. The EPICS V was installed on board ship with standard immobilization precautions and the computer was placed on a rubber pad for vibration protection. Simple tests of instrument performance with standard fluorescent microspheres revealed no obvious deleterious effects of engine vibrations; underway ship motion had only a slight effect on sample analysis rate (10° rolls produced oscillations in rate but not in magnitude of signals).

Forward light scatter (1 to 19°) (FLS), which reflects particle size (HUTTER and EIPEL, 1978), and autofluorescence at two wavelengths were measured simultaneously on each particle. We defined fluorescence emission windows in the orange (550 to 590 nm) and red (660 to 700 nm) regions of the spectrum to quantify autofluorescence of phycoerythrin (characteristic of certain cyanobacteria) and chlorophyll, respectively (YENTSCH and YENTSCH, 1979). Although these pigments were not necessarily the sole sources of the fluorescence signals we measured, they were the dominant ones; for convenience we refer to the signals in the following discussion as PE and CHL. Identification of cell types corresponding to specific areas of the flow cytometric signatures was accomplished by using the cell sorting capabilities of the EPICS V, which allows one to deflect physically the particles of interest, and collect them for microscopic examination. This is accomplished by imposing a high frequency vibration on the flow cell which causes the stream to break into droplets after the point of analysis; at typical sample concentrations no droplet will contain more than a single particle. When the preset fluorescence and/or light scatter criteria have been met, individual droplets (containing the particle of interest) can be charged and electrostatically deflected out of the main stream for collection. Uniform fluorescent beads (diameter 0.9 μm with peak emission in the green; Duke Scientific Co.) at a known concentration were added to each sample as internal standards, so that cell concentrations could be calculated (cell ml^{-1} = cell count × [beads ml^{-1}/bead count]), and so that relative size and fluorescence values could be compared from sample to sample. These standard beads were visible in the PE vs FLS plots of the unconcentrated samples as sharply-defined peaks with high light scatter.

Synechococcus were also counted manually by epifluorescence microscopy of aliquots of

the same samples concentrated onto 0.2-μm pore size Nuclepore filters (WATERBURY *et al.*, 1979).

RESULTS

The phytoplankton at the Gulf Stream station was dominated numerically by the small (≤1 μm diameter) cyanobacterium *Synechococcus*, distinguished by its characteristic autofluorescence at about 580 nm due to phycoerythrin (WATERBURY *et al.*, 1979; LI *et al.*, 1983). These cells appeared as large clusters of signals in each of the two-dimensional contour plots (Fig. 1A, B, C). They had lower FLS signals than the 0.9-μm standard beads, high PE (orange) fluorescence, and low to moderate CHL (red) fluorescence. The clusters were less distinct in the CHL vs FLS plots because of the presence of other cells without phycoerythrin, but analysis by sorting revealed that they dominated the central low CHL/intermediate FLS region of this plot (Fig. 1B). In all cases the identification of these clusters as *Synechococcus* was verified by cell sorting. The CHL distribution of *Synechococcus* overlapped that of other, non-PE-containing phytoplankton, as shown by the continuous distribution of signals bisecting the FLS/CHL plot and extending beyond the range of *Synechococcus* in terms of light scatter (Fig. 1B).

The concentration of *Synechococcus* at each depth was determined by integrating the number of counts in the central cluster of the PE vs FLS plot in Fig. 1A (FLS between channels 5 to 50 of 64 full scale; PE greater than channel 15). Our numbers were comparable to those from microscopic counts, although a systematic bias is present (Fig. 2A). In estimating the concentration of 'other phytoplankton' we considered only particles with CHL signals greater than channel 20 (out of 64 full scale); this was the upper limit for CHL in the cluster in Fig. 1C identified by sorting as *Synechococcus*. *Synechococcus* was by far the most abundant organism in this low CHL range: the number of cells with FLS and CHL signals in the *Synechococcus* range in Fig. 1B was nearly the same as the number of *Synechococcus* as determined from Fig. 1A. It is possible, however, that we might have missed CHL-containing cells if their FLS signals were much lower than those of *Synechococcus*.

The low FLS signals associated with many particles with high CHL signals in the CHL vs FLS plots on unconcentrated samples (Fig. 1B) were later shown to be artifacts resulting from high noise levels from the logarithmic amplifier for FLS. Lowering the gain on this amplifier, as in the analyses of the concentrated samples (Fig. 1E) alleviated this problem.

Dramatic changes in the abundance of both *Synechococcus* and the other phytoplankton populations were observed in the depth profile (Fig. 2). The most obvious feature was the decrease (by an order of magnitude) in the concentration of *Synechococcus* across the thermocline; at the same time the mean fluorescence per cell approximately doubled (Fig. 2A). Examination of the PE vs FLS plots (Fig. 1A) at 60, 80, and 100 m reveals that this increase was due to the appearance of a distinct subpopulation of cells with four-fold brighter PE fluorescence; both populations appeared to be larger than the cells nearer the surface. These two populations may represent different strains of *Synechococcus* (WOOD *et al.*, 1985) or different stages of photoadaptation of cells of a single strain (COHEN-BAZIRE and BRYANT, 1982; KURSAR *et al.*, 1981; ALBERTE *et al.*, 1984).

In contrast to *Synechococcus*, the concentration of other phytoplankton did not decrease across the thermocline (Fig. 2C).There was a minimum in cell concentration at 100 m, with an increase below this depth; this increase was accompanied by an increase in

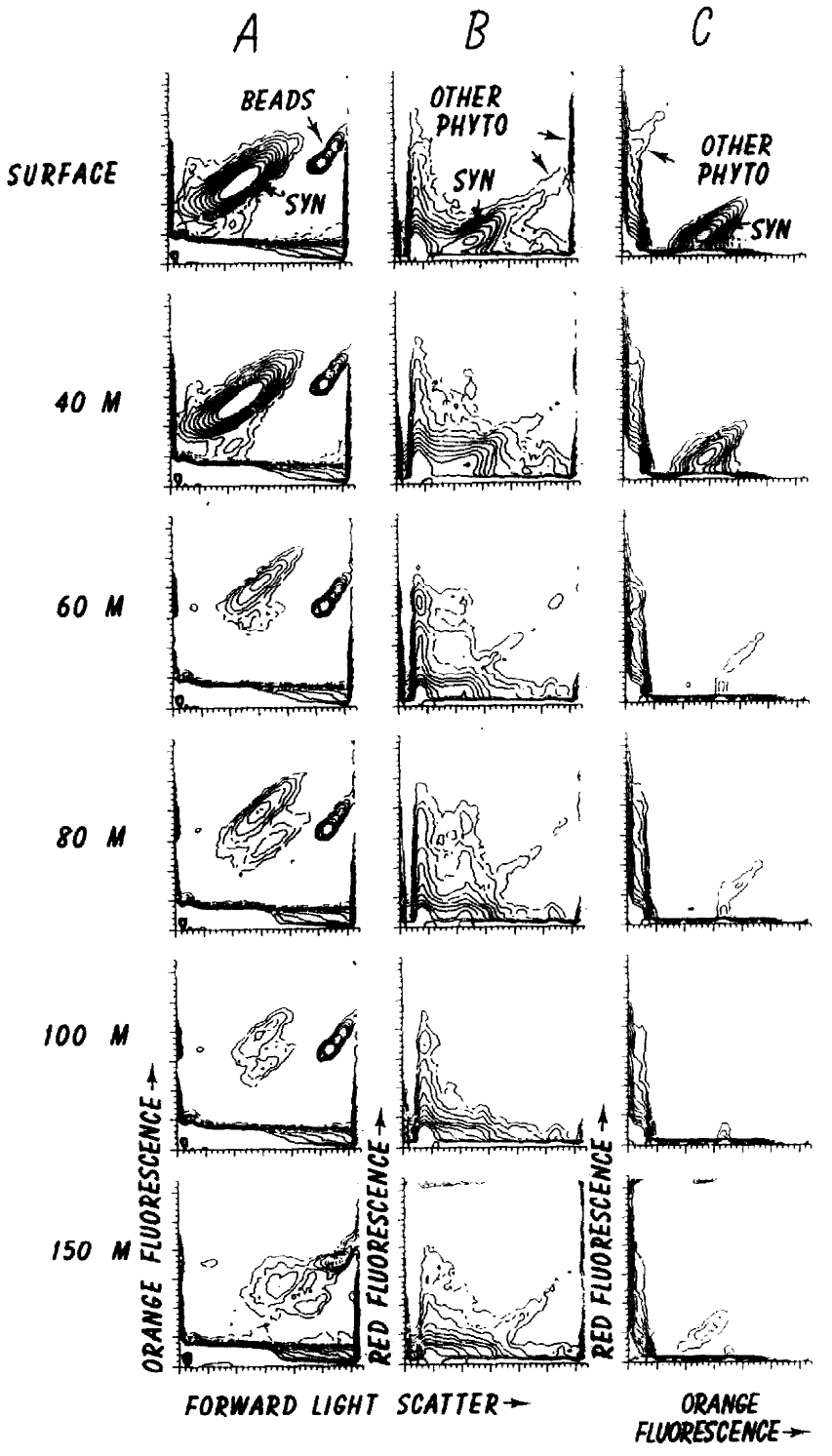
A
B
C
BEADS
SYN
OTHER PHYTO
SYN
OTHER PHYTO
SYN
SURFACE
40 M
60 M
80 M
100 M
150 M
ORANGE FLUORESCENCE
RED FLUORESCENCE
RED FLUORESCENCE
FORWARD LIGHT SCATTER
ORANGE FLUORESCENCE

Fig. 1A, B, C.

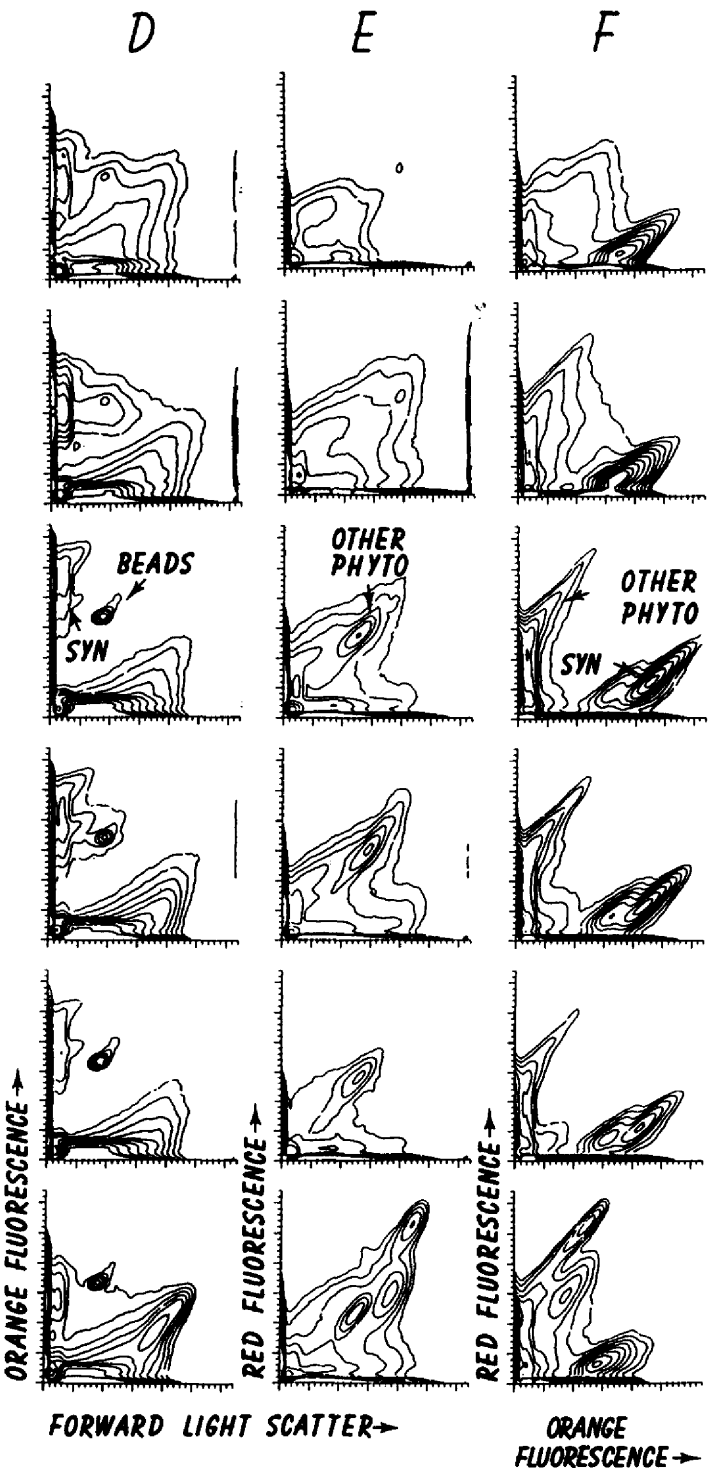

Fig. 1. Flow cytometric analyses of particles from a depth profile at a station in the Gulf Stream. Water samples were analyzed either without treatment (A, B, C) or after concentrating the particles onto a 1-μm pore-diameter Nuclepore membrane filter and resuspending them in seawater (D, E, F). The data are presented as contour plots of orange fluorescence (PE) vs forward light scatter (FLS) (A,D), red fluorescence (CHL) vs FLS (B, E), and CHL vs PE (C, F). Contour levels for A to C are 2, 4, 7, 12, 20, 30, 50, 80, 120, 180 particles; levels for D to F are 10, 20, 40, 100, 180, 400, 800, 1200, 1800, 2500 particles. Uniform fluorescent microspheres were added as internal standards, and can be seen as a distinct signal in some frames. The signals from 3-decade logarithmic amplifiers were divided into 64 linear channels for each axis.

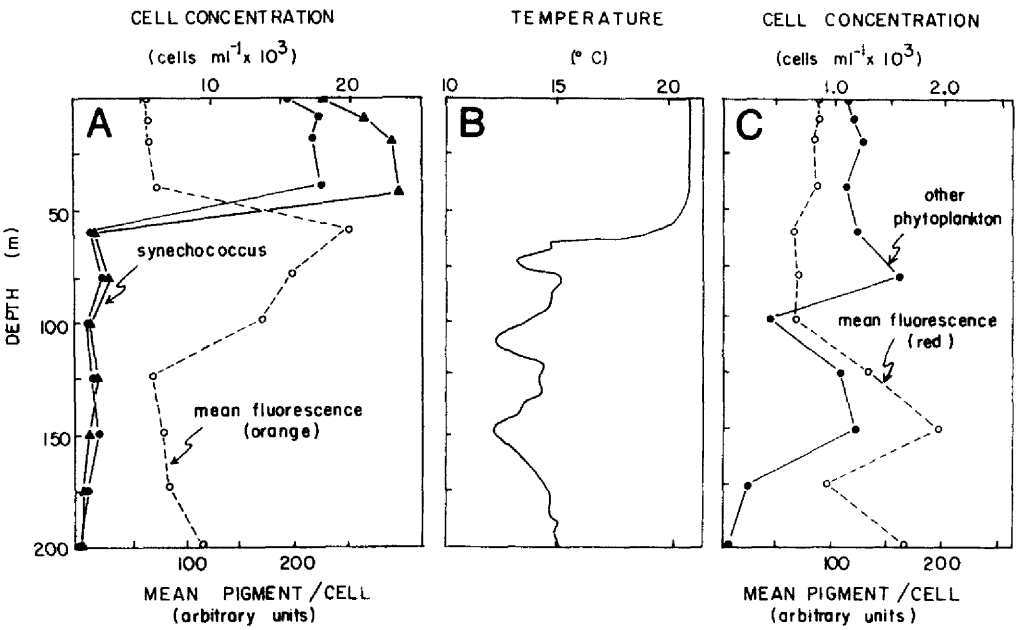

Fig. 2. Depth profiles of cell concentrations and mean pigment fluorescence obtained from the data in Fig. 1. (A) *Synechococcus* cell concentration (flow cytometric counts = solid circles; microscope counts = triangles) and mean phycoerythrin fluorescence per cell (open circles); (B) temperature; and (C) the concentration (solid circles) and mean chlorophyll fluorescence per cell (open circles) of 'other phytoplankton'. Temperature was obtained from an XBT trace. Pigment fluorescence values are in relative units and have been converted from the original logarithmic scale to a linear one.

the mean pigment fluorescence at 150 m to over twice that in near-surface water. The ratio of *Synechococcus* cell numbers to other phytoplankton varied with depth from about 20:1 near the surface to about 1:1 at 150 m.

To better characterize the less-frequent and larger phytoplankton cells, i.e. the 'other phytoplankton', water from the same samples was concentrated on 1-μm filters and analyzed using a lower signal gain for light scatter measurement (compare the bead positions in the PE vs FLS plots between Fig. 1A and D). In the upper waters these particles appear to be quite diverse; there is little correlation between light scatter and fluorescence and no clearly defined population structure, except for clusters of *Synechococcus* cells in the PE vs FLS and CHL vs PE plots (Fig. 1D and F). The mode PE fluorescence of the *Synechococcus* retained on the filter is about two-fold higher than those from the unconcentrated water samples (although this is not obvious because of the log scale); this probably reflects the selective retention of larger *Synechococcus* cells by the 1-μm pore size filter. The increase in fluorescence below the thermocline, with appearance of a second, brighter subpopulation of *Synechococcus*, is again clearly visible in the samples from 60 to 100 m.

Below the thermocline the number of particles with high orange fluorescence decreases (Fig. 1D) and the fluorescence and scatter signals become more tightly correlated. It should be noted that 'orange fluorescence' in these larger cells is undoubtedly not due to

phycoerythrin but probably to spill-over from a poorly characterized green autofluorescence that we have observed in many cell types. The samples between 60 and 100 m in Fig. 1D show a single phytoplankton cluster (other than *Synechococcus*), but sorting of this region revealed that a variety of species were actually present, including several small flagellated organisms. In the 150 m sample, where a temperature minimum occurred, three distinct subpopulations are visible in the CHL vs FLS plot (Fig. 1E). One of these subpopulations was sorted for examination, and proved to be composed of small colonial cells, which we were not able to identify.

DISCUSSION

The changes in the phytoplankton population structure, reflected by the flow cytometric signatures, appear to be related to the temperature structure of the water column. Dramatic changes in *Synechococcus* population structure and the disappearance of a component of the larger phytoplankton occurred across the main thermocline at the station examined. The appearance of distinct populations of larger phytoplankton also coincided with a cold layer at 150 m. This suggests that distinct water masses, each with its own community of phytoplankton, can be detected by flow cytometric analysis.

In our minds, one of the most significant results of this first exploration of the use of flow cytometry for analyzing plankton assemblages is demonstration of our ability to reveal distinct subpopulations of very closely-related cells, most obviously in the case of *Synechococcus*. Although we cannot resolve the origin of these subpopulations (i.e. different water masses vs different physiological states), they seem to be a common feature of *Synechococcus* populations; we have seen similar structure and transitions in subsequent sampling of both vertical profiles and horizontal transects.

The results reported here indicate that shipboard flow cytometry can be a powerful new tool for biological oceanographers. The capacity for multiparameter analysis on single cells allows one to resolve changes in the community structure that would be obscured by traditional methods of analysis in which only average characteristics of samples are measured. As indicated by the successful operation of the flow cytometer on this cruise, the operational aspects of sea-going flow cytometry are not difficult. The major challenges confronting us now are the improvement of resolution through more specific methods of sample analysis (including the use of fluorescent molecular probes) and the expansion of experience in sorting and identification. Most importantly, the development of more sophisticated data analysis techniques is now needed to realize the potential of this technology for the study of plankton in the sea.

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