**Characterization of a high lipid-producing thermotolerant marine photosynthetic pico alga from genus *Picochlorum* (Trebouxiophyceae)**

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**Abstract:**

A new marine strain of picoplanktonic algae, PMPFPPE4, was isolated from a mixed net-phytoplankton sample taken from the upper euphotic layer of the southeastern Adriatic Sea. Evaluation of the new strain included morphological investigation (by light and electron microscopy), phylogenetic analysis (utilizing plastid 16S rRNA and nuclear 18S rRNA genes), and physiological characterization (screening of pigment/lipid composition and capturing photosynthesis measurements). The new strain was proven to belong to the genus Picochlorum and the lipid composition revealed an unexpected accumulation of triacylglycerols, indicating an evolutionary adaptation for growth under unfavourable conditions. In addition, lipid remodelling in the exponential to stationary growth phase was characterized by an increased share of membrane-forming digalactosyldiacylglycerols and phosphatidylcholines. Maximum photosynthetic activity measured was at 30°C, but the most rapid increase of photosynthetic activity was at lower temperatures (15–20°C). Moreover, the thermotolerant strain did not exhibit photoinhibition below 40°C and survived a one-month cultivation period in complete darkness. The strain’s survival in low light and dark conditions suggests a potential shift from autotrophy to mixotrophy under unfavourable growth conditions. Thus, the unique physiological attributes represented by a high growth rate, thermotolerance, phototolerance and high triacylglycerol synthesis may render the strain highly attractive for biofuel production and growth in large outdoor systems.

**Key words:** morphology; *Picochlorum*; photosynthetic picoeukaryotes; phylogeny; physiology.

**Introduction**

Photosynthetic picoeukaryotes (PPEs), with a cell size less than 3 μm, represent a significant portion of picophytoplankton (Dı́ez *et al.*, 2001). Although bacteria, cyanobacteria and archaea living in vast ocean are dominant in abundance, PPEs can greatly contribute to the global carbon cycling due to larger cell volume (Li, 1994; Richardson & Jackson, 2007).

Recently, PPE diversity research has intensified by applying various molecular approaches and metabarcoding of environmental DNA (high-throughput sequencing of DNA markers) mainly with the variable regions of the nuclear 18S rRNA and plastid 16S rRNA gene. This allows for an improved understanding of the diversity and importance of the pico-world (Fuller *et al.* 2006a; Fuller *et al.* 2006b; Decelle *et al.* 2015; de Vargas *et al.*, 2015) and we owe today’s vast knowledge to cross-oceanic expeditions such as Malaspina, *Tara* Oceans, Biosope, and Ocean Sampling Day (OSD) (Grob *et al.*, 2007; Claustre *et al.*, 2008; Bork *et al.*, 2015; Duarte, 2015; de Vargas *et al.*, 2015; Tragin & Vaulot, 2018). However, in addition to environmental metabarcoding, cloning of environmental DNA, fingerprinting and tag-sequencing, and single-cell genomics methodologies are also utilized in approaching protist diversity (Sieracki *et al.* 2019).

According to the results obtained from the above-mentioned cross-oceanic expeditions, the most diverse eukaryotic organisms belong to the piconanoplankton, i.e. ultraplankton (cells in diameter ≤ 5µm). Trebouxiophyceae, a class of green algae containing the highly diversified marine coccoid genus *Picochlorum* W.J. Henley *et al.* within the ˝core˝ chlorophytes, comprise approximately one third of the sequences in oceanic temperate areas and are more abundant along upwellings and nutrient rich coastal zones (Tragin *et al.*, 2016). Recently, genus *Picochlorum* was recognized as the most abundant trebouxiophyte in temperate areas of the US and the European Atlantic coast with the highest number of reads of major operational taxonomic units (OTUs) found within OSD dataset (Tragin & Vaulot, 2018). During the Mediterranean PROSOPE cruise, Dı́ez *et al.* (2001) and Massana *et al.* (2004) both showed a considerable diversity of PPEs, including putative photosynthetic representatives from a wide range of classes (e.g. Chrysophyceae, Cryptophyceae, Prasinophyceae and Prymnesiophyceae; Vaulot *et al.*, 2008). Green algae in the southern Adriatic Sea comprised about 26% of the photosynthetic reads, but genus *Picochlorum* has not been confirmed among 18S rRNA reads yet (Tragin & Vaulot, 2018).

Algae culturing is one of the most important methods in examining not solely for just the diversity of these organisms, but also their role in the ecosystem and for future application. Physicochemical approaches can be useful in applied biology studies, such as biotechnology. At the simplest level, photosynthetic pigments (the key taxonomic diagnostic feature for microalgae) allow for distinguishing green, brown and red algae, but the photosynthetic pigment signature is often indicative of the algal class level (Guillou *et al.*, 1999). Biotechnological approaches for algal studies mainly focus on lipids and fatty acids since the target is often for biofuel production (Hu *et al.*, 2008; Liu *et al.*, 2011) and algae that synthesize and accumulate large quantities of neutral lipids are good candidates for biofuels and biomaterials production (Hu *et al.*, 2008). However, screening of cell lipid or fatty acid components can reveal much about their lifestyle and environment (Galloway & Winder, 2015) and useful information regarding physiology may also emerge from photosynthesis measurements.

The process of photosynthesis depends mainly on abiotic factors such as pH, carbon availability, light intensity and temperature (Kirk, 1994). The widely accepted P-I model describes the relationship between photosynthetic activity and light intensity (Webb *et al.*, 1974; Platt *et al.*, 1980) and provides parameters for biomass specific photosynthetic activity, photoinhibition, photoadaptation, light utilization or respiration. Since growth continues by the consumption of photosynthetic products, the photosynthetic parameters can help us understand the species behaviour in the environment.

Here we describe a newly isolated strain of pico green alga showing high competitiveness and survival in culture conditions that has caught our attention. It was isolated along with pennate diatoms from the oligotrophic southeastern Adriatic Sea. During fieldwork, the photosynthetic signal in the aphotic zone was noticed and later recognized as PPEs maxima in darkness at a depth of 280 m, while viable diatoms were observed up to 500 m (Babić *et al.*, 2017; Bosak *et al.*, 2016). In the cultivation effort of monoclonal PPEs and pennate diatoms, pico green algae (later recognized as different strains of *Picochlorum*, including PMFPPE4) often outgrew other cultures and survived harsh conditions (neglected and/or mixed culturing). We isolated it in a monoclonal culture and characterized by morphological investigation (using light and transmission electron microscopy), phylogenetic analysis (using plastid 16S rRNA and nuclear 18S rRNA genes), pigment and lipid screening, and conducting photosynthesis measurements under various temperature and light regimes.

**Materials and methods**

*Isolation and cultivation*

The water sample taken from the southeastern Adriatic Sea at station M300 (42.49 N; 17.29 E) during the February/March 2015 (28 February - 3 March 2015) BIOTA (Bio-tracing Adriatic Water Masses) cruise utilized a 20 µm-mesh-size phytoplankton net immersed to a depth of 20 m brought to the surface. The environmental conditions and nutrient concentrations of the researched area are given in Babić *et al.* (2017). Upon arrival in the laboratory, the sample was inoculated n Guillard’s f/2 Marine Water Enrichment Solution (Sigma-Aldrich, United Kingdom) and a mixture culture was grown. The culture contained mostly diatoms, dinoflagellates, cryptophytes, haptophytes and heterotrophic nannoflagelates. Unknown PPE cells were filtered through 3.0-µm-pore-size Nucleopore polycarbonate membrane filters into a fresh medium (Whatman, United Kingdom) with a syringe and a filter holder. Following filtration, isolation continued by the dilution method of transferring, in a repeated fashion, a sub-volume of a culture (1/10 of the medium volume) to a fresh medium (9/10 of the medium volume) in order to obtain one cell per tube at the end of the series (Knight-Jones, 1951; Throndsen, 1978). The xenic culture of strain PMFPPE4 was established and subsequently transplanted to fresh medium biweekly in order to maintain the cells in an exponential growth phase. The strain was later cultivated in ASN III medium (Stanier *et al.*, 1979), a synthetic medium utilized due to the significant need in photosynthetic experiments. The PMFPPE4 strain is deposited in Roscoff Culture Collection under number RCCXXXX.

*Morphology*

The fresh culture samples were investigated under an Olympus BX51TF (Olympus corporation, Japan) inverted microscope (Artray Co. Ltd, Japan) and a Zeiss Axioimager A2 light microscope (Carl Zeiss, Oberkochen, Germany) equipped with DIC, phase contrast, and Axiocam 305 camera with which the images were obtained. Before examination, PMFPPE4 cells were settled on glass slides for 20 min and then examined using a 1000× magnification.

For transmission electron microscopy (TEM), cultured cells were fixed in 1% (w/v) glutaraldehyde in a 50 mM cacodylate buffer (pH 7.2) for 30 min at 5°C and pelleted by centrifugation at 500 × g for 5 min. Cells were re-suspended with an ice-cold 50 mM cacodylate buffer (pH 7.2) and embedded in 2% agarose. The cell embedded agarose was cut into small pieces and twice washed with an ice-cold 50 mM cacodylate buffer (pH 7.2) and then post-fixed with 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4°C, followed by a 10 min wash in ice-cold water. After dehydration in a graded series of ethanol (25%; 50%; 75%; 80%; 90%; 100%), the material was placed in absolute ethanol overnight. On the next day, the material was initially placed in a 1:1 mixture of absolute ethanol and 100% acetone for 30 min proceeded by an additional 30 min in 100% acetone. Thereafter, the material was placed in a 1:1 mixture of Spurr's medium and acetone of ⅓ Spurr's and ⅔ acetone for 30 min, ½ Spurr's and ½ acetone for 30 min, and ⅔ Spurr's and ⅓ acetone for 30 min, respectively, and subsequently in Spurr's medium for 2 hours at 45°C. The material was then set into a plastic mold and polymerized in Spurr's medium at 65°C for a 48 hour period. After that, ultrathin sections were made with an ultra-microtome Leica Ultracut R initially stained with 4% aqueous uranyl acetate for 10 min, then with lead citrate, pH 12.0, for 10 min (Reynolds, 1963). and examined under a a FEI Morgagni 268D transmission electron microscope (Eindhoven, The Netherlands) at 70 kV.

*DNA extraction and PCR amplification*

DNA extraction was performed with a DNeasy Plant Mini Kit (Qiagen), in accordance with manufacturer instructions, using 50 mL of PMFPPE4 culture in the exponential growth phase. Purity of the DNA extraction was assessed with the NanoDrop spectrophotometer (BioSpec-nano; Shimadzu, Tokyo, Japan) and the plastid 16S rRNA and nuclear 18S rRNA genes were amplified by PCRs. For the 16S rRNA gene, algal plastid biased primers utilized PLA491F (5’-GAGGAATAAGCATCGGCTAA-3’) (Fuller *et al.*, 2006a) as the forward primer and OXY1313R (5’-CTTCAYGYAGGCGAGTTGCAGC-3’) (West *et al.*, 2001) as the reverse. In regards to the 18S rRNA gene, Euk63F (5’-CGCTTGTCTCAAAGATTA-3’) and Euk1818R (5’-ACGGAAACCTTGTTACGA-3’) were utilized as the forward and reverse primers, respectively (Lepère *et al.*, 2011).

The PCR mixture for 16S rRNA gene amplification (50 µL) contained 10 µL 1 × GoTag® Flexi green Buffer (Promega), 2.5 µL magnesium chloride (1.25 mM MgCl2, Promega), 1 µL dNTP mix (1.25 mM, Promega), 2.5 µL of each primer (10 µM), 0.25 µL GoTaq® DNA polymerase (100 U, Promega), 3 µL of template DNA and 28.25 µL of miliQ H20. The PCR was performed under the following conditions: initial denaturation at 95°C for 5 min; followed by 40 cycles at 95°C for 45 s, 60°C for 45 s, 72°C for 1.15 min; and final extension at 72°C for 7 min.

For the 18S rRNA gene amplification, the PCR mixture (50 µL) contained EmeraldAmpMax PCR Master Mix© (Takara Bio, USA) in volume of 25 µL, miliQ H20 (17 µL), 2 µL of each of the primers (10 µM), and 4 µL of template DNA. The PCR reaction was conducted under the following conditions: initial denaturation at 98°C for 30 s; followed by 35 cycles at 98°C for 10 s, 55°C for 30 s, 72°C for 1.00 min, and 72°C for 10 min. The PCR products were quality-assessed on agarose gels prior to purification with the StartaPrep PCR Purification Kit (Agilent Technologies, Inc.) The purified products were Sanger-sequenced by a commercial provider (Macrogen, The Netherlands).

*Sequence processing, multiple sequence alignments and phylogeny*

Partial sequences of both 16S rRNA and 18S rRNA genes were checked, edited, and paired (5’–3’ and 3’–5’ ends) using Sequencher 4.1.4 (Gene Code Corporation, USA). BLAST analysis was completed for all sequences using the blastn tool available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Sequences of strain PMFPPE4 are deposited in GenBank under accession numbers KU843868.1 for 16S rRNA gene and MH010869.1 for 18S rRNA gene.

In addition to BLAST identification of strain PMFPPE4, small phylogenetic analyses were performed to identify PMFPPE4 placement and grouping among other *Picochlorum* strains. A total of 20 sequences for each gene were included (Supplementary information, Table S1) with the exception of the 18S rRNA gene Bayesian inference phylogeny which utilized 16 unique sequences. Four sequences were proven genetically identical (accession numbers: KT860852, KT860853, KT860854 and JQ315636) and therefore omitted from the alignment prior to analysis. The outgroup in the 16S rRNA gene dataset contained four sequences belonging to the marine coccoid prasinophytes *Pycnococcus provasolii* R.R.L. Guillard and *Pycnococcus* sp. while the outgroup in the 18S rRNA gene dataset included the freshwater auto-sporulating species *Marvania coccoides* and *Marvania geminata*. Multiple sequence alignments were performed in AliView ver. 1.18 using the Muscle algorithm under default parameters (Larsson, 2014). The 16S rRNA gene alignment had a length of 1322 nucleotides, of which 229 were marked as variable, 182 as parsimony-informative sites, and 47 as singleton sites. The 18S rRNA gene alignment had a length of 1816 nucleotides, of which 159 were marked as variable, 89 as parsimony-informative sites, and 68 as singleton sites. Alignments were visually inspected, and no sites were manually excluded. Alignments are available at zenodo link: <https://zenodo.org/deposit/1186231>.

A separate phylogenetic analysis was performed on each gene dataset and began by first identifying the best model of nucleotide substitution and rate variation across sites using a model-selection routine available in the program IQtree v. 1.5.5, with the specified command -TESTNEWONLY (Nguyen *et al.*, 2014). Model selection was completed using the Bayesian information criterion (BIC) penalizing for the number of model parameters helping to avoid overfitting. Phylogenies were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) in IQtree v. 1.5.5 (Nguyen *et al.*, 2014) and MrBayes v. 3.2.6. (Ronquist *et al.*, 2012), respectively. Clade support was assessed using IQtree’s UltraFast bootstrap routine (Minh *et al.*, 2013) with 1000 pseudoreplicates specified by the use of the -bb 1000 command. Bayesian analyses were carried out with a mixed-model strategy in which various variants of the Generalized time-reversible model (GTR) were sampled in proportion to their posterior probability (MrBayes option ‘nst=mix’). The among-site rate variation in MrBayes was accommodated via a Γ distribution with four rate categories (Γ4) and estimation of the proportion of invariant sites (I). Four simultaneous Markov chain Monte Carlo (MCMC) simulations were completed wherein each was composed of one cold and three heated chains for a total of 10 million generations with a sampling frequency of one thousand generations. Among the MCMC runs, stationarity and convergence were assessed from the MrBayes output (standard deviation of split frequencies and potential scale reduction factor) and by inspection of the posterior distributions in the program Tracer v. 1.6 (Rambaut *et al.* 2007). The first 25% of the sampled posterior distributions were discarded as burn-in. Unique sequences omitted from 18S rRNA gene Bayesian phylogeny were reintroduced into the Newick tree by manually modifying its Newick file.

*Pigment analysis*

In addition to the cell counts, pigment analysis was performed daily with high-performance liquid chromatography (HPLC) using a 1 mL of exponentially growing PMFPPE4 culture filtered through 0.7-μm-pore-size GF/F filters (Whatman, United Kingdom) then freshly frozen in liquid nitrogen. Extraction into 4 mL of cold 90% acetone was conducted by sonication, and the extract was cleaned by centrifugation. Pigment separation by a reversed phase HPLC followed the protocols of Barlow *et al.* (1997) and Šilović *et al.* (2011).

*Lipid analysis*

For lipid class determination, 50 mL of the PMFPPE4 culture, in the mid-exponential and stationary growth phase, was filtered through pre-combusted GF/F filters. Filters were stored for three days prior to the extraction of particulate lipids using a modified one-phase solvent mixture of dichloromethane-methanol-water (Bligh & Dyer, 1959). To each sample, N-nonadecanone (KET) was added as an internal standard in order to estimate the recoveries in subsequent steps of the sample analysis. Extracts evaporated to dryness under a nitrogen atmosphere were re-dissolved in 24 µL dichloromethane and lipid classes were determined by thin-layer chromatography-flame ionization detection (TLC-FID; Iatroscan MK-VI, Iatron, Japan). Representing total lipids, eighteen lipid classes were separated on Chroma rods SIII and quantified by an external calibration using a standard lipid mixture with a hydrogen flow of 160 mL min-1 and air flow of 2000 mL min-1. The quantified lipid classes included: hydrocarbons (HC); lipid degradation indices (fatty acid methyl esters (ME); free fatty acids (FFA); alcohols (ALC); 1,3-diacylglycerols (1,3DG); 1,2-diacylglycerols (1,2DG) and monoacylglycerols (MG)); wax and steryl esters (WE/SE, later discussed as SE since WE presumed absent in phytoplankton monocultures as they represent zooplankton storage lipids; Kattner, 1989), phytoplankton energy reserves (triacylglycerols (TAG)); membrane lipids including three phospholipids (phosphatidylglycerols (PG), phosphatidylethanolamines (PE) and phosphatidylcholines (PC)); three glycolipids (sulfoquinovosyldiacylglycerols (SQDG) monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG)); sterols (ST); and pigments (PIG). For this study, we did not take into discussion lipid degradation indices or hydrocarbons and remaining lipid classes represent cell lipids. The standard deviation determined from duplicate runs accounted for 1-8% of the relative lipid class abundance with total lipid concentrations obtained by summing cell lipid classes. A detailed description of the procedure is published in Gašparović *et al.* (2015; 2017). Total percentages of determined lipid classes within cells were displayed as a pie-chart plotted using the R software (version 3.4.3) (R Development Core Team, 2008) ˝ggplot2˝ package (Wickham, 2011).

*Photosynthesis measurement*

Photosynthesis measurements carried out in a laboratory photosynthetron developed by Üveges *et al.* (2011) examined the photosynthetic characteristics of PMFPPE4 under 72 combinations of temperature and light intensities. Temperature varied in 5°C increments from 5 to 40°C in conjunction with adjustments for light intensity (0; 15; 40; 125; 230; 250; 500; 800 and 1300 µmol m-2 s-1) For the cultivation and photosynthesis measurements, PMFPPE4 cells were maintained in ASN-III medium (Stanier *et al.*, 1979). Photosynthesis measurements and carbon uptake calculations were completed as described by Lengyel *et al.* (2015) and photosynthetic parameters for the absence of photoinhibition were completed according to Webb *et al.* (1974). In the instance that photoinhibition was in fact observed, β (photoinhibition parameter) and other parameters were calculated according to Platt *et al.* (1980). Both the photosynthesis-light intensity (P-I) and the photosynthetesis-temperature (P-T) curves as well as the Gaussian curves were scrutinized to find the temperature optima of the photosynthetic parameters. with the calculation of the photosynthetic variables carried out in GraFit 7.0 software (Leatherbarrow, 2009).

*Growth experiments*

The growth rate experiment lasted 20 days with PMFPPE4 cultivated under the following conditions: temperature 22±0.5°C; light 30 μmol photons m-2 s-1, in a photoperiod of 16 h of light: 8 h of dark with continuous shaking on an Orbital Shaker OR100 (Cole Parmer, UK) for 12 h during the light period. The starting culture consisted of ca. 106 cells mL-1 established in 200 mL of f/2 medium in Erlenmeyer flasks in triplicates. Cells were counted daily in triplicates using a Birken-Türk haemocytometer. The number of cells mL-1 was calculated and the standard deviation (SD) was included in generating the growth rate graph plotted using the R software (version 3.4.3) (R Development Core Team 2008) ˝ggplot2˝ package (Wickham, 2011). The PMFPPE4 net growth rate (k) was calculated by the equation: k = ln(X1-X0)/(T1-T0); for which X1 = number of cells at the end of exponential phase; X0 = number of cells at the beginning of exponential phase; T1 = number of days at the end of exponential phase; and T0 = number of days at the beginning of exponential phase.

A dark cultivation experiment was also performed with the strain kept at a stable temperature of 22±0.5 in complete darkness for one month in 4 replicates. No shaking nutrient or oxygen intake intervention was provided and each week, one replicate was examined under a light microscope.

**Results**

The cells of the isolated algal strain PMFPPE4 are green, spherical to oval in shape,1.5 - 3.0 μm (n=50) in diameter and include a lateral U-shaped chloroplast occupying approximately two thirds of the cell (Fig. 1). Cells have a smooth surface (Figs. 1 and 2) with no observation of flagella or any other type of cell appendices. Cells divide by autosporulation into two daughter cells. Neither zoospore formation nor sexual reproduction was observed. TEM revealed that cells contain one nucleus, one mitochondrium, and a single lateral U-shaped chloroplast lacking a pyrenoid, and starch grains present primarily within the chloroplast (Fig. 2).

The phylogeny of the 16S rRNA gene confirmed PMFPPE4 placement in the paraphyletic genus *Picochlorum* [Bayesian posterior probability (BPP)/Bootstrap proportion (BS), BPP/BS=1/92 (Figure 3a)] along with other cultured *Picochlorum* strains without species affiliation, BPP/BS=0.59/80 (Figure 3a). All strains most similar to PMFPPE4 were from the Roscoff Culture Collection (RCC), isolated from the Pacific Ocean (RCC1034, RCC289 and RCC13) and the Mediterranean Sea (RCC9) thus indicating the cosmopolitan presence of this coccoid pico green alga (Figure 3a). The sister clade containing two additional unknown RCC strains of *Picochlorum* sp. (RCC846 and RCC945) diverged from the PMFPPE4 clade with great support BPP/BS=1/99 (Figure 3a) and the outgroup represented *Pycnococcus provasolii* and three unidentified *Pycnococcus* species as root to the tree (BPP/BS=1/100; Figure 3a).

The phylogeny of the 18S rRNA gene also confirmed the PMFPPE4 placement to the monophyletic genus *Picochlorum* (BPP/BS=0.99/99, Figure 3b) by grouping it with five other unknown cultured *Picochlorum* strains (BPP/BS=0.73/52; Figure 3b). Besides the RCC strains (RCC9, 13 and 14), the included strains of KMMCC C-275 and KMMCC 44 were isolated from the Yellow Sea (Figure 3b). Here, RCC289, *Picochlorum oklahomense* and *Picochlorum oklahomense* strain Xmm7W6 branch off the PMFPPE4 clade, albeit with high support (BPP/BS=1/98, Figure 3b). Furthermore, the genus consists of a smaller clade presented with *P. oklahomense* strain DHmm4W1 and *P. maculatum* DHmm1W1 (BPP/BS=0.87/74; Figure 3b), *Picochlorum* sp. strain KMMCC C-93 branches off with BPP/BS = 0.99/55 and a clade consisting of *Picochlorum* sp. RCC475 and *P. eukaryotum* (PP/BS=0.99/99; Figure 3b). The tree root is comprised of the *Micractinium pusillum* strain SAG 13.81 and the *Chlorella vulgaris* strain SAG 211.11b (Figure 3b).

HPLC analyses of pigment content revealed chlorophylls *a* and *b*, lutein, β-carotene, violaxanthin and neoxanthin.

Considerable amounts of particulate lipids were detected in the exponential growth phase (day 12) and stationary growth phase (day 20) with 753 and 2529 µg L-1 on average, respectively (Fig. 4, values in rectangles). The most abundant class were TAGs (27.2% in the exponential and 17.3% in the stationary) (Fig. 4) and among membrane forming lipids during the exponential phase, the most abundant were phospholipids PG (21.1%) and glycolipids SQDG (12.2%). The stationary phase was characterized by the increased share of glycolipids DGDG (15.8%) and phospholipids PC (12.8%) (Fig. 4).

The photosynthesis-light intensity (P-I) curves of PMFPPE4 (Supplementary information, Figure S1) showed photosynthetic activity increase with temperature. The highest value was measured at 30°C and the calculated photosynthetic variables (Table 1) demonstrated temperature dependence. The biomass specific maximal photosynthetic activity (PBmax) increased with temperature (Fig. 5). The highest value recorded at 30°C although the optimal temperature was 27.7°C (Table 1). PBmax increased in an order of magnitude along the temperature gradient. At 5°C and 30°C, PBmax was recorded at 0.304 and 3.222 µg C µg-1 Chl *a* h-1, respectively. Because photoinhibition was only detected at 40 °C, maximum production obtained in the absence of photoinhibition (Ps) differs from PBmax only at the highest temperature. The photoadaptation parameter (Ik) of the species also increased with temperature, and the highest value was calculated at 30°C (114.3 µmol m-2 s-1). Good light utilization (α) of the strain was found along the temperature gradient, and highest values were observed between 20-35°C. Biomass specific respiration of the species increased with increasing temperature and reached a maximum at ~32°C, then slightly declined.

The P-T curves show a similar level of photosynthetic activity within the light intensity range of 230-1300 µmol m-2 s-1. The maximum biomass specific photosynthetic activity varied between 2.89 and 3.10 µg C µg-1 Chl a g Chl *a* h-1 (Fig. 6, left panel) and the photosynthesis optimal temperature in this light intensity range varied between 27.3 and 27.8°C. At lower light intensities, there was a remarkable decrease observed in photosynthetic activity and in the optimal photosynthesis temperature. At 125 µmol m-2 s-1, the maximum level of photosynthetic activity dropped to 2.1 µg C µg-1 Chl a g Chl *a* h-1 and the optimal temperature declined to just below 27°C. From the measurements of the two lowest light intensities, the decrease was even more pronounced. At 40 and 15 µmol m-2 s-1, the highest photosynthetic activity recorded was at 25.4 and 24.9°C, respectively, and 0.92 and 0.28 C µg-1 Chl a g Chl *a* h-1, respectively.

PMFPPE4 cells demonstrated acclimatization and steady growth during the first 7 days of the growth experiment. The average abundance was 9 × 106 cells mL-1 per day and the net growth rate (k) of was very high: 2.90 d-1. Two weeks after inoculation, the culture moved into a stationary phase, and cells began to aggregate at the base of the Erlenmeyer flasks. During the last five days of the experiment, the cell count stabilized to average values of 4 × 107 ± 2 × 106 cells mL-1 (Fig. 7).

Results from the dark cultivation experiment revealed survival of the PMFPPE4 strain is possible even without performing photosynthesis. After a one-month growth period in darkness, the strain survived, though cell size was generally smaller (minimum cell diameter was 1.5 µm) in comparison to those grown in 16:8 h light: dark periods.

**Discussion**

***Morphological, ecological and genetical comparisons to similar taxa***

Among the known algal genera, *Picochlorum* sp. PMFPPE4 is morphologically similar to genus *Neocystis* Hindák in cell shape and parietal chloroplast, but differs in the number of autospores and the lack of a mother cell wall around vegetative cells (Ettl & Gartner, 2014). Similarities with genus *Chlorella* and ˝*Chlorella*-like˝ organisms were high, however the species belonging to *Chlorella* possess a pyrenoid and is absent in genus *Picochlorum* (Bock *et al.*, 2011) Moreover, even higher similarities were noted with genus *Nannochloris* Naumann though without a designated type species, the validity of this genus is questionable (Guiry & Giury, 2019).

Recently, several species from the genus *Nannochloris* were transferred to *Picochlorum*, indicating there is an insufficient number of distinguishable characteristics to separate the two genera, thus leading taxonomists to recognize just the genus *Picochlorum* (Henley *et al.*, 2004; D. Vaulot, personal communication). So, with this information in conjunction with the discussion of Hepperle & Krienitz (2001) regarding identification difficulties of the so-called ˝*Chlorella*-˝ and ˝*Nannochloris*-like˝ algae, Henley *et al.* (2004) used molecular support (18S rDNA phylogeny) and managed to move 13 marine/saline isolates from ˝*Nannochloris*-like˝ algae into *Picochlorum* gen. nov. W.J. Henley, J.L. Hironaka, L. Guillou, M.A. Buchheim, J.A. Buchheim, M.W. Fawley & K.P. Fawley.

Genus *Picochlorum* is comprised of five species, three of which are taxonomically accepted: *P. oklahomense* Hironaka (as a type species), *P. maculatum* (Butcher) Henley *et al.* and *P. atomus* (Butcher) Henley *et al*. (Guiry & Guiry, 2019). There are few marine Trebouxiophyceae genera besides the mostly freshwater, *Picochlorum*: *Chlorella.* However, there are a few known marine species (1.5 to 10 µm in diameter), *Elliptochloris* Tschermak-Woess (5 to 10 µm in diameter), *Carolibrandtia* R.Hoshina & T.Nakada (symbiotic green coccoid in ciliates) and *Phyllosiphon* J.G.Kühn (biofilm-associated siphonus parasitic green algae (Motti *et al.*, 2005; Procházková *et al.*, 2015; Tragin *et al.*, 2016; Hoshina *et al.*, 2018)) for which genus *Picochlorum* differs significantly in cell size, ecology, and the absence of a pyrenoid. Genus *Pseudochloris* B.Somogyi, T.Felföldi & L.Vörös is an additional trebouxiophyte genus described by Somogyi *et al.* (2013), comprised of a single species, *P. wilhelmii*, isolated from aquarium seawater. Morphology of this genus resembles *Piccohlorum* sp. PMFPPE4 in the spherical to oval cell shape, but cells seem larger (1.6-3.9 µm wide and 1.8-4.0 µm long). The cell ultrastructure of *Pseudochloris* contains peroxisomes and numerous plastoglobuli, not observed in *Picochlorum* sp. PMFPPE4 (Somogyi *et al.* 2013). Both genera divide by autosporulation (Somogyi *et al.* 2013) and pigment composition is similar and contain chl *a* and *b*, lutein, violaxanthin and neoxanthin with the add-on of antheraxanthin, zeaxanthin and α-carotene in *Pseudochloris.* Phylogeny based on 18S rRNA gene defines *P. wilhelmii* as sequences belonging to the previously misidentified *Nannochloris eucaryotum* strain UTEX 2505 (JX235961), *N. eucaryotum* strain SAG 66.87 (JX235962), *Chlorella minutissima* strain -1 1.9 (X56102) and *C. minutissima* SAG 1.80 (AB006046) which directly describes misidentification problems with *Clorella*- or *Nannochloris*-like organisms (Somogyi *et al.* 2013).

In the paper, Somogyi *et al.* 2013 represent genus *Picochlorum* in their phylogeny as monophyletic, branched off the newly described genus *Pseudochloris*, therefore acknowledging their genetic differences. Other classes of algae also have pico-sized non-motile coccoid representatives in marine environments, additionally complicating identification of *Picochlorum*. First example within class Mamiellophyceae are the cosmopolitan and numerically important genera in coastal sea areas: *Bathycoccus* W.Eikrem & J.Throndsen and *Ostreococcus* C.Courties & M.-J.Chrétiennot-Dinet (Moreau *et al.* 2012). Morphological differences with genus *Picochlorum* are significant since *Bathycoccus* contains specific spider-web-like scales on the cell surface produced by Golgi apparatus (Moreau *et al.* 2012). On the other hand, the smallest known single-celled eukaryotic genus *Ostreococcus*, species *O. tauri* has greater morphological similarities with *Picochlorum* sp. PMFPPE4 in terms of a simple cell ultrastructure containing a single chloroplast with one starch granule without a pyrenoid, and a mitochondrion located between nucleus and chloroplast (Chrétiennot-Dinet *et al.* 1995). The pigment composition of *O. tauri* cells is quite different from *Picochlorum* sp. PMFPPE4 cells. *O. tauri* cells, contain chl *c*-like, siphonaxanthin-like 1 and siphonaxanthin-like 2, antheracanthin, zeaxanthin, and α-carotene which are all absent in *Picochlorum* sp. PMFPPE4 (Chrétiennot-Dinet *et al.* 1995). Class Pinguiophyceae with marine coccoid genus *Pinguiococcus* R.A.Andersen, D.Potter, D.& J.C.Bailey is comprised of one species *P. pyrenoidosus* collected and described from an aquaculture tank and also morphologically resembles *Picochlorum* sp. PMFPPE4 cells with spherical to irregular cell shapes containing one parietal chloroplast seen in light microscopy (Andersen *et al.* 2002). The cell ultrastructure, though, when examined by TEM shows that *P. pyrenoidosus* cells include one stalked pyrenoid in the chloroplast and numerous vacuoles (Andersen *et al.* 2002). Cells protrude the surface as a result of growing vacuoles, and in older cultures, cells have fine extensions of the protoplasm and large storage vacuoles (Andersen *et al.* 2002), as observed in a growing culture. Furthermore, the genera *Pycnococcus* (Pyramimonadophyceae), *Prasinoderma* (Palmophyllophyceae) and *Nannochloropsis* (Eustigmatophyceae) all resemble *Picochlorum* in LM. *Pycnococcus provasolii* resembles *Picochlorum* sp. PMFPPE4 with spherical, subspherical or ovoid cells ranging from 1.5-4.0 µm in diameter containing one cup-shaped chloroplast (Guillard *et al.* 1991) but differs in the cell ultrastructure observed in TEM. One to two pyrenoids are present and the outer mitochondrion membrane protrudes into chloroplast region of the pyrenoid (Guillard *et al.* 1991). *Prasinoderma singularis* Jouenne, a solitary coccoid prasinophyte isolated from south-east Pacific Ocean, can be misidentified in LM to *Picochlorum* sp. PMFPPE4 due to cell size (2.2-5.5 µm in diameter) and a single bilobed cupuliform chloroplast, but the starch sheath covered pyrenoid is usually well visible in this species, which is a delimitating factor concerning identification of cells belonging to *Picochlorum* (Jouenne *et al.* 2011). The pigment of this species varies from *Picochlorum* sp. PMFPPE4 by containing MG 3,8-divinylphaeoporphyrine a5 monomethyl ester, prasinoxanthin, uriolide, micromonol, zeaxanthin and antheraxanthin (Jouenne *et al.* 2011). Finally, cells belonging to *Nannochloropsis granulata* B.Karlson & D.Potter,isolated and described from northeast Atlantic Ocean, are commonly mistaken with *Picochlorum* cells. In LM, the two are similar in both cell size (2-4 µm in diameter) and a chloroplast absent of a pyrenoid (Karlson *et al.* 1996). However, sometimes cells belonging to *N. granulata* can have two chloroplasts and always possess up to 5 refractile granules in cytoplasm (Karlson *et al.* 1996). *N. granulata* also has different pigments than *Picochlorum* sp. PMFPPE4 which include vaucheriaxanthin like pigment, zeaxanthin and canthaxanthin (Karlson *et al.* 1996). Examples of genus defining within chlorophytes based on multilayer approaches are common (Chrétiennot-Dinet *et al.* 1995; Bock *et al.* 2011 and references therein; de la Vega *et al.* 2011; Somogyi *et al.* 2013; Gonzalez-Esquer *et al.* 2018). Physicochemical characteristics of cultured PPE representatives can help the taxonomic positioning of certain strains, as Dahmen *et al.* (2014) showed with the identification of *Picochlorum* sp. strain CTM 20019, or in examples of genera *Ostreococcus*, *Pycnococcus*, *Prasinoderma*, *Nannochloropsis*, etc. discussed above. Molecular data is mostly used tool in defining unknown picoalgal strain as *Picochlorum* (de la Vega *et al.*, 2011; Gonzalez-Esquer *et al.* 2018; Watanabe & Fujii, 2016) or other genera, such as *Pseudochloris* (Somogyi *et al.* 2013).

 In both phylogenies, PMFPPE4 was most similar to other *Picochlorum* strains from the RCC, but none have yet been investigated in depth. In 18S rRNA phylogeny, *Picochlorum* strains RCC13 (Pacific Ocean), RCC14 (Atlantic Ocean), RCC9 (Mediterranean Sea), and KMMCC C-275 and KMMCC 44 (Yellow Sea) are genetically identical and likely belonging to one species. PMFPPE4 is grouped with this genetically identical clade and thus suggests possible species conspecifity. Further investigation would be needed to provide such a conclusion since species conspecifity within genus *Picochlorum* has yet to be thoroughly investigated as with some other coccoid genera (*Chlorella*, *Choricystis*, *Marvania* or *Mychonastes*), though it is possible (Krienitz *et al.* 1996; Henley *et al.* 2004; Krienitz *et al.* 2019). The lack of taxonomical identification within the genus suggests the existence of a new and undescribed species with the subsequent need for future description. A more extensive morphological effort (thorough TEM examination of all genetically similar strains in vegetative cells and cell divisions) would be required to correctly link those genetically very similar strains. In the 16S rRNA gene phylogeny, not one *Picochlorum* sequence is identified to the species level and in the 18S rRNA gene phylogeny, only three sequences of the taxonomically accepted *Picochlorum* species are available. The remaining are unknown strains indicating poor sequence coverage, lack of taxonomical studies within the genus, and the usage of gene markers not providing sufficient resolution for species delimitation. A possible upgrade in the genetic identifications of *Picochlorum* species would be the usage of another gene marker, such as ITS. ITS has already been proven excellent in delimitating genetically highly similar organisms due to secondary structure comparisons (Škaloud *et al.* 2016; Garcia da Silva *et al.* 2017; Temraleeva & Moslalenko 2019). Underappreciation of this genus up until now is most certainly due to its minute size, the impossibility in identifying cells in environmental samples by their autofluorescence (i.e. flow cytometry), difficult cultivation, and the fact that molecular genetic research on minute coccoid algae is scarce (Barcytė *et al.*, 2017). Though, potentially undescribed microorganisms can be obtained from current field samples, re-examination of established cultures from public collections such as the Roscoff Culture Collection (RCC), the National Center for Marine Algae and Microbiota (NCMA) or the Culture Collection of Algae at the University of Texas Austin (UTEX) is of extreme importance and would be the next step following our study.

***Physiology and biotechnological potential of Picochlorum sp. PMFPPE4***

The Adriatic *Picochlorum* strain PMFPPE4 entered its exponential phase of growth between days 7 and 14, and the stationary phase after day 15 when cells started to aggregate at the base of the Erlenmeyer flasks. It took approximately 18 days for the *P. oklahomense* cultures to reach the maximum biomass concentration in the medium, under the conditions examined by Zhu & Dunford (2013). Shortly after maximum was achieved, the *P. oklahomense* biomass concentration started to decrease and abundance began to decline after day 15, thus signalling the need for biomass harvesting as soon as the maximum biomass concentration is realized (Zhu & Dunford, 2013). Rapidly growing *Picochlorum* strains are well known from past research (de la Vega *et al.* 2011; Watanabe & Fujii, 2016; Gonzalez-Esquer *et al.* 2019) again illustrating the necessity of biomass harvesting in the exponential growth phase for purposes of further culturing or biofuel production.

During lipid screening, the *Picochlorum* sp. PMFPPE4 strain was cultivated under nutrient rich conditions in a standard 16:8 light: dark regime. The observation of a high TAG level, particularly found in the exponential phase, was unexpected. These TAGs represent algae energy storage lipids and accumulation in phytoplankton is mainly attributed to nitrogen deprivation, as discovered in both oligotrophic seas and nitrogen depleted phytoplankton monocultures (e.g. Parrish & Wangersky, 1987; Bourguet *et al.*, 2009; Guschina & Harwood, 2009; Novak *et al.*, 2019). Specifically, photosynthetic picoeukaryotes are a dominant phytoplankton share in oligotrophic habitats since the high surface to volume ratio favours nutrient uptake (Maranon, 2009). The observed TAG accumulation may be explained as a life strategy by increasing the surface to volume ratio under conditions of abundant nutrient supply in order to optimize nutrient uptake (Maranon, 2009). We can also hypothesize that PMFPPE4 accumulation of TAGs may indicate an evolutionary preparation of the strain for growth under unfavorable conditions. Similar high lipid producing strains of *Picochlorum* sp. investigated by Tran *et al.* (2014) and other microalgal strains investigated by Hu *et al.* (2008) revealed greater lipid production is a direct consequence of growth in unfavourable conditions as somewhat of a stress factor (15°C, was not photosynthesis optimum for investigated strain). Therefore, the results of this study become even more interesting. The most favourable photoperiod for cell growth, nutrient consumption and lipid production as well as the general growth rate in *P. maculatum* was confirmed under the same 16:8 light: dark regime in Kumar *et al.*, 2019 as was in our experiment.

Furthermore, during transition from the exponential to stationary growth phase, lipid remodelling took place characterized by the increased proportion of the membrane forming DGDG and PC. Glycolipids DGDGs and MGDGs are the major class of lipids in the membranes of plastids not only required as bulk constituents of photosynthetic membranes, but also for the photosynthetic reaction itself (Kobayashi *et al.*, 2007). The bilayer forming DGDG plays an important role in the structural organization of the photosynthetic apparatus (Härtel *et al.*, 1997) by stabilizing the thylakoid bilayer structure. And, MGDG supports the fluidity of the thylakoid membrane and hence the velocity of electron flow (Mock & Kroon, 2002). We assume that TAGs stored during the exponential phase are utilized to build DGDG and PC, important for physiological processes and cell integrity during stationary growth phase while medium nutrients are still plentiful. Phospholipid PC is among the major structural lipids in the outer membrane with zwitterionic PC and PE representing up to 68–80% of the structural phospholipids (van Meer *et al.*, 2008). Lipid remodelling from the exponential, early stationary and late stationary phases is also observed for diatoms (Su *et al.*, 2013). The lipid composition of the PMFPPE4 strain is congruent with other algal species but, it should be noted that lipid composition of plankton cells varies according to environmental factors (Guschina & Harwood, 2009).The significant amount of particulate lipids in *Picochlorum* sp. PMFPPE4 cells in which TAGs are identified as dominant in both the exponential and stationary growth phases and phospholipids in membranes during the exponential growth phase, makes this strain exceptional in the investigations made thus far (de la Vega *et al.* 2011; Dahmen *et al.* 2014; Tran *et al.* 2014; Kumar *et al.* 2019; Gonzalez-Esquer *et al.* 2019; Dahlin *et al.* 2019). Therefore, the high TAG content and high growth rate suggests PMFPPE4 to be of interest for biofuel production and growth in outdoor systems. Dahmen *et al.* (2014) emphasized the biotechnological potential of genus *Picochlorum* and demonstrated the feasibility of using a wild *Picochlorum* sp. as feedstock for aquaculture, human nutrition, or biodiesel production. In addition, de la Vega *et al.* (2011) demonstrated the great potential and survival ability of *Picochlorum* sp. HM1 under adverse conditions, making it a good candidate for outdoor cultivation. *Picochlorum* sp*. ‘soloecismus’* was also acknowledged as a resilient strain holding great potential as a platform for production of biofuels and bioproducts (Gonzalez-Esquer *et al.* 2019). The genome-sequenced *Picochlorum* strain, *Picochlorum* sp. ‘*renovo*, proved to be a high-growth halophilic thermotolerant strain suggested for outdoor growth and systems and biofuel production (Dahlin *et al.* 2019).

The increasing photosynthetic activity of *Picochlorum* strain PMFPPE4 along the temperature gradient is consistent with the research on sister algal genera, such as *Chlorella* (Yun & Park, 2003; Lee *et al.*, 2018) and with other species from different phyla (e.g. Bacillariophyta, Cyanobacteria, Rhodophyta (Coles & Jones, 2000; Üveges *et al.*, 2012; Lengyel *et al.*, 2015; Pálmai *et al.*, 2018)). The photosynthetic activity along temperature gradient of *Picochlorum* sp. PMFPPE4 is well below the values of some river cyanobacteria species (Coles & Jones, 2000) or the dominant species of tropical alkaline saline lakes (Schagerl *et al.*, 2015), but similar to some dominant saline diatom species (Lengyel *et al.*, 2015). Also, the temperature optimum for photosynthetic activity in *Picochlorum* sp. PMFPPE4 was measured at exactly 27.7°C consistent with the optimum temperature of *P. maculatum* with maximum nutrient consumption at 28°C, in a photoperiod of 18h light and 6h dark and 150 µmol m-2 s-1 (Kumar *et al.*, 2019). Additional *Picochlorum* strains exhibited optimal growth rates (although photosynthetic activity was not measured) at similar temperatures (25°C – 30°C), and at higher temperatures, the growth rate started to decline (de la Vega *et al.*, 2011; Foflonker *et al.*, 2016). The potential mixotrophy of the species could be the reason for moderate photosynthetic activity under unfavorable environmental conditions (e.g. low temperature). Green algae typically prefer higher light intensities (~80-500 µmol m-2 s-1 (Reynolds, 1988; Padisák, 2004)) than compared to our findings, but there are some species with a preference for shade (e.g. *Picocystis salinarum* (Roesler *et al.* 2002)). The low light intensity preference along temperature gradient with good light utilization suggests PMFPPE4 can tolerate or even prefer a light limited habitat. On the other hand, the higher DGDG:MGDG ratio found in our strain indicates toleration and growth under higher light conditions (Mock & Kroon, 2002). Other *Picochlorum* strains also show tolerance to wide range of light intensities, such as *Picochlorum* sp. HM, *Picochlorum* sp*. ‘soloecismus’* and *Picochlorum* sp*. ‘renovo’* (de la Vega *et al.* 2011; Gonzalez-Esquer *et al.* 2019; Dahlin *et al.* 2019). Our findings, together with survival of *Picochlorum* sp. PMFPPE4 in dark conditions for a long period, further acknowledge the broad ecological niche of this picoalga in regard to light regimes and temperature in which it manages to store enough reserve TAGs to survive unfavourable conditions. Additionally, a record of PPEs below the photic zone (Babić *et al.*, 2017) during the BIOTA cruise suggests a possible switch of *Picochlorum* sp. PMFPPE4 to a mixotrophic lifestyle under an unfavourable growth environment. The accumulation of PPEs at 280 m of depth was not significantly correlated with any of the environmental variables (e.g. salinity, nutrients or temperature) suggesting that they were found outside their ecological optima (Babić *et al.*, 2017). Vertical density gradients were relatively strong in that area and geostrophic currents indicated a strong vertical shear (Babić *et al.*, 2017). The shear may have cause vertical instabilities that may have turned surface waters to deep layers. This may be the reason for the occurrence of maximum PPEs at depths below the euphotic zone. This indeed may be supported by the fact that selective pressures for preserving photoautotrophic machinery can be relaxed under certain conditions for which the energy costs of maintaining the photosynthetic apparatus outweigh the benefits of its products, or by the fact that picoeukaryotes use phagocytosis in the case of mixotrophy/heterotrophy (Massana & Logares 2013) with mixotrophy achieved through osmotrophy (the uptake of dissolved organic substrates (Pringsheim, 1963; Glibert & Legrand, 2006)). Given the general morphology of *Picochlorum* sp. PMFPPE4 strain (no observed flagella or apparatus for catching prey) and no record of toxin production for prey immobilization and/or disability in available literature, we can assume that in unfavorable conditions this organism acts like osmotroph.

This study identified a pico green alga isolated from the southeastern Adriatic Sea, strain PMFPPE4, as a member of the genus *Picochlorum*, a largely disregarded but widespread and molecularly diversified genus of Trebouxiophyceae. Identification of small coccoid algae can be achieved through a multilayer approach, considering morphology, phylogeny and physiology. The ecological preferences of *Picochlorum* sp. PMFPPE4 is wide: from shaded (and complete darkness) cooler marine environments (5 – 10°C) where it can act as an osmotroph; to higher light intensity (114.3 µmol m-2 s-1) and higher temperature (30 – 40°C)where it has photosynthesis activity maxima and finally photoinhibition (> 40°C). The low light intensity preference along temperature gradient with good light utilization suggests this strain can tolerate or even prefer a light limited habitat. The most important finding in regards to lipid screening is the strain’s ability to synthesize large amounts of triacylglycerids, important in biofuel feedstock and the food industry, and its higher DGDG/MGDG signals preference for higher light conditions. This exceptional strain can also serve as a model green alga for diverse ecological experiments due to its longevity in cultured conditions.

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**Supplemetary information:**

Table S1: List of all taxa with strain information and GenBank accession numbers used for phylogeny inference in this study

Figure S1: Photosynthesis-light intensity curves of *Picochlorum* sp. PMFPPE4 strain

**Author contributions:**

M. Mucko: original concept, writing and editing manuscript, morphological analyses, culture establishment and experiments and phylogeny analyses and plotting; J. Padisák: photosynthetic activity measurements, writing and editing manuscript; M. Gligora Udovič: photosynthetic activity measurements, writing and editing manuscript; T. Pálmai: photosynthetic activity measurements and plotting, writing and editing manuscript; T. Novak: lipid analysis, writing and editing manuscript; N. Medić: lipid analysis, writing and editing manuscript; B. Gašparović: lipid analysis, writing and editing manuscript; P. Peharec Štefanić: sample preparation and examination for transmission electron microscopy, editing manuscript; S. Orlić: DNA isolation, sequence processing and editing manuscript; Z. Ljubešić: original concept, writing and editing manuscript.

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**Tables:**

**Table 1.** Photosynthetic parameters of a *Picochlorum* sp. PMFPPE4 at different temperatures.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **T (°C)** | **5** | **10** | **15** | **20** | **25** | **30** | **35** | **40** |
| Ps[[1]](#footnote-1) | 0.304 | 0.614 | 0.812 | 1.890 | 2.491 | 3.222 | 2.131 | 0.586 |
| PBmax[[2]](#footnote-2) | 0.304 | 0.614 | 0.812 | 1.890 | 2.491 | 3.222 | 2.131 | 0.470 |
| Ik[[3]](#footnote-3) | 25.504 | 40.105 | 49.182 | 61.768 | 81.931 | 114.259 | 89.895 | 38.172 |
| α[[4]](#footnote-4) | 0.012 | 0.015 | 0.017 | 0.031 | 0.030 | 0.028 | 0.024 | 0.012 |
| β[[5]](#footnote-5) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.001 |
| RB[[6]](#footnote-6) | 0.027 | 0.063 | 0.087 | 0.263 | 0.347 | 0.527 | 0.400 | 0.377 |

**Figure legends:**

**Figures 1-2.** Light (LM) and transmission electron (TEM) micrographs of *Picochlorum* sp. PMFPPE4 strain. **1**: LM micrographs of strain PMFPPE4. White arrows indicate U-shaped chloroplasts and black arrows indicate dividing autospores. **2**: TEM micrographs of strain PMFPPE4. N: nucleus; M: mitochondrion; C: chloroplast; S: starch inclusions. Scale bars: Fig. 1, 5 µm; Fig. 2, 300 nm.

**Figure 3.** Consensus phylograms inferred with Bayesian inference (BI) and Maximum Likelihood (ML) for 16S rRNA (a) and 18S rRNA gene (b). Bayesian posterior probability (PP) and Maximum likelihood bootstrap values (BS) over 0.5/50 are indicated above branches. All taxa names consist of genus and species name, then strain (if specified in literature).

**Figure 4.** Distribution of *Picochlorum* sp. PMFPPE4 strain lipid classes during exponential and stationary growth phase. Total lipid concentrations are given in µg L-1 in rectangle (right bottom corner), whereas the relative importance is given in % of total lipids. Abbreviations: **TAG**: triacylglycerols, **DGDG**: digalactosyldiacylglycerols, **PC**: phosphatidylcholines, **PG**: phosphatidylglycerols, **SQDG**: sulfoquinovosyldiacylglycerols, **PE**: phosphatidylethanolamines, **MGDG**: monogalactosyldiacylglycerols, **ST**: sterols, **PIG**: pigments, **SE**: steryl esters.

**Figure 5.** Temperature dependence of 4 photosynthetic parameters of *Picochlorum* sp. PMFPPE4 strain in laboratory experiment. **PBmax**: Biomass specific maximal photosynthetic production (µg C µg-1 Chl *a* h-1), **Ps**: Maximal production obtained in the absence of photoinhibition; without photoinhibition it is equal to PBmax (µg C µg-1 Chl *a* h-1), **Ik**: photoadaptation parameter (µmol m-2 s-1), **RB**: biomass specific respiration (µg C µg-1 Chl *a* h-1).

**Figure 6.** Photosynthesis-temperature (P-T) curves of the *Picochlorum* sp. PMFPPE4 strain measured in different light intensity ranges.

**Figure 7.** Line graph of average *Picochlorum* sp. PMFPPE4 strain cell abundances with standard deviation measured by haemocytometer during over a period of 20 days.

1. maximal production obtained in the absence of photoinhibition; without photoinhibition it is equal to PBmax (µg C µg-1 Chl *a* h-1) [↑](#footnote-ref-1)
2. biomass specific maximal photosynthetic production (µg C µg-1 Chl *a* h-1) [↑](#footnote-ref-2)
3. photoadaptation parameter (µmol m-2 s-1) [↑](#footnote-ref-3)
4. light utilization parameter ((µg C µg-1 Chl *a* h-1) (µmol m-2 s-1)-1) [↑](#footnote-ref-4)
5. photoinhibiton parameter ((µg C µg-1 Chl *a* h-1) (µmol m-2 s-1)-1) [↑](#footnote-ref-5)
6. biomass specific respiration (µg C µg-1 Chl *a* h-1) [↑](#footnote-ref-6)