PHYTOPLANKTON COMPOSITION AND ECOLOGICAL TOLERANCE OF THE AUTOTROPHIC PICOPLANKTON IN ATANASOVSKO LAKE (BLACK SEA COASTAL LAGOON, BULGARIA)

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Abstract. Atanasovsko Lake is a coastal lagoon used for salt extraction for many years. Its salinity varies within a wide range (from 6.3 to > 72‰). Despite these extreme conditions, phytoplankton blooms are often observed. Therefore, our aim was to study the key abiotic drivers and ecological optimum of the picoplankton in a 5-year period (2013-2017). The taxonomic composition, long-term dynamics and abiotic drivers of the autotrophic picoplankton (APP) has been investigated in North Saltern of the coastal lagoon in Atanasovsko Lake. The APP species were identified by using a polyphasic approach based on molecular and phylogenetic analyses of 16S/18S rDNA in combination with cytomorphological and ecological characterization. *Picochlorum oklahomense* (Trebouxiophyceae, Chlorophyta) and marine *Synechococcus sp*. (Synechococcales, Cyanobacteria) were the dominant genotypes within the APP. The relative biovolume of the APP to the total phytoplankton biovolume was strongly variable (from 36 to 99%) as the salinity and temperature were found to be the main abiotic drivers of its dynamics. Salinity between 30-59‰ and mid-summer temperature above 24°C provide an ecological optimum for the APP development at a relative biovolume of 93%. This is the first record of *Picochlorum oklahomense*/*Synechococcus* sp. assemblage within the APP composition of a European coastal lagoon.
Keywords: *ecology, hypersaline lakes, Synechococcus, Picochlorum oklahomense, 16S/18S DNA*

Introduction

Over the last decade, a number of studies have focused on the autotrophic picoplankton (APP) in saline inland waters of Europe (Felföldi et al., 2009; Somogyi et al., 2011, 2014; Keresztes et al., 2012; Paranhos et al., 2017). Unusually high abundance of APP with extraordinarily high picoplankton productivity was registered in soda lakes in the Carpathian Basin (Felföldi et al., 2009). The APP abundance in hypersaline lakes is higher than those in freshwater and marine environments with similar trophic conditions, and the contribution of the APP to the total phytoplankton community can reach up to 90-100% (Somogyi et al., 2014). The selective advantage of pico-sized primary producers in turbid waters is related to the more effective utilization of the light and nutrients (Agustí, 1991; Reynolds, 2006; Felföldi et al., 2009) and reducing grazing pressure (Paranhos et al., 2017). These results confirmed the assumption of Krienitz et al. (2012) that APP has a key position as a highly productive

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primary producer in saline environments and therefore, more attention should be paid to its distribution.

Saline waters also include coastal lagoons and salterns. Coastal lagoons are shallow brackish or marine water bodies separated from the ocean by a barrier island, spit, reef, or sand bank (Barnes, 1980; Kennish and Paerl, 2010; Kennish, 2015). They are formed on shallow banks and are only 12% of the coastal shorelines worldwide (Kennish, 2015). Most of them are situated along African coast (17.9% from shoreline) and North America (17.6%), and only 5.3% are on European territory (Kennish, 2015). Lagoons are characterized with spatial and temporal natural variability, which is not typical for the other aquatic ecosystems (Barnes, 1980). This variability is represented as a strong ecological stress, which forms the community structure. Due to the rarity of these habitats and species that they sustain, coastal lagoons are of primary conservational importance.

Studies on the picophototrophic communities in coastal lagoons and salterns are scarce (Ayadi et al., 2004; Estrada et al., 2004; Elloumi et al., 2009; Schapira et al., 2010). The available results analyzed mainly the dynamics of APP abundance under the influence of abiotic and biotic drivers (Elloumi et al., 2009; Schapira et al., 2010). The reports about the taxonomic composition of APP are even rarer. By applying molecular-genetic methods, a new species *Picocystis salinarum* (Prasinophyceae) has been described from the San Francisco salterns, California, USA (Lewin et al., 2001). Annual domination of the green algae *Picochlorum sp*. and *Picocystis sp*. was recorded in a coastal lagoon in San Diego, California, USA (Wang et al., 2014).

The object of our study was Atanasovsko Lake, a coastal lagoon located on the Black Sea coast in Southeast Bulgaria. The major part of the lake was modified as salterns. Atanasovsko Lake includes a variety of habitats and it is located along the second largest migration route of birds from Europe to Africa, Via Pontica. Our aim was to study the key abiotic drivers and ecological optimum of the picoplankton towards abiotic drivers in a 5-year period.

This is the first study on the taxonomic composition and long-term dynamics of the abundance of APP in a European coastal lagoon.

Materials and methods

Site description

Atanasovsko Lake is a coastal lagoon separated from Black Sea via sand spit, about 1 km long (*Fig. 1A,B*). It is below the sea level (-1.5 m a.s.l.). The volume of the lake is 3.2 mil. m^3, the average depth is 0.3 m, and the surface is 10.9 km^2 (Gecheva et al., 2017).

The lake has been used for salt production since 1906 and was separated by dykes on small basins for salt extraction. The key factor for the lake is the mechanism of salt production and sea-freshwater exchange (Hubenov et al., 2015). Freshwater supply is via atmospheric deposition (rainfall) and river waters (Azmaka and Vetrenska rivers). The inflow of seawater is achieved via sluice from April and continues with varying intensity throughout the summer until the autumn. Due to the decrease in rainfall and the continuous evaporation of water from the crystallization basins, salinity increases almost twice from spring to autumn every year (Ivanov et al., 1964), reaching extreme values in some years – up to 169‰ (Botev, 1997).

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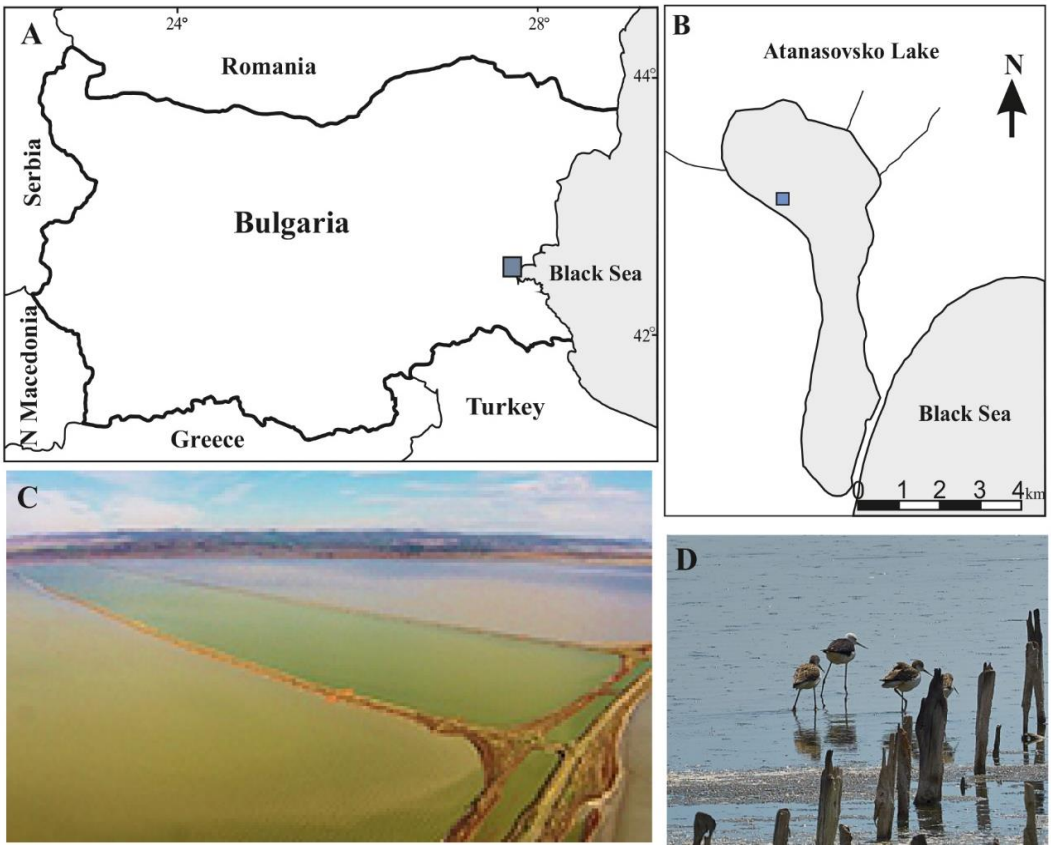

Figure 1. *Atanasovsko Lake.* ***A:*** *Map of Bulgaria (the square shows the location of Atanasovsko Lake);* ***B:*** *Map of Atanasovsko Lake (the square shows the location of the study pond North Saltern);* ***C:*** *Study pond North Saltern with an intensive bloom of APP;* ***D:*** *Habitat of APP, with a high abundance of waterfowl*

Our study was focused on a small basin in the North Saltern (N 42°34'16.4"; E 27°28'04.4") for the period 2013-2017 (*Fig. 1C*). The pond is characterized by variable salinity and intensive blooms of picoplankton. Atanasovsko Lake is one of the most significant wetlands for conregations of waterfowl along the Bulgarian Black Sea coast (*Fig. 1D*).

Sample techniques

Water samples for physico-chemical analyzes were taken five times per each year (2013, 2014, 2015, 2016 and 2017) during the months March, June, July, September and October in the pond North Saltern. The parameters pH, conductivity, temperature and dissolved oxygen/oxygen saturation were measured *in situ* with Multi 3410 SET B TetraCON 952-3 (WTW). Nutrients were analyzed by pHotoFlex STD (WTW) following adopted standards (N-NO_2: EN 26777; N-NO_3: ISO 7890-1; N-NH4: ISO 7150/1; P-PO_4: EN ISO 6878). During the study period (2013-2017) twenty-five water samples were collected for physico-chemical analyzes. Chlorophyll-*a* concentration was determined spectrophotometrically (M107 Visible Spectrophotometer, Spectronic Camspec Ltd., Leeds, UK) according to ISO 10260: 2002. North Saltern waters were classified as mixo- to hypersaline according to the Venice System (Anonymous, 1958).

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The phytoplankton sampling was performed three times per year (2013-2017) during the summer season from June to September. In 2015, phytoplankton samples were collected two times instead three times. Due to the shallow depth and lack of vertical salt gradient in the lake, samples were collected with a Meyer bottle from the sub-surface layer and fixed *in situ* with formaldehyde (4% final concentration). During the study period (2013-2017), 14 phytoplankton samples were analyzed.

Isolation and cultivation of the APP species

The APP species were isolated by streaking cells across agar plates (Andersen and Kawachi, 2005) from living natural samples in 2016 when were registered unusual highly-dense phytoplankton blooms. The strain PACC 8945 was isolated from a bloom sample in June 2016 (T=27°C; S=30.3‰) and cultured on liquid medium with seawater (Schlösser, 1982), modified by addition of Na_2CO_3 (pH=8.5). The strain PACC 8946 was isolated from a bloom sample in July 2016 (T=27.6°C; S=59‰) and cultured on liquid medium with artificial seawater (Eddy, 1956). Algal cultures were maintained in a laboratotory at room temperature (22°C±2°C) and a 15:9 hour light:dark cycle. Two unialgal strains (PACC 8945, PACC 8946) were deposited in Plovdiv Algal Culture Collection (PACC) of Plovdiv University in Bulgaria.

Extraction of DNA, PCR amplification and sequencing of the prokaryotic species (16S DNA)

Genomic DNA was extracted from the cultured cyanobacterial mass by the Proteinase-K extraction method. The concentration of the DNA and its purity was measured on a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The integrity of the extracted DNA was visualized on an agarose gel by ethidium bromide staining and UV transillumination (MiniBIS Pro gel documentation system, DNR Bio-Imaging Systems Ltd., Jerusalem, Israel). Isolated DNA was used to perform PCR amplification of 16S DNA. 16S DNA was amplified by using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'). PCR reactions were conducted using PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare, Buckinghamshire, UK) where the final mixture contained 1.5 U of Taq DNA polymerase, 10 mM of Tris–HCl pH 9, 50 mM of KCl, 1.5 mM of $MgCl_2$, 200 µM of each dNTP, 5 pmol of each of the two primers, 100 ng of genomic DNA, and water to a final volume of 25 µl. Amplification was done in a TC-412 thermal cycler (Techne, Cambridge Ltd., UK) using the following program: initial denaturation for 5 minutes at 94°C, followed by 30 cycles of 60 seconds at 95°C, 60 seconds at 53°C, 2 minutes at 72°C, and final elongation step of 10 minutes at 72°C. All PCR products were analyzed by electrophoresis in a 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer with GeneRuler™ 100 bp DNA Ladder Plus as a size marker (Fermentas Life Sciences), stained with ethidium bromide, and visualized under UV trans-illumination. After visualizing the bands under UV light, amplified product was cut out of the gel and purified using a DNA extraction kit (Fermentas Life Sciences).

The purified product of 16S DNA was sent for direct sequencing (Eurofins MWG Operon, Ebersberg, Germany). Sequencing was done employing the same primers used for PCR amplification. The nucleotide sequence of 16S rRNA gene obtained from DNA sequencing was compared with other cyanobacterial sequences from the NCBI database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The sequence was deposited in

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the GenBank (Nacional Center for Biotechnology Information, NCBI) under accession number MN061337.

Extraction of DNA, PCR amplification and sequencing of the eukaryotic species (18S DNA)

The dominant eukaryotic species were cultured under aseptic conditions and genomic DNA was isolated. Five milliliters of microalgae culture were harvested by centrifugation at 14 000 RPM on Eppendorf 5424R centrifuge and used for DNA preparation. Genomic DNA from the strain PACC 8946 was isolated using QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer requirements. The DNA concentration and quality was determined spectrophotometrically at a wavelength of 260 nm using Epoch microtiter plate reader and T3 plate protocol. Ribosomal DNA encoding genes were amplified according Yamamoto et al. (2003). Briefly, the reaction was carried out in a 50 µl reaction mixture that contained 1x reaction buffer, 200 µM dNTPs, 0.2 µM 5'-PCR 1-primer (ACCTGGTTGATCCTGCCAGT), 0.2 µM 3'-PCR 3-primer (CCTTCYGCAGGTTCACCTAC), 100 ng microalgal genomic DNA, and 1 unit of Q5 High Fidelity DNA polymerase (New England Biolabs). The samples were placed in a thermocycler and subjected to 30 PCR cycles of denaturation at 95°C for 60 s, annealing at 66°C for 60 s, and extension at 72°C for 60 s. There was a final extension step at 72°C for 10 min. PCR amplicon was separated on a 0.8% agarose gel and purified with the QIAquick Gel Extraction Kit (QIAGEN). The Microsynth Company (Switzerland) performed the sequencing using ABI automated sequencers. Chromatograms were corrected manually with DNA Star software (Lasergene, USA). The resulted nucleotide sequence was deposited in the GenBank under accession number MN088860, subjected to Blastn analysis and compared with other related sequences from the NCBI database. Thirty-three sequences with highest similarity to the query were chosen for further phylogenetic analysis.

Phylogenetic analysis

Multiple sequence alignment of the prokaryotic and eukaryotic species/strains was performed separately by using the ClustalW program built in the phylogenetic software MEGA 7 (Kumar et al., 2016). Phylogenetic trees were computed by MEGA 7 using the maximum-likelihood (ML), and neighbor-joining (NJ) algorithms (Nei and Kumar, 2000). All algorithms were performed with 1000 bootstrap replicates. Nucleotide positions containing gaps and missing data were eliminated from the data set (complete deletion option). *Escherichia coli* str. K-12 (NR102804) was used as an outgroup for the prokaryotic species/strains. The analysis of the eukaryotic species/strains involved 34 nucleotide sequences.

Phytoplankton counting and biovolumes

The taxonomic composition of the nano- and microplankton was determined with a light microscope Amplival (magnification up to 1000 x). Phytoplankton counting was performed on an inverted microscope by the method of Utermöhl (1958). The algal biovolume was determined on the basis of formulas for geometric shapes (Hillebrand et al., 1999). Nano- and microplankton cell size measurements were performed on each species at least on ten individuals. The cell biovolume of picoplankton species were calculated by measuring of 50 cells. The total phytoplankton biovolume was calculated

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as the sum of the biovolumes of all species in each sample. The relative biovolume of the APP community was determined as a percentage of the total biovolume. We accept that the APP community (pro- and eukaryotic members) was dominant when there is a relative biovolume of more than 50% of the total biovolume, according to Somogyi et al. (2014).

Statistical analysis

The methodology of generalized additive models (GAMs) (Hastie and Tibshirani, 1990; Stasinopolus et al., 2016) was used to determine the empirical relationships between abundance of APP, total phytoplankton biovolume and abiotic factors. The required computations were performed with the gam and gamlss R packages developed by these authors (R core team 2019). GAMs allow non-linear relationships between the covariates (predictors) via smoothing spline functions and response as opposed to the traditional generalized linear models in which the predictors are included linearly.

Results

Environmental conditions and trophic status

The lagoon is characterized by high summer temperatures, with average values ranging from 19.2 to 24.2°C (*Table 1*). Lowest temperature (7.8°C) was measured in October 2014, while highest summer temperature (29.8°C) was measured in July 2015. North Saltern waters are alkaline, with pH ranging from 8.6 to 9.1. During the summer season, super oxygen saturation was observed, reaching >200% in June 2016 as a result of the intensive blooming of APP. Nutrients had constant high levels. The ammonium nitrogen ($N\text{-}NH_4$) had highest values in the mid-summer (>1.5 mg L^{-1}). Nitrite nitrogen ($N\text{-}NO_2$) increased from spring to summer, while the nitrate nitrogen ($N\text{-}NO_3$) was usually under the detection limit. The levels of $P\text{-}PO_4$ were high, which indicates that there is no P-limitation of the phytoplankton.

Table 1. *Environmental conditions and chlorophyll-a in 2013-2017*

		Parameter										
Year		**T, ºC**	**pH**	**DO, mg L^{-1}**	**OS, %**	**C, mS cm^{-1}**	**S, ‰**	**$N\text{-}NO_2$, mg L^{-1}**	**$N\text{-}NO_3$, mg L^{-1}**	**$N\text{-}NH_4$, mg L^{-1}**	**$P\text{-}PO_4$, mg L^{-1}**	**Chl-*a*, µg L^{-1}**
2013	**min**	17.2	8.36	7.5	89.6	45.9	30.1	0.11	<0.2	0.13	0.60	8.7
	max	25.1	8.7	15.4	158.9	78.3	54.3	0.21		>1.5	4.5	26.7
	mean	**21.1**	**8.6**	**11.1**	**124.8**	**65.3**	**43.6**	**0.15**		**n.a.**	**1.97**	**17.7**
2014	**min**	7.8	8.4	10.0	116.1	60.9	39.9	0.11	<0.1	0.14	0.74	9.4
	max	29.3	9.4	14.9	147.2	71.7	49.3	0.61	0.78	>1.5	1.8	37.8
	mean	**19.2**	**8.8**	**11.9**	**127.6**	**66.1**	**44.5**	**0.26**	**n.a.**	**n.a.**	**1.3**	**25.2**
2015	**min**	10.0	8.4	8.1	97.3	11.4	6.3	0.08	<0.1	0.05	0.15	1.5
	max	29.8	8.9	14.8	129.0	60.3	40.2	0.17		0.74	0.85	2.1
	mean	**19.6**	**8.6**	**10.5**	**109.8**	**31.6**	**19.8**	**0.12**	**n.a.**	**0.33**	**0.43**	**1.8**
2016	**min**	18.6	8.6	6.4	79.9	41.3	26.1	0.12	<0.2	0.14	0.27	256.3
	max	27.6	9.5	19.7	>200	83.1	59	0.18	19.3	>1.5	0.69	305.6
	mean	**24.1**	**9.1**	**12.1**	**97.9**	**59**	**40**	**0.14**	**n.a.**	**n.a.**	**0.48**	**280.9**
2017	**min**	17.5	8.7	7.9	72.6	62.8	41.8	0.078	<0.2	0.12	0.08	331.7
	max	29.6	9.1	16.6	172.5	128.6	>72	0.153		>1.5	0.21	400.6
	mean	**24.2**	**8.9**	**11.3**	**136.4**	**97.6**	**n.a.**	**0.12**	**n.a.**	**n.a.**	**0.16**	**366.2**

Legend: Temperature (T), Dissolved oxygen (DO), Oxygen saturation (OS), Conductivity (C), Salinity (S), Chlorophyll-a (Chl-a), n.a. - *not applicable*

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Chlorophyll-*a* varied in an extremely wide range from 1.8 to 366.2 μg L^{-1}, but in the prevailing part of the study period indicated hypertrophic conditions according to the fixed boundary system on an OECD report (OECD, 1982). Salinity was controlled by the technological requirements for salt production and naturally increases from spring to autumn. Due to the shallow nature of the lagoon, salinity was strongly influenced by the meteorological conditions, e.g. after a rainy spring in 2015, the salinity reached 40.2‰ only in September. The same year, the mid-seasonal salinity had a minimum value of 19.8‰, which corresponds to the mixosaline zone. With exception of 2015, the average salinity of North Saltern was above 40‰, which is typical for the hypersaline waters. The extreme variations of the salinity in North Saltern over the five-year study period ranged from 6.3 to > 72‰.

Identification of the prokaryotic APP

The 16S DNA sequence analysis of the strain PACC 8945 showed a high percentage of identitties with NCBI 16S rDNA sequences belonging to genus *Synechococcus*. Since the taxonomy of the order Synechococcales and in particular of the genus *Synechococcus* is still problematic, the obtained in this study nucleotide sequence (MN061337) was also used for phylogenetic analyses. For the purpose of the phylogenetic analyses, sequences of representatives of the genera *Synechococcus* (freshwater, marine and species inhabiting waters with very high salinity), *Prochlorococcus*, *Cyanobium* and the newly proposed *Parasynechococcus* were selected.

Determination of the phylogenetic position of different taxa is the first and most important criterion in the classification process. The most commonly used molecular-genetic marker that provides an excellent framework for testing phylogenetic hypotheses is the 16S rRNA gene. The phylogenetic tree obtained from the ML analysis in this study is shown in *Figure 2*. Four distinct clades could be distinguished in the phylogenetic reconstruction, here named Clade 1, Clade 2, Clade 3, and Clade 4 (*Fig. 2*). The first clade (Clade 1) comprises *Synechococcus* marine strains and strains suggested as members of a new genus *Parasynechococcus* (Coutinho et al., 2016). The representatives of genus *Parasynecococcus* are not monophyletic, but rather showed polyphyly, being scattered in the phylogenetic tree. These phylogenetic positions do not confirm the separation of *Parasynecococcus* as a new genus. The topology demonstrates the need of new markers that, in combination with 16S DNA, could contribute to solving such problems.

The second clade (Clade 2) includes the studied PACC 8945 strain, which is positioned close to a strain isolated from a hypersaline water reservoir in Romania and strains isolated from the Red Sea and the Pacific Ocean, each with salinity between 36 and 55 g L^{-1} (Keresztes et al., 2012; Coutinho et al., 2016).

Clade 3 combines representatives of the genus *Prochlorococcus* (*Prochlorococcus marinus*). The phylogenetic tree shows pronounced monophily and potential for distinguishing between low light intensity (LLI) and high light intensity (HLI) strains of genus *Prochlorococcus*. Clade 4 consists of freshwater cyanobacterial strains of the genera *Synechococcus* and *Cyanobium*. The phylogenetic reconstruction based on the neighbor-joining (NJ) algorithm showed a similar topology.

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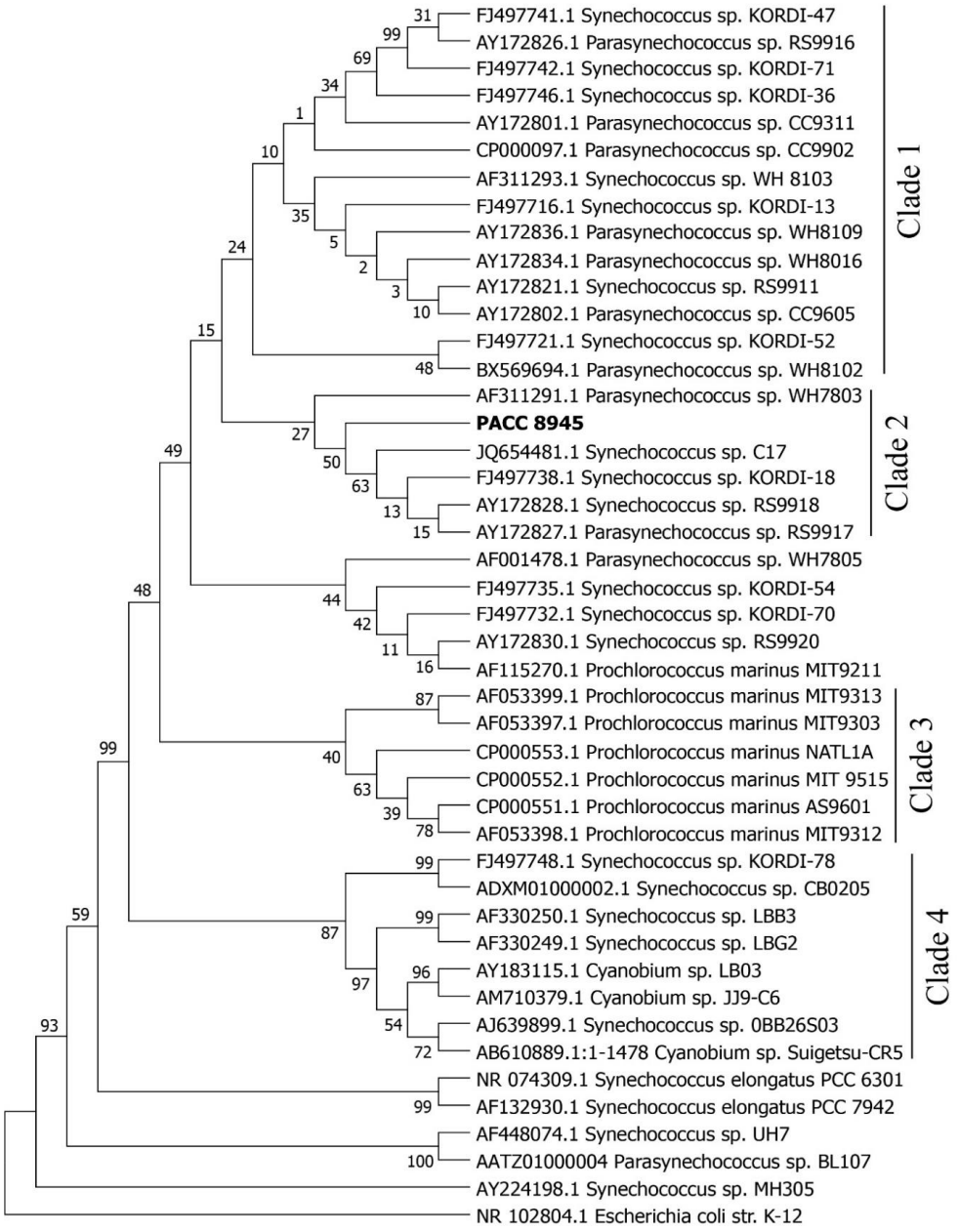

Figure 2. *A Maximum-Likelihood (ML) phylogenetic tree based on 16S DNA sequences. The analysis includes 16S DNA sequences of cyanobacterial strains from the genera Synechococcus, Prochlorococcus, Cyanobium, Parasynechococcus. Numbers show the bootstrap support. The 16S DNA sequence of E coli str. K-12 was used as an outgroup. Access numbers in GenBank are given before the name of the species*

Identification of the eukaryotic APP

For analysis of the phylogenetic relationships and for construction of the phylogenetic tree, 18S DNA sequences from different genera that have been shown homology with the target nucleotide sequence were selected.

The applied selection included the following genera *Picochlorum, Nanochlorum, Nannochloris, Pseudochloris, Marvania, Chlorella, Auxenochlorella* and *Coccomyxa*.

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The phylogenetic tree (*Fig. 3*) consisted of 2 clades: the main clade, including species from genera *Picochlorum, Nanochlorum* and *Nannochloris*, and clade 2, which contains species of the taxonomically distant genera *Pseudochloris, Marvania, Chlorella* and *Auxenochlorella*.

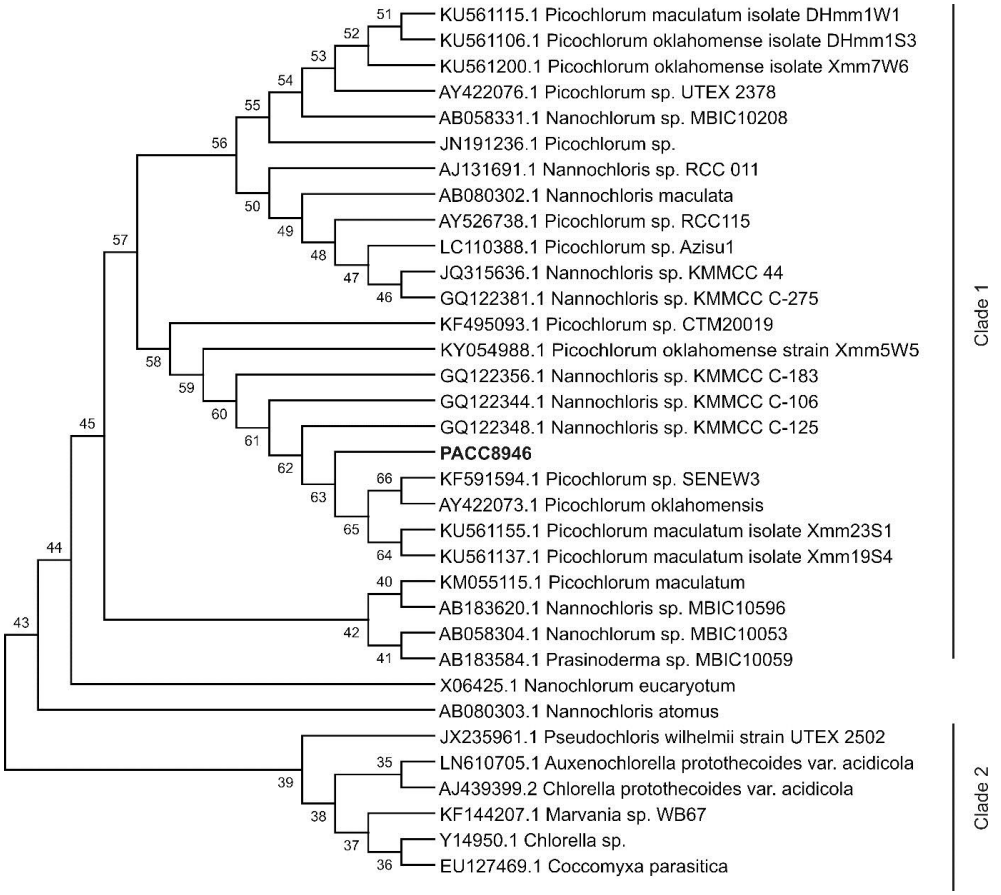

Figure 3. *Molecular phylogenetic analysis based on 18S DNA sequences of species from the genera Picochlorum, Nanochlorum, Nannochloris, Prasinoderma, Pseudochloris, Auxenochlorella, Chlorella, Marvania and Coccomyxa. The tree with the highest log likelihood is shown*

Studied isolate PACC 8946 indicated practical identity of the 18S DNA sequence with the holotype of *Picochlorum oklahomense* (GenBank accession AY422073) and *Picochlorum* sp. SENEW3 (GenBank accession KF591594) from a poikilohaline pond of San Elijo Lagoon, USA (Henley et al., 2004; Wang et al., 2014).

The cytomorphological features of the eukaryotic species (strain PACC 8946) revealed also similarity with *Picochlorum oklahomense.* We observed small rounded cells (d = 2-3 μm) with one laterale chloroplast, without pyrenoide (*Fig. 4A,B*). Reproduction was carried out by autosporulation into two or four daughter cells (*Fig. 4C,D*). The mother cells reached approximately 5 μm in size.

Following the polyphasic approach of the current classification and taking in account the genetic similarity of strain PACC 8946 with *Picochlorum oklahomense*, and similarity in the cytomorphological and ecological markers, we identified the eukaryotic assemblage of APP from the North Saltern as *Picochlorum oklahomense* Hironaka.

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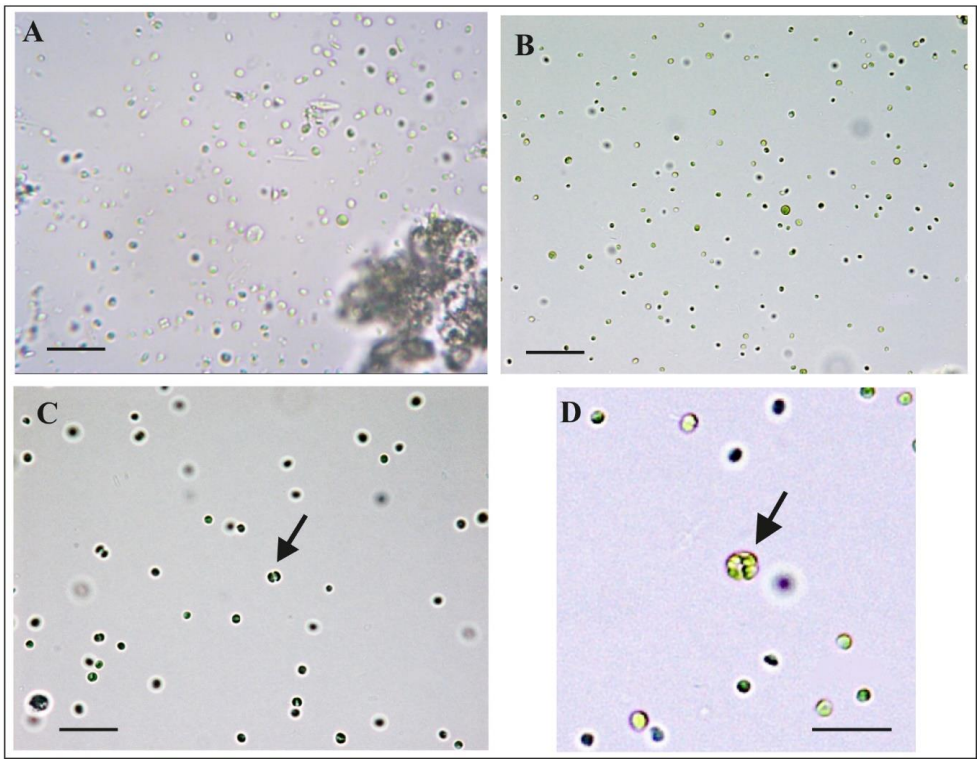

Figure 4. *Photomicrographs of Picochlorum oklahomense.* ***A:*** *Field sample, containing spherical cells of Picochlorum, Synechococcus and pennate diatom;* ***B, C, D:*** *Picochlorum oklahomense, strain PACC 8946, isolated from field sample, collected in Atanasovsko Lake;* ***C:*** *Autosporulation into two daughter cells;* ***D:*** *Autosporulation into four daughter cells. The arrow indicates autosporulation. Scale bars = 10 μm*

Taxonomic composition, abundance and biovolume of the phytoplankton community

During the study period, we found a small number of species (from 10 to 19), which belonged to seven taxonomic groups: Cyanobacteria, Prasinophyceae, Trebouxiophyceae, Chlorophyceae, Bacillariophyceae, Dinophyceae and Cryptophyceae (*Table 2*). Common species that accompanied the APP throughout the study period were diatoms (*Cocconeis placentula*, *Cylindrotheca closterium*, *Triblionella punctata* and *Planothidium delicatulum*), green algae (*Oocystis sp*.) and the dinoflagellate *Katodinium fungiforme*.

The total biovolume of the phytoplankton varied within a very wide range - from 0.6 to 121.7 mm^3 L^{-1} (2015 and 2016, respectively) (*Table 3*). With exception of 2015, APP community was dominant, forming above 50% of the total biovolume. Highest mid-seasonal levels of the total biovolume (65.4 mm^3 L^{-1}) were recorded in 2016, when the APP formed over 93% from the biovolume. The contribution of the APP to the total phytoplankton biovolume reached a maximum value of 99.3% in July 2016 (T=27.6°C; S=59‰). During the same month, the APP was in a state of intensive blooming, with an extremely high abundance of 240.1×10^6 cells mL^{-1}. We found that a key factor for the intensive blooms of APP in 2016 was the salinity range 30-59‰, combined with the higher average annual temperature of 24.1°C (*Table 1*). Conversely, in 2015, the relative biovolume of APP accounted for only 1/3 of the total biovolume and the abundance of APP was significantly lower: mean 0.16×10^6 cells mL^{-1}.

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Table 2. *Main cyanobacterial and algal morphotypes in Atanasovsko Lake during the period 2013-2017*

Year	2013	2014	2015	2016	2017
Cyanobacteria					
Prokaryotic APP	●	●	●	●	●
Komvophoron breve	●	●			●
Merismopedia punctata			●		
Phormidium bulgaricum	●				●
Phormidium chlorinum			●		
Pseudanabaena minima		●	●	●	●
Prasinophyceae					
Polyblepharides amylifera			●		
Pyramimonas micron		●		●	●
Trebouxiophyceae					
Eukaryotic APP	●	●	●	●	●
Closteriopsis acicularis			●		
Oocystis sp.	●	●	●	●	●
Provasoliella ovata					●
Chlorophyceae					
Monoraphidium griffithii			●		
Monoraphidium arcuatum			●		
Bacillariophyceae					
Amphora sp.		●		●	●
Cocconeis placentula		●	●	●	●
Cyclotella caspia		●	●	●	●
Cylindrotheca closterium	●	●	●	●	●
Navicula capitatoradiata		●			●
Nitzschia longissima	●	●	●	●	●
Planothidium delicatulum	●	●	●	●	●
Triblionella punctata	●	●	●	●	●
Dinophyceae					
Katodinium fungiforme	●	●	●	●	●
Cryptophyceae					
Hillea fusiformis		●	●	●	●
Teleaulax acuta				●	●

Table 3. *Parameters of the phytoplankton community in Atanasovsko Lake during the period 2013-2017*

Date	TB, $mm^3\ L^{-1}$	CAPPTB, %	AbAPP, $\times 10^6$ cells mL^{-1}
12 June 2013	8.7	58.0	1.62
29 July 2013	16.8	72.2	4.62
01 Sept.2013	26.7	83.8	5.43
10 June 2014	4.1	75.4	0.74
19 July 2014	15.8	88.0	2.63
13 Sept. 2014	12.3	59.2	2.12
17 June 2015	0.6	36.6	0.13
27 July 2015	0.8	35.1	0.18
09 June 2016	62.2	97.0	32.30
27 July 2016	121.7	99.3	240.11
16 Sept. 2016	12.4	84.1	5.48
10 June 2017	36.1	98.5	7.10
21 July 2017	5.3	13.8	1.15
11 Sept. 2017	55.5	51.1	6.00

Abbreviations: TB (Total biovolume); CAPPTB (Contribution of APP to the total phytoplankton biovolume); AbAPP (Abundance of APP)

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Statistical analysis

Marked correlations in bold (*Table 4*) are significant at p-value < 0.05. There is a strong linear relationship between the response of variables C (Conductivity) and S (Salinity), total phytoplankton biovolume (TB) and abundance of APP (AbAPP). The correlations between the explanatory variables (covariates) T (temperature), C, S, N-NO_2, N-NO_3, N-NH_4, P-PO_4 and the response variables TB, AbAPP, CAPPTB (Contribution of APP to the total phytoplankton biovolume) is not significant. This means that the relationship between the response and covariates is not linear.

Table 4. *Pearson correlation matrix*

	T	**C**	**S**	**N-NO_2**	**N-NO_3**	**N-NH_4**	**P-PO_4**	**AbAPP**	**TB**	**CAPPTB**
T	1.00	0.18	0.13	−0.03	−0.25	0.18	**−0.61**	0.18	0.09	−0.36
C	0.18	1.00	**0.96**	0.12	−0.01	0.48	−0.15	0.13	0.33	−0.04
S	0.13	**0.96**	1.00	0.06	0.02	0.50	−0.13	0.22	0.34	0.03
N-NO_2	−0.03	0.12	0.06	1.00	0.38	0.22	0.13	0.34	0.36	−0.03
N-NO_3	−0.25	−0.01	0.02	0.38	1.00	0.22	−0.07	−0.09	−0.11	−0.08
N-NH_4	0.18	0.48	0.50	0.22	0.22	1.00	0.26	0.17	0.06	−0.13
P-PO_4	**−0.61**	−0.15	−0.13	0.13	−0.07	0.26	1.00	−0.10	−0.11	0.18
AbAPP	0.18	0.13	0.22	0.34	−0.09	0.17	−0.10	1.00	**0.87**	0.40
TB	0.09	0.33	0.34	0.36	−0.11	0.06	−0.11	**0.87**	1.00	**0.54**
CAPPTB	−0.36	−0.04	0.03	0.03	−0.08	−0.13	0.18	0.40	**0.54**	1.00

Abbreviations: T (Temperature); C (Conductivity); S (Salinity), AbAPP (Abundance of APP), TB (Total biovolume); CAPPTB (Contribution of APP to the total phytoplankton biovolume)

The generalized additive models (GAMs) allow non-linear relationships between the responses AbAPP, TB and smoothing spline functions of the covariates T, S, N-NO_2, N-NO_3, N-NH_4 and P-PO_4. Only the cubic spline functions of the covariates T and S are highly significant and the response TB within GAMs model with gamma distribution and log-link function. Deviance explained by this model is 87% whereas the correlation between measured and model predicted values is 0.71. Similarly, the GAMs model with log-link function, response AbAPP and cubic spline functions of the covariates T and S explain 88% of the deviance whereas the correlation between measured and model predicted values is 0.76. The parameter estimates, standard errors, parameter estimates divided by their estimated standard errors, p-value and significant codes are given in *Table 5*. The AbAPP and TB measured and predicted values are presented in *Table 6*.

Table 5. *GAMs model: response variables TB and AbAPP, cubic spline models of the covariates temperature (T) and salinity (S)*

Gamma distribution terms	**Estimate**	**Std. error**	**t value**	**Pr (>|t|)**	**Sc.**	**Estimate**	**Std. error**	**t value**	**Pr(>|t|)**	**Sc.**
	TB					**AbAPP**				
μ link function: log										
Intercept	2.115	0.854	2.476	0.076		2.022	1.245	1.624	0.178	
cs (T)	−0.099	0.032	−3.134	0.042	*	−0.135	0.048	−2.843	0.047	*
cs (S)	0.064	0.008	7.780	0.002	**	0.059	0.012	4.875	0.008	**
Scale link function - log										
Intercept	−0.821	0.183	−4.483	0.014	*	−0.440	0.178	−2.482	0.068	

Significance codes (Sc): '**' 0.01 '*' 0.05

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Table 6. *The measured and GAMs model predicted values with response variables TB and AbAPP, and cubic spline functions of the covariates temperature (T) and salinity (S)*

Case	Observed TB	Predicted TB	Observed AbAPP	Predicted AbAPP
1	8.73	11.69	1.62	1.57
2	16.81	16.97	4.62	4.99
3	26.68	27.99	5.43	5.91
4	4.14	4.213	0.74	0.77
5	15.76	14.89	2.63	4.53
6	12.31	10.93	2.12	1.44
7	0.58	0.73	0.13	0.14
8	0.81	0.94	0.18	0.24
9	62.21	37.71	32.30	23.37
10	121.73	78.50	240.11	107.35
11	12.38	81.18	5.48	102.89
12	36.08	30.09	7.10	6.92
13	5.34	5.496	1.15	0.87
14	55.52	57.47	6.00	14.69

These GAMs results are preliminary of an ongoing and extended study and should be considered with caution because of the small time-series sample size.

Discussion

North Saltern waters are alkaline, rich in ammonium nitrogen (often above the detection range) and phosphate (*Table 1*). Similar high nutrient levels have been reported by Wang et al. (2014) for the San Elijo lagoon, California. The high biogenic load of the lagoons is due to the long water stay and thus the repeated nutrient recycling (Kennish and Paerl, 2010). An additional source of nitrogen and phosphorus loading may be wintering and migrating aquatic birds (Boros et al., 2008), which have high abundance in Atanasovsko Lake as part of the Via Pontica migration route. Salinity of the North Saltern increased naturally from spring to autumn every year, and this cyclic pattern is also characteristic of other lagoons (Wang et al., 2014). Due to the shallow nature of the lake (0.3 m), salinity was strongly influenced by the meteorological conditions (Hubenov et al., 2015). In the last two years, the average seasonal values of chlorophyll-*a* have been in the range of hypereutrophic conditions (*Table 1*). According to Kennish and Paerl (2010) many coastal lagoons are eu- and even hypertrophic.

The APP community in North Saltern was represented by small (2-3 μm) spherical to oval or cylindrical cells (*Fig. 4A*), which cannot be identified by using the classical optical miscroscopy. The reviewed literature showed that pico-sized taxa (< 3 μm) are evaluated via convergent evolution and are characterized by extremely high phylogenetic and physiological diversity (Krienitz et al., 2012; Somogyi et al., 2013). Following the application of molecular genetic methods for Class Trebouxiophyceae, a number of marine or saline picoplankton species belonging to the genera *Picochlorum*, *Chloroparva* and *Pseudochloris* have been identified (Henley et al., 2004; Somogyi et al., 2011, 2013). A large variety of marine coccoid picoplankton lines was also found in Prasinophyceae (Krienitz et al., 2012). *Mychonastes* spesies (Chlorophyceae) were described from brackish and fresh waters.

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The conducted molecular-genetic and phylogenetic analyses proved that the APP in Atanasovsko Lake is an assemblage of two basic genotypes: *Picochlorum oklahomense* Hironaka (Chlorophyta, Trebouxiophyceae) and marine *Synechococcus* sp. (Synechococcales, Cyanobacteria). This finding was confirmed by cytomorphological charateristics of the cultures and ecological data. At present, the species *Picochlorum oklahomense* have been reported from very distinct and isolated habitats. They are parts of limno- and halosphere, such as the saline pool in Salt Plains National Wildlife Refuge of Oklahoma, USA (Henley et al., 2004), inland hypersaline lakes in Romania (Keresztes et al., 2012) and the coastal lagoon in southern California (Wang et al., 2014). These isolated habitats provide specific conditions for development of ensembles dominated by *Picochlorum oklahomense* and marine *Synechococcus* sp. (in Transylvanian lakes, Romania and Atanasovsko Lake, Bulgaria) or *Picocystis salinarum* (San Elijo lagoon, California). Confirmed habitats have the following common characteristics: shallow, with variable salinity, hypertrophic and with high summer temperatures. Three of them (San Elijo lagoon, salt pans of Oklahoma and Atanasovsko lake) maintain high abundance from waterfowl. This confirmed the suggestion of Keresztes et al. (2012) that the distribution of picoplankton species between isolated habitats is via waterfowl transfer.

The low number of associated phytoplankton species included representatives from 7 taxonomic groups (*Table 2*). Most of these species are cosmopolitan in marine or salty waters, euryhaline – e.g. diatoms *Cylindrotheca closterium*, *Triblionella punctata*, prasinophycean *Polyblepharides amylifera*, *Pyramimonas micron* (Ettl, 1983; Popovský and Pfiester, 2008). They were reported from coastal, inland haline lakes or freshwater basins with high mineralization. In 2015, the year with decreased salinity – 19.8‰ (*Table 1*), we identified species that are widespread in freshwater (sometimes also in slight salty) habitats as cyanobacteria *Merismopedia punctata*, *Phormidium chlorinum* and green algae *Monoraphidium griffithii*, *M. arcuatum* and *Closteriopsis acicularis* (Komárek and Fott, 1983; Komárek and Anagnostidis, 1999, 2005). According to Vadrucci et al. (2013), the large gradient of salinity and biogenic load are the most important factors that select taxonomic composition in coastal lagoons. Phytoplankton communities in coastal lagoons exhibit high inter- and intra-ecosystem taxonomic heterogeneity due to their spatial and temporal variability in the hydrogeological and geomorphological characteristics. This makes harder the development of indices for assessment of the ecological status of coastal lagoons.

During the 5-year period, the phytoplankton community in North Saltern was dominated by APP, which often reached extremely high abundance – from 10^6 to 10^8 cells mL^{-1} (*Table 3*), to a state of visible blooming of water (*Fig. 1C*). The high abundance of APP in shallow, turbid lakes is explained by the increased surface-to-volume ratio of the cells, which provides better utilization of light and more efficient absorption of nutrients (Felföldi et al., 2009). Similar abundance of the APP (10^6 to 10^8 cells mL^{-1}) and share in total biomass 90-100% was reported by Felföldi et al. (2009) for eutrophic shallow lakes similar to the Atanasovsko Lake. The results showed that the contribution of APP to the total biovolume is highly variable (from 36 to 99%) - *Table 3*. Concerning the ecological factor abundance of APP a nonparametric regression model is proposed between the response abundance of APP and the salinity and temperature (*Table 5*) as the main abiotic drivers. In a study of picoplankton dynamics in an Australian lagoon, Schapira et al. (2010) also identified salinity as a key factor in the abundance of APP.

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We registered highest APP abundance in 2016 at salinity range 30-59‰ and average summer temperatures above 24°C (*Tables 1 and 3*). These conditions stimulated the blooms of the picoplankton with a contribution of APP to the total phytoplankton biovolume > 93%. As an ecological optimum for APP predominance (over 99% of total biovolume) we defined salinity 59‰ and temperature 27.6°C, at which the abundance of APP reached values similar with the reported maximum (1.08×10^8 cells mL^{-1}) in extremely shallow turbid soda lakes (Somogyi et al., 2009). Temperature values in the lake during 2016 were close to the optimum growth temperature of 30°C in cultures of *Picochlorum* sp., strain SENEW3 (Wang et al., 2014).

Concerning salinity, the wide-ranging halotolerance of *Picochlorum oklahomense* is well known and accepted as an important species-specific ecological characteristic (Henley et al., 2004; Wang et al., 2014). Although in culture experiments Wang et al. (2014) found that the growth rate of *Picochlorum* sp. (SENEW3) decreases at salinity values above 50‰, in our samples from Atanasovsko Lake the optimum salinity appears to be higher (59‰). In natural samples from Cabdic Lake (Romania), the coexistence *Picochlorum oklahomense/Synechococcus* sp. was found at similar to the Atanasovsko Lake physico-chemical conditions: temperature = 28.2°C and salt concentration 55 g L^{-1} (Keresztes et al., 2012).

Conclusions

Due to the extremely small size (2-3 μm) and the lack of sufficient morphological features for taxonomic identification, the APP species in North Saltern of Atanasovsko Lake were identified by molecular and phylogenetic analyzes of 16S/18S DNA. The APP species showed genotype identity with marine *Synechococcus* sp. (Synechococcales, Cyanobacteria) and *Picochlorum oklahomense* (Trebouxiophyceae, Chlorophyta). Our analysis demonstrated the possibility to apply these molecular methods for identification of picoplankton species from natural sample isolates.

By using a polyphasic approach, based on molecular, morphological and ecological characteristics of natural samples and isolates, we collected data for the *Picochlorum oklahomese/Synechococcus* sp. assemblage. This is the first report for *Picochlorum oklahomese* and marine *Synechococcus* sp. as part of APP in a European coastal lagoon. North Saltern is characterized by a continuous salinity gradient in the limits of mixo- to hypersalinity zone (6.3 to 72‰). Waters are alkaline, with high levels of nutrients (ammonia, phosphate) and high summer temperatures.

The main abiotic drivers that control the taxonomic composition and abundance of the APP are salinity and temperature. Salinity between 30 and 59‰ and an average summer temperature above 24°C provide an ecological optimum for growth of APP at a relative biovolume > 93%.

We think that APP, in which *Picochlorum oklahomese* is associated with *Synechococcus* sp. or *Picocystis salinarum* is distributed in more coastal lagoons or inland hypersaline lakes with similar to the Atanasovsko Lake characteristics. We suggest that the distribution of the picoplankton species is related to the waterfowl and these picoplankton species may be found in more habitats with a high abundance of waterfowl or along their migration routes.

Future research on the taxonomic composition of autotrophic picoplankton in coastal lagoons and salt marshes needs to be expanded, since it plays a key role as a primary producer.

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