**Bacterioneuston and bacterioplankton structure and abundance in two trophically distinct marine environments – a marine lake and the adjacent coastal site on the Adriatic coast**

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**Abstract**

Marine surface microlayer (SML) is large and extreme marine environment with an important role in biogeochemical cycling and climate regulation. We explored seasonal structure and abundance of bacterial assemblages in SML (bacterioneuston) and underlying water layer (ULW) (bacterioplankton) in eutrophic marine Rogoznica Lake and more oligotrophic coastal area of the adjacent Adriatic Sea. SML and ULW in each site were similar in pH, salinity, dissolved oxygen, oxygen saturation and temperature. Rogoznica Lake was colder in winter and warmer in summer compared to the Adriatic Sea. Regarding nutrients, SML and ULW were notably different environments. SML was consistently enriched in nitrate, nitrite, orthophosphate and total organic carbon than ULW in both investigated environments. Except in spring in Rogoznica Lake, bacterial abundance in SML was also significantly higher (*p* <0.05) than in ULW. Both layers and sites show prominent seasonal variability. High-throughput 16S rRNA gene sequencing of DNA and cDNA revealed a considerable difference in bacterial assemblage structure, although study sites were <200 m apart. Heterotrophs were predominant in both layers with pronounced spatial and temporal structural differences, except in autumn in Rogoznica Lake when, autotrophs became dominant fraction under oxygen-deprived conditions. All these variations were driven by *in situ* conditions, the most important ones being total organic carbon and temperature (and additionally dissolved oxygen in Rogoznica Lake). This is especially important in terms of ongoing eutrophication, warming and deoxygenation, noticed not only in the Adriatic Sea and Rogoznica Lake, but globally as well. Therefore, further structural and physiological changes in bacterioneuston and bacterioplankton assemblages can be expected.

**Key words:** microbial ecology, Adriatic Sea, Rogoznica Lake, marine surface microlayer, eutrophication

**INTRODUCTION**

Since oceans cover ∼71% of the Earth’s surface, marine surface microlayers are evidently large ecosystems, yet very specific. Surface microlayers (SML) represent the uppermost 1 – 1,000 µm thick air-water interface, which is physically and chemically distinct from the underlying water layer (ULW) [1]. This boundary between the atmosphere and the hydrosphere serves as both, a source, and a sink for a broad range of compounds, since every substance entering or leaving the ocean must pass through this interface [2, 3]. This feature gives SML a key role in controlling the gas, energy and organic matter exchange between air and sea, thereby directly influencing global elemental cycling and climate change [4]. Therefore, better understanding, characterization and description of spatial and temporal dynamics in bacterial community structure in this environment is considered as the central goal of marine microbial ecology [5]. On the other hand, intensive solar and ultraviolet radiation, temperature and salinity fluctuations, high heavy metals and other pollutant concentrations, make SML quite inhospitable habitat [6]. Like in many other extreme ecosystems, life in SML is dominated by microorganisms [2], which are regarded as *neuston*, whereas those present in the underlying water layer are considered as *plankton* [7]. Due to the pivotal role of bacteria and archaea in biogeochemical cycles and productivity, understanding of their dynamics is essential in accessing the functioning of marine ecosystems, in general [8, 9]. Observations from the studies focusing on bacterioneuston and bacterioplankton assemblages are disparate. Analysis of 16S rRNA gene clone libraries, from the samples collected from the North Sea, Fjord mesocosm (Norway) and Blyth Estuary (UK) showed distinct bacterioneuston communities compared to the ones present in the subsurface layer [10-12]. The similar results were also obtained for bacterial and archaeal communities in Montane Lakes (Spain) [13, 14]. However, studies conducted on the Peruvian Coast and Mediterranean Sea, showed no significant differences between SML and ULW samples [15, 16]. Which environmental factors control bacterioneuston diversity and activity is not entirely clear. However, it is suggested that it is not random, but ecologically regulated [17]. The foremost environmental conditions influencing bacterioneuston include meteorological conditions (primarily wind), organic matter concentration, aerosol deposition and UV light [16, 18, 19], but its seasonal variations are rarely taken into account [16]. Correlation between environmental parameters and community structure over space and time can provide useful links to ecosystem function [11] in order to better understand the biological, chemical and physical processes at the surfaces of marine environments. This is also important in elucidation of ocean-climate feedbacks [2].

We hypothesized that the bacterial assemblage structure in SML and ULW will depend on the trophic status of their environment and, since it is under the high influence of the air-water exchange, it will show pronounced temporal variability. Therefore, the aims of this study were to thoroughly compare and investigate, for the first time, seasonal fluctuations of total (DNA) and indicatively active (RNA) bacterioneuston and bacterioplankton assemblages, in two trophically distinct, but close coastal marine environments; a highly eutrophic marine system of Rogoznica Lake, and the adjacent coastal area of the oligotrophic Adriatic Sea. We applied high-throughput sequencing and quantitative PCR (qPCR) assays, targeting 16S rRNA phylogenetic marker in both DNA and RNA gene pools. Complementary environmental data were used to identify key factors influencing these assemblages, their temporal and spatial distribution, abundance and structure.

**METHODS**

**Study sites**

The Adriatic Sea is the northernmost marginal sub-basin of the Mediterranean Sea, whose physical and biological characteristics have been extensively studied in the past [20]. Based on its morphological features, the Adriatic Sea is conventionally divided in northern, central and southern Adriatic, each respectively showing distinct physical and biological characteristics. The study area was located on the eastern coast of Central Adriatic (43⁰ 15’ 53’’ N, 15⁰ 57’ E) (Fig. 1). This part shows oligotrophic characteristics with occasional eutrophication episodes in coastal zones of urban areas [21]. On the other hand, Rogoznica Lake is a naturally eutrophic and highly stratified marine lake, situated on the eastern coast of Central Adriatic on the Gradina peninsula (43° 32’ N, 15° 58’ E). The lake has a maximum depth of approx. 15 m and covers an area of 10,276 m2. The water column of the lake is stratified into oxic mixolimnion, chemocline (where the sharp changes of many physico-chemical parameters occur) and anoxic monimolimnion. Stratification was maintained throughout most of the investigated years, with occasional occurrence of holomictic conditions [22]. Oxic mixolimnion harbors a relatively low number of phyto- and zooplankton species, but their abundance is higher than in the adjacent Adriatic Sea [23]. Chemocline is the layer of intense primary production, conducted by a dense population of anoxygenic phototrophic bacteria [24]. Produced organic matter accumulates in the monimolimnion, causing anoxia and biological production of hydrogen sulfide (H2S) [25]. As a result of degradation processes, this layer is also characterized by high dissolved organic carbon (up to 6 mg/L), ammonium (up to 329 µM) and phosphate (up to 22 µM) concentrations [26].

**Sampling and measurements of physico-chemical parameters**

Samplings were conducted on August 27th 2019, October 23rd 2019, January 21st 2020 and May 6th 2020, representing summer, autumn, winter and spring season, respectively.

Microlayer was sampled using a metal mesh screen, previously cleaned with 70% ethanol and rinsed with ultrapure Mili-Q water. The thickness of the collected microlayer, determined from the ratio of the sample volume and the surface area of the mesh screen [27], was calculated to be 144-201 µm. Underlying water samples were collected directly by submerging a sterile glass bottle (1 L) ̴ 15 cm below the surface. Samples were filtered through 0.22 µm pore-size mixed cellulose esters membrane filters and immediately flash-frozen in liquid nitrogen. The filtered volume of all samples was 1L, except for the SML samples from the Adriatic Sea collected in spring (300 mL) and summer (500 mL) and SML samples from Rogoznica Lake collected in spring (300 mL), summer (250 mL) and autumn (200 mL). In that case, multiple filters were combined for extraction until the final volume of 1L was reached.

Environmental parameters of temperature, pH, dissolved oxygen (DO), oxygen saturation and salinity of ULW were measured *in situ* by HQ40D multimeter probe (Hach Lange, Germany), whereas SML samples were measured *ex situ* by immersing the probes in an aliquot sample immediately after it was collected. Samples for total organic carbon (TOC) analysis were collected in 20 mL glass vials, conserved with 100 µL of mercury (II)-chloride (10 mg/L) and closed with teflon-lined screw caps. Measurements were conducted in duplicates by high temperature catalytic oxidation (HTCO) method and non-dispersive infrared (NDIR) CO2 detection on TOC-Vcph instrument (Shimadzu, Japan) with platinum on silica as a catalyst. Samples for nitrate (NO3-), nitrite (NO2-) and orthophosphate (PO43-) analysis were directly stored at -20 ⁰C (without filtration) and analyzed according to Strickland and Parsons method [28].

**Nucleic acids co-extraction and cDNA synthesis**

Nucleic acids were co-extracted according to a slightly modified method described by Griffiths et al. (2000) [29]. Briefly, filters were cut and placed in bead tubes with 0.5 mL of 2% (w/v) cetyl trimethylammonium bromide (CTAB) buffer and 0.5 mL phenol:chloroform:isoamyl alcohol (25:24:1). Cells were lysed by vortexing at maximum speed for 5 min. After centrifugation (13,300 rpm for 10 min), the supernatant was transferred to a new tube along with an equal volume of chloroform:isoamyl alcohol (24:1), gently mixed and then centrifuged again (13,300 rpm, 10 min). Total nucleic acids were precipitated from the extracted aqueous layer with 0.1 volume 3M sodium acetate and 1 volume of ice-cold isopropanol. After the final centrifugation (12,500 rpm, 30 min) pelleted nucleic acids were washed in ice-cold 70% (v/v) ethanol, air-dried and resuspended in 50 μL of molecular grade water. The quality of DNA and RNA was determined by electrophoretic separation on 1% (w/v) agarose gel, and the concentrations were determined using a Qubit fluorometer (Invitrogen, USA).

Aliquot samples (10 µL) were treated with one unit of DNase I (Sigma Aldrich, USA) at 37 ⁰C for 15 min in order to remove co-extracted DNA. Remaining RNA (5 µL) was mixed with 1 µL of random hexamers and 1 µL of dNTP mixture and incubated at 65 ⁰C for 5 min. Conversion to cDNA was done using the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio Inc., Japan). Reactions were incubated at 30 ⁰C for 10 min, and at 42 ⁰C for 50 min, followed by inactivation of reverse transcriptase at 95 ⁰C for 5 min. Possible DNA contamination of RNA templates and successful cDNA synthesis were checked by PCR assays targeting 16S rRNA genes in which RNA or cDNA aliquots were used as templates.

**Quantitative PCR (qPCR)**

qPCR was used to estimate the abundance of bacterial 16S rRNA genes in template DNA. The absence of measured inhibitors in templates was confirmed by an inhibition test. A known amount of the plasmid pGEM-T Easy Vector (Promega, France) was mixed with the DNA templates or water before running a qPCR with plasmid-specific T7 (5’-TAATACGACTCACTATAGGG-3’) and SP6 (5’-ATTTAGGTGACACTATAG-3’) primers, as described previously [30]. Reaction conditions were: 4 min. at 94 ⁰C; 35 cycles of denaturation during 45 s at 94 ⁰C, annealing for 45 s at 55 ⁰C and the final extension at 72 ⁰C for 45 s. In case the measured cycle threshold (Ct) values of the DNA samples and the water controls were significantly different, samples were additionally diluted, until no significant difference was obtained, indicating no inhibition.

Reactions were conducted on AB7300 Real Time PCR System (Applied Biosystems, USA). Each reaction tube contained 10 µL of SYBR Green PCR Master Mix (Applied Biosystems, USA), 1.5 µL of each primer (10 mM) and 1 ng of DNA template in a final volume of 20 µL. Cycling conditions were: initial denaturation step for 15 min. at 95 °C; 30 cycles of denaturation during 15 s at 95 °C; annealing for 30 s at 60 °C; and the final extension at 72°C for 30 s. The abundance of targeted 16S rRNA gene was assessed by 341f (5’- CCTACGGGAGGCAGCAG-3’) and 534r (5’-ATTACCGCGGCTGCTGGCA-3’) primer set [31]. Bacterial 16S rRNA standard was made from the cloned amplicon from the collected environmental sample. Targeted gene was ligated into the pGEM-T Vector System (Promega, France) and cloned into *Escherichia coli* JM109 competent cells, according to the manufacturer’s instruction. Plasmid DNA was extracted from isolated positive transformant using the GenElute Plasmid Miniprep Kit (Sigma Aldrich, USA) and linearized with SalI restriction endonuclease (Thermo Fisher Scientific, USA). Reactions were performed in triplicates against a range of standards established by ten-fold serial dilution of plasmid (0.5 × 102 to 0.5 × 108 gene copy/µL). The efficiency of assay was 106.04 % with *R2* > 0.99. The specificity of reactions was checked by melting curves. Detected gene numbers were normalized to the DNA extraction yields, assuming 100 % extraction efficiency.

**High-throughput sequencing, bioinformatics and statistics**

Triplicate DNA and cDNA samples were pooled (5 ng/µL of each replicate) and sent for high-throughput sequencing of partial 16S rRNA gene on an Illumina MiSeq platform at FISABIO (Valencia, Spain). The hypervariable V4 region was accessed by 515F (5’-GTGYCAGCMGCCGCGGTAA-3’) [32] and 806R (5’-GGACTACNVGGGTWTCTAAT-3’) [33] primer set. In total, 7 097 618 sequences (average length 292 bp) were obtained from 32 samples and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME2) pipeline [34]. Raw sequence data were demultiplexed and quality filtered by q2-demux plugin, followed by joining, denoising and chimera removal with DADA2 [35]. Sequences with < 250 bp in length and those with quality score < 30 were removed from the further analysis. After corrections, unique sequences were distinguished as amplicon sequencing variants (ASVs) and taxonomically assigned against the Silva (r138.1) database [36]. This approach corrects sequencing errors at single-nucleotide level, thus enabling better resolution than the OTU method [37]. The relative abundances of taxa were calculated as the percentage of sequences assigned to an ASV from total sequences in a sample. Alpha diversity metrics and Shannon-Wiener diversity index, were estimated after the samples were rarefied due to unequal numbers of sequences in the samples. The raw sequence data from this study have been deposited in the European Nucleotide Archive (ENA) database under the project accession number PRJEB45256.

Abundances of 16S rRNA genes in samples were compared pairwise by non-parametric multiple comparison procedures using Student-Newman-Keuls test (SNK), since normality in the distribution of individual datasets was not met. Analysis was performed in SigmaPlot software (v 11.0). Data are presented as average ± standard deviation (SD). Hierarchical cluster analysis was conducted using PRIMER 6 v.6.1.11 and PERMANOVA +v.1.0.1 statistical software packages [38]. Similarities were based on the Bray-Curtis (dis)similarity index using a group average method. To identify the most important environmental factors (explanatory variables) influencing variability in bacterial assemblage structure (response variables), principal component analysis (PCA) was conducted using the Canoco v.5 software [39].

**RESULTS**

**Environmental conditions**

In the SML of the Adriatic Sea temperature ranged from 13.9 ⁰C in winter to 28.4 ⁰C in summer, with an annual average of 20.45 ± 6.25 ⁰C (Fig. 2a). Opposite trend was observed for DO, which was lowest in summer (9.3 mg/L) and highest in winter (14.1 mg/L) (average 11.5 ± 2.0 mg/L), however, oxygen saturation minimum was recorded in summer (101.3 %) and maximum in autumn (133.3 %), with an average of 112.83 ± 15.03 %. The SML of the Adriatic Sea was quite stable in terms of salinity (average of 37.3 ± 0.5) and pH (average of 8.03 ± 0.12). Similar values were also observed in ULW (Fig. 2b). Temperature minimum was observed in winter (13.6 ⁰C) and maximum in summer (28.4 ⁰C), with an average of 20.33 ± 6.41 ⁰C. Values of DO (average 11.59 ±1.66 mg/L), oxygen saturation (113.05 ± 14.94 %), salinity (37.25 ± 0.59) and pH (8.06 ±0.11) were almost identical to the ones observed in SML.

In the SML of Rogoznica Lake temperature varied from 7.1 ⁰C in winter to 31.6 ⁰C in summer (average of 20.23 ± 10.05 ⁰C). DO was continuously decreasing from winter (12.3 mg/L) to autumn, when it approached hypoxic value of 4.3 mg/L (annual average of 8.05 ±3.44 mg/L). In contrast to the Adriatic Sea, oxygen saturation in the SML of Rogoznica Lake reached the maximum in spring (102.7 %) and the minimum in autumn (47.5 %) (average of 84.93 ± 25.54 %). Also, salinity varied more in Rogoznica Lake than in the Adriatic Sea, ranging from 22.8 in winter to 34.2 in autumn in SML (average 29.05 ± 5.08). It is important to stress that the atmospheric wet precipitation is the only source of freshwater in this marine lake system. pH was relatively stable, with an average of 8 ± 0.3 (Fig. 2a). In the ULW of Rogoznica Lake seasonal trends of investigated parameters were identical, with averages of temperature (20.1 ±9.74 ⁰C), DO (8.13 ± 3.4 mg/L), oxygen saturation (84.75 ± 25.75 %), salinity (29.05 ± 5.13) and pH (8.06 ± 0.31) very similar to the ones recorded in SML (Fig. 1b).

The Adriatic Sea SML was enriched in NO2-, NO3-, PO43- and TOC when compared to the ULW (Fig. 2c). The lowest NO2- concentration in SML was recorded in autumn (0.21 µM) and the highest was recorded in spring (0.62 µM). The minimum of NO2- in ULW was also found in autumn (0.07 µM), whereas the maximum of 0.031 µM was found in winter. The same trend in SML was found for NO3- that was minimal in autumn (0.01 µM) and maximal in spring (3.314 µM). In ULW, NO3- were under the detection limit in autumn, while the highest concentration was recorded in summer (1.51 µM). The same trend was observed for PO43- in the SML of the Adriatic Sea with the lowest concentration in autumn (0.47 µM) and highest in summer (0.28 µM). Seasonal variations of TOC were well expressed in the SML of the Adriatic Sea (ranging from 2.369 mg/L in winter to 9.442 mg/L in summer). Concentrations of TOC were generally lower in ULW and ranged from 1.163 mg/L in autumn to 4.525 mg/L in summer.

In Rogoznica Lake these parameters were generally higher than in the Adriatic (Fig. 2c). The concentration of NO₂⁻ in the SML of the lake was lowest in spring (0.213 µM) and highest in autumn (1.15 µM). The same trend was observed in ULW (0.051 µM in spring and 0.62 µM in autumn). The minimum of NO3- in the SML was also recorded in spring (0.486 µM), but the maximum was recorded in summer (2.30 µM). In ULW, NO₃⁻ ranged from 0.067 µM in spring to 2.22 µM in summer. The highest value of PO43- in the SML of the lake was recorded in winter (3.164 µM) and the lowest one in spring (0.284 µM). In ULW, PO43- were also the lowest in spring (0.051 µM), while the highest concentration was recorded in autumn (0.85 µM). Concentration of TOC in SML was lowest in autumn (2.332 mg/L) and reached the extreme value of 13.735 mg/L in summer. The same trend was observed for ULW as well (minimum of 1.29 mg/L in autumn and maximum of 2.111 mg/L in summer).

**Abundance of bacterial 16S rRNA genes**

The abundance of 16S rRNA genes in the Adriatic Sea was significantly higher in SML than in ULW (SNK, *p* < 0.05) in all seasons (Fig. 3). The lowest abundance in SML was observed in winter (8.3 × 105 ± 2.91 × 105 gene copy/mL), followed by an increase in spring (2.29 × 106 ± 3.84 × 105 gene copy/mL) and summer (2.86 × 106 ± 5.97 × 105 gene copy/mL), whereas the maximum was recorded in autumn (8.04 × 106 ± 1.06 × 106 gene copy/mL). This trend was slightly different in the ULW samples of the Adriatic Sea. Minimum was also recorded in winter (1.24 × 105 ± 1.35 × 104 gene copy/mL), followed by an increase in spring (2.05 × 105 ± 2.37 × 104 gene copy/mL) and maximum in summer (4.72 × 105 ± 1.32 × 104 gene copy/mL), whereas in autumn 1.83 × 105 ± 3.71 × 103 gene copy/mL were recorded. Annual averages in the Adriatic were 3.51 × 106 ± 3.14 × 104 gene copy/mL in SML and 2.46 × 105 ± 1.54 × 105 gene copy/mL in ULW.

In Rogoznica Lake, SML also contained significantly more (SNK, *p* < 0.05) bacterial 16S rRNA genes than ULW, except in spring (Fig. 3). Same as in the Adriatic, the lowest gene number in the SML of Rogoznica Lake was detected in winter (1.82 × 106 ± 1.62 × 105 gene copy/mL) and the maximum was observed in spring (1.08 × 107 ± 1.29 × 105 gene copy/mL), followed by a decrease in summer (7.47 × 106 ± 4.07 × 104 gene copy/mL) and autumn (2.17 × 106 ± 4.51 × 104 gene copy/mL). The same trend was recorded in ULW. Minimum of 5.45 × 105 ± 9.21 × 103 gene copy/mL was found in winter, maximum of 9.82 × 106 ± 9.62 × 105 gene copy/mL was reached in spring, whereas lower abundances were in summer (1.52 × 106 ± 3.86 × 104 gene copy/mL) and autumn (7.70 × 105 ± 1.03 × 105 gene copy/mL). The average abundance of 16S rRNA genes in the SML of Rogoznica Lake was 5.56 × 106 ± 4.34 × 106 gene copy/mL and 3.16 × 106 ± 4.46 × 106 gene copy/mL in ULW.

Pairwise comparison of average values in SML and ULW between the Adriatic Sea and Rogoznica Lake showed a significantly higher abundance (S-N-K, *p* < 0.05) of 16S rRNA genes in Rogoznica Lake in both investigated layers.

**Taxonomic structure of bacterioneuston and bacterioplankton assemblages**

**Taxonomic structure of the total bacterial assemblages**

The taxonomic structure of bacterial assemblages in the SML and the ULW of the Adriatic Sea and Rogoznica Lake marine system was assessed using phylogenetic annotations of the 16S rRNA gene (DNA) sequence reads generated from HTS. The most frequent class from both study sites was Gammaproteobacteria, followed by Alphaproteobacteria (details are given in Supplement file).

At the lower family/genus level (Fig. 4a), *Alteromonas*-related DNA sequences were the most frequently retrieved from the Adriatic Sea samples (12.39 ± 19.6%). During winter and spring they comprised < 2% of total assemblages in both layers. However, in summer and autumn they comprised 47.03% and 40.68% of total bacteria in SML, respectively. *Vibrio* was also lowly represented (< 2%) in winter and spring, reaching maximum in summer ULW (22.01%) and decreasing in autumn (average, 6.63 ± 8.03%). On the other hand, *Rhodobacteraceae* were relatively equally distributed seasonally and between the layers, accounting on average 5.36 ± 3.8% of DNA sequences. SAR11 (mainly represented by clade I) was also detected throughout the year and was more abundant in ULW, especially in winter (14.14%) (average, 5.09 ± 5.77%). Similarly, SAR86 clade was also more abundant in ULW than in SML and comprised up to 13.07% in winter ULW (average, 4.76 ± 5.17). Cyanobacterial sequences were represented by *Synechococcus* CC9902 strain, which was more prominent for ULW and comprised on average 2.72 ± 2.92% of all sequences, and *Phormidesmis* which was detected only in spring SML sample (6.06%). It is also interesting to point out the development of *Rubritalea* in spring SML (19.02%), *Pseudoalteromonas* (15.22%) in autumn SML and *Oleibacter* (13.19%) in summer SML.

In Rogoznica Lake, SAR11 (clade I) was on average the most frequent (11.38 ± 11.07) and slightly more abundant in ULW, with the maximum relative abundance in winter ULW (35.99%). Similarly, as in the Adriatic Sea, *Alteromonas* was the most abundant in the summer SML (49.62%) of Rogoznica Lake, but was not detected in autumn, whereas in winter it reached 5.46% of total sequences (average 7.43 ± 17.14%) (Fig. 4a). Winter was also the only season in which *Pseudohongiella* (2.23% in SML and 21.85 in ULW) and *Paraperlucidibaca* (42.8% in SML and 15.88% in ULW) occurred. The relative abundance of Rhodobacteraceae was relatively stable year-round, with a minimum of 1.81% in winter ULW and maximum of 9.53% in spring SML (average, 6.2 ± 2.87%). Dynamics of *Synechococcus* CC9902 in Rogoznica Lake was opposite to one observed in the Adriatic. It was found dominant only in summer (7.78 %in SML and 17.24% in ULW) and autumn samples (10.37% in SML and 15.26% in ULW) with an average of 6.82 ± 6.9% of the total DNA sequences. Season-specific were also NS4 and NS5 marine group within Flavobacteriacae, which occurred in spring (4.21% in SML, 3.91% in ULW – NS4 marine group; 6.66% in SML and 6.65 in ULW – NS5 marine group), as well as genus *Persicirhabdus* (9.2 in SML and 4.34 in ULW) and SAR86 clade (4.77% in SML and 8.32% in ULW). Autumn season in Rogoznica Lake was also specific in terms of development of *Spongiibacter* (11.9% - SML; 10.91% - ULW), Ectothiorhodospiraceae (8.36% - SML; 11.59% - ULW), Planctomycetota (4.25% - SML; 4.54% - ULW).

**Taxonomic structure of the indicatively active bacterial assemblages**

Taxonomic structure indicating the active bacterial assemblages was inferred from the 16S rRNA transcripts (cDNA sequences) and was similar to the one observed in DNA gene pool. In the Adriatic Sea as well as in Rogoznica Lake Gammaproteobacteria and Alphaproteobacteria were the most abundant classes (details are given in Supplement file).

On the lower taxonomic level, *Alteromonas* was the most frequently retrieved genus from cDNA sequences in the Adriatic Sea, with pronounced seasonality (Fig. 4b). It comprised 4.68% of assemblages in winter SML, disappearing during spring and developing an abundant population in SML during summer (37.06%) and autumn (33.35), accounting on average 10.57 ± 15.43% of potentially active bacteria. Rhodobacteraceae was the second most abundant family, which was, however, detected in all seasons (ranging from 3.24% in winter SML to 13.96% in autumn ULW), comprising on average 7.25 ± 4.3% of cDNA reads. Cyanobacteriacae, represented by *Synechococcus* CC9902 strain, were most abundant in winter (6.65% in SML and 14.62% in ULW), whereas in the rest of the year comprised a minor ( ̴ 3%) cDNA fraction found only in ULW. Cyanobacterial genus *Phormidesmis* comprised 13.67% of cDNA reads and was found exclusively in spring SML sample. Interestingly, SAR 11 clade accounted for < 5% of cDNA assemblages in all samples from the Adriatic Sea. *Vibrio*-related cDNA sequences were found abundant in summer (7.53% in SML and 9.66% in ULW) as well as Saccharospirillaceae, i.e. *Oleibacter* (19.05% in SML and 10.82% in ULW). On the contrary, *Rubritalea* was found only in spring (16.84% in SML and 2.24 in ULW), whereas *Pseudoalteromonas* was characteristic for autumn SML (9.21%). Two taxa, AEGEAN 169 marine group and Helieaceae (OM60/NOR5 clade) were specific for the ULW. AEGEAN 169 marine group was relatively stable throughout the year ( ̴ 3%), whereas OM60/NOR5 clade reached maximum of 17.03% of cDNA sequences in spring.

On average, most of the cDNA sequences from Rogoznica Lake were taxonomically assigned to *Synechococcus* CC9902 strain, accounting for 16.3 ± 16.3% of all reads (Fig. 4b). This strain showed pronounced seasonal dynamics. While it was completely absent from winter samples, in autumn it comprised 30.64% and 44.01% of indicatively active assemblages in SML and ULW, respectively. Distribution of Rhodobacteraceae was stable through the seasons and layers, comprising on average 6.88 ± 1.99% cDNA reads. Clade SAR 11 was more abundant in Rogoznica Lake than in the Adriatic Sea (average, 5.2 ± 5.75%), especially in the winter SML sample (17.34%). On the other hand, *Alteromonas* was lowly represented in winter (<6%), and absent from all the other layers and seasons, except in summer SML when it dominated cDNA assemblages with the relative abundance of 28.16%. Winter was a specific season in Rogoznica Lake in terms of development of Moraxellaceae, i.e. *Paraperlucidibaca* and *Pseudohongiella*. *Paraperlucidibaca* accounted for 21.74% of cDNA reads in SML sample and 48.64% in the ULW sample. *Pseudohongiella* was specific for the SML sample (19.71%). Both taxa were absent in other seasons and the Adriatic Sea. In spring, however, many other taxa occurred in both layers, but accounted minority of ̴ 3%. These taxa include: Flavobacteriaceae (NS4 and NS5 marine group), SAR11, SAR116 and SAR 86 clades, *Persicirhabdus*, *Litoricola*, Methylophilaceae (OM43 clade), Cryomorphaceae, Helieaceae (OM60/NOR5 clade) and KI89A clade within Gammaproteobacteria. Members of Spongiibacter (4.91% in SML; 5.69% in ULW), Ectothiorhodospiraceae (3.74% in ULW), Bdellovibrionaceae (OM27 clade; 3.5% in ULW), Ectothiorhodospiraceae (3.74% in ULW) and Hyphomonadaceae (6.22% in SML) were exclusively found in autumn sample of Rogoznica Lake.

**Bacterioneuston and bacterioplankton diversity**

In total, 826 593 DNA and 1 005 208 cDNA were successfully taxonomically assigned from the Adriatic Sea SML samples, whereas 896 580 DNA and 874 771 cDNA sequences were obtained from ULW samples (Table 1). Number of ASVs in the SML varied from 881 in autumn to 2 578 in spring DNA gene pool (average, 1 805 ± 795) and from 1 417 in summer to 2 771 in spring cDNA samples (average, 1 968 ± 659). Shannon-Wiener index (H’), based on DNA sequences, was the lowest in summer SML (5.2), and the was maximum recorded in winter (9.25), comprising an average of 7.55 ± 2.3 in SML. Shannon-Wieners’ minimum in ULW was observed in spring (7.96 in DNA and 7.3 in cDNA), whereas maximum was reached in winter (8.46 in DNA and 8.64 in cDNA). There was no statistically significant difference (one-way ANOVA; *p* > 0.05) in Shannon-Wiener diversity between SML and ULW in both DNA and cDNA pools.

Number of taxonomically assigned sequences from ULW samples of Rogoznica Lake varied from 182 202 and 220 433 in spring (DNA and cDNA) to 217 564 and 305 659 in autumn DNA and cDNA samples, respectively. Minimum number of sequences in ULW was retrieved in winter (183 219 in DNA and 210 616 in cDNA), whereas the maximum was found in autumn (234 014 in DNA and 263 723 in cDNA). The number of ASVs in Rogoznica Lake was generally lower than in the Adriatic Sea. The lowest number of ASVs in the ULW was observed in winter (759 in DNA and 465 in cDNA), whereas the highest numbers were found in autumn (1 355 in DNA and 1 338 in cDNA) (average, 943 ± 277 in DNA and 916 ± 366 in cDNA). However, in ULW samples the fewest ASVs were found in spring (501 in DNA and 513 in cDNA), with the most (873 in DNA and 623 in cDNA) found in autumn, comprising an average of 640 ± 168 and 587± 65 ASVs in DNA and cDNA gene pools, respectively. The Shannon-Wiener index in SML of Rogoznica Lake was the lowest in winter (5.2 in DNA and 4.88 in cDNA) and the highest in autumn (7.53 in DNA and 6.86 in cDNA). The lowest diversity in ULW was also recorded in winter (4.56 in DNA and 4.52 in cDNA), but the highest values were reached in summer DNA (7.02) and spring cDNA (6.59).

**Table 1:** Sequence counts, number of amplicon sequencing variants (ASVs) and Shannon-Wiener index (H’) of individual DNA and cDNA samples collected seasonally from the sea surface microlayer (SML) and underlying water (ULW) in the Adriatic Sea and Rogoznica Lake.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | | WINTER | | SPRING | | SUMMER | | AUTUMN | |
| DNA | cDNA | DNA | cDNA | DNA | cDNA | DNA | cDNA |
| ADRIATIC SEA | No. of sequences | SML | 177 317 | 226 508 | 219 914 | 293 987 | 236 995 | 262 706 | 192 367 | 222 007 |
| ULW | 225 153 | 212 553 | 261 393 | 196 157 | 204 338 | 197 683 | 205 696 | 268 378 |
| No. of ASVs | SML | 2 346 | 2 245 | 2 578 | 2 771 | 1 413 | 1 417 | 881 | 1 440 |
| ULW | 2 062 | 1 971 | 17 42 | 1 428 | 1 189 | 836 | 1 759 | 1 975 |
| Shannon-Wiener index (H') | SML | 9.25 | 9.15 | 9.79 | 9.20 | 5.20 | 6.07 | 5.95 | 6.36 |
| ULW | 8.46 | 8.64 | 7.96 | 7.30 | 8.12 | 8.04 | 8.29 | 8.53 |
| ROGOZNICA LAKE | No. of sequences | SML | 213 821 | 217 564 | 182 202 | 171 986 | 216 588 | 2491 90 | 220 433 | 305 695 |
| ULW | 183 219 | 210 616 | 185 614 | 248 250 | 146 373 | 245 178 | 234 014 | 263 723 |
| No. of ASVs | SML | 759 | 465 | 860 | 934 | 800 | 926 | 1 355 | 1 338 |
| ULW | 536 | 554 | 501 | 513 | 649 | 657 | 873 | 623 |
| Shannon-Wiener index (H') | SML | 5.20 | 4.88 | 7.26 | 7.45 | 5.32 | 6.21 | 7.53 | 6.86 |
| ULW | 4.56 | 4.52 | 6.73 | 6.59 | 7.02 | 6.47 | 6.81 | 5.81 |

In order to investigate structural seasonal differences present on two study sites and in two layers a hierarchical cluster analysis was conducted, based on relative abundances of all taxa (including those with abundance of < 2%) (Fig. 5). A dendrogram clearly clustered samples from the Adriatic Sea and Rogoznica Lake at the Bray-Curtis similarity level of 44.5%. Within each cluster, there is evident seasonal grouping (similarity level between 50 and 60%), with the exception of SML and ULW winter samples from Rogoznica Lake, which separated from all other samples at 37.5% similarity level. Similarities between SML and ULW samples were generally around 70%, with the clearest structural differences observed in summer in the Adriatic Sea and in winter in Rogoznica Lake. Detailed similarity matrix between the samples is given in Supplement file (Fig. 2).

**Bacterial assemblages in relation to environmental conditions**

PCA analysis was conducted in order to summarize the variability across the samples in relation to analyzed environmental factors. For the Adriatic Sea samples, the first two axes of the analysis cumulatively explained 78.46% of the variation, indicating high contribution of the measured environmental factors to the observed variability (Fig. 6a). The highest positive value for the first axis was found for TOC (F = 0.88) and temperature (F = 0.78), followed by NO3- (F = 0.75), NO2- (F = 0.68) and PO43- (F = 0.53). Strong negative values, were observed for dissolved oxygen (F = -0.91) and salinity (F = -0.73). For the second axis, which explained 24.95% of the variation, pH showed high negative score (F = -0.83).

For Rogoznica Lake, PCA analysis cumulatively explained 67.37% of the variation in samples’ variability (Fig. 6b). The most important environmental factor for the first axis was TOC (F = 0.96), DO (F = 0.84) and temperature (F = 0.61). Strong negative scores showed salinity (F = -0.85), PO43- (F = -0.79) and NO2- (F = -0.81). For the second axis, which explained 20.34% of the variation, the most important factor was NO3- (F = 0.89), whereas pH (F = -0.22) was not found important in none of the two axes.

**DISCUSSION**

For the first time, this study explored in detail seasonal variations of bacterial assemblages along with environmental conditions in SML and ULW of two coastal Adriatic Sea areas; a highly eutrophic marine system of Rogoznica Lake and the adjacent coastal zone of the Adriatic Sea. Both investigated layers were similar in temperature, oxygen (concentration and saturation), salinity and pH values. Trends in these parameters were, however, different between two study sites. The Adriatic Sea was more saline than Rogoznica Lake throughout the year. Oxygen concentrations were slightly higher in the Adriatic, but saturation was lower than in Rogoznica Lake for most of the year, except in autumn when stratification of the water column in the lake started to break down, and oxygen concentration approached hypoxic values in the surface layer. Temperature shows that the lake is warming and cooling faster than the Adriatic, being colder in winter and warmer in summer, reflecting its small size and isolated character. In terms of organic matter and nutrient concentrations SML and ULW were two noticeably different environments. At both investigated sites, SML was consistently enriched in NO₂⁻, NO₃⁻ and PO₄3⁻ when compared to ULW. Nevertheless, these values were higher in Rogoznica Lake than in the Adriatic Sea, throughout the year. Organic carbon concentrations were also substantially higher in SML than in ULW in both study sites during all investigated seasons, especially in summer when TOC reached the extreme value of almost 14 mg/L in the SML of Rogoznica Lake, exceeding the values observed in other highly eutrophic marine environments, as well as in the anoxic monimolimnion of the lake [40, 41].

All this reflected bacterial abundance, which was significantly higher (*p* < 0.05) in SML than in ULW, except in the spring season in Rogoznica Lake. The maximum in the Adriatic Sea was reached in autumn SML, whereas the maximum in ULW was in summer. Bacterial abundances in the Adriatic Sea station were comparable to the ones recorded in surface layers of other coastal sites in Central Adriatic [42], but for an order of magnitude lower than in SML. In Rogoznica Lake, the maximum of bacterial abundance was found in spring in both investigated layers (SML and ULW). These values were from one to two orders of magnitude lower than in hypoxic chemocline and anoxic monimolimnion of the lake [24], but in the range of values observed during a holomictic event [22].

Besides the abundance, bacterioplankton and bacterioneuston structure and dynamics were spatially and seasonally conspicuous. The stability of the SML in the Adriatic Sea, in terms of bacterial assemblage structure, was least pronounced in winter. In both layers SAR86 and SAR11 clades dominated in DNA assemblages, whereas cDNA was dominated by *Synechococcus* CC9902 strain. Both these clades are ubiquitous in the surface ocean, and SAR11 clade was also found very abundant in the euphotic zone of the open Adriatic Sea [43, 44]. However, its relative abundance was not as nearly as low as in coastal site investigated here, especially within the indicatively active fraction where it accounted for < 5% year-round. On the other hand, *Synechococcus* CC9902 was also typical for the coastal region in Central and Southern Adriatic, especially in winter [45]. At the same time in Rogoznica Lake *Paraperlucidibaca* was the most relatively abundant genus, accounting for almost 50% of cDNA sequences, followed by *Pseudohongiella* and *Glaciecola*, which were not detected at the Adriatic Sea study area. *Paraperlucidibaca* was also found abundant in Antarctic waters as an important degrader of semi-labile organic matter [46]. On the other hand, the role of *Pseudohongiella* cannot be discussed with certainty since it has been isolated relatively recently, with no studies on their ecological functions [47]. However, it has been found abundant in the coastal seawater with large loads of organic matter from municipal wastewater [48], which is usually enriched in PO43- as was the case in Rogoznica Lake SML winter sample. It was shown that *Glaciecola* genus is well adapted to low temperature, and that their growth is tightly related to phytoplankton-derived DOC, especially diatoms [49], which are the most dominant phytoplankton group in the lake, especially during colder seasons [23]. This can explain its development in the winter season in the lake. Its absence, as well as the absence of *Paraperlucidibaca* and *Pseudohongiella* from the Adriatic Sea samples indicate that, besides the concentration, there are also possible variances in the type of TOC in these two environments during winter.

Differences between SML and ULW samples during spring in the Adriatic Sea were more evident than in winter. For the spring SML sample *Rubritaela*, *Rhodobacteraceae* were specific and abundant, as well as cyanobacterial genus *Phormidesmis*, which accounted for almost 14% of cDNA assemblages. This is surprising since this genus is characteristic and well adapted to cold polar and alpine habitats [50]. To the best of our knowledge, this is its first record in temperate area. The composition of the ULW sample remained relatively similar to the one observed during winter, with the exception of development of SAR116 clade, common and widespread marine bacteria, ecologically and metabolically similar to SAR11 clade [51], as well as *Halieaceae* (formerly *Alteromonadaceae*), which are important marine hydrocarbon utilizers [52]. Development of *Rubritaela* and *Rhodobacteraceae* indicates that the Adriatic Sea SML during spring was probably enriched in polysaccharides since both genera use them as substrate, and *Rhodobacteraceae* were also found as important members of biofilms in coastal seawater [53, 54]. Spring SML and ULW samples from Rogoznica Lake did not differ noticeably in structure. In contrast to the Adriatic Sea study site, no *Rubritaela* and *Phormidesmis*-related sequences were detected and clade SAR11 was still present in both layers of the lake. Also, *Synechococcus* CC9902, which was absent in winter, started to develop.

Summer was the season when stability of SML was most pronounced, as expected due to meteorological conditions. *Alteromonas* was predominant at both investigated sites and peculiar to SML. At the Adriatic Sea station, it comprised 47.03% of DNA and 37.06% of cDNA sequences, whereas in Rogoznica Lake the relative abundance of *Alteromonas* reached 49.62% of DNA and 28.16% of cDNA sequences. It is a widespread copitrophic genus of marine Gammaproteobacteria with a strong capability for various polysaccharide degradation in both, dissolved and particulate form [55], therefore high TOC values favored its development in summer. Along with *Alteromonas*, *Oleibacter* also developed in summer, representing approx. 20% of potentially active assemblages in the Adriatic Sea, but only 4% in the lake. This genus is an important degrader of petroleum aliphatic hydrocarbons [56], so its development might be contributed to increased maritime traffic. ULW samples in this season were dominated by *Vibrio* spp. in the Adriatic and *Synechococcus* CC9902 in Rogoznica Lake. Some *Vibrio* spp*.* are well-known waterborne human and/or animal pathogens [57]. This requires a special attention since this genus was found specific for the summer season during which the Adriatic Sea is the area of increased touristic and fishing activities. It is noteworthy to mention that summer ULW samples from the Adriatic Sea also contained a very few *Escherichia-Shigella* – related sequences (0.015 % in DNA and 0.48 % in cDNA). Apart from this, investigated sites showed a high microbial water quality.

Autumn samples from the Adriatic Sea remained relatively similar to the summer ones. In contrast the samples collected from Rogoznica Lake showed alternation in structure. *Alteromonas* disappeared and the abundance of *Synechococcus* CC9902 started to increase in both SML and ULW samples, reaching almost a half of indicatively active assemblages in ULW. This season in the lake was characterized by low oxygen concentration (4.3 mg/L) and saturation (< 50%), which was probably the consequence of high TOC degradation during summer. *Synechococcus* CC9902 was also found active in the anoxic monimolimnion in the lake due to its metabolic diversity and capability to thrive under these extreme conditions [24], and forms the basis for re-oxygenation of the surface layer.

Alpha diversity indices showed that the bacterial diversity was higher in the SML of the Adriatic Sea during winter and spring, while ULW was more diverse than SML in the warmer seasons (summer and autumn) and comparable to the values observed in the open Adriatic waters [44]. On the other hand, SML in Rogoznica Lake had higher diversity than ULW in all seasons except in summer, but these values were much lower than in deeper chemocline and monimolimnion layers [24]. Reduced diversity in summer can be attributed to high UV radiation, under which only highly adapted taxa can thrive. Indeed, *Alteromonas*, which was found to be resilient to solar radiation [58] was predominant genus in the SML during summer in both investigated sites. However, while it remained abundant in the Adriatic during autumn, it was absent from Rogoznica Lake when oxygen concentration and saturation decreased.

All these spatial and temporal changes in bacterioplankton and bacterioneuston were strongly associated to temperature and TOC, and additionally to DO in Rogoznica Lake, as shown by the PCA. This is particularly important since the warming and accumulation of organic matter have already been recorded in both the Adriatic Sea [20, 59] and Rogoznica Lake, as a direct consequence of global changes. This study showed that season- and habitat-specific heterotrophic bacteria dominate SML and ULW, but under oxygen-deprived conditions autotrophs became the most abundant bacterial fraction within the cDNA sequences. Therefore, structural and functional changes in bacterioplankton and bacterioneuston assemblages can be expected. Since they are in direct contact to the atmosphere, they are likely first to respond to environmental fluctuations, which can affect carbon and nitrogen cycling due to their essential role in it. However, in future studies it would be important to connect seasonal changes in microbial community composition not only to concentration, but also to the exact type of the organic matter, since the dominant taxa in each season greatly differ and show preferences towards different substrates.

Cluster analysis showed considerable differences in bacterial assemblage structure in SML and ULW of the Adriatic Sea and Rogoznica Lake although the two investigated sites were only approx. 180 m apart and connected through porous karst system [23], confirming our hypothesis that the trophic status of the environment as well as the sampling season are important drivers of these changes. This also shows that in coastal areas and small and isolated ecosystems, meteorological conditions did not have a crucial role in bacterial assemblage structure during the investigated period, but rather that it was influenced by *in situ* environmental parameters. However, in atypical years, in which an abrupt decrease in air temperature occurs, stratification in the Lake starts to break down causing anoxigenic holomixis, which consequently drastically changes bacterial assemblage structure and diversity [22, 60]. In addition to here analyzed abiotic parameters, for comprehensive investigations of changes in bacterial assemblages, biotic factors, including viruses, should be assessed in future, since they have been also shown as important drivers of bacterial abundance and diversity variations in Rogoznica Lake [61] as well as in the coastal area of the Central Adriatic [62]. Besides, upcoming studies should evaluate terrestrial impact and weathering from the surrounding rocks on bacterial assemblages, especially on the enclosed ecosystem of the Lake, as well as bacterial dispersal via marine aerosols. Taking into account the vicinity between the stations, aerosol transmission is inevitable [63-66], especially during winter and early spring, which are characterized by strong northern to north-eastern wind episodes. After being transmitted by the atmosphere, factors continue selecting taxa with adaptations to maintain their activity in a particular environment, confirming the old and famous Baas Becking hypothesis that “everything is everywhere but the environment selects.” Marine aerosols from this area have been detected and chemically characterized, showing high sea salt content and sulfate concentration [67]. However, aerosol microbiome has not been studied yet, so this hypothesis should be subjected to future studies.

**COMPLIANCE WITH ETHICAL STANDARDS**

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**Code Availability:** Not applicable.

**Contributions:** MČ – conceived the study, conducted sampling and experimental work and wrote the manuscript;

MDS – conducted bioinformatical analysis; IDR – conducted nutrient analysis; IC – contributed substantially to

manuscript drafting, project administration and funding acquisition. All authors have read the manuscript and approved its publication.

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**FIGURE CAPTIONS**

**Fig. 1:** Geographic location of two investigated stations. A – Adriatic Sea; R – Rogoznica Lake

**Fig. 2:** Seasonal variations of environmental parameters of temperature, salinity, pH, dissolved oxygen and oxygen saturation in the sea surface microlayer (SML) (a), and underlying water layer (ULW) (b) and nitrate (NO3-), nitrite (NO2-), orthophosphates (PO43-) and total organic carbon (TOC) (c) in the Adriatic Sea and Rogoznica Lake.

**Fig. 3:** Seasonal abundances of bacterial 16S rRNA genes in sea surface microlayer (SML) and underlying water layer (ULW) of the Adriatic Sea and Rogoznica Lake determined by qPCR. Identical letters above the bars indicate samples among which no statistical significance was found according to the Student-Newman-Keuls test (*p* > 0.05). Vertical error bars represent standard deviation.

**Fig. 4:** Hierarchical cluster dendrogram based on pairwise Bray-Curtis (dis)similarity matrix, taxonomic structure and seasonal distribution of bacterial assemblages in the sea surface microlayer (SML) and underlying water layer (ULW) of the Adriatic Sea and Rogoznica Lake, based on DNA (●) and cDNA (○) 16S rRNA gene sequences. Taxa with the relative abundance <2% is presented as ‘’other’’.

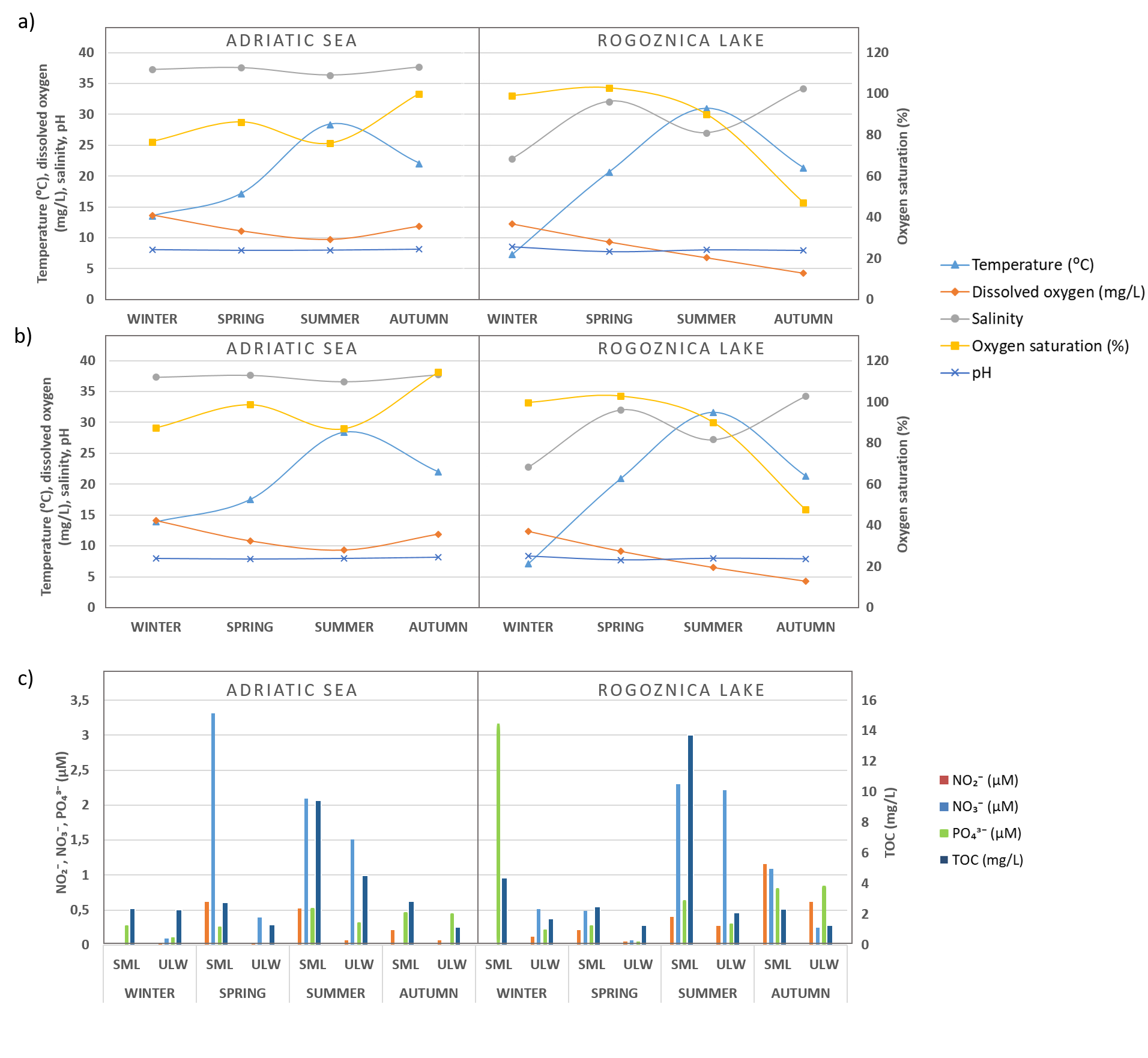
**Fig. 5:** Principal component analysis (PCA) ordination diagram of DNA and cDNA samples collected from the sea surface microlayer (SML) and underlying water layer (ULW) for the Adriatic Sea (a) and Rogoznica Lake (b) throughout the investigated year. Environmental variables of temperature (T), total organic carbon (TOC), dissolved oxygen (DO), orthophosphate (PO₄3⁻), nitrate (NO₃⁻), nitrite (NO₂⁻) salinity (S) and pH are presented as arrows. Eigenvalues (a): Axis 1: 0.5351, Axis 2: 0.2495, Axis 3: 0.1412, Axis 4: 0.0541. Eigenvalues (b): Axis 1: 0.4703, Axis 2: 0.2034, Axis 3: 0.1834, Axis 4: 0.1274.

**FIGURES**

**Fig. 1**

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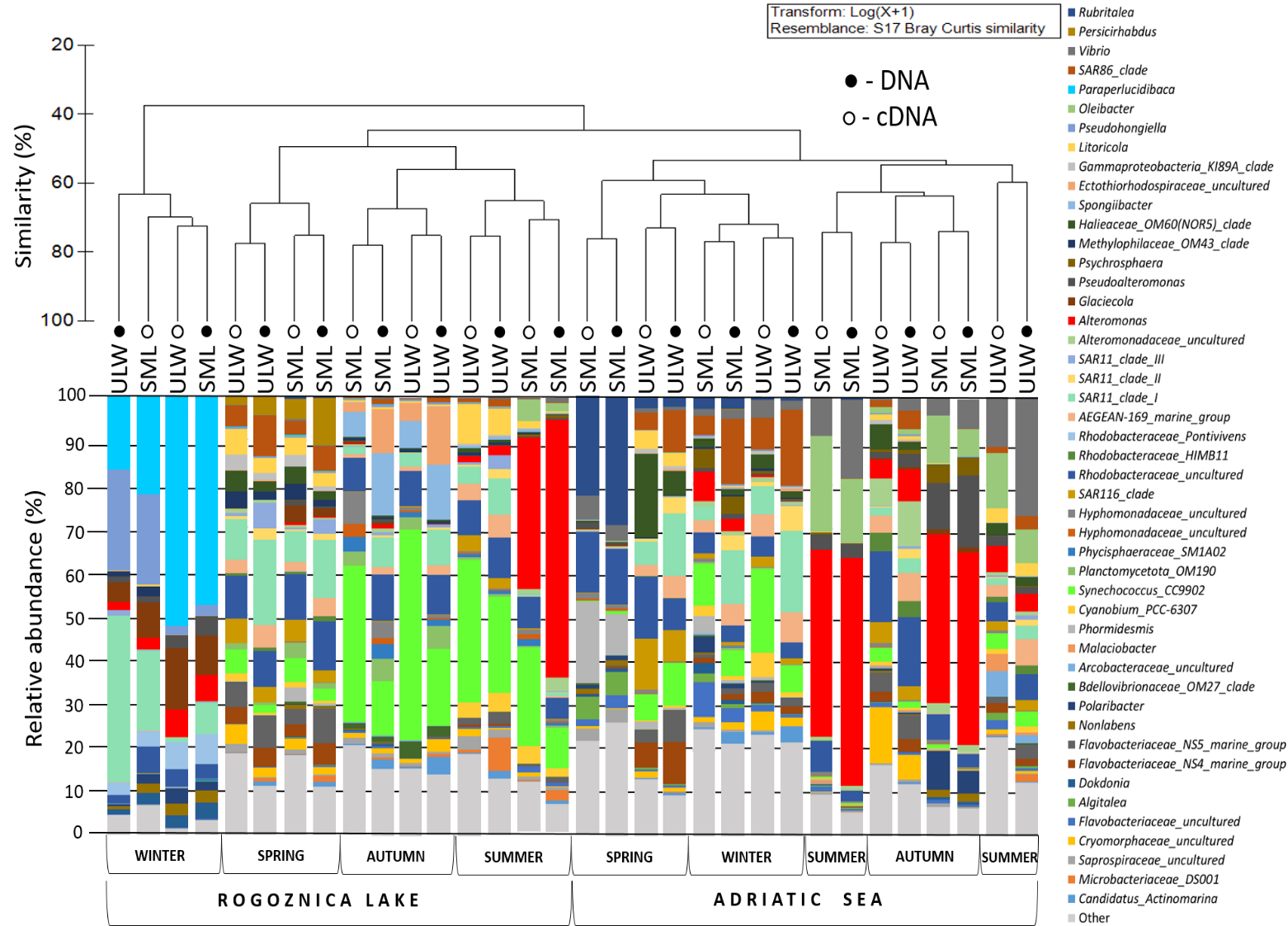
**Fig. 2**

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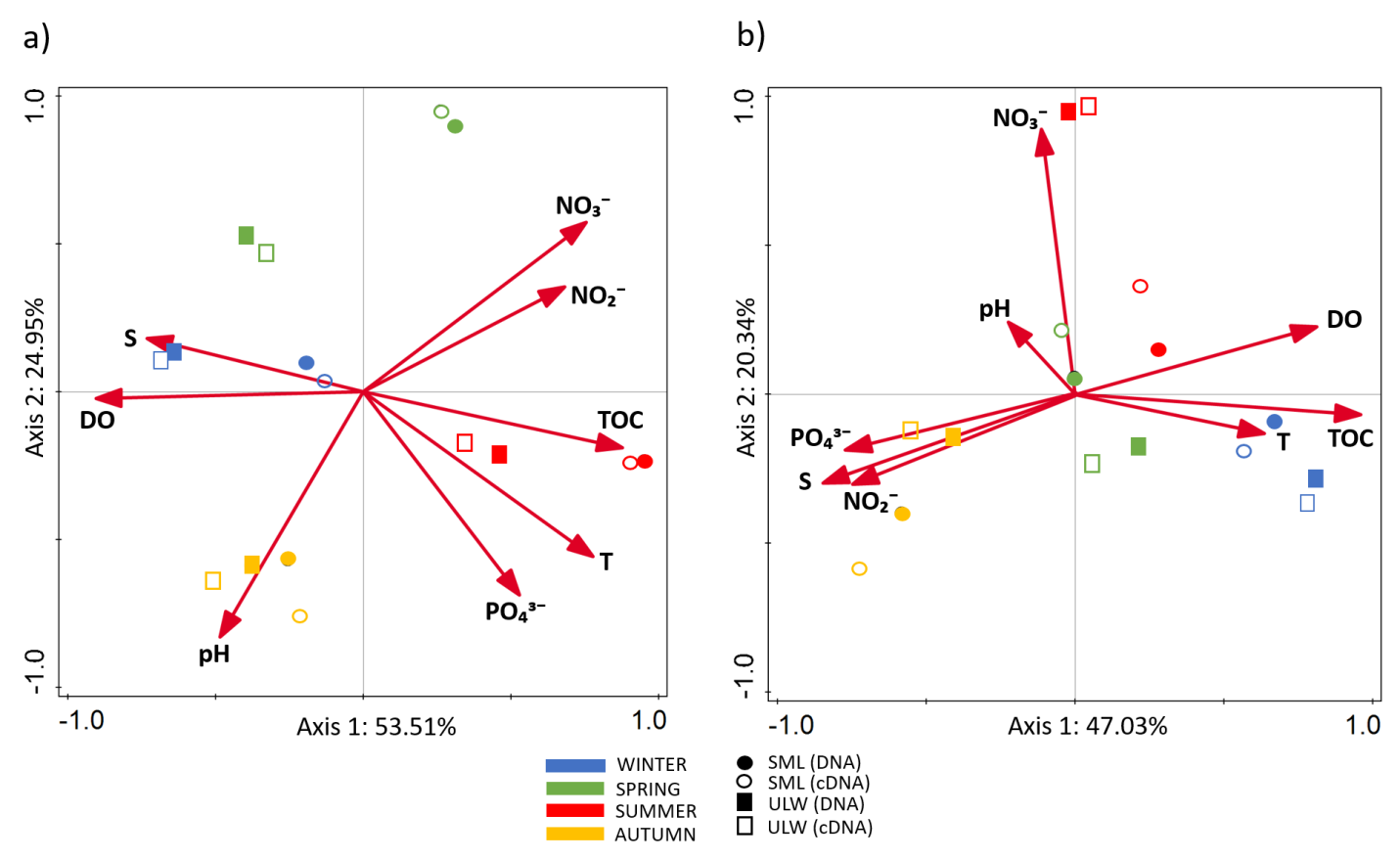
**Fig. 3**

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**Fig. 4**

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**Fig. 5**

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