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Identification and Characterisation of Microbial Populations Using Flow Cytometry in the Adriatic Sea

Danijela Šantić and Nada Krstulović
Institute of Oceanography and Fisheries
Croatia

1. Introduction

Synechococcus, *Prochlorococcus* and picoeukaryotes have an important role in primary production and also represent significant food resource for protists and small invertebrates (Callieri & Stockner, 2002), thus participating in the role of prey in the energy flow at higher trophic levels. Together with mentioned primary producers, heterotrophic bacteria are important components of marine plankton communities (Azam & Hodson, 1977). On one hand heterotrophic bacteria are consumers of dissolved organic matter (DOM), and as such they are links in the chain of matter and energy flow through an ecosystem (Cole et al., 1988). On the other hand, they decompose organic matter and transform inorganic compounds in forms suitable for primary producers (Ducklow et al., 1986).

Until recently, most determinations of bacterial abundance were usually performed by epifluorescence microscopy of DAPI or Acridine Orange stained samples (Hobbie et al., 1977; Porter & Feig, 1980). During the 1990's flow cytometry was introduced in oceanography (Darzynkiewicz & Crissman, 1990; Allman et al., 1993; Fouchet et al., 1993; Troussellier et al. 1993; Shapiro, 1995; Davey & Kell, 1996; Porter et al., 1997; Collier & Campbell, 1999). Use of flow cytometry in marine microbiology resulted in the discovery of several bacterial groups based on different content of DNA and different amount of fluorescence (Li et al., 1995; Marie et al., 1997): high nucleic acid content group with high amount of fluorescence (HNA) and group with low nucleic acid and low amount of fluorescence (LNA) content (Gasol & Moràn, 1999; Gasol et al., 1999); and with discovery of cyanobacteria *Prochlorococcus* (Chisholm et al., 1988). So, due to endogenous fluorescence (fluorescing photopigments) and exogenous fluorescence (DNA dyes) it is possible to distinguish the picoplankton cells from other particles in the water column. Detailed, stained heterotrophic bacteria can be detected and discriminated from other non-bacterial particles with a combination of light scatter, green (DNA dyes), orange or red fluorescence (fluorescing photopigments). In addition, the combination of these parameters allows better resolution of the different subpopulation (HNA and LNA) within the heterotrophic bacterial group (Figure 2). Autotrophic picoplankton cells contain plant pigments in a broad of variety, with chlorophyll *a* as the major compound and single source of the red fluorescence. The chlorophyll fluorescence is the principal factor used for discriminating autotrophic cell from other particles, so heterotrophic bacterial cells can easily be distinguished from

autotrophic cells in a plot Red vs. Green fluorescence. Further, the orange fluorescence can be used to detect second important fluorescing photopigment respectively phycoeritrin. Phycoeritrin is typical in many *Synechococcus* spp. and some picoeukaryotes, so *Synechococcus*, *Prochlorococcus* and picoeukaryotes can easily be discriminated in a plot Red vs. Orange fluorescence (Figure 3). Flow cytometry also significantly reduces the time employed in each of these determinations (multiparameter analysis of individual cells); increases the level of resolution and provides new insights into the structure and functioning of plankton communities that simply cannot be obtained with conventional epifluorescence microscopy (Li et al., 1995; Marie et al., 1996; Marie et al., 1997). Flow cytometry has been routinely used for the analysis of marine samples and now is commonly accepted as a reference technique in oceanography and for the analysis of bacterial community (Monger & Landry, 1993).

Flow cytometry in our studies contributes to better understanding of prokaryotic roles in the Adriatic Sea as a separate ecosystem and as an important part of the Mediterranean Sea. Studies of prokaryotic community by flow cytometry in the eastern part of Adriatic Sea started in year 2003. The first studies were carried out for purposes of comparing two direct counting methods for bacterioplankton (Šantić et al., 2007). The accuracy of epifluorescence microscopy (EM) was assessed against direct counts made by flow cytometry (FCM). Furthermore, flow cytometry is used for investigation and characterization of heterotrophic prokaryotic community (Šolić et al., 2008; Šolić et al., 2009; Šolić et al., 2010) and autotrophic prokaryotic community (Vilibić & Šantić, 2008; Šantić et al., 2011). Autotrophic picoplankton community, including *Prochlorococcus* and picoeukaryotes, in the eastern part of Adriatic was described for the first time in the northern Adriatic Sea (Radić et al., 2009).

2. Material and methods

For comparing the two counting methods, epifluorescence microscopy and flow cytometry, samples were collected in two geographically different areas: Adriatic Sea, part of the Mediterranean Sea (Figure 1) and English Channel, part of the Atlantic Ocean (50°15' N, 4° 15' W, off shore station 6 km off Plymouth, and four shore stations from Plymouth Sound UK). From the Adriatic Sea a total of 919 samples comprising both offshore and shore areas were collected on a monthly basis from 29 sites during 2005. From the English Channel (N = 132) samples were collected at weekly to monthly intervals during winter 2006 from one offshore and four shore sites. In addition, for the purpose of testing repeatability and counting precision, four replicates were made by both direct counting methods, for each sample through vertical profile collected from shore and off shore sites from the Adriatic Sea and the English Channel. For the comparison of the share of biomass within the microbial community samples were collected on a monthly basis from the Adriatic Sea (N = 110) from one coastal (ST103) and one open sea (CA009) site during 2010. Seawater samples from the Adriatic Sea sites and offshore site in the English Channel were collected by Niskin bottles through vertical profile. At four shore sites from the English Channel samples were collected manually from the surface. All samples were fixed with formaldehyde (2% final concentration), kept in the dark at 4 °C and analyzed within two weeks. For epifluorescence microscopy (EM) preserved samples were stained with 4'-6-diamidino-2-phenylindole (DAPI) (1 µg mL⁻¹ final concentration) for 5 minutes and were filtered through 0.2 µm pore diameter black polycarbonate filters (Millipore, Ireland). Filters were then mounted on microscope slides and stored at 4 °C where they were kept until observation with an Olympus microscope under UV light (Porter & Feig, 1980) at magnification of 1000. From

100 to 400 bacteria were counted per sample, depending on concentration. For flow cytometry analysis (FCM), fixed samples were stained with SYBR GREEN I (add dye at a final concentration of 5 parts in 100 000 and incubated 15 min at room temperature in the dark) (Molecular probes Inc.) (Marie et al., 1997; Lebaron et al., 1998). Samples from the Adriatic Sea were analyzed on a Beckman Coulter EPICS XL-MCL with a high flow rate from 1 to 1.2 $\mu\text{L}/\text{sec}$. Fluorescent beads were added (Level-II Epics DIVISION of Coulter Corporation Hialeah, Florida) for calibration of fluorescence intensity. Samples from the English Channel were analyzed on a flow cytometer FACSort. Beckman Coulter flow set beads at known concentration were used to calibrate the flow rate. Bacterial abundance was determined in scatter plots of particle side scatter versus SYBR GREEN I fluorescence related to cellular nucleic acid content to discriminate bacteria from other particles (Figure 2).

Abundances of *Synechococcus*, *Prochlorococcus* and picoeukaryotes were determined using flow cytometry (Marie et al., 1997), and different populations were distinguished according to light diffraction, red emission of cellular chlorophyll content and orange emission of phycoerythrin-rich cells (Figure 3). Samples were preserved in 0.5% glutaraldehyde, frozen at -80°C and stored until analysis. Samples were analysed on a Beckman Coulter EPICS XL-MCL with a high flow rate from 1 to 1.2 $\mu\text{L sec}^{-1}$. Fluorescence beads were added to calibrate the cells' fluorescence intensity (Level-II Epics Division of Coulter Corporation Hialeah, Florida).

Biomasses of *Synechococcus*, *Prochlorococcus*, picoeukaryotes and heterotrophic bacteria were calculated by using the following volume-to-carbon conversion factors: 250 fgCcell^{-1} for *Synechococcus*, 53 fgCcell^{-1} for *Prochlorococcus*, 2100 fgCcell^{-1} picoeukaryotes and 20 fgCcell^{-1} for heterotrophic bacteria (Zhang et al., 2008).

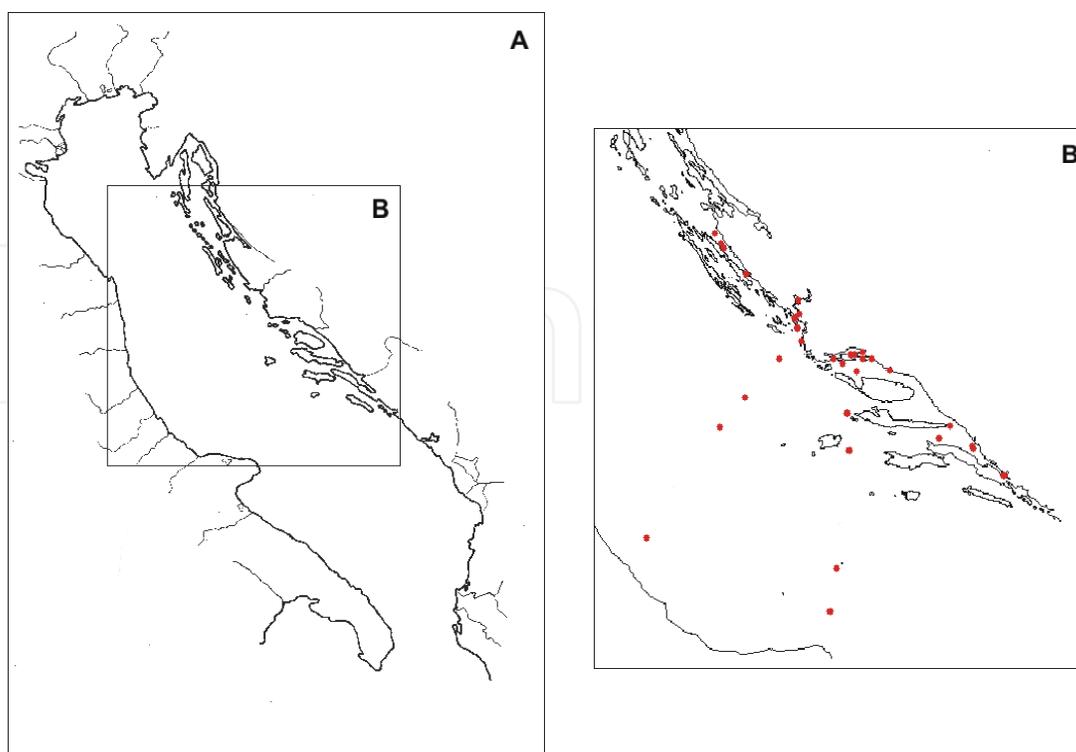


Fig. 1. (A) The Adriatic Sea (B) Locations of the investigated sites

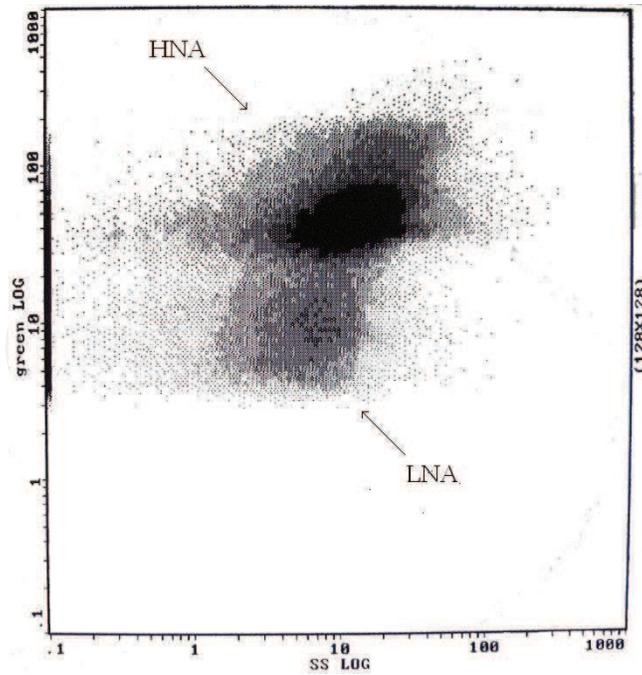


Fig. 2. Two-parametric citogram of heterotrophic prokaryotes

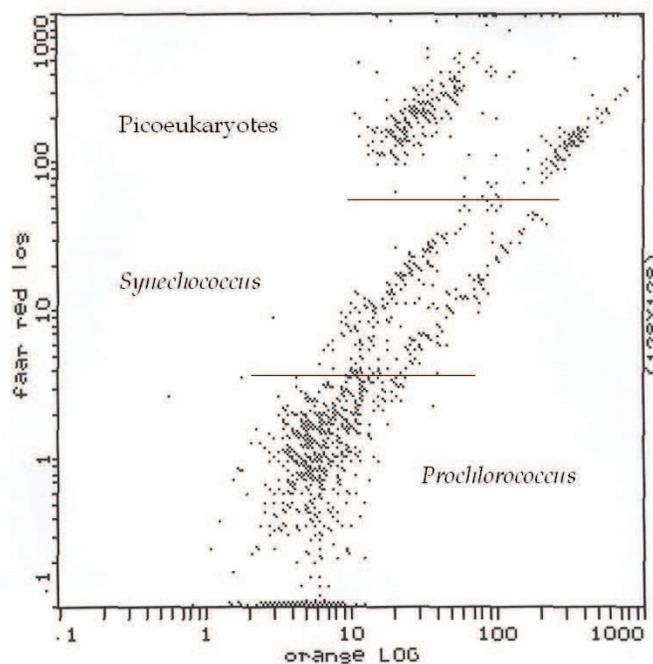


Fig. 3. Two-parametric citogram of autotrophic prokaryotes

3. Results and discussion

Detailed comparison results of two direct counting methods for bacterioplankton in the field samples from different oceanographic regions- the Adriatic Sea and the English Channel showed statistically significant correlation between bacterial counts measured with microscopy and flow cytometry for samples collected in the Adriatic Sea ($r = 0.61$, $n = 919$, $P < 0.001$) and in the English Channel ($r = 0.64$, $n = 33$, $P < 0.001$). Similar significant

correlations ($R^2 > 0.8$) were also found in the north-western Mediterranean Sea (Lebaron et al., 1993, 1998; Gasol et al., 1999). Bacterial counts obtained by flow cytometry and microscopy were more similar in the Adriatic Sea than in the English Channel and replicate experiments in both investigated areas showed that coefficients of variation were lower for bacterial counts estimated by FCM than by microscopy (Figure 4).

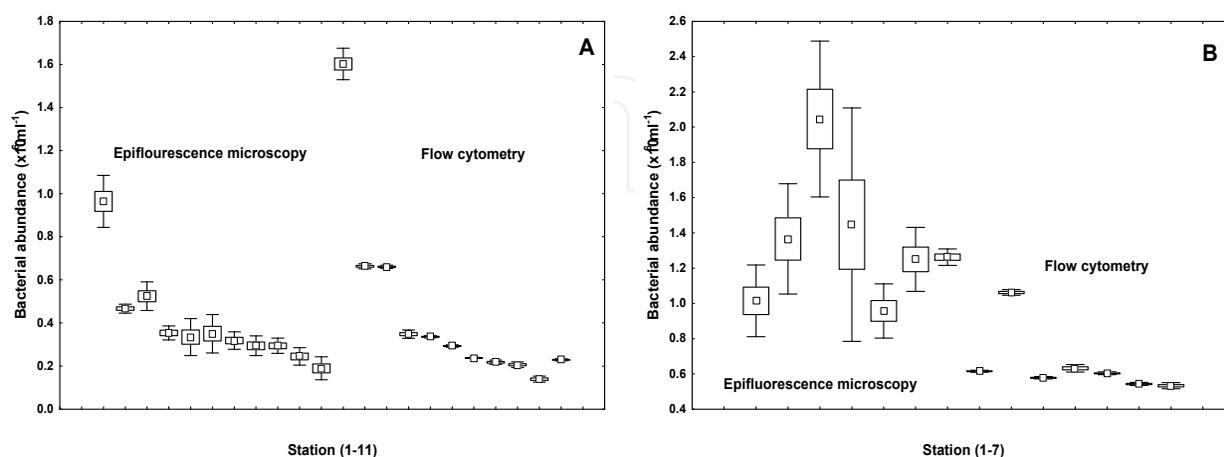


Fig. 4. Box- Whiskers (mean; 50 % conf. int.; std. dev) plot of bacterial abundance obtained by epifluorescence microscopy and flow cytometry from (A) the Adriatic Sea and (B) the English Channel

Noted significant variations in bacterial abundance obtained by microscopy can be explained by the fact that presence of organic and mineral particles and the small sizes of most marine bacteria may result in lower bacterial discrimination (Lebaron et al., 1993; Gasol & Morán, 1999). Use of flow cytometry deals better with that problem because flow cytometer is separating bacteria from other particles on the basis of light scatter (size) and pigment content and has greater precision than microscopy counting (Sieracki et al., 1995; Monger & Landry, 1993; Joachimsthal et al., 2003; Chisholm et al., 1988).

Abundance of heterotrophic bacteria obtained by flow cytometry in the investigated coastal area and in the open Adriatic Sea area ranged from 10^5 to 10^6 cells mL^{-1} and results are similar to previous values obtained by epifluorescence microscopy reported for the eastern coast of Adriatic Sea (Krstulović et al., 1995; Krstulović et al., 1997). Seasonality in the bacterial community in the most investigated coastal areas (Figure 5), with maxima in the spring-summer period and minima during winter was also determined, as in the previous reports on central Adriatic (Krstulović, 1992; Šolić et al., 2001). The average proportion of HNA bacteria in the central and southern coastal area ranged approximately from 20 % to 90 % and LNA bacteria from 10 % to 80 %, while in the open sea HNA and LNA ranged from 30 % to 70 %. In our research the prevalence of the LNA group over HNA was determined, as also established in oligotrophic areas of world's seas and oceans (Zubkov et al., 2001; Jochem et al., 2004; Andrade et al., 2007). In our research of the Adriatic Sea area, the prevalence of the HNA bacterial group in the water column was shown at stations which have a higher trophic level and our finding is consistent with studies that found that the dominance of the HNA over the LNA group in eutrophic areas directly influenced by river inflow (Li et al., 1995; Šolić et al., 2009). The predominance of the LNA group in oligotrophic conditions can be explained by the high surface area to volume ratio of cell and therefore the successful survival in poor conditions (Jochem et al., 2004).

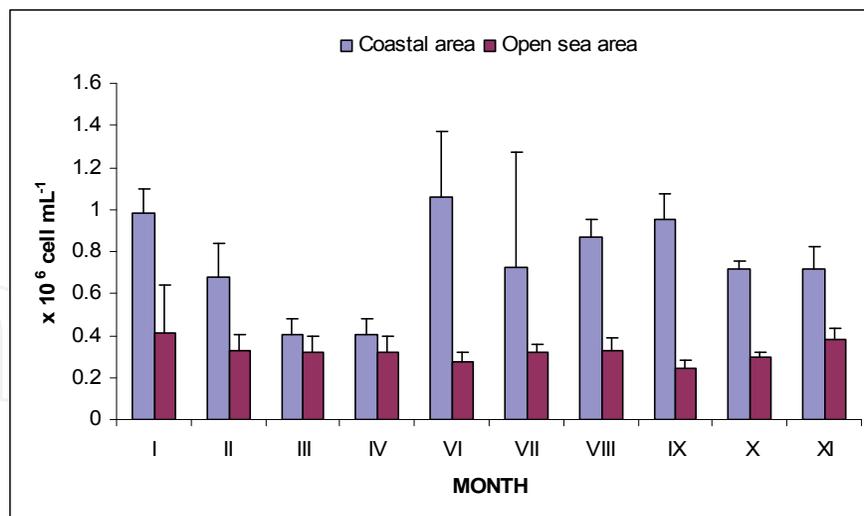


Fig. 5. Seasonal fluctuations of heterotrophic bacteria in the coastal and open sea area. Average values (column) and positive standard deviation (bars) are presented

Average abundance of *Synechococcus* in the central and southern coastal area obtained by flow cytometry ranged from 10^2 to 10^5 cells mL^{-1} , while in the open sea area it ranged from 10^3 to 6.3×10^4 cells mL^{-1} . When comparing all investigated areas, the highest individual number of *Synechococcus* was found at the coastal station and was recorded as 4.6×10^5 cells mL^{-1} (Figure 6). Abundance of *Synechococcus*, determined in the range of 10^2 to 10^5 cells mL^{-1} , is consistent with previous results obtained by epifluorescence microscopy and reported by Ninčević Gladan *et al.* (2006). According to the literature, similar ranges of *Synechococcus* abundance (10^3 to 10^5 cells mL^{-1}) have also been obtained by flow cytometry in the north-western Mediterranean (Bernardi Aubry *et al.*, 2006), eastern Mediterranean (Uysal & Köksalan, 2006) and the northern Adriatic Sea (Paoli & Del Negro, 2006; Radić *et al.*, 2009).

Our investigations revealed the presence of *Synechococcus* over a wide temperature range in the coastal area, as well as in the open sea. Moreover, increased numbers of *Synechococcus* were found during the warmer seasons, except in the eutrophic coastal area where high values were observed during the colder seasons. Although many authors describe these cyanobacteria as eurythermal organisms (Waterbury *et al.*, 1986; Shapiro & Haugen, 1988; Neuer, 1992), seasonal distribution of *Synechococcus* in the north-western Mediterranean Sea (Agawin *et al.*, 1998) and the northern Adriatic Sea (Fuks *et al.*, 2005) have shown an increased abundance of this genus during the warmer seasons and a lower abundance during the colder seasons. Our research determined abundance of *Synechococcus* over a wide temperature range and showed *Synechococcus* as eurythermal organisms in accordance with the earlier studies (Waterbury *et al.*, 1986; Shapiro & Haugen, 1988; Neuer, 1992). The average cell abundance of *Prochlorococcus* in the central and southern coastal area of eastern Adriatic Sea ranged from 0 to 10^4 cells mL^{-1} , while the average abundance ranged from 10^3 to $x 10^4$ cells mL^{-1} in the open sea. Similar to *Synechococcus*, variations in the abundances of *Prochlorococcus* were more pronounced in the coastal areas compared to the open sea area (Figure 7). When comparing all investigated areas, the highest individual number of *Prochlorococcus* was found at the station located at the mouth of river Krka and was 7.1×10^4 cells mL^{-1} (Figure 7). This is consistent with the high abundance of *Prochlorococcus* recorded in the Mediterranean coastal and open sea waters, where abundance was shown to range, in

average order of magnitude, from 10^3 to 10^4 cells mL^{-1} (Sommaruga *et al.*, 2005; Garczarek *et al.*, 2007). For *Prochlorococcus* our research results indicate that cells are detectable within the temperature range of 6.33 °C to 26.93 °C, similar to some reports for the northern Atlantic and north-western Mediterranean Sea (Buck *et al.*, 1996; Agawin *et al.*, 2000; Vaultot *et al.* (1990).

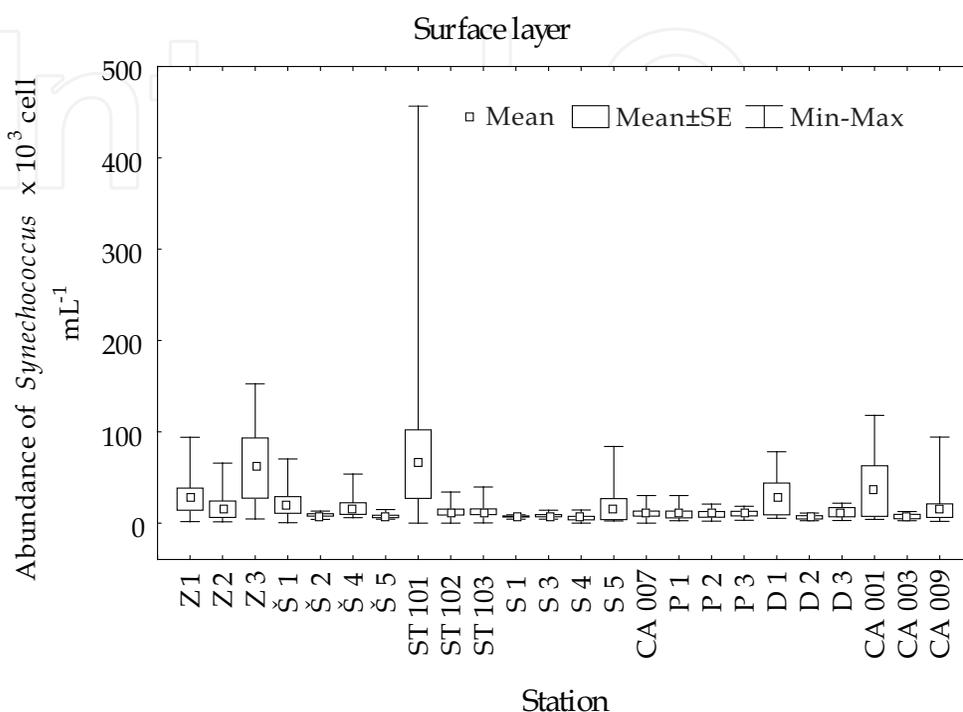


Fig. 6. Abundance of *Synechococcus* at the surface layer

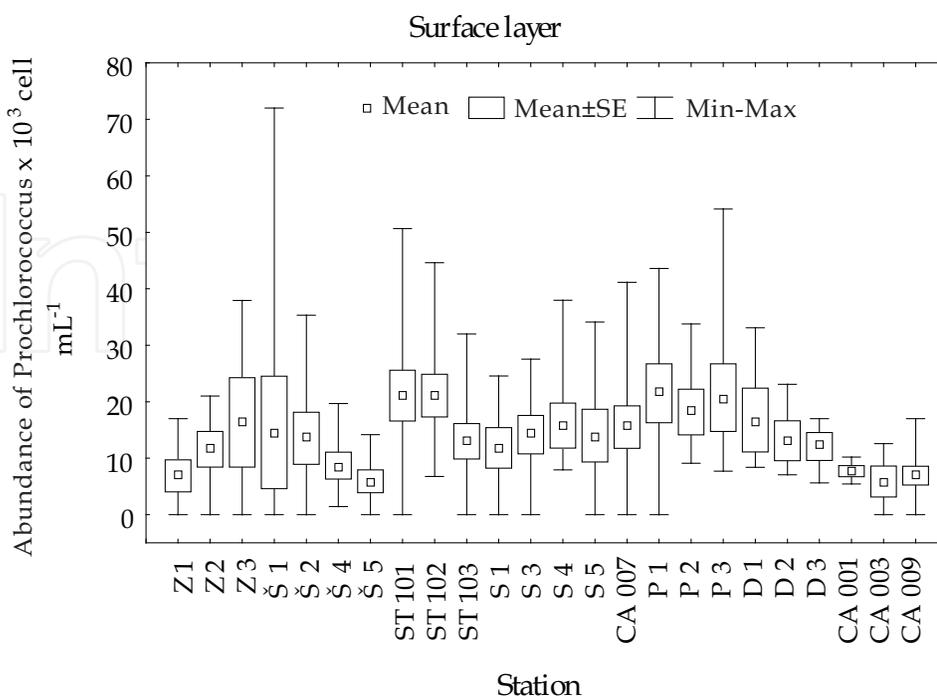


Fig. 7. Abundance of *Prochlorococcus* at the surface layer

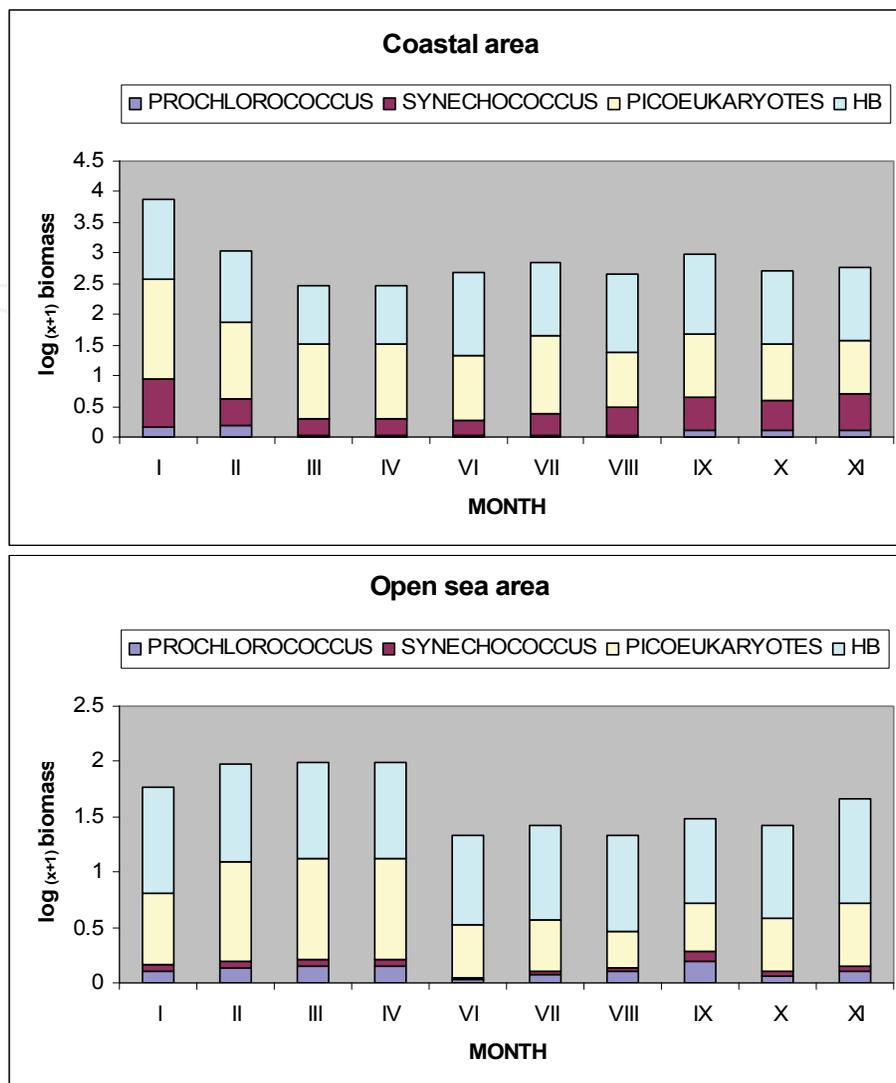


Fig. 8. Seasonal fluctuations of *Synechococcus*, *Prochlorococcus*, picoeucaryotes and heterotrophic bacterial biomasses in the coastal and open sea area

Use of the flow cytometry, in addition to noted understanding of the abundance and seasonal distribution of *Synechococcus* and *Prochlorococcus*, resulted in the data of vertical distribution of cyanobacteria in the open Adriatic Sea. At deep open sea stations, the high abundance of *Synechococcus* was found in the bottom layers, which agrees with the results of Uysal & Köksalan (2006) for the eastern Mediterranean Sea and of Bernardi Aubry *et al.* (2006) for the northern Adriatic Sea. Therefore, high abundance of *Synechococcus* in the bottom layer is consistent with the finding that *Synechococcus* can successfully live in environments with limited light (Waterbury *et al.*, 1986; Wehr, 1993), due to different pigment ecotypes (Olson *et al.*, 1988). The maximum depth at which *Prochlorococcus* was found in the investigated area of Adriatic Sea was 200 metres at one station located in the Jabuka Pit, with an abundance of 10^3 cells mL^{-1} in February under mixed water column conditions. Previous investigations have revealed high *Prochlorococcus* abundance in deeper layers of the euphotic zone (Wehr, 1993), even at depths of 150 to 200 metres (Partensky *et al.*, 1999a). The most likely reason for their occurrence at this depth is vertical mixing of the water column (Bernardi Aubry *et al.*, 2006) or perhaps the existence of two *Prochlorococcus*

ecotypes that inhabit the shallow and deeper euphotic layer (Moore *et al.*, 1998; Partensky *et al.*, 1999a). Our results generally showed that in the investigated microbial community autotrophic component was dominant over the heterotrophic component during the winter season, while dominance of heterotrophic component in the microbial community was observed during the warmer seasons. Further, within the prokaryotic community heterotrophic prokaryotes were mostly dominant throughout the studied area. It is also important to point out that autotrophic prokaryotic community was mostly dominated by the *Synechococcus* biomass, and it was also observed that the biomass of *Prochlorococcus* was higher in the open sea area in comparison with the coastal site (Figure 8).

Owing to the ability to analyze around ten thousand cells in few minutes, flow cytometry can really reduce the time needed for determination of microbial abundances and offer new insights into the structure and functioning of microbial communities that can not be obtained with conventional epifluorescence microscopy.

The future research of microbial communities in the Adriatic Sea, in addition to the characterisation of the microbial community by analysing endogenous fluorescence (chlorophyll and phicoerythrin fluorescence) and exogenous fluorescence (DNA dyes), should also introduce the methods of single cell analysis by cytometry. Introduction of activity probes, nucleic acid probes and immunofluorescent probes will expand the knowledge about functioning within the specific communities and between different ones.

4. Conclusions

In conclusion, the results reported herewith show a significant relationship between epifluorescence microscopy and flow cytometry, but coefficients of variation were considerably lower for bacterial counts estimated by flow cytometry than epifluorescence microscopy. Generally, the use of flow cytometry in marine microbiology reduces the processing time of the sample and increases the number of processed samples. Also, the use of this method provides more information about microbial community members, especially for *Prochlorococcus*, HNA and LNA bacterial groups (cells are not visible by epifluorescence microscopy). Thanks to flow cytometry, first data for abundances of *Prochlorococcus*, HNA and LNA bacteria were published, and this method increases the knowledge about microbial community members and their relationships in the Adriatic Sea.

5. Acknowledgments

This research was supported by the Croatian Ministry of Science, Education and Sport as part of the research program 'Role of plankton communities in the energy and matter flow in the Adriatic Sea' (project no 001-0013077-0845). Also thank Olja Vidjak and Marin Ordulj to help.

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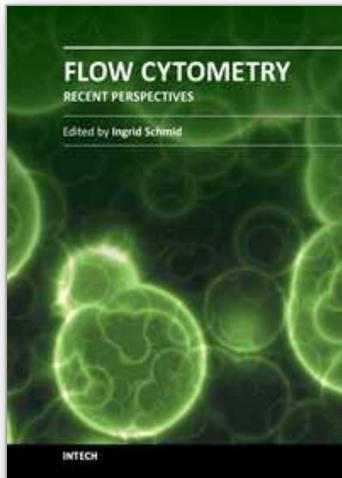
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Flow Cytometry - Recent Perspectives

Edited by M.Sc. Ingrid Schmid

ISBN 978-953-51-0626-5

Hard cover, 500 pages

Publisher InTech

Published online 13, June, 2012

Published in print edition June, 2012

"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Danijela Santic and Nada Krstulovic (2012). Identification and Characterisation of Microbial Populations Using Flow Cytometry in the Adriatic Sea, *Flow Cytometry - Recent Perspectives*, M.Sc. Ingrid Schmid (Ed.), ISBN: 978-953-51-0626-5, InTech, Available from: <http://www.intechopen.com/books/flow-cytometry-recent-perspectives/identification-and-characterisation-of-microbial-populations-using-flow-cytometry-in-the-adriatic>

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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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