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Possible bloom of free trichomes in the Bay of Marseille, NW Mediterranean Sea: an anomaly evidenced by flow cytometry

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The composition of ultraphytoplankton (<10 μm) sampled in surface waters (1 m depth) was investigated during summer 2010 in the Bay of Marseille (NW Mediterranean) using flow cytometry. In addition to groups of *Synechococcus*, *Prochlorococcus*, picoeukaryotes and nanoeukaryotes, an unidentified additional cluster was observed. The particles forming this cluster had a high abundance ($>7 \times 10^4$ events mL^{-1}), with both orange and red fluorescence intensities like *Synechococcus*, but with a size signal larger than that of 2 μm fluorospheres (beads). These unknown particles were sorted out by flow cytometry and then observed by epifluorescence microscopy: they appeared to be chain-forming microorganisms, just like trichomes of some diazotroph cyanobacteria with one heterocyst, but not unambiguously distal. The chains observed after cell sorting were not straight, but rather folded. To our knowledge, this is the first report of a possible bloom of such free trichomes. The small sample volume available for the delayed analysis and the limited resolution of our photomicrography did not allow for species identification. The brief occurrence of free trichomes in the Bay of Marseille with such a high abundance remains to be explained. High temperature in summer, induced stratification of the water column and nitrate depletion may be related to this event. High-frequency surveys of ultraphytoplankton assemblages at the single cell level appear to be necessary to observe such phenomenon more efficiently and document their dynamics.

KEYWORDS: ultraphytoplankton; trichomes; Bay of Marseille; flow cytometry; cell sorting

INTRODUCTION

Marine phytoplankton is the major component responsible for primary production. Although it represents only 2% of global biomass, it is responsible for about 50% of global primary production. In coastal areas, it can represent up to 30% of the global oceanic primary production (Gattuso *et al.*, 1998). Phytoplankton is thus at the very basis of the trophic network, and fuels the

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entire ecosystem. In marine environments, nitrogen is a limiting factor for phytoplankton growth in coastal and oceanic waters (Capone, 2008). Diazotrophs are microorganisms capable of fixing atmospheric nitrogen (N_2) dissolved in the water into a more usable form (such as ammonia for instance). As a consequence, they are able to grow without external sources of fixed nitrogen (i.e. nitrates, nitrites). They also support the non-diazotrophic phytoplankton community and primary production by releasing this fixed nitrogen into the marine environment, and thus play an important role in oligotrophic environments. For instance, in tropical waters, the non-heterocystous cyanobacterium *Oscillatoria* (previously identified as *Trichodesmium*) is largely responsible for nitrogen fixation (Carpenter and Romans, 1991; Karl *et al.*, 2002). The diazotroph heterocystous cyanobacteria *Richelia* spp. and *Calothrix* spp. also provide substantial inputs of nitrogen in tropical and subtropical oceans (Carpenter *et al.*, 1999; Foster *et al.*, 2008).

Richelia spp. and *Calothrix* spp. have morphologically similar filaments (called trichomes), comprising several vegetative cells and one terminal heterocyst. They segregate nitrogen fixation spatially from photosynthesis, fixing dissolved atmospheric N_2 during daytime. *Richelia* spp. and *Calothrix* spp. have been usually found growing either as a common endosymbiotic association within several diatom genera including *Rhizosolenia, Guinardia* and the more ubiquitous *Hemiaulus*, or, less frequently, as an episymbiont attached to *Chaetoceros* spp. (Venrick, 1974; Villareal, 1991; Foster *et al.*, 2008; Zeev *et al.*, 2008). The differences between *Richelia* and *Calothrix* trichomes concern their length, location, number per diatom host and phylogeny (Jason *et al.*, 1999; Foster and Zehr, 2006; Foster *et al.*, 2011). *Richelia* is considered to be the most widespread heterocystous cyanobacteria in marine pelagic environments (Jason *et al.*, 1999). The presence of *Richelia* has been widely reported in the Atlantic Ocean (Villareal, 1991, 1994; Carpenter *et al.*, 1999; Foster *et al.*, 2007), Pacific Ocean (Mague *et al.*, 1974; Jason *et al.*, 1999; Gómez *et al.*, 2005), Caribbean Sea (Janson *et al.*, 1995, Jason *et al.*, 1999) and Northern Arabian Sea (Padmakumar *et al.*, 2010). In the Mediterranean, *Richelia* was also been reported by Zeev *et al.* (Zeev *et al.*, 2008) and Crombet *et al.* (Crombet *et al.*, 2011) in recent years. To the best of our knowledge, only one report (Gómez *et al.*, 2005) mentioned *Richelia* in a free state, and this was in the western Pacific Ocean.

In the present paper, we report a brief occurrence of abundant-free trichomes in July 2010 in the Bay of Marseille (NW Mediterranean Sea). They were detected by flow cytometry and then observed by epifluorescence microscopy after flow cytometry cell sorting. Due to a small sample volume (about 1.5 mL) available for the delayed analysis, a clear identification has not been possible even though independent experts in phytoplankton have been contacted. However, to our knowledge, this is the first time that free trichomes have been observed in such high abundance. The possible conditions leading to such a burst of abundance are discussed.

METHOD

Study site and sampling

The study site (Fig. 1) is located in the Gulf of Lion, near Marseille, France (NW Mediterranean Sea). Sampling was performed at the long-term coastal observation station SOFCOM (43°14′30″N, 5°17′30″E) which is part of the French national monitoring program (SOMLIT, Service d'Observation en Milieu LITtoral) of the coastal environment initiated and conducted by the INSU/CNRS. The survey station is located between the Frioul and Maïre islands and has a water column of 60 m depth.

Seawater was collected from the surface (~1 m depth) during daytime (in the morning) with a Niskin Bottle (12 L). According to the protocol defined by the SOMLIT network, one subsample (1.5 mL) was fixed onboard with Glutaraldehyde (1% final concentration) (Marie *et al.*, 2000a, b), kept in an ice box and then stored in liquid nitrogen in the laboratory until flow cytometry analysis a few months later.

Temperature and salinity were measured *in situ* with a conductivity temperature depth profiler (SBE, Sea-Bird Electronics). Seawater samples were also collected for nutrients (NO_3^-, NO_2^- and PO_4^{3-}) with 100 mL polyethylene flasks and analysed with a Technicon autoanalyzer (Technicon III, Brian and Luebbe, Axflow) according to Tréguer and LeCorre (Tréguer and LeCorre, 1975). Samples for NH_4^+ were collected in 100 mL glass flasks and concentrations were determined by absorption spectrometry (UV 160A, Shimadzu). Total chlorophyll *a* concentrations were determined by spectrofluorometry (Neveux and Panouse, 1987) on a 10.005R, Turner Designs spectrofluorometer after filtering 1 L of seawater on GF/F filter and acetone extraction.

Meteorological data (temperature, rainfall) were recorded at the Meteorological station of the MIO (formerly COM) installed on the Frioul Island (43°15′59.85″N, 5°17′38.06″E; altitude: 25 m), at about 3 km from the sampling station.

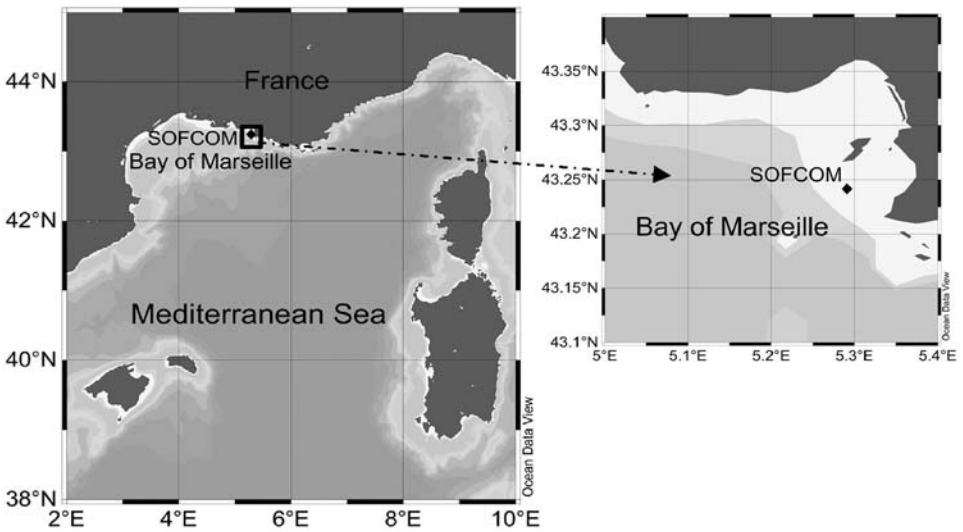

Fig. 1. Location of the sampling station (SOFCOM) in the Bay of Marseille, north western Mediterranean Sea.

Flow cytometry

Ultraphytoplankton (<10 μm) analyses at the single cell level were performed at the Regional Flow Cytometry Platform for Microbiology (PRECYM, http://precym.com.univ-mrs.fr/) with a BD Influx™ Mariner (BD Biosciences) high-speed cell sorter equipped with three laser lines (488, 561 and 355 nm). In the present study, ultraphytoplankton cells were only excited with the 200 mW solid state 488 nm laser (Coherent Sapphire) and characterized by five optical signals: forward scatter (FSC) related to cell size, side scatter (SSC) related to cell structure and shape, green fluorescence (FLG, $510 < \lambda < 550$ nm), orange fluorescence (FLO, $565 < \lambda < 595$ nm) related to phycoerythrin and red fluorescence (FLR, $\lambda > 630$ nm) related to chlorophyll *a*. Before analysis, seawater samples were thawed at room temperature (about 20 min) in the dark. A nozzle with a 100 μm orifice (diameter) was used, and a 27 psi sheath fluid (0.5 × PBS in the distilled water) pressure was applied. The sample was injected into the sheath vein with a sample pressure of 29 psi. For each sample analysed, 960 μL of seawater were supplemented with 10 μL of 2 μm (Fluoresbrite YG, Polyscience) and 30 μL of Trucount™ bead solution (500 μL of distilled water per Trucount™ tube, Becton Dickinson). The beads were used both as an internal standard and to control the flow rate. In addition, the sample flow rate was determined for each set of analyses by weighing the sample uptake three times during a 5 min analysis under the same experimental setting.

All data were collected in log scale, and stored in list mode files using the Spigot™ software (BD Biosciences), and then analysed with the Summit™ software (Dakocytomation).

Epifluorescence microscopy

Particles of interest were sorted by flow cytometry directly on a microscope slide, and then immediately observed on the Olympus (BX61) epifluorescence microscope of the PRECYM platform, equipped with a 40× objective and a cooled CCD digital camera (RETIGA SRV, QImaging, USA). Images were recorded by using ImagePro+ software (MediaCybernetics, USA).

Phytoplankton taxonomy and enumeration

Seawater samples, collected for taxonomic composition of phytoplankton, were fixed with a neutral Lugol's solution and analysed under an inverted binocular microscope after sedimentation of an appropriate volume in settling chambers following the classical Utermöhl technique (Lund *et al.*, 1958; Hasle, 1978). The sample volume analysed was adapted to the cell concentrations, generally settling chambers of 100 mL, considering the oligotrophic conditions prevailing in Marseille nearshore waters.

Four hundred individuals were systematically counted for the most abundant species as recommended by Uehlinger (Uehlinger, 1964). Taxa were identified at the species level when possible by optical microscopy.

RESULTS AND DISCUSSION

Flow cytometry analysis of seawater from the Bay of Marseille usually resolved four ultraphytoplankton groups identified as *Synechococcus*, *Prochlorococcus*, picoeukaryotes and nanoeukaryotes as reported by Grégori *et al.* in a 2-year survey (Grégori *et al.*, 2001). However, in the present study and with the sampling protocol described above, *Prochlorococcus* was poorly detected as shown in Fig. 2A and C. The major differences between these two protocols are the fixative (1% glutaraldehyde instead of 2% paraformaldehyde, final dilution) and deep freezing schedule (onboard direct freezing in the Grégori *et al.* investigation, about 5 h later in the present case).

On 12 July 2010, the composition of the ultraphytoplankton assemblage, as determined by flow cytometry, was very unusual. Surprisingly, in addition to *Synechococcus*, picoeukaryotes and nanoeukaryotes, *Prochlorococcus* was clearly observed on that day (6.66×10^3 cells mL^{-1}, Fig. 2B), in contrast to the analyses run on samples from 28 June and 26 July 2010 (Fig. 2A and C), the previous and next sampling cruise, respectively. However, the most striking feature was the optical resolution by flow cytometry of an unknown cluster formed of highly concentrated ($>7 \times 10^4$ events mL^{-1}) particles exhibiting both orange and red fluorescence like *Synechococcus*, but in a far wider range of intensity (Fig. 2B), and a forward scatter signal (related to size) larger than that of 2 μm beads as shown in Fig. 3A. This unidentified group was immediately sorted out by using the Influx Mariner cell-sorting capacity and observed straight away by microscopy, despite the very low amount of sample (1.5 mL) initially available. Even

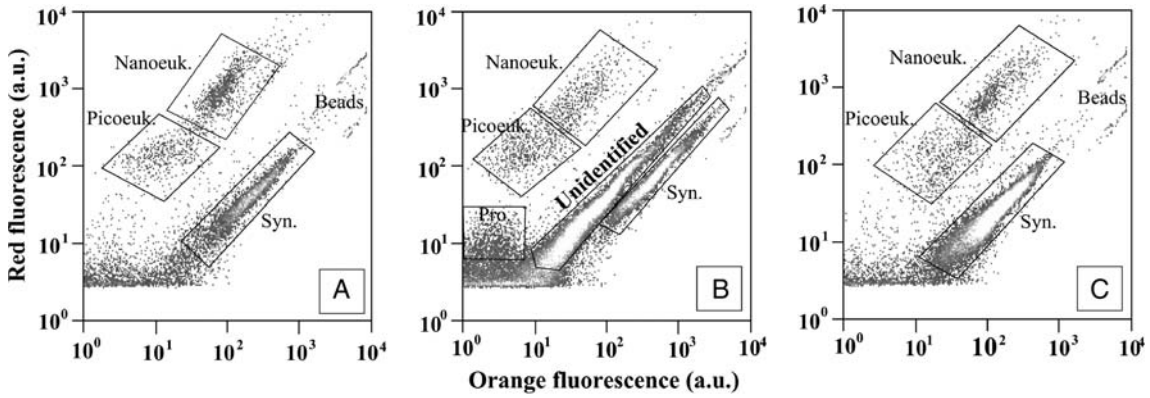

Fig. 2. Resolution by flow cytometry of the ultraphytoplankton assemblage composition in the Bay of Marseille. Panels (**A**) and (**C**) display cytogrammes of red fluorescence (chlorophyll *a*) versus orange fluorescence (phycoerythrin) resolving three cell groups: *Synechococcus* (Syn.), picoeukaryotes (Picoeuk.) and nanoeukaryotes (Nanoeuk.) in samples collected on 28 June and 26 July 2010, respectively. In panel (**B**), the cytogram red fluorescence versus orange fluorescence reveals the presence of *Prochlorococcus* (Pro.) and an additional and unidentified cell group in the sample collected on 12 July 2010.

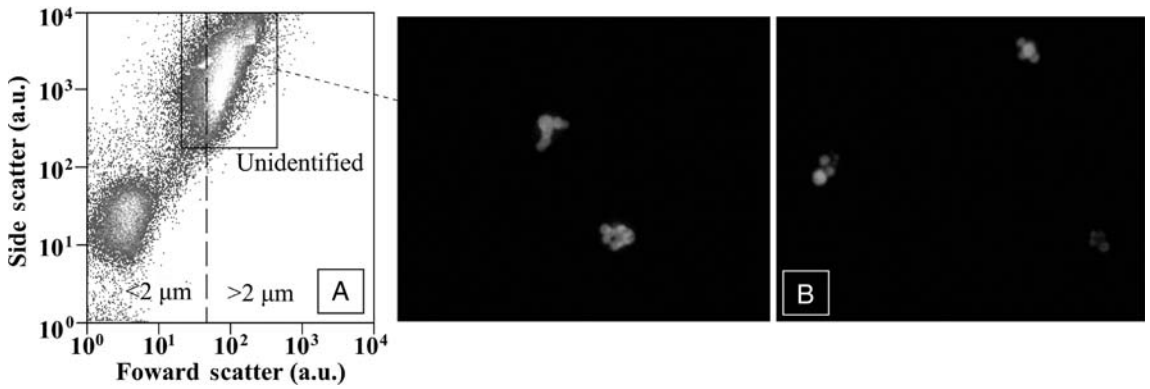

Fig. 3. Observation by epifluorescence microscopy of the unidentified group after flow cytometry sorting. Panel (**A**) side scatter (related to structure) versus forward scatter (related to size) cytogram for the sample collected on 12 July 2010. Particles composing the unidentified group exhibit a size signal larger than that of 2 μm beads. Panel (**B**) epifluorescence microscopy reveals that the sorted particles composing the unidentified group are small chains with one heterocyst that could be distal, specific features of free trichomes (magnification: objective ×40).

though the quality of the images is not perfect, the pictures obtained, like the ones shown in Fig. 3B, are consistent with chain-forming cells containing one heterocyst. They suggest that the sorted organisms are free trichomes. They are reminiscent of *Richelia*, known to form small chains with a terminal heterocyst, but from the available data we cannot unambiguously ascertain that the heterocysts of the free trichomes were in distal position. In addition, the sorted chains are not straight as they look like in symbiont trichomes (Zeev *et al.*, 2008; Padmakumar *et al.*, 2010), but rather folded. That might be explained by their free-state and the flow cytometry sorting procedure (hydrodynamic focusing and/or droplet formation). The size of the chains, the physiological state (i.e. the amount of pigments per cell) and the way they flow through the interrogation point of the flow chamber (folded or not, along various angles) can explain the wide range of orange and red fluorescence intensities observed (Fig. 2B). However, they cannot be considered clusters of *Synechococcus* as (i) they clearly display a heterogeneous cell which looks like a heterocyst (such as present in *Richelia*); (ii) clusters of *Synechococcus* would not appear at the same location on the flow cytometry plots (cytograms) but rather on the right side of the regular *Synechococcus* cluster, with higher intensities (several cells flowing together would appear as a "superparticle" with thus higher red and orange fluorescence intensities). However, due to the folded shape of the trichomes, it is not possible to prove that the heterocyst occupies a distal position, characteristic of *Richelia*. Furthermore, we do not think that the observed trichomes could be considered to be *Calothrix* cyanobacteria. *Calothrix* trichomes make chains much longer, with at least 10 vegetative cells and they are less present in surface water (Foster *et al.*, 2008). As shown in Fig. 3B, the trichomes observed contained less than 10 vegetative cells. They could not be considered Pleurocapsids either, since Pleurocapsids are essentially linked to fresh water. We also checked the pictures of pleurocapsalean cyanobacteria published by Waterbury and Stanier (Waterbury and Stanier, 1978), and could not find a similar picture.

To our knowledge, only Gómez *et al.* (Gómez *et al.*, 2005) have reported unattached filaments of *R. intracellularis*. They were in the western Pacific Ocean and were primarily considered recently released filaments from the plasmalemma surface of symbiotic diatoms lacking a frustule. Diatoms with plasmalemma bearing *R. intracellularis* filaments and lacking the frustule were also detected in the presence of free-living *R. intracellularis* in the same regions where the *Richelia-Chaetoceros* consortia were observed. The authors hypothesized that unattached filaments of *R. intracellularis* originating from *Rhizosolenia clevei* were able to colonize senescent colonies of *Chaetoceros* lacking epiphytic *R. intracellularis*. However, this interpretation was questioned by Foster and Zehr (Foster and Zehr, 2006) who suggested that Gómez *et al.* probably misidentified *Calothrix* as *R. intracellularis*. Janson *et al.* (Janson *et al.*, 1995) observed that *R. intracellularis* growing as an endosymbiont in *R. clevei* lacked gas-vesicles and concluded that the cyanobacterium cells might be unable to regulate their position in the water column and, therefore, should not be viable in the absence of a host.

In the present study, the amount of seawater sample (1.5 mL) assigned to flow cytometry analysis was used up by the analysis of picophytoplankton, the one of heterotrophic prokaryotes and then the sorting of this unidentified cluster for epifluorescence microscopy observation. Unfortunately, the flow cytometry analysis was carried out a few months after sampling. Consequently, it was not possible to perform further analysis to clearly identify the sorted species (by molecular biology techniques or electron microscopy for instance). The poor quality of the photomicrography achieved immediately after sorting could only suggest the presence of free trichomes with what looks like a heterocyst but could not support an unambiguous identification. It remains unclear why free trichomes developed in the Bay of Marseille with such a high abundance (7.05×10^4 events mL^{-1}). We also compared the sample collected 12 July with samples collected on 28 June (the previous sampling cruise) and 26 July (the next sampling cruise). The cluster made of free

Table I: Physical, chemical and biological variables determined before, during and after the possible bloom of free trichomes

	Before bloom 28 June 2010	Bloom 12 July 2010	After bloom 26 July 2010
Temperature (°C)	22.16	24.72	15.18
Salinity	37.81	37.61	37.99
O_2 (mL L^{-1})	5.09	4.97	5.51
NO_3^-(μM)	0.38	0.02	0.1
NO_2^- (μM)	0.09	0	0.03
NH_4^+ (μM)	0.32	0.33	0.02
PO_4^{3-} (μM)	0.01	0	0
SiO_4^{2-} (μM)	1.37	1.6	1.51
NOP (μg L^{-1})	18.08	13.53	17.12
Chl *a* (μg L^{-1})	0.72	0.48	0.31
Synechococcus (cells mL^{-1})	12356	25886	68756
Prochlorococcus (cells mL^{-1})	nd	6662	nd
Picoeukaryotes (cells mL^{-1})	1216	2286	1097
Nanoeukaryotes (cells mL^{-1})	2441	925	2047

nd: not detected.

trichomes is apparent only on 12 July, tending to prove that the possible bloom of free trichomes was brief. All the available physical, chemical and biological features collected before, during and after the event are reported in Table I.

All the samples were taken in the morning. Surface temperature was 22.16, 24.72 and 15.18°C, respectively, and the variation of salinity between the three dates was less than 1%. A large decrease in nitrate concentration (from 0.38 down to 0.02 μM, Table I) coincided with the possible bloom of free trichomes and the occurrence of *Prochloroccoccus*, the doubling of *Synechococcus* and picoeukaryote abundances and a 2.6-fold decrease in nanoeukaryote concentration (Table I). The depletion of nitrate thus shifted the composition of the ultraphytoplankton assemblage towards cyanobacteria and picoeukaryotes.

Table II: Abundance of diatom species before, during and after the possible bloom of free trichomes

Diatom species	Before bloom 28 June 2010	Bloom 12 July 2010	After bloom 26 July 2010
Guinardia delicatula (cells L^{-1})	0	0	40
Rhizosolenia styliformis (cells L^{-1})	0	20	0

Diatoms were not abundant during the study (Table II). Only *Rhizosolenia* (20 cells L^{-1}) was found on 12 July and *Guinardia* (40 cells L^{-1}) was observed on 26 July. To assign the presence of the observed trichomes to their release after lysis of their diatom host, known to contain 1 or 2 trichomes only (Zeev *et al.*, 2008), would require an initial diatom abundance of about 3.5×10^4cells mL^{-1} which is unrealistic.

The presence of the free trichomes did not last for long. This can be easily accounted for by their basic nature as symbionts, not suited for living outside their host. Indeed, the possible bloom of free trichomes was very brief since no free trichomes were detected 2 weeks before the bloom, nor 2 weeks after (Fig. 2). According to the meteorological data (wind speed and direction) (Fig. 4A), there was no wind around 12 July, which favoured a temperature increase and a stronger stratification (less than 5 m) (Fig. 4B). A few days later, a

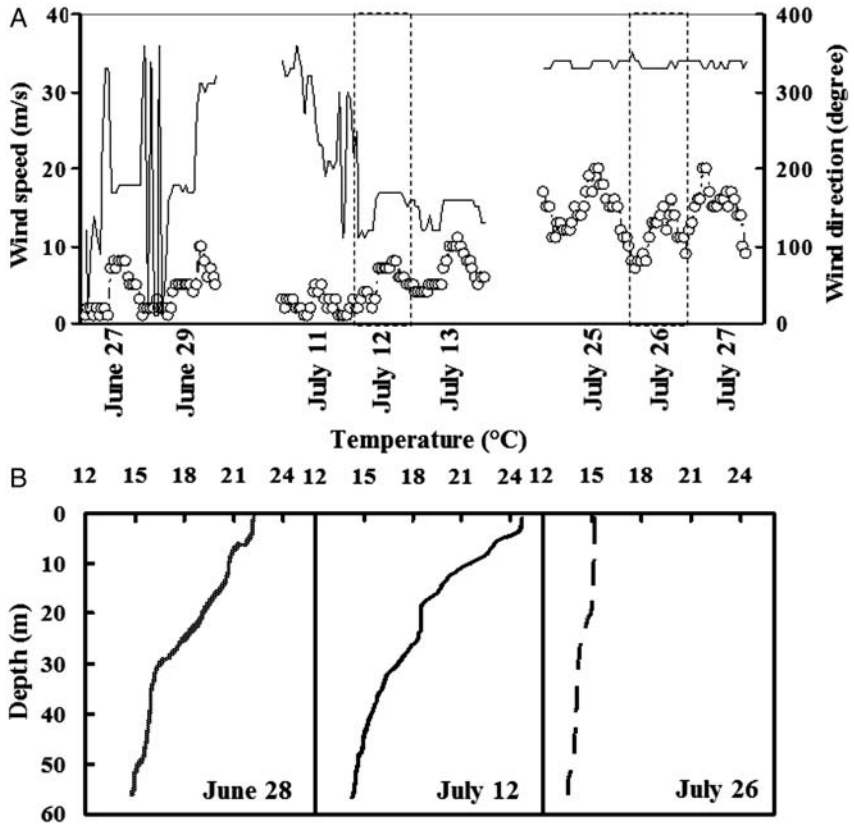

Fig. 4. (**A**) Wind speed (dots) and direction (line); (**B**) vertical temperature at the SOFCOM station in the Bay of Marseille in summer 2010.

strong (between 10 and 20 m.s^{-1}) and persistent wind from the north occurred and lasted well after 26 July. With such an intensity, this wind ("Mistral") disrupted the thermohaline stratification and resulted in a homogenized water column as illustrated by Fig. 4B (temperature profile). By blowing from the north, the Mistral pushed the surface water further south and it was replaced by colder deep water (as demonstrated by the 10°C drop in temperature on 26 July (Fig. 4B)). This might explain why the free trichomes were not detected at the SOMLIT station in late July.

In contrast, the coincidence of the free trichomes in large abundance with nitrate depletion (Table I) and a bloom of *Prochlorococcus* (Fig. 2B) may suggest that specific conditions linked to the oligotrophic nature of the bay (Travers and Travers, 1972), long periods of high temperature particularly in summer, might induce such a bloom in the Bay of Marseille. The diazotrophy would provide a clear advantage to the trichomes over other phytoplankton species dependent on the inorganic nitrogen availability in the medium.

The possibility of a fresh water origin for the observed trichomes is very unlikely. Indeed, there is no river flowing out in the Bay of Marseille. The outflow of the Rhône River, west of Marseille, is moved westward by the Ligurian current. Only strong and lasting north wind can reverse the Rhône river outflow eastward, which was not the case on 12 July (Fig. 4A). There was also no storm with flash floods in the month preceding the 12 July sampling. From the recordings of the Meteorological station on the Frioul Island in the Bay of Marseille, the closest heavy rain observed before 12 July occurred on 15 June as shown on Fig. 5.

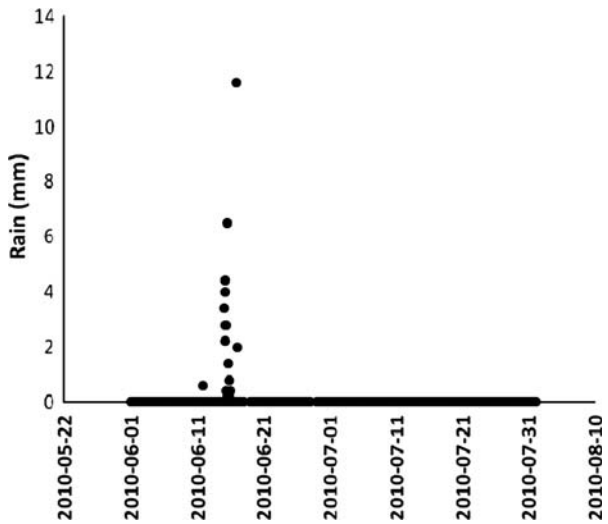

Fig. 5. Rainfall recorded at the Meteorological station of the MIO on Frioul Island, during the study period.

The fact that such large abundance of free trichomes has not been reported before is very likely due to the short duration of this phenomenon. According to this study, it occurs as a sporadic and sudden event. To better monitor such occurrences requires high-frequency observation. Regarding ultraphytoplankton assemblages, high-frequency monitoring techniques at the single cell level are now available and should be more widely applied to detect and understand such phenomenon (Dubelaar and Gerritzen, 2000; Thyssen *et al.*, 2008, 2010).

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