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Navigating the Future

Distribution and diversity of marine picocyanobacteria community: Targeting of *Prochlorococcus* ecotypes in winter conditions (southern Adriatic Sea)

Ivana Babić [a,*,1], Ines Petrić [b,*,1], Sunčica Bosak [a], Hrvoje Mihanović [c], Iris Dupčić Radić [d], Zrinka Ljubešić [a]

[a] Department of Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia
[b] Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia
[c] Physical Oceanography Laboratory, Institute for Oceanography and Fisheries, Šetalište I. Meštrovića 63, 21000 Split, Croatia
[d] Institute for Marine and Coastal Research, University of Dubrovnik, Kneza Damjana Jude 12, 20000 Dubrovnik, Croatia

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ABSTRACT

Adriatic, the northernmost part of the Mediterranean Sea, due its oligotrophy, topography, and hydrology dynamics, and complex circulation patterns, was suggested as an important study site for rapid climatology impacts. Its southern part is mainly oligotrophic and dominated by picophytoplankton, with cyanobacteria as main representatives. Diversity and distribution patterns of different *Prochlorococcus* ecotypes were investigated by molecular tools and flow cytometry during the winter convection event in the southern Adriatic (BIOTA winter cruise; February/March 2015). Phylogenetic diversity based on clone libraries of the 16S–23S ribosomal DNA ITS region, as well as flow cytometry (histograms of red fluorescence), indicated presence of 2 different *Prochlorococcus* in the Adriatic. HLI, as a typical clade for Mediterranean Sea, was likewise found to be dominant *Prochlorococcus* in the Adriatic, followed by less abundant LLI clade. In addition, *Prochlorococcus* were found to co-occur with diverse *Synechococcus* population (53% and 47% of obtained ITS sequences, respectively). Different *Prochlorococcus* ecotypes had similar patterns of vertical distribution, predominantly occupying upper 100 m depth layer, but their distribution was clearly affected by the heterogeneity of hydrological conditions, nitrogen concentration and temperature along vertical and horizontal sampling points. Different studies pointed out that, as a consequence of climate changes, serious alteration of biological and ecological patterns are already taking place Therefore, understanding of the distribution and abundance of picophytoplankton in Adriatic, being still limited, is much needed baseline for predicting possible biogeochemical impact of future environmental changes.

1. Introduction

Marine unicellular pico-cyanobacteria *Prochlorococcus*, the smallest known photosynthetic prokaryotes, dominate oligotrophic, warm, euphotic zones of open ocean waters (Chisholm et al., 1988; Chisholm et al., 1992; Partensky et al., 1999a; Biller et al., 2015). Annual mean global abundance of *Prochlorococcus* in the world oceans reaches $2.9 \pm 0.1 \times 10^{27}$ cells, accounting for 50% of the total chlorophyll in open ocean surfaces (Partensky et al., 1999a; Flombaum et al., 2013). Alongside *Synechococcus*, representing second dominate picophytoplankton genera, these picocyanobacteria have an important role in oceanic carbon fixation and nutrient cycling (Partensky et al., 1999b; Zwirglmaier et al., 2008; Mella-Flores et al., 2011; Flombaum et al., 2013). Geographical, as well as vertical distributions of these two lineages, is constrained by the light, temperature, nutrients, and chlorophyll *a* concentration. *Prochlorococcus* prefers warm (>15 °C) oligotrophic waters, whereas *Synechococcus* dominates in coastal and more temperate/mesotrophic open ocean waters (Partensky et al., 1999a).

Prochlorococcus group members share >97% similarity of their 16S rRNA genes and were traditionally designated as a single microbial 'species'. However, extensive molecular analyses (Moore and Chisholm, 1999; Partensky et al., 1999a; West et al., 2001; Rocap et al., 2002; Martiny et al., 2009) showed that this group is composed of multiple phylogenetically and physiologically distinct clades, divided into two separate groups — high-light-adapted (HL) and low-light-adapted (LL) ecotypes. In flow cytometric signatures, two ecotypes are usually well discriminated as "dim" and "bright" populations of *Prochlorococcus*, with dim populations corresponding to HL and bright to LL ecotypes (West and Scanlan, 1999). HL ecotypes were shown to dominate upper regions of the euphotic zone, while LL ecotypes were shown to be the most abundant in the lower euphotic zone (Rocap et al., 2002;

* Corresponding authors.
E-mail addresses: ibos254@gmail.com, ivana.bosnjak@biol.pmf.hr (I. Babić), ipetric@irb.hr (I. Petrić).
[1] Equal contribution.

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Johnson et al., 2006 and Martiny et al., 2009). Analysis of the variable 16S–23S rRNA internal transcribed spacer (ITS) sequences (Rocap et al., 2002 and Johnson et al., 2006) allowed further differentiation into 12 different clades. Six of them, namely HLI, HLII, LLI, LLII, LLIII and LLIV, are well known and described, while three new HL clades (HLIII, HLIV and HLV) and three new LL-adapted clades (NC1, LLV and LLVI) were only recently described (West et al., 2001; Rocap et al., 2002; Huang et al., 2012; Malmstrom et al., 2013; Biller et al., 2015). *Synechococcus* have also high genetic diversity, with group members subdivided into three major Subclusters, 5.1, 5.2 and 5.3 (Dufresne et al., 2008; Scanlan et al., 2009). The most abundant Subcluster 5.1, found throughout the euphotic zone, comprises of at least 20 recognizable clades and is phylogenetically closely related to *Prochlorococcus* (Rocap et al., 2002; Fuller et al., 2003; Ahlgren and Rocap, 2006; Zwirglmaier et al., 2008; Choi and Noh, 2009; Ahlgren and Rocap, 2012).

Southern Adriatic, the deepest part of the northernmost Mediterranean sub-basin, is characterized by general cyclonic circulation (Gačić et al., 2002). The basin is connected to the Eastern Mediterranean (EM) through the Strait of Otranto. Waters inflow from the Ionian Sea into the Adriatic by the East Adriatic Current that partly belongs to the South Adriatic Gyre (Artegiani et al., 1997). During winter, strong convection processes cause the mixing of fresher Adriatic and more saline Ionian waters, resulting in the formation of Adriatic Dense Water (AdDW). Bottom layers of the pit are seldom influenced by convection processes and are primarily under influence of colder and denser water formed in the Northern Adriatic (Gačić and Civitarese, 2012). Southern Adriatic is highly oligotrophic being influenced by the inflow of saline and warmer water from the Eastern Mediterranean, represented mostly by the Levantine Intermediate Water (LIW) (Zore-Armanda, 1969). In these conditions picophytoplankton community dominates while microphytoplankton communities are limited to low-salinity surface layers and deep chlorophyll maximum depths (DCM) (Cerino et al., 2012).

Different studies acknowledged that *Prochlorococcus* (as well as *Synechococcus*) resides in the Adriatic (Radić et al., 2009; Šantić et al., 2011; Šilović et al., 2011; Najdek et al., 2014 and Korlević et al., 2015), however due to inconsistencies in the results, distribution patterns and ecotype diversity of *Prochlorococcus* in the Adriatic are yet to be revealed. With the goal to reveal spatial and temporal distribution of *Prochlorococcus* population in the Adriatic, we carried out a research cruise in the southern Adriatic Sea. By combining flow cytometry and molecular approach based on 16S–23S rRNA ITS region, we aimed to explore full extent of the *Prochlorococcus* diversity and, ultimately, to understand its relationship to the dynamics of the marine environment.

Studies focusing on the consequences of climate change on species distribution and ecosystems structure have showed that a serious alteration of biological and ecological patterns is taking place worldwide (Vilibić and Supić, 2005; Lejeusne et al., 2010; Albouy et al., 2014). Due to its characteristics (depth, deep-water turnover and endemism) it is expected that in the Mediterranean, in comparison with other seas, potential amplification of impacts and earlier changes in biodiversity due to the climate change would occur (Marine Board Special Report, 2011). Any changes in biodiversity may affect ecosystem functioning, even in the case of invasions by a single species. This all makes this system an ideal model for investigating biodiversity response to direct and indirect effects of climate-related variables. *Prochlorococcus* considering its high abundances and contributions to primary production may have large impacts on biogeochemical cycles and marine ecosystems. Therefore, studies on *Prochlorococcus* as a model system, would not only allow us to advance our understanding on microbial ecology of the Adriatic Sea but would eventually allow us to propose *Prochlorococcus* as an indicator organism for future changes in this ecosystem, introduced by the projected climate changes.

2. Materials and methods

2.1. Study site and sampling design

The BIOTA 2015 winter cruise was conducted in eastern part of the southern Adriatic between 28 Feb and 3 Mar 2015. Stations sampled during the winter BIOTA 2015 cruise are shown in the Fig. 1 and consisted of 7 stations on P transect and 5 stations on M transect. Water temperature, salinity and oxygen at all sampling sites were measured using a CTD probe (SBE 19plus, SEA-Bird Electronics Inc., USA), additionally equipped with WET Labs FLNTU for measurement of chlorophyll *a* fluorescence (Chl *F*) and Biospherical Instruments photosynthetically active radiation (PAR) sensor. Factory calibration was used to convert Chl *F* measurements into nominal Chl *a* concentration ($\mu g\ L^{-1}$; Cetinić et al., 2015), while euphotic zone depth (i.e. 1% PAR depth) was determined from the corresponding PAR measurements (Table 1).

Subsamples for nutrient measurement: nitrite (NO_2), nitrate (NO_3) and phosphate (PO_4) were frozen (−20 °C) and analyzed in laboratory with standard oceanographic methods, using spectrophotometer PerkinElmer Lambda 15 (Ueberlingen, Germany) (Strickland and Parsons, 1972). Subsamples (50 mL) for ammonium analysis were preserved with 2 mL of phenol-ethanol solution ($1\ mol\ L^{-1}$), kept at 4 °C in dark and analyzed within a month according to Ivančić and Degobbis (1984).

2.2. Flow cytometry sample processing and analysis

Subsamples of 3 mL seawater were preserved with 0.1% glutaraldehyde (final conc.), incubated for 10 min in the dark, deep frozen in liquid nitrogen and stored at −80 °C. Analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California) equipped with standard filter setup and with a 15 mW argon laser (488 nm excitation). Data acquisition was done at a high flow rate (approximately $90\ mL\ min^{-1}$) for 5 to 7 min depending on the concentration of the target population, using high gain settings (FSC E01, SSC 380, FL1 400, FL2 585, FL3 640) and red fluorescence as threshold parameters. Data were acquired in a log mode. Fluorescence polystyrene calibration beads (Partec Calibration Beads 1 μm, ref. no. 05-4007) were added to all samples as an internal standard. For sample processing, BD FACSFlow sheath fluid (ref. 342003) was used. Acquisition was carried out with the CellQuest Pro software (Becton Dickinson) and data analysis with FlowJo software (Tree Star, Inc., Ashland, Oregon). Discrimination of photosynthetic groups (*Prochlorococcus* and *Synechococcus*) and enumeration of populations was made using a three dimensional gate based on red fluorescence, orange fluorescence and side scatter signals. Only particles that appear in the selected regions in these three plots were counted. *Prochlorococcus* "dim" and "bright" populations were discriminated from the histograms of red fluorescence.

2.3. Sample collection for molecular analysis

Three stations (P150A, P600 and M300) were chosen for molecular-based studies based on their physico-chemical profiles of the water column. P150A was chosen as a representative of neritic stations and the impact of land was visible in lower salinity in the upper layer, while P600 and M300 were representatives of pelagic stations, both of them under high influence of intrusion of LIW in Adriatic (Table 1, Fig. 1).

Water samples were collected at multiple depths (Table 1) with 5 L Niskin bottles. For each sampling depth triplicate samples were taken. 1.0 L of each water sample was pre-filtered through 20 μm-pore-size filters following filtration onto 0.2 μm-pore-size mixed cellulose ester membrane filters (47 mm, Whatman, UK). The filters were placed in 1 mL of sucrose-lysis buffer (50 mM Tris–HCl, pH 8; 40 mM EDTA, pH 8; 0.75 M Sucrose) and immediately stored in liquid nitrogen

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