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Investigating the Potential of Newly Isolated Microalgae Strains from the Ionian Sea (Greece) Cultured in an Open Raceway Pond

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Abstract: Microalgae are a subject of interest not only for fundamental research but for various biotechnological applications as well. In this study, the ability of newly isolated strains, i.e., *Picochlorum costavermella* VAS2.5, *Picochlorum oklahomense* SAG4.4, *Microchloropsis gaditana* VON5.3, and *Nephroselmis pyriformis* PAT2.7, to grow when cultured in an open raceway pond under laboratory conditions and produce various metabolites of high-added value was evaluated. *N. pyriformis* PAT2.7 and *P. costavermella* VAS2.5 were the greatest in biomass production (exceeding 0.4 g/L), while *P. costavermella* VAS2.5 and *M. gaditana* VON5.3 were the greatest in lipid production (reaching approximately 18%, wt/wt). The lipid fraction of glycolipids and sphingolipids was predominant (43.6–55.4%, wt/wt), followed by neutral lipids (27.1–50.1%, wt/wt) and phospholipids (6.9–17.4%, wt/wt). *Picochlora* and *M. gaditana* VON5.3 lipids were rich in $^{\Delta 5,8,11,14,17}$C20:5 and/or $^{\Delta 9,12,15}$C18:3, while *N. pyriformis* PAT2.7 synthesized $^{\Delta 9}$C16:1 in large quantities (30–40%, wt/wt). All strains showed remarkable yields in polysaccharide and protein production, demonstrated a well-balanced amino acid profile, and synthesized pigments in amounts comparable to other studies. The biochemical profiles of these strains showcased their suitability for use primarily in the aquaculture sector.

Keywords: *Picochlorum*; *Microchloropsis*; *Nephroselmis*; raceway pond culture; aquaculture; biomass production; total lipid and lipid fraction fatty acid composition; proteins and essential amino acids; polysaccharides; pigments

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1. Introduction

Microalgae are oxygenic photosynthetic microorganisms that possess features that enlist them as a group of high ecological and commercial significance [1–4]. In general, microalgae can grow on simple nutrient media and in relatively simple set-ups, occupy little space, and adapt to a wide range of conditions regarding temperature, salinity, or nutrient supply. More importantly, microalgae can produce metabolites of high-added value, including lipids, usually containing polyunsaturated fatty acids (PUFA) in significant quantities; proteins and essential amino acids for humans or fish; and polysaccharides,

mainly starch-like, which can be used in various applications, e.g., as a food additive or in biofuel production [2]. Microalgae are also widely used as a source of pigments [5–7]. For example, microalgae carotenoids are widely used in the food industry, and fish feed since they improve the appearance of fish flesh, as well as in cosmetics and pharmaceuticals for their antioxidant capacity [2,8].

Since the 1950s, various microalgae have been increasingly used in large-scale applications. Aquaculture is one of the major sectors that utilize microalgae, as these are essential for the diet of zooplankton, which is used in the larvae feed [9,10]. Additionally, they can be incorporated into fish feed to replace part of fishmeal or to supply the highly unsaturated fatty acids (HUFA), especially n-3 eicosapentaenoic acid (EPA, $^{\Delta 5,8,11,14,17}$C20:5) and n-3 docosahexaenoic acid (DHA, $^{\Delta 4,7,10,13,16,19}$C22:6), which are known for their beneficial effects against neurological disorders, inflammation, cardiovascular disease, and even cancer [11,12]. In a different light, microalgal lipids that contain more saturated fatty acids can be channeled toward biodiesel manufacture since such lipids are resistant to oxidation and yield more energy during combustion [13]. Unfortunately, only a few strains belonging to the genera of *Chlorella*, *Dunaliella*, or *Haematococcus* (Chlorophyta) are cultivated on a large scale due to their generally high production costs, so research into optimizing microalgae cultivation has intensified over the last 10 years [14–17].

A sustainable alternative for large-scale applications of microalgae could be utilizing locally sourced (indigenous) strains, which might be more sustainable compared with relying on commercial strains [18–20]. In contrast to commercial strains, indigenous strains are expected to have developed special characteristics to be well-adapted to their natural environment, which could potentially offer benefits such as better adaptation to local conditions, and, therefore, reduced costs for their cultivation due to less reliance on external inputs like fertilizers, freshwater, etc., and enhanced environmental sustainability [21]. More importantly, the risk of altering the local microbiota is significantly reduced when indigenous strains are used, and at the same time, the balance of the local ecosystem is protected. Furthermore, the study of new strains can further expand the current knowledge, even in terms of basic research.

The aim of the present research was to assess the biotechnological potential of selected marine microalgae strains isolated from coastal areas of the Ionian Sea (Greece) for large-scale applications. Microalgae like *Picochlorum costavermella* and *Nephroselmis pyriformis* (Chlorophyta) are not well-studied; thus, this research can offer new data regarding their physiology, while the need to find strains of known others that mark high yields, like *Microchloropsis gaditana* (formerly known as *Nannochloropsis gaditana*) (Eustigmatophyceae), is of high importance, as stated earlier. The microalgae strains were cultured in a laboratory-scale paddle wheel open raceway pond-simulating photobioreactor in order to study their ability to grow in this type of bioreactor, which is quite popular for large-scale applications.

2. Materials and Methods

2.1. Biological Material and Culture Conditions

The microalgal strains *Picochlorum costavermella* VAS2.5, *Picochlorum oklahomense* SAG4.4, *Microchloropsis gaditana* VON5.3, and *Nephroselmis pyriformis* PAT2.7, which were previously isolated from coastal areas of the Ionian Sea of Greece and molecularly identified [21], were used as biological material in this study. In brief, filtration of seawater samples of 0.5 L through membranes of diameter 47 mm and pore size 0.2 μm occurred, which were transferred onto the surface of modified Artificial Sea Water (mASW) (Table 1), which was solidified with agar (Biolab Zrt, Budapest, Hungary) added at 1.5% (wt/vol) and contained in 90 mm diameter Petri dishes. After 7 days of incubation at 25 ± 1 °C and under constant illumination of 300 μE/m^2·s, provided by linear fluorescent daylight tubes T5, 8 W,

6500 K, cells from green colonies were transferred to new Petri dishes containing solidified mASW, following the streak plate method. This procedure was repeated consecutively (i.e., up to 10 times) until mono-algal but non-axenic strain isolation. It is noted that the use of antibiotics (i.e., ampicillin and streptomycin sulfate, both at concentration 0.05 g/L) was quickly discontinued since it turned out to defective in the elimination of the diverse bacterial populations, while microalgae seemed to be stressed after their extended use. The extermination of rotifers, when present, was accomplished by adding 0.08 g/L urea in the medium, and diatoms' growth was inhibited through the addition of 0.01 g/L GeO_2. During the time of treatment (i.e., approximately 3 days) with these chemicals, microalgal growth did not seem to be affected. Molecular identification of the microalgal isolates was performed by PCR amplification of the 18S rRNA and ITS (internal transcribed spacer) gene markers. Both isolation and molecular characterization processes are described in detail in [21].

Table 1. The composition of the modified Artificial Sea Water (mASW) used as growth medium for all cultures performed in this study. The growth medium has a 33 ‰ salinity.

Compound	**Supplier**	**Concentration (g/L)**
NaCl	PENTA (Prague, Czech Republic)	27.0
$MgSO_4 \cdot 7H_2O$	PanReac AppliChem (Darmstadt, Germany)	6.6
$CaCl_2$	PENTA	1.5
KNO_3	Scharlau (Barcelona, Spain)	1.0
KH_2PO_4	Himedia (Mumbai, India)	0.07
$FeCl_3 \cdot 6H_2O$	BDH (Poole, UK)	0.014
Na_2EDTA	Merck (Darmstadt, Germany)	0.019
Microelement solution Compound	**Supplier**	**Concentration (mg/L)**
$ZnSO_4 \cdot 7H_2O$	Merck	40.0
H_3BO_3	Fluka (Steinheim, Germany)	600.0
$CoCl_2 \cdot 6H_2O$	Sigma–Aldrich (St. Louis, MO, USA)	1.5
$CuSO_4 \cdot 5H_2O$	BDH	40.0
$MnCl_2$	Sigma–Aldrich	400.0
$(NH_4)_6MO_7O_{24} \cdot 4H_2O$	Sigma–Aldrich	370.0

A paddle wheel, open raceway pond (ORWP) photobioreactor was used as an open-type pilot-scale reactor for the mono-algal but non-axenic cultures of the aforementioned microalgal strains under non-aseptic conditions (Figure 1). The photobioreactor had the following dimensions: an outside length of 110.5 cm and an inside length of 80 cm, with a width of 61 cm. Its total depth was 20 cm, while the culture depth specifically measured 7.5 cm. The photobioreactor had a capacity of 100 L and a working volume (V_w) of 40 L. This reactor simulated an open pond with mechanical agitation through the rotation of a double 4-bladed paddlewheel driven by an electric motor rotating at 35 rpm. Inside the reactor, there was a horizontal partition 80 cm long, creating two hollow partitions and two circulation channels (Figure 1). This photobioreactor was made of stainless steel, which is resistant to corrosion from salt water.

Pre-cultures were performed at a scaled level, initially from 0.1 L (in 0.25 L Erlenmeyer flasks) to aquarium-type tanks V_w = 6 L prior to inoculation of the cultures at 40 L with initial culture density of $1.5 \cdot 10^6$ cells/mL (i.e., 0.01–0.03 g/L of dry biomass, depending on the microalgal strain). The growth medium was modified Artificial Sea Water (mASW),

which was sterilized in an autoclave at 121 °C for 20 min. The composition of mASW is presented in Table 1.

Figure 1. Cultures of selected microalgae in an open raceway pond photobioreactor (ORWP) (V_w = 40 L). The photobioreactor has the following dimensions: an outside length of 110.5 cm and an inside length of 80 cm, with a width of 61 cm. Its total depth is 20 cm, while the culture depth specifically measured 7.5 cm. Culture conditions: modified artificial seawater (mASW), pH: 8.5, temperature: 23 ± 1 °C, photoperiod: 24:0, light intensity: 27–34 μE/(m^2·s), agitation: 35 rpm by using a double 4-bladed paddlewheel.

In all experiments, the pH remained at the value of about 8.5 with the addition of NaOH 2.5 N. The temperature was maintained at 23 ± 1 °C using a submersible stainless steel aquarium heater of type BPS-6084 Waterproof Aquarium Heater 50 W Stainless Steel 20 cm with 2 Suction Cups (BPS, Toledo, Spain). Both pH and temperature values were monitored through the use of a Hanna Instruments HI8424N Portable pH/ORP/Temperature Meter (Hanna Instruments, Woonsocket, RI, USA). Continuous illumination of 27–34 μE/(m^2·s) was provided to the cultures from three LED lamps (Phillips, Eindhoven, The Netherlands) (120 cm, 36 W). It is noted that the variation in light intensity was due to the internal lighting of the room that housed the bioreactor as well as the natural sunlight entering the room. KERN Analog Refractometer was used for salinity measurement. It is noted that the V_w, thus the culture depth of 7.5 cm, of each experiment was maintained constant by recording the height of the culture daily and adding sterile deionized water to balance the volume loss due to evaporation.

2.2. Growth and Biomass Determination

Growth of microalgae was estimated through total dry biomass concentration (expressed as x, g/L) determination, which was held by filtration of 10 mL of the culture through a 0.45 μm membrane filter. The estimation of growth parameters was held by ap-

plying the experimental data on the integrated version of Verhulst's model (i.e., Sigmoidal Logistic function) (1):

$$x_t = x_{max}/(1 + b \cdot e^{1/\mu \cdot t}) \quad (1)$$

where x represents dry biomass, in g/L, of microalgae at time t, b is a positive constant equal to $(x_{max} - x_0)/x_0$, where x_0 is the initial dry biomass in g/L), x_{max} is the carrying capacity of the system, and μ is the specific growth rate (1/d). The parameter values were determined by fitting Equation (1) to the experimental data using the Levenberg–Marquardt method for parameter value optimization. The minimization of the residual root mean square error between the experimental and model-predicted data, quantified by the values of the coefficient of determination R^2, was used as a criterion for parameter optimization.

Biomass determination at selected timepoints (i.e., 10 and 19 days) of the culture was held after harvesting of the microalgal cells by centrifugation at 7455× g for 10 min at 4 °C (NÜVE NF 800R, Ankara, Turkey) since its collection in quantity was essential for the rest of the carried out analyses regarding the biochemical profile of the isolates. Prior to the gravimetric determination of dry biomass, the harvested cells were washed twice with deionized water followed by centrifugation under the aforementioned conditions, collected, and dried at 80 °C until constant weight.

2.3. Lipid Extraction and Purification

Total microalgal lipids were extracted using a 2:1 (vol/vol) mixture of chloroform (PENTA)/methanol (Fisher Chemical, Hampton, VA, USA), in accordance with a modified version of the Folch et al. (1957) [22] method, as adapted by Dourou et al. (2017) [23]. The extracts were filtered through Whatman No. 1 paper and washed with a 0.88% (wt/vol) KCl (Merck) solution to remove non-lipid components, such as lipoproteins and pigments. It is noted that for prevention of the sample from moisture interference, Na_2SO_4 (Sigma–Aldrich) was added. Following, the solvent was evaporated under vacuum using a Rotavapor R-210 evaporator (BUCHI, Flawil, Switzerland), enabling the gravimetric quantification of total cellular lipids, expressed as a percentage of dry biomass (L/x%, wt/wt).

2.4. Lipid Fractionation

Approximately 100 mg of microalgal lipids were dissolved in 1 mL of chloroform and separated through fractionation using a 25 × 100 mm column packed with 1 g of silicic acid (Fluka). It is noted that prior to this, silicic acid had been activated by heating at 80 °C overnight. The fractionation process involved sequential washes with 100 mL of dichloromethane (Sigma–Aldrich), 100 mL of acetone (Fluka), and 50 mL of methanol (Sigma–Aldrich) to obtain neutral lipids (NL), glycolipids and sphingolipids (G+S), and phospholipids (P), respectively [24]. Each lipid fraction (NL, G+S, and P) was quantified gravimetrically and expressed as a percentage of the total lipids after the solvents were evaporated under vacuum.

2.5. Fatty Acid Composition of Cellular Lipids

Total lipids and their fractions were converted into fatty acid methyl esters (FAMEs) and examined using an Agilent 7890A Gas Chromatography (GC) system (Agilent Technologies, Shanghai, China) to determine their composition in fatty acids. The GC apparatus featured a flame ionization detector set at 280 °C and an HP-88 column (J&W Scientific, Folsom, CA, USA) measuring 60 m × 0.32 mm. Helium served as the carrier gas at a flow rate of 1 mL/min while the analysis proceeded at 200 °C. Identification of peaks of FAMEs was carried out by comparing them to authentic standards.

2.6. Polysaccharide Determination

To determine polysaccharide content, 20–25 mg of fat-free biomass (x_f) was hydrolyzed with 5 mL of 2.5 M HCl (Sigma–Aldrich) at 100 °C for 60 min. After cooling at room temperature, the hydrolysate was neutralized using 2.5 M KOH (Sigma–Aldrich). Following, the hydrolysate was filtered through Whatman No. 1 paper so as to remove cellular debris. The reducing sugars, expressed as glucose, were analyzed in line with the DNS method [25]. Intracellular polysaccharides, including both storage and structural forms, were quantified as glucose equivalents and expressed as a percentage of dry biomass (S/x%).

2.7. Protein and Amino Acid Profile Determination

The total cellular protein content was assessed following the biuret method, as detailed in a previous study [24]. Protein concentration was quantified in terms of albumin equivalents and expressed as a percentage of dry biomass (P/x%).

The amino acid composition of the microalgae was determined following acid hydrolysis (6 N, 110 °C, 24 h) and derivatization using AccQ-Tag™ Ultra, in line with the amino acid analysis application solution (Waters Corporation, Milford, MA, USA) and Kotzamanis et al. (2020) [26]. DL-Norvaline (Sigma) 2.5 mM was used as an internal standard. Ultra-Performance Liquid Chromatography (UPLC) was conducted on an Acquity system (Waters Corporation) equipped with a photodiode array (PDA) detector, with the detection wavelength set to λ = 260 nm. A BEH C18, (100 mm × 2.1 mm i.d., 1.7 μm) from Waters was used, with a flow rate of 0.7 mL/min and the column temperature was maintained at 55 °C. Peak identification and integration were carried out using Empower v.2.0 software (Waters), employing Amino Acid Standard H (Thermo Scientific, Waltham, MA, USA) as an external standard. All analyses were performed in duplicate. If the replicate values did not meet the standardized acceptance criteria based on mean and standard deviation (<5%), new duplicate analyses were carried out in accordance with established protocols. It is noted that tryptophan was not quantified due to its sensitivity to acid hydrolysis, while cysteine reacted with cysteine, forming a disulfide bond to generate cystine.

In addition, during acid hydrolysis, asparagine is converted to aspartate (i.e., the ionic form of aspartic acid) and glutamine to glutamate (i.e., the ionic form of glutamic acid). Therefore, the reported values for these amino acids represent the sum of both amino acids.

2.8. Pigment Estimation

Approximately 0.5 g of wet biomass was dissolved in 10 mL of 95% ethanol (vol/vol, Fisher Chemical) prepared using Milli-Q water (Honeywell, Charlotte, NC, USA). The mixture was centrifuged at 13,300× g for 15 min at 4 °C. Subsequently, 0.5 mL of the supernatant was collected and mixed with 4.5 mL of the solvent [27]. Eventually, spectrophotometric analysis of the final solution at λ = 470 nm, λ = 649 nm, and λ = 664 nm occurred to determine chlorophyll a, chlorophyll b, and carotenoid concentrations using a quartz cuvette (1 cm^2), with the ethanol solution serving as the blank sample.

Equations (2)–(4) used for the pigment quantification (in μg/mL) were the following [27]:

$$C_a = (13.36 \cdot A_{664}) - (5.19 \cdot A_{649}) \quad (2)$$

$$C_b = (27.43 \cdot A_{649}) - (8.12 \cdot A_{664}) \quad (3)$$

$$C_{x+c} = ((1000 \cdot A_{470}) - (2.13 \cdot C_a) - (97.63 \cdot C_b))/209 \quad (4)$$

where A represents Absorbance and C_a, C_b, and C_{x+c} stand for chlorophyll a, chlorophyll b, and carotenoids, respectively. Pigments, in terms of Total Chlorophylls (TCh) and Total Carotenoids (TC), were expressed as percentages in the dry biomass.

It has to be taken into account that since *M. gaditana* VON5.3 contains only chlorophyll a [28], the results provided in Table 1 refer only to chlorophyll a and not the sum of chlorophyll a and chlorophyll b.

2.9. Phosphorus and Nitrogen Uptake

Samples of the microalgae cultures were filtered through a 0.45 μm membrane filter (0.45 μm Millipore filters, GN-6 Metricel Grid 47 mm, Pall Corporation) to remove cells and analyzed for orthophosphate (PO_4^{3-}) and nitrate (NO_3^--N) concentration according to Standard Methods (APHA) [29]. Orthophosphate and nitrate concentrations were determined according to the ascorbic acid method (4500-PE) and the Ultraviolet Spectrophotometric Method (4500-NO_3^- B), respectively. More specifically, for the nitrate determination the samples were properly diluted, and then the absorbance at 220 nm was measured using a quartz cuvette. For the orthophosphate determination, the samples were appropriately diluted, and then for every sample (i.e., 50 mL), 8 mL of combined reagent, consisting of sulfuric acid 5 N, antimony potassium tartrate solution 0.004 M, ammonium molybdate solution 0.032 M, and ascorbic acid solution 0.1 M, were added to form the reduced blue antimony–phospho–molybdate complex. The absorbance at 880 nm was measured after stirring (10–20 min) in a plastic cuvette. Artificial saline water with the same salinity (i.e., 33 ‰) as the culture medium was used for the construction of both the nitrate and the orthophosphate standard curves.

2.10. Microscopy

Cell morphology and size were observed using an optical Euromex Microscope BioBlue BB.4267 (Arnhem, The Netherlands), equipped with a digital camera and the ImageFocus Plus v2.4.9.0 capture and analysis software.

2.11. Data Treatment & Statistical Analysis

The experimental data from microalgal cultures and growth kinetics were treated using OriginPro 2021 9.8.0.200®, 1991–2020 (OriginLab Corp., Northampton, MA, USA).

3. Results and Discussion

3.1. Growth and Biomass Production

Cultures were carried out in an ORWP photobioreactor for selected microalgal strains, namely *P. costavermella* VAS2.5, *M. gaditana* VON5.3, *P. oklahomense* SAG4.4, and *N. pyriformis* PAT2.7. Specifically, the selection of the four strains, previously isolated and cultured under laboratory conditions [21], was based on their interesting biochemical features as well as the lack of sufficient relative literature data. These cultures had been carried out in bioreactor volumes ranging from 0.1 L to 2 L.

The ORWP photobioreactor was chosen because it is the most commonly used for commercial microalgae production, as the shear stress exerted on the cells is limited due to the reactor configuration and the low rotational speeds of the paddles of the wheel. In addition, an ORWP has a simple design, low construction, maintenance, and repair costs, and does not have any special technology or energy demands [30]. Intriguingly, the mono-algal but non-axenic cultures performed herein proved resistant to external infections regarding protozoa, rotifers, etc., indicating well-adaptation of the microbial communities that were isolated from their natural habitats. The mASW used as a growth medium had a high salinity (i.e., 33‰), simulating natural seawater (i.e., the salinity of 31–37.9‰) of the Amvrakikos Gulf and the Gulf of Patras, two of the major gulfs of the Ionian Sea, from where most of the strains used herein were originally isolated [31,32]. Also, mASW had an N–P ratio ≅ 9, which was expected to be sufficient for biomass

production and synthesis of high-added-value compounds in considerable amounts [33]. As shown in Table 2, nitrate uptake did not exceed 40%. Low nitrate uptake rates were also recorded when *Chlorella vulgaris* CCAP 211/11B was cultured under different conditions on a nitrogen-rich medium [34]. Nevertheless, it must be taken into consideration that the photometric method used for the nitrate determination potentially poses a risk of inaccuracy in measurements since, at 220 nm, interference with several other substances may occur. Regarding orthophosphate uptake, rates in the range of 60–80% were observed (Table 2), which are similar to those reported by Patel et al. (2012) [35]. These results suggest that a growth medium with lower initial nitrogen and phosphorus concentrations and a lower N–P ratio, compared with the growth medium used in this study, could be used without affecting biomass growth and bioproduct synthesis and accumulation.

Microalgal growth, biomass production, and reserve material synthesis were examined after 10 and 19 days of culture (approximately 450 h) (Figure 2, Table 2). *N. pyriformis* PAT2.7 and *P. costavermella* VAS2.5 produced similar levels of biomass (i.e., 0.42 ± 0.08 g/L and 0.41 ± 0.00 g/L, respectively). Biomass production by *M. gaditana* VON5.3 was slightly lower (0.36 ± 0.04 g/L) compared with the above-mentioned strains, while *P. oklahomense* SAG4.4 exhibited the lowest biomass yield (0.21 ± 0.01 g/L). The aforementioned observations can be correlated with the cell size of each strain, which possibly affects the weight of total biomass. As shown in Figure 3, *Nephroselmis* cells are almost twice the size and up to 1.8 times larger compared with *Picochlorum* and *Microchloropsis* cells, respectively, which might partially explain why *N. pyriformis* PAT2.7 gained the highest final biomass production.

P. costavermella VAS2.5 exhibited lower biomass productivity rates compared with other strains of the genus, while even lower yields were recorded for *P. oklahomense* SAG4.4. For example, the biomass productivity of *Picochlorum renovo* NREL39-A8 and *Picochlorum celeri* TG2-WT-CSM/EMRE cultivated in an ORWP of V_w = 100 L, having 0.2 m of culture depth, was 16.1 g/(m^2·d) and 22.1 g/(m^2·d), respectively [36]. The authors of the above study recorded high biomass productivities during the warm season when sunlight was abundant and the cultures were supplied by 1500–2000 μmol/(m^2·s) (i.e., equal to 2000 μE/(m^2·s)). Association with the season of cultivation was recorded for another *P. celeri* strain as well [37]. On the other hand, when a *Picochlorum* sp. was cultivated in a 1 L Duran bottle and an ORWP having the available light intensity of 600 μE/(m^2·s) and 2256 μE/(m^2·s), respectively, the former culture produced significantly higher amounts of biomass [38]. The authors of the aforementioned investigation attributed these observations to the small optical path and the more efficient mixing in the Duran bottle.

As regards *M. gaditana* VON5.3, the strain used in the current paper noted similar or higher productivity rates when compared with other *Microchloropsis* (i.e., *Microchloropsis gaditana* and *Microchloropsis salina*) and *Nannochloropsis* strains. For example, when *Microchloropsis salina* (formerly known as *Nannochloropsis salina*) CCMP 1776 was cultivated in F/2 medium in a conventional raceway pond, p_x = 0.0013 g/(L·d) was recorded even though the culture was exposed to up to 2000 μE/(m^2·s) during clear and sunny days [39]. Biomass production of *M. gaditana* VON5.3 was similar to the *Nannochloropsis oculata* used by Millán-Oropeza and Fernández-Linares (2017) [6] during a short time during the winter season and during spring. In contrast, *Nannochloropsis* sp. growing in a medium enriched, or not, with a combination of vitamin supplements gained biomass in the range of 0.9 g/L, although microalgal growth seemed to be unaffected by the presence of vitamins in the growth medium [38]. Additionally, in a recent study, water-submerged LEDs were placed at the bottom of an ORWP, as artificial lighting poses as one of the major growth-controlling factors in microalgae cultures in an ORWP and is also a promising solution to limit dark zones and enhance microalgal productivity for the culture of *Nannochloropsis oceanica*. How-

ever, the extra lighting affected biomass production only to a limited extent and under low sunlight irradiances [5].

Lastly, to our knowledge, strains that belong to *Nephroselmis* have not been cultured in ORWP. However, *N. pyriformis* PAT2.7 presented similar or even higher yields regarding biomass production in comparison to various lab-scale investigations. For instance, *Nephroselmis* sp. produced biomass that ranged from 0.09 to 0.13 g/L when was cultured under different values of illumination, salinity, and nitrogen content in the growth medium [40]. Similar biomass production (i.e., x = 0.45 g/L) was obtained when *Nephroselmis* sp. KGE2 was cultivated on livestock wastewater effluent (LWE) and was slightly higher under growth medium supplementation with acid mine drainage in 5% and 10% (i.e., x = 0.59 g/L and x = 0.58 g/L, respectively) [41]. In contrast, when *Nephroselmis* sp. KGE1 was cultivated in 500 mL Erlenmeyer flasks on BBM growth medium under white fluorescent light illumination at 100 μE/(m^2·s) and 27 °C for 3 weeks, it gained more than 1 g/L of biomass [42].

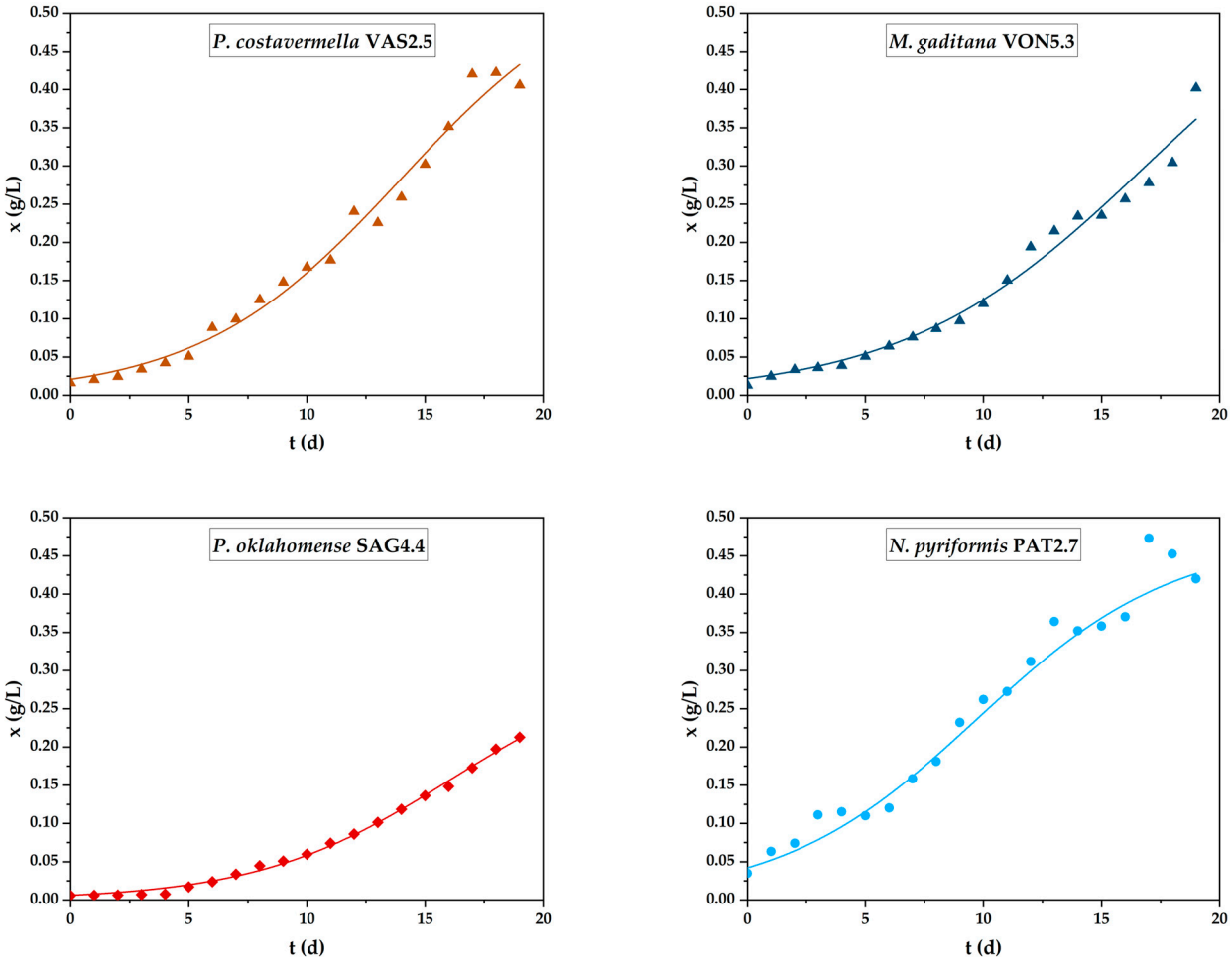

Figure 2. Growth curves of the four microalgal strains for cultivation in an open raceway pond photobioreactor (ORWP) (V_w = 40 L). The name of the microalgae strain is indicated in the footnote of the individual graphs per case. Culture conditions: modified artificial seawater (mASW), pH: 8.5, temperature: 23 ± 1 °C, photoperiod: 24:0, light intensity: 27–34 μE/(m^2·s), agitation: 35 rpm by using a double four-bladed paddlewheel.

Table 2. Biomass production, reserve material accumulation, growth parameters, and nutrient uptake from the cultures performed in an open raceway pond photobioreactor (V_W = 40 L) after 10 and 19 days of culture. Culture conditions: modified artificial seawater (mASW), pH: 8.5, temperature: 23 ± 1 °C, photoperiod: 24:0, light intensity: 27–34 μE/(m^2·s), agitation: 35 rpm by using a double four-bladed paddlewheel.

Strain	t (d)	Biomass (x)		Lipids (L)				Polysaccharides (S)	Proteins (P)	Pigments		Growth Parameters			Nutrients Uptake	
		x	p_x	L/x	Lipid Fractions (%)			S/x	P/x	TCh/x	TC/x	x_{max}	μ	R^2	NO_3^--N	PO_4^{3-}
		(g/L)	(g/L·d)	(%)	NL	G+S	P	(%)	(%)	(%)	(%)	(g/L)	(1/d)		(%)	(%)
P. costavermella VAS2.5	10	0.21 ± 0.04	0.02 ± 0.00	12.0 ± 2.1	UND	UND	UND	7.3 ± 0.3	24.8 ± 0.9	UND	UND	0.57 ± 0.06	0.23 ± 0.02	0.98	UND	UND
	19	0.41 ± 0.00		18.5 ± 1.4	27.1 ± 0.9	55.4 ± 6.4	17.4 ± 5.6	7.7 ± 0.1	25.4 ± 2.1	3.2 ± 0.1	1.5 ± 0.3				23.0 ± 6.9	60.4 ± 0.8
P. oklahomense SAG4.4	10	0.17 ± 0.00	0.01 ± 0.00	23.2 ± 0.9	UND	UND	UND	11.6 ± 1.3	21.8 ± 2.0	UND	UND	0.31 ± 0.02	0.24 ± 0.01	1.00	UND	UND
	19	0.21 ± 0.01		13.2 ± 1.1	38.0 ± 6.4	44.6 ± 8.5	17.4 ± 2.1	14.7 ± 0.7	23.4 ± 2.5	5.0 ± 0.6	1.6 ± 0.1				14.8 ± 3.1	63.6 ± 8.3
M. gaditana VON5.3	10	0.20 ± 0.02	0.02 ± 0.00	18.7 ± 1.5	UND	UND	UND	10.7 ± 1.4	41.4 ± 6.1	UND	UND	0.60 ± 0.15	0.19 ± 0.03	0.98	UND	UND
	19	0.36 ± 0.04		18.3 ± 2.3	32.4 ± 2.2	51.9 ± 0.5	15.7 ± 1.7	9.0 ± 0.2	29.8 ± 1.7	4.0 ± 0.5	1.3 ± 0.2				37.8 ± 3.9	79.2 ± 4.1
N. pyriformis PAT2.7	10	0.18 ± 0.01	0.02 ± 0.00	7.2 ± 0.4	UND	UND	UND	10.5 ± 0.5	47.3 ± 2.3	UND	UND	0.47 ± 0.04	0.23 ± 0.03	0.97	UND	UND
	19	0.42 ± 0.08		7.3 ± 0.3	50.1 ± 0.4	43.6 ± 0.7	6.9 ± 0.2	14.3 ± 0.3	49.7 ± 4.9	1.0 ± 0.2	0.2 ± 0.0				29.9 ± 4.2	80.0 ± 5.1

Abbreviations: t (d), days; x (g/L), dry biomass; p_x (g/L·d), volumetric biomass productivity; L/x (%), lipids on dry biomass; NL (%), neutral lipid fraction on total lipids; G+S (%), glycolipid and sphingolipid fraction on total lipids; P (%), phospholipid fraction on total lipids; S/x (%), intracellular polysaccharides on dry biomass; P/x (%), intracellular proteins on dry biomass; TCh/x (%), total chlorophyll (chlorophyll a and b) on dry biomass; TC/x (%), total carotenoids on dry biomass; x_{max} (g/L), carrying capacity of the system; μ (1/d), maximum specific growth rate; R^2, R-squared statistical measure; and UND, undetermined.

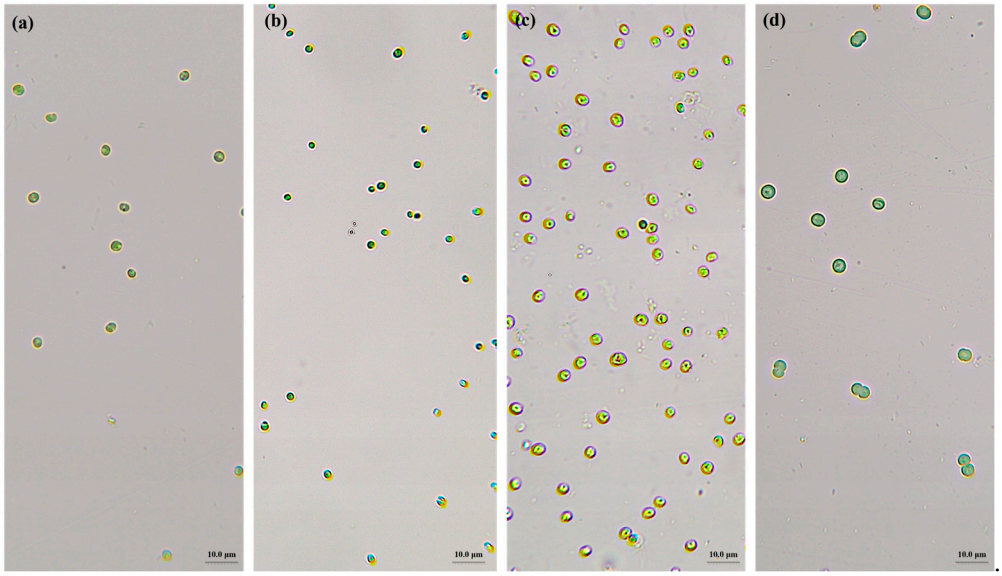

Figure 3. Morphology of cells of the four microalgal strains under an optical microscope after their cultivation in mASW. (**a**) *Picochlorum costavermella* VAS2.5; (**b**) *Picochlorum oklahomense* SAG4.4; (**c**) *Microchloropsis gaditana* VON5.3; and (**d**) *Nephroselmis pyriformis* PAT2.7. Total magnification: 400×.

On the whole, the above should not come as a surprise, as illumination, a key factor for microalgal growth, is not very intense for bioreactors of photosynthetic microorganisms as they increase in scale, especially when light is supplied from the top of the ORWP, as in our set-up, and it is also strongly correlated with "mutual shading" of the cells [43]. It must be noted, though, that microalgal growth can be saturated in relatively low light intensities as well [44]. Another hypothesis for the relatively low productivity yields can be linked to the fact that in the carried-out cultures, no extra carbonate dissolved in the growth medium or gas CO_2 was provided in the cultures, which, in combination with the probable relatively low mixing rate of the cultures, could have led to carbon limitation.

Regardless, the cultures that were carried out led to the production of adequate biomass quantities, which enabled an extensive biochemical characterization, including the determination of (a) total lipids and their content in neutral and polar lipids (phospholipids, glycolipids, and sphingolipids) and also the fatty acid composition of total lipids and each lipid fraction, (b) protein content and amino acid profile, (c) polysaccharide content, and (d) pigment content, i.e., chlorophyll a, chlorophyll b, and carotenoids.

3.2. Synthesis of Storage Lipids and Fatty Acid Composition of Total Lipids and Lipid Fractions

The lipid content was at similar levels for *P. costavermella* VAS2.5 (L/x% = 18.5 ± 1.4%, wt/wt) and *M. gaditana* VON5.3 (L/x% = 18.3 ± 2.3%, wt/wt). *P. oklahomense* SAG4.4 also exhibited satisfactory lipid accumulation, though lipid reserves were decreased from L/x% = 23.2 ± 0.9% (wt/wt) at 10 days to L/x% = 13.2 ± 1.1% (wt/wt) by the end of the culture. This phenomenon has been observed elsewhere and was attributed to the reduction in photosynthesis due to the increase in cell density of the culture [24]. Thus, cells turned to the reserves, which can be used for energy conservation and/or further growth. The lowest lipid accumulation rate was recorded for *N. pyriformis* PAT2.7, which was L/x% = 7.3 ± 0.3% (wt/wt). In general, these results are very encouraging and indicate the potential of these newly isolated strains to produce even higher yields. Furthermore, it must be taken into account that the microalgae were grown on mASW, which was neither nitrogen nor phosphorus-limited (Table 2). Nitrogen and/or phosphorus limitation or absence from the growth medium usually triggers lipid accumulation [24]. Other influential factors for lipid content are temperature and light intensity, though in a species-

and strain-dependent manner [45]. For example, when *N. oculata* was cultured in an ORWP containing modified F/2 medium with ASW that had as a nitrogen source $NaNO_3$ 0.25 g/L (i.e., 0.18 g/L of nitrate) instead of KNO_3 1 g/L (i.e., 0.61 g/L of nitrate) of this study (Table 1), lipid accumulation ranged from 26 ± 1% to 44 ± 4% (wt/wt) and from 42 ± 5% to 44 ± 7% (wt/wt) during the winter and spring seasons, respectively [6]. Another important parameter affecting lipid biosynthesis is the growth phase of culture. For instance, the biomass of *N. oceanica* was mainly composed of protein and carbohydrates, with relatively low lipid contents at the late exponential phase [5]. According to the authors, low lipid contents suggested that the cells' growth occurred in the absence of nutrient stress, which was correlated with the physiological phase of the culture. Herein, as shown in Figure 2, most of the strains used at the time of biomass harvesting were in the exponential or late exponential phase.

Additionally, in Table 2, the percentages (%, wt/wt) of lipid fractions relative to total lipids are presented. The predominant lipid fraction was that of G+S (43.6–55.4%, wt/wt), followed by NL (27.1–50.1%, wt/wt) and finally P (6.9–17.4%, wt/wt). The predominance of the polar structural lipids (i.e., G+S and P) over the main storage neutral lipids was anticipated since it seems to be a common feature for many microalgae of various genera, like *Chlorella*, *Tetraselmis* (Chlorophyta), and *Nannochloropsis* (Eustigmatophyceae) [24,46]. The above-mentioned can be answered by the fact that glycolipids and phospholipids are the major components of chloroplast and thylakoid membranes, while sphingolipids constitute a diverse group of membrane lipids of high importance for various cell functions, including the control of cell division or communication with the cell's environment [47,48]. Both G+S and P lipid fractions are considered important sources of omega-3 fatty acids, mainly of $^{\Delta 9,12,15}$C18:3, $^{\Delta 5,8,11,14,17}$C20:5, and $^{\Delta 4,7,10,13,16,19}$C22:6. PUFAs, due to their exceptional structure, possess a key role in the regulation of membrane fluidity and function [49–52]. Moreover, PUFAs are of great commercial interest for the food, animal feed, and pharmaceutical industries, as they present beneficial effects against various threats to human and animal health, like cardiovascular and neurodegenerative diseases, while other studies have investigated their potential as anticancer agents with promising results [53–56]. In any case, several studies have concluded that glycolipid content could be increased under conditions of nitrogen or phosphorus deficiency [57–59]. In contrast, the dominant lipid fraction of *N. pyriformis* PAT2.7 was the NL fraction (i.e., 50.1 ± 0.4%, wt/wt), which represents mainly storage lipids, followed by the G+S fraction (i.e., 43.6 ± 0.7%, wt/wt). Some microalgae alter their lipid biosynthetic pathways towards the synthesis of more neutral lipids under stress conditions, as has occurred for *Scenedesmus* sp. (Chlorophyta), *Isochrysis galbana* (Coccolithophyceae), and *Diacronema lutheri* (formerly known as *Pavlova lutheri*) (Pavlovophyceae) under phosphorus limitation [60,61]. Moreover, in general, low light intensities favor polar lipid synthesis, while the opposite occurs under high light intensities [60]. Thus, it might be an indication that, herein, the supplied illumination for *N. pyriformis* PAT2.7 was relatively high. As mentioned previously, though, during the growth cycle, lipid content varies in quantity and quality as well. For example, during the stationary phase, as happened for the harvested biomass of *N. pyriformis* PAT2.7 in this study, an increased TAG level is often observed [45].

The composition of total lipids and their lipid fractions in fatty acids at the end of the cultures of the four microalgal strains are presented in Table 3.

Table 3. Fatty acid composition of total lipids (TL) and their lipid fractions (neutral lipids—NL, glycolipids—G, and phospholipids—P) of the selected four microalgal strains that were cultured in an open raceway pond photobioreactor (V_w = 40 L) after 19 days of culture. Culture conditions: modified artificial seawater (mASW), pH: 8.5, temperature: 23 ± 1 °C, photoperiod: 24:0, illumination: 27–34 µE/(m^2·s), agitation: 35 rpm by using a double four-bladed paddlewheel.

Strain	Lipid Fraction	Composition of Total Lipids and Lipid Fractions in Fatty Acids (%, wt/wt)											
		C14:0	**$^{\Delta 9}$C14:1**	**C16:0**	**$^{\Delta 9}$C16:1**	**C17:0**	**C18:0**	**$^{\Delta 9}$C18:1**	**$^{\Delta 9,12}$C18:2**	**$^{\Delta 9,12,15}$C18:3**	**$^{\Delta 6,9,12,15}$C18:4**	**$^{\Delta 13}$C20:1**	**$^{\Delta 5,8,11,14,17}$C20:5**
P. costavermella VAS2.5	TL	6.1 ± 0.2	3.1 ± 0.2	15.7 ± 0.1	27.9 ± 0.5	<0.5	<0.5	6.6 ± 0.0	3.0 ± 0.0	ND	ND	3.1 ± 0.0	23.4 ± 3.9
	NL	5.7 ± 0.6	0.9 ± 0.1	23.3 ± 1.0	33.0 ± 3.4	ND	2.5 ± 2.0	14.7 ± 3.9	2.2 ± 0.1	ND	ND	2.9 ± 0.9	10.0 ± 2.5
	G	8.5 ± 1.5	2.9 ± 0.2	16.8 ± 0.1	26.8 ± 1.3	<0.5	1.9 ± 0.4	6.5 ± 0.6	2.1 ± 0.6	ND	ND	2.0 ± 0.0	25.6 ± 1.6
	P	1.8 ± 0.2	1.0 ± 0.2	11.2 ± 0.3	23.8 ± 0.7	0.6 ± 0.1	1.6 ± 0.2	14.1 ± 0.7	5.9 ± 0.2	ND	ND	6.6 ± 0.1	31.1 ± 0.9
P. oklahomense SAG4.4	TL	1.7 ± 0.2	4.0 ± 0.0	14.8 ± 0.0	3.4 ± 0.2	7.3 ± 0.2	<0.5	13.8 ± 1.4	29.6 ± 2.0	16.6 ± 0.6	1.3 ± 0.7	ND	ND
	NL	5.0 ± 0.4	13.1 ± 0.4	9.3 ± 1.1	1.6 ± 0.1	1.4 ± 0.0	2.7 ± 1.0	6.9 ± 1.1	10.9 ± 0.9	9.4 ± 1.6	3.8 ± 1.9	ND	ND
	G	<0.5	0.5 ± 0.3	14.5 ± 2.8	2.1 ± 0.1	15.7 ± 0.1	<0.5	16.6 ± 0.3	17.6 ± 1.8	18.9 ± 0.8	2.6 ± 0.3	ND	ND
	P	<0.5	<0.5	16.4 ± 0.6	5.4 ± 0.1	2.6 ± 0.3	3.5 ± 1.7	11.2 ± 2.3	27.3 ± 1.7	14.7 ± 1.4	3.0 ± 0.0	ND	ND
M. gaditana VON5.3	TL	6.8 ± 0.1	3.1 ± 0.1	15.5 ± 1.1	31.5 ± 0.5	<0.5	<0.5	7.2 ± 0.3	2.6 ± 0.0	<0,5	ND	3.6 ± 0.2	22.7 ± 0.8
	NL	3.0 ± 0.7	0.9 ± 0.1	15.7 ± 3.3	17.9 ± 4.9	ND	<0.5	4.7 ± 0.2	1.8 ± 0.5	<0,5	ND	2.7 ± 0.8	7.6 ± 2.2
	G	8.2 ± 0.1	3.2 ± 0.0	15.3 ± 1.3	23.8 ± 1.8	ND	1.0 ± 0.2	3.5 ± 1.2	2.7 ± 1.3	ND	ND	2.0 ± 0.1	23.7 ± 0.7
	P	2.3 ± 0.1	0.5 ± 0.1	16.0 ± 0.9	23.5 ± 0.9	ND	0.9 ± 0.1	11.0 ± 0.4	4.5 ± 0.4	ND	ND	6.5 ± 1.0	22.2 ± 2.0
N. pyriformis PAT2.7	TL	32.7 ± 1.0	5.3 ± 0.0	8.9 ± 0.4	41.4 ± 1.6	ND	0.5 ± 0.2	3.3 ± 0.1	0.6 ± 0.0	ND	ND	ND	ND
	NL	35.8 ± 1.5	6.1 ± 0.2	10.6 ± 0.2	42.0 ± 1.2	ND	<0.5	2.1 ± 0.6	1.4 ± 0.0	ND	ND	ND	ND
	G	31.9 ± 0.2	6.0 ± 0.1	7.8 ± 0.4	39.1 ± 1.0	ND	0.9 ± 0.2	18 ± 0.7	<0.5	ND	ND	ND	ND
	P	16.2 ± 0.3	0.5 ± 0.0	14.8 ± 1.7	30.0 ± 2.8	ND	6.9 ± 1.3	21.7 ± 3.0	1.4 ± 1.0	ND	ND	ND	ND

Abbreviations: ND: not detected. Note: only glycolipids (i.e., G fraction) are mentioned as the amide bond of sphingolipids resists methanolysis during methyl esterification.

Regarding *P. costavermella* VAS2.5, the predominant fatty acid in TL is $^{\Delta 9}$C16:1 (27.9 ± 0.5%, wt/wt), followed by a significant amount of $^{\Delta 5,8,11,14,17}$C20:5 (23.4 ± 3.9%, wt/wt) and C16:0 (15.7 ± 0.1%, wt/wt). As shown in Table 3, $^{\Delta 9,12}$C18:2 was almost consumed, while $^{\Delta 9,12,15}$C18:3 was not detected. This is likely due to the activity of Δ6 and Δ5 desaturases and Δ6 elongase that led to the production of $^{\Delta 5,8,11,14,17}$C20:5 in significant amounts [1]. The NL lipid fraction showed a similar pattern to that of TL, except for the percentage of $^{\Delta 5,8,11,14,17}$C20:5, which was about half (10.0 ± 2.5%, wt/wt), whereas the percentage of $^{\Delta 9}$C18:1 was higher (i.e., 14.7 ± 3.9%, wt/wt against 6.6 ± 0.0, wt/wt, in TL). Similar composition and content to TL were noted for the lipid fractions G and P, with the difference of a higher percentage of $^{\Delta 5,8,11,14,17}$C20:5 (31.1 ± 0.9%, wt/wt) in the P fraction. In the case of *P. oklahomense* SAG4.4, the fatty acid $^{\Delta 9,12}$C18:2 was the dominant one in TL (i.e., 29.6 ± 2.0%, wt/wt), followed by significant amounts of $^{\Delta 9,12,15}$C18:3 (16.6 ± 0.6%, wt/wt), C16:0 (14.8 ± 0.0%, wt/wt), and $^{\Delta 9}$C18:1 (13.8 ± 1.4%, wt/wt). Intriguingly, in this *Picochlorum* strain, it seems that lipid biosynthesis practically was terminated in $^{\Delta 9,12,15}$C18:3, which can be considered as an indication of the absence of activity regarding Δ6, Δ5 desaturases, and Δ6 elongase [1]. In the lipid fraction of NL, the aforementioned fatty acids were present in low proportions, whereas $^{\Delta 9}$C14:1 was present in higher amounts compared with TL (i.e., 13.1 ± 0.4%, wt/wt against 4.0 ± 0.0%, wt/wt). In general, a similar picture was found for both G and P fractions, but in the case of the lipid fraction G, the percentage of C17:0 (15.7 ± 0.1%, wt/wt) is remarkable. The fatty acid C17:0 is a rare fatty acid that possesses pheromonic and allomonic properties [62]. The presence of such C17:0 has been established for microalgae, though its relatively high percentage is rather interesting, as usually the content of C17:0, or other odd-chain and branched-chain fatty acids, does not exceed 3–5% of all FAs. However, it must be noted that C17:0 has been considered a marker of bacterial contamination of microalgae and cyanobacteria cultures [63], which probably was the case in our open culture bioreactor as well. The fatty acids occurring in higher percentages in the TLs of *M. gaditana* VON5.3 were, in order, $^{\Delta 5,8,11,14,17}$C20:5 (22.7 ± 0.8%, wt/wt), $^{\Delta 9}$C16:1 (31.5 ± 0.5%, wt/wt), and C16:0 (15.5 ± 1.1%, wt/wt). In general, these fatty acids were the dominant ones in all lipid fractions and at similar levels. However, in the case of the NL lipid fraction, $^{\Delta 5,8,11,14,17}$C20:5 and $^{\Delta 9}$C16:1 were detected at significantly lower percentages compared with the TL (i.e., 7.6 ± 2.2%, wt/wt, and 17.9 ± 4.9%, wt/wt, for $^{\Delta 5,8,11,14,17}$C20:5 and $^{\Delta 9}$C16:1, respectively), while in the G and P lipid fractions, higher values of $^{\Delta 9}$C16:1 compared with the NL fraction were recorded. Finally, no production of any PUFA was recorded in the lipids of *N. pyriformis* PAT2.7 under these culture conditions. Therefore, the lipids of this strain are a more suitable source for biodiesel production in comparison with the lipids of the other strains. Predominant fatty acids in both total lipids and their lipid fractions were $^{\Delta 9}$C16:1 (exceeding 40%, wt/wt in TL) and C14:0. The great percentage of $^{\Delta 9}$C16:1 is a finding of high interest, as it is a fatty acid that, in recent investigations, was attributed, among other things, with anti-inflammatory properties and the ability to reprogram gut microbiota [64]. Lastly, it is worth mentioning the high proportion of $^{\Delta 9}$C18:1 (21.7 ± 3.0%) in the lipid fraction of P.

3.3. Synthesis of Polysaccharides, Proteins, and Amino Acid Profile

Regarding the ability to accumulate polysaccharides and proteins under these culture conditions, all strains showed remarkable yields (Table 2). The highest concentrations of both classes of cellular materials were achieved by *N. pyriformis* PAT2.7, with S/x% = 14.3 ± 0.3% (wt/wt) (similar results were also recorded for *P. oklahomense* SAG4.4) and P/x% = 49.7 ± 4.9% (wt/wt) at the end of culture. Although polysaccharides are synthesized through anabolic pathways competing with lipid biosynthesis [1], in this study, both metabolites were produced in significant quantities, indicating that the supply of light

and nutrients was sufficient for the cultures of the selected microalgae. The results of the current study were comparable to those obtained from Gao et al. (2023) [36], who recorded carbohydrate content of 5.2–20.6% for the two cultivated *Picochlorum* strains in outdoor raceway ponds. Indoor and outdoor cultivation of *Nannochloropsis* sp. for 8 days led to carbohydrate accumulation of 22 ± 1.4% and 23 ± 1.3%, respectively [38]. The protein content of the four examined microalgal strains was comparable to those examined by Das et al. (2015) [38] and Gao et al. (2023) [36], as previously mentioned. Notably, the very high protein content of *N. pyriformis* PAT2.7 in ORWP as well is a strong indication for the utilization of this microalga as a food/feed supplement.

The nutritional value of microalgae, in terms of protein quality, depends significantly on its amino acid composition [65]. Regarding the amino acid profile of the tested microalgal strains, *N. pyriformis* PAT2.7 presented the highest total content of essential (EAA) and non-essential amino acids (NEAA). However, the EAA to NEAA ratio remained balanced at approximately 1:1 for all the cultured microalgae, which is also observed in several microalgae species [10]. The most abundant EAA in all selected species was leucine, followed by lysine and arginine.

The highest lysine values were observed at *P. costavermella* VAS2.5 (3.5 ± 0.0%) and the lowest in *P. oklahomense* SAG4.4 (2.3 ± 1.0%). The differences between the microalgae of the same genus could be explained by the considerable variability in amino acid profile, which is due to the different algal species, environmental growth conditions, nutrient content of the feeding medium, and the stage of the algal life cycle [66]. *N. pyriformis* PAT2.7 showed the highest concentration of methionine (1.0 ± 0.1%), while the rest of the tested strains showed levels of approximately 0.6%. A similar trend appeared regarding the threonine concentration. The essential amino acid profile comparisons presented in the studies conducted by León-Vaz et al. (2023) [67] and Čmiková et al. (2024) [68] revealed similarities among the Chlorophyta phylum. In this study, *P. costavermella* VAS2.5, *P. oklahomense* SAG4.4, and *N. pyriformis* PAT2.7 were included. The presence of high leucine content was also highlighted in other Chlorophyta and Ochrophyta species, such as *Nannochloropsis granulata*, *Nannochloropsis oculata*, *Tetraselmis chui,* and *Tetraselmis suecica* [69,70].

Cerri et al. (2021) [71] reported lower levels of arginine in the Heterokontophyta (e.g., *Nannochloropsis oceanica*) and Chlorophyta (e.g., *Tetraselmis suecica*) microalgae when investigating the potential of microalgal strains as a dietary supplement for aquafeeds, in comparison to the findings of the present study.

Regarding the NEAA, glutamic acid was the most prevalent amino acid, followed by aspartic acid and glycine; analogous dominance in glutamic acid content was also observed in the research of León-Vaz et al. (2023) [67], where 56 different microalgal strains were analyzed. Similar results for glutamic acid were also demonstrated in the research of Čmiková et al. (2024) [68], where *Nannochloropsis* sp. (Eustigmatophyceae), *Tetraselmis chui* (Chlorophyta), and other species were cultured and analyzed for the amino acid profile. Aspartic acid was the second most abundant NEAA, in agreement with the available literature for Chlorophyta and Heterokontophyta [68,71]. Regarding taurine, hydroxyproline, and cysteine, either minimal quantities were found, or they were not detected in the tested microalgal strains. The results of the amino acid profile analysis are shown in Tables 4–6.

Overall, the tested microalgae species demonstrated a well-balanced amino acid profile, with high levels of lysine and threonine but low levels of methionine. This makes them an excellent alternative protein source for fish feeds, especially when combined with other protein sources or methionine supplementation.

Table 4. Essential amino acid composition (g/100 g of dry biomass) of the selected four microalgal strains that were cultured in an open raceway pond photobioreactor. The presented values are the average of two batches.

	Essentials AA (g/100 g Dry Biomass)								
	Lysine	**Methionine**	**Histidine**	**Isoleucine**	**Leucine**	**Phenylalanine**	**Threonine**	**Valine**	**Arginine**
P. costavermella VAS2.5	3.5 ± 0.0	0.6 ± 0.1	0.8 ± 0.0	2.1 ± 0.1	4.4 ± 0.1	2.0 ± 0.1	2.3 ± 0.1	2.6 ± 0.0	2.3 ± 0.1
P. oklahomense SAG4.4	2.3 ± 1.0	0.7 ± 0.2	1.2 ± 0.4	1.9 ± 0.2	3.9 ± 0.6	3.5 ± 1.1	2.7 ± 0.2	2.7 ± 0.3	3.0 ± 0.8
M. gaditana VON5.3	2.4 ± 0.2	0.6 ± 0.0	0.9 ± 0.1	1.9 ± 0.0	4.0 ± 0.0	2.4 ± 0.2	2.4 ± 0.0	2.5 ± 0.0	2.5 ± 0.1
N. pyriformis PAT2.7	3.3 ± 0.1	1 ± 0.1	1.4 ± 0.4	3.4 ± 0.0	5.5 ± 0.0	3.2 ± 0.3	3.6 ± 0.3	3.4 ± 0.0	3.9 ± 0.0

Table 5. Non-essential amino acid composition (g/100 g of dry biomass) of the selected four microalgal strains that were cultured in an open raceway pond photobioreactor. The presented values are the average of two batches.

	Non-Essentials AA (g/100 g Dry Biomass)									
	Taurine	**Tyrosine**	**Cysteine**	**Hydroxyproline**	**Serine**	**Alanine**	**Proline**	**Glutamic Acid**	**Aspartic Acid**	**Glycine**
P. costavermella VAS2.5	0.0 ± 0.0	1.3 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	2.0 ± 0.1	3.3 ± 0.0	2.1 ± 0.0	5.4 ± 0.0	4.5 ± 0.0	2.4 ± 0.1
P. oklahomense SAG4.4	0.2 ± 0.2	2.0 ± 0.7	0.3 ± 0.1	0.3 ± 0.2	2.5 ± 0.3	3.3 ± 0.6	2.4 ± 0.1	5.5 ± 0.8	4.6 ± 1.4	3.0 ± 0.5
M. gaditana VON5.3	0.0 ± 0.0	1.5 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	2.1 ± 0.1	3.1 ± 0.1	2.0 ± 0.0	5.1 ± 0.0	4.1 ± 0.1	2.6 ± 0.0
N. pyriformis PAT2.7	0.0 ± 0.0	2.8 ± 0.4	0.2 ± 0.0	0.0 ± 0.0	3.3 ± 0.1	4.6 ± 0.0	2.3 ± 0.0	7.7 ± 0.0	6.5 ± 0.2	3.1 ± 0.1

Table 6. Essential and non-essential amino acid ratio of the microalgal strains that were cultured in an open raceway pond photobioreactor. The presented values are the average of two batches.

	Sum of EAA	Sum of NEAA	EEAA/NEAA
P. costavermella VAS2.5	20.6 ± 0.6	21.2 ± 0.2	1.0 ± 0.0
P. oklahomense SAG4.4	21.8 ± 0.5	24.1 ± 0.5	0.9 ± 0.0
M. gaditana VON5.3	19.6 ± 0.3	20.6 ± 0.0	1.0 ± 0.0
N. pyriformis PAT2.7	28.7 ± 1.1	30.4 ± 0.5	0.9 ± 0.0

3.4. Synthesis of Pigments

Finally, in the tested strains, similar levels regarding the content of total chlorophyll and carotenoids (i.e., TCh/x% = 3.2–5.0%, wt/wt, and TC/x% = 1.3–1.6%, wt/wt) were observed. The *Picochlorum* strains and *M. gaditana* VON5.3 synthesized pigments in amounts comparable to other studies. For example, the chlorophyll content of *N. oceanica* was 1.84 ± 0.11% and carotenoids 0.42 ± 0.02% in the study of Carneiro et al. (2022) [5]. *N. oculata* used by Millán-Oropeza and Fernández-Linares (2017) [6] produced chlorophyll a, which is almost equal to the chlorophyll content of the present study, and carotenoids at 4.9 ± 0.1%–11.6 ± 1.1% and 0.8 ± 0.01%–2 ± 0.2% during the winter season and 3.3 ± 0.5%–6.2 ± 0.6% and 0.5 ± 0.1%–1.1 ± 0.1% during the spring season, respectively. These results align with the findings of Patrinou et al. (2022) [7]. Specifically, total chlorophyll content dropped from 5.1 ± 1.2% to 2.7 ± 0.05% and carotenoid content from 0.51 ± 0.10% to 0.27 ± 0.05% when *Tetraselmis striata* (Chlorophyta) CTP4 was cultivated at 25 ± 1 °C and 28 ± 1 °C, respectively. These findings reveal a correlation between pigment synthesis and temperature of incubation. However, pigment synthesis depends on many factors, with the most significant being the provided illumination and "mutual shading," which should be taken into consideration as it affects the cells of the low layers of the cultivation. On the other hand, *N. pyriformis* PAT2.7 pigment content was lower in all relevant measurements. However, it should be mentioned that *Nephroselmis* strains are well-known producers of carotenoids like neoxanthin, lutein, and siphonaxanthin, which can be used in the pharmaceutical and food/feed industries [8]. Therefore, it is crucial to find ways to optimize carotenoid synthesis.

4. Conclusions

The strains of *Picochlorum costavermella*, *Picochlorum oklahomense*, *Microchloropsis gaditana,* and *Nephroselmis pyriformis* were easily cultivated in an open raceway pond under controlled conditions, producing significant amounts of cellular metabolites. Intriguingly, strains such as *P. costavermella* VAS2.5 and *N. pyriformis* PAT2.7, for which the available literature is limited, seem to be potential new candidates and subjects for biotechnology study. Specifically, *Picochlorum* strains and *M. gaditana* VON5.3 produced notable amounts of lipids, which were dominated by polar lipids that were rich in PUFAs, especially $^{\Delta 5,8,11,14,17}$C20:5 and $^{\Delta 9,12,15}$C18:3. Therefore, even though the stage of the current research does not yet establish the suitability of the aforementioned strains for utilization in large-scale applications, these strains pose as enticing candidates for further trials. On the other hand, *N. pyriformis* PAT2.7 proved to be weaker in lipid production compared with to the other tested strains, and its lipids were dominated by neutral ones, which, though, contained large quantities of $^{\Delta 9}$C16:1, a fatty acid of great biotechnological interest. Moreover, all tested strains accumulated great amounts of polysaccharides and proteins, and overall, they demonstrated a well-balanced amino acid profile with high levels of lysine and threonine. Therefore, these strains have proved to be excellent alternative protein sources for fish feed. Lastly, the two *Picochlorum* strains and *M. gaditana* VON5.3 synthesized pigments in amounts comparable to other studies, while *N. pyriformis* PAT2.7 pigment

production was lower. Overall, the results of the present study provided strong evidence for the validity of the hypothesis of exploitation and potential in the use of indigenous microalgal strains for the development of high-interest applications.

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Abbreviations

The following abbreviations are used in this manuscript:

PUFA	Poly-Unsaturated Fatty Acids
HUFA	Highly-Unsaturated Fatty Acids
EPA	n-3 Eicosapentaenoic Acid
DHA	n-3 Docosahexaenoic Acid
ORWP	Open Raceway Pond
mASW	modified Artificial Sea Water
NL	Neutral Lipids
G+S	Glycolipids + Sphingolipids
P	Phospholipids

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