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Winter presence of prochlorophytes in surface waters of the northwestern Mediterranean Sea

Abstract—Cells with characteristics similar to the recently discovered oceanic prochlorophytes have been detected with ship-board flow cytometry during winter in the surface waters of the northwestern Mediterranean Sea as well as in the low-salinity dilution zone of the Rhône River. Maximum abundances reached 50,000 cells ml^{-1} at the surface and were only slightly lower than those observed previously at the bottom of the euphotic zone in the Atlantic and Pacific Oceans. The concentration, light scatter, and pigment fluorescence of these cells correlated tightly with those of *Synechococcus* spp. cyanobacteria. These results point out that the ecological niches of oceanic prochlorophytes are probably more diverse than initially thought.

Within the past 2 yr, a previously uncharted population of very abundant and very small, red-fluorescing cells has been detected with flow cytometry near the bottom of the euphotic zone in the Atlantic and Pacific Oceans (Chisholm et al. 1988; Li and Wood 1988; Neveux et al. 1989; Olson et al. in press). The cells observed initially (Chisholm et al. 1988) were described as very small coccoid procaryotes (typically 0.6–0.8 μm in diameter), assigned to Prochlorophyta on the basis of their pigment composition: a Chl *a*-like pigment, Chl *b*, α-carotene, and zeaxanthin. This pigment composition differed somewhat, however, from that of the other known members of this division (Lewin 1984; Burger-Wiersma et al. 1986): Chl *a* absorption exhibited a spectral shift toward longer wavelengths, similar to that of divinyl Chl *a* (Bazzaz and Brereton 1982) and α-carotene replaced β-carotene.

The existence of oceanic prochlorophytes was also hypothesized independently in the Banda Sea by Gieskes et al. (1988) on the basis of water-column pigment signature. The organisms described by Chisholm et al. (1988) appear similar to the "very small red fluorescent bodies" observed by Li and Wood (1988) and to the "green photosynthetic bacteria" reported by Neveux et al. (1989) in the Sargasso Sea, although in the latter case both Chl *a* and Chl *b*-like pigments displayed shifts in their absorption and fluorescence excitation spectra. The unambiguous classification of these organisms clearly awaits further work on pure cultures and, at the present time, they are best referred to as presumptive oceanic prochlorophytes.

Initial observations of these oceanic populations were made at the bottom of the euphotic zone in stratified pelagic waters; they could not be detected in the upper euphotic zone or in mixed coastal waters (Chisholm et al. 1988; Gieskes et al. 1988; Li and Wood 1988; Neveux et al. 1989). A pigment similar to divinyl Chl *a* was first reported several years ago in Atlantic surface waters (Gieskes and Kraay 1983), however, and recent observations made over longer time scales and with more sensitive flow cytometry have established that they are indeed present and abundant in Sargasso Sea surface waters in winter (Olson et al. in press). In open oceanic waters, their esti-

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mated contribution to the total depth-integrated biomass and productivity reach 40 and 15%, respectively (Chisholm et al. 1988; Neveux et al. 1989). Their localization in the nitracline could make them responsible for a large part of the water-column "new" production—the most relevant quantity from the point of view of global biogeochemical fluxes.

We report here on the winter presence of oceanic prochlorophytes in the northwestern Mediterranean basin. These organisms were detected throughout the entire euphotic zone in a variety of habitats, including stratified offshore waters, well-mixed nearshore waters, and low salinity, river-influenced coastal waters. Their abundances and cellular properties are compared with those of prochlorophyte populations from the deep chlorophyll maximum (DCM) measured during a previous cruise in the Sargasso Sea (Neveux et al. 1989), and their contribution to the total photosynthetic biomass is estimated.

Sixteen stations were occupied in the northwestern Mediterranean Sea between 29 December 1988 and 8 January 1989 during the second leg of cruise 179 of the RRS *Discovery*, part of the EROS 2000 project. From 1 to 10 depths were sampled with 10-liter GoFlo bottles, depending on bottom depth and on the shape of the density and fluorescence profiles measured with a CTD. Eight subsurface samples were retrieved by pumping while the ship was mapping the extent of the Rhône River outflow. Five surface samples were retrieved with a small rubber boat in the Rhône estuary itself. Sargasso Sea samples were collected during the CHLOMAX cruise of the *N.-O. Suroît* in September–October 1987, as described by Neveux et al. (1989).

At six stations (ZA3–ZA8), located on an offshore transect to the southeast of the Rhône estuary, discrete vertical profiles of Chl *a* were measured by reverse-phase, ion-pairing HPLC. For these samples, volumes ranging between 2 and 4 liters were GF/F filtered. At one station (ZF0) surface samples were prefractionated by passage through 2- and 0.6-μm Nuclepore filters in order to separate specific cell populations. Analyses were performed on board. Pigments were extracted with 2.5 ml of 90% acetone and, after mixing with ion-pairing agents, 100 μl were injected in a 3-cm column of reverse-phase 3-μm C_{18} (Pecosphere, Perkin Elmer). Elution and detection were as described by Mantoura and Llewellyn (1983). Pigment identification was by coelution with pigments from well-characterized phytoplankton cultures and by UV-vis spectral matching with an HP1040 diode array detector. This set-up did not allow us to distinguish zeaxanthin from lutein.

At two stations (ZA6, ZD1) 2-liter surface samples were fractionated by passage through 5-, 2-, 1-, 0.8-, and 0.6-μm Nuclepore, and finally GF/F filters, which were stored in liquid nitrogen until extraction in 90% acetone. Chl *a*, *b*, *c* and the fluorescence excitation peak of the whole extract were determined as described by Neveux and Panouse (1987) with a Perkin Elmer MPF66 spectrofluorometer.

All samples were immediately analyzed on-board ship with an EPICS 541 flow cytometer (Coulter Electronics). Samples were illuminated with the 488-nm and, for a few, the 515-nm lines of a 6-W argon laser (Coherent, Palo Alto) focused through confocal optics. Laser power was 1.3 W. Duplicate 0.25-ml volumes were analyzed with the Biosense flow cell, which provides a higher sensitivity than the standard flow cell. Orange fluorescence (from phycoerythrin) was collected through a 585±10-nm band-pass interference filter (Glen Spectra Ltd.) and red fluorescence (from Chl) through a 690-nm long-pass filter after reflection on a short-pass 640-nm dichroic filter (MTO). The four measured parameters, forward- and right-angle light scatter (FALS and RALS) plus orange and red fluorescence, were recorded on 3-decade logarithmic scales, stored in list mode, and analyzed with a custom-designed software (CYTOPC).

Cell populations related to oceanic prochlorophytes (*see below*) were discriminated from picoeucaryotes (probably small chlorophytes) by their much smaller scatter and red fluorescence (Fig. 1A) and from cyanobacteria (*Synechococcus* spp.) by their smaller scatter (Fig. 1A) and their lack of orange phycoerythrin fluorescence (Fig. 1B). For these cells, we were usually able to mea-

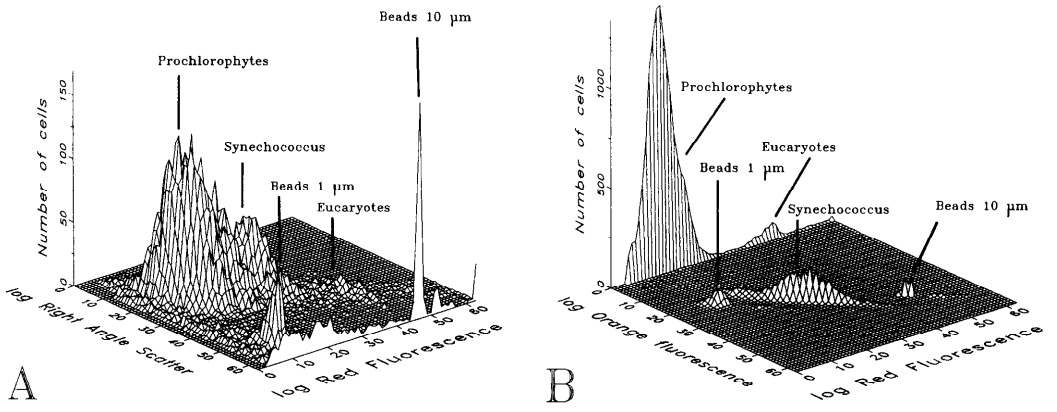

Fig. 1. Cytograms obtained for a 250-µl sample taken at 10-m depth from station ZC3 (northwestern Mediterranean Sea off the French-Spanish border, 42°44'N, 4°20'E). A. Right-angle scatter (RALS) vs. red fluorescence. B. Orange vs. red fluorescence. All parameters are recorded on 3-decade logarithmic scales. The vertical axis corresponds to the number of events recorded. All the chlorophyll-containing cells are characterized by their red fluorescence (A) and *Synechococcus* by the presence of orange fluorescence, in contrast to its absence in prochlorophytes and picoeucaryotes (B); 1- and 10-µm beads were added to the sample to serve as internal standards.

sure RALS but not FALS, since the FALS detector of our instrument lacked sufficient sensitivity. To account for day-to-day variations in instrument settings, two types of beads were mixed with each analyzed sample (Fig. 1): 1-µm beads (Polysciences) to normalize FALS and RALS, which were expressed after normalization in bead scatter units (bsu), and 10-µm, 2% Fullbright beads (Coulter) to normalize fluorescence, expressed in bead fluorescence units (bfu). This normalization allowed back-calibrating the red fluorescence of beads to Chl *a* by comparing for the same sample total red fluorescence per unit of volume (measured by flow cytometry) and Chl *a* concentration (measured by spectrofluorometry).

For *Synechococcus* we used cultures of three strains isolated from the Sargasso Sea during the CHLOMAX cruise (Neveux et al. 1989), which gave a conversion factor of 25 fg Chl *a* per bfu. For Mediterranean prochlorophytes, we used fractions of two field samples passing a 0.6-µm Nuclepore but retained on a GF/F filter at stations ZD1 and ZF0, where these cells contributed, respectively, 88 and 65% of the total red fluorescence, providing an average conversion factor of 93 fg Chl *a* per bfu. For Sargasso Sea prochlorophytes, we also used filter-fractionated samples and obtained a conversion factor of 24 fg bfu^{-1}.

In all samples collected in winter in the euphotic zone of the northwestern Mediterranean Sea, we detected by flow cytometry a population of cells smaller and fainter than *Synechococcus* (Fig. 1A) and, in contrast to the latter, lacking any orange fluorescence characteristics of phycoerythrin (Fig. 1B). This flow cytometric signature led us to relate these cells to the oceanic prochlorophytes initially described by Chisholm et al. (1988), similar to those we observed previously in the Sargasso Sea DCM with the same instrumental set-up (Neveux et al. 1989). Both populations had very similar mean right-angle scatter of the order of 0.04–0.05 bsu (Table 1). On average, 60% of these cells passed through a 0.8-µm Nuclepore membrane, 28% through 0.6 µm, but all were retained by a GF/F filter, in agreement with the figures of Chisholm et al. (1988). The ratio of forward- to right-angle light scatter (FALS : RALS) was 4 times lower for this population than for *Synechococcus,* as observed previously in the Sargasso Sea (Neveux et al. 1989): this result indicates that their average diameter was about 1.4 times smaller since, in this size range, FALS : RALS is proportional to the fourth power of the diameter and does not depend on the refractive index, although both FALS and RALS taken independently do (Guillard 1988; Morel pers. comm.).

HPLC pigment analyses of the 0.6-μm to GF/F size fraction at station ZF0 yielded only Chl *a*, Chl *b*, and zeaxanthin-lutein—a pigment signature consistent with that obtained by Chisholm et al. (1988) for oceanic prochlorophytes. The HPLC set-up used (reverse phase, 3-cm column, wide absorbance band detector) did not permit distinction between normal Chl and divinyl Chl-like pigments, in contrast to Gieskes and Kraay's (1983) method.

Spectrofluorometric analysis of acetone extracts of the same size fraction at station ZD1, where prochlorophytes accounted for 88% of the total red fluorescence, however, revealed a shift of 4 nm in the Chl *a* fluorescence excitation peak, strongly suggesting the presence of divinyl Chl *a*-like Chl (Neveux et al. 1989). The Chl *b* : Chl *a* ratio (0.23) was lower than observed for a typical DCM population (1.07; Chisholm et al. 1988); in contrast, the zeaxanthin-lutein : Chl *a* ratio was higher (1.12 vs. 0.32 in the DCM; Chisholm et al. 1988). The observed zeaxanthin-lutein clearly was not coming from *Synechococcus* cyanobacteria because the 0.6-μm to GF/F fraction at station ZF0 accounted for 56% of the zeaxanthin-lutein and 35% of the prochlorophyte abundance of the total sample, but <2% of the *Synechococcus* abundance. α-carotene, also reported by Chisholm et al. (1988), was not detected, possibly because of its very low contribution to the carotenoids.

Mediterranean surface cells emitted on average 4 times less red fluorescence than Sargasso Sea DCM cells (Table 1). This difference, however, did not reflect so much a difference in cellular Chl *a* content (Table 1), which was also similar to the figure of 2.17 fg cell^{-1} obtained by Chisholm et al. (1988), as a lower fluorescence yield, probably linked to dissimilarities in the proportions of accessory pigments (Olson et al. 1989). The ratio of red fluorescence excited at 488 nm to that excited at 515 nm, an index of accessory chlorophyll composition (Olson et al. 1989), was higher than obtained for cultures of Chl *c*-containing algae and higher than for the chlorophyte *Dunaliella primolecta,* confirming that the accessory Chl was Chl *b*-like rather than Chl *c*-like (Neveux et al. 1989; Olson et al. 1989).

Table 1. Comparison of abundances and cellular properties of prochlorophytes at the surface of the Mediterranean Sea and at the deep chlorophyll maximum (DCM) of the Sargasso Sea (Neveux et al. 1989). Right-angle light scatter is expressed in bead scatter units (bsu) relative to 1-μm Polysciences beads; red fluorescence is expressed in bead fluorescence units (bfu) relative to 10-μm, 2% Fullbright Coulter beads. Cell Chl *a* was obtained by multiplying cell fluorescence by a conversion factor determined as explained in the text. SD—Standard deviation.

	Mediterranean surface (n = 17)	Sargasso DCM (n = 21)
Period	Dec–Jan	Sep–Oct
Abundances (10^3 × cells ml^{-1})		
Mean ± SD	19±16	73±16
Range	1–52	39–95
Size-related parameters		
Right-angle scatter (bsu)		
Mean ± SD	0.041±0.013	0.051±0.014
Range	0.026–0.077	0.027–0.083
Pigment-related parameters		
Red fluorescence (bfu)		
Mean ± SD	0.021±0.007	0.080±0.033
Range	0.012–0.031	0.027–0.083
Conversion factor (fg bfu^{-1})	93	24
Chl *a* content (fg cell^{-1})		
Mean ± SD	2.1±0.7	1.9±0.8
488 : 515 excitation ratio		
Mean ± SD	2.0±0.4*	3.1±0.5†

* n = 10.
† n = 14.

This ratio was nonetheless lower than for Sargasso Sea DCM populations (Table 1), suggesting a lower Chl *b* : Chl *a* ratio in the Mediterranean case, in agreement with spectrofluorometric measurements.

Although this set of evidence (flow cytometric signature, size range, presence of divinyl Chl *a* and of Chl *b*) points to a close relation between the cells we observed in the Mediterranean Sea and oceanic prochlorophytes, it must be stressed that, since we were unable to perform transmission electron microscopy, these cells might indeed be eucaryotes, belonging probably to the Chlorophyceae in view of their pigment composition. In this case they would be significantly smaller than one of the smallest coccoid eucaryotic species maintained in culture, *Micromonas pusilla* (Shapiro and Guillard 1986), since their average RALS

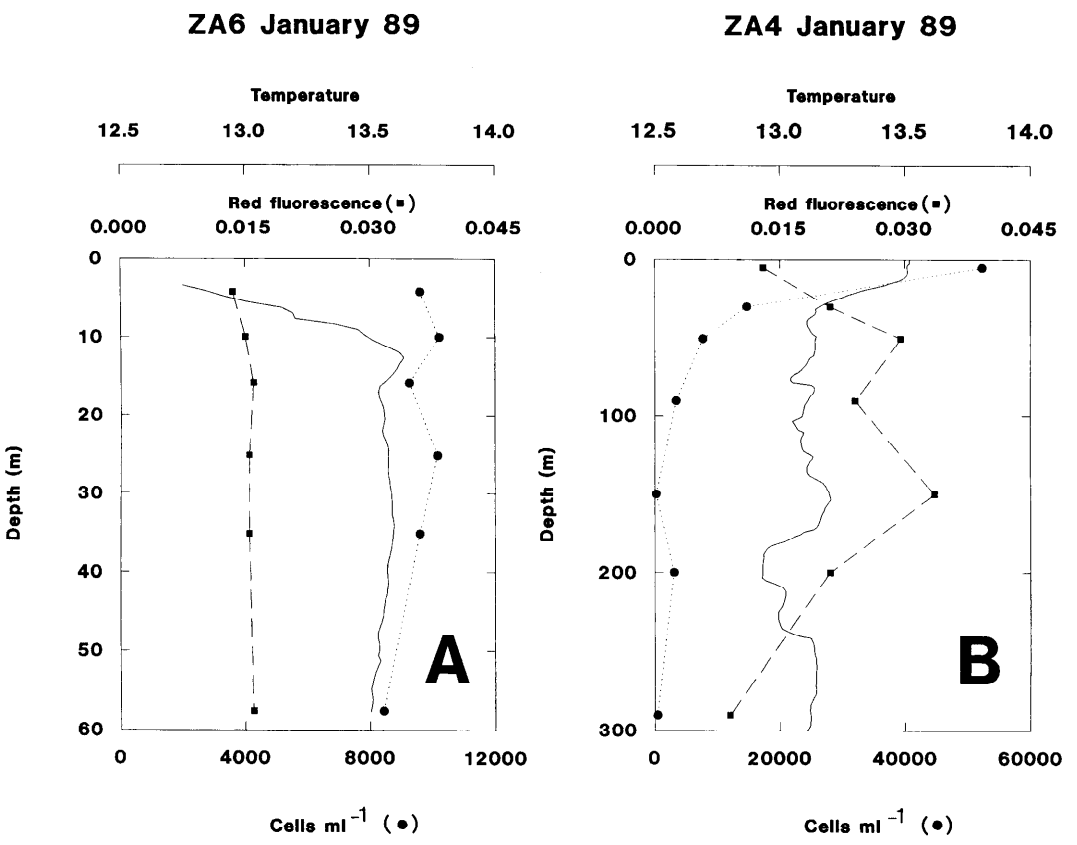

Fig. 2. Vertical profiles of temperature (——), prochlorophyte cell concentration, and Chl red fluorescence normalized to 10-μm, 2% Fullbright Coulter beads. A. Station ZA6 at the edge of the Rhône River plume (43°17′N, 4°49′E; bottom depth, 60 m). B. Station ZA4 offshore (42°50′N, 5°2′E; bottom depth, 1,460 m). Note the influence of the Rhône River at station ZA6 marked by a sharp decrease in temperature near the surface.

is about 10 times lower than for the latter species (D. Vaulot unpubl. data). Alternatively, it is also possible that populations that present similar flow cytometric signatures across the study zone are heterogenous, some having a different pigment composition from the one we determined on a limited number of samples. With these caveats in mind, we refer to these cells as prochlorophytes in the remainder of the text.

In sharp contrast to the observations of Chisholm et al. (1988), we found that Mediterranean Sea prochlorophytes were not restricted to offshore waters but were present at all stations, including coastal waters (Fig. 2) and the Rhône River estuary at salinities as low as 1.2‰. Maximum concentrations were always observed at the surface and not at depth (Fig. 2). The vertical structure of these prochlorophyte populations (Fig. 2) closely reflected the physical structure of the water column, with uniform abundance and fluorescence in coastal waters (Fig. 2A) and decreasing abundance coupled with increasing fluorescence below the thermocline in stratified offshore areas (Fig. 2B), as observed for cyanobacteria (Olson et al. 1990).

Surface abundances ranged from 10^3 to 5×10^4 cells ml^{-1} (Table 1 and Fig. 3) and maxima occurred in stratified offshore waters. The maximum cell concentration was only 2.5 times lower than observed in the Sargasso Sea DCM in late summer (Table 1) and similar to that found in winter in the DCM off California (Chisholm et al. 1988). From fluorescence calibration achieved with size fractions separated by filtration, prochlorophytes were estimated to contribute between 3.7×10^{-4} and 1.3×10^{-1} μg liter^{-1} Chl *a* (mean, 2.2×10^{-2} μg liter^{-1}; $n = 65$)

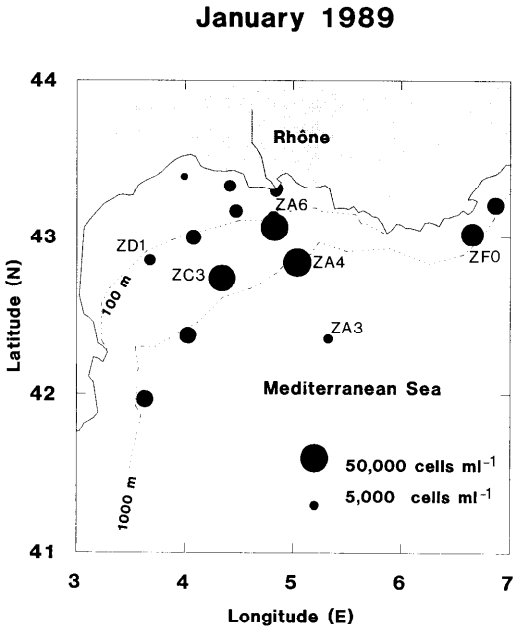

Fig. 3. Surface cell concentrations (proportional to circle area) of prochlorophytes in the northwestern Mediterranean Sea in winter. Only stations mentioned in the text are located.

and to represent up to 31% of the total Chl *a* (mean, 7.8%; $n = 22$). In terms of C, assuming an average size of 0.8 μm as determined from differential filtrations and a C content of 133 fg μm^{-3} (Simon and Azam 1989), the average contribution of prochlorophytes was equal to 0.40 μg C liter^{-1}.

In the Sargasso Sea in summer and in the Pacific Ocean, prochlorophytes and *Synechococcus* display divergent vertical distributions, the former occupying the DCM and the latter the upper euphotic zone (Chisholm et al. 1988; Li and Wood 1988; Olson et al. in press), but in the Mediterranean Sea the concentrations of both cell types were extremely well correlated in winter (Fig. 4A). Moreover, both the RALS and pigment fluorescence of prochlorophytes

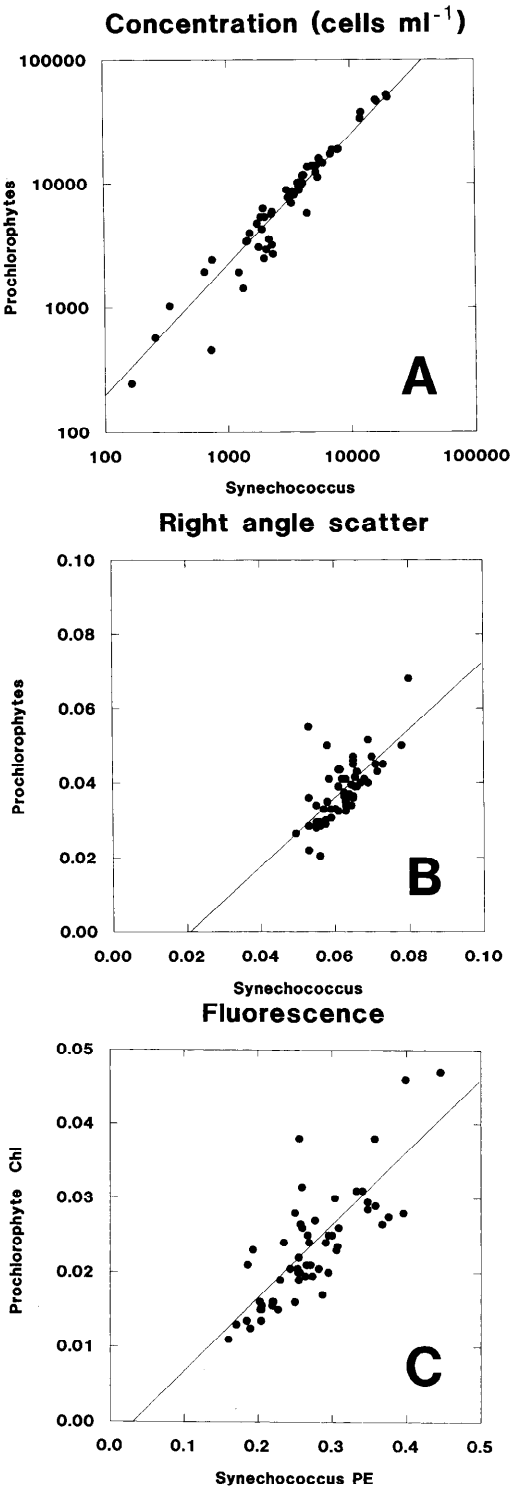

Fig. 4. Relation between prochlorophytes and *Synechococcus*. A. Concentrations (log scales), $y = 1.6x^{1.0}$ ($r = 0.96$, $n = 65$, $P < 0.001$). B. Right-angle scatter, $y = 0.92x - 0.02$ ($r = 0.69$, $n = 57$, $P < 0.001$, samples with <250 cells counted not included). C. Pigment fluorescence (phycoerythrin for *Synechococcus*, chlorophyll for prochlorophytes), $y = 0.098x - 0.003$ ($r = 0.80$, $n = 57$, $P < 0.001$, samples with <250 cells counted not included).

were significantly correlated with the same cellular parameters measured on *Synechococcus* (Fig. 4B,C), whereas the RALS and Chl fluorescence of prochlorophytes were only weakly correlated together ($r = 0.30$, $n = 57$, $P = 0.021$). These observed relationships imply a fairly constant ratio of prochlorophyte to cyanobacterial biomass equal to 1.5 in terms of Chl *a* and 0.6 in terms of C.

The western Mediterranean basin is oligotrophic (McGill 1965). This characteristic translates into low surface Chl levels in summer (0.05–0.5 μg liter^{-1}) and the appearance of spatially and temporally localized DCM (e.g. Coste et al. 1977). Several peculiarities, however, make the Mediterranean Sea stand apart from the oligotrophic areas of the Atlantic and Pacific Oceans. Deep convective motions affect the middle of the northwestern Mediterranean basin in winter and induce large fluxes of nutrients to the surface layer, causing massive phytoplankton blooms and annual maxima of productivity in late winter–early spring (Jacques et al. 1973). Nutrient-rich rivers such as the Rhône or the Ebro fertilize the northern margins (Coste et al. 1977).

The occurrence of prochlorophytes in the Mediterranean Sea at widely different stations indicates that these organisms are able to survive and probably proliferate in various environmental conditions, some resembling those where they have been reported previously (Chisholm et al. 1988; Li and Wood 1988; Neveux et al. 1989), but others are quite different, including vertically mixed waters and river plumes. In the latter case, it is not clear whether these organisms are able to grow in low-salinity waters or are brought there by simple mixing of coastal populations, as the linear relation between cell concentration and salinity in the Rhône estuary suggests (data not shown). Their estimated contribution to the total photosynthetic biomass (8% in terms of Chl), although small, is not negligible. Within the procaryotic phytoplankton, their importance is comparable to cyanobacteria, previously assumed to dominate the Mediterranean picoplankton (Hagström et al. 1988), since they account for ~60% of procaryotic Chl and 40% of procaryotic photosynthetic carbon. It is likely that this contribution is increased in summer when a DCM develops offshore.

The observation of high prochlorophyte concentrations in surface waters contrasts also with initial observations which detected them only at depth (Chisholm et al. 1988; Gieskes et al. 1988; Li and Wood 1988; Neveux et al. 1989). These surface populations are characterized by a lower fluorescence yield, a lower Chl *b* : Chl *a* ratio, and a higher zeaxanthin-lutein : Chl *a* ratio than DCM populations. These indices suggest photoadaptative (SooHoo et al. 1986) and photoprotective (Kana et al. 1988) mechanisms in response to surface light levels. Throughout the investigated area, surface NO_3^- concentrations were of the order of 0.1–3 μM, with higher values near the Rhône estuary (Woodward and Owens 1989), in contrast to typical values encountered in the surface layer of the Atlantic and Pacific Oceans, which are below the level of detection of classical analytical methods. This difference probably allows prochlorophytes to proliferate in winter in Mediterranean surface waters.

Another intriguing observation is the strong correlation between the abundances and cell properties of prochlorophytes and those of *Synechococcus*. At this period of the year, water-column-integrated *Synechococcus* concentrations are regulated by temperature, while their detailed vertical distribution is controlled by density stratification (Vaulot and Partensky 1989). *Synechococcus* light scatter is thought to be regulated by nutrient status and growth rate (Glibert et al. 1986), but pigment fluorescence is essentially a function of light conditions (Olson et al. 1990). If prochlorophytes respond to similar controls, then the correlations we observed between prochlorophytes and cyanobacteria would mean that both types of organisms responded similarly in winter to various factors, including temperature, nutrient status, and light. The fact that prochlorophytes are also observed in low-light environments such as the DCM, where *Synechococcus* is usually absent (Olson et al. 1990), suggest that the former may have more ecological plasticity with respect to their light requirement. In contrast, *Syn-*

echococcus might more easily adapt to very low nutrient levels, probably because of its capacity to store N (Wyman et al. 1985), explaining its success in oligotrophic surface waters.

The sharp differences in habitats between the populations of the Mediterranean Sea and the Atlantic and Pacific Oceans lead to the question whether they belong to different taxa. This hypothesis could find some support in the multiple forms that prochlorophytes seem able to assume (endosymbiotic, filamentous, coccoid), as well as in the chemical disparity of the Mediterranean Sea with respect to other oceanic areas, such as its high inorganic N:P ratio (McGill 1965). In contrast, if they belong to similar taxa, this variability could express plasticity on a seasonal scale, as the recent observation of prochlorophytes in winter in Sargasso Sea surface waters (Olson et al. in press) suggests.

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A high-sensitivity flow cytometer for studying picoplankton

Abstract—We developed a relatively small, inexpensive, laser-based flow cytometer suitable for studying marine picoplankton. The instrument uses an air-cooled He-Cd laser with low power consumption and a commercially available, square quartz flow cell. Sensitivity and resolution are sufficient to detect marine prochlorophytes in forward light scatter and autofluorescence and marine bacteria in forward light scatter and stained DNA fluorescence.

The technology of flow cytometry was introduced into oceanography less than a decade ago (Yentsch et al. 1983; Olson et al. 1983). Most instruments currently in use were designed for biomedical research and clinical applications and have shortcomings for oceanography. Large commercial instruments have been modified to analyze picoplankton and marine bacteria (Robertson and Button 1989; R. Olson pers. comm.), but such analysis usually requires the use of high-power (usually 208 V, 50 amp, 3 phase lines are necessary), water-cooled lasers. The power and water requirements as well as the large size of these instruments make shipboard use inconvenient. Furthermore, the high cost of purchasing and maintaining these instruments usually precludes ownership by a single investigator.

Smaller and more portable clinical flow cytometers use low-power, air-cooled lasers, but they lack the flexibility and sensitivity needed in oceanographic research. Changing optical filters or lasers in these

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