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Effect of sunlight on prokaryotic organic carbon uptake and dynamics of pigments relevant to photoheterotrophy in the Adriatic Sea

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ABSTRACT: Marine photoheterotrophic microorganisms are capable of using light to meet their energy requirements and organic compounds as both carbon and energy sources. We still have little knowledge of the extent to which stimulation of these microorganisms by light could affect the estimates of organic carbon uptake. We evaluated the light and dark prokaryotic organic carbon uptake (³H-leucine) rates in a grid of stations covering the whole Adriatic Sea during winter and late summer. Light-exposed (photosynthetically active radiation and ultraviolet radiation [UVR]) surface samples were either unaffected, photostimulated or photoinhibited without any clear geographical or seasonal pattern. Light-enhanced leucine uptake occurred only in 23% of assays, suggesting that photoheterotrophy is not a major metabolic strategy and/or it is often counterbalanced by negative effects caused by UVR. Concentrations of bacteriochlorophyll *a*, zeaxanthin and divinyl chlorophyll *a* were measured in order to relate ³H-leucine uptake to the distribution of aerobic anoxygenic phototrophs (AAPs), total *Cyanobacteria* and *Prochlorococcus* populations, respectively, together with direct estimates of total prokaryotes and *Synechococcus* abundance. No relationship between light-enhanced leucine uptake and presence of picocyanobacteria or AAPs was evident. Divinyl chlorophyll *a* concentration was below the limit of detection in February, whereas in late summer the highest values were found around 50 m depth in the central and southern basins. In contrast, bacteriochlorophyll *a* concentration was correlated to total prokaryote abundance and dissolved organic carbon. Since locally leucine uptake in the light was markedly different from dark controls, *in situ* light incubations should be more appropriate than dark incubations when carbon budget calculations are intended.

KEY WORDS: Prokaryotic production · Bacteriochlorophyll *a* · Divinyl chlorophyll *a* · Zeaxanthin · Light

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INTRODUCTION

The discovery of 'photoheterotrophy' has recently brought new insights into biogeochemical processes and C fluxes in the sea (Zubkov 2009). Photoheterotrophs are capable of using light to meet their energetic requirements and organic compounds as both their carbon and energy sources (Béjã & Suzuki 2008). Prokaryotic photoheterotrophs are divided into 3 main groups: aerobic anoxygenic phototrophic bacteria (AAPs), proteorhodopsin-containing bacteria (PR) and aerobic oxygenic phototrophic cyanobacteria displaying heterotrophic-like behaviour (Béjã & Suzuki 2008). AAPs are known to generally represent less than 10% (though occasionally up to 25%; Lami et al. 2007) of total prokaryotes in the marine photic layer (Lamy et al. 2011, Ferrera et al. 2014, Kirchman et al. 2014), PR have been estimated to represent up to 50% of the microorganisms living in the North Atlantic Ocean (Campbell et al. 2008), and the contribution of *Prochlorococcus* to leucine uptake in the North Atlantic has been found to be up to 25% within the whole prokaryotic community (Mary et al. 2008). Given the importance of these metabolic strategies, our view of carbon fluxes has dramatically changed over the past 15 yr (Gasol et al. 2008, Ruiz-González et al. 2013).

Despite their abundance and widespread distribution, it is not clear to what extent these organisms influence the path of carbon through aquatic trophic webs (Ruiz-González et al. 2013). In general, the effect of solar radiation on heterotrophic processes is still a matter of debate, since a large number of studies presented contrasting results partly due to the difficulty in separating its direct and indirect effects (Ruiz-González et al. 2013 and references therein). In the past years, the majority of the studies dealing with the role of light on bacterial growth have focussed on ultraviolet radiation (UVR), reporting either its direct (Sieracki & Sieburth 1986, Herndl et al. 1993) or indirect effect (Obernosterer et al. 1999). Subsequent studies started to include the role of visible light or photosynthetically active radiation (PAR) on bacterial activity, reporting both photostimulation (e.g. Church et al. 2004, 2006, Mary et al. 2008) or photoinhibition (e.g. Sommaruga et al. 1997, Morán et al. 2001, Pakulski et al. 2007) on natural prokaryotic communities. In a recent review, Ruiz-González et al. (2013) summarized the results of 26 studies on the effect of sunlight on leucine uptake rates and reported an average minimum reduction in incorporation of 62% by UVR and a mean maximum stimulation of 100% by PAR. The effect of PAR and UVR

has also been studied in specific bacterial groups, with evidence indicating that prokaryotic community structure strongly determines its response to sunlight (Alonso-Sáez et al. 2006, Ruiz-González et al. 2012b). Recent experiments indicated that leucine uptake by AAPs is not affected by light either in the Delaware estuary (Stegman et al. 2014) or in Antarctic coastal waters (Kirchman et al. 2014), whereas by focussing on *Cyanobacteria*, Sommaruga et al. (2005) found a negligible effect of PAR and UVR on *Synechococcus* populations, in contrast to a pronounced negative influence on *Prochlorococcus* cell growth in the NW Mediterranean Sea. Nevertheless, one of the hypotheses for light-enhanced amino acid uptake highlights the pivotal role of *Cyanobacteria* (mainly *Prochlorococcus*) in the differences between light- and dark-treated prokaryotic carbon production samples (Church et al. 2004, 2006, Michelou et al. 2007).

In the Adriatic Sea, Sommaruga et al. (1997) evaluated the uptake of thymidine and leucine in light and dark treatments and reported a strong (70%) photo-inhibition of radiolabelled thymidine and leucine assimilation. In particular, they found that UV-A inhibited the incorporation of both tracers after short-term experiments, while no effect of UV-B was detected by their approach. Moreover, PAR was also reported to inhibit thymidine and leucine incorporation by up to 30%. However, it must be taken into account that the distribution of *Prochlorococcus* in the Adriatic Sea is largely understudied, and the current literature reports extremely low abundance both in the northern ($\leq 3\%$ of total picophytoplanktonic biomass; Radić et al. 2009) and in the southern basins ($< 10\%$ of total picophytoplankton abundance; Šilović et al. 2011). For this reason, the photoinhibition of radiotracer uptake could be the result of a direct negative effect of light coupled with the low abundance of this important organic carbon utilizer. On the other hand, *Synechococcus* is abundant and widely distributed, accounting for, on a yearly average, up to 16% of total picoplanktonic biomass in the northern basin (Pugnetti et al. 2008). To date, no data on AAP abundance or bacteriochlorophyll *a* (BChl *a*) concentration have been presented in the literature about the Adriatic Sea.

Here, we evaluated the light and dark leucine incorporation rates in surface samples in a grid of stations covering the whole Adriatic. We tested the potential stimulation (or inhibition) of organic carbon uptake under natural sunlight conditions (UVR included) both in winter (February) and late summer (October). ^3H -leucine uptake rates were related to

the concentration of divinyl chl *a* (as a signal for *Prochlorococcus* distribution), *Synechococcus* cell counts, zeaxanthin (as a signal for *Cyanobacteria* distribution) and BChl *a* (as a signal for AAPs), and the spatial and temporal dynamics of these pigments were investigated. Furthermore, since vertical water displacement in the Adriatic Sea is an important hydrological phenomenon (Gačić et al. 2001) potentially affecting the structure and functions of surface pelagic assemblages (Gačić & Civitarese 2012 and references therein), the hypothesized photoinhibition or photostimulation of communities from intermediate and bottom water samples collected from depths of 5 to 1215 m were also tested.

MATERIALS AND METHODS

Sampling strategy

Experimental work was done during 2 surveys of the whole Adriatic Sea in February and October 2008. Water samples were collected for ³H-leucine incorporation expts, total prokaryotes and *Synechococcus* abundances, cyanobacterial community structure (see the Supplement at www.int-res.com/articles/suppl/a074p235_supp.pdf), pigment concentration (chl *a*, BChl *a*, divinyl chl *a*, zeaxanthin) and dissolved organic carbon (DOC). Up to 4 prokaryotic organic carbon uptake (PCU) experiments were set up at each station, whereas more complete profiles were performed for cell abundance, pigments and DOC concentrations. Water samples and temperature and salinity data were collected by a rosette sampler for 24 Niskin bottles (10 l each) equipped with a SBE 911 CTD probe. Table 1 (also see Table S2 in the Supplement) shows sampling depths and station coordinates (for sampling station locations, see Fig. 1).

DOC

Water samples, collected directly from the Niskin bottles, were filtered

on sterile cellulose acetate 0.2 µm filters (Sartorius), in 20 ml amberglass vials with teflon caps, previously combusted at 450°C for 4 h, rinsed with MilliQ water and oven dried. The vials were rinsed 3 times with filtered seawater before the collection of the sample. The samples were stored in the dark at 4°C until analysis.

DOC concentrations were measured using a Shimadzu TOC 5000 Analyzer equipped with 1.2% platinum on silica as the catalyst at 680°C (Cauwet 1994). Samples were acidified (pH 2) with 2 µmol l⁻¹ HCl and purged with pure air for 10 min immediately

Table 1. Sampling stations and depth (m) for prokaryotic organic carbon uptake (PCU) experiments. **Bold** text indicates October experiments; s: surface. PAR is the photosynthetically active radiation, shown as the average of measurements taken every 10 min in the incubators during PCU experiments

Stn	Longitude (°E)	Latitude (°N)	PCU experiments	PAR (µmol photons m ⁻² s ⁻¹)	Start of incubation (h, local time)
A01	12.9203	45.4010	s,5,10,24	212	14:00
A02	12.9658	44.9748	s,5,15,32	137	13:20
A03	12.9920	44.4233	s,10,20,39	340	12:05
A04	13.2652	44.1003	s,20,57	216	08:30
A05	13.9212	43.3975	s,10,30	448	11:07
A06	14.0412	43.4510	s,10,62	415	11:15
A07	14.2322	43.5592	s,20,87 s,80	385 1619	11:40 10:45
A10	15.1667	43.0413	s,20,250	386	8:42
A11	16.0118	42.6002	s,168 s,165	87 207	15:08 16:05
A12	16.6160	42.3445	s,152	1743	12:50
A14	17.1043	41.2783	s,150	142	07:55
A15	17.4080	41.5468	s,983	125	11:35
A16	17.7082	41.8202	s 1139	149 664	16:18 16:00
A17	17.9447	42.026	s 1215 s 1205	1847 1536 2075 1744	14:15 11:55 11:20 13:05
A18	18.164	42.2008	s,1040	694	15:10
A19	18.3500	41.1987	s,1040 s,100 1035	261 186 1203	08:00 16:45 15:05
A20	18.7507	40.7508	s,845 s 100,840	1910 1785 1577	13:00 10:50 9:30
A22	18.7485	39.8315	s,360	1411	11:32
A23	18.9722	39.8282	s 887 s 100,853	474 348 58 581	08:06 07:00 17:00 15:45
A24	19.2002	39.8302	s,100 1011	1245 681	11:15 12:45

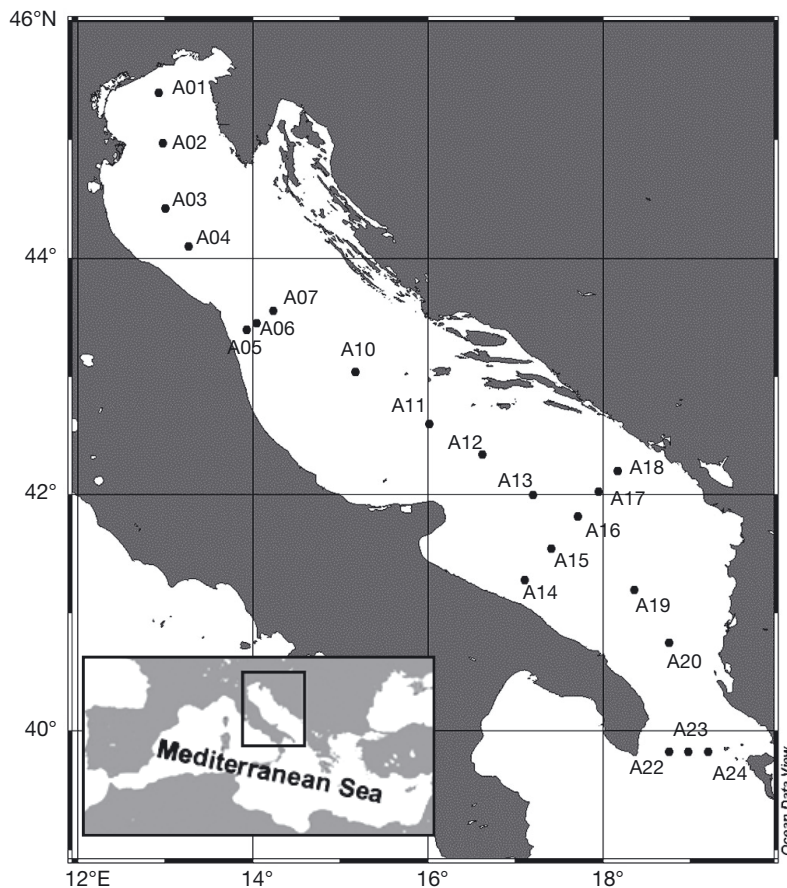


Fig. 1. Sampling stations in the Adriatic Sea. Image created with the software Ocean Data View (<http://odv.awi.de>)

prior to analysis. DOC concentrations were calculated by subtracting the system blanks and dividing by the slope of the calibration curve (Thomas et al. 1995). Potassium hydrogen phthalate was used for the primary stock standard and for the 4-point calibration curve. An aliquot (100 μ l) of each sample was injected for each analysis, and the concentration was calculated as the average of 3 to 5 replicates until the analytical precision was within 2% relative standard deviation. The accuracy was estimated by periodic analysis of seawater samples certified for their DOC content (DOC-CRM University of Miami).

Total prokaryotes and *Synechococcus* abundances

Total prokaryotes and *Synechococcus* abundances were determined by preserving 60 ml of seawater with 2% final concentration borate-buffered formalin (pre-filtered through a 0.2 μ m Acrodisc filter). Samples were stained for 15 min with 4'6 diamidino-2-

phenylindole (DAPI, Sigma) at 1 μ g ml⁻¹ final concentration (Porter & Feig 1980) and filtered in triplicate onto 0.2 μ m black polycarbonate filters (Nuclepore). Filters were then mounted on microscope slides, between layers of non-fluorescent immersion oil (Cargille), and counted by epifluorescence microscopy (LEICA DM2500) at 1000 \times magnification under a UV excitation Band Pass [BP] 340–380 nm, emission Barrier filter [BA] 430 nm and a green (BP 515–560 nm, BA 590 nm) filter set for total prokaryotes (DAPI) and *Synechococcus* (Syn), respectively. A minimum of 300 cells was counted for each filter in at least 20 randomly selected fields.

PCU

PCU was estimated by the incorporation of ³H-leucine (Kirchman et al. 1985). Quadruplicate 1.7 ml aliquots and 2 killed controls (5% trichloroacetic acid [TCA], final concentration) were amended with a 20 nM radiotracer and incubated at seawater surface temperature both in the dark and under natural solar irradiation (PAR + UVR) in on-deck opaque incubators. *In situ* surface temperature was maintained by continuous pumping of

seawater collected under the ship's hull (~2 m depth). Incubations were performed in transparent polypropylene tubes, kept at ~0.5 m below the water-atmosphere interface within incubators. The experiments were divided in 3 groups according to the perturbation to which resident assemblages were subjected during incubation: surface samples did not undergo light intensity or temperature changes compared to *in situ* conditions; photic layer samples collected between 5 and 100 m were exposed to higher radiation and, in summer, to higher temperature (6–8°C warmer than *in situ*); deep samples (collected below 100 m) were experimentally exposed to light (the aphotic zone was below this depth according to PAR profiling in February; data not shown) and to higher temperature in summer. Before the first cruise, different incubation times (1, 2 and 6 h) for leucine uptake were tested with surface seawater samples from a coastal northern Adriatic station. Incubations were stopped with 5% TCA (final concentration). As no significant differences were observed among the different incubation times (Kruskal-Wallis

ANOVA among light incubations $p = 0.49$; among dark incubations $p = 0.58$), consistent with Sommaruga et al. (1997), we chose 1 h as the onboard experimental time interval. Incident light intensity was measured in proximity to polypropylene tubes (~0.5 m depth, within the incubators) every 10 min with a QSL-100 quantameter (Biospherical Instruments). Extraction with 5% TCA and 80% ethanol was carried out using the microcentrifugation method (Smith & Azam 1992). Activity in the samples was determined by a β -counter (Packard Tri-Carb 2900TR) after the addition of 1 ml scintillation cocktail (Ultima Gold MV; Packard).

Chl *a*, BChl *a*, divinyl chl *a* and zeaxanthin concentrations

Seawater samples for pigment analysis were filtered through 25 mm \varnothing Whatman GF/F glass-fibre filters (nominal pore size = 0.7 μm). The filtered volume varied between 1 and 4.3 l. The filters were placed into cryotubes, frozen in liquid nitrogen and stored at -80°C until laboratory analysis at the Laboratoire d'Océanographie de Villefranche (France).

Pigment extraction and analysis were performed in the laboratory according to a procedure derived from that of Van Heukelem & Thomas (2001). Briefly, samples were extracted in 3 ml HPLC-grade methanol for 2 h minimum and then stored at -20°C until analysis. Analyses were performed within 24 h of extraction. The extracts, clarified by vacuum filtration through Whatman GF/F filters, were placed in a refrigerated (4°C) auto-sampler until injected onto a reversed phase C8 Zorbax Eclipse column (dimension: 3×150 mm, 3.5 μm pore size). Instrumentation comprised an Agilent Technologies 1100 series HPLC system, equipped with diode array detection at 450, 667 and 770 nm. A comprehensive description of the method is available in Ras et al. (2008). About 25 separate pigments could be quantified, and detection limits were low (3 times signal:noise ratio; e.g. 0.1 ng l^{-1} for chl *a*, 0.1 to 0.05 ng l^{-1} for accessory pigments), enabling a reliable determination of very low pigment concentrations. The HPLC injection precision was estimated to be 0.4%.

Chl *a*, BChl *a*, divinyl chl *a* and zeaxanthin concentrations were calculated from the peak areas with an internal standard correction (Vitamin E acetate, Sigma) and external calibration standards provided by DHI Water and Environment (Denmark) and Sigma.

Note that the use of GF/F filters may underestimate the actual pigment concentration, since cells smaller

than 0.7 μm (i.e. *Prochlorococcus* and AAPs) can pass through the membrane.

Statistical analyses

All statistical analyses were performed with the software STATISTICA (StatSoft). Nonparametric Mann-Whitney tests were performed for each experiment among leucine incorporation rates measured in the dark (4 replicates) and those exposed to sunlight (4 replicates) in order to detect significant differences.

Relationships among parameters were evaluated by means of the Spearman's correlation coefficient (ρ) of rank correlation. In particular, this test was used to detect positive or negative correlations between PCU experiment outcomes and physico-chemical/biological variables (abundance of total prokaryotes and *Synechococcus*, pigment concentrations). The differences (ΔLeu) calculated as data obtained after light incubation (mean of 4 replicates) minus data from dark incubations (mean of 4 replicates) and the percent change of light Leu uptake rates (mean of 4 replicates) over its dark uptake (% change) rates (mean of 4 replicates) were computed and related to the other parameters by means of the Spearman test. ΔLeu is positive if light incubations lead to faster uptake rates, negative if photoinhibition occurs and 0 in case of no difference. ΔLeu is of importance for carbon budget computations, being more dependent on the absolute leucine incorporation values than on the degree of photostimulation/inhibition. On the other hand, the percent change from dark incubation was calculated to test the effect of light on prokaryotic metabolism. In the latter case, a value of 100 implies no differences due to light or dark, a value >100 indicates photostimulation, and a value <100 denotes photoinhibition.

A principal component analysis (PCA) was performed on both cruise datasets in order to depict the relationship between pigment dynamics (zeaxanthin, BChl *a*, divinyl chl *a*, chl *a*) and other physical (temperature, salinity), chemical (DOC) and biological (total prokaryotes) variables. PCA rotates a cluster of data points such that the maximum variability is visible in order to identify the most important gradients. All data were standardised for the analysis by subtracting the mean and dividing the obtained value by the standard deviation. An arbitrary value of 0.5 ng l^{-1} was assigned to pigment values for which concentration was lower than the instrumental detection limit.

RESULTS

Background information

Strong latitudinal temperature gradients characterised the sampled area in February and in October (Fig. 2; more details are provided in the Supplement)

DOC concentration in the upper 150 m (Fig. 2) showed stronger gradients in October than in February in the surveyed area. In winter, the highest values were found at the northernmost stations (average

of all samples collected between Stns A01 and A04 = 74.4 ± 18.7 [SD] $\mu\text{mol l}^{-1}$), especially at Stn A02 (maximum value = $115.9 \mu\text{mol l}^{-1}$). Relatively low DOC concentrations characterised the southern stations (average of all determinations from Stns A07 to A20 = $50.5 \pm 6.3 \mu\text{mol l}^{-1}$), with the exception of surface samples collected at Stns A16 and A23, which showed 89.1 and $67.1 \mu\text{mol l}^{-1}$, respectively. Below 200 m, DOC concentration was lower (average of all data = $47.0 \pm 11.0 \mu\text{mol l}^{-1}$) than in the upper water column. The same latitudinal gradient characterised

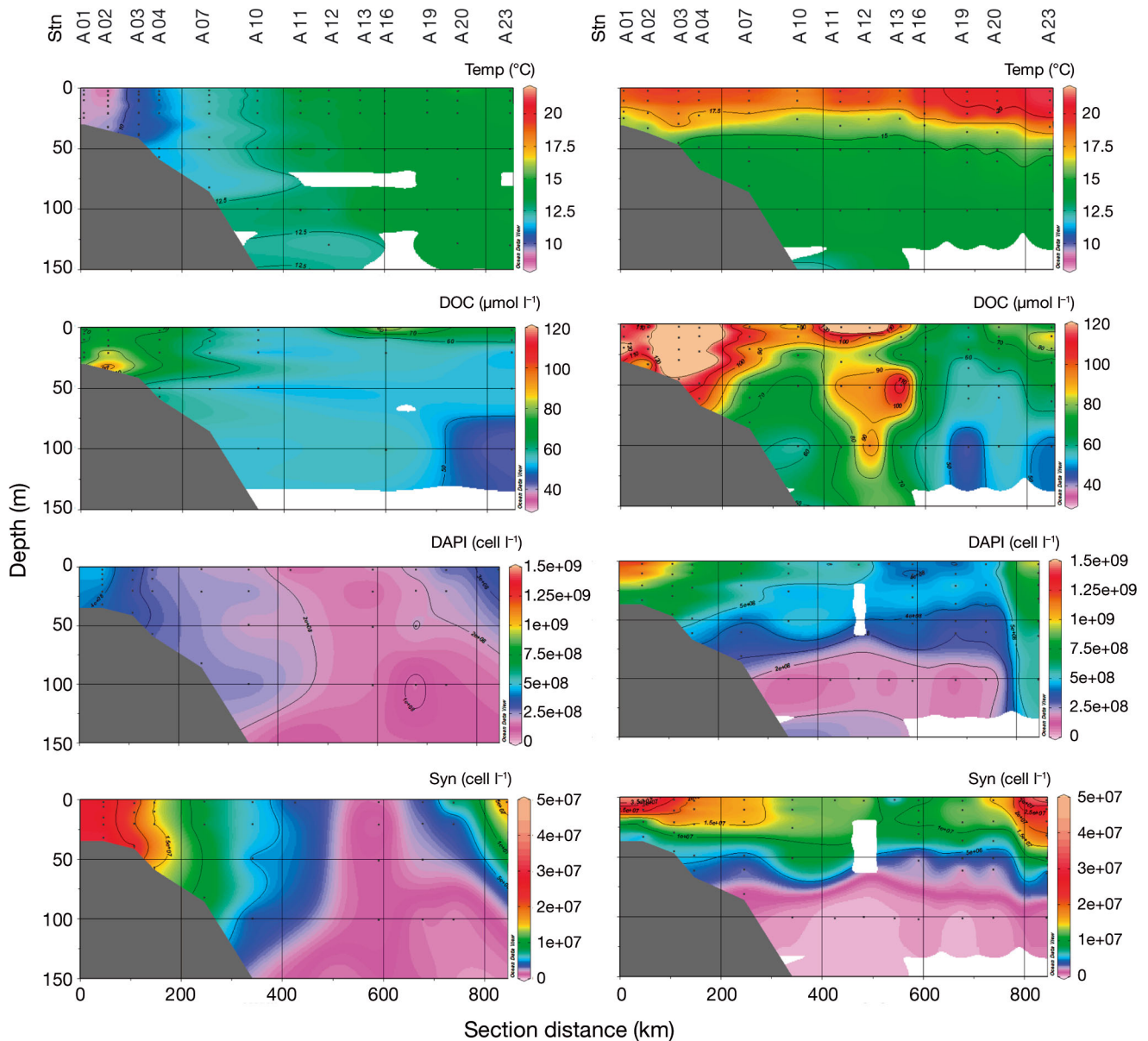


Fig. 2. Temperature (Temp), dissolved organic carbon (DOC), DAPI-stained prokaryotes (DAPI) and *Synechococcus* (Syn) data along a latitudinal transect covering the whole Adriatic Sea in February (left plots) and October (right plots) 2008. Station numbers are shown along the top

October surface waters (upper 100 m), with higher values in the northern basin ($105.2 \pm 35.0 \mu\text{mol l}^{-1}$ from Stns A01 to A07) compared to the central and southern ones ($73.5 \pm 19.5 \mu\text{mol l}^{-1}$ from Stns A10 to A23). In the deepest layers, a DOC accumulation was observed at Stns A13 and A16 (average value of samples below 200 m = $72.8 \pm 24.1 \mu\text{mol l}^{-1}$), in contrast to that detected at the southernmost sites (from Stns A19 to A23 $48.3 \pm 6.2 \mu\text{mol l}^{-1}$).

Total prokaryotes and *Synechococcus* abundances

Total prokaryote abundances along the latitudinal transect in February and October are shown in Fig. 2. In February, a general north-to-south decreasing gradient was observed. In the upper 150 m, the highest value was detected at Stn A02 at 6 m depth ($5.3 \pm 0.2 \times 10^8 \text{ cell l}^{-1}$), while the lowest abundance was observed at Stn A19 at 100 m ($4.7 \pm 0.3 \times 10^7 \text{ cell l}^{-1}$). Below 150 m, DAPI-stained prokaryotes were, on average, $1.25 \pm 0.8 \times 10^8 \text{ cell l}^{-1}$. In October, DAPI-stained prokaryotes ranged from $6.4 \pm 0.3 \times 10^7 \text{ cell l}^{-1}$ (Stn A20, 840 m) to $1.4 \pm 0.1 \times 10^9 \text{ cell l}^{-1}$ (Stn A02, 2 m). A decrease in abundances from the surface to the bottom layer was observed, with a clear stratification in abundance distributions in the upper 150 m (Fig. 2).

The distribution of *Synechococcus* along the upper 200 m of the water column in February and October is shown in Fig. 2. In February, a north to south

decreasing trend was found, with an increase at Stn A23, similar to that observed for total counts; in contrast to the evident horizontal variation, the abundance decrease with depth appeared less pronounced, with uniform values along the water column in the northern continental shelf (Stns A01 to A07) and weak stratification characterising the southern stations. Abundances ranged from $1.9 \pm 0.1 \times 10^5 \text{ cell l}^{-1}$ (Stn A16, 20 m depth) to $4.0 \pm 0.2 \times 10^7 \text{ cell l}^{-1}$ (Stn A03, 39 m depth). Similarly, in October a north to south decrease in cell numbers was encountered, with a southern increase at Stn A23. In contrast to the late winter scenario, the vertical distribution along the transect appeared clearly stratified. Observed values ranged from $1.1 \pm 0.3 \times 10^5 \text{ cell l}^{-1}$ (Stn A19, 100 m depth) to $4.9 \pm 0.2 \times 10^7 \text{ cell l}^{-1}$ (Stn A02, 2 m depth).

Prokaryotic organic carbon uptake experiments

PCU values are reported in Figs. 3 & 4. In February, surface PCU (Fig. 3) was generally highest in the northernmost (Stns A01 to A07) and in the southernmost stations (Stns A18 to A23), whereas in the central basin, slow uptake rates were measured ($<100 \text{ pmol Leu l}^{-1} \text{ h}^{-1}$). In October, no evident geographical gradient was detected. The experiment performed with surface waters showed that less than half of the samples (11 out of 26) had significantly different uptake rates under light and dark conditions (Mann-Whit-

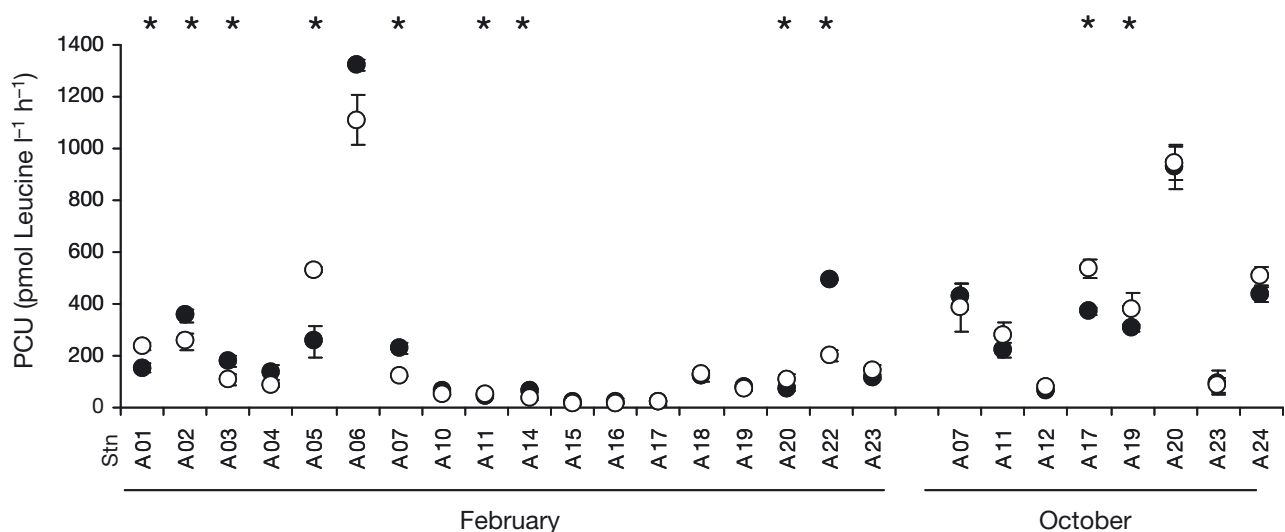


Fig. 3. Prokaryotic organic carbon uptake (PCU) in February and October 2008 in Adriatic Sea surface waters. Open circles indicate light incubations; filled circles indicate dark incubations. Data are means \pm SD of 4 replicates. Asterisks indicate significant differences between light and dark incubations (Mann-Whitney test, $p < 0.05$). Where not visible, filled circles are hidden behind open circles

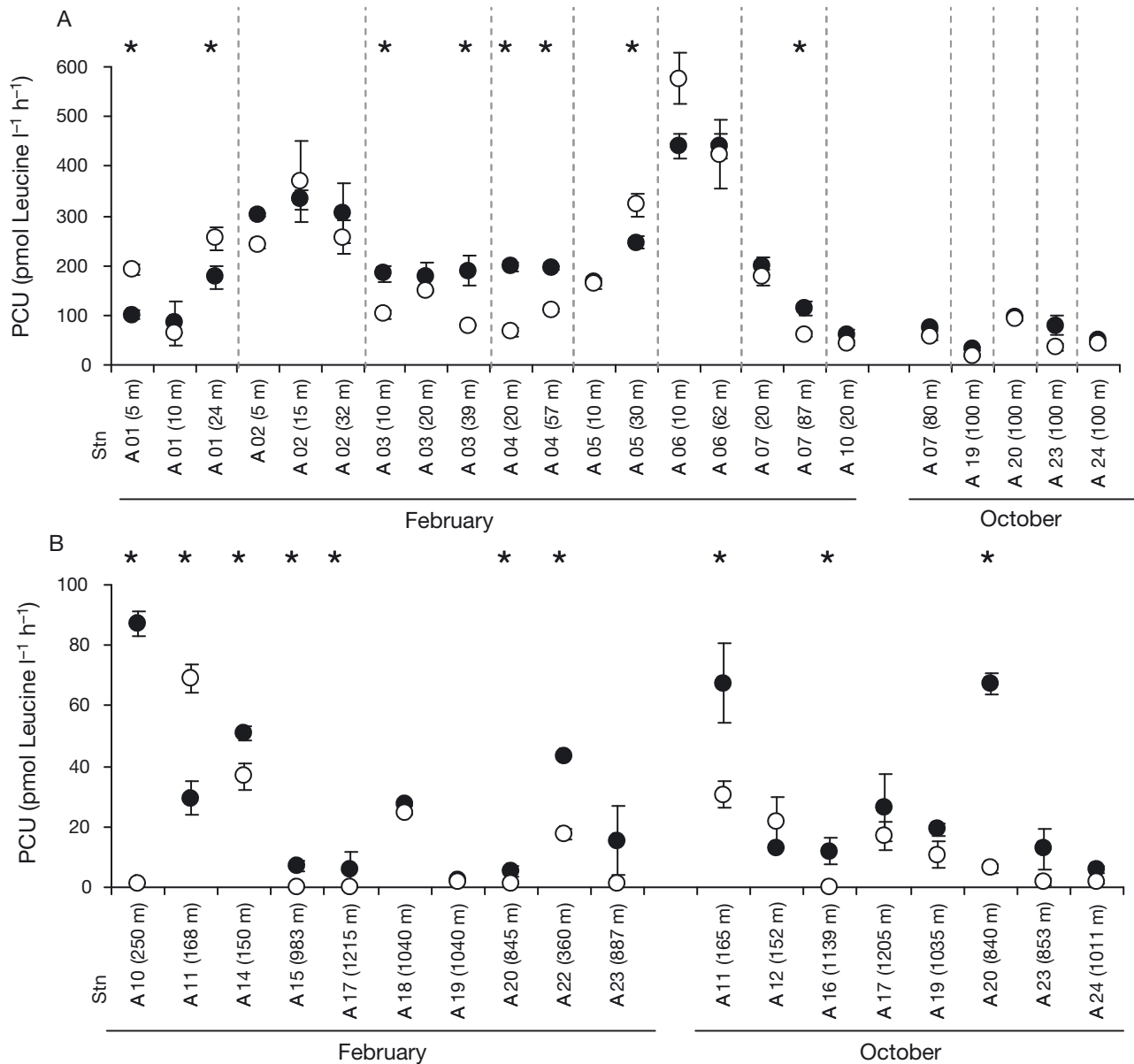


Fig. 4. Prokaryotic organic carbon uptake (PCU) in February and October 2008 in Adriatic Sea samples collected in (A) the photic zone (surface samples excluded) and (B) the aphotic zone. Open circles indicate light incubation; filled circles indicate dark incubation. Data are means \pm SD of 4 replicates. Asterisks indicate significant differences between light and dark incubations (Mann-Whitney-test, $p < 0.05$)

ney test, $p < 0.05$), and these differences were found mainly in the northern Adriatic in February. In winter, about half of the experiments showing significant differences (5 out of 9) displayed photoinhibition, whereas in October, most of the samples (6 out of 8) were unaffected by light and only 2 were photostimulated.

Two-thirds of the samples collected in the photic layer (surface excluded) did not show any significant response to light, including all of the samples collec-

ted in October (Fig. 4); within the remaining experiments, 3 displayed photostimulation (Stn A01: 5 m, 24 m; Stn A05: 30 m) and 5 showed photoinhibition (Stn A03: 10 m and 39 m; Stn A04: 20 m and 57 m; Stn A07 87 m). Samples collected in the aphotic layer always displayed faster leucine uptake when incubated in the dark, except for the bottom (168 m) of Stn A11 in February (Fig. 4).

ΔLeu in February ranged from -294.85 to 277.89 pmol Leu $l^{-1} h^{-1}$, with a mean value of -18.13 ;

in summer, the range was less variable (from -61.01 to $165.88 \text{ pmol Leu l}^{-1} \text{ h}^{-1}$), with a positive average of $7.33 \text{ pmol Leu l}^{-1} \text{ h}^{-1}$). Winter percent change from dark incubations varied between 0 (when light incubations revealed undetectable leucine uptake) and 234.46% (average 84.25%). Similarly, a bulk light inhibition was found in summer, with an average of 85.89% and a range of 9.41 to 165.19%.

Pigment concentration

Chl *a* concentrations (Fig. 5) were highest in February, coinciding with freshwater inputs, with values

reaching $0.8 \mu\text{g l}^{-1}$ at the bottom of Stn A02. All other determinations (from Stns A03 to A20) showed consistently low concentrations around $0.27 \pm 0.09 \mu\text{g l}^{-1}$, being rather constant along the upper water column (100 m). Similarly, in October, Stn A02 showed the highest phototrophic biomass (chl *a* maximum = $1.7 \mu\text{g l}^{-1}$ at the surface), whereas lower chl *a* concentrations characterised the rest of the basin ($0.2 \pm 0.1 \mu\text{g l}^{-1}$). Differently from what we detected during the February cruise, a deep chlorophyll maximum (DCM) was observed at $\sim 50 \text{ m}$ from Stns A03 to A20.

BChl *a* concentrations along the latitudinal transect in February and October are shown in Fig. 5. A latitudinal gradient with the highest concentrations

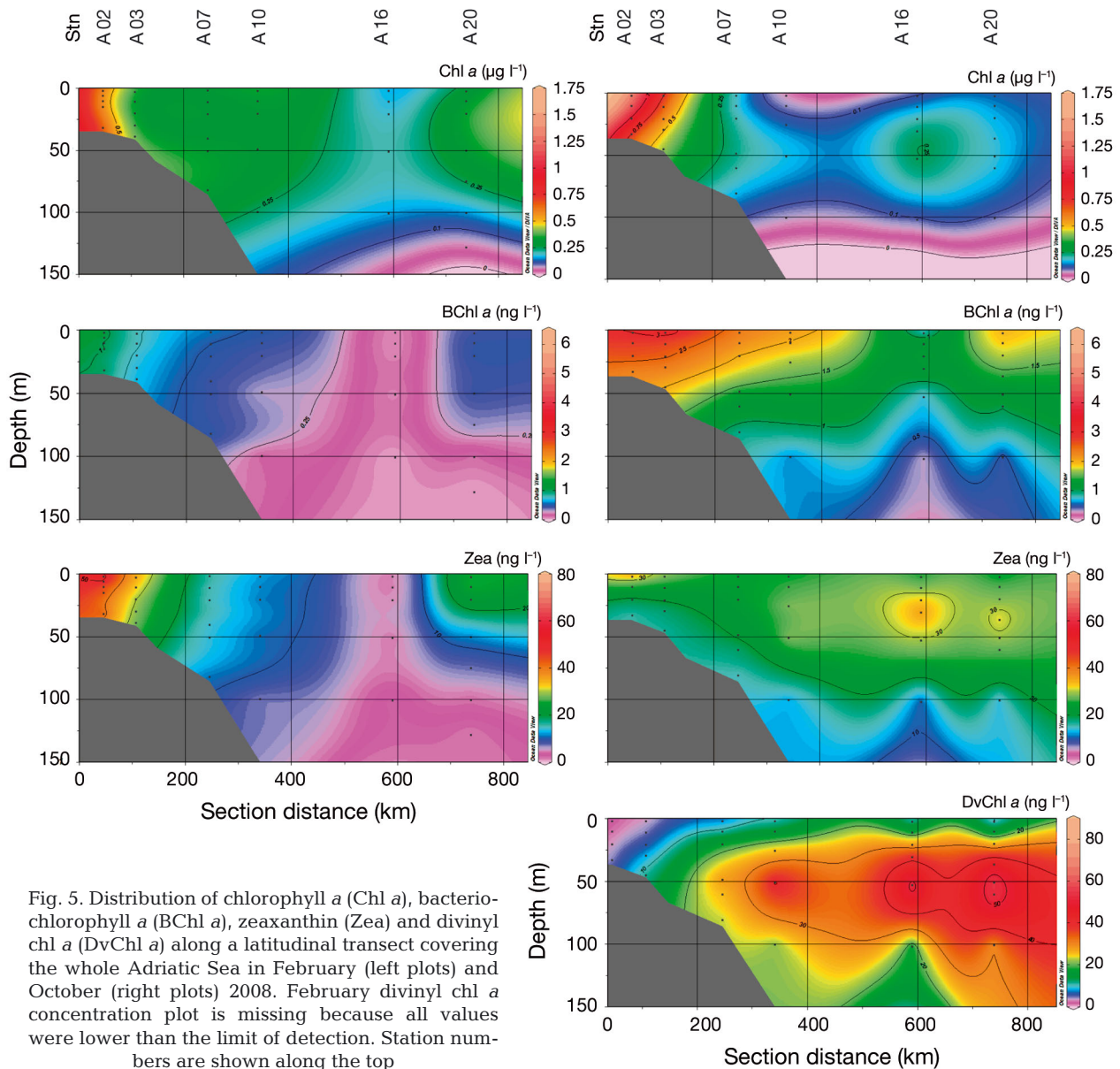


Fig. 5. Distribution of chlorophyll *a* (Chl *a*), bacteriochlorophyll *a* (BChl *a*), zeaxanthin (Zea) and divinyl chl *a* (DvChl *a*) along a latitudinal transect covering the whole Adriatic Sea in February (left plots) and October (right plots) 2008. February divinyl chl *a* concentration plot is missing because all values were lower than the limit of detection. Station numbers are shown along the top

northward, evenly distributed along the upper water column was evident in winter, whereas in summer the BChl *a* concentration was vertically stratified with surface maxima. In February, the highest values were detected at the northern station between 15 and 30 m depth (1.5 ng l^{-1}), while the lowest (below limit of detection) were observed through the whole water column of Stn A16 (central basin) and below 100 m at Stn A20 (southern basin). In October, BChl *a* ranged from 0 (< limit of detection) at 50 m and below at Stns A07, A10, A16 and A20 to 6.1 ng l^{-1} (Stn A03, at the surface).

In February, zeaxanthin concentrations were homogeneous along the water column, showing a north-to-south decreasing gradient (Fig. 5): the highest values were found at the northern station (A02; $61.5 \pm 23.7 \text{ ng l}^{-1}$), whereas the minima were detected at Stn A16 ($1.92 \pm 1.02 \text{ ng l}^{-1}$). In contrast, Stn A20 presented a stratified distribution, ranging from 27.3 ng l^{-1} at the surface to 0.9 ng l^{-1} at 130 m. In October, the highest concentrations were found at the surface at the northernmost station (71.6 ng l^{-1}) and between 30 and 50 m in the central/southern sector of the Adriatic ($40.7 \pm 10.1 \text{ ng l}^{-1}$).

Divinyl chl *a* concentrations were always below detection limits during February, whereas in October (Fig. 5), detectable values ranged between 4.4 ng l^{-1} (Stn A10, surface) and 84.4 ng l^{-1} (Stn A10, 50 m). Concentrations higher than 50 ng l^{-1} were detected at the DCM between 30 and 60 m at the southern stations (Stn A07 to A20).

Statistical analysis

Results from Spearman's correlation test to detect positive or negative correlations between PCU expt outcomes and physicochemical/biological variables are shown in Table 2. The calculation of Spearman's coefficient did not highlight any positive or negative relationship between environmental variables and PCU percent change; the only significant correlation was between temperature and ΔLeu ($\rho = 0.39$; $N = 26$; $p < 0.05$). PCU rates measured after light and dark incubations were correlated negatively to salinity and positively to BChl *a*, prokaryote abundance (DAPI) and *Synechococcus* abundance.

The multivariate analysis results are reported in Fig. 6. In this case, the first 2 components explained 68.75% of the total variance. The rotation of variables highlighted that chl *a* and zeaxanthin were negatively related to salinity, BChl *a* was tightly connected to DOC concentration and to total prokaryote abundance, and divinyl chl *a* was in opposition to temperature. The plotting of sampling sites highlighted 6 main clusters: (I) samples collected in October in an area of strong freshwater influence (Stn A02) abundant in chl *a* and zeaxanthin, without traces of divinyl chl *a*; (II) samples collected in October in a northern area influenced by freshwater, with absence of *Prochlorococcus*, presenting a high load of DOC, total prokaryotes and BChl *a*-containing organisms; (III) samples collected in October in surface/sub-surface waters of the central and southern sectors of the Adriatic Sea, presenting high temperature and relatively low chl *a* and zeaxanthin concentrations; (IV) samples collected in the central-southern Adriatic, both in February and October, showing high salinity, low pigment concentration, low DOC concentration and low prokaryote numbers; (V) samples collected in October at the DCM, presenting the highest divinyl chl *a* content; (VI) samples collected in February at the northern stations, with abundant chl *a* and zeaxanthin concomitantly with low salinity values. The 6 clusters of sampling sites were delineated only for descriptive purposes and have no statistical significance.

Table 2. Relationship (Spearman's coefficient ρ) between results from prokaryotic organic carbon uptake (PCU) experiments and selected parameters considering the whole surface dataset. DOC: dissolved organic carbon, BChl *a*: bacteriochlorophyll *a*; Zea: zeaxanthin; DvChl *a*: divinyl chlorophyll *a*; DAPI: DAPI-stained (total) prokaryotes; PAR: photosynthetically active radiation measured during incubations; PCU % change: PCU measured under light conditions as a percentage of dark incubations; PCU dark: PCU measured after dark incubations; PCU light: PCU measured after light incubations; ΔLeu : PCU light-PCU dark. ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

	PCU % change	PCU ΔLeu	PCU dark	PCU light
Temperature	ns	0.39* (N = 26)	ns	ns
Salinity	ns	ns	-0.48** (N = 27)	-0.43* (N = 25)
DOC	ns	ns	ns	ns
BChl <i>a</i>	ns	ns	0.96*** (N = 9)	0.92*** (N = 8)
Zea	ns	ns	ns	ns
DvChl <i>a</i>	ns	ns	ns	ns
Chl <i>a</i>	ns	ns	ns	ns
<i>Synechococcus</i>	ns	ns	0.64*** (N = 25)	0.64*** (N = 24)
DAPI	ns	ns	0.64*** (N = 25)	0.69*** (N = 24)
PAR	ns	ns	ns	ns

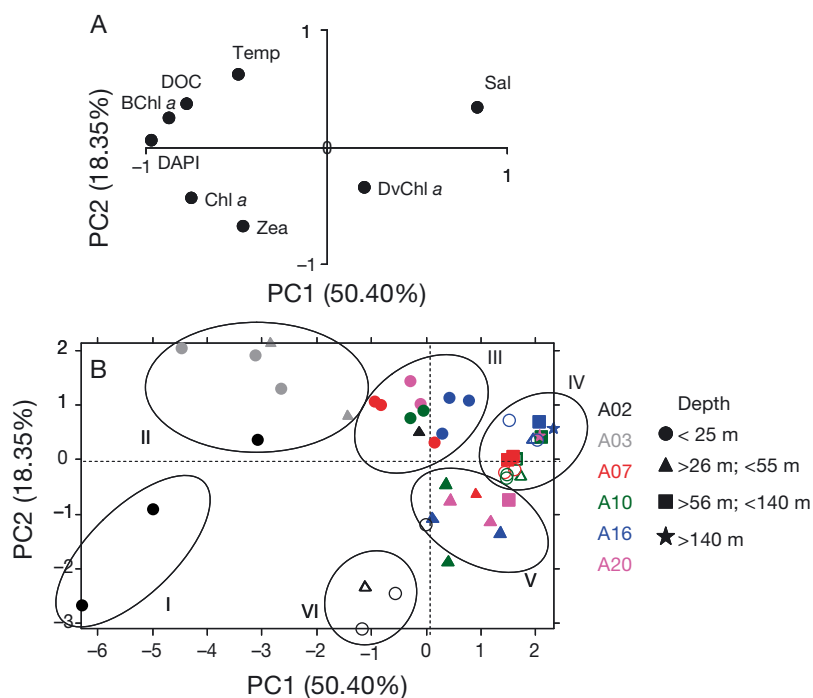


Fig. 6. Principal component analysis biplot of (A) rotated variables and (B) sampling sites applied to pigments and environmental parameters determined in February (open symbols) and October (full symbols). Different colours indicate different stations, different symbols refer to different water column layers. The percentage of variance for each component (PC1 and PC2) is indicated. The characterisation of the 6 distinct clusters (I–VI) is provided in the ‘Results’. The 6 groups were identified only for descriptive purposes and have no statistical significance. Samples collected at Stns A03 and A20 in February were not included in the analysis due to the lack of dissolved organic carbon (DOC) data. Temp: temperature; Sal: salinity; BChl a: bacteriochlorophyll a; Zea: zeaxanthin; DvChl a: divinyl chl a; DAPI: DAPI-stained total prokaryotes

DISCUSSION

Effect of sunlight on PCU

In this study, we examined the differences that occur between light and dark incubations for PCU measurements in samples subjected to different light and temperature perturbations relative to the natural *in situ* conditions. Our results revealed different behaviours of prokaryotic communities located at different sites throughout the Adriatic Sea (Figs. 3 & 4). Light-exposed samples for PCU were either photo-enhanced, photoinhibited or unaffected without any defined geographical or seasonal pattern. Most of the experiments carried out in surface waters in the central basin did not show any difference between light and dark incubations, and these corresponded to the lowest measured leucine uptake rates, whereas a positive effect of light on production processes was

found in a minority of experiments (6 out of 26) carried out both in the northern and in the southern basins. The lack of a consensus among these results is in agreement with a recent review by Ruiz-González et al. (2013). By summarizing results from more than 25 studies, the authors pinpointed both leucine uptake stimulation and inhibition in natural samples exposed to different radiations, stressing the negative effect of UVR on heterotrophic production.

Despite all of the studies performed thus far, there are still many uncertainties regarding the role of photoheterotrophs in light-enhanced amino acid uptake in natural systems. Photo-enhanced leucine uptake was found, for example, by Michelou et al. (2007) when partially screening samples from UVR in surface waters along 2 transects in the North Atlantic Ocean, and in the North Pacific Ocean, similar observations have been reported for in-depth integrated PCU rates (Church et al. 2006). Both studies highlighted the potential pivotal importance of photoheterotrophic growth by *Prochlorococcus*. These organisms are not present or are very low in abundance in the Adriatic Sea, representing $\leq 3\%$ of picophytoplanktonic biomass in the northern basin (Radić et al. 2009) and

<10% of total picophytoplankton abundance along the south-eastern coast (Šilović et al. 2011). On the other hand, *Synechococcus* is present in the whole basin (e.g. Šilović et al. 2011, 2012), potentially contributing to leucine utilization (e.g. Paoli et al. 2008, Ruiz-González et al. 2012b). In our study, *Prochlorococcus*-indicating pigments were not detected by HPLC measurements in February (divinyl chl a, Fig. 5), and only a few ribotypes were observed through DGGE in the central and southern basins (see Fig. S1 and Table S1 in the Supplement), at stations where PCU was unaffected by light incubations. As revealed by Spearman’s correlation coefficients applied to surface data, the percent change from dark incubations and ΔLeu were neither related to pigment concentrations nor *Synechococcus* or total prokaryote abundance. As evidenced by Ruiz-González et al. (2012b), single microbial groups or taxa (represented here by *Cyanobacteria* and AAPs)

can uptake more organic carbon (C) when exposed to light even in the presence of UVR (e.g. *Synechococcus*), but the overall community response can still be negative when this photostimulation is not enough to compensate for the reduction in activity of other dominant phylotypes. Although the experiment outcomes revealed no clear seasonality in the differential uptake of leucine in light and dark conditions (Fig. 3), the positive correlation between ΔLeu and temperature (Table 2) indicates that the overall rate of light-enhanced C uptake was higher in October than in February, possibly as a consequence of faster absolute PCU (e.g. Celussi et al. 2011).

Besides the restricted presence of *Prochlorococcus* and the detrimental effect of UVR in our unscreened incubators, understanding why surface photoheterotrophic stimulation of PCU is a weak or absent phenomenon in the Adriatic Sea (evidencing heterotrophy as the primary metabolism involved in prokaryotic organic matter uptake) is not easy. One possible explanation might be connected to inputs of land-derived nutrients which may represent an important source of C and energy (directly via allochthonous organic material and indirectly by fuelling primary productivity) which would favour heterotrophic prokaryotes or heterotrophic processes (Paoli et al. 2006). If systems for photophosphorylation in non-cyanobacterial photoheterotrophs are not necessary because of a high load of reduced C in the environment, then the light-enhanced C uptake would be negligible (Kolber et al. 2000, Lamy et al. 2011). Even though DOC concentrations in this area (especially in the northern basin; see also Giani et al. 2005) are generally higher than those measured at several oceanic locations (Ogawa & Tanoue 2003), no significant direct relationship was found between ΔLeu (and % change) and DOC, implying that organic C content is not per se responsible for the lack of photostimulation. On the contrary, DOC concentrations were positively correlated to both light and dark-measured PCU (Spearman $\rho = 0.49$ and 0.38 , respectively; $p < 0.05$; $n = 53$), suggesting a prevalently heterotrophic metabolism, even in presence of light.

Furthermore, we did not find any direct relationship between light intensity (Table 1) and light-enhanced/inhibited PCU (Table 2). By excluding UVR and utilizing different approaches, Pakulski et al. (2007) and Church et al. (2004), for example, found direct relationships between irradiance and light-enhanced amino acid uptake. In the present study, different assemblages (collected at different stations) were used, each being exposed to different irradiances according to different sampling times

and different weather conditions. The ecological relationship between light-enhanced/inhibited PCU and irradiance is still not clear. Differential responses to light can be the result of diverse assemblage compositions (Ruiz-González et al. 2012b) which can affect the sensitivity of the community to harmful radiation competing with the benefits coming from different light-harvesting pigments (or proteins). Previous comparisons of PCU estimates obtained under light or dark conditions in the northern Adriatic Sea revealed strong photoinhibition (30% of estimates) caused by PAR (Sommaruga et al. 1997), whereas most of the studies carried out worldwide highlight the detrimental role of UVR (e.g. Ruiz-González et al. 2012a,b and references therein).

Samples collected below the surface layer (both in the photic and aphotic zones) and incubated under surface light and temperature conditions also typically did not show any enhancement of leucine uptake in the light, with only few exceptions (Fig. 4). On the contrary, when a significant difference between light and dark treatment was found, in most of the cases leucine uptake was inhibited. This outcome could be related to the 'light exposure history' (Ruiz-González et al. 2013), according to which exposing a natural sample to an optical spectrum different from the *in situ* one would interfere with the dynamics of photoacclimation (which are linked to community composition and metabolism), eventually enhancing the negative effect of harmful radiation (i.e. UVR).

Dynamics of pigments relevant to photoheterotrophy

Even though most of the research performed to date has not detected a direct relationship between the abundance of specific photoheterotrophic organisms and the bulk of leucine uptake in the light (Ruiz-González et al. 2013), understanding the dynamics of photoheterotrophs in a given environment can be relevant since they can occasionally determine significant differences between the light and dark C flow through the biological system. This has been observed in particular for *Prochlorococcus* (e.g. Church et al. 2004, 2006, Michelou et al. 2007, Mary et al. 2008), whereas to date no study has highlighted any light-enhanced bulk leucine uptake related to AAPs (e.g. Kirchman et al. 2014, Stegman et al. 2014).

It is important to point out that at the time of sampling no published data about *Prochlorococcus* in the Adriatic Sea were available in the scientific litera-

ture, and for this reason our sampling scheme was exploratory rather than focused. The few data that became subsequently available do not allow a comprehensive description of spatial and temporal dynamics of *Prochlorococcus* in this basin. It is generally more abundant around 50 m depth in the southern basin from spring (Šilović et al. 2011) to late summer, whereas in the northern sector, abundant populations have been observed in summer (Radić et al. 2009), either in surface (October 2006) or shallow bottom waters (June 2006). Divinyl chl *a* data presented here (those in the Mediterranean Sea have been shown to follow *Prochlorococcus* cell abundance; Garczarek et al. 2007) highlight the extremely scarce presence of this taxon throughout the whole Adriatic in winter, whereas in late summer it was found especially at 50 m depth in the central and southern basins (cluster V in Fig. 6) where AAPs (BChl *a* data) and *Synechococcus* populations were not very abundant. Since no relationship between divinyl chl *a* and leucine uptake was found in the surface layer, it is plausible that the contribution of *Prochlorococcus* in photoheterotrophic processes is more pronounced in intermediate waters (30–70 m) in summer. On the other hand, *Synechococcus* was present throughout the whole Adriatic Sea during both cruises, potentially contributing to leucine utilization, as already observed elsewhere (Paoli et al. 2008, Ruiz-González et al. 2012b). Zeaxanthin concentration, highly correlated with *Synechococcus* abundance (Spearman rank correlation = 0.67; $p < 10^{-6}$; $n = 54$), was also in close relationship with chl *a*. Previous investigations have highlighted that in the southern Adriatic Sea picocyanobacteria constitute the major planktonic fraction contributing to phototrophic biomass (Cerino et al. 2012), whereas in the northern basin higher nutrient availability makes microphytoplankters the dominating size class in terms of biomass (Pugnetti et al. 2008). Considering absolute values, however, *Cyanobacteria* are usually more abundant in the north (Gallina et al. 2011), as a consequence of the decreasing north-to-south trophic gradient which characterises the Adriatic and affects all phytoplankton (Cerino et al. 2012 and references therein). Our zeaxanthin data confirm this kind of distribution since its highest concentrations were found in nutrient-rich surface diluted waters (northern stations) both in February and October (clusters I and VI in Fig. 6: Spearman's ρ between salinity and zeaxanthin = -0.35 ; $p < 0.008$; $n = 56$). When considering only the October zeaxanthin distribution, it was evident that the concentrations of this pigment were more pronounced in deep samples

where divinyl chl *a* was maximal and where *Synechococcus* numbers were lower compared to the surface (Fig. 2), suggesting that occasionally high zeaxanthin at depth is associated with the *Prochlorococcus* population.

BChl *a* distribution along the Adriatic followed total prokaryote abundance ($\rho = 0.88$; $p < 10^{-6}$; $n = 56$), indicating that AAPs are probably a constant fraction of bacterioplankton in the upper water column (close relationship between DAPI and BChl *a* in Fig. 6). The close relationship with DOC ($\rho = 0.74$; $p < 10^{-6}$; $n = 54$) indicates that AAPs preferentially rely on available organic carbon rather than photosynthesis, as already evidenced in other Mediterranean waters (Lamy et al. 2011). BChl *a* maxima, consistently higher than those reported by Lamy et al. (2011), were retrieved in the northern basin in summer (cluster II in Fig. 6), where PCU incubation experiments have not been performed, whereas minima were found in the deepest layers of the southern stations where lowest DOC and total prokaryotes were estimated (cluster IV in Fig. 6). These findings are in agreement with results reported by Ferrera et al. (2014), who found a defined seasonal pattern with summer maxima and winter minima both in AAP abundance and BChl *a* concentration. However, it must be kept in mind that we have presented BChl *a* data and not AAP abundances and therefore only tentative extrapolations can be made to describe AAP distribution considering that (1) per cell concentration of BChl *a* can be very variable and (2) a previous study in the Mediterranean Sea showed a highly significant correlation between pigment concentration and cell numbers (Lamy et al. 2011). Bearing this in mind, a possible transformation of BChl *a* data into AAP abundance, using the per cell range provided by Lamy et al. (2011) ($0.05\text{--}0.44\text{ fg cell}^{-1}$), would indicate that in our study AAPs might represent up to 10% of total prokaryotes. This estimate is rather low when compared to other studies which identified a maximum 25% contribution of AAPs to total bacteria (e.g. Lami et al. 2007) and is fairly similar to the general percent ratio of picocyanobacteria to total bacteria in the Adriatic (Radić et al. 2009, Gallina et al. 2011, Šilović et al. 2011). This would indicate that in this basin, photoheterotrophs (*Cyanobacteria* and AAPs) can be as high as 20% of total prokaryotes, notably lower than estimates reported in studies where a clear signal of photoheterotrophy was found, such as, for example, ~25% of *Prochlorococcus* over non-pigmented bacteria in lit oceanic waters (Church et al. 2006, Mary et al. 2008).

CONCLUSIONS

This study represents the first attempt to understand the prokaryotic organic matter dynamics under full sunlight conditions (light vs. dark) over the whole Adriatic Sea. Light-exposed samples were mainly unaffected by solar radiation, implying either a counterbalance between UVR-related negative effects and positive responses of the photoheterotrophic communities and/or a general reliance of prokaryotes on the abundant DOC load in this semi-enclosed basin. Even though we did not find any relationship between photostimulation/photoinhibition and the abundance of specific photoheterotrophic groups, locally leucine uptake in the light was significantly different from dark controls, suggesting that when these measurements are meant to compute C budgets, *in situ* light incubations could be more appropriate than dark ones. Direct cell counts indicate that *Cyanobacteria* can represent up to 10% of total prokaryotes, and derived data suggest that the same percentage can be applicable to AAPs. The resulting 20% (*Cyanobacteria* + AAPs) is indeed notably lower than estimates obtained in previous case studies where clear photoheterotrophy signals have been evidenced.

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