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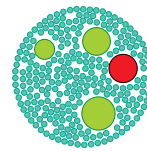
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Characterization of a lipid-producing thermotolerant marine photosynthetic pico-alga in the genus *Picochlorum* (Trebouxiophyceae)

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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.

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Introduction

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

Recently, PPE diversity research has been intensified by applying various molecular approaches and metabarcoding of environmental DNA (high-throughput sequencing of DNA markers), mostly variable regions of the nuclear 18S rRNA and plastid 16S rRNA gene, allowing better understanding of the diversity and importance of the pico-world (Fuller *et al.*, 2006a, 2006b; Decelle *et al.*, 2015; de Vargas *et al.*, 2015). 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 & Vault, 2018). In addition to environmental

metabarcoding approaches, cloning of environmental DNA, fingerprinting and tag-sequencing and single-cell genomics approaches are also being used to appraise protist diversity (Sieracki *et al.*, 2019).

The most diverse eukaryotic organisms in the above-mentioned cross-oceanic expeditions always belong to the piconanoplankton, i.e. ultraplankton (cells with diameter ≤ 5 µm). Trebouxiophyceae, a class of green algae containing the highly diversified marine coccoid genus *Picochlorum* within the 'core' chlorophytes, comprise approximately one-third of the sequences in oceanic temperate areas and are more abundant in upwelling and in nutrient-rich coastal zones (Tragin *et al.*, 2016). Recently, the genus *Picochlorum* was recognized as the most abundant trebouxiophyte in temperate areas of the USA and European Atlantic coast with the highest number of reads of major operational taxonomic units (OTUs) found in the OSD dataset (Tragin & Vault, 2018). In the Mediterranean, during the PROSOPE cruise, Díez *et al.* (2001) and Massana *et al.* (2004) found a large diversity of PPEs, including

putative photosynthetic representatives from a wide range of classes (e.g. Chrysophyceae, Cryptophyceae, Prasinophyceae and Prymnesiophyceae; Vaultot *et al.*, 2008). Green algae in the southern Adriatic Sea comprised roughly 26% of photosynthetic reads, but the genus *Picochlorum* has not yet been confirmed among 18S rRNA reads (Tragin & Vaultot, 2018).

Algae culture is one of the most important methods for studying the diversity of these organisms and their role in the ecosystem, as well as for future applications. 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 us to distinguish green, brown and red algae, but the photosynthetic pigment signature is often also indicative of the algal class (Guillou *et al.*, 1999). Biotechnological approaches to algal studies mostly focus on lipids and fatty acids, since their target is often biofuel production (Hu *et al.*, 2008; Liu *et al.*, 2011). 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 algae for their cell lipid or fatty acid components can also tell us much about their lifestyle and environment (Galloway & Winder, 2015). Useful information about physiology may also emerge from photosynthesis measurements. The process of photosynthesis mainly depends 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 such as biomass-specific photosynthetic activity, photoinhibition, photoadaptation, light utilization or respiration. Since growth continues by the consumption of photosynthetic products, photosynthetic parameters can help us understand the behaviour of a species in the environment.

Here we describe a newly isolated pico green algal strain showing high competitiveness and survival in culture conditions. It was isolated together with pennate diatoms from the oligotrophic southern Adriatic Sea. During the fieldwork, a photosynthetic signal in the aphotic zone was noticed that was later recognized as representing photosynthetic picoeukaryotes (PPEs) in the dark at a depth of 280 m, while viable diatoms were observed up to 500 m (Bosak *et al.*, 2016; Babić *et al.*, 2017). When cultivating 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 monoclonal culture and characterized it by morphological analysis (using light and transmission electron microscopy), phylogenetic analysis (using plastid 16S

rRNA and nuclear 18S rRNA genes), pigment and lipid screening, and photosynthesis measurements under different temperature and light regimes.

Materials and methods

Isolation and cultivation

The water sample was taken from 20 m depth to the surface during the 28 February–3 March 2015 BIOTA (Bio-tracing Adriatic Water Masses) cruise conducted in the south-eastern Adriatic Sea at station M300 (42.48N, 17.28E), using a 20 µm-mesh-size phytoplankton net. Environmental conditions and nutrient concentrations of the area are given in Babić *et al.* (2017). Immediately upon arrival in the laboratory, the sample was inoculated in Guillard's f/2 Marine Water Enrichment Solution (Sigma-Aldrich, UK). After a mixed culture had grown (containing mostly diatoms, dinoflagellates, cryptophytes, haptophytes and heterotrophic nanoflagellates), unknown PPE cells were filtered into a fresh medium through 3.0µm-pore-size Nucleopore polycarbonate membrane filters (Whatman, UK) with a syringe and a filter holder. Following filtration, isolation continued by the dilution method; repeated transfer of 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; Thronsen, 1978). A xenic culture of strain PMFPPE4 was established and subsequently transplanted biweekly to fresh medium, thus keeping the cells in the exponential phase of growth. The strain was later cultivated in ASN III medium (Stanier *et al.*, 1979), a synthetic medium used in photosynthetic experiments. The PMFPPE4 strain is deposited in the Roscoff Culture Collection under number RCC6905.

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 and phase contrast, and an AxioCam 305 camera with which images were obtained. Before examination, PMFPPE4 cells were allowed to settle on glass slides for 20 min. The cells were examined using a 1000× magnification.

For transmission electron microscopy (TEM), cultured cells were fixed in 1% (w/v) glutaraldehyde in 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 ice-cold 50 mM cacodylate buffer (pH 7.2) and embedded in 2% agarose.

The agarose with the embedded cells was cut into small pieces and washed twice with ice-cold 50 mM cacodylate buffer (pH 7.2). The cells were 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 placed in a mixture of 1:1 absolute ethanol and 100% acetone for 30 min, then in 100% acetone for a further 30 min. The material was then placed in a mixture of Spurr's medium and acetone: initially in 1/3 Spurr's and 2/3 acetone for 30 min, followed by 1/2 Spurr's and 1/2 acetone for 30 min and lastly in 2/3 Spurr's and 1/3 acetone for 30 min. This was followed by placing the material in Spurr's medium alone for 2 h at 45°C. Finally, the material was placed in a plastic mould and polymerized in Spurr's medium at 65°C for 48 h. Ultrathin sections were made with an ultra-microtome Leica Ultracut R and stained with 4% aqueous uranyl acetate for 10 min, then with lead citrate, pH 12.0 for 10 min (Reynolds, 1963) and examined using 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's instructions, using 50 ml of PMFPPE4 culture in the exponential growth phase. Purity of the extracted DNA was assessed with the NanoDrop spectrophotometer (BioSpec-nano; Shimadzu, Tokyo, Japan). The plastid 16S rRNA and nuclear 18S rRNA genes were amplified by PCRs. For the 16S rRNA gene, we used algal plastid biased forward and reverse primers PLA491F (5'-GAGGAATAAGCATCGGCTAA-3') (Fuller *et al.*, 2006a) and OXY1313R (5'-CTTCAYGYAGGCG AGTTGCAGC-3') (West *et al.*, 2001), respectively. For the 18S rRNA gene, we used Euk63F (5'-CGCTTGT CTCAAAGATTA-3') as forward and Euk1818R (5'-ACGGAAACCTTGTTACGA-3') as reverse primers (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 GoTag® DNA polymerase (100 U, Promega), 3 µl of template DNA and 28.25 µl of miliQ H₂O. 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 25 µl EmeraldAmpMax PCR Master Mix® (Takara Bio, USA), miliQ H₂O (17 µl),

2 µl of each of the primers (10 µM) and 4 µl of template DNA. The PCR reaction was performed 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 and 72°C for 1 min; and final extension at 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 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 with the blast 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, we performed small phylogenetic analyses to identify placement and grouping of PMFPPE4 with other *Picochlorum* strains. A total of 20 sequences for each gene were included in the phylogenetic analyses (Supplementary table 1), with the exception of the 18S rRNA gene Bayesian inference phylogeny which used 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 included 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 *M. 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 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 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) which penalizes for the number of parameters and helps 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 with the -bb 1000 command. Bayesian analyses were carried out with a mixed-model strategy, whereby various variants of the Generalized time-reversible model (GTR) were sampled in proportion to their posterior probability (MrBayes option 'nst=mix'). Among-site rate variation in MrBayes was accommodated via a Γ distribution with four rate categories (Γ_4) and by estimating the proportion of invariant sites (I). Four simultaneous Markov chain Monte Carlo (MCMC) simulations were run, each composed of one cold and three heated chains for a total of 10 million generations with a sampling frequency of one thousand generations. Stationarity and convergence among the MCMC runs were assessed from the MrBayes output (standard deviation of split frequencies and potential scale reduction factor) and by inspecting 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 1 ml of exponentially growing PMFPPE4 culture filtered through 0.7- μ m-pore-size GF/F filters (Whatman, UK), then freshly frozen in liquid nitrogen. Extraction in 4 ml of cold 90% acetone was performed by sonication, and the extract was cleaned by centrifugation. Pigments separation by 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 phases was filtered through precombusted GF/F filters. The filters were stored for 3 days at -80°C before lipid extraction. Particulate lipids were extracted using a modified one-phase solvent mixture of dichloromethane-methanol-water (Bligh & Dyer, 1959). Nonadecanone (KET) was added to each sample as

an internal standard to estimate the recoveries in the subsequent steps of the sample analysis. Extracts evaporated to dryness under a nitrogen atmosphere were re-dissolved in 24 μ l dichloromethane and lipid classes were determined by thin-layer chromatography-flame ionization detection (TLC-FID; Iatroscan MK-VI, Iatron, Japan). Eighteen lipid classes, constituting total lipids, were separated on Chroma rods SIII and quantified by an external calibration with a standard lipid mixture, with a hydrogen flow of 160 ml min^{-1} and air flow of 2000 ml min^{-1} . 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 sterol esters (WE/SE, henceforth discussed as SE (WE represent zooplankton storage lipids (Kattner, 1989) and should not be present in phytoplankton monocultures); 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 do not discuss lipid degradation indices or hydrocarbons and the remaining lipid classes represent cell lipids. The standard deviation determined from duplicate runs accounted for 1–8% of the relative abundance of lipid classes. Total lipid concentrations were 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 R software (version 3.4.3) (R Development Core Team, 2008) using the 'ggplot2' package (Wickham, 2011).

Photosynthesis measurement

Measurements of the photosynthetic characteristics of PMFPPE4 were carried out in a laboratory photosynthetron developed by Ūveges *et al.* (2011) using 72 combinations of temperature and light intensity. Temperature varied, in 5°C increments, from 5 to 40°C and light was adjusted to nine light intensities (0; 15; 40; 125; 230; 250; 500; 800 and $1300\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$). For cultivation and photosynthesis measurements PMFPPE4 cells were maintained in ASN-III medium (Stanier *et al.*, 1979). Photosynthesis measurements and carbon uptake calculations were performed as described by Lengyel *et al.* (2015). Photosynthetic parameters were calculated in the absence of photoinhibition according to Webb *et al.* (1974) and, if photoinhibition was observed, β (photoinhibition parameter) and the other parameters were calculated according to Platt *et*

al. (1980). Curves (photosynthesis-light intensity (P-I), photosynthesis-temperature (P-T) and Gaussian curves to find temperature optima of photosynthetic parameters) and the calculation of the photosynthetic variables were carried out in GraFit 7.0 software (Leatherbarrow, 2009).

Growth experiments

The growth-rate experiment lasted for 20 days throughout which PMFPPE4 was grown in the following conditions: temperature $22 \pm 0.5^\circ\text{C}$; light $30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a photoperiod of 16 h of light: 8 h of dark and continuous shaking on an Orbital Shaker OR100 (Cole Parmer, UK) for 12 h during the light period. The starting culture, consisting of $\sim 10^6$ cells ml^{-1} , was established in 200 ml of f/2 medium in Erlenmeyer flasks in triplicate. Cells were counted daily in triplicates using a Birken-Türk haemocytometer. The number of cells ml^{-1} was then calculated and the standard deviation (SD) was included in generating the growth rate graph, which was plotted using R version 3.4.3 (R Development Core Team, 2008) with the 'ggplot2' package (Wickham, 2011). Net growth rate (k) of PMFPPE4 was calculated using the equation: $k = \ln(X_1 - X_0)/(T_1 - T_0)$; X_1 = Cell number at the end of exponential phase; X_0 = cell number at the beginning of exponential phase; T_1 = number of days at the end of exponential phase; T_0 = number of days at the beginning of exponential phase.

Additionally, a dark cultivation experiment was performed. The strain was kept in darkness for one month in four replicates, without shaking or any intervention regarding nutrient or oxygen intake, at a stable temperature of $22 \pm 0.5^\circ\text{C}$. Each week, one replicate was examined under light microscope.

Results

The cells of the isolated algal strain PMFPPE4 are green, spherical to oval, $1.5\text{--}3.0 \mu\text{m}$ ($n = 50$) in diameter, with a lateral U-shaped chloroplast occupying approximately two-thirds of the cell (Fig. 1). Cells have a smooth surface (Figs 1, 2). No flagella or any other kind of cell appendices were observed. Cells divide by autosporulation into two daughter cells. Neither zoospore formation nor sexual reproduction was observed. TEM revealed that cells contain one nucleus, one mitochondrion, a single, lateral U-shaped chloroplast lacking a pyrenoid, and starch grains are mostly present within the chloroplast (Fig. 2).

The phylogeny of the 16S rRNA gene confirmed placement of the strain PMFPPE4 in the paraphyletic genus *Picochlorum* (Bayesian posterior probability (BPP)/Bootstrap values (BS), BPP/BS = 1/92 (Fig. 3a)), together with other cultured *Picochlorum* strains without species affiliation, BPP/BS = 0.59/80

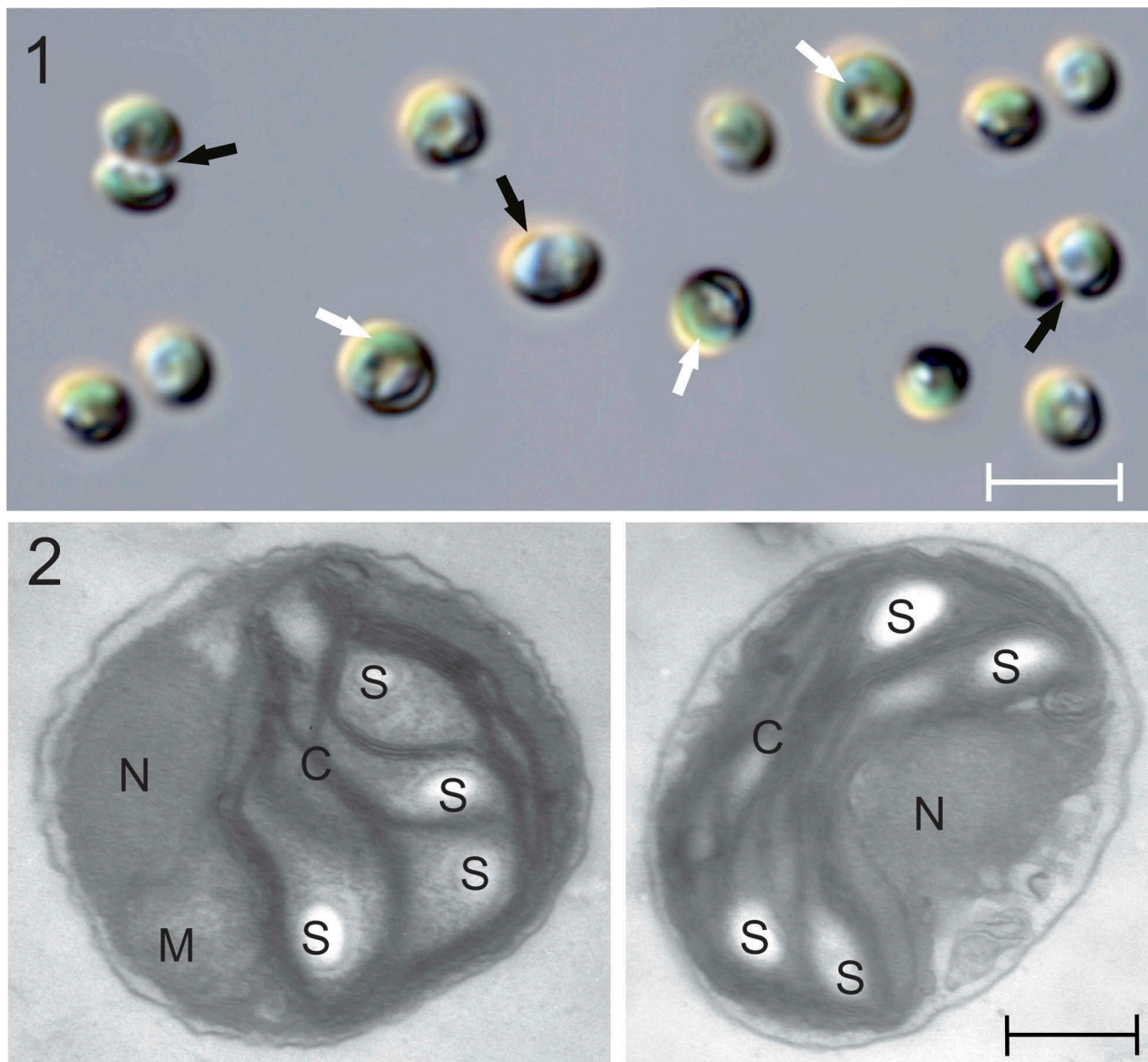
(Fig. 3a). The most similar strains to PMFPPE4 were all from the Roscoff Culture Collection (RCC), and isolated from the Pacific Ocean (RCC1034, RCC289 and RCC13) and the Mediterranean Sea (RCC9), indicating the cosmopolitan presence of this coccoid pico green alga (Fig. 3a). The sister clade containing two additional unknown RCC strains of *Picochlorum* sp. (RCC846 and RCC945) diverged from the PMFPPE4 clade with great support BPP/BS=1/99 (Fig. 3a). The outgroup represented *Pycnococcus provasolii* and three unidentified *Pycnococcus* species as root to the tree (BPP/BS=1/100; Fig. 3a).

The phylogeny of the 18S rRNA gene also confirmed taxonomic assignment of the strain PMFPPE4 to the monophyletic genus *Picochlorum* (BPP/BS = 0.99/99, Fig. 3b) by grouping it with five other unknown cultured *Picochlorum* strains (BPP/BS = 0.73/52; Fig. 3b). Besides the RCC strains (RCC9, 13 and 14), these included strains KMMCC C-275 and KMMCC 44 isolated from the Yellow Sea (Fig. 3b). The strain RCC289, *Picochlorum oklahomense* and *P. oklahomense* strain Xmm7W6 branched off the PMFPPE4 clade with high support (BPP/BS = 1/98, Fig. 3b). Furthermore, the genus consists of a smaller clade represented by *P. oklahomense* strain DHmm4W1 and *P. maculatum* DHmm1W1 (BPP/BS = 0.84/74; Fig. 3b); *Picochlorum* sp. strain KMMCC C-93 branching off with BPP/BS = 0.99/55; and a clade consisting of *Picochlorum* sp. RCC475 and *P. eukaryotum* (BPP/BS = 0.94/93; Fig. 3b). The tree root was comprised of the *Micractinium pusillum* strain SAG 13.81 and the *Chlorella vulgaris* strain SAG 211.11b (Fig. 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 (day 12) and stationary (day 20) growth phases (753 and $2529 \mu\text{g l}^{-1}$ on average; Fig. 4). The most abundant class was TAG (27.2% and 17.3% in the exponential and stationary growth phase, respectively; Fig. 4). Regarding membrane-forming lipids, the most abundant were the phospholipids PG (21.1%) and the glycolipids SQDG (12.2%) during the exponential phase, and the stationary phase was characterized by an increased share of the glycolipids DGDG (15.8%) and phospholipids PC (12.8%) (Fig. 4).

The photosynthesis-light intensity (P-I) curves of PMFPPE4 (Supplementary fig. 1) showed an increase in photosynthetic activity with increasing temperature. The highest value was measured at 30°C . The calculated photosynthetic variables (Table 1) show temperature dependence. Biomass-specific maximal photosynthetic activity ($P_{\text{max}}^{\text{B}}$) increased with temperature (Fig. 5), the highest value was recorded at 30°C , although the temperature optimum was at 27.7°



Figs 1–2. Light (LM) and transmission electron (TEM) micrographs of *Picochlorum* sp. PMFPPE4 strain. **Fig. 1:** LM micrographs of strain PMFPPE4. White arrows indicate U-shaped chloroplasts and black arrows indicate dividing autospores. **Fig. 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.

C (Table 1). $P_{\text{max}}^{\text{B}}$ increased by an order of magnitude along the temperature gradient: at 5°C $P_{\text{max}}^{\text{B}}$ it was 0.304 and at 30°C it was 3.222 $\mu\text{g C } \mu\text{g}^{-1} \text{ Chl } a \text{ h}^{-1}$. Since photoinhibition was detected only at 40°C, maximal production obtained in the absence of photoinhibition (P_{s}) differs from $P_{\text{max}}^{\text{B}}$ only at the highest temperature. The photoadaptation parameter (I_{k}) also increased with temperature, and the highest value was calculated at 30°C (114.3 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Good light utilization (α) of the strain was found along the temperature gradient, and highest values were observed at temperature range 20–35°C. Biomass-specific respiration of the strain increased with increasing temperature, reaching a maximum at ~32°C, then slightly decreasing.

The P-T curves showed a similar level of photosynthetic activity in the light intensity range of 230–1300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Maximum biomass-

specific photosynthetic activity varied between 2.89 and 3.10 $\mu\text{g C } \mu\text{g}^{-1} \text{ Chl } a \text{ h}^{-1}$ (Fig. 6). The temperature optimum for photosynthesis in this light intensity range varied between 27.3 and 27.8°C. At lower light intensities, a remarkable decrease in photosynthetic activity and optimum temperature was observed. At 125 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ the maximum photosynthetic activity dropped to 2.1 $\mu\text{g C } \mu\text{g}^{-1} \text{ Chl } a \text{ h}^{-1}$ and the temperature optimum decreased to just below 27°C. At the two lowest light intensities the decrease was even more pronounced: at 40 and 15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ the highest photosynthetic activity was at 25.4 and 24.9°C, respectively, with values of 0.92 and 0.28 $\mu\text{g C } \mu\text{g}^{-1} \text{ Chl } a \text{ h}^{-1}$, respectively.

PMFPPE4 cells showed acclimatization and steady growth during the first 7 days of the growth experiment, with an average abundance of 9×10^6 cells ml^{-1} per day and net growth rate (k) was very high (2.90

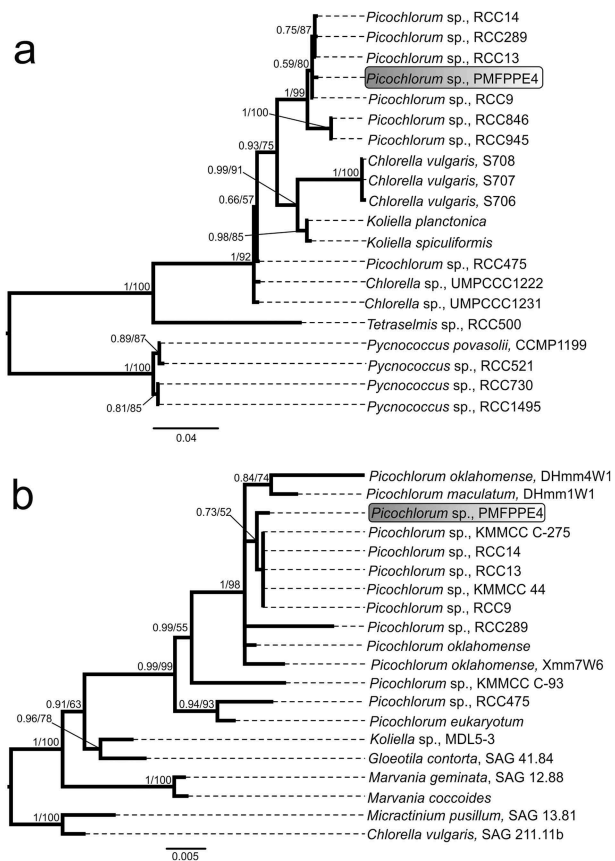


Fig. 3. Consensus phylograms inferred with Bayesian inference (BI) and Maximum Likelihood (ML) for 16S rRNA (a) and 18S rRNA gene (b). Bayesian posterior probability (BPP) and Maximum likelihood bootstrap values (BS) over 0.5/50 are indicated above branches. All taxa names consist of genus and species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

day⁻¹). Two weeks after inoculation the culture moved into the stationary growth phase and cells began to aggregate at the bottom of the Erlenmeyer flasks. Cell count stabilized during the last 5 days of the experiment to average values of $4 \times 10^7 \pm 2 \times 10^6$ cells ml⁻¹ (Fig. 7).

Dark cultivation showed that survival of the PMFPPE4 strain was possible without photosynthesis. After a one-month growth period in darkness the strain survived, although cell size was generally smaller (minimum cell diameter was 1.5 µm) in comparison with those grown in 16:8 h light:dark periods.

Discussion

Morphological, ecological and genetic comparisons with similar taxa

Picochlorum sp. PMFPPE4 is morphologically similar to the 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 to the genus

Chlorella and ‘*Chlorella*-like’ organisms were high, except that species belonging to *Chlorella* always possess a pyrenoid, which is absent in the genus *Picochlorum* (Bock *et al.*, 2011). There were even greater similarities to the genus *Nannochloris* Naumann, but without a designated type species, the validity of this genus is questionable (Guiry & Guiry, 2019).

To date several species from the genus *Nannochloris* have been transferred to *Picochlorum*, indicating there are insufficient distinguishable characters to separate these two genera and leading taxonomists to recognize only the genus *Picochlorum* (Henley *et al.*, 2004; D. Vaultot, personal communication). The lack of distinguishing characteristics and the discussion of Hepperle & Krienitz (2001) regarding difficulties in identification of the so-called ‘*Chlorella*-’ and ‘*Nannochloris*-like’ algae, led Henley *et al.* (2004), using molecular support (18S rDNA phylogeny), 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. At present, the genus *Picochlorum* comprises 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 *Picochlorum*: *Chlorella*, which is mostly freshwater, but there are few known marine species (1.5–10 µm in diameter); *Elliptochloris* Tschermak-Woess (5–10 µm in diameter); *Carolibrandtia* R. Hoshina & T. Nakada (symbiotic green coccoid in ciliates); and *Phyllosiphon* J.G. Kühn (biofilm-associated siphonous parasitic green algae (Motti *et al.*, 2005; Procházková *et al.*, 2015; Tragin *et al.*, 2016; Hoshina *et al.*, 2018), from which the genus *Picochlorum* differs significantly in cell size, ecology and absence of a pyrenoid. *Pseudochloris* B. Somogyi, T. Felföldi & L. Vörös is an additional trebouxiophyte genus described by Somogyi *et al.* (2013), comprising one species: *P. wilhelmii*, isolated from aquarium seawater. *Pseudochloris* is morphologically similar to *Picochlorum* sp. PMFPPE4 in having spherical to oval cells, but the cells seem to be larger (1.6–3.9 µm wide and 1.8–4.0 µm long). The cell ultrastructure of *Pseudochloris* includes peroxisomes and numerous plastoglobuli, which were not observed in *Picochlorum* sp. PMFPPE4 (Somogyi *et al.*, 2013). Both genera divide by autospore formation (Somogyi *et al.*, 2013) and pigment composition is similar – Chl *a* and *b*, lutein, violaxanthin and neoxanthin (with the addition of antheraxanthin, zeaxanthin and α -carotene in *Pseudochloris*). Phylogeny based on the 18S rRNA gene defines *P. wilhelmii* as sequences belonging to 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 clearly illustrates the problems with

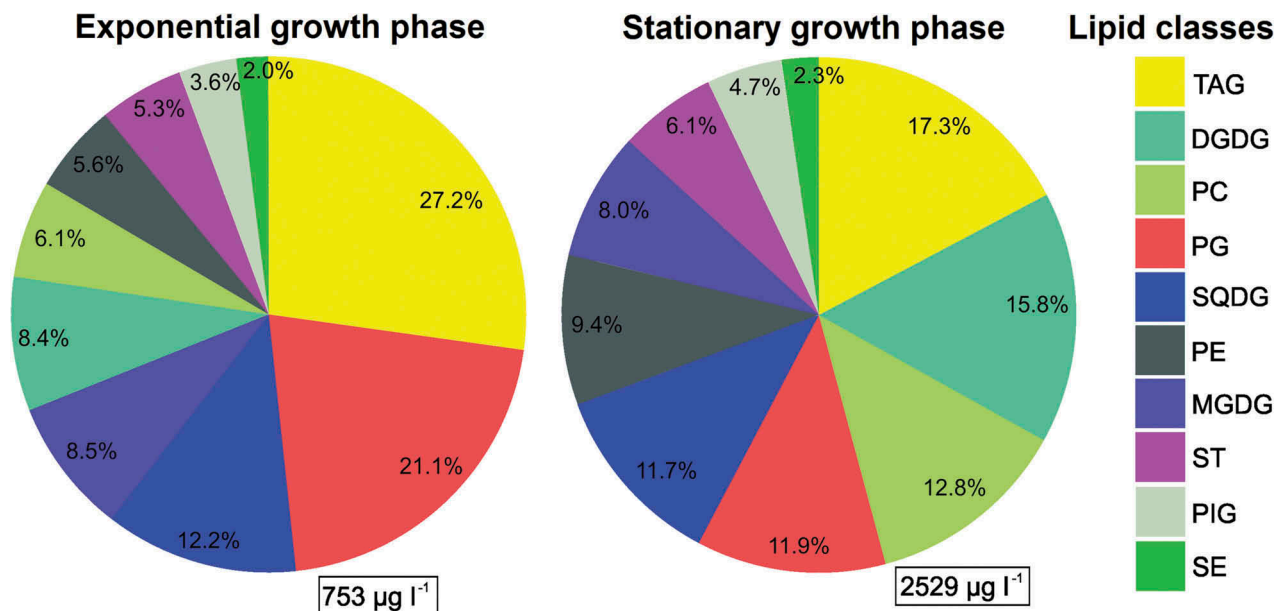


Fig. 4. Distribution of *Picochlorum* sp. PMFPPE4 strain lipid classes during exponential and stationary growth phase. Total lipid concentrations are given in $\mu\text{g l}^{-1}$ in rectangle (right bottom corner), whereas the relative importance is given in % of total lipids. Abbreviations: TAG: triacylglycerols,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

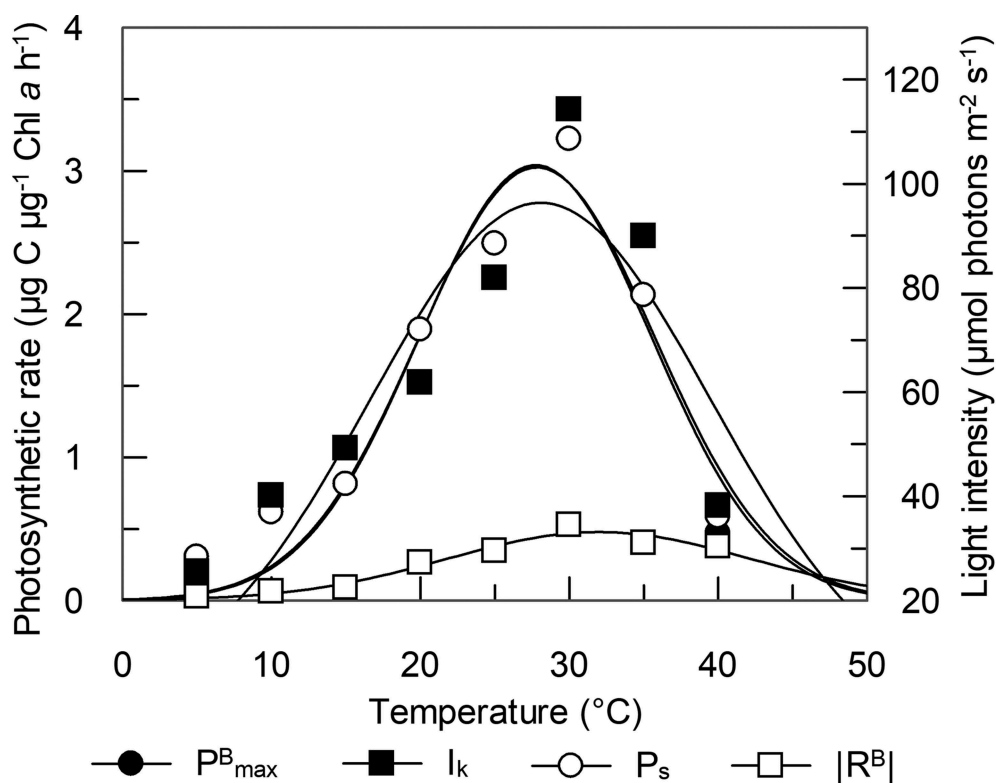


Fig. 5. Temperature dependence of 4 photosynthetic parameters of *Picochlorum* sp. PMFPPE4 strain in laboratory experiment. P^B_{max} : Biomass specific maximal photosynthetic production ($\mu\text{g C } \mu\text{g}^{-1} \text{ Chl a h}^{-1}$), P_s : Maximal production obtained in the absence of photoinhibition; without photoinhibition it is equal to P^B_{max} ($\mu\text{g C } \mu\text{g}^{-1} \text{ Chl a h}^{-1}$), I_k : photoadaptation parameter ($\mu\text{mol m}^{-2} \text{ s}^{-1}$), R^B : biomass specific respiration ($\mu\text{g C } \mu\text{g}^{-1} \text{ Chl a h}^{-1}$).

misidentification of *Clorella*- or *Nannochloris*-like organisms (Somogyi *et al.*, 2013).

Somogyi *et al.* (2013) represent the genus *Picochlorum* in their phylogeny as monophyletic, branched off the newly described genus *Pseudochloris*, therefore

acknowledging their genetic differences. Other classes of algae have also pico-sized non-motile coccoid representatives in the marine environment, which further complicates the identification of *Picochlorum*. Within the class Mamiellophyceae are genera cosmopolitan and

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

| T (°C) | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 |
|------------|--------|--------|--------|--------|--------|---------|--------|--------|
| P_s^1 | 0.304 | 0.614 | 0.812 | 1.890 | 2.491 | 3.222 | 2.131 | 0.586 |
| P_s^B | 0.304 | 0.614 | 0.812 | 1.890 | 2.491 | 3.222 | 2.131 | 0.470 |
| I_k^3 | 25.504 | 40.105 | 49.182 | 61.768 | 81.931 | 114.259 | 89.895 | 38.172 |
| α^4 | 0.012 | 0.015 | 0.017 | 0.031 | 0.030 | 0.028 | 0.024 | 0.012 |
| β^5 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.001 |
| R^{B6} | 0.027 | 0.063 | 0.087 | 0.263 | 0.347 | 0.527 | 0.400 | 0.377 |

¹Maximal production obtained in the absence of photoinhibition; without photoinhibition it is equal to P_s^B max ($\mu\text{g C } \mu\text{g}^{-1} \text{ Chl } a \text{ h}^{-1}$); ²biomass specific maximal photosynthetic production ($\mu\text{g C } \mu\text{g}^{-1} \text{ Chl } a \text{ h}^{-1}$); ³photoadaptation parameter ($\mu\text{mol m}^{-2} \text{ s}^{-1}$); ⁴light utilization parameter ($(\mu\text{g C } \mu\text{g}^{-1} \text{ Chl } a \text{ h}^{-1}) (\mu\text{mol m}^{-2} \text{ s}^{-1})^{-1}$); ⁵photoinhibition parameter ($(\mu\text{g C } \mu\text{g}^{-1} \text{ Chl } a \text{ h}^{-1}) (\mu\text{mol m}^{-2} \text{ s}^{-1})^{-1}$); ⁶biomass specific respiration ($\mu\text{g C } \mu\text{g}^{-1} \text{ Chl } a \text{ h}^{-1}$).

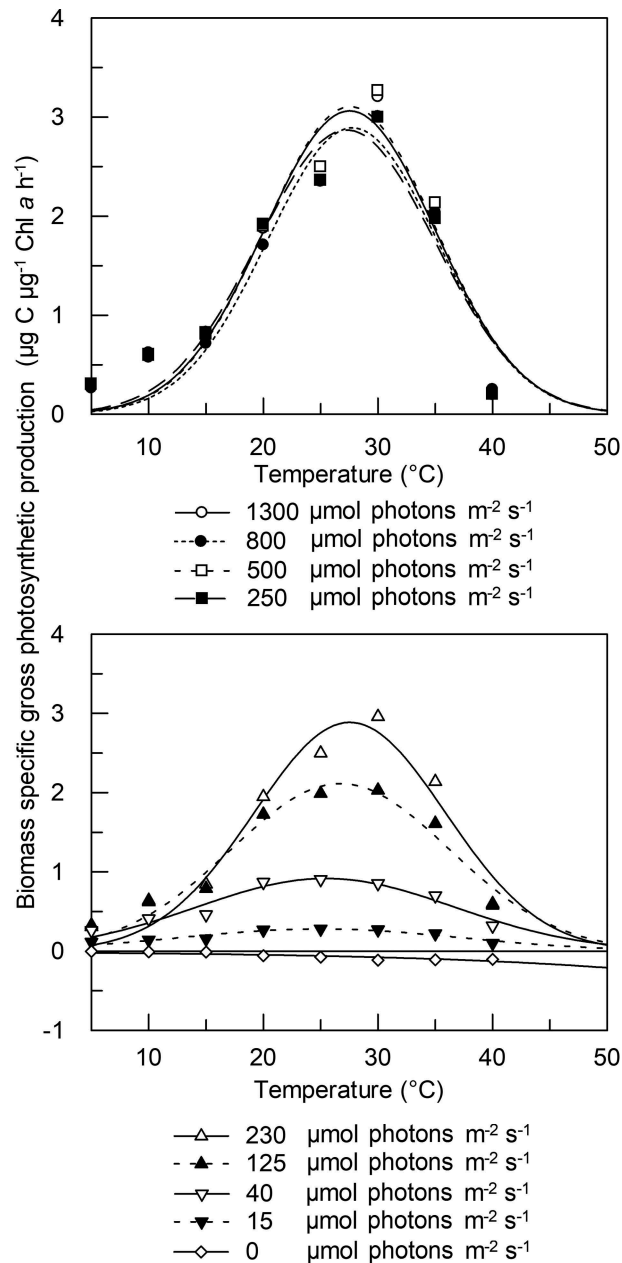


Fig. 6. Photosynthesis-temperature (P-T) curves of the *Picochlorum* sp. PMFPPE4 strain measured in different light intensity ranges.

numerically important genera in coastal areas: *Bathycoccus* W.Eikrem & J.Thronksen and *Ostreococcus* C.Courties & M.-J.Chrétiennot-Dinet (Moreau *et al.*, 2012). Morphological differences to the genus

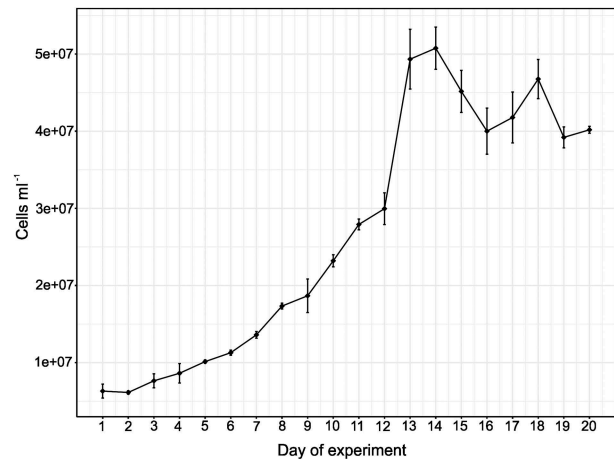


Fig. 7. Line graph of average *Picochlorum* sp. PMFPPE4 strain cell abundances with standard deviation measured by hemocytometer during over a period of 20 days.

Picochlorum are significant for *Bathycoccus*, which has specific spider-web-like scales, produced by the Golgi apparatus, on the cell surface (Moreau *et al.*, 2012). The smallest known single-celled eukaryotic genus *Ostreococcus*, species *O. tauri* has more morphological similarities to *Picochlorum* sp. PMFPPE4, in terms of simple cell ultrastructure, containing a single chloroplast with one starch granule without pyrenoid and a mitochondrion located between the nucleus and chloroplast (Chrétiennot-Dinet *et al.*, 1995). However, pigment composition of *O. tauri* cells is quite different to *Picochlorum* sp. PMFPPE4 cells, *O. tauri* cells contain chl *c*-like, siphonaxanthin-like 1 and siphonaxanthin-like 2, antheraxanthin, zeaxanthin and α -carotene, which are absent in *Picochlorum* sp. PMFPPE4 (Chrétiennot-Dinet *et al.*, 1995). Class Pinguiphyceae contains the marine coccoid genus *Pinguicoccus* R.A.Andersen, D.Potter, D.& J.C. Bailey comprised of one species, *P. pyrenoidosus* (collected and described from an aquaculture tank), which also morphologically resembles *Picochlorum* sp. PMFPPE4 in having spherical to irregular cells with one parietal chloroplast seen in light microscopy (Andersen *et al.*, 2002). However, examining cell ultrastructure in TEM, *P. pyrenoidosus* cells have one stalked pyrenoid in the chloroplast and numerous vacuoles (Andersen *et al.*, 2002). Additionally, when observed in growing culture, cells of *P. pyrenoidosus* have surface protuberances

caused by growing vacuoles, and in older cultures cells develop fine extensions of the protoplasm and large storage vacuoles (Andersen *et al.*, 2002). Furthermore, the genera *Pycnococcus* (Pyramimonadophyceae), *Prasinoderma* (Palmophyllophyceae) and *Nannochloropsis* (Eustigmatophyceae) all resemble *Picochlorum* in LM. *Pycnococcus provasolii* most closely resembles *Picochlorum* sp. PMFPPE4 in having spherical, subspherical or ovoid cells ranging from 1.5–4.0 µm in diameter with one cup-shaped chloroplast (Guillard *et al.*, 1991). However, the cell ultrastructure observed in TEM differs as one or two pyrenoids are present and the outer mitochondrial membrane protrudes into the chloroplast region of the pyrenoid (Guillard *et al.*, 1991). *Prasinoderma singularis* Jouenne, a solitary coccoid prasinophyte isolated from the South-east Pacific Ocean, can be misidentified in LM as *Picochlorum* sp. PMFPPE4 owing to cell size (2.2–5.5 µm in diameter) and a single bilobed cupuliform chloroplast. However, the starch-sheath-covered pyrenoid is usually clearly visible in this species, which is a delimiting factor concerning identification of cells belonging to *Picochlorum* (Jouenne *et al.*, 2011). In addition, the pigment of *P. singularis* differs from *Picochlorum* sp. PMFPPE4 as it contains Mg-3,8-divinylphaeoporphyrin 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 the North-east Atlantic Ocean, are commonly mistaken for *Picochlorum* cells, as in LM they are similar in size (2–4 µm in diameter), chloroplast and absence of a pyrenoid (Karlson *et al.*, 1996). However, cells belonging to *N. granulata* can have two chloroplasts and they always possess up to five refractile granules in the cytoplasm (Karlson *et al.*, 1996). *N. granulata* also has different pigments to *Picochlorum* sp. PMFPPE4: vaucherixanthin like pigment, zeaxanthin and canthaxanthin (Karlson *et al.*, 1996).

Examples of defining genera within chlorophytes using 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 with positioning certain strains, as Dahmen *et al.* (2014) showed with the identification of *Picochlorum* sp. strain CTM 20019, or in the examples of *Ostreococcus*, *Pycnococcus*, *Prasinoderma*, *Nannochloropsis*, etc. discussed above. However, molecular data is the most common tool used to define unknown picoalgal strains such as *Picochlorum* (de la Vega *et al.*, 2011; Watanabe & Fujii, 2016; Gonzalez-Esquer *et al.*, 2018) or other genera, such as *Pseudochloris* (Somogyi *et al.*, 2013).

In both phylogenies, PMFPPE4 was most similar to other *Picochlorum* strains from the RCC, of which none has yet been investigated thoroughly. In the 18S rRNA phylogeny, *Picochlorum* strains RCC13 (Pacific Ocean), RCC14 (Atlantic Ocean) and RCC9 (Mediterranean Sea) and KMMCC C-275 and KMMCC 44 isolated from the Yellow Sea were genetically identical and probably belong to the same species. PMFPPE4 grouped with this genetically identical clade, suggesting possible conspecificity. However, further investigation would be needed, since conspecificity within the genus *Picochlorum* has yet to be thoroughly investigated, although it is possible (Krienitz *et al.* 1996, 2009; Henley *et al.* 2004). The lack of taxonomic identification within the genus suggests the existence of new and undescribed species and a need for their description in future. However, more extensive morphological effort (thorough TEM examination of all genetically similar strains in vegetative cells and cell divisions) will be needed in order to correctly link those genetically very similar strains. In the 16S rRNA gene phylogeny, not one *Picochlorum* sequence is identified to species level, and in the 18S rRNA gene phylogeny, only three sequences of taxonomically accepted *Picochlorum* species are available. The remainder are unknown strains, indicating both poor sequence coverage, lack of taxonomic studies within the genus and usage of gene markers which do not provide sufficient resolution for species delimitation. A possible upgrade in genetic identification of *Picochlorum* species would be use of another gene marker, such as ITS which already proved to be excellent in delimiting genetically highly similar organisms due to secondary structure comparisons (Škaloud *et al.*, 2016; Garcia da Silva *et al.*, 2017; Temraleeva & Moslalenko, 2019). Under-appreciation of *Picochlorum* until now is most certainly due to its minute size, the inability to identify cells in environmental samples by their autofluorescence (i.e. flow cytometry), the difficulty in cultivation, and the fact that molecular genetic research on minute coccoid algae is scarce (Barcytė *et al.*, 2017). Although potentially undescribed microorganisms can be obtained from current field samples, re-examination of established cultures from public collections such as RCC, NCMA or UTEX is of extreme importance and would be the next step following our study.

Physiology and biotechnological potential of Picochlorum sp. PMFPPE4

Picochlorum strains are known to grow rapidly (de la Vega *et al.*, 2011; Watanabe & Fujii, 2016; Gonzalez-Esquer *et al.*, 2019). PMFPPE4 entered its exponential phase between days 7–14 and the

stationary phase after day 15. In *P. oklahomense*, maximum biomass concentration was reached at around 18 days, after which it declined (Zhu & Dunford, 2013). These results combined illustrate the necessity to harvest biomass of *Picochlorum* species as soon as the maximum biomass concentration is reached, in the exponential phase.

During lipid screening, *Picochlorum* sp. PMFPPE4 strain was cultivated under nutrient-rich conditions and standard 16:8 light:dark regime. The high TAG level observed, particularly in the exponential phase, was unexpected. TAGs are algal energy-storage lipids. The accumulation of TAG in phytoplankton is usually attributed to nitrogen deprivation, as found 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). PPEs are a dominant phytoplankton fraction in oligotrophic habitats, as high surface to volume ratio is favourable for nutrient uptake (Marañón, 2009). The observed TAG accumulation can be explained as a life strategy to increase surface to volume ratio under conditions of abundant nutrient supply to optimize nutrient uptake (Marañón, 2009). We can also hypothesize that accumulation of TAG by PMFPPE4 could indicate an evolutionary preparation by the strain for growth under unfavourable conditions. A similar high-lipid-producing strain of *Picochlorum* sp. investigated by Tran *et al.* (2014), and other microalgal strains investigated by Hu *et al.* (2008), showed higher lipid production as a direct consequence of growth in unfavourable conditions (at 15°C, which was not the photosynthesis optimum for the investigated strain), therefore the results of this study become even more interesting. Furthermore, during the transition from the exponential to stationary growth phase lipid remodelling took place, characterized by increased proportion of membrane-forming DGDG and PC. The glycolipids DGDGs and MGDGs are the major class of lipids in the membranes of plastids, where they are required not only 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, while MGDG supports the fluidity of the thylakoid membrane and therefore the velocity of electron flow (Mock & Kroon, 2002). We assume that TAGs stored during the exponential phase were used to synthesize DGDG and PC, which are more important for physiological processes and cell integrity, during the stationary growth phase when nutrients are still plentiful in the medium. The phospholipid PC is one of the major structural lipids in the outer membrane, where zwitterionic PC and PE represent up to 68–80% of the structural phospholipids (van Meer *et al.*, 2008). Lipid remodelling in 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 mostly congruent with other algal species, but it should be noted that lipid composition of plankton cells varies according to environmental factors (Guschina & Harwood, 2009). However, the high amount of particulate lipids in cells of *Picochlorum* sp. PMFPPE4 (among which were TAGs, identified as dominant in cells both in the exponential and stationary growth phases, and phospholipids in membranes during the exponential growth phase) makes this strain exceptional in investigations so far (de la Vega *et al.*, 2011; Dahmen *et al.*, 2014; Tran *et al.*, 2014; Gonzalez-Esquer *et al.*, 2019; Dahlin *et al.*, 2019; Kumar *et al.*, 2019). Therefore, the high TAG content and high growth rate suggest PMFPPE4 could be of interest for biofuel production and growth in outdoor systems. Dahmen *et al.* (2014) emphasized the biotechnological potential of the genus *Picochlorum* and demonstrated the feasibility of using a wild *Picochlorum* sp. as feedstock for aquaculture, human nutrition or biodiesel production. Additionally, de la Vega *et al.* (2011) demonstrated the great potential for *Picochlorum* sp. HM1 to grow and thrive under adverse conditions, making it a good candidate for outdoor cultivation. *Picochlorum* sp. 'solocismus' was also noted as a resilient strain with great potential as a platform for production of biofuels and bioproducts (Gonzalez-Esquer *et al.*, 2019). Another *Picochlorum* strain, the genome sequenced *Picochlorum* sp. 'renovo', proved to be a high-growth, halophilic, thermotolerant strain and was also suggested for outdoor growth systems and biofuel production (Dahlin *et al.*, 2019).

Increasing photosynthetic activity of *Picochlorum* strain PMFPPE4 with temperature is consistent with results from research on sister algal genera, such as *Chlorella* (Yun & Park, 2003; Lee *et al.*, 2018) and also 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 for some river cyanobacteria species (Coles & Jones, 2000) and the dominant species in tropical alkaline saline lakes (Schagerl *et al.*, 2015), but is similar to some dominant saline diatom species (Lengyel *et al.*, 2015). The photosynthetic temperature optimum for *Picochlorum* sp. PMFPPE4 was measured at exactly 27.7°C, which is congruent with that of *P. maculatum*, which had maximum nutrients consumption at 28°C with a photoperiod of 18h light and 6h dark and 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Kumar *et al.*, 2019). Other *Picochlorum* strains showed optimum growth rates (although photosynthetic activity was not measured) at similar temperatures (25–30°C), and at higher temperatures growth rate declined (de la Vega *et al.*, 2011; Foflonker *et al.*, 2016). The potential mixotrophy of the species could be a reason for the moderate photosynthetic

activity under unfavourable environmental conditions (e.g. low temperature). Green algae typically prefer higher light intensities ($\sim 80\text{--}500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$; Reynolds, 1988; Padišák, 2004) than our findings, but there are also species with a preference for shade (e.g. *Picocystis salinarum*; Roesler *et al.*, 2002). The low light intensity preference along the temperature gradient, with good light utilization, suggests PMFPPE4 can tolerate or even prefer a light-limited habitat. Conversely, the higher DGDG:MGDG ratio found in our strain indicates it can grow under higher light conditions (Mock & Kroon, 2002). Other *Picochlorum* strains, such as *Picochlorum* sp. HM, *Picochlorum* sp. 'solocismus' and *Picochlorum* sp. 'renovo' also show tolerance of a wide range of light intensities (de la Vega *et al.* 2011; Dahlin *et al.* 2019; Gonzalez-Esquer *et al.* 2019). Our findings, including survival of *Picochlorum* sp. PMFPPE4 in dark conditions for an extended period, further suggest that the ecological niche of this picoalga is wide, in regard to both light regimes and temperature, and that it stores sufficient TAGs in reserves to survive unfavourable conditions. Additionally, a record of PPEs below the photic zone (Babić *et al.*, 2017) during the BIOTA cruise suggest a possible switch of *Picochlorum* sp. PMFPPE4 to a mixotrophic lifestyle in an unfavourable growth environment. The accumulation of PPEs at 280 m depth was not significantly correlated with any 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 caused vertical instabilities and transport of water parcels from surface to deep layers, which could have been responsible for the occurrence of the maximum of PPEs at depths below the euphotic zone. Selective pressures on preserving photoautotrophic machinery can be relaxed under certain conditions, such as when the energy costs of maintaining the photosynthetic apparatus outweigh the benefits of its products. Picoeukaryotes can use phagocytosis in cases of mixotrophy/heterotrophy (Massana & Logares, 2013) and additionally, mixotrophy can be achieved through osmotrophy (the uptake of dissolved organic substrates; Pringsheim, 1963; Glibert & Legrand, 2006). Given the general morphology of the *Picochlorum* sp. PMFPPE4 strain (no observed flagella or apparatus for catching prey) and that there is no record of toxin production for prey immobilization and/or disability in the available literature, we can assume that in unfavourable conditions this organism acts as an osmotroph.

This study identified a pico green alga isolated from the south-eastern Adriatic Sea, strain PMFPPE4, as 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 are broad: from shaded (and completely dark) and cooler marine environments ($5\text{--}10^\circ\text{C}$), where it may act as an osmotroph; to higher light intensity ($114.3 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and temperature ($30\text{--}40^\circ\text{C}$), where it has photosynthesis activity maxima and finally photoinhibition ($> 40^\circ\text{C}$). The low light intensity preference along a temperature gradient, with good light utilization, suggests this strain can tolerate a light-limited habitat. In regard to lipids, the most important finding was the ability of the strain to synthesize large amounts of triacylglycerids, which are important for biofuel feedstock and the food industry, and its high DGDG/MGDG ratio which indicates it can tolerate high light conditions. This exceptional strain can also serve as a model green algae in diverse ecological experiments, due to its longevity in cultured conditions.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at [10.1080/09670262.2020.1757763](https://doi.org/10.1080/09670262.2020.1757763)

Supplementary table 1: List of all taxa with strain information and GenBank accession numbers used for phylogeny inference in this study

Supplementary fig. 1: 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).

Author contributions

M. Mucko: original concept, writing and editing manuscript, morphological analyses, culture establishment and experiments and phylogeny analyses and plotting; J. Padišák: 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 phylogeny; Z. Ljubešić: original concept, writing and editing manuscript.

Supplementary Material

Supplemental data for this article can be accessed [here](#).

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