



Seasonal biochemical fingerprints of outdoor cultivated *Spirulina* (*Limnospira platensis*) and *Microchloropsis gaditana*: insights into compositional and functional shifts

Diana Lopes^{a,1,*}, Ana S.P. Moreira^{a,1,*}, Tiago Conde^a, Andreia Ferreira^b, Kayane Oliveira^b, Alexandra Conde^c, Helena R. Rocha^h, Marta Coelho^h, Ana Gomes^h, Manuela Pintado^h, Ana Alexandra Ramos^d, Hugo Pereira^d, Sónia P.M. Ventura^c, Cláudia Nunes^e, Manuel A. Coimbra^b, Natacha Coelho^{f,g}, Alexandre M.C. Rodrigues^f, Maria do Rosário Domingues^a

^a CESAM - Centre for Environmental and Marine Studies, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

^b LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

^c CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

^d GreenCoLab, Associação Oceano Verde, Universidade do Algarve, Campus de Gambelas, 8005-139, Faro, Portugal

^e CICECO - Aveiro Institute of Materials, Department of Materials and Ceramic Engineering, University of Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

^f Necton S.A., Belamandil, 8700-152, Olhão, Portugal

^g MED (Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento) & CHANGE - Global Change and Sustainability Institute, Faculdade de Ciências e Tecnologia, Campus de Gambelas, Universidade do Algarve, Ed. 8, 8005-139, Faro, Portugal

^h CBQF—Centro de Biotecnologia e Química Fina—Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Diogo Botelho, 1327, Porto, 4169-005, Portugal

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ABSTRACT

Microalgae are increasingly recognized as sustainable bioresources with applications in food, feed, nutraceuticals, and aquaculture. Among them, *Spirulina* (*Limnospira platensis*) and *Microchloropsis gaditana* (formerly *Nannochloropsis gaditana*) stand out due to their rich biochemical composition with industrial applications and high market value. This study separately investigates the seasonal variability in the biochemical composition of *L. platensis* cultivated in open raceway ponds and *M. gaditana* grown in closed photobioreactors.

The results suggest a response to seasonal changes. *L. platensis* exhibited higher protein content in spring (47.82%) and summer (45.48%), while carbohydrate accumulation peaked in winter (50.85%). *M. gaditana* showed increased lipid (21.67%) and carbohydrate (14.56%) content in autumn compared to winter and summer, but with lower variations in response to seasonal environmental changes. This microalga exhibited higher FA 20:5 *n*-3 (EPA) levels in winter compared to summer. The dominant carbohydrate residue in both microalgae, present throughout all seasons, was glucose, which could be associated with the presence of β -glucans, in addition to glycogen, reinforcing their bioactive potential.

Pigment composition was also seasonally influenced in *L. platensis*, which accumulated more chlorophyll and phycocyanin in spring and summer, whereas *M. gaditana* showed elevated levels of neoxanthin in autumn and winter, and violaxanthin in spring.

Although limited by a single sampling per season and the different cultivation systems size, this study provides preliminary observations of seasonal variations in biomass composition, highlighting that biochemical composition varies with season in a species-specific manner. Understanding these variations improves our knowledge of microalgal biochemical plasticity under outdoor conditions.

* Corresponding authors.

E-mail addresses: dianasalzedaslopes@ua.pt (D. Lopes), ana.moreira@ua.pt (A.S.P. Moreira).

¹ These authors contributed equally to this work.

1. Introduction

Microalgae are widely recognized for their potential as sustainable bioresources, finding diverse applications in food, feed, cosmetics, and pharmaceuticals. They have the ability to produce valuable bioactive compounds and nutrient-rich biomasses [1]. Furthermore, microalgae play a significant role in carbon capture and climate change mitigation, underscoring their pivotal contribution to environmental sustainability [2,3].

Among the vast diversity of photosynthetic microalgae and cyanobacteria, the Monodopsidaceae family (Eustigmatophytes including *Nannochloropsis* sp. and *Microchloropsis* sp.) and the cyanobacteria *Spirulina* (*Limnospira* sp.) are two attractive groups with broad applications, such as food, feed, and nutraceuticals [4,5]. In this study, we focused on the widely used species, *Limnospira platensis* and *Microchloropsis gaditana* (formerly known as *Nannochloropsis gaditana* [6]).

Considering both microalgae and cyanobacteria, *Spirulina* is the most widely produced and consumed globally, with a global production of 56,208 tons in 2019 and a market size of €533 million in 2023 [7,8]. This cyanobacterium is renowned for its exceptional protein content, rich profile of macro- and micronutrients, including essential amino acids, lipids, vitamins, and minerals, as well as potent antioxidant capacity [9]. In Europe, the commercial uses of *Limnospira* sp. biomass are mainly for human food, supplements, and nutraceuticals [10]. While *Spirulina* (*Limnospira* sp.) is approved for human consumption worldwide, *Nannochloropsis* sp. and *Microchloropsis* sp. are not yet authorised for use within the European Union. Nevertheless, *Nannochloropsis* sp. and *Microchloropsis* sp. are widely exploited in aquaculture feed to enhance the nutritional value of aquafeeds [11].

Monodopsidaceae species are highly valued for their lipid content and rich composition in omega-3 polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic acid (EPA, 20:5 *n*-3). EPA is widely used as a dietary supplement and is considered essential for human health [12]. Beyond its nutritional benefits, EPA derived from *Nannochloropsis* and *Microchloropsis* also enhances the oxidative stability of functional foods. Compared to PUFA supplements from fish or krill, *Nannochloropsis*- and *Microchloropsis*-based extracts exhibit greater stability and a longer shelf life, mainly due to the co-extraction of lipids and pigments with antioxidant activity [13–15]. The global market demand for *Nannochloropsis* is projected to reach USD 4.2 million by 2028, with an annual growth rate of 8.5% from 2021 to 2028 [15].

Both *Spirulina* and Monodopsidaceae can be cultivated year-round using open raceway ponds (ORPs) and closed photobioreactors (PBRs). ORPs offer advantages such as ease of scale-up, low energy consumption, and relatively low construction and operating costs [16]. However, closed PBRs have gained increasing attention due to their ability to control growth conditions more effectively, minimise contamination risks, and achieve higher biomass-to-substrate conversion ratios [17]. In both cases, large-scale outdoor microalgae production faces seasonal challenges, particularly due to temperature and light fluctuations [18], which alter their biochemical composition and influence their value for final applications.

Seasonal environmental fluctuations can impact the growth of microalgae species, affecting the productivity and nutritional composition of the biomass [18]. Temperature shifts of ± 10 °C from the optimal conditions reduce growth rates or completely inhibit growth in *N. oceanica* [18]. For *Spirulina*, optimal growth occurs at 35 °C [9,19], with light intensities between 250 and 350 W.m⁻², maximizing biomass production, whereas intensities above 400 W.m⁻² inhibit growth [19]. Indeed, *Limnospira* spp. can withstand a wide range of environments; however, growth and productivity are affected by temperature [9,19]. Growth was reported as practically nil at low temperatures (below 17 °C) [9]. Similarly, light intensity plays a crucial role in *Limnospira* growth and chemical composition — higher intensities can boost *Limnospira*'s growth. In contrast, lower intensities can enhance pigment and protein production, but at the expense of overall productivity [19].

Seasonal variations affect outdoor cultivation, with self-shading reducing productivity in summer and lower temperatures mitigating this effect in winter and spring [19]. For instance, *Spirulina* cultivated at 25 °C, 30 °C, and 37 °C showed a higher biomass production at higher temperatures, with cultivation at 30 °C enhancing the synthesis of pigments such as chlorophyll *a*, carotenoids, C-phycoerythrin, and total phycobiliproteins, as well as proteins and carbohydrates [20]. Similarly, an average temperature of 30 °C, light irradiance of 100 $\mu\text{mol photons.m}^2.\text{s}^{-1}$, and orbital agitation of 136 rpm resulted in maximum biomass production and protein content after 7 days of cultivation [21].

Regarding *Nannochloropsis* species, such as *N. oceanica*, they thrive at 25–29 °C, with growth inhibited above 31 °C or below 9 °C [22]. In particular, *M. gaditana* reached its peak biomass production at 25 °C and 120 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ under a 16 h/8 h light cycle [23,24]. Variations in temperature and light have a significant influence on the biochemical and nutritional profiles of Monodopsidaceae species, particularly in their fatty acid (FA) composition. Suboptimal temperatures, especially below 20 °C, have been associated with enhanced EPA production. For instance, *M. salina* exhibited the lowest growth rate but the highest EPA content at 5 °C [25], while *N. oculata* showed a 158% increase in EPA accumulation at 10 °C (12.8 mg.L⁻¹) compared to 25 °C [26]. In contrast, higher temperatures promoted lipid accumulation, with *M. gaditana* achieving a maximum lipid content at 30 °C [27]. Additionally, increased light intensity (500 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) stimulated lipid and carotenoid production in *N. oceanica*, but it reduced protein and sugar content [28].

Carbohydrate synthesis in microalgae is strongly influenced by external factors such as light intensity, temperature, and nutrient availability, and during colder or low-light conditions (e.g., winter) [29]. In particular, *Limnospira platensis* tends to accumulate a higher total carbohydrate content (up to 45% dw), acting as an energy reserve or cryoprotectant [30]. During warmer and high-light seasons, carbohydrate biosynthesis may decline in favor of protein or lipid synthesis. Glucose residues often dominate the carbohydrate profile, acting as structural components (cellulose) or storage polysaccharides, that correspond to glycogen in cyanobacteria such as *L. platensis* and β -glucans in eukaryotic microalgae. The β -glucans are recognized as bioactive compounds due to their immunomodulatory and prebiotic properties [31]. *Limnospira platensis* also synthesizes a complex sulphated heteropolysaccharide, mainly composed by rhamnose, xylose, and galactose, which has been reported to exhibit antiviral and immunomodulatory properties [32–34]. Similarly, *Nannochloropsis* species are described to have ($\beta 1 \rightarrow 3$)-glucans and smaller amounts of sulphated heteropolysaccharides. However, the seasonal variation in these polysaccharides remains poorly understood, with only the stimulation of total carbohydrate being described.

Although seasonal variations, particularly those related to temperature and light fluctuations, are well known to influence microalgae biomass production, their impact on biochemical composition remains poorly understood. To the best of the authors' knowledge, no studies have investigated these seasonal effects in *M. gaditana* and *L. platensis* with a comprehensive biochemical characterization. Addressing this gap is essential for characterising seasonal biochemical trends. A deeper understanding of seasonal shifts improves awareness of how environmental fluctuations impact biochemical composition.

This non-comparative study aims to investigate seasonal variations in the biochemical profiles of outdoor industrially produced *L. platensis* and *M. gaditana* subjected to ambient conditions of each season (autumn, winter, spring, and summer). The biochemical analyses include proximate composition, fatty acid profiles, pigments, carbohydrates, and amino acids to explore the potential of season-specific biochemical profiles.

2. Material and methods

2.1. Outdoor industrial biomass production

The industrial production of *L. platensis* took place at the facilities of Allmicroalgae - Natural Products S.A. in Pataias, Portugal. The biomass was cultivated in ORPs (Supplementary Fig. 1A), covering areas of 1000 m² (in autumn and winter) and 4000 m² (in spring and summer), within a transparent plastic greenhouse that allowed exposure to natural light. During autumn and winter, *L. platensis* cultures are kept in maintenance mode and thus kept in smaller ORPs. The culture medium used was organic medium [35] and pH was set at 10. Continuous agitation was maintained by paddlewheels, with one operating in the 1000 m² ponds and two in the 4000 m² ponds. The culture depth varied between 15 and 20 cm, depending on seasonal fluctuations and evaporation rates. To compensate for water loss due to evaporation, fresh water was added daily. An internal ventilation system was activated when needed to maintain the culture's temperature under 35 °C, in order to avoid the collapse of the cultures. However, below 35 °C, the system allows temperature variations according to the external temperature. Samples were taken from the biomass harvested on October 24, 2023 (autumn); January 19, 2024 (winter); April 17, 2024 (spring); and July 10, 2024 (summer).

The production of *M. gaditana* occurred outdoors at Necton S.A. in Olhão, Portugal, in aerated Flat-Panel Photobioreactors (FP-PBRs) of 2700 L and horizontal flow-through TPBRs of 19,000–27,000 L (Supplementary Fig. 1B). The culture medium used was Nutribloom® Plus (Necton S.A.) and the concentration of nitrates was kept between 2 and 4 mM by daily monitoring and adjustment with fresh medium. Throughout cultivation, pH was automatically maintained below 8.5 via the injection of pure CO₂, while culture temperature was maintained below 30 °C through the spray of underground water over the reactors. Samples were taken from the biomass harvested at 10 am on October 30, 2023 (autumn – TPBR); January 22, 2024 (winter – TPBR); April 22, 2024 (spring – TPBR); and July 17, 2024 (summer – FP-PBR).

Biomass concentration was inferred through daily measurements of culture's optical density. Culture's productivity (g.L⁻¹.d⁻¹) was calculated over the 7 days preceding harvesting. The harvest days corresponded to the peak of the 4 seasons of the year. The harvests were all carried out at the same time of the day, 10 am, so that the effect of biochemical fluctuations that occur during the day would not affect this comparison. Harvesting was done by a sieve (*L. platensis*) or by centrifugation (*M. gaditana*). The obtained paste was immediately frozen followed by freeze dried using a LyoMicron lyophilizer (Coolvacuum, Spain) operating at an absolute pressure of 0.04 mbar and a condenser temperature of –70 °C for 4 days. Samples were collected from industrial batches of each microalga, produced during each season. For biochemical analyses, analytical replicates were performed using different portions of each batch.

It is important to note that the two raceway pond systems used for *L. platensis* (1000 m² with a single paddle vs. 4000 m² with two paddles) may differ in hydrodynamics, which may lead to system-specific effects. Likewise, *M. gaditana* was cultivated in two photobioreactor configurations (FP-PBRs and horizontal flow-through TPBRs), which differ in geometry and mixing characteristics. These differences cannot be fully standardised and represent an intrinsic limitation of working under industrial cultivation conditions.

2.2. Elemental analysis and protein estimation

The CHN composition (Table S1) was determined by elemental analysis (Vario EL III®, Elemental Analyser System; GmbH, Hanau, Germany) according to the procedure provided by the manufacturer. The total protein was determined by multiplying the percentage of nitrogen by the factor 4.78 [36]. This conversion factor is widely accepted for microalgae and commonly applied in the scientific literature [37,38]

ensuring comparability with previous studies. All analyses were performed in triplicate, and the average values were used for data interpretation.

2.3. Determination of ash content

Ash content was determined through gravimetry by burning 50 mg of the sample dry biomass at 550 °C for 4 h, in a muffle furnace (NABERTHERM, LE62K17N1). After the combustion of the biomass, the final weight corresponds to the ash content. All analyses were performed in triplicate, and the average values were used for data interpretation.

2.4. Amino acid analysis

Microalgae samples were subjected to acid hydrolysis to determine the total amino acid content. Approximately 100 mg of each sample was weighed into SPME glass vials, and 2 mL of 6 M HCl was added (≥37%, ACS grade, Sigma-Aldrich, USA). The mixture was vortexed for proper mixing. A needle was inserted into the vial septum to release oxygen, while another needle connected to a nitrogen stream was introduced. Nitrogen gas was bubbled through the sample for 1 min, after which the oxygen-release needle was removed, and nitrogen bubbling continued for an additional 3 min. The nitrogen inlet was removed, and the vial was tightly sealed with a PTFE tape. These vials were placed in an oven (Carbolite, UK) at 115 °C for 20 h to complete the hydrolysis. Afterwards, the vials were cooled to room temperature, diluted with 5 mL of ultra-pure water (18.2 MΩ·cm, Milli-Q, Merck Millipore, USA), and the pH was adjusted to 3.5 using 10 M NaOH (≥98%, Sigma-Aldrich, USA). The solution was transferred to a 10 mL Class-A volumetric flask, diluted to the mark with ultra-pure water, and filtered through a 0.22 μm PTFE syringe filter.

A liquid chromatograph (Thermo Scientific Vanquish, USA) equipped with Vanquish Pump, Vanquish Autosampler, Vanquish Diode Array Detector (DAD), and Vanquish Fluorescence Detector (FLD) was used for the detection and quantification of amino acids. The chromatographic separation was performed on a Chromolith Performance RP18e column (100–4.6 mm, Merck, Germany). Two eluents were prepared for HPLC analysis: i) eluent A: a solution of 2.8 g Na₂HPO₄ (≥99%, analytical grade, Merck, Germany), 7.6 g Na₂B₄O₇·10 H₂O (≥99.5%, Merck, Germany), and 0.064 g NaN₃ (≥99%) dissolved in ultra-pure water, adjusted to pH 7.5 with HCl, and made up to 2 L; eluent B: a mixture of 450 mL methanol (HPLC grade, Merck, Germany), 450 mL acetonitrile (HPLC grade, Merck, Germany), and 100 mL ultra-pure water, prepared to a total volume of 1 L. The elution program included a flow rate increasing from 0.9 mL.min⁻¹ to 1.2 mL.min⁻¹ at set intervals and a gradient starting at 10% eluent B, increasing to 100% eluent B, and returning to the initial conditions. The program duration was 55 min, including stabilization phases. Fluorescence detection was conducted at an excitation wavelength of 356 nm and an emission wavelength of 445 nm, with a sensitivity factor of 1. The data collection rate was 2 Hz, and the column temperature was maintained at 25 °C.

To ensure proper amino acid derivatization, additional reagents were prepared: i) borate buffer (pH 9.5): 6.2 g of H₃BO₃ (≥99.5%, Merck, Germany) was dissolved in ultra-pure water, pH adjusted to 9.5 with 4 M NaOH (≥98%) and diluted to 1 L; ii) internal standard (IS): a stock solution of 200 mg.L⁻¹ homoserine (≥98%, VWR, USA) in 0.1 M HCl was prepared by dissolving 0.01 g of homoserine in 50 mL 0.1 M HCl, then diluted tenfold to a working solution of 20 mg.L⁻¹; iii) reagent A: a mixture of 3 mL IS solution (20 mg.L⁻¹), 100 mL mercaptoethanol (Fluka Analytical, USA), and 0.5 g sodium tetraphenylborate (Merck, Germany), diluted to 25 mL with borate buffer and stored cold for up to a week; iv) reagent B: a solution of 0.6 g iodoacetic acid (Sigma-Aldrich, USA) in 15 mL borate buffer, pH adjusted to 9.5 with 4 M NaOH, and diluted to 20 mL; v) reagent C: 0.175 g o-Phthalaldehyde (OPA, ≥99%, Millipore Sigma, Germany) dissolved in 5 mL methanol (HPLC grade), mixed with 0.5 mL mercaptoethanol, diluted to 25 mL with borate

buffer, and bubbled with nitrogen gas. This solution was stored cold and protected from light. A calibration curve was created using a 100 mg.L⁻¹ amino acid standard mixture (MilliporeSigma, Germany) prepared in 0.1 M HCl. Standard solutions (1–30 mg.L⁻¹) were prepared by diluting the stock solution in 0.1 M HCl.

2.5. Carbohydrate analysis

The total carbohydrate content and profile were determined in the different microalgae biomass. Neutral sugars were analysed by gas chromatography-flame ionization detection (GC-FID) (Perkin Elmer Clarus 400), after conversion into their alditol acetates, as previously reported by Ferreira et al. [39]. The samples (1–2 mg) were pre-hydrolysed with H₂SO₄ (0.2 mL, 72% w/w, 95–97%; PanReac, Spain) for 3 h at room temperature, with occasional stirring, followed by a hydrolysis in H₂SO₄ (2.2 mL, 1 M, 95–97%; PanReac, Spain) for 2.5 h at 100 °C to release the monosaccharides from the polysaccharides. 2-Deoxyglucose (0.2 mL, 1.0 mg/mL, >99%; Sigma-Aldrich, USA) was used as internal standard. Monosaccharides were reduced with NaBH₄ (0.1 mL, 15% w/v in 3 M NH₃, >99%; Scharlau, Spain) for 1 h at 30 °C and acetylated with acetic anhydride (3 mL, >99%; Carlo Erba, Italy), using methyl-imidazole (0.45 mL, >99%; Thermo Scientific, USA) as a catalyst, for 30 min at 30 °C.

Free alditols in dry biomass were also analysed by converting them into their respective alditol acetates through acetylation, using 2-deoxyglucitol (0.2 mL, 1.0 mg/mL, >99%; Sigma-Aldrich, USA) as internal standard.

The alditol acetates were analysed using a GC-FID Perkin Elmer Clarus 400 equipped with a DB-225 column (30 m × 0.25 mm and 0.15 µm of film thickness (J&W Scientific). The operating temperatures of the injector and the detector were 220 °C and 240 °C, respectively. The oven temperature program was used as follows: the initial temperature was set to 220 °C, hold for 7 min, and then raised to 240 °C at 10 °C.min⁻¹. The carrier gas (H₂) had a flow rate of 1.7 mL.min⁻¹. Uronic acids (UAs) were determined using the *m*-phenylphenol colorimetric method [40]. Briefly, after pre-hydrolysis with H₂SO₄ (0.2 mL, 72% w/w, 95–97%; PanReac, Spain) for 3 h at room temperature, the samples were hydrolysed in 1 M H₂SO₄ for 1 h at 100 °C. The hydrolysed samples were diluted (1:3, v/v) and boric acid (1 mL, 200 mM, >99%; Sigma-Aldrich in H₂SO₄ 98% w/v) was added to 0.1 mL of each sample. The mixture was heated at 100 °C for 10 min, cooled on ice, and *m*-phenylphenol solution (20 µL; 0.15% w/v; 90%, Acros Organics, USA) was added. Absorbance was measured at 520 nm (BioTek - Eon Microplate Reader). A calibration curve was prepared with D-galacturonic acid (0–100 µg/mL, 7 points, >97%; Sigma-Aldrich, USA), and results were expressed as GalA equivalents.

2.6. Lipid analysis

2.6.1. Lipid extraction

Lipids of *L. platensis* and *M. gaditana* were extracted from 25 mg of freeze-dried biomass using a modified Folch's method [41,42]. All the reagents used for lipid extraction are HPLC grade. The biomass was mixed with dichloromethane (DCM):methanol (MeOH) (2:1, v/v), vortexed, and incubated for 30 min. After centrifugation (Pro-Analytical C400R; centurion Scientific, UK), the organic phase was collected, and the process was repeated three times. The extract was dried under nitrogen and re-dissolved in DCM:MeOH (2:1, v/v). Phase separation was achieved by adding Milli-Q water, vortexing, and centrifugation, with the organic phase collected and the aqueous phase re-extracted twice using DCM. Combined organic phases were filtered, dried under nitrogen, transferred to pre-weighed amber vials, and stored at –20 °C. The lipid content was determined gravimetrically as a percentage of dry biomass.

2.6.2. Fatty acids analysis

Fatty acid methyl esters (FAME) were prepared from lipid extracts of *L. platensis* and *M. gaditana* using alkaline trans-esterification [43]. Lipid extracts were combined with an internal standard solution (methyl nonadecanoate in *n*-hexane 99%), followed by KOH solution (2.0 M in methanol), and vortexed. A saturated NaCl solution (1 g in 100 mL of Milli-Q water) was added to induce phase separation, and the organic phase was collected, dried under nitrogen, and dissolved in *n*-hexane 99% for GC–MS analysis (Agilent Technologies 8860 GC System, USA). For analysis, 2 µL of the prepared FAME solution was injected into a GC–MS equipped with a DB-FFAP column with the following specifications: 30 m of length, 0.32 mm internal diameter, and 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA) [44]. The injector and detector temperatures were set at 220 °C and 230 °C, respectively. The oven temperature followed a programmed gradient, starting at 58 °C (held for 2 min), increasing to 160 °C at a rate of 25 °C.min⁻¹, then to 210 °C at 2 °C.min⁻¹, and finally reaching 225 °C at 20 °C.min⁻¹. The system operated in electron impact mode with helium as the carrier gas. FAME were identified by comparing their retention times and mass spectra with standard references (Supelco 37 Component FAME Mix) and databases (NIST Library, “Lipid Web”). Results were processed using Agilent MassHunter software.

2.7. Pigment analysis

2.7.1. Quantification of carotenoids and chlorophyll content

Carotenoids (i.e. beta-carotene, canthaxanthin, zeaxanthin, fucoxanthin, neoxanthin, violaxanthin, astaxanthin, and lutein) and chlorophyll were extracted from 5 mg of freeze-dried biomass using organic solvents (i.e. methanol for carotenoids and acetone for chlorophyll) following the methods described by Couso et al. and Schüler et al. [45,46]. These solvents were added until complete extraction was achieved, aided by cell disruption through tungsten bead milling. The quantification of carotenoids was performed using high-performance liquid chromatography (HPLC) on a Chromaster System (Hitachi, VWR), equipped with a diode array detector (5430 DAD, Hitachi, VWR) and a Purospher® STAR RP-18 chromatographic column (Merck, 250 × 2.1 mm, 5 µm). The analysis was conducted at a temperature of 27 °C, with a flow rate of 1 mL.min⁻¹ over 40 min, and an injection volume of 50 µL. Compound identification took place at a wavelength of 450 nm, using standards for comparison. The concentrations of the carotenoids were calculated based on their respective calibration curves [45,46]. Additionally, the chlorophyll content was determined spectrophotometrically from acetone extracts, with concentrations calculated according to the equations established by Lichtenthaler & Wellburn [47] using a HITACHI spectrophotometer (model UH5300). All analyses were performed in triplicate, and the average values were used for data interpretation.

2.7.2. Phycocyanin content

Phycocyanin was extracted using distilled water from the dry biomass of various *Spirulina* samples. The extraction conditions were consistent across all three biomasses, with a solid-liquid ratio (SLR) of 0.03 g of dry biomass per mL of solvent, distilled water, at a constant temperature of 25 °C, and stirring for 30 min in an orbital rotor at 80 rpm. After extraction, the samples were centrifuged (Hettich® MIKRO 200/200R) for 15 min at 15,000 rpm. The supernatant was then collected for analysis. The solvent was added to the recovered pellet, and the extraction process was repeated until the supernatant became colorless. The absorbance spectra of the various extracts were measured between 230 and 700 nm using a UV–Vis microplate reader (SpectraMax, USA). The calibration curve was prepared using a pure standard of phycocyanin (lyophilized powder) from Sigma-Aldrich, measured at 615 nm.

2.8. Statistical analysis

The statistical analysis was performed to identify significant differences between seasons for each microalga. Univariate statistical analyses were performed using GraphPad Prism 8.0.1. software. The Shapiro–Wilk test was performed to verify normality of the dataset. When normality was observed, significant differences between groups were evaluated using One-Way ANOVA with Tukey's post hoc test, considering differences significant at p -value <0.05 . Whenever normality was not verified, the Kruskal–Wallis test, followed by Dunn's post-hoc comparisons was employed.

3. Results and discussion

3.1. Meteorological and productivity data

To enhance the understanding and correlation between the biochemical profile and environmental factors, meteorological and productivity data were obtained from the producers of the microalgae under study. Specifically, temperature and external radiation levels recorded on the day of harvest (Supplementary Figs. S2 and S3) were compared across the different seasons. Because each species was grown in the industrial system in which it is routinely cultivated, system-specific effects may overlap with seasonal responses. For this reason, the present work does not aim to compare cultivation systems or species, but to describe seasonal biochemical trends within each system.

For *L. platensis*, temperature variations were observed across the seasons, with the lowest values recorded in winter (18.4 °C) and spring (19.0 °C). Autumn presented a moderate temperature of 23.1 °C, while summer exhibited the highest temperature at 33.3 °C (Fig. S2). On the other hand, the external radiation recorded during production of *L. platensis* was higher in spring (483.1 W.m⁻²) and summer (321.0 W.m⁻²), and lower in winter (30.5 W.m⁻²) and autumn (90.9 W.m⁻²) (Fig. S2).

A similar seasonal trend was observed for *M. gaditana*, though with less pronounced fluctuations. The lowest temperatures occurred during winter (20.9 °C) and spring (22.2 °C), whereas autumn and summer recorded slightly higher values of 24.8 °C and 26.1 °C, respectively (Fig. S3). The external radiation recorded during production of *M. gaditana* (Fig. S3) increased from winter (627.6 W.m⁻²) to summer (987.5 W.m⁻²), with intermediate values recorded in autumn (830.4 W.m⁻²) and spring (917.0 W.m⁻²).

In terms of culture's productivity, for *L. platensis* the highest values were achieved in spring (0.04 g.L⁻¹.d⁻¹) and summer (0.03 g.L⁻¹.d⁻¹), while productivity declined to 0.01 g.L⁻¹.d⁻¹ in autumn and was null in winter. During autumn and winter, *L. platensis* cultures are kept in maintenance mode, and no actual production was performed. Because no biomass growth occurred in autumn and winter, the biochemical composition reported for these seasons reflects maintenance biomass and cannot be interpreted in a production context.

M. gaditana presented higher productivity values in autumn (0.22 g.L⁻¹.d⁻¹) and spring (0.25 g.L⁻¹.d⁻¹) compared to winter (0.14 g.L⁻¹.d⁻¹). The productivity of *M. gaditana* during the summer was null due to several constraints. Therefore, summer biochemical profile may not be representative.

Microalgae growth is affected by temperature and light. The lower (or null) productivity values observed in winter could be correlated with lower radiation levels, shorter photoperiods, and colder temperatures. A previous work demonstrated the influence of these parameters on *L. platensis* growth and productivity [48]. Similarly, the productivity of *Nannochloropsis oculata* cultivated in an outdoor pilot-scale OPR was influenced by seasonal variations, resulting in increases of 264% and 90% during summer compared to winter and autumn-spring, respectively [49].

3.2. Proximal analysis of microalgae biomass grown across the different seasons

To evaluate the effects of seasonality on the biochemical composition, we first investigated the changes in the proximal composition. In *Spirulina* (*L. platensis*), the proximal analysis (Fig. 1 and Table S2) revealed that proteins were the predominant component during autumn, spring, and summer, ranging from 43.34 ± 0.13% in autumn to 47.82 ± 0.74% in spring. In contrast, protein content declined markedly in winter, reaching 27.01%, suggesting a seasonal influence on protein accumulation. *Spirulina* is recognized for its high protein content, which generally ranges between 46 and 63%, and can reach as high as 70% in nitrogen-supplemented media [50]. The influence of temperature and light intensity, two environmental factors that fluctuate across the four seasons, can impact the protein content of *Spirulina*. A decrease in the protein content of *Spirulina* under high light (500 μE.m⁻².s⁻¹) and high temperature (35 °C) was reported [51], while the protein content of *Spirulina* grown in thermostated PBRs between May and September was higher at 35 °C (64–68%) when compared to 25 °C (57–62%) [52]. In the present study, protein levels remained stable in spring (47.82 ± 0.74%) and summer (45.48 ± 1.24%), reflecting more favorable growth conditions during these seasons. A possible explanation for the highest protein content observed in spring and summer may be linked to higher external radiation recorded during these seasons (Supplementary Fig. S2). Previous studies with *Spirulina* demonstrated that high light can reduce glutamine synthetase (GS) activity, which in turn enhance the activity of nitrate reductase (NR) and proteases involved in nitrogen assimilation. This higher nitrogen assimilation may contribute to protein production and could partly explain the protein accumulation observed in our study [53].

The carbohydrate content, quantified by the sum of neutral sugars, uronic acids and alditols, was also affected by seasonality (Fig. 1 and Table S2), with winter exhibiting the highest levels (50.85 ± 1.47%) and spring the lowest (10.32 ± 0.05%). Previous studies indicate that the carbohydrate content of *Spirulina* is typically between 10 and 27% [54].

The marked increase during winter may reflect metabolic adjustments to low-temperature stress. In plants, cold acclimation triggers reprogramming of the transcriptome, proteome, and metabolome, with carbohydrates playing a central role in this process [55]. A study in *Spirulina platensis* demonstrated an accumulation of soluble sugars under low temperatures, associated with the up-regulation of enzymes involved in gluconeogenesis and carbohydrate metabolism, including fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (PEPCK) [56]. The enhancement of gluconeogenesis under cold stress thus contributes to increased soluble sugar production. The increase of soluble carbohydrates under lower temperature may have contributed to the increase of total carbohydrates observed during the winter season.

In addition, other study with outdoor cultivation has reported that carbohydrate content in *Arthrospira platensis* can rise to 44–45% of the dry weight during winter, which is associated with reduced protein synthesis, promoting carbon storage in polysaccharides due to low temperatures, decreased light intensity, and nutrient limitation [30]. Overall, these findings suggest that the elevated carbohydrate content observed in our study during winter represents an adaptive metabolic strategy to cope with combined environmental stresses.

Regarding the lipid content of *L. platensis*, fluctuations were observed across the different seasons, with lower values found in winter (4.56 ± 0.49%) and summer (6.30 ± 1.54%) and higher values in autumn (8.63 ± 0.52) and spring (9.51 ± 0.35%). The lipid content in *L. platensis* typically varies between 4 and 9% [50], and can also be affected by changes in environmental factors across different seasons. In a study with *Spirulina platensis*, lipid accumulation was observed by increasing the temperature (from 25 to 30 °C) and light intensity (from 2500 to 5000 lx) [57]. However, many microalgae species accumulate lipids in response to low light and temperatures [58]. In fact, studies have reported a decrease in transcript levels associated with lipid metabolism,

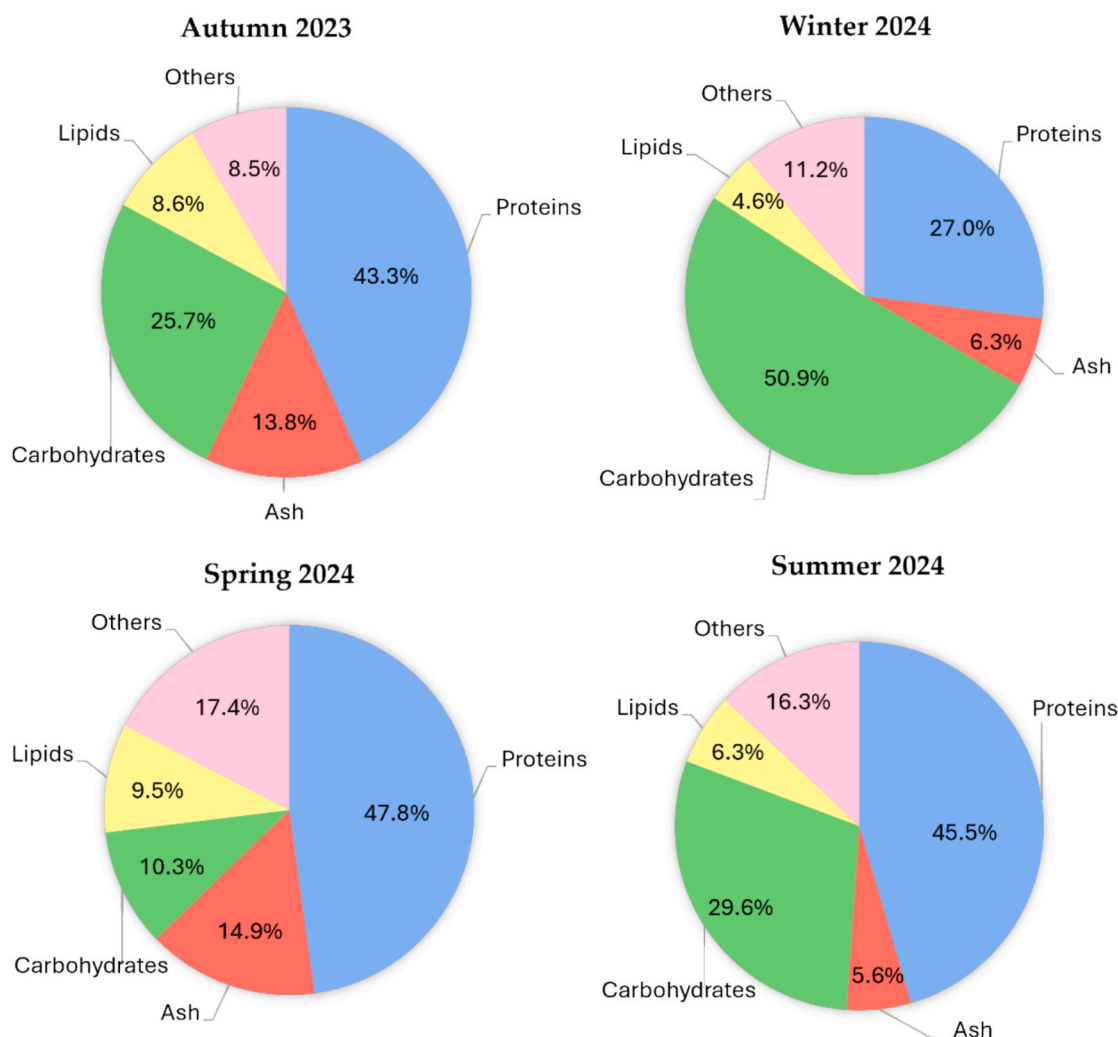


Fig. 1. Proximal composition of *L. platensis* biomass across the seasons (autumn, winter, spring, and summer), including the percentage (% of dried biomass) of proteins, ashes, carbohydrates and lipids content. The results of statistical analysis are presented in Table S2.

namely *gpsA* gene (GO:0004367) and *glpA* gene (GO:0004368), during exposure to high temperature, which in turn can result in a decrease in lipid content [59]. This effect in gene expression related to lipid metabolism may corroborate the decrease of lipids between spring and summer.

The ash content of *L. platensis* also varied seasonally, peaking in spring ($14.92 \pm 0.13\%$) and dropping significantly in winter ($6.34 \pm 0.11\%$) and summer ($5.63 \pm 0.08\%$). The “others” fraction (Fig. 1), estimated by difference, also changed across the four seasons, ranging from $8.53 \pm 0.89\%$ in autumn to $17.43 \pm 0.33\%$ in spring. In *M. gaditana* (Fig. 2 and Table S2), proteins represented always the predominant fraction, ranging from $30.71 \pm 2.99\%$ in winter to $37.36 \pm 0.75\%$ in spring. The protein content reported for *M. gaditana* varies between 20 and 45% [60]. Similar to what has been observed in *Spirulina*, the higher protein content recorded in spring may be associated with the increased external radiation during this season (Supplementary Fig. S2). Elevated radiation has been shown to reduce GS activity, thereby inducing NR and protease activities involved in nitrogen assimilation, which could contribute to enhanced protein accumulation.

The total carbohydrate content ranged from $11.23 \pm 0.12\%$ in winter to a maximum of $14.56 \pm 0.69\%$ in autumn. This range is in accordance with values previously reported for the *Microchloropsis* (formerly *Nannochloropsis*) genus (5–30%) [61,62]. Light intensity can affect carbohydrate content, as higher light intensities promote carbohydrate accumulation in *N. gaditana* grown under different light

intensities [63]. A previous study on *Nannochloropsis oceanica* also revealed an up-regulation of proteins involved in glycolysis under high light intensity [64]. Accordingly, we hypothesize that the increase in light intensity registered between winter and spring (Supplementary Fig. S3) contributed to the higher amounts of carbohydrates found in spring than in winter (Fig. 2 and Table S2).

The lipid content in *M. gaditana* varied between seasons, with higher levels found in autumn ($21.67 \pm 1.35\%$) and winter ($18.92 \pm 0.42\%$). The lower values recorded in spring and summer were $15.80 \pm 0.96\%$ and $15.93 \pm 0.60\%$, respectively. These values are in range with those previously reported for *M. gaditana* (13.50% and 15.45%) [65].

Several studies have shown that lipid accumulation in microalgae can increase under low-temperature conditions, as this helps maintain membrane fluidity and ensures normal cellular function [66,67]. Stress conditions such as low temperature can trigger the formation of reactive oxygen species (ROS). At moderate levels, ROS act as signaling molecules that activate pathways regulating lipid biosynthesis, thereby promoting lipid accumulation [68]. Furthermore, under low-temperature stress, the antioxidant defense system of algal cells is stimulated, leading to up-regulation of antioxidant-related enzymes and increased amino acid biosynthesis, which can provide additional precursors for lipid synthesis [69]. Overall, these mechanisms likely contribute to the higher lipid content observed in *M. gaditana* during winter (colder season) compared to spring and summer seasons.

The ash content varied between $11.41 \pm 0.12\%$ in autumn and 18.23

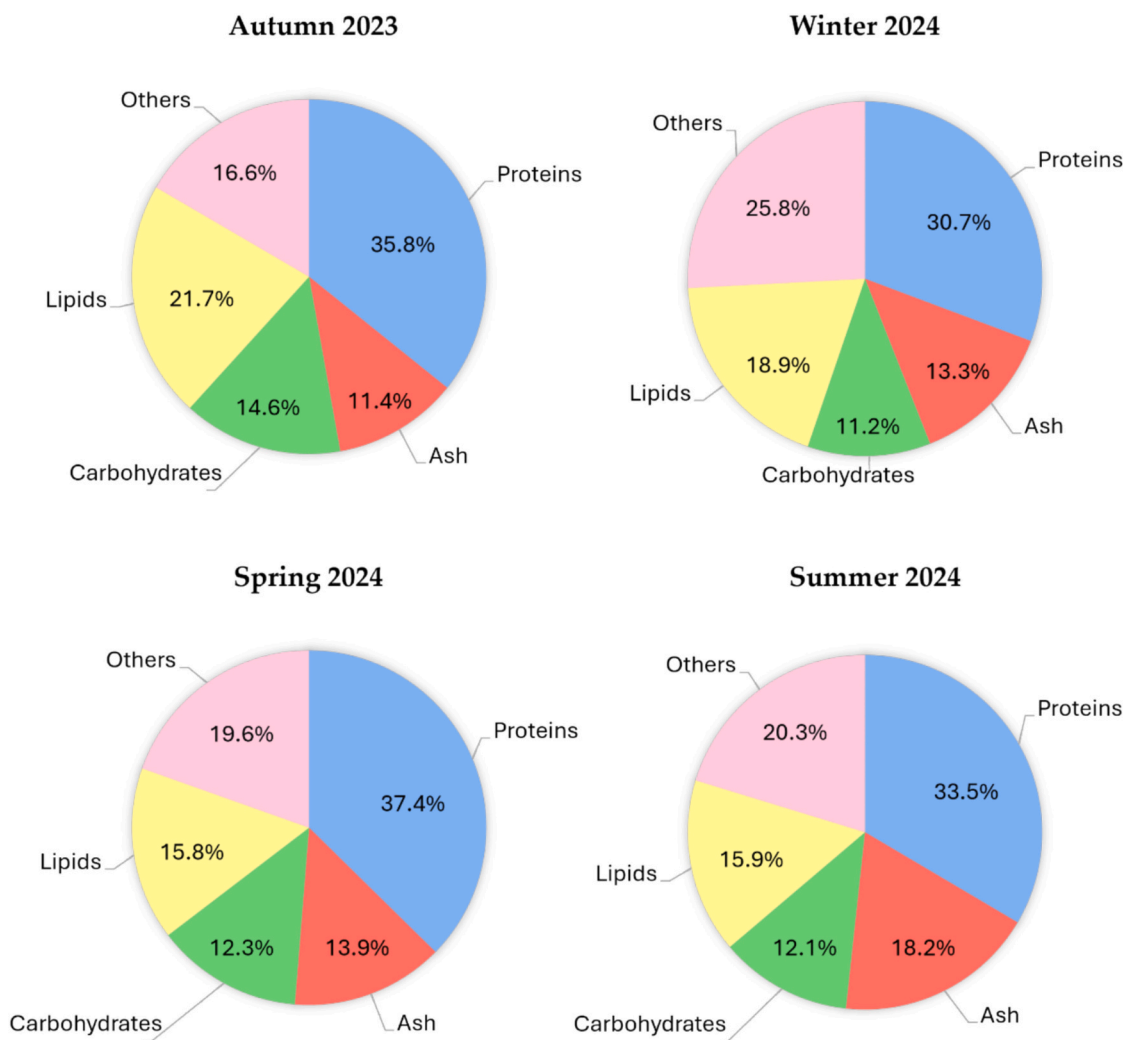


Fig. 2. Proximal composition of *M. gaditana* biomass across the seasons (autumn, winter, spring, and summer), including the percentage (% of dried biomass) of proteins, ashes, carbohydrates and lipids content. The results of statistical analysis are presented in Table S2.

$\pm 0.74\%$ in summer, representing a lower value compared to a previous report for spray-dried *M. gaditana* (21.26%) [70]. The “others” fraction (estimated by difference) ranged from $16.59 \pm 1.28\%$ in autumn to $25.84 \pm 3.79\%$ in winter.

It is important to emphasise that not all seasons supported growth for both species. *L. platensis* did not grow in autumn and winter, and *M. gaditana* exhibited no productivity during summer. Therefore, biochemical data from these non-productive seasons do not reflect feasible production outputs and cannot be used to identify optimal cultivation periods.

3.3. Amino acids profile of microalgae biomass grown across the different seasons

The amino acid content of *L. platensis* and *M. gaditana* was also analysed across the four seasons. Regarding *L. platensis* (Fig. 3 and Supplementary Table S3), the most abundant amino acids identified were methionine, threonine and tyrosine. Spring exhibited the highest total amino acid content ($47.15 \pm 0.11 \text{ g}\cdot 100 \text{ g}^{-1}$ DW biomass), followed by summer ($46.09 \pm 0.32 \text{ g}\cdot 100 \text{ g}^{-1}$ DW biomass). This is in accordance with the highest total protein contents found in these seasons. The higher abundance in spring and summer was particularly observed in essential amino acids, such as methionine and threonine. The increased metabolic activity due to higher temperatures and

prolonged sunlight exposure enhances *Spirulina*'s protein production [71]. This was observed when *Spirulina* sp. was exposed to temperatures ranging from 25 to 40 °C, where protein content was higher at 30 °C followed by 35 °C [72], which were in the range of the temperature recorded in summer (33.3 °C) (Supplementary Fig. S2). However, excessively high temperatures could also impact specific amino acids due to thermal degradation [73]. Contrarily, during winter, the total amino acid content was the lowest ($30.94 \pm 0.17 \text{ g}\cdot 100 \text{ g}^{-1}$ DW biomass) (Table S8). The reduced metabolic activity of *L. platensis* in colder temperatures likely contributes to this decrease in amino acid synthesis. *L. platensis* relies on photosynthesis for energy, and in colder conditions, its growth rate decreases because lower temperatures can impair photosynthetic efficiency. This means that less energy and fewer resources are available for amino acid and protein synthesis [71].

Although the impact of seasonal environmental changes on *L. platensis* amino acids has not been previously addressed, several studies have reported these changes in plants and algae in response to changes in factors such as temperature and radiation. For instance, one study addressed the impact of different temperature regimes on *Chlorella* and *Nannochloropsis*, and an increase in amino acids content was observed from 25 to 35 °C [74]. Another study, focusing on the diatom *Skeletonema costatum*, demonstrated that environmental conditions, including temperature and light, significantly impact its growth and biochemical composition. This work noted that exposure to high levels

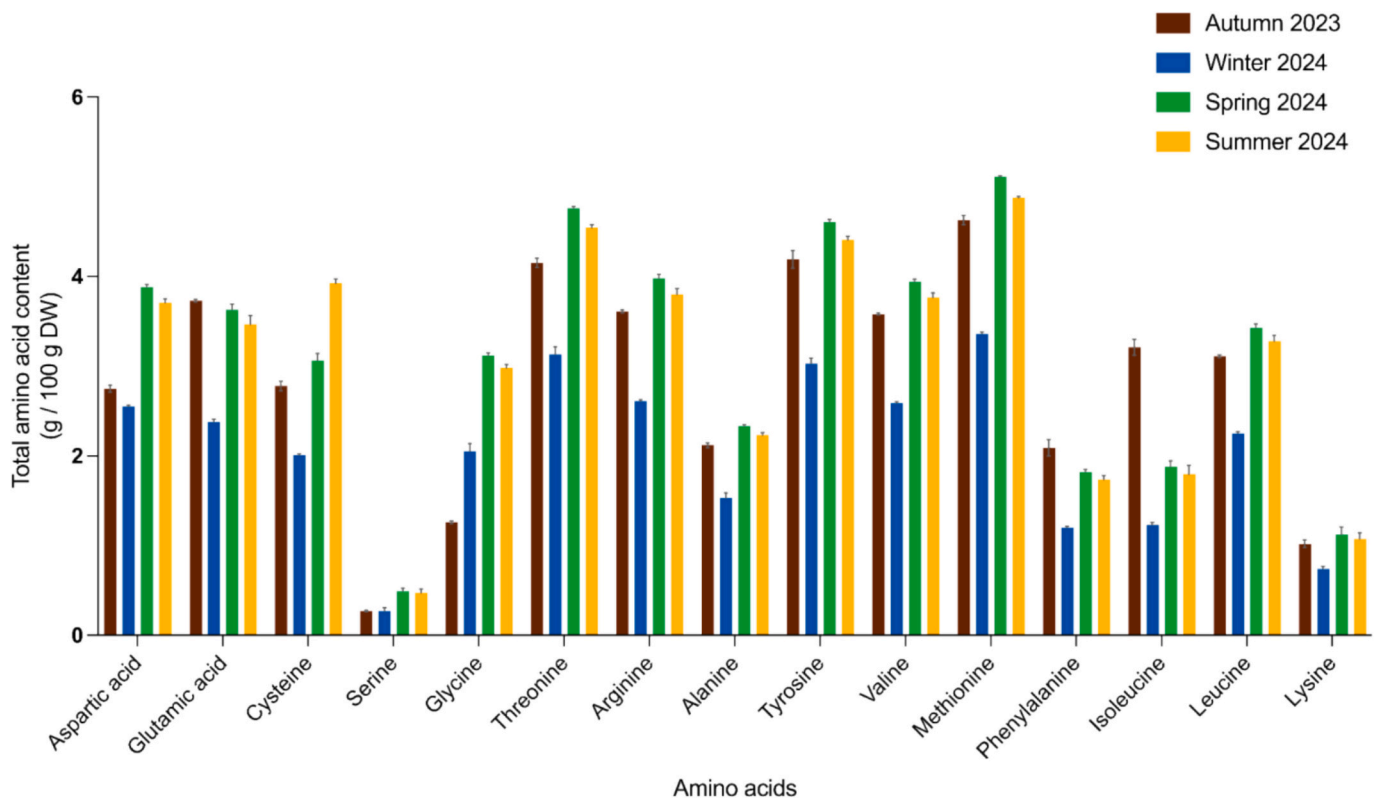


Fig. 3. Amino acids content (g.100 g⁻¹ DW biomass) of *L. platensis* along the seasons. This analysis includes both essential (threonine, valine, methionine, phenylalanine, isoleucine, leucine, and lysine) and non-essential (aspartic acid, glutamic acid, cysteine, asparagine, glutamine, glycine, arginine, alanine, and tyrosine) amino acids. Values are three replicates' mean ± standard deviation (SD) (n = 3). Results of statistical analysis are presented in Supplementary Table S8.

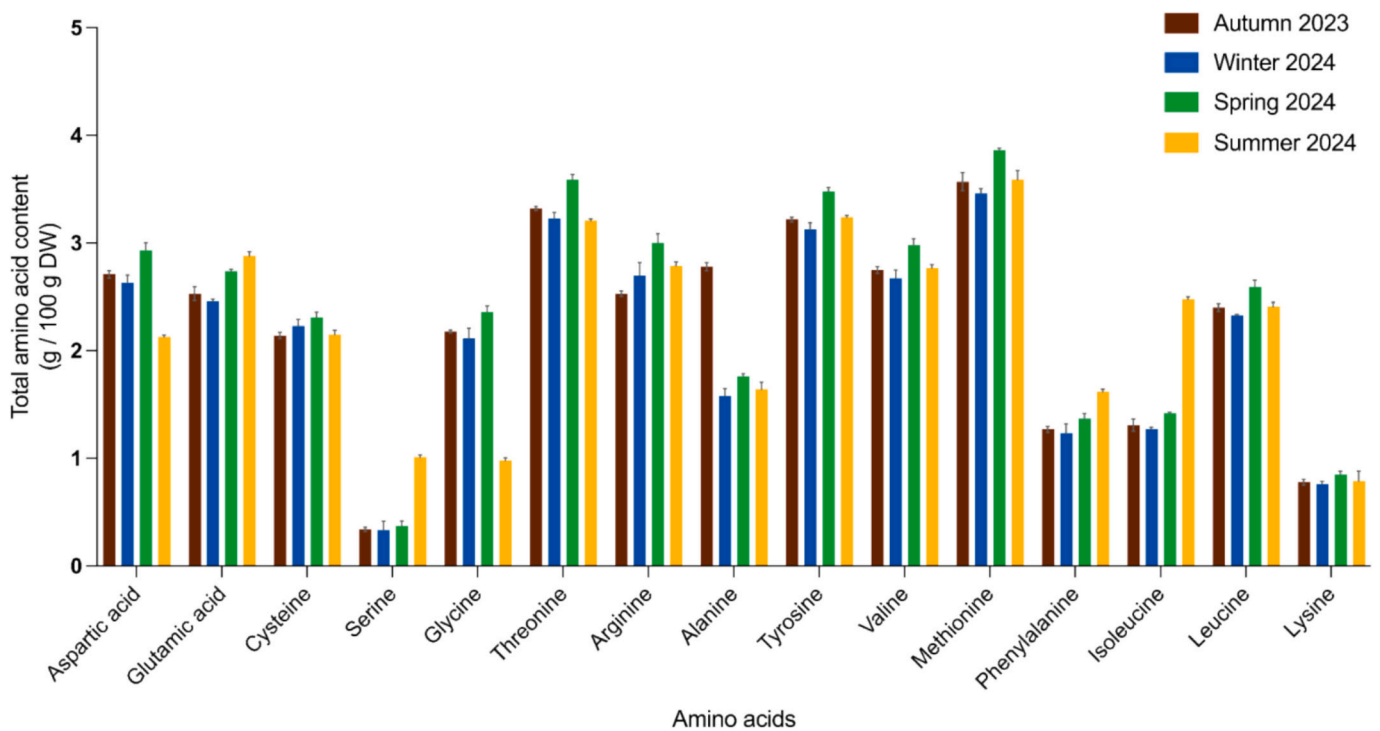


Fig. 4. Amino acids content and profile (g.100 g⁻¹ DW biomass) of *Microchloropsis gaditana* along the seasons. This analysis includes both essential (threonine, valine, methionine, phenylalanine, isoleucine, leucine, and lysine) and non-essential (aspartic acid, glutamic acid, cysteine, asparagine, glutamine, glycine, arginine, alanine, and tyrosine) amino acids. Values are three replicates' mean ± standard deviation (SD) (n = 3).

Table 1

Carbohydrate profile (mol%) of *L. platensis* biomass across autumn, winter, spring, and summer seasons. Values are the mean \pm standard deviation (SD) of three replicates ($n = 3$). Values in the same row followed by the same letter are significantly different at $p < 0.05$.

Monosaccharides (mol%)	Autumn 2023	Winter 2024	Spring 2024	Summer 2024
Rha	1.96 \pm 0.09 ^{a,b,c}	1.22 \pm 0.16 ^{a,d,e}	6.37 \pm 0.51 ^{b,d,f}	2.76 \pm 0.05 ^{c,e,f}
Fuc	0.64 \pm 0.07 ^a	0.48 \pm 0.01 ^b	2.42 \pm 0.21 ^{a,b,c}	0.73 \pm 0.04 ^c
Rib	1.91 \pm 0.2 ^{a,b}	0.93 \pm 0.08 ^{a,c}	5.82 \pm 0.35 ^{b,c}	1.3 \pm 0.32
Ara	0.74 \pm 0.11 ^{a,b}	0.49 \pm 0.03 ^{a,c,d}	2.3 \pm 0.35 ^{b,c,e}	0.95 \pm 0.06 ^{d,e}
Xyl	1.26 \pm 0.09 ^{a,b}	1.13 \pm 0.04 ^{a,c,d}	7.06 \pm 0.27 ^{b,c,e}	2.75 \pm 0.12 ^{d,e}
Man	1.63 \pm 0.16 ^{a,b}	0.25 \pm 0.02 ^{a,c,d}	2.2 \pm 0.27 ^{c,e}	0.59 \pm 0.22 ^{b,d,e}
Gal	5.08 \pm 0.27 ^{a,b}	1.75 \pm 0.1 ^{a,c,d}	15.04 \pm 1.03 ^{b,c,e}	5.45 \pm 0.12 ^{d,e}
Glc	79.52 \pm 1.81 ^{a,b}	90.12 \pm 0.79 ^{a,c,d}	48.08 \pm 2.33 ^{b,c,e}	81.82 \pm 1.03 ^{d,e}
UA	7.26 \pm 1.77 ^a	3.63 \pm 1.08 ^{a,b}	10.72 \pm 1.15 ^{b,c}	3.64 \pm 0.4 ^c

Abbreviations: Rha, rhamnose; Fuc, fucose; Rib, ribose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acids.

of UVB radiation led to a decrease in amino acid content, indicating that environmental factors can influence amino acid content in algae [75]. This suggests that amino acids biosynthesis is particularly sensitive to environmental conditions, with higher temperatures (30–35 °C), and prolonged light exposure favouring it. From a nutritional perspective, *L. platensis* harvested in spring provides a richer source of essential amino acids, making it the ideal season for maximizing its protein content.

The most abundant amino acids detected in *M. gaditana* were also methionine, threonine and tyrosine (Fig. 4 and Table S3). The data revealed that while seasonal differences exist, the fluctuations in total amino acid content for *M. gaditana* are less pronounced compared to *L. platensis*. The highest total amino acid content was observed for *M. gaditana* produced in spring (35.56 \pm 0.11 g.100 g⁻¹ DW biomass), followed by summer (33.67 \pm 0.09 g.100 g⁻¹ DW biomass) and autumn (33.86 \pm 0.25 g.100 g⁻¹ DW biomass). The lowest content found in winter (32.13 \pm 0.14 g.100 g⁻¹ DW biomass) may be attributed to lower metabolic activity due to decreased temperature and light availability, which could impact protein biosynthesis [76]. However, the extent of reduction is not as extensive as observed in *L. platensis*, suggesting relatively stable protein metabolism in *M. gaditana* even under less favorable environmental conditions. The observed differences in amino acid variability between *L. platensis* and *M. gaditana* can be attributed to their distinct metabolic strategies and ecological adaptations. *L. platensis*, as a cyanobacterium, relies predominantly on photoautotrophic growth, making its metabolic processes highly responsive to fluctuations in environmental factors such as light intensity and temperature. This dependence results in significant variations in amino acid composition under different environmental conditions. For instance, studies have shown that the amino acid content in *Spirulina* varies widely, with total amino acids ranging from 11.49 to 56.14 mg.100 g⁻¹ DW biomass across different samples [77]. In contrast, *M. gaditana*, a eukaryotic microalga, exhibits greater metabolic plasticity, particularly through its ability to modulate lipid metabolism under stress conditions. Under nutrient-replete conditions, certain mutants of *M. gaditana* have demonstrated improved lipid productivity, indicating a capacity to adjust metabolic pathways to enhance lipid accumulation without compromising growth [78]. This metabolic flexibility likely contributes to a more stable amino acid profile, as the organism can reallocate resources to maintain essential functions despite environmental fluctuations. A more stable amino acid profile in *M. gaditana* implies a more robust metabolic regulation, allowing it to sustain protein synthesis more evenly throughout the year.

From a nutritional perspective, while *L. platensis* harvested in spring offers the highest total amino acid content, *M. gaditana* provides a more consistent protein profile throughout the year. This stability may be advantageous for applications where a steady protein composition is desired, regardless of seasonal variations. The findings suggest that while both species respond to environmental changes, *M. gaditana* exhibits greater resilience and metabolic balance across seasons, making it a reliable source of amino acids in varying conditions.

These findings suggest that while external factors influence both microalgae, *L. platensis* demonstrates greater susceptibility to environmental changes in amino acid levels, whereas *M. gaditana* maintains a more stable biochemical composition. Understanding these differences is crucial for optimising cultivation strategies, particularly in biotechnological applications where consistent protein yields are desirable. Further research exploring the molecular mechanisms underlying these metabolic adjustments could provide deeper insights into how microalgae regulate amino acid biosynthesis in response to environmental stressors.

3.4. Carbohydrates profile of microalgae biomass grown across the different seasons

The carbohydrate profile was determined for *L. platensis* and *M. gaditana* biomass cultivated throughout the different seasons of the year. In addition to the variation observed in the total content (Fig. 1 and Table S2), *L. platensis* showed a significant variation on the carbohydrate profile depending on the time of the year (Table 1). In winter, *L. platensis* was predominantly composed by glucose (90.12 \pm 0.79 mol%), with minor amounts (< 5 mol%) of uronic acids, galactose, rhamnose, xylose, ribose, fucose, arabinose, and mannose residues. Contrastingly, in spring, the glucose residues only represented half of the total carbohydrates (48.08 \pm 2.33 mol%), increasing the relative percentage of the other sugar residues, mainly galactose (15.04 \pm 1.03 mol%), uronic acids (10.72 \pm 1.15 mol%), xylose (7.06 \pm 0.27 mol%), and rhamnose (6.37 \pm 0.51 mol%). In autumn and summer, this cyanobacterium presented a carbohydrate profile similar to winter profile's, with glucose as major monosaccharide representing 79.52 \pm 1.81 mol% and 81.82 \pm 1.03 mol%, respectively, and with other sugar residues appearing in minor amounts (< 7 mol%). Despite this similar trend, significant differences were found between these seasons (Table 1).

Glucose residues are associated with the presence of storage carbohydrates, namely glycogen and 1,3/1,6-linked β -glucans [31,79]. Similar to other prokaryotes, cyanobacteria accumulate glycogen, a highly branched glucose polymer with (α 1,4) glycosidic bonds for linear chains and (α 1,6) at branch points. *L. platensis* and other cyanobacteria can also produce 1,3:1,6- β -glucans, β -linked glucose molecules linked by 1–3 glycosidic bonds with 1–6 glycosidic branches, similarly to some microalgae, mushrooms, yeast, and bacteria [79,80]. β -glucans are well known for their bioactive properties, such as antitumor, antioxidant, immunomodulatory, and antibacterial activities, and are therefore highly interesting as bioactive ingredients for the development of functional foods [31].

The higher cells' accumulation of carbohydrates, namely glucans, was observed in winter, where lower light intensity and lower temperatures (18.4 °C) were registered (Supplementary Fig. S2). This increase in carbohydrate synthesis was previously observed for *L. platensis* grown at a suboptimal temperature of 25 °C, compared with the optimal temperature of 35 °C (in summer), where the productivity of *L. platensis* was superior [81]. According to the literature, high light irradiance has a

Table 2

Carbohydrate profile (mol%) and free alditols (mg.g⁻¹ DW biomass) of *M. gaditana* biomass across autumn, winter, spring, and summer seasons. Values are the mean \pm standard deviation (SD) of three replicates ($n = 3$). Values in the same row followed by the same letter are significantly different at $p < 0.05$.

Monosaccharides (mol%)	Autumn 2023	Winter 2024	Spring 2024	Summer 2024
Rha	6.12 \pm 0.19	5.85 \pm 0.32	6.35 \pm 0.16	5.71 \pm 0.38
Fuc	1.62 \pm 0.05	1.46 \pm 0.03	1.31 \pm 0.19	2.51 \pm 1.58
Rib	4.35 \pm 0.22 ^a	2.67 \pm 0.8 ^{a,b}	6.29 \pm 0.22 ^{b,c}	3.16 \pm 0.10 ^c
Ara	1.57 \pm 0.05	4.33 \pm 2.01 ^a	2.22 \pm 1.19	0.85 \pm 0.06 ^a
Xyl	1.43 \pm 0.26 ^a	2.04 \pm 0.28 ^b	4.10 \pm 0.43	1.73 \pm 0.07
Man	0.54 \pm 0.11 ^a	3.00 \pm 0.75	2.13 \pm 0.40	3.46 \pm 0.78
Gal	19.50 \pm 0.44	20.19 \pm 0.79	16.79 \pm 0.72	16.53 \pm 1.23
Glc	64.87 \pm 0.49	60.45 \pm 1.31	60.81 \pm 1.04	66.04 \pm 0.87

Abbreviations: Rha, rhamnose; Fuc, fucose; Rib, ribose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose.

positive influence on the synthesis of carbohydrates in *L. platensis* [81,82]. However, seasonal environmental stress in winter, manifested in colder temperatures, reduced light, and nutrient limitations, could lead *L. platensis* to redirect carbon into carbohydrate reserves, significantly elevating its content (~44–45% DW), as observed for other cyanobacteria. Conversely, the more favorable spring and summer conditions support growth-focused metabolism, resulting in lower carbohydrate accumulation (~15–25% DW) [30].

The existence of other sugar residues (e.g., galactose, uronic acids, xylose, rhamnose, fucose, and mannose) with a higher production ratio in spring in relation to glucans could be related to the presence of sulphated polysaccharides [32]. These polysaccharides have been associated with bioactive properties, namely antioxidant and antimicrobial activities, from sulphated polysaccharides isolated from *L. platensis* [83], and calcium spirulan, which exhibits antiviral properties [84].

Regarding *M. gaditana*, the biomass of each season showed little change in its total carbohydrate content (Fig. 2 and Table S2), but also differences in the amount of each individual monosaccharide (Table 2). Glucose residues were predominant in all seasons (60.45–66.04 mol%), in accordance with the literature for Eustigmatophytes (e.g., *Microchloropsis* and *Nannochloropsis*) [62,85]. Glucose residues found in these genera can be associated with the presence of β -glucans, namely laminaran in *N. oceanica*, β -1,3/ β 1,4-glucans [29] and β -1,3-/ β 1,6-glucans were described in *N. oculata* [86] and a soluble and low molecular weight β -1,3-glucan with limited β -1,6-branching in *M. gaditana* [87].

The sugar residues, galactose (16.53–20.19 mol%), rhamnose (5.71–6.35 mol%), mannose (0.55–3.46 mol%), ribose (2.67–6.29 mol%), xylose (1.43–4.10 mol%), fucose (1.31–2.51 mol%), and arabinose (0.85–4.33 mol%) are also present in minor amounts in *M. gaditana* across all seasons, in accordance with the literature for Eustigmatophytes [29,62,85]. In contrast to glucose, galactose, rhamnose, and fucose, other sugar residues showed significant changes throughout the year (Table 2).

According to literature, total carbohydrates production can be stimulated increasing the temperature from 15 to 30 °C in *N. oculata* [88], which could explain the lower carbohydrate production in winter (112.27 \pm 1.25 mg.g⁻¹ DW biomass) in relation to the autumn (145.55 \pm 6.92 mg.g⁻¹ DW biomass) and spring (133.52 \pm 5.57 mg.g⁻¹ DW biomass) and summer (120.8 \pm 2.49 mg.g⁻¹ DW biomass) seasons (Fig. 2). Higher light intensities (160 μ mol m⁻².s⁻¹) promoted the carbohydrate content in *M. gaditana* [23], which agrees with the higher carbohydrate content in autumn, spring and summer in relation to winter, where the light availability was lower (Fig. S2).

The free polyol mannitol also significantly decreased in summer (2.80 \pm 0.08 mg.g⁻¹ DW biomass), when compared with autumn (28.25 \pm 0.69 mg.g⁻¹ DW biomass), winter (20.00 \pm 3.51 mg.g⁻¹ DW biomass), and spring (33.65 \pm 2.65 mg.g⁻¹ DW biomass). Mannitol is an osmoregulatory compound found in Eustigmatophytes, that allows a rapid acclimation to variable osmolarity [89]. Besides salinity-driven changes in the intracellular content, high light intensity also reduces the content of osmoprotectants, such as mannitol, in *N. oceanica* [90],

consistent with the lower mannitol levels observed in this study during summer.

3.5. Fatty acids profile of microalgae biomass grown across the different seasons

The profile of esterified FA was identified for *L. platensis* and *M. gaditana* to determine changes across the seasons (Supplementary Table S4).

In *L. platensis*, the most abundant FAs throughout each season were FA 16:0, FA 18:2 *n*-6, and FA 18:3 *n*-6 (Fig. 5), which is in agreement with previous studies [91,92]. The total content of saturated fatty acids (SFA) was higher in autumn (15.31 \pm 0.41 mg.g⁻¹ biomass) compared to winter (5.32 \pm 0.46 mg.g⁻¹ biomass). SFA levels in spring (12.72 \pm 2.60 mg FA.g⁻¹ biomass) and summer (9.79 \pm 2.05 mg FA.g⁻¹ biomass) were similar. Regarding the impact of seasonality in the SFA, a previous study with *L. platensis* under controlled conditions indicated that an increase in temperature from 25 to 38 °C potentially increases the proportion of SFA, while an increase in light intensity from 170 to 1400 μ mol photons.m⁻².s⁻¹ is associated with a decrease in the relative abundance of SFA [93]. In the present study, the decrease in SFA observed between autumn and winter could be connected to the decrease in the temperatures registered (23.1 °C in autumn and 18.4 °C in winter).

Regarding the PUFA, namely FA 18:2 *n*-6 (linoleic acid, LA) and FA 18:3 *n*-6 (gamma-linolenic acid, GLA), they exhibited their lowest levels in winter (2.53 \pm 0.43 and 5.15 \pm 0.06 mg FA.g⁻¹ biomass, respectively). In contrast, their levels were higher and comparable across autumn (6.95 \pm 0.03 and 8.82 \pm 0.30 mg FA.g⁻¹ biomass, respectively), spring (6.90 \pm 1.41 and 7.37 \pm 1.46 mg FA.g⁻¹ biomass, respectively), and summer (5.78 \pm 1.40 and 6.51 \pm 1.58 mg FA.g⁻¹ biomass, respectively). The study with *L. platensis* subjected to different controlled conditions of temperature and light intensity indicated that, contrary to SFA, the proportion of PUFA decreases at higher temperatures, while higher light intensities seem to stimulate higher relative abundances of PUFA [93]. While the absolute amount of GLA was lower in winter compared to the other seasons (Supplementary Table S3), the relative abundance of this PUFA was higher in comparison (Supplementary Table S4). Thus, the changes in temperature and light intensity across the different seasons promoted a decrease in the proportion of GLA in biomasses grown under warmer seasons.

Both omega-6 FA (LA and GLA) are considered high-value compounds and are sought out as dietary components [94,95]. GLA was the most abundant PUFA reported in *L. platensis* in the present study. This omega-6 PUFA is obtained through dietary intake, typically from milk, and can play an essential role in the regulation of inflammation. In particular, supplementation of GLA is associated with anti-inflammatory properties [95], as GLA helps balance arachidonic acid (ARA, FA 20:4*n*-6) levels, and can be converted to dihomo- γ -linolenic acid (FA 20:3*n*-6), a precursor for the anti-inflammatory prostaglandin PGE1 [96]. Another study indicated that dietary supplementation with GLA can improve the skin's protective barrier, preventing dry skin and managing mild atopic

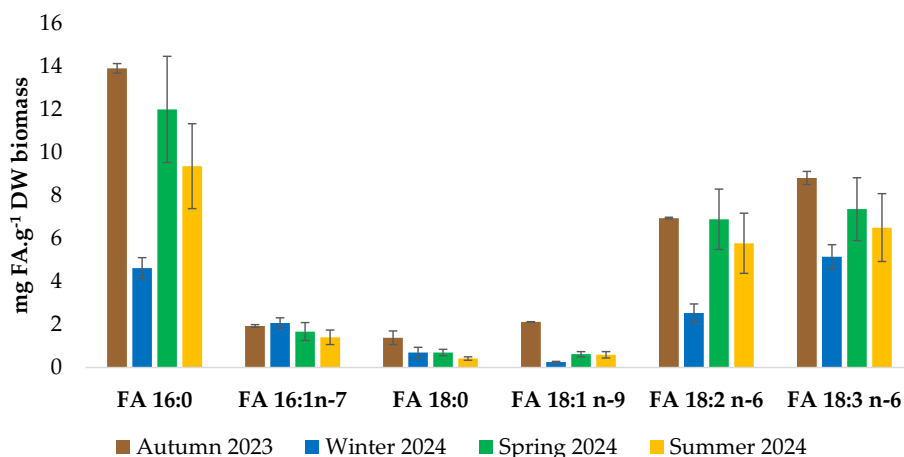


Fig. 5. Composition of the most abundant fatty acids ($\text{mg}\cdot\text{g}^{-1}$ DW biomass) of *L. platensis* determined by gas chromatography–mass spectrometry (GC–MS). Values are the mean \pm standard deviation (SD) of three replicates ($n = 3$). Results of statistical analysis are presented in Supplementary Table S3.

dermatitis [94]. On the other hand, LA (FA 18:2n-6) is an essential FA that mammals must obtain from the diet, as it serves as a precursor to longer-chain, more unsaturated fatty acids such as ARA (FA 20:4n-6), which plays a role in cell function and the biosynthesis of eicosanoids with pro-inflammatory and vasomodulatory actions [97]. Observational data further support an inverse relationship between LA intake and the incidence of cardiovascular diseases (particularly coronary artery disease) and metabolic disorders such as type 2 diabetes [98]. Given its role in human health, an adequate dietary supply of LA is essential. The seasonal variation observed in *L. platensis* suggests that biomass collected during autumn, spring, and summer, when LA and GLA levels are highest, could serve as a valuable natural source of LA and GLA for the production of nutraceuticals with anti-inflammatory and skin-improvement properties.

In *M. gaditana* (Fig. 6 and Supplementary Table S6), the most abundant FA across all seasons were FA 16:0, FA 16:1 n-7, and FA 20:5 n-3. This is in accordance with the FA profile reported for this microalga [99]. Regarding seasonal variations, the SFA content, namely FA 14:0, FA 16:0, and FA 18:0, remained relatively stable throughout the year. In contrast, the levels of unsaturated FA, especially of FA 20:5 n-3, exhibited seasonal fluctuations. The levels of FA 20:5 decreased significantly between winter (31.64 ± 3.69 $\text{mg}\cdot\text{g}^{-1}$ biomass) and summer (11.44 ± 1.19 $\text{mg}\cdot\text{g}^{-1}$ biomass), which corresponded to the seasons with the lowest (20.9 °C) and highest (26.1 °C) registered temperatures (Fig. S2).

This pattern aligns with previous results with *M. gaditana*, where higher temperatures (33 °C) promoted a substantial decrease in PUFA

content, particularly in EPA (FA 20:5 n-3) [100]. Other studies have indicated that light also affects EPA accumulation in *Nannochloropsis* species, with low light intensity being associated with higher yields of EPA [101]. Thus, it was expected that seasons with lower light intensities and lower temperatures, such as winter, would provide biomass enriched in this omega-3 FA. At lower temperatures, microalgae typically increase PUFA production to maintain membrane fluidity, ensuring optimal cellular function under colder conditions [102–104]. The seasonal variations in *M. gaditana* indicated that EPA levels were higher in winter.

This study highlights the high EPA content in *M. gaditana*, surpassing levels reported in other microalgae species such as *Pavlova gyrans* and *Tetraselmis striata*, both of which are also used as sources of PUFA and EPA for supplements and aquafeed [105,106]. Notably, the EPA content observed here are consistent with values reported for the same species [107] and other *Nannochloropsis* sp. [92,108,109], reinforcing its potential as a sustainable and efficient source of this essential FA. The health benefits of EPA are well established, with numerous studies highlighting their role in reducing the risk of coronary diseases, lowering triglyceride levels, and supporting overall well-being [110]. While *M. gaditana* has potential as a food supplement, it is still not approved for human consumption within the European Union. However, its primary application has been in the aquaculture sector due to its high nutritional value, particularly in terms of protein and EPA content [111]. The aquaculture industry heavily depends on fish oil as a primary source of omega-3 PUFA, including EPA, which are essential for many fish species and critical for maintaining the nutritional value of farmed

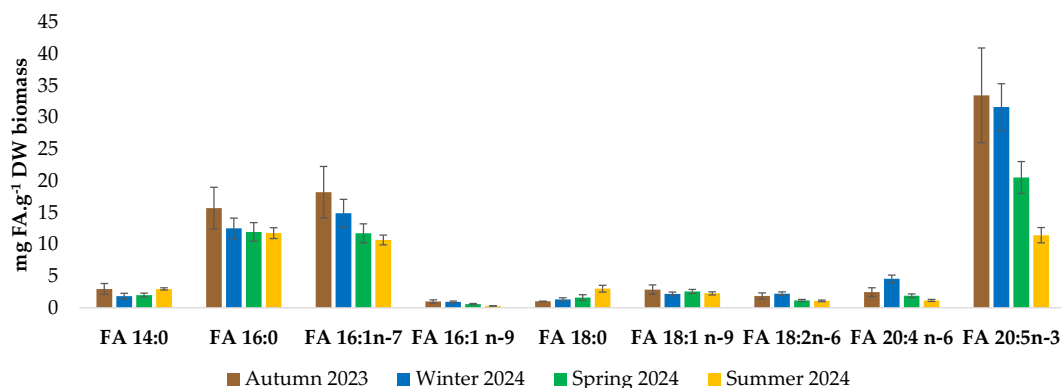


Fig. 6. Composition of the most abundant fatty acids ($\text{mg}\cdot\text{g}^{-1}$ DW biomass) of *M. gaditana* determined by gas chromatography–mass spectrometry (GC–MS). Values are the mean \pm standard deviation (SD) of three replicates ($n = 3$). Results of statistical analysis are presented in Supplementary Table S4.

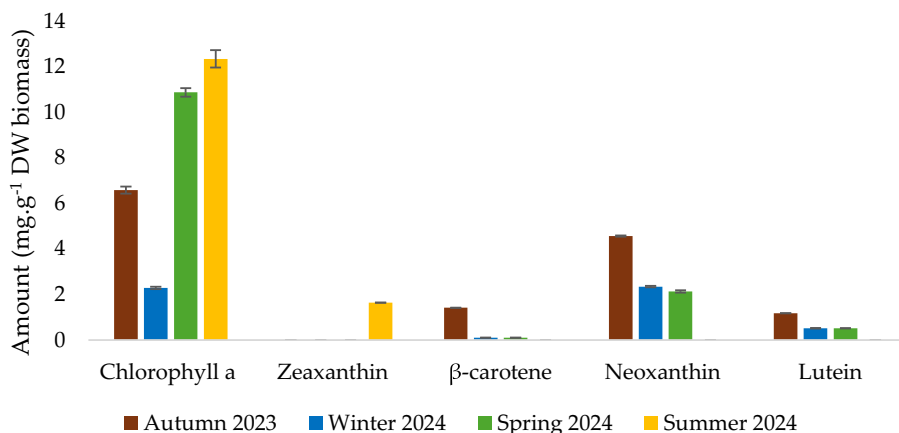


Fig. 7. Profile of chlorophylls and carotenoids ($\text{mg}\cdot\text{g}^{-1}$ DW biomass) in *L. platensis* along the seasons determined by HPLC. Values are the mean \pm standard deviation (SD) of three replicates ($n = 3$). Results of statistical analysis are included in Supplementary Table S5.

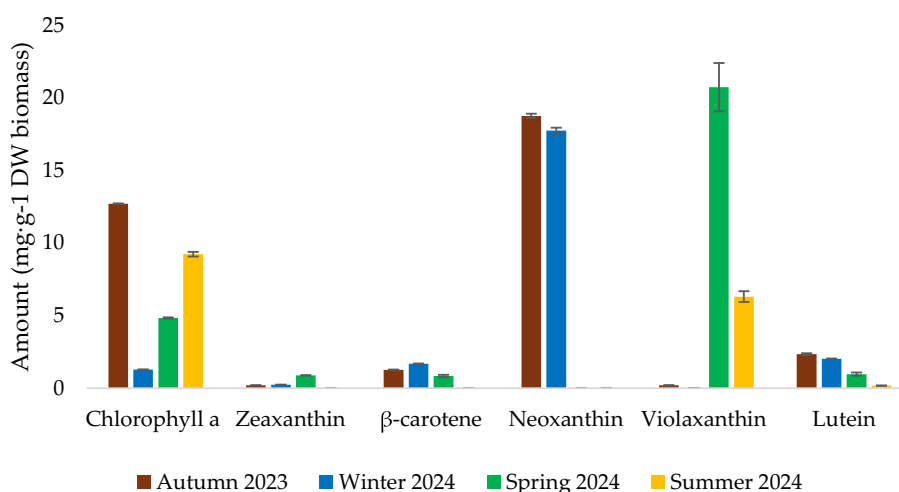


Fig. 8. Profile of chlorophylls and carotenoids ($\text{mg}\cdot\text{g}^{-1}$ DW biomass) in *M. gaditana* along the seasons determined by HPLC. Values are the mean \pm standard deviation (SD) of three replicates ($n = 3$). Results of statistical analysis are included in Supplementary Table S6.

seafood, supporting growth performance and ensuring fillet quality for consumers [112]. The supplementation of aquafeed with EPA-enriched sources is fundamental to increase the available EPA in the produced fish, thus *M. gaditana* grown in winter can support the requirements of feed-enriched in this healthy FA.

3.6. Pigments of microalgae biomass grown across the different seasons

The pigment profiles, including chlorophylls and carotenoids, of *L. platensis* (Fig. 7 and Table S8) and *M. gaditana* (Fig. 8 and Table S9), and phycocyanin levels of *L. platensis* (Supplementary Table S7) were evaluated across the four seasons.

For *L. platensis* (Fig. 7 and Table S8), chlorophyll *a* was the most abundant pigment identified across all four seasons, with levels ranging between $2.29 \pm 0.05 \text{ mg}\cdot\text{g}^{-1}$ DW biomass in winter to $12.35 \pm 0.38 \text{ mg}\cdot\text{g}^{-1}$ DW biomass in summer. Chlorophyll *a*, with its blue-green color is valuable as natural food colorants (E140 and E141), a trend driven by consumer demand for clean-label products [113]. Furthermore, these pigments offer a range of health benefits, including antioxidant, anti-cancer, and anti-inflammatory properties [113]. The results from our study suggest that chlorophyll content were higher in summer.

Carotenoid levels were also affected by seasonality. Neoxanthin was the most abundant carotenoid species identified in *L. platensis*, reaching peak production in autumn ($4.55 \pm 0.02 \text{ mg}\cdot\text{g}^{-1}$ DW biomass) and being completely absent in summer. Similarly, β -carotene and lutein followed

a similar pattern by peaking in autumn (1.42 ± 0.01 and $1.18 \pm 0.00 \text{ mg}\cdot\text{g}^{-1}$ DW biomass) and disappearing in summer. These carotenoids provide not only interesting antioxidant compounds for applications as nutraceuticals, pharmaceuticals, and cosmeceuticals, but also a wide color spectrum of natural dyes from yellow to purple, while exhibiting excellent stability under reducing conditions, positioning them as potential natural alternatives to synthetic dyes [33]. Thus, autumn samples exhibited higher carotenoid levels.

On the other hand, zeaxanthin was only detected in the summer ($1.64 \pm 0.02 \text{ mg}\cdot\text{g}^{-1}$ DW biomass), indicating its synthesis under high-light and high-temperature conditions. Although it has not been reported in microalgae yet, studies in *Arabidopsis thaliana* have reported the accumulation of zeaxanthin under excess light [34]. This carotenoid is known to enhance photoprotection and mitigate light-induced stress [34], and has a robust antioxidant activity that can protect eye cells from light-induced damage and help prevent retinal impairment [114].

For *L. platensis* we have also quantified phycocyanin (Supplementary Table S10), a pigment-protein complex that is highly abundant in this cyanobacterium [115]. We observed a similar seasonal trend to chlorophylls, with a significantly higher content produced during summer ($110.06 \pm 1.22 \text{ mg}\cdot\text{g}^{-1}$ DW biomass) and a lower content obtained in winter ($36.1 \pm 1.59 \text{ mg}\cdot\text{g}^{-1}$ DW biomass) (Supplementary Table S8). The coordinated accumulation of both chlorophylls and phycocyanin is expected, as this fluorescent protein acts as an antenna, capturing light and directing it towards the chloroplasts, where chlorophylls are

located, thereby contributing directly to biomass growth [116]. The higher light irradiance during the summer season stimulates production of phycocyanin, resulting in higher phycocyanin content. Phycocyanin exhibits similar beneficial properties to chlorophyll *a*, including anti-obesity, anti-melanogenic, and anti-neurodegenerative effects, making it valuable across various industries, from food as a natural colourant to pharmaceuticals as a biofluorescent marker or an active anti-tumoral agent. However, the stability of this pigment is often considered a drawback [115].

Regarding the pigment profile of *M. gaditana* (Fig. 8 and Supplementary Table S9), chlorophyll *a* peaked at $12.70 \pm 0.01 \text{ mg}\cdot\text{g}^{-1}$ DW during autumn.

Neoxanthin was especially abundant in autumn ($18.75 \pm 0.15 \text{ mg}\cdot\text{g}^{-1}$ DW biomass) and winter ($17.73 \pm 0.16 \text{ mg}\cdot\text{g}^{-1}$ DW biomass), but it was absent in spring and summer. In contrast, violaxanthin peaked in spring ($20.73 \pm 1.36 \text{ mg}\cdot\text{g}^{-1}$ DW biomass), indicating optimal production conditions during this period. Elevated temperatures, especially when combined with high light intensity, can enhance the activity of the xanthophyll cycle, leading to an increase in the conversion of violaxanthin into other pigments [117], which can explain the decrease in this carotenoid from spring to summer. Regarding neoxanthin, further studies are still required to understand how this carotenoid is produced during seasons associated with lower temperatures and light intensities. The remaining carotenoids, zeaxanthin, β -carotene, and lutein were found in lower content throughout the year, with lutein steadily decreasing from $2.34 \pm 0.06 \text{ mg}\cdot\text{g}^{-1}$ DW in autumn to $0.17 \pm 0.00 \text{ mg}\cdot\text{g}^{-1}$ DW in summer.

The seasonal pigment profile of *M. gaditana* highlights its potential as a source of commercially valuable carotenoids, particularly violaxanthin and neoxanthin, which are currently underutilized in industrial applications. While carotenoids, such as β -carotene, lutein, and zeaxanthin, are widely used as colorants in food and nutraceuticals in human health, violaxanthin and neoxanthin remain largely unavailable on the market, despite their presence in higher plants and their potential health benefits [118–121]. Violaxanthin, in particular, has been identified in various microalgae, and has exhibited bioactive properties, including antioxidant, anti-proliferative, anti-inflammatory, and pro-apoptotic effects against human cancer cells, reinforcing its potential for medical, nutraceutical, and functional food applications [118,119]. Similarly, neoxanthin has shown biological activities, including a suppressive effect on the differentiation of 3 T3-L1 adipose cells, leading to reduced lipid accumulation, an effect not observed with other carotenoids evaluated in the study [120]. Neoxanthin also demonstrated anticancer potential by inducing apoptosis in PC-3 human prostate cancer cells [121]. These findings highlight the relevance of *M. gaditana* as a promising source of bioactive carotenoids with potential applications in health-promoting and therapeutic products, positioning all year-round produced biomasses as sources of high-value compounds.

4. Conclusions

This study suggests that seasonal variations may have a significant influence on the biochemical composition of microalgae and cyanobacteria, reflecting their metabolic plasticity and adaptability to changing environmental conditions.

L. platensis exhibited seasonal fluctuations, particularly in protein, carbohydrate, and ash contents. Protein levels peaked in spring and summer, with essential and non-essential amino acids showing pronounced increases during this season. On the other hand, carbohydrate accumulation was highest in winter, likely as a response to the stress of lower temperatures. Glucose was the dominant carbohydrate residue, indicating the presence of storage polysaccharides, such as glycogen and β -glucans. These polysaccharides are also predominant in autumn and summer. Furthermore, spring featured a more diverse sugar profile, including galactose, uronic acids, and rhamnose, potentially indicative of bioactive sulfated polysaccharides. Lipid content remained relatively

stable, with elevated LA levels in autumn, spring, and summer, reinforcing its potential as a dietary source of essential FA.

M. gaditana maintained a more stable biochemical profile throughout the year, with lipid and carbohydrate content increasing in colder seasons. PUFA content, especially EPA, peaked in autumn and winter. This microalga also showed glucose as the main sugar associated to the presence of β -glucans and cellulose (structural component). The protein content of *M. gaditana* was relatively stable throughout the year, which is reflected in its amino acid profile.

L. platensis exhibited higher chlorophyll and phycocyanin content in spring and summer, and carotenoids such as β -carotene, neoxanthin, and violaxanthin peaked in autumn, while *M. gaditana* displayed significant seasonal variations in violaxanthin and neoxanthin.

Overall, these preliminary findings highlight that seasonal variation should be considered when interpreting biochemical composition of outdoor cultures. However, these results cannot be used to determine optimal production seasons, as productivity was not sustained in all periods for either species. Seasons with no growth (autumn/winter for *L. platensis* and summer for *M. gaditana*) provide biochemical information but do not represent viable production scenarios.

Further studies with different sampling times within each season would also be valuable to determine whether the observations presented here, based on a single sampling per season, are consistent across seasons and to evaluate possible intra-seasonal variations. Also, larger datasets combined with multivariate analyses would allow for a more robust and comprehensive evaluation of the seasonal variations. Finally, as each species was cultivated in its standard industrial system, system-specific effects cannot be completely separated from seasonal influences, and no comparison between cultivation systems is intended.

CRediT authorship contribution statement

Diana Lopes: Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Ana S.P. Moreira:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Tiago Conde:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Andreia Ferreira:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Kayane Oliveira:** Writing – review & editing, Investigation, Data curation. **Alexandra Conde:** Writing – review & editing, Investigation, Data curation. **Ana Alexandra Ramos:** Writing – review & editing, Validation, Investigation, Data curation. **Hugo Pereira:** Writing – review & editing, Investigation, Data curation. **Cláudia Nunes:** Writing – review & editing, Validation, Investigation, Data curation. **Sónia P.M. Ventura:** Writing – review & editing, Validation, Investigation, Data curation. **Natacha Coelho:** Writing – review & editing, Investigation, Data curation. **Alexandre M.C. Rodrigues:** Writing – review & editing, Validation. **Helena R. Rocha:** Validation, Investigation, Data curation. **Marta Coelho:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Ana Gomes:** Writing – review & editing, Validation. **Manuela Pintado:** Writing – review & editing, Validation, Funding acquisition. **Manuel A. Coimbra:** Writing – review & editing, Validation, Funding acquisition. **Maria do Rosário Domingues:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2026.104520>.

Data availability

Data will be made available on request.

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