Phyto- and bacterioplankton abundance and viability and their relationship with phosphorus across the Mediterranean Sea

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ABSTRACT: In a synoptic cruise performed during early summer, the composition and viability of pelagic communities of the Mediterranean Sea were studied across the different sub-basins and straits in relation to nutrient regimes and hydrological conditions. The picoplankton fraction dominated the pelagic community across the study region. Bacterioplankton was the most abundant (mean $\pm$ SE = $7.73 \pm 0.39 \times 10^5$ cells ml⁻¹) component of the plankton, reaching the highest abundance in the Marmara and Black Seas, and its abundance was positively related to phosphate concentration. *Synechococcus* spp. was abundant ($3.70 \pm 0.87 \times 10^4$ cells ml⁻¹), and was most frequent in the surface layers of the West Basin and the Black Sea. Two populations of *Synechococcus* (*Synechococcus* sp. 1 and sp. 2) were identified, with contrasting distributions along the Mediterranean Sea. *Prochlorococcus* spp. was also present ($3.77 \pm 0.36 \times 10^4$ cells ml⁻¹) and was associated with the deep chlorophyll maximum. Diatoms dominated the microphytoplankton except in the Black Sea, where dinoflagellates and flagellates became predominant. The percentage of living cells (%LC) of *Synechococcus* sp. 1 displayed a negative relationship with temperature, whereas that of *Synechococcus* sp. 2 increased with increasing temperature. *Prochlorococcus* spp. %LC was negatively related to inorganic nitrogen, but positively related to salinity. The viability of heterotrophic bacteria, dinoflagellates and *Synechococcus* sp. 1 increased with increasing phosphate, underlining the important role of phosphorus in the Mediterranean Sea. Our *in situ* observations relating survival to environmental forcing allowed the identification of competition and niche segregation within the coexisting populations.

KEY WORDS: Phytoplankton · Bacterioplankton · Viability · Phosphorus · Mediterranean Sea

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INTRODUCTION

The Mediterranean Sea is a semi-enclosed sea composed of various sub-basins interconnected through straits (Astraldi et al. 1999, Candela et al. 1999). Limited riverine input and a net nutrient export to the Atlantic Ocean render the Mediterranean Sea an oligotrophic, nutrient-limited ecosystem (Béthoux et al. 1998, Moutin & Raimbault 2002, Ribera d'Alcalà et al. 2003), with increasing P limitation towards its eastern basin (Krom et al. 1991, Turley et al. 2000, Crispi et al. 2001). Nutrient availability influences phytoplankton biomass and production

(Dugdale 1967) as well as community structure, with small phytoplankton forms (picophytoplankton) dominating under oligotrophic conditions (Platt et al. 1983, Agawin et al. 2000). The Mediterranean Sea presents a seasonal variability in phytoplankton communities (Margalef 1985), with a dominance of diatoms during winter–spring periods, when mixing increases nutrient availability, and a dominance of picophytoplankton and small flagellates in the summer, when strong stratification and oligotrophic conditions prevail (Duarte et al. 1999). In addition, the Mediterranean Sea differs in phytoplankton production and community structure among basins (Turley 1999, Ignatiades

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et al. 2009), as oligotrophic conditions increase toward the eastern basin.

Bacterioplankton is also an important component of the planktonic communities in oligotrophic areas (Hagström et al. 1988, del Giorgio & Cole 1998), where they experience nutrient limitation (Pinhassi et al. 2006). Indeed, bacterial production has been reported to be P limited across the Mediterranean Sea (Thingstad et al. 1998, Christaki et al. 2001, Van Wambeke et al. 2002, Pitta et al. 2005). Heterotrophic bacteria and phytoplankton may thus compete for the limiting inorganic resource when it is scarce (i.e. orthophosphate in the Mediterranean Sea). Experimental studies (Zohary & Robarts 1998, Joint et al. 2002, Mindl et al. 2005) have concluded that bacteria often dominate the uptake of free orthophosphate and are thus superior competitors to algae for P at low concentrations.

The composition of planktonic communities in the Mediterranean Sea could be strongly affected by the competitive capacity of their different groups for nutrients, especially phosphate. Although different mechanisms to allow the coexistence of different planktonic species have been experimentally defined in mesocosms for oligotrophic conditions (e.g. Thingstad et al. 2005), in natural communities these mechanisms remain unidentified. The relative abundance of different components does not suffice to evaluate the competitive success of a species, because grazing pressure may reduce the abundance of the best competitor (Thingstad et al. 2005). However, a useful indicator of the status of different planktonic components under *in situ* environmental conditions is cell viability status, as indicated by the proportion of living cells in the populations comprising the

natural communities (Agustí 2004, Alonso-Laita & Agustí 2006), using methods available for autotrophs (Agustí & Sánchez 2002) and heterotrophic bacteria (Grégori et al. 2001).

In the present study we examined the distribution and status of heterotrophic bacteria and planktonic autotrophs in the different sub-basins and straits of the Mediterranean and Black Seas and their relationship with nutrient availability and hydrological conditions. The status of the planktonic populations was characterized by the proportion of living cells of the major populations comprising the community. We did so using data derived from a research cruise, Thresholds 1, performed in June to July 2006 across the Mediterranean Sea and entering the Marmara and Black Seas, providing a unique opportunity to investigate these patterns at the basin scale.

MATERIALS AND METHODS

Sampling and chemical analysis. The study was conducted during the Thresholds-1 cruise aboard the RV 'García del Cid' from 6 June to 6 July 2006. A total of 36 stations were sampled between the Balearic Islands, in the Western Mediterranean Sea, to the Black Sea (Fig. 1), including some coastal and open water stations. At each station, vertical profiles of temperature, salinity and fluorescence down to 200 m depth were performed using a Seabird 911 CTD. Water samples were collected using 5 l Niskin bottles attached to a Rosette-CTD system at 5 to 12 depths, depending on the station, from surface waters to 200 m. Samples for

Fig. 1. Stations sampled during the Thresholds-1 cruise along the Mediterranean Sea. Numbers represent the different sampling stations

nutrient (total phosphorus [TP]; total nitrogen [TN]; dissolved inorganic nitrogen [DIN], nitrate+nitrite; dissolved inorganic phosphorus [DIP]; and silicate) analyses were collected at each depth and kept frozen until analyzed in a Bran Luebe AA3 autoanalyzer using standard methods (Hansen & Koroleff 1999). Analyses of total nutrients were measured using alkaline persulfate and UV digestion (Oms et al. 2003) followed by analysis of the inorganic products. Samples of 200 ml of water were filtered through Whatman GF/F filters to estimate total chlorophyll *a* (chl *a*) concentration and extracted for 24 h in 90% acetone for fluorometric determination (Turner Designs fluorometer) following Parsons et al. (1984).

Phytoplankton communities and viability of populations. For quantification of nano- and microphytoplankton abundance, samples were collected at the surface (5 m) and the deep chlorophyll maximum (DCM). Samples of 2 to 3 l were concentrated onto 50 to 70 ml samples by using a Millipore cell concentrator chamber. This concentration system has been used in previous studies (Agustí & Sánchez 2002, Alonso-Laita & Agustí 2006) with accurate results for microphytoplankton, with no effect on the viability or other cell properties (e.g. movement for flagellated cells, integrity of frustules). Ten ml aliquots (duplicates) of the concentrated sample were filtered onto 2 µm pore-size black polycarbonate filters, fixed with glutaraldehyde (1% final concentration) and stored frozen at -80°C until counting. Phytoplankton cells were counted using an epifluorescence microscope (Zeiss® Axioplan Imaging), and were classified into 3 majors groups, flagellates, dinoflagellates and diatoms; the latter were further separated into pennate and centric. Autotrophic picoplankton abundance was assessed using flow cytometry. At each station, duplicated 2 ml fresh samples from 5 depths were counted on-board (duplicated counts) using a FACSCalibur flow cytometer (Beckton Dickinson). An aliquot of a calibrated solution of 1 µm diameter fluorescent beads (Polysciences) was added to the samples as an internal standard for the quantification of cell concentration. Red (FL3, bandpass filter >670 nm), green (FL1, bandpass filter 530 nm) and orange (FL2, bandpass filter 585 nm) fluorescence as well as the forward and side scattering signals of the cells and beads were used to detect picoplanktonic populations of *Synechococcus* spp., *Prochlorococcus* spp. and eukaryotes (Marie et al. 2005).

The proportion of living cells in the autotrophic communities examined was quantified by applying a cell membrane permeability test, the cell digestion assay (CDA) (Agustí & Sánchez 2002). The CDA consists of the exposition of the phytoplankton communities to an enzymatic cocktail (DNase and Trypsin) that enters the cytoplasm and digests cells with compromised

membranes, i.e. dead or dying cells, which are removed from the sample. The cells remaining in the samples after the CDA are the living cells, which were then counted by flow cytometry or epifluorescence microscope, as described in this section.

The CDA was applied to the prepared concentrates of nano- and microphytoplankton cells to quantify total cell abundance. Specifically, the CDA was applied to duplicated 10 ml aliquots of cell concentrate by adding 2 ml of DNase I solution (400 µg ml⁻¹ in Hanks' Balanced Salt Solution [HBSS]), followed by 15 min incubation at 35°C in a Digital Dry Bath (Labnet®). After this time, 2 ml of Trypsin solution (1% in HBSS) were added, followed by 30 min incubation at 35°C. Samples were then placed in ice to stop the enzymatic cell digestion process and then were filtered onto polycarbonate 2 µm pore diameter black filters, washed several times with filtered seawater, fixed with glutaraldehyde (1% final concentration) and stored frozen at -80°C until counting by epifluorescence microscopy.

Fresh samples to quantify the proportion of living picophytoplankton cells were sampled from the 5 same depths selected to estimate total picoplankton abundance at each station. Duplicated 1 ml samples were run with the CDA, by first adding 200 µl of DNase I solution and then, after 15 min incubation at 35°C, 200 µl of Trypsin solution. Treated samples were incubated for 30 min at 35°C and were finally placed in ice to cease enzymatic activity. Samples were then counted by flow cytometry as described for total picophytoplankton abundance estimates.

The %LC was calculated as the ratio between the concentration of cells after applying the CDA, which represents the living cell abundance, and total population abundance, which includes both living and dead cells (Agustí & Sánchez 2002).

Bacterioplankton abundance and viability. The proportion of living and dead heterotrophic bacteria were quantified using the nucleic acid double staining (NADS, Grégori et al. 2001) flow cytometric protocol. This technique consists of the use of 2 nucleic acid fluorescent dyes, SYBR Green I (SGI; Molecular Probes) and propidium iodide (PI; Sigma Chemical). Bacterial membranes are permeable to SG1, whatever their cell viability, resulting in green fluorescence when stained. However, living or viable cells, with intact plasmic membranes, are impermeable to PI. Thus only compromised or damaged cells are stained with PI (Barbesti et al. 2000), showing red fluorescence as described in Falcioni et al. (2008). Subsamples were analyzed immediately after collection. Samples (1 ml) were stained with 10 µl of PI (1 mg ml⁻¹ stock solution), reaching a final concentration of 10 µg ml⁻¹, and incubated for 30 min in the dark at room temperature. Ten µl of SGI (10-fold dilution of 10 000× commercial

solution in dimethyl sulfoxide) were added to the sub-sample and incubated for an additional 10 min. SGI and PI fluorescence were detected using a FACSCalibur flow cytometer (Beckton Dickinson) in the green (FL1) and red (FL3) cytometric channels, respectively. Bivariate plots of green versus red fluorescence allowed the discrimination of live (green fluorescent, impermeable to PI) from dead cells (red fluorescent membrane-compromised cells, stained by PI and SGI) (Fig. 2). Bacterial concentration was calculated using a 1 μm diameter fluorescent bead (Polysciences) solution as an internal standard.

Total heterotrophic bacterial abundance (cells ml^{-1}) was calculated as the sum of red and green fluorescent cell abundance, while living bacterial cell abundance was determined from the green fluorescent cell counts.

Statistical analysis. Spearman's rank coefficients were used to determine the correlation between physical, chemical and biological variables, which departed from normality (Siegel & Castellan 1988). The different parameters were averaged within each sub-basin, and the statistical significance of the differences between average values were tested using Student's *t*-test, with a critical *p*-value of <0.05 . Data on nutrients, temperature, salinity and population abundance and viability were grouped by bins of nutrients concentration, temperature and salinity to search for patterns. Linear regression analyses were applied to binned data. Means are presented $\pm$ SE throughout.

Fig. 2. Flow cytometer dot plot of red (FL3) versus green (FL1) fluorescence of Mediterranean bacteria stained with SYBR Green I and propidium iodide (nucleic acid double staining method). Living cells (L) showed high green fluorescence but low red fluorescence, and differentiated well from dead (D) cells, which showed lower green fluorescence

RESULTS

Hydrology

A thermocline ranging in depth from 15 to 50 m, with surface temperatures above 19°C , was evident across the study region (Fig. 3), with a strong stratification observed in the Black Sea, while the West and East Mediterranean Basins showed a similar degree of stratification (Fig. 3). The Marmara and Black Seas presented a shallower thermocline (<20 m) and lower surface water temperature and salinity (Table 1). An intrusion of Deep Atlantic Water ($<15^{\circ}\text{C}$) was observed below the thermocline west of the Strait of Sicily (Fig. 3). Surface water salinity increased from the Western Mediterranean, with values ranging from 37 to 38.2, to the eastern basin, where salinity increased from 37.9 to 39.3 from the Ionian Sea to the Aegean and Cretan Seas (Table 1). The lowest surface salinity values, 17.2 to 21.6, were found in the Eastern Seas, which consist of the Marmara and Black Seas.

Low nutrient values, consistent with the oligotrophic nature of the Mediterranean Sea, were observed across the study region, although nutrient concentrations were somewhat increased in the Strait of Sicily and in the western region of the Ionian Sea (Fig. 3, Table 1). DIP concentrations were low throughout the study region, but displayed elevated values entering the Ionian Sea through the Strait of Sicily, and reached rather high ($>2 \mu\text{mol P l}^{-1}$) values in the Marmara and Black Seas (Table 1, Fig. 3). DIP concentrations were significantly, negatively related with temperature and salinity ($r_s = -0.31$, $p < 0.0005$, and $r_s = -0.36$, $p < 0.0001$, respectively). High concentrations of TN were found in the Ionian Sea, where the maximum was observed in deep layers (Table 1). DIN concentrations tended to be low in surface waters, but increased in the vicinity of the Strait of Sicily and displayed maximum values in the Eastern Seas (Fig. 3, Table 1). Silicate concentrations were generally low, increasing in the Ionian Sea and reaching relatively high values in the Eastern Seas, which presented elevated nutrient concentrations relative to the other regions (Table 1).

Plankton abundance and community distribution

Chl *a* values ranged from 0.047 to $6.98 \text{ mg chl } a \text{ m}^{-3}$ (Table 1), with an average of $0.61 \pm 0.06 \text{ mg chl } a \text{ m}^{-3}$ across the study region. A DCM was observed at 50 to 80 m depth along the Western Mediterranean, deepening east of the Strait of Sicily to reach 110 m depth at the Aegean Sea (Fig. 4). In the Black and Marmara Seas, chl *a* concentration values were high at the surface layer, showing values above $1 \text{ mg chl } a \text{ m}^{-3}$ in the

Fig. 3. Vertical distribution of (A) temperature ($^{\circ}\text{C}$), (B) dissolved inorganic phosphorus (DIP, $\mu\text{mol P l}^{-1}$) and (C) dissolved inorganic nitrogen (DIN, $\mu\text{mol N l}^{-1}$). Sampling depths along the different areas are represented by dots. Sub-basins are marked as follows: West Basin (WB); Strait of Sicily (SS); Ionian Sea (IS); Aegean and Cretan Seas (AC); Marmara Sea (MS); and Black Sea (BS)

Table 1. Average $\pm$ SE (range) hydrological properties, chlorophyll *a* (chl *a*) concentration and nutrient concentration in Mediterranean basins. Eastern Seas consist of the Marmara and Black Seas. Average values for sub-basins connected by different letters are significantly different ($p < 0.05$)

Variable	West Basin	Strait of Sicily	Ionian Sea	Aegean & Cretan Seas	Eastern Seas
Temperature ($^{\circ}\text{C}$)	16.9 ± 0.6 (13.4–24.5)	16.7 ± 0.5 (13.6–24.5)	17.2 ± 0.6 (14.5–24.8)	16.4 ± 0.4 (13.9–23.2)	12.9 ± 0.8 (6.9–19.8)
Salinity	37.4 ± 0.1 (36.9–38.2)	37.7 ± 0.1 (37.3–38.7)	38.4 ± 0.1 (37.9–38.9)	38.5 ± 0.1 (33.9–39.3)	23.6 ± 1.5 (17.2–38.7)
Chl <i>a</i> ($\text{mg chl } a \text{ m}^{-3}$)	0.86 ± 0.23 (0.12–6.98)	0.58 ± 0.10 (0.14–3.57)	0.46 ± 0.07 (0.09–1.96)	0.40 ± 0.05 (0.09–1.36)	0.92 ± 0.15 (0.05–2.06)
Dissolved inorganic N ($\mu\text{mol N l}^{-1}$)	0.81 ± 0.23^b (0.03–4.58)	0.99 ± 0.21^b (0.05–3.57)	0.68 ± 0.14^b (0.05–2.78)	0.56 ± 0.10^b (0.05–2.81)	2.01 ± 0.55^a (0.05–9.06)
Total N ($\mu\text{mol N l}^{-1}$)	$5.97 \pm 0.88^{b,c}$ (0.25–15.10)	6.71 ± 0.61^b (0.66–17.72)	10.43 ± 1.29^a (0.05–23.19)	3.22 ± 0.39^c (0.05–10.12)	5.93 ± 0.73^b (0.05–16.18)
Phosphate ($\mu\text{mol P l}^{-1}$)	0.02 ± 0.01^b (0.01–0.13)	0.07 ± 0.01^b (0.01–0.39)	0.05 ± 0.01^b (0.01–0.21)	0.07 ± 0.02^b (0.01–0.49)	5.99 ± 1.91^a (0.01–26.37)
Total P ($\mu\text{mol P l}^{-1}$)	0.32 ± 0.02^b (0.19–0.51)	0.32 ± 0.02^b (0.18–0.79)	0.28 ± 0.01^b (0.14–0.52)	0.25 ± 0.02^b (0.05–0.50)	0.79 ± 0.19^a (0.18–5.18)
Silicate ($\mu\text{mol Si l}^{-1}$)	0.64 ± 0.09^b (0.22–2.2)	0.78 ± 0.10^b (0.05–2.12)	1.07 ± 0.22^b (0.13–5.44)	0.53 ± 0.04^b (0.15–1.39)	9.91 ± 2.15^a (0.05–41.51)

upper mixed layer (Fig. 4). Chl *a* concentrations were significantly, negatively correlated with temperature and salinity ($r_s = -0.50$, $p < 0.0001$, and $r_s = -0.23$, $p < 0.005$, respectively), and weakly, but positively, correlated with silicate and DIN concentrations ($r_s = 0.19$, $p < 0.05$, and $r_s = 0.20$, $p < 0.05$, respectively).

The phytoplankton community showed important differences across sub-basins. Picophytoplankton was composed of populations of *Synechococcus* spp., *Prochlorococcus* spp. and picoeukaryotes, the latter group showing sparse populations across the study

region. We were able to identify 2 different *Synechococcus* spp. populations (*Synechococcus* sp. 1 and *Synechococcus* sp. 2) across the study region as indicated by the different signals of phycoerythrin/phyco-cyanin (orange fluorescence, FL2) and chlorophyll (red fluorescence, FL3) observed at the flow cytometer (Fig. 5). *Synechococcus* sp. 2 displayed higher orange and red fluorescence than *Synechococcus* sp. 1 (Fig. 5), and were less abundant, reaching maximum abundances 1 order of magnitude lower than *Synechococcus* sp. 1 (Fig. 6). The 2 identifiable populations of

Fig. 4. Vertical distribution of chlorophyll *a* concentration ($\text{mg chl } a \text{ m}^{-3}$) across the study region. Sampling depths and locations are represented by dots. Abbreviations as in Fig. 3

Fig. 5. Flow cytometry signatures, as the relationship between orange and red fluorescence per cell of picophytoplankton populations in the vicinity of the Strait of Sicily (Stn 3), at (A) 5, (B) 40 and (C) 52 m depths, showing the distinctive signals of *Synechococcus* sp. 1 and sp. 2

Synechococcus exhibited distinct distributions (Fig. 6), with *Synechococcus* sp. 1 more abundant in the West Basin and the Eastern Seas (Fig. 5), and *Synechococcus* sp. 2 more abundant in deep layers, especially in the Ionian and Eastern Seas (Fig. 6). The 2 populations co-occurred at the Strait of Sicily (Stn 3); however, they could be differentiated by their fluorescence signals in the cytogram (Fig. 5), confirming that they corresponded to 2 distinct populations and not to the result of differences in the cell fluorescence signals due to photoadaptation of a unique *Synechococcus* sp. population. The abundance of *Synechococcus* sp. 1 increased significantly with salinity ($r_s = 0.31$, $p < 0.0001$) and DIP ($r_s = 0.29$, $p < 0.001$).

The abundance of *Prochlorococcus* spp. closely followed the DCM distribution (Fig. 6), with maximum values between 50 to 80 m depth in the West Basin and 110 m in the eastern basin, but decreased strongly in the Marmara and Black Seas (Fig. 6). The average *Prochlorococcus* spp. abundance ($3.77 \pm 0.36 \times 10^4$ cells ml^{-1}) was similar to that of *Synechococcus* sp. 1 ($2.74 \pm 0.74 \times 10^4$ cells ml^{-1}). The abundance of both *Synechococcus* sp. 1 and *Prochlorococcus* spp. were positively correlated with the total phytoplankton biomass, measured as chl *a* concentration ($r_s = 0.33$, $p < 0.0001$, and $r_s = 0.31$, $p < 0.0001$, respectively), while *Synechococcus* sp. 2 was not significantly correlated with chl *a* ($p > 0.05$). However, *Prochlorococcus* spp. was positively related with *Synechococcus* sp. 2 ($r_s = 0.18$, $p < 0.05$) but not with *Synechococcus* sp. 1 ($p > 0.05$).

Heterotrophic bacterial abundance was relatively uniform, ranging from 1.80×10^5 to 3.92×10^6 cells ml^{-1} throughout the study region, with maximum values measured in the Marmara and Black Seas, and relatively high abundance in the vicinity of the Strait of Sicily (Fig. 6). Bacterial abundance decreased with depth and was not significantly related to chl *a* concentration ($p > 0.05$), nor with the abundance of the different picophytoplankton groups. A significant, positive relationship between bacterial abundance and phosphate concentration was found ($r_s = 0.33$, $p < 0.0001$).

Microphytoplankton communities displayed an average of 1531 ± 242 cells l^{-1} , showing generally higher abundance at the DCM than at the surface and reaching maxima in excess of 3000 cells l^{-1} at 3 stations (Stns 1, 23 and 13 in the West Basin and the Aegean and Marmara Seas, respectively; Fig. 7). Diatoms were the dominant microphytoplanktonic group throughout the study region, with an average of 860 ± 216 cells l^{-1} , dominating the maxima observed at the West Basin and Aegean Sea (Stns 1 and 23; Fig. 7), where *Guinardia* sp. and *Chaetoceros* spp. were the most abundant genera, respectively. The pennate genera *Nitzschia* spp. was the dominant diatom in the shallow Marmara Sea (Fig. 7). Dinoflagellates were preferentially

Fig. 6. Abundance (cells ml^{-1}) distribution of (A) *Synechococcus* sp. 1, (B) *Synechococcus* sp. 2, (C) *Prochlorococcus* spp. and (D) heterotrophic bacterioplankton across the study region. Abbreviations as in Fig. 3

Fig. 7. Distribution of microphytoplankton abundance (cells l^{-1}) at the surface (5 m) and the deep chlorophyll maximum (DCM). The dominant species at the 3 stations (Stns 1, 13 and 23) are indicated. Sub-basins are marked as follows: West Basin (WB); Strait of Sicily (SS); Ionian Sea (IS); Aegean and Cretan Seas (AC); and Eastern Seas (ES, including the Marmara and Black Seas; dashed lines separate the different sub-basins)

located at the surface and displayed low abundance (mean = 52 ± 15 cells l^{-1}). The abundance of naked dinoflagellates (*Gymnodinium* spp.) increased considerably at the Black and Marmara Seas, reaching a maximum of 3500 cells l^{-1} (Fig. 7). Autotrophic flagellates were also present and displayed abundances ranging from 50 to 1000 cells l^{-1} across the study region, with the highest values found in the Black Sea and at the stations corresponding to the middle of the Ionian Sea (Fig. 7). Microphytoplankton abundances displayed weak relationships with nutrient concentration, but flagellate and dinoflagellate abundance was

positively related to phosphate concentration ($r_s = 0.36$, $p < 0.05$, and $r_s = 0.83$, $p < 0.0001$, respectively). No significant relations were observed between silicate concentrations and diatom abundance.

Living cells

Picoplankton %LC ranged widely across the study region both for autotrophic and heterotrophic cells (Table 2), although the average %LC for heterotrophic bacterioplankton was significantly higher. The %LC of the different picoplankton groups didn't follow the distribution of the abundance of the population. Important differences in the average %LC were found across the Mediterranean Sea (Fig. 8). Both *Synechococcus* sp. 1 and sp. 2 displayed the highest average %LC in the Aegean Sea (Fig. 8). *Synechococcus* sp. 1 and sp. 2 populations presented opposite trends, showing high and low %LC, respectively, in the Ionian Sea (Fig. 8). Indeed, high %LC values of *Synechococcus* sp. 1 were found in deeper waters, while maximum *Synechococcus* sp. 2 %LC values were observed in the upper layers. The populations of *Synechococcus* sp. 1 and sp. 2 differed clearly in their response to temperature, as indicated by opposite relationships with temperature, with the %LC of *Synechococcus* sp. 1 decreasing with increasing temperature ($R^2 = 0.69$, $p < 0.05$; Fig. 9), whereas the %LC of *Synechococcus* sp. 2 increased with increasing temperature ($R^2 = 0.59$, $p < 0.05$; Fig. 9).

Prochlorococcus spp. %LC decreased to reach very low values in the Eastern Seas (Fig. 8), but presented similar average values of about 70%LC in the West Basin and the Ionian and Aegean Seas, with significantly lower percentages in the vicinity of the Strait of Sicily. *Prochlorococcus* spp. %LC values differed across the water column, with lower values found at surface waters than in deeper layers. *Prochlorococcus* spp. %LC was strongly and positively related to salin-

Table 2. Percentage of living cells (mean $\pm$ SE) of picoplankton and microphytoplankton groups quantified across the Mediterranean Sea. DCM: deep chlorophyll maximum

Pico-/microphytoplankton group	% living cells	
	Mean $\pm$ SE	Range
<i>Synechococcus</i> sp. 1	59.8 ± 2.6	3.8–99.5
<i>Synechococcus</i> sp. 2	58.7 ± 3.5	1.2–100
<i>Prochlorococcus</i> spp.	56.8 ± 2.6	3–98.9
Heterotrophic bacterioplankton	70.4 ± 1.5	3.4–96.2
Diatoms surface	60.3 ± 3.9	29.8–87.8
Diatoms DCM	56.6 ± 5.7	5.9–100
Flagellates	68.2 ± 2.1	37.7–99.3
Dinoflagellates	56.6 ± 4.4	13.5–99.5

Fig. 8. Distribution of the viability (percentage of living cells) of picoplankton populations within sub-basins. Boxes show the lower and upper quartiles, median, minimum and maximum values, and outliers (open circles). Boxes connected by same letter are not significantly different ($p < 0.05$). Abbreviations as in Fig. 7

ity ($R^2 = 0.82$, $p < 0.001$; Fig. 7) and temperature ($R^2 = 0.58$, $p < 0.05$). Moreover, *Prochlorococcus* spp. viability was strongly, but negatively, related to DIN concentrations ($R^2 = 0.89$, $p < 0.0005$; Fig. 10).

Heterotrophic bacteria populations showed significantly higher average %LC in the Eastern Seas ($86.1 \pm 1.7\%$) than in the other sub-basins, where the differences in the %LC of bacterioplankton were low. Heterotrophic bacteria viability increased with increasing phosphate concentration ($R^2 = 0.29$, $p < 0.0001$), but decreased with increasing temperature ($R^2 = 0.57$, $p < 0.05$; Fig. 11).

Fig. 9. Relationship between the average ($\pm$ SE, represented as error bars) percentage of living cells of *Synechococcus* sp. 1 (●, solid line) and *Synechococcus* sp. 2 (□, dashed line) binned by 1°C intervals and water temperature (°C). Solid and dashed lines represent the fitted regression equations

Fig. 10. Relationship between the average ($\pm$ SE) percentage of living cells (%LC) of *Prochlorococcus* spp. (binned by 1 salinity unit intervals and $0.5 \mu\text{mol N l}^{-1}$ dissolved inorganic nitrogen [DIN] intervals) and salinity (O, solid line) and DIN concentrations ($\square$, dashed line)

The %LC values of autotrophic flagellates were relatively homogeneous across the Mediterranean Sea, with somewhat higher values in the West Basin and the Sicily Strait (Fig. 12). In contrast, the average %LC of autotrophic dinoflagellates was lower ($32 \pm 6\%$) in the Strait of Sicily than in the other sub-basins (Fig. 12). The maximum %LC values of autotrophic dinoflagellates were observed in the Aegean Sea, although no significant differences were found in the averaged values (Fig. 12). The %LC of diatoms was about 60% in surface waters (Fig. 12), with somewhat lower values in the Aegean and Eastern Seas and higher values in the Ionian Sea, declining to a minimum of $53 \pm 6\%$ LC in the Aegean Sea. Diatoms presented greater variability in %LC at the DCM, with the maximum average %LC observed in the Ionian Sea ($73 \pm 12\%$), and decreasing %LC values towards the eastern ($36 \pm 17\%$) and western Mediterranean sub-basins ($42 \pm 11\%$).

DISCUSSION

The surface hydrology of the Mediterranean Sea showed the expected increase in salinity (Astraldi et al. 1999) and more oligotrophic conditions (Turley 1999, Crispi et al. 2001) toward the east. Seawater temperature followed similar patterns, with higher temperature in the eastern basin in the Ionian Sea, whereas the

Fig. 11. Relationship between (A) the average ($\pm$ SE) percentage of living heterotrophic bacterioplankton binned by 1°C intervals and temperature ($^\circ\text{C}$) and (B) bacterial viability (log viable bacteria ml^{-1}) and dissolved inorganic phosphorus concentration (DIP, log $\mu\text{mol P l}^{-1}$)

eastern Marmara and Black Seas displayed lower values (Pinet 2006, Oguz et al. 1994), as expected from the high freshwater inputs that exceed evaporation in that area (Özsoy & Ünlüata 1997). Nutrient concentrations were generally low and declined toward the eastern basin (Turley 1999, Crispi et al. 2001). However, higher nitrate and phosphate concentrations were found in the area crossing the Strait of Sicily and in the Eastern Seas. The Strait of Sicily constitutes a key feature affecting the thermohaline circulation between the western and eastern basins of the Mediterranean Sea (Béranger et al. 2004), displaying complex mesoscale features in surface waters (Gasparini et al. 2005). High

Fig. 12. Distribution of microphytoplankton cell viability across sampled sub-basins. Boxes present the lower and upper quartiles, median, minimum and maximum values, and outliers. Boxes connected by same letter are not significantly different ($p < 0.05$). Abbreviations as in Fig. 7

nutrient concentrations in the Strait of Sicily may derive from intense remineralisation in the shallow platforms, as well as upwelling of deeper waters at either side of the strait (Astraldi et al. 2001). Elevated nutrient concentrations in the Marmara and Black Seas are likely derived from the substantial riverine inputs of nutrients in this basin (Murray 1991, Mee et al. 2005).

As expected for an oligotrophic sea (e.g. Agawin et al. 2000), picoplankton plays a key role in Mediterranean waters. The heterotrophic bacterioplankton abundance reported here was similar to previous reports for the Mediterranean Sea (Zohary & Roberts 1998, Van Wambeke et al. 2002, Morgan et al. 2006, Mével et al. 2008). In agreement with previous reports, bacterial abundance decreased with depth (Turley & Stutt 2000, Tanaka & Rassoulzadegan 2004), with this decrease being more pronounced in the Black Sea

(Morgan et al. 2006). Picoautotrophs dominated phytoplankton biomass in the Mediterranean Sea, as reported earlier for late spring (e.g. Mura et al. 1996). Microphytoplankton abundance was low across the Mediterranean, consistent with previous reports in the Mediterranean Sea for the same season (Moncheva et al. 2001, Ignatiades et al. 2009). Diatoms were the dominant microphytoplankton group throughout the cruise except in the Black Sea, where autotrophic dinoflagellates and flagellates dominated the biomass, a reported consequence of increased P inputs to the Black Sea in the 1980s (Humborg et al. 1997).

Prochlorococcus spp. and *Synechococcus* spp. were the dominant picophytoplankton genera and coexisted across the Mediterranean Sea. *Prochlorococcus* spp. was present across the Mediterranean, but its abundance decreased strongly in the Marmara and Black Seas, consistent with the low abundance of this organ-

ism in cool, eutrophic waters (e.g. Vaulot & Partensky 1992, Partensky et al. 1999a). *Synechococcus* concentrations were high, comparable to previous reports in the Mediterranean (Agawin et al. 1998, Jacquet et al. 1998, Van Wambeke et al. 2002, Garczarek et al. 2007), particularly so in surface layers (Campbell & Vaulot 1993, Agawin et al. 2000), as expected from their sensitivity to low light and resistance to UV radiation (Agustí 2004, Llabrés & Agustí 2006). In contrast, *Prochlorococcus* spp. was most abundant in deeper layers, particularly at the DCM, as previously reported (Scanlan & West 2002).

The results presented here provide evidence of 2 distinct populations of *Synechococcus*, i.e. *Synechococcus* sp. 1 and sp. 2, which not only differ in their fluorescence signatures but are also differentially distributed across the water column and between the 2 major Mediterranean basins. *Synechococcus* sp. 1 dominated in the West Basin and was preferentially located in surface waters, whereas *Synechococcus* sp. 2 occupied deeper layers, especially at the Ionian Sea stations. These 2 populations, characterized here (by their distinct signals at the flow cytometer), have not been described before, probably because previous studies focused on specific Mediterranean basins. In contrast, sampling across the Mediterranean Sea in the present study, from its West Basin to the Eastern Seas, allowed the identification of the 2 different populations. Diversity in populations of *Synechococcus* in the Mediterranean Sea may be expected when considering that genetic studies have identified important differences within the Mediterranean strains (Scanlan & West 2002). Veldhuis & Kraay (1993) have described the coexistence of 2 different *Synechococcus* populations in the Red Sea, similar to those described here.

The result of a positive correlation between *Synechococcus* sp. 2 and *Prochlorococcus* spp. and the lack of a relationship between *Synechococcus* sp. 1 and sp. 2 suggests that similar controls may regulate the abundance of *Synechococcus* sp. 2 and *Prochlorococcus* spp. in the Mediterranean Sea. In fact, %LC increased as seawater temperature increased for both *Prochlorococcus* spp. and *Synechococcus* sp. 2, indicating a similarity in their temperature preferences. *Prochlorococcus* spp. is expected to be associated with warm waters, as this genera is excluded from cold waters (Partensky et al. 1999a), and Alonso-Laita & Agustí (2006) demonstrated an increase in *Prochlorococcus* spp. viability in the subtropical Atlantic when seawater temperature exceeded 21°C. *Synechococcus* sp. 1, however, showed an inverse pattern with respect to *Prochlorococcus* spp. and *Synechococcus* sp. 2, with %LC decreasing as temperature increased. *Synechococcus* is also described as a warm-water genus, although it is also present in cold waters (Shapiro &

Haugen 1988, Partensky et al. 1999b), suggesting a higher tolerance of some strains to low temperatures. Our results, showing an increase in *Synechococcus* sp. 1 viability as temperature decreased, are in contrast with the results of Alonso-Laita & Agustí (2006), who found an increase in the viability of *Synechococcus* in the subtropical Atlantic at temperatures above 21°C. *Synechococcus* populations from Blanes Bay (NW Mediterranean) also showed an increase in growth rate and cell division as seawater temperature increased (Agawin & Agustí 1997, Agawin et al. 1998). The low-temperature preference of *Synechococcus* sp. 1 may be explained by the positive relationship between *Synechococcus* sp. 1 abundance and phosphate concentration, as *Synechococcus* sp. 1 was most abundant in the cool ($12.9 \pm 0.8^\circ\text{C}$), P-rich waters of the Black Sea. Regarding N, neither *Synechococcus* populations were related to changes in its concentration, which is in contrast with the general positive response of *Synechococcus* to increasing N concentration (Waterbury et al. 1986). Also, the %LC of *Synechococcus* in the Atlantic Ocean increased in areas where N concentration increased (Agustí 2004, Alonso-Laita & Agustí 2006), in contrast with our results that emphasize the important role of P in the Mediterranean Sea. This contrasting result is in agreement, however, with the large genetic diversity reported by Scanlan et al. (2009) for *Synechococcus* spp.

Although the abundance of *Prochlorococcus* spp. was independent of nutrient concentration, there was a strong negative relationship between the percentage of living *Prochlorococcus* spp. cells and DIN concentrations. This negative relationship is consistent with the described incapacity of *Prochlorococcus* spp. to use nitrate, which may even be toxic for this species (Moore et al. 2002). The consistency between viability patterns and physiological constraints highlights the importance of the analysis of viability in natural populations to identify the environmental niches of phytoplankton species (Agustí 2004, Alonso-Laita & Agustí 2006), free of the confounding effects top-down controls confer to abundance patterns as indicators of environmental affinities. The proportion of living cells was also a useful indicator to examine the relationships between heterotrophic bacteria and environmental factors. Whereas most studies have focused on the abundance, production and metabolic activity of heterotrophic bacteria (Schumann et al. 2003, Gasol et al. 2008, Mével et al. 2008), the percentage of living bacteria in the ocean has been shown to be informative on their response to environmental factors (e.g. del Giorgio et al. 1996, Alonso-Sáez et al. 2006). Several methods have been applied to evaluate the viability of bacterial assemblages, with the NADS method used here being the more widely

used (Grégori et al. 2001, Alonso-Sáez et al. 2006, Ortega-Retuerta et al. 2008), as it has been shown to be highly efficient for natural planktonic bacteria (Falcioni et al. 2008). Here the quantification of the percentage of living bacteria allowed us to underline the consistent relationship between bacterioplankton and phosphate. Indeed, bacterial abundance and cell viability both increased with increasing phosphate concentration, in agreement with the results identifying phosphate as an important nutrient limiting bacterial production and growth in the Mediterranean Sea (Thingstad & Rassoulzadegan 1995, Thingstad et al. 1998, Zohary & Robarts 1998, Van Wambeke et al. 2002). These results confirm that bacterioplankton may be as dependent, or more so, as autotrophic plankton on nutrient availability (e.g. Gasol et al. 2008). Although the availability of inorganic nutrients has been shown to constrain the abundance and growth efficiency of bacterioplankton (Alonso-Sáez et al. 2007, Gasol et al. 2008), few studies have explored the relationship between bacterial viability and nutrients, most studies focusing on the relationship between bacterial viability and phytoplankton production and biomass (e.g. Ortega-Retuerta et al. 2008) or solar radiation (Alonso-Sáez et al. 2006),

The abundance of autotrophic dinoflagellates and flagellates was also positively related to phosphate concentration. This suggests bacteria and autotrophic flagellates to be strongly regulated for the same resource, although bacterioplankton have been shown to be superior competitors for P over autotrophic phytoplankton (Joint et al. 2002). The positive relationship between the viability of autotrophic dinoflagellates and phosphate remained high ($r_s = 0.59$, $p < 0.01$), even when excluding the Black Sea, where phosphorus concentrations were very high. Low ratios of Si to N and P in the Black Sea (Humborg et al. 1997) may exclude diatoms from the phytoplankton communities, as diatoms displayed the lowest %LC in the Black Sea. Major phytoplankton representatives in the Black Sea belonged to the dinoflagellate order Gymnodiniales and to the flagellate groups, which often present mixotrophic (Stoecker 1999) and heterotrophic forms (Fenchel 1982) and a high demand for P. High water-column stratification in the Black Sea may also represent an advantage for flagellates and dinoflagellates, in contrast to diatoms, which are favoured by intense mixing (Smayda 1997). In fact, the extent of stratification acts together with nutrient availability to determine microphytoplankton community structure (Margalef 1978, Thomas & Gibson 1990, Schöllhorn & Granéli 1996), and may also affect cell viability. Indeed, high turbulence and nutrient concentration in the Strait of Sicily (Candela et al. 1999) and the Aegean Sea (Balopoulos et al. 1999) was associated with high

diatom cell viability, approaching 100 % in the Strait of Sicily. In contrast, dinoflagellates presented the lowest %LC in the vicinity of the Strait of Sicily and high variability in cell viability in the Aegean and Cretan Seas, where the variability in water-column structure was high.

The relationships between the abundance and cell viability of planktonic organisms and temperature reported here is particularly relevant as a basis to predict the possible responses of the planktonic community to current warming of the Mediterranean Sea (Béthoux et al. 1990, Béthoux & Gentili 1996, Marbà & Duarte 1997, Vargas-Yáñez et al. 2008), which is particularly vulnerable to climate change (Giorgi 2006, Giorgi & Lionello 2008). Warming is expected to favour *Synechococcus* sp. 2 and *Prochlorococcus* spp., which showed increased cell viability with increasing temperature, and negatively affect *Synechococcus* sp. 1 and bacteria, which displayed negative relationships between cell viability and temperature. Climate warming is also expected to affect phytoplankton communities through its effect on increased water column stratification (Behrenfeld et al. 2006, Marcos & Tsimplis 2008, Tsimplis et al. 2008). Indeed, the longer stratification period described for the Mediterranean Sea due to a lengthening of summer conditions (Coma et al. 2009) may clearly lead to delays in the shifts of community due to seasonal succession. Increased stratification is expected to favour dinoflagellates and small flagellates and suppress diatoms (Béthoux et al. 2002, Tunin-Ley et al. 2009), as our results on cell viability concur.

In summary, the present study provides evidence for clear patterns of change in the abundance and cell viability of planktonic organisms across the Mediterranean basin. Our results identify, in particular, water temperature and phosphate concentrations as key determinants of the viability of planktonic organisms in the Mediterranean, thereby supporting existing evidence that both phytoplankton and bacteria tend to be P-limited in the Mediterranean Sea (Krom et al. 1991, Zweifel et al. 1993, Thingstad & Rassoulzadegan 1995, Pitta et al. 2005). Examination of the proportion of living cells, which indicates the survival success of the populations, allowed us to provide evidence of niche segregation of populations, such as those of *Prochlorococcus* spp., and *Synechococcus* sp. 2, that coexist in the Mediterranean Sea with bacteria and other groups, but that are affected differently by environmental conditions. The relationships between temperature and phosphate concentrations and the cell viability of various plankton components provide a basis for hypotheses regarding how climate warming may affect planktonic communities across the Mediterranean Sea.

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