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## SHORT COMMUNICATION

### ***Synechococcus* and *Prochlorococcus*-like populations detected by flow cytometry in a eutrophic reservoir in summer**

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**Abstract.** Particles with characteristics similar to marine free-living prochlorophytes have been detected by flow cytometry during summer in a eutrophic reservoir in the south of Spain. The *Prochlorococcus*-like particles showed a vertical distribution similar to *Synechococcus*. Both populations displayed a subsurface maximum at 5 m.

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Abundant populations of marine free-living prochlorophytes were first identified in water samples from the North Atlantic and Pacific with the help of flow cytometry (Chisholm *et al.*, 1988). Since then, many other researchers have reported the presence of this group of prokaryotic picoplankton in the euphotic layer of the world's tropical and temperate oceans [Chisholm *et al.* (1992) and references therein]. After isolation, culture, and genetic and ecophysiological characterization, they were named *Prochlorococcus marinus* (Chisholm *et al.*, 1992). Flow cytometry has been essential in the discovery of this organism (Chisholm *et al.*, 1988), in the realization of its ubiquitous presence in the ocean (Olson *et al.*, 1990; Vaultot *et al.*, 1990; Veldhuis and Kraay, 1990; Chavez *et al.*, 1991) and in the assignment of its quantitative role as a primary producer in different geographical locations (Campbell *et al.*, 1994; Li, 1994; Vaultot *et al.*, 1995). This was due to the unmatched capacity of flow cytometry to analyse small cells in terms of sensitivity and time, and to the fact that both *Synechococcus* and *Prochlorococcus* showed a very characteristic signature when analysed by flow cytometry.

So far, free-living prochlorophytes have not been reported to occur in fresh water. In this study, we report the presence of small coccoid cells with red auto-fluorescence (as seen in fluorescence microscopy) in La Concepción, a eutrophic reservoir in the south of Spain. This cell population presented a flow cytometric signature strikingly similar to that of the marine free-living prochlorophyte and co-occurred with *Synechococcus* in the same period of the annual stratification cycle.

The freshwater samples were obtained in the course of an annual study in La Concepción reservoir, located in the south of Spain (36°32'N, 45°63'W). La Concepción is a eutrophic reservoir with a capacity of 61 hm<sup>3</sup> and a surface area of 2.14 km<sup>2</sup>. During this study, its maximum depth was 40 m. This mass of water has been defined as warm monomictic (Lucena and Rodríguez, 1984). A single sampling station, located at the deepest area, was chosen to monitor a number of

ecological variables during the stratification cycle in 1993. Water samples were collected at about noon by means of 5 l Van Dorn bottles triggered at the surface, Secchi disc depth (SD), at  $2 \times$  SD, around the maximal temperature gradient, at the middle depth of the hypolimnion and near the bottom. Temperature and dissolved oxygen concentration were measured throughout the entire water column by means of a YSI-GRANT 3800 water quality logger.

Water samples were taken as quickly as possible to the laboratory and processed (1–2 h after collection). For chlorophyll determination, 500 ml of water were filtered on Whatman GF/F glass fibre filters. Pigment was extracted in darkness, in  $\text{MgCO}_3$  neutralized acetone for 18–24 h at  $4^\circ\text{C}$ . The chlorophyll concentration was measured spectrophotometrically (Beckman DU-7) according to Talling and Driver (1963).  $\text{NO}_3^-$  was measured by a TRAACS 800 (Bran and Luebbe) autoanalyser in water samples filtered by Whatman GF/F and stored frozen ( $-20^\circ\text{C}$ ) until analysis. The protocol used was based on Wood *et al.* (1967).

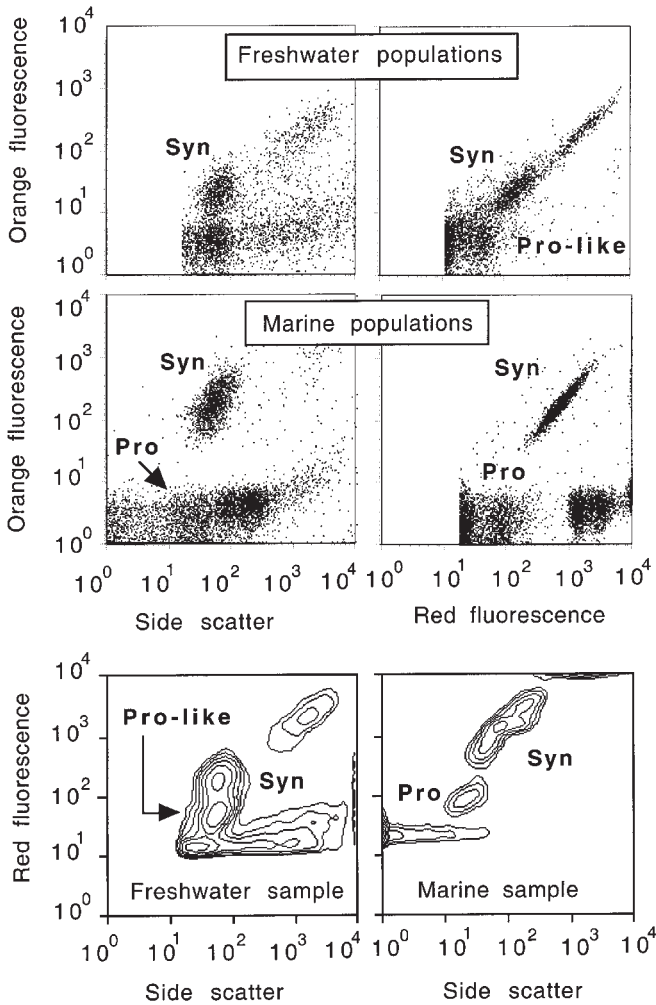
Freshwater samples for flow cytometry were handled as follows: 3 ml of water were fixed with 2% glutaraldehyde and immediately stored in liquid  $\text{N}_2$  until their analysis 1–2 weeks later (Vaulot *et al.*, 1989). Samples (freshwater and marine) were analysed in a FACSCAN flow cytometer (Becton Dickinson). The marine sample shown in this study for comparison is considered a typical one; it was obtained from 5 m depth in an oceanographic cruise in the Almería–Oran front (December 1996, Discovery cruise, Omega project, MAST, EC). However in this case, the sample was not fixed and was analysed, on board, immediately after extraction from the continuous flow of the ship. Both samples were analysed with the same gains (FSC = E01, SSC = 400, FL1 = 555, FL2 = 555, FL3 = 650) and red fluorescence was used as threshold parameter. *Prochlorococcus*-like and *Synechococcus* populations were enumerated using a three-dimensional gate based on red fluorescence versus side scatter, orange fluorescence versus side scatter and orange fluorescence versus red fluorescence plots. Only particles that appear in the selected regions in these three plots were counted as *Prochlorococcus*-like or *Synechococcus*.

The pigment composition of marine *Synechococcus* and *Prochlorococcus* produces a very characteristic signature in flow cytometry, which permits easy identification (Chisholm *et al.*, 1988; Li, 1989; Olson *et al.*, 1990; Vaulot *et al.*, 1990). Both cell populations present a similar level of both forward light scatter and side scatter, although *Synechococcus* tends to give a slightly higher signal. However, *Synechococcus* cells are easily recognized by the large orange fluorescence due to their phycobilins, compared to prochlorophytes, which lack it. During the maximum stratification period, we have identified in samples from the La Concepción reservoir two populations with a similar signature to that of marine *Synechococcus* and *Prochlorococcus* when analysed by flow cytometry (Figure 1). Both populations appeared in almost the same relative positions in the bivariate plots obtained from the marine and freshwater samples. Both populations presented almost identical levels of side scatter in both samples. However, the orange and red fluorescence levels per cell were higher in the marine sample (Figure 1). Although both samples were analysed using the same flow cytometer and the same gains, the marine sample was analysed on board immediately after

its collection, while the freshwater sample was fixed and stored in liquid N<sub>2</sub> until its analysis. Besides differences in the treatment prior to the analysis which may affect the signal level, taxonomic differences might exist between the freshwater and the marine populations and/or differences in their physiological states. Photoadaptation and nitrogen limitation have been reported to affect fluorescence and side scatter (Vaulot *et al.*, 1990). *Synechococcus* has been previously reported to occur in freshwater environments (McMurter and Pick, 1994; Maeda *et al.*, 1992); however, as far as we know, the *Prochlorococcus*-like population which co-occurs in La Concepción with *Synechococcus* has never been reported before. Fahnenstiel *et al.* (1991) reported a population of red-fluorescing phototrophic picoplankton (RFPP) in the size range of cyanobacteria in lakes Huron and Michigan. However, they were able to prove that RFPP was a population of small eukaryotic cells. In the work of Fahnenstiel *et al.* (1991), RFPP show a level of red fluorescence similar to *Synechococcus*, or even larger. This is a major difference with our *Prochlorococcus*-like population; they always show less red fluorescence than *Synechococcus*, as do the marine prochlorophytes (Figure 1). Ecological differences also exist that will be discussed later. Interestingly, Vaulot *et al.* (1990) reported the presence of *Prochlorococcus* in the low-salinity dilution zone of the Rhône River at salinities as low as 1.2‰. To date, three genera of prochlorophytes have been described: *Prochloron* (symbiotic), *Prochlorococcus* and *Prochlorothrix*. Only *Prochlorothrix* is known to grow in fresh water (Burger-Wiersma *et al.*, 1986); however, they form unbranched trichomes of cylindrical cells (3–15 µm long with a diameter of 0.5–3 µm). Even isolated cells of these colonies would probably produce a flow cytometric signature very different from the one described here (Figure 1).

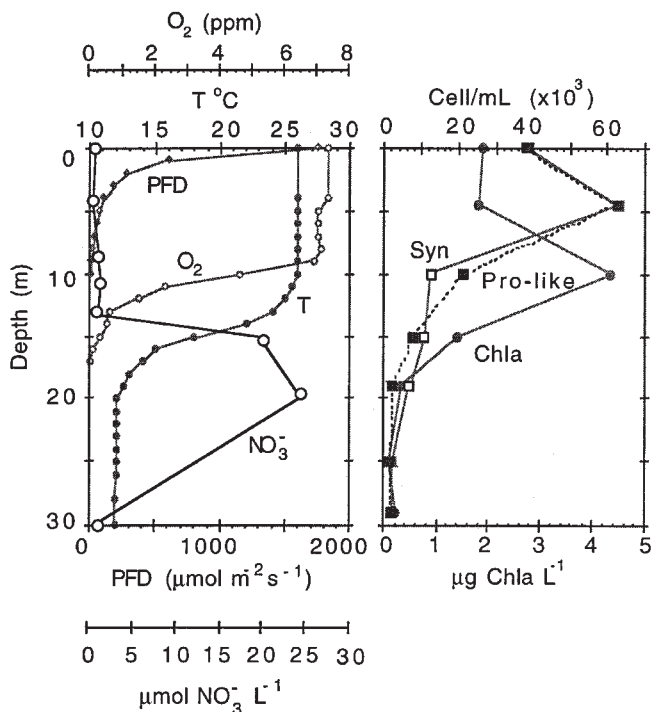
The evidence presented here is based on their flow cytometry signature and therefore it is not possible to ensure unambiguously the presence of *Prochlorococcus* in this reservoir. However, the flow cytometric signature of both *Synechococcus* and *Prochlorococcus* is considered to be very characteristic, mainly when both populations appear in the same sample due to the relative level of their orange and red fluorescence and side scatter signals (Li, 1989; Campbell and Vaulot, 1993). If the *Prochlorococcus*-like population found in this reservoir turns out to be an entirely different type of cell, we should be careful in the general 'identification' of *Prochlorococcus* presence in marine samples when this is only based on its flow cytometric signature.

Both cell populations appeared in summer, when the water column was highly stratified. In this respect, they resemble marine *Prochlorococcus* and *Synechococcus*, which are more abundant in highly stratified waters in summer (Chisholm, 1992). *Synechococcus* started to appear from the beginning of July; however, we only have evidence of the presence of the *Prochlorococcus*-like population from 2 August when we decided to increase the sensitivity of our cytometer by increasing its gains to detect *Synechococcus* better. From this date on, we analysed the water samples using the gains reported in this study. We could detect abundant populations of both *Synechococcus* and *Prochlorococcus*-like cells until 16 September. For the following sampling date (4 October), both populations have already disappeared. However, RFPP were found to be abundant throughout the



**Fig. 1.** Representative bivariate plots in arbitrary units of orange fluorescence versus side scatter, orange fluorescence versus red fluorescence and red fluorescence versus side scatter (contour plot) of phytoplanktonic assemblage analysed by flow cytometry in La Concepción reservoir during August (1993). Two cell populations with a flow cytometric signature similar to that of marine *Synechococcus* (Syn) and *Prochlorococcus* (Pro) were present. For comparison, we show a typical marine sample collected from the surface in the Almería–Oran front (December 1996, Discovery cruise, Omega Project, MAST-III, EU). Both samples were analysed using the same flow cytometer (FACSCAN) and the same gains; however, the marine sample was analysed on board immediately after its collection, while the freshwater sample was fixed and stored in liquid N<sub>2</sub> until its analysis.

year with maximum abundance during the late spring period of isothermal mixing to the onset of thermal stratification (Fahnenstiel *et al.*, 1991). The typical ecological conditions in which the *Synechococcus* and the *Prochlorococcus*-like population were found in La Concepción are shown in Figure 2. The water



**Fig. 2.** Vertical profiles of temperature, oxygen, photon flux density (at noon), nitrate, chlorophyll *a* and the *Synechococcus* and *Prochlorococcus*-like populations in La Concepción in August (1993).

column was highly stratified, with a constant water temperature of 26°C in the epilimnion. The hypolimnion remained anoxic during a large fraction of the stratification cycle. The nitrate concentration was below 1 μmol l<sup>-1</sup> (Figure 2), and NO<sub>2</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup> and PO<sub>4</sub><sup>3-</sup> were undetectable or close to their detection limit in the epilimnion (results not shown). On 18 August, the *Synechococcus* and *Prochlorococcus*-like populations showed similar cell densities and vertical distributions. Maximum cell densities (60 × 10<sup>3</sup> cells ml<sup>-1</sup>) were found at 5 m (4% of incident photon flux density) and did not coincide with the subsurface chlorophyll maximum located just above the thermocline (Figure 2). Microscopic observation revealed that the diatom *Melosira granulata* and several species of Cryptophyta were largely responsible for this subsurface chlorophyll maximum (García-Ruíz, 1995).

*Prochlorococcus* production represents a variable (5–82%) but important fraction of total primary production in different areas of the world's oceans (Campbell *et al.*, 1994; Li, 1994; Vaulot *et al.*, 1995; Liu *et al.*, 1997). The *Prochlorococcus*-like population in La Concepción seems to be present only during summer, but according to their cell densities they may account for a significant, so far neglected, fraction of primary production during stratification in freshwater ecosystems. An effort should be made to look for this population of

cells in different freshwater environments and to isolate and unambiguously identify them.

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