Bacterial diversity in the South Adriatic Sea during a strong deep winter convection year

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Abstract

The South Adriatic Sea is the deepest part of the Adriatic Sea representing a key area for both the Adriatic Sea and the deep Eastern Mediterranean. It has a role in dense water formation for the Eastern Mediterranean deep circulation cell and it represents an entry point for water masses originating from the Ionian Sea. The biodiversity and seasonality of bacterial picoplankton before, during and after deep winter convection in the oligotrophic South Adriatic waters were assessed combining comparative 16S rRNA sequence analysis and catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH). The picoplankton communities reached their maximum abundance in the spring euphotic zone when the maximum value of chlorophyll a was recorded in response to deep winter convection. The communities were dominated by Bacteria while Archaea were a minor constituent. A seasonality of bacterial richness and diversity was observed, with minimum values in the winter convection and spring post-convection period, and maximum values during summer stratified conditions. SAR11 was the main constituent of the bacterial communities and reached the maximum abundance in the euphotic zone in spring after the convection episode. Cyanobacteria were the second most abundant group strongly depending on the convection event when minimal cyanobacterial abundance was observed. Euphotic zone in spring and autumn was characterized by Bacteroidetes and Gammaproteobacteria. The Bacteroidetes clades NS2b, NS4 and NS5 and the gammaproteobacterial SAR86 clade were detected to co-occur with phytoplankton blooms. SAR324, SAR202 and SAR406 were present in the deep layer exhibiting different seasonal variations in abundance. Overall, our data demonstrate that the abundances of particular bacterial clades and the overall bacterial richness and diversity are greatly impacted by strong winter convection.
Introduction

The Adriatic Sea is a semi-enclosed basin in the Northeastern Mediterranean Sea. The South Adriatic Pit (SAP), the deepest part of the Adriatic Sea (1200 m maximum depth), represents a key area for both the Adriatic Sea and the entire Eastern Mediterranean basin. The role of the Adriatic as a dense water source and driving engine of the Eastern Mediterranean deep circulation cell is well known (1). Open-ocean winter convection is responsible for the dense water production, generating a mixture of the Adriatic less saline waters with the more saline and warmer waters originating from the Ionian Sea (2). The circulation in the South Adriatic is characterized by a cyclonic South Adriatic Gyre (SAG, Fig. 1). The East Adriatic Current (EAC), that brings warmer and more saline waters from the Ionian Sea and Levantine basin, and West Adriatic Current (WAC), that transports less saline waters out of the Adriatic along the western border, are characterizing the cyclonic surface circulation (3). The Levantine Intermediate Water (LIW) and Ionian Surface Water (ISW) flow into the Adriatic along the South Adriatic eastern border making this part of the Adriatic the entry point for water masses. The impact of LIW inflow on the biogeochemical cycles in the Adriatic Sea is substantial, and fluctuation of a number of physical, chemical and biological parameters in the Adriatic Sea was attributed to the LIW ingress (4, 5). However, very little is known if and how the inflow of LIW impacts the diversity and structure of picoplankton communities on a seasonal basis.

In oceanic oligotrophic waters temporal dynamics of prokaryotic communities was mainly described from time-series sites, which often focused exclusively on SAR11 (6–8). Only few studies describing temporal changes of bacterial and archaeal communities in the Mediterranean sea have been conveyed to date (9, 10). Studies describing Adriatic picoplankton diversity were mainly focused on coastal waters (11, 12). In addition, temporal studies were exclusively done in the northern coastal waters (13–16). Deep winter convective mixing was shown to shape the community structure via transport of nutrients i.e. phosphorus and nitrogen, to the euphotic zone consequently triggering blooms of photosynthetic microorganisms in early spring (17). For the southern part of the Adriatic total prokaryotic picoplankton abundances were determined during the winter convection episode in 2008 but without any diversity estimation (18, 19). Also several studies have reported seasonal changes in picoplankton heterotrophic production and bacterial community metabolic capacity suggesting the importance and influence
of the deep convection event intensity on the annual cycle and productivity of this area (20, 21).

In the winter season 2011/2012, a strong deep winter convection event occurred in the southern part of the Adriatic resulting in the highest record-breaking densities ever observed for this region (22, 23). Deep convective mixing reached down to 600 m water depth, bringing phosphorus and nitrogen nutrients into the euphotic zone and thus triggering a bloom of photosynthetic microorganisms in early spring (21). We investigated, for the first time in South Adriatic offshore waters, (i) the diversity and seasonal dynamics of bacterial and archaeal communities and (ii) their response to a strong deep winter convection event, over a period of one year, using a combination of 454 pyrosequencing of the 16S rRNA gene and catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) techniques.
**Materials and methods**

**Sampling and environmental parameters estimation**

Samples for bacterial and archaeal community structure analysis were taken on-board the R/V *Naše more* on 3 October 2011, 18 February 2012, 29 March 2012 and 10 September 2012 at two stations. The stations were P-300 (42°27'32'' N, 17°56'02'' W), outside the SAP, and P-1200 (42°13'01'' N, 17°42'50'' W), inside the SAP (Fig. 1). Samples were taken with Niskin bottles at 10 depths (0 m, 10 m, 75 m, 100 m, 200 m, 400 m, 600 m, 800 m, 1000 m, 1200 m) at station P-1200 and 6 depths (0 m, 10 m, 75 m, 100 m, 200 m, 300 m) at station P-300. Chlorophyll *a* (Chl *a*) concentrations were determined from samples taken at the same dates and additionally on 12 January and 30 May 2012. 500 ml of seawater was filtered on GF/F filters (Whatman, UK). Filters were frozen at -18°C and Chl *a* concentration was determined by a fluorometric procedure (24). Temperature and salinity were recorded with a SBE25 (SEA-Bird Electronics Inc., USA) CTD probe continuously throughout the water column at the same dates when samples for Chl *a* were taken.

454-pyrosequencing

Aliquots of 1 l of seawater were filtered on 0.2 µm Nucleopore polycarbonate membrane filters (Whatman, UK) with a peristaltic pump. Filters were stored in 1 ml sucrose buffer (40 mM EDTA, 50 mM Tris-HCl and 0.75 M sucrose) in liquid nitrogen and afterwards at -80°C. The DNA was extracted according to Massana et al. (25). The bacterial V1-V2 16S rRNA region was sequenced at MR DNA (http://www.mrdnalab.com, Shallowater, TX, USA) using the bacterial 16S-based tag-encoded FLX amplicon pyrosequencing (bTEFAP) method (26), and Roche/454 FLX titanium instruments and reagents following the manufacturer’s guidelines. Primers used for the target 16S rRNA sequence amplification were 27Fmod (5'-AGRGTGTGATCMTGCTCAG-3') and 519Rmodbio (5'-GTTACNGCGGCKGCTG-3').

Sequence analysis

Obtained Standard Flowgram Format (SFF) files were extracted using a sff_extract script (available at http://bioinf.comav.upv.es/sff_extract/index.html) applying the sff_extract -c command that allows sequence quality checking. Fasta files were split according to the barcode.
Sequence using mothur (27). Sequences containing any differences in the barcode or primer sequence were removed in the barcode splitting step. Multifasta files were processed by the SILVAngs pipeline (https://www.arb-silva.de/ngs) (28) as described in Ionescu et al. (29). Briefly, sequences were aligned against the SILVA SSU rRNA SEED using the SILVA Incremental Aligner (SINA) (30). Sequences with low alignment quality (50 alignment identity, 40 alignment score reported by SINA) were removed (putative contaminations and artifacts). An additional quality check was done by removing all sequences shorter than 200 nucleotides, with more than 2% of ambiguities or 2% of homopolymers. Identical sequences were identified (de-replication) and clustered (Operational Taxonomic Units [OTU]) at 97% sequence identity using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit) (31) running in accurate mode and ignoring overhangs. The representative OTU sequence was classified against the SILVA SSU Ref dataset (release 115; http://www.arb-silva.de) using blastn (version 2.2.22+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings (Dataset S1) (32). Sequences obtained in this study have been submitted to the European Nucleotide Archive (ENA) under accession numbers ERS536204-ERS536263.

454 pyrosequencing of 60 samples yielded a total of 837,192 pyrotags (13,953 ± 9841 pyrotags on average per sample) that ranged from 2,895 to 41,363 pyrotags. The average pyrotag length was 412 ± 31 bp with a minimum length of 200 bp. On average, 36 ± 46 pyrotags were rejected in the process of quality control. The vast majority of pyrotags were successfully taxonomically assigned (13634 ± 9676 pyrotags per sample). Each sample, on average, contained 1,541 ± 918 OTUs (97% sequence identity) of which 33% on average were singletons (Table 2, S1). The sequencing effort applied was insufficient to determine the whole bacterial richness as could be observed in the rarefaction curves that did not level off even for the samples with the greatest number of pyrotags (Fig. S1, S2).

CARD-FISH

Water samples were fixed on-board with formaldehyde (2% v/v final concentration) for 24 h at 4°C. Upon arriving in the laboratory 120 ml of water sample was filtered on 0.2 µm Isopore polycarbonate membrane filter (diameter 47 mm, pore 0.2 µm; GTTP Millipore, USA) and stored at -20°C. Catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) using specific probes (Table 1) was performed according to Pernthaler et al. (33) with a slight
Endogenous peroxidases were inactivated by incubating the filters in methanol supplemented with 0.15% H$_2$O$_2$ for 30 min. Hybridization was done in 400 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 10% dextrane sulfate [wt/vol; Sigma-Aldrich, USA], 0.01% sodium dodecyl sulfate [SDS], 1% Blocking Reagent [Roche, Switzerland]) supplemented with a probe specific % of formamide (X vol/vol; Fluka, Germany; Table 1) and 1.3 µl of HRP probe solution (8.4 pmol µl$^{-1}$ HRP labeled probe in TE buffer; Biomers, Germany) for 2 h at 46°C. Unhybridized probes were washed by incubating the filter cuts in 50 ml of washing buffer (X mM NaCl [specific for each probe; Table 1], 5 mM EDTA [pH 8.0], 20 mM Tris-HCl [pH 7.5], 0.01% SDS [wt/vol]) for 15 min at 48°C. After a washing step in 1X PBS (pH 7.6, RT, 15 min) tyramide signal amplification was performed by incubating the filter cuts in a substrate mix for 45 min at 46°C. The substrate mix was prepared by adding 1 part of Alexa488 solution (Invitrogen, USA) to 1000 parts of amplification buffer (0.8X PBS [pH 7.6], 0.08% Blocking reagent, 1.6 M NaCl, 8% dextrane sulfate) supplemented by freshly prepared 0.0015% H$_2$O$_2$ in 1X PBS. Filter cuts were washed in prewarmed (46°C) 1X PBS (10 min, RT). All filter cuts were counted manually (minimum of 1,000 DAPI signals) on a Nikon Eclipse 50i microscope (Dataset S2).

**Data analyses**

Observed richness, richness estimators (Chao1 and ACE [Abundance-based Coverage Estimator]) and Shannon’s Diversity Index were calculated after normalization for sampling effort across samples. 2521 pyrotags, corresponding to the smallest sampling effort in the dataset, were randomly re-sampled through rarefaction. OTUs that were classified as chloroplast were not taken into account. Community turnover was estimated on re-sampled data by simple accounting the proportion of shared OTUs or taxa in two consecutive seasons or layers. Differences in richness or diversity among seasons were tested with one-way ANOVA (Systat 12, Systat Software Inc., USA). Normality and homogeneity of variances were tested by Lilliefors and Levene’s tests, respectively. Significant ANOVA results (p<0.05) were followed with post hoc Tukey-HSD multiple comparison tests to investigate which of the means were different. Differences among seasons and stations in respect to pyrotag relative contribution and cell abundances (CARD-FISH derived data) of SAR11, Gammaproteobacteria, Bacteroidetes and Cyanobacteria were tested with two-way ANOVA (Systat 12). For the pyrotag and CARD-
FISH data, only samples from the euphotic zone (<200 m) were considered as they were retrieved from the same depths at the two stations, inside and outside the SAP.

To estimate the influence of singletons (OTUs that are present in the whole dataset with only one sequence), “No relative” sequences and pooling of sequences at different taxonomic levels on bacterial diversity estimates, different datasets were constructed. Datasets containing no singletons (OTU-singl.) and only taxonomically assigned sequences (OTUannot., without the “No relative” sequences) were build. In addition, to compare the 454 pyrosequencing and CARD-FISH approach relative abundances of different taxa targeted by the set of probes used in CARD-FISH (Table 1) were extracted from the 454 dataset and compared with the relative abundances of the same taxa detected by CARD-FISH (expressed as % of EUB338I-III signals). OTUs that were classified as chloroplast were not taken into account. Pairwise distance matrices were calculated from the relative abundance data using the Bray-Curtis dissimilarity index (34). Dissimilarity matrices were compared with the Pearson’s product moment correlation coefficient and the significance was determined using the Mantel test followed by the Bonferoni correction.

In addition, cluster analysis was performed on datasets containing relative cell abundances detected by CARD-FISH (expressed as % of EUB338I-III singals) and relative contribution of pyrotags taxonomically assigned to the same group. Samples were compared using the Bray-Curtis similarity coefficient followed by a cluster analysis using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean). All analyses were done in the R software environment (http://www.r-project.org/) using the package vegan (http://cran.r-project.org/web/packages/vegan/index.html) and custom scripts. Analysis testing the differences in richness, among seasons and stations were done in Systat 12 (Systat Software, Inc., USA).

To estimate the correlations between community and environmental parameters the Pearson’s correlation coefficients were calculated. Bacterial relative abundances data derived from CARD-FISH and relative contribution of 454 pyrotags to a specific group were transformed using the Hellinger transformation while the environmental parameters were logarithm-transformed. False discovery rates (q values) based on the observed p values were calculated to ensure more stringent criteria. Correlations that match the criteria of \( p<0.007, q<0.1 \) and \( r>0.45 \) were taken into account. All correlations were performed in R using the package vegan, Hmisc (http://cran.r-project.org/web/packages/Hmisc/index.html) and Bioconductor.
software (http://www.bioconductor.org/packages/release/bioc/html/qvalue.html). Cytoscape was used to visualize the network (35).
Results

Hydrography

Temperature stratification was observed throughout the whole year inside and outside the pit, except in winter 2012 when a deep-convection episode has occurred (Fig. 2a) (22). Due to heating, in spring 2012, stratification was re-established in a thin layer close to the surface (Fig. 2a). Intensity of the deep-convection inside the pit could be discerned from the uniform temperature (13.75°C) recorded from 10 m to 500 m water depth (Fig. 2a). Correspondingly, during this event the salinity was uniform from surface down to 500-600 m at this station, while outside the uniform salinity reached only to 200 m. In autumn, before the convection event, only the station outside the pit was under the influence of saltier LIW (Fig. 2b). After the convection episode, in spring and summer, an intensification of LIW introgression was observed in the whole region, affecting both investigated stations (Fig. 2b). Chl a concentration varied between <0.01 µg l⁻¹ and 4.87 µg l⁻¹. Before and after the convection episode Chl a could be detected only in the euphotic zone, with concentration maximums at the Deep Chlorophyll Maximum (DCM) between 50-100 m; however, during the convection Chl a could be detected down to 600 m (Fig. 2c). The maximum value of Chl a was measured in spring 2012 at 35 m inside the pit (4.87 µg l⁻¹) while a much lower Chl a concentration maximum was detected outside the pit, at 75 m (0.88 µg l⁻¹), in the same month (Fig. 2c).

Water mass characteristics

Three types of water layers i.e. euphotic zone, deep layer and mixed layer, which differed in temperature, salinity and Chl a concentrations were identified outside and inside the pit. In autumn, a euphotic zone (<100 m; T: 17.93 ± 3.26°C; S: 38.71 ± 0.11; Chl a >0.01 µg l⁻¹) and a deep layer (>100 m; T: 13.86 ± 0.63°C; S: 38.71 ± 0.07; Chl a <0.01 µg l⁻¹) were observed. Winter water column, characterized by a deep winter convection that reached down to 600 m, could be divided into a mixed layer (<600 m; T: 13.68 ± 0.19°C; S: 38.67 ± 0.04; Chl a >0.01 µg l⁻¹) and a deep layer (>600 m; T: 13.48 ± 0.17°C; S: 38.71 ± 0.01; Chl a <0.01 µg l⁻¹). Spring water column, when the stratification was established again, could be divided into the euphotic zone (<200 m; T: 14.30 ± 0.56°C; S: 38.77 ± 0.08; Chl a >0.01 µg l⁻¹) and a deep layer (>200 m; T: 13.61 ± 0.24°C; S: 38.70 ± 0.03; Chl a <0.01 µg l⁻¹). Summer, characterized by a strong
stratification, was also defined by a euphotic zone (<200 m; T: 16.07±2.69°C; S: 38.85±0.07; Chl a >0.01 µg l⁻¹) and a deep layer (>200 m; T: 13.63±0.44°C; S: 38.72±0.06; Chl a <0.01 µg l⁻¹). Further detailed description of the water masses, including nutrient concentrations is given in Najdek et al. (21). Briefly, nutrients concentrations were lower in the euphotic zone and higher in the deep layer with the exception in winter when a homogenous nutrient distribution was observed in the mixed layer.

**Seasonal bacterial variation in richness, diversity and community turnover**

Different datasets were compared using the Pearson’s correlation coefficient in order to determine the similarity between community structure at different taxonomic levels, as well as the influence of the removal of singletons or sequences that were not taxonomically assigned (“No relative” group). The community structure showed almost no change upon singleton or “No relative” sequences removal (Fig. S3a). In contrast, when the OTU level was compared with higher taxonomic levels (Genus-Phylum) a drop in the correlation was observed (Fig. S3a).

Bacterial richness, Chao1, ACE, and Shannon diversity showed significantly difference according to season (p<0.05; Fig. 3; Table S3). In the euphotic zone (including the mixed layer) diversity slightly increased from autumn (Chao1=1,121) to winter (Chao1=1,228) and reached lowest numbers in spring (Chao1=792). Maximum values were detected in summer (Chao1=2,108). The pattern in the deep layer was similar, however with autumn being similarly high like summer and substantially low values for winter and spring.

Community turnover at different taxonomic levels was calculated to determine how much of the bacterial community is seasonally changing in the euphotic zone/mixed layer and deep layer (Fig. S4). The highest turnover was detected in winter and summer in the euphotic zone. The shared proportion of OTUs in any two subsequent seasons, both in the euphotic zone/mixed layer and deep layer was very low (<1.5%) while at higher taxonomic levels, as expected, the proportion of shared taxa was much higher (11-100%). A similar pattern was observed in the euphotic zone/mixed layer and deep layer. The only observed difference between the two layers was a lower proportion of shared higher taxonomic levels (genus-phylum) between autumn and winter in the deep layer in comparison to the euphotic zone. In addition, the number of shared OTUs between two water layers in each season was calculated (data not shown) and also resulted
in a very low number of shared OTUs (<2%) reflecting the high variability of microbial communities at the OTU level. A slightly higher number of shared OTUs between the spring euphotic zone and deep layer was observed (4.14%) reflecting the consequence of the mixing event.

**Seasonal bacterial and archaeal variation**

Seasonal changes in picoplankton communities were analyzed by classifying each reference OTU sequence (using the SILVAngs pipeline; Table S5) and by performing the quantitative CARD-FISH analysis of major taxonomic groups (Table S6). We used the 454 pyrosequencing to get an in-depth analysis of diversity and CARD-FISH to quantify the cell numbers (absolute and relative). Two-way ANOVA for the tested phylogenetic groups (SAR11, Gammaproteobacteria, SAR86, Bacteroidetes and Cyanobacteria; Table S4) showed significant (p<0.05) seasonal but no spatial (inside versus outside SAP) differences (supplementary results section) for the CARD-FISH and 454 data.

Total picoplankton cell numbers ranged 0.94-8.6 x 10^5 cells ml^{-1} (Fig. 5a). In autumn, the cell number decreased from the euphotic zone (3.6 x 10^5 ml^{-1}) to the deep layer (1.5 x 10^5 ml^{-1}). Cell numbers were at the minimum in winter, during the deep convection (Fig. 5a). The mixed layer (2.7 x 10^5 ml^{-1}) contained higher cell numbers than the deep layer (1.1 x 10^5 ml^{-1}). In spring, characterized by a high Chl a concentration in the euphotic zone, the cell number reached a maximum with a higher number in the euphotic zone (4.5 x 10^5 ml^{-1}) than in the deep layer (2.0 x 10^5 ml^{-1}). Summer was also characterized with a higher cell number in the euphotic zone (3.1 x 10^5 ml^{-1}) than in the deep layer (1.8 x 10^5 ml^{-1}, Fig. 5a).

**Bacteria**, compared to **Archaea**, were dominating the communities throughout the whole water column during all seasons, with higher abundances in the euphotic zone/mixed layer than in the deep layer (Fig. S9a). On average, **Bacteria** comprised more than 60% of the communities with highest abundances in the euphotic zone in spring and summer (74%). **Bacteria** abundances in the two layers, i.e. mixed and deep layer, were relatively more similar in winter. **Archaea** were less abundant than **Bacteria**, with higher abundances in the deep layer, especially in autumn (15%) and summer (13%, Fig. 5b).
Members of the alphaproteobacterial clade SAR11 were dominating the communities during the entire study period (Fig. 4, 5c). In the euphotic zone and mixed layer they accounted for half of the picoplankton cells in autumn (50%), winter (50%), spring (48%) and summer (50%). In the deep layer SAR11 were slightly less abundant (Fig. 5c). The high dominance of SAR11 was also reflected in the high proportion of SAR11-related pyrotags (Fig. S6).

Cyanobacteria were characteristic of the euphotic zone (Fig. 4). In autumn, spring and summer they accounted for 18%, 5.5% and 9.1% of the communities, respectively while in the deep layer they comprised <1% of the communities. In winter, Cyanobacteria accounted for <1% of the communities in the mixed layer while in the deep layer there were no signal detection (Fig. 6a). We tried to derive the seasonal dynamics of the two dominant cyanobacterial genera Synechococcus and Prochlorococcus from pyrotag frequencies. In autumn, Synechococcus-related pyrotags were present at a higher proportion above 75 m, while Prochlorococcus-related pyrotags proportionally increased with depth (Fig. S5). In winter, almost only Prochlorococcus-related pyrotags where found. Spring was characterized by an approximately equal relative abundance of Prochlorococcus and Synechococcus-related pyrotags in the euphotic zone while in summer Prochlorococcus-related pyrotags were highly dominant in the deep chlorophyll maximum (DCM) layer (75 m; Fig. S5).

CARD-FISH indicated that Gammaproteobacteria were prevalent in the euphotic zone in autumn, spring and summer with 12%, 9.5% and 7.4%, respectively, while in the deep layer they accounted for <3% of the communities on average (Fig. 4, 6b). During the deep convection they accounted for <3% in the mixed layer while in the deep layer no signal was observed (Fig. 6b). The main gammaproteobacterial clade detected throughout this study was SAR86 (Fig. S7). Members of the SAR86 clade were characteristic of the euphotic zone/mixed layer with the highest abundance in autumn summer (4.0%), while in the deep layer they accounted on average for <1% (Fig. S9c).

Distribution of Bacteroidetes was similar to that of Gammaproteobacteria, with higher abundances in the euphotic zone/mixed layer and with abundance peaks in autumn (6%) and spring (7.5%) (Fig. 4, 6c). Pyrotags related to the order Flavobacteriales were abundant with high frequencies of clades NS2b, NS4 and NS5 (Fig. S8).
Members of the deltaproteobacterial SAR324 clade were abundant in the deep layer with an abundance peak in the summer deep samples (8.0%). In the phase of stratification they always comprised <2% of total prokaryotes in the photic layer (Fig. S10a). SAR202 clade members were characteristic of deep layer and winter mixed layer while in the euphotic zone they comprised <2% of the total prokaryotes (Fig. S10b). The maximum abundance was observed in the autumn (8.7%) and winter (9.3%) deep layers. The SAR406 clade, similarly to SAR324 and SAR202, was more abundant in the deep layer especially in summer when it accounted for 12% of the total prokaryotes (Fig. S10c).

A comparison between the CARD-FISH and 454 data sets using the Pearson’s correlation coefficient was performed to estimate if the CARD-FISH and 454 approaches are showing the same community structure (Fig. S3b). Relative abundances of different taxonomic groups detected by CARD-FISH were compared with the relative abundances of the same taxonomic groups found in the 454 dataset and with the different taxonomic levels of the 454 dataset. A weak positive correlation between the CARD-FISH and 454 dataset (R=0.21, p<0.05) was observed indicating that the two methods are showing the same direction of the underlying patterns but that the bacterial structure revealed by the two methods is not the same (Fig. S3b). In addition, a cluster analysis was performed to reveal the differences in the clustering of the samples using CARD-FISH and 454 data (Fig. S12). The dendrogram based on the CARD-FISH data showed two clusters, one containing mainly samples from the euphotic zone and one containing samples from the deep/mixed layer. On the other hand, 454 data showed the clustering of winter and summer samples in one cluster and of autumn and spring samples in the other, with an additional cluster containing samples with a high contribution of Sphingomonadales related pyrotags.

Discussion

In this study we present findings on the seasonal dynamics of picoplankton communities in the South Adriatic, through the whole water column and in a period of one year, in which a strong deep convection occurred. Our results are based on a combination of two molecular techniques, the high-resolution 454 pyrosequencing and the quantitative CARD-FISH. Only a weak positive correlation was found between the CARD-FISH and 454 pyrosequencing data. In addition, CARD-FISH and 454 pyrosequencing data were used to build cluster dendrogram that showed a
different clustering of a part of the samples. Discrepancies in compositional information obtained by 454 pyrosequencing and CARD-FISH data likely result from the distortion of 454 read frequencies by PCR bias, problems with library construction, clade-specific differences in the rRNA operon number and DNA extraction efficiency. In addition, the inactive physiological status of the cells can lead to discrepancies. Half of the picoplankton community is composed of SAR11, which low rRNA content especially at greater depths can lead to an underestimation of SAR11 in CARD-FISH counts (36).

Deep water convection event, typical for mid-latitude ecosystems, is one of the most important factors influencing the seasonal picoplankton dynamics (e.g. cell numbers, metabolic activities) in the South Adriatic (6, 7, 17). The observed convection is peculiar and stronger than in other mid-latitude systems because of the local meteorological (strong winds) and hydrographic conditions needed for dense water formation (22, 23). This strong convection event greatly influenced the distribution of different picoplankton clades by transporting typical deep water clades such as SAR324, SAR202 and SAR406 to the surface, but also indirectly by supplying nutrients for phytoplankton blooms. In addition, the convection event influenced the bacterial diversity and composition both in the euphotic zone and deep layer.

Alpha-diversity of euphotic bacterial communities in the South Adriatic varied strongly according to season, with highest abundances detected in summer and lowest in the post convection period, i.e. spring. The observed increase in richness in the euphotic zone to summer maxima was different from the Western English Channel surface waters where a winter maximum and summer minimum was observed (37, 38) and from the BATS where an opposite pattern was detected with higher winter richness values during the mixing and summer lowers during the stratified conditions (17). The diversity minimum in spring, during the maximum productivity, could be explained in the availability of a series of new ecological niches due to phytoplankton-derived organic matter production in which specialist could bloom (39). In addition, the maximum observed richness in summer could be explained by the intensification of the gyre (in summer both stations were under the influence of LIW) that caused a stronger introgression of LIW, possible source of new LIW specific subclades (21, 40).

During the entire year, the euphotic zone and the mixed layer were dominated by Bacteria, while Archaea were only a minor constituent, as was found elsewhere (41, 42). SAR11
was the most abundant phylogenetic group that regularly accounted for more than 40% of the communities. A similar seasonal pattern of post convection SAR11 maxima was found in the NW Sargasso Sea (7) while in a coastal oligotrophic Mediterranean system a spring-summer higher relative abundance of SAR11 was detected (9). However, in both studies the relative contribution of SAR11 to the whole community was lower than in our study. This could, however, also be attributed to the lower number of SAR11-specific probes used in these studies, as here a highly sensitive combination of six FISH probes, one helper oligonucleotide and signal amplification via CARD-FISH was applied for the quantification of SAR11. Worldwide distribution and high annual abundance of this clade (43), supported with genomic data (44, 45), suggests that SAR11 clade plays a major role in the oxidation of low-molecular-weight dissolved organic matter in oligotrophic systems.

The seasonality of Cyanobacteria, as the second most abundant group, strongly depended on the convection event when a strong decrease in their relative abundance was observed. A similar strong decrease in Prochlorococcus abundances due to a seasonal deep convection was observed at BATS, which was attributed to a selective elimination of low light adapted Prochlorococcus ecotypes (LL) incapable of coping with turbulent deep winter mixing (46). In autumn, Synechococcus specific pyrotags were dominant above 75 m water depth, while below Prochlorococcus specific pyrotags were more abundant. In other seasons, only Prochlorococcus specific pyrotags or the same abundance of pyrotags specific to both genera could be found at all depths (47). The observed increase of Bacteroidetes and Gammaproteobacteria in spring detected here is atypical of oligotrophic offshore waters (41). However, in coastal and eutrophic systems spring increase in the abundance of these two groups has been linked to phytoplankton blooms and was related to the break-down of biomass of phytoplankton blooms (39, 48). Furthermore, it was suggested that Bacteroidetes are increasingly being replaced by Gammaproteobacteria in the post-bloom period (39, 48). A similar pattern could be observed in our data in autumn and spring. The response of these groups to a phytoplankton bloom could be observed especially in spring when, in a post convection period, the highest Chl a concentration was observed mainly attributed to Chaetoceros ssp. and Guinardia striata diatom bloom (S. Ljubimir, pers. comm.). In coastal waters, Ulvibacter spp., Formosa-related and Polaribacter species from the order Flavobacteriales, phylum Bacteroidetes, were found to increase during or shortly after a diatom bloom (39). Although Bacteroidetes pyrotags from the South Adriatic were
mainly assigned to *Flavobacteriales*, none of above groups were found in high numbers. Instead, the NS2b, NS4 and NS5 clades were the main *Flavobacteriales* groups indicating that they could be better adapted to oligotrophic conditions. In the same study, *Reinekea* spp. and SAR92 gammaproteobacterial groups were found to increase after the diatom bloom and as a response to algal decay (39). Similarly to the *Bacteroidetes* groups, no *Reinekea* spp. and SAR92 specific pyrotags in high proportion were observed, but instead SAR86 was dominating the *Gammaproteobacteria* indicating SAR86 as an analog of *Reinekea* spp. and SAR92 in oligotrophic offshore waters.

The deep layer samples showed a higher relative abundance of *Archaea*, especially in autumn and summer, than the euphotic and mixed layers. A similar abundance of *Archaea* was observed in the Atlantic mesopelagic waters (41, 49) and Eastern Mediterranean deep waters (50). The greater abundance of *Archaea* in the deep layer in autumn and summer are pointing to a response of *Archaea* to processes in the euphotic zone (51). In winter at 800 m and 1000 m and in spring at 600 m, an unexpected dominance of *Sphingomonadales*-related pyrotags, almost completely belonging to a single genus (*Sphingobium*), was observed. Blooms of a single species from the order *Sphingomonadales* have been previously reported in a coastal lagoon co-occurring with a bloom of filamentous cyanobacteria (52). The SAR202 clade exhibits a similar abundance pattern like *Archaea* being more abundant in the deep layer in autumn and summer with the exception of winter when it was uniformly distributed through the water column (41, 53). The SAR406 clade compromised ~6% of the deep layer communities through the year with the exception of summer when its abundance increased at more than 10%. The deltaproteobacterial SAR324 clade, similarly to SAR406, peaked in the deep layer in summer with a secondary peak in the winter mixed layer (41). In the same paper patchy distributions of SAR406 along the Atlantic transect was observed that was explained by a possible natural variation due to seasonality. The same explanation could be applied to explain the summer seasonal peak of SAR406 in our study.

The measured environmental parameters did not reveal any clear insight into the factors that shape the bacterial distribution in the South Adriatic (supplementary results section). Of all abundant taxa, only *Deferribacteres* significantly correlated to nitrate, as previously found in the Western English Channel but apart from that bacterial groups were mainly correlating between
themselves indicating the difficulties in explaining bacterial distribution and dynamic using correlation methods especially in cases of seasonal sampling campaigns (38, 54).

Overall, we could show in this study that the strong winter convection of 2012 had profound consequences for the bacterial and archaeal picoplankton communities of the South Adriatic Sea, both with respect to species richness, diversity and the abundances of different phylogenetic groups. The convection episodes are obviously changing the biogeochemistry and microbiology of the whole Adriatic. High frequency temporal sampling might in the future reveal further detail of the surprisingly dynamics succession of picoplankton clades in oligotrophic offshore waters.

Acknowledgments

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References


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Figure captions

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FIG 3 Total number of bacterial OTUs (a), richness predicted by the Chao1 (b) and ACE (c) estimators and Shannon’s diversity index (d) in the euphotic zone/mixed layer and deep layer of the South Adriatic. The seasonal differences were tested with ANOVA. Different letters represent significant differences (p<0.05, Tukey-HSD test) between seasons; *variance non-homogeneous.

FIG 4 Taxonomic classifications and relative contribution of the most common bacterial pyrotags (>2%) inside and outside of the SAP; NA, data not available.

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FIG 6 Vertical and seasonal distribution of Cyanobacteria (a), Gammaproteobacteria (b) and Bacteroidetes (c) inside and outside of the SAP. Relative cell abundances are represented as % DAPI counts.

Supplementary figure captions

FIG S1 Rarefaction curves for the South Adriatic bacterial communities sampled at different depths in autumn 2011 (a, b) and winter 2012 (c, d) inside (a, c) and outside (b, d) the SAP.

FIG S2 Rarefaction curves for the South Adriatic bacterial communities sampled at different depths in spring 2012 (a, b) and summer 2012 (c, d) inside (a, c) and outside (b, d) the SAP.

FIG S3 Pearson’s correlation coefficient was used to compare the community structure dataset obtained by 454 pyrosequencing and CARD-FISH. Communities were compared after removing singletons (OUT-singl.) and “No relative” sequences (OTUannot.) and after pooling sequences at different taxonomic levels (a). Taxa detected by CARD-FISH were compared with the same taxa from the 454 dataset (labeled as 454; red square) and with the different taxonomic levels of the 454 dataset (b). The correlation coefficient was calculated from the distance matrices resulted in relative abundances of sequences or CARD-FISH counts (expressed as % of EUB338I-III signals). Correlation significances were determined by the Mantel tests with 1000 matrix permutations. All R values were significant after Bonferroni correction.

FIG S4 Turnover of bacterial communities between consecutive seasons at different taxonomic levels of the euphotic zone/mixed layer (a) and deep layer (b). The percentage of shared communities was calculated after randomly sub-sampling the data matrix to the smallest sampling effort and pooling the sequences from all the samples belonging to a specific layer.
OTUall represents taxonomically assigned OTUs together with the OTUs belonging to the “No relative” group.

FIG S5 Taxonomic classifications and relative contribution of the most common bacterial pyrotags within the *Cyanobacteria* inside and outside of the SAP. The relative contribution of *Cyanobacteria* related pyrotags are stated near the columns. For simplicity, only clades with more than 2% of pyrotags in a given sample are shown. All clades below the threshold value are summed as “Other *Cyanobacteria*”; NA, data not available.

FIG S6 Taxonomic classifications and relative contribution of the most common bacterial pyrotags within the *Alphaproteobacteria* inside and outside of the SAP. The relative contribution of *Alphaproteobacteria* related pyrotags are stated near the columns. For simplicity, only clades with more than 2% of pyrotags in a given sample are shown. All clades below the threshold value are summed as “Other *Alphaproteobacteria*”; NA, data not available.

FIG S7 Taxonomic classifications and relative contribution of the most common bacterial pyrotags within the *Gammaproteobacteria* inside and outside of the SAP. The relative contribution of *Gammaproteobacteria* related pyrotags are stated near the columns. For simplicity, only clades with more than 2% of pyrotags in a given sample are shown. All clades below the threshold value are summed as “Other *Gammaproteobacteria*”; NA, data not available.

FIG S8 Taxonomic classifications and relative contribution of the most common bacterial pyrotags within the *Bacteroidetes* inside and outside of the SAP. The relative contribution of *Bacteroidetes* related pyrotags are stated near the columns. For simplicity, only clades with more than 2% of pyrotags in a given sample are shown. All clades below the threshold value are summed as “Other *Bacteroidetes*”; NA, data not available; *, no *Bacteroidetes* related pyrotags were obtained.

FIG S9 Vertical and seasonal distribution of *Bacteria* (a), *Roseobacter* (*Alphaproteobacteria*) (b) and SAR86 (*Gammaproteobacteria*) (c) inside and outside of the SAP. Relative cell abundances are represented as % DAPI counts.

FIG S10 Vertical and seasonal distribution of SAR324 (*Deltaproteobacteria*) (a), SAR202 (*Chloroflexi*) (b) and SAR406 (*Deferribacteres*) (c) inside and outside of the SAP. Relative cell abundances are represented as % DAPI counts.

FIG S11 Cluster analysis dendrogram of the bacterial communities in the South Adriatic derived from CARD-FISH and 454 pyrosequencing. The analysis was performed on the datasets containing relative bacterial cell abundances detected by CARD-FISH (expresses as % of EUB338I-III signals) and on the relative contribution of pyrotags taxonomically assigned to the same groups. Samples were compared using the Bray-Curtis similarity coefficient followed by clusters analysis using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean).

FIG S12 Correlation network for the bacterial community (454 dataset) and environmental parameters. Bacterial taxa that were present >2% in at least one sample were chosen for analysis. Only strong correlation were plotted (r<0.45 or r>0.45, p<0.007, q<0.1). Grey nodes-environmental parameters; blue nodes-bacterial taxa, solid lines-positive correlation, dashed lines-negative correlation, correlation coefficient are given on the edge lines.
### TABLE 1 Probes and hybridization conditions applied for CARD-FISH.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target organisms</th>
<th>Sequence (5’→3’)</th>
<th>FA (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NaCl in washing buffer (mM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARCH915</td>
<td>Archaea</td>
<td>GTGCTCCCCCGCAATTCCT</td>
<td>35</td>
<td>80</td>
<td>(55)</td>
</tr>
<tr>
<td>EUB338</td>
<td>Bacteria</td>
<td>GCTGCCTCCGCTAGGAGT</td>
<td>35</td>
<td>80</td>
<td>(56)</td>
</tr>
<tr>
<td>EUB338-II</td>
<td>Supplement to EUB338</td>
<td>GCAGCCACCCGTAGGGT</td>
<td>35</td>
<td>80</td>
<td>(57)</td>
</tr>
<tr>
<td>EUB338-III</td>
<td>Supplement to EUB338</td>
<td>GCTGCCACCCGTAGGGT</td>
<td>35</td>
<td>80</td>
<td>(57)</td>
</tr>
<tr>
<td>NON338</td>
<td>Control</td>
<td>ACTCCTACGGGAGGCAGC</td>
<td>35</td>
<td>80</td>
<td>(58)</td>
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<tr>
<td>CF319a</td>
<td>Bacteroidetes</td>
<td>TGGTCCGTGTCTCAGTAC</td>
<td>35</td>
<td>80</td>
<td>(59)</td>
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<tr>
<td>SAR202-312R</td>
<td>SAR202 clade</td>
<td>TGTCGTCCGCCCTCTGCT</td>
<td>40</td>
<td>56</td>
<td>(60)</td>
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<tr>
<td>CYA664</td>
<td>Cyanobacteria</td>
<td>GGAATTTCCCTTCGCCC</td>
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<td>SAR406-97</td>
<td>SAR406 clade</td>
<td>CACCCGTTGCCGATTTA</td>
<td>40</td>
<td>56</td>
<td>(62)</td>
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<tr>
<td>RO537</td>
<td>Roseobacter</td>
<td>CAACGCTAACGCCCTCC</td>
<td>35</td>
<td>80</td>
<td>(63)</td>
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<tr>
<td>SAR11-152&lt;sup&gt;c&lt;/sup&gt;</td>
<td>SAR11 clade</td>
<td>ATTAGACAAATTTTCTTCCGCT</td>
<td>25</td>
<td>159</td>
<td>(43)</td>
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<tr>
<td>SAR11-441&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SAR11 clade</td>
<td>TACAGTGATTTTTTCTTCCGAC</td>
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<td></td>
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<tr>
<td>SAR11-441&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SAR11 clade</td>
<td>TACGGTCATTTTCTTCCGAC</td>
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<td>SAR11-487&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SAR11 clade</td>
<td>CGGACCTTCTATTCCGGG</td>
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<td>SAR11-542&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SAR11 clade</td>
<td>TCCGAACCTACAGCTAGGTC</td>
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<td>SAR11-732&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SAR11 clade</td>
<td>GTCAGTAATAGATCCAGAAAGC</td>
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<tr>
<td>SAR324-1412</td>
<td>SAR324 clade</td>
<td>GCCCCTTGTAACACCCCAT</td>
<td>35</td>
<td>80</td>
<td>(41)</td>
</tr>
<tr>
<td>GAM42a&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Gammaproteobacteria</td>
<td>GCCCTCCCACATCGTTT</td>
<td>35</td>
<td>80</td>
<td>(64)</td>
</tr>
<tr>
<td>SAR86-1245</td>
<td>SAR86 clade</td>
<td>TTAGCRTCCGCCTGAT</td>
<td>35</td>
<td>80</td>
<td>(65)</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> Formamide concentration (vol/vol) in CARD-FISH hybridization buffer.

<sup>b</sup> NaCl concentration (mM) in washing buffer.

<sup>c</sup> A mixture of 6 probes to detect the SAR11 clade; includes an unlabelled helper SAR11-487-h3 (5′-CGGCTGCTGGCACGAAGTTAGAC-3′).

<sup>d</sup> Including an unlabelled competitor probe Bet42a (5′-GCCTCCCACATCGTTT-3′) (64).
### TABLE 2 Sequencing information

<table>
<thead>
<tr>
<th></th>
<th>Average per sample</th>
<th>Standard deviation</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrotag length (bp)</td>
<td>412</td>
<td>±31</td>
<td>200-833</td>
</tr>
<tr>
<td>Number of pyrotags</td>
<td>13,953</td>
<td>±9,841</td>
<td>2,895-41,363</td>
</tr>
<tr>
<td>Number of classified pyrotags</td>
<td>13,634</td>
<td>±9,676</td>
<td>2,663-40,498</td>
</tr>
<tr>
<td>Number of “No relative” pyrotags</td>
<td>283</td>
<td>±229</td>
<td>31-1,079</td>
</tr>
<tr>
<td>Number of rejected pyrotags</td>
<td>36</td>
<td>±46</td>
<td>2-234</td>
</tr>
<tr>
<td>Number of OTUs (97% sequence identity)</td>
<td>1,541</td>
<td>±918</td>
<td>234-4,209</td>
</tr>
<tr>
<td>Number of singletons*</td>
<td>521 (33%)</td>
<td>±359</td>
<td>53-1,578 (15%-48%)</td>
</tr>
</tbody>
</table>

*Percentage of total OTU number is given in parentheses.
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