Algal Research 93 (2026) 104468

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Bioremediation of drainwater from soilless cultivation by *Tetradesmus obliquus* and *Raphidonema monicae*: growth performance and biochemical composition from lab to industrial scale

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ARTICLE INFO

Keywords:
Microalgae
Bioremediation
Industrial scale
Soilless cultivation
Drainwater

ABSTRACT

To meet the demands of the growing population, agricultural practices have been increasing and putting a strain on land and freshwater usage. Soilless agriculture has emerged as a more sustainable practice to mitigate this issue but still generates nutrient-rich drainwater that can harm the environment if not properly managed. In this context, this study explores a circular economy approach to reuse the drainwater of soilless farming as a culture medium for microalgae production. For this, the growth performance of four strains, *Chlorella* sp., *Nannochloropsis limnetica*, *Raphidonema monicae* and *Tetradesmus obliquus*, was assessed using drainwater under summer and winter conditions at lab-scale. Based on productivity, protein and polyunsaturated fatty acids (PUFA) contents, *T. obliquus* and *R. monicae* were selected for comparison in pilot-scale flat-panel photobioreactors (FP-PBR) and raceway ponds (RW). *T. obliquus* presented significantly higher growth in FP-PBR (0.11 g L^{-1} d^{-1}) compared to RW (0.09 g L^{-1} d^{-1}), with complete nitrate removal in both systems. *R. monicae* showed similar growth across systems, removing 19 % of nitrate in FP-PBR and 56 % in RW. Principal component analysis indicated species-specific traits drive biochemical profiles, with limited influence from the cultivation system. Both species were cultivated in 19-m^3 tubular photobioreactors, with improved productivities (*T. obliquus* with 0.23 g L^{-1} d^{-1} and *R. monicae* with 0.13 g L^{-1} d^{-1}) until stationary phase or legal nitrate limits, yielding about 20 kg of dry weight each. The biomass produced in drainwater was biochemically characterized, showing it was rich in proteins (>30 %), PUFA (>55 %) and phenolics, highlighting their potential application in various sectors, including aquaculture and agriculture. Overall, this study demonstrates the potential of these strains for drainwater treatment, promoting a circular economy by converting waste into valuable biomass.

1. Introduction

About 37.9 % of the world's land and 70 % of the total freshwater supply is exploited for agricultural purposes [1]. To accommodate the demands of a continuously growing global population, there has been an intensification of agricultural production, resulting in increased land and freshwater use [2]. In Portugal, approximately 1 million hectares are used for agriculture, and it is estimated that around 3000 ha are utilized by greenhouse facilities, an area expected to increase in the coming years [3,4]. Along with other southern European countries [5,6], Portugal is situated in an arid or semi-arid region, experiencing an increase in prolonged drought periods due to climate change, which

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https://doi.org/10.1016/j.algal.2025.104468
Received 19 August 2025; Received in revised form 8 November 2025; Accepted 4 December 2025

leads to freshwater scarcity.

Over the last few decades, there has been a shift in agricultural practices towards reducing the environmental impact and reliance of agriculture on arable land, while improving productivity and minimizing resource' usage. Among various approaches, soilless cultivation refers to a method of crop cultivation that does not require soil, where plants are kept in a neutral substrate, such as coconut peat, and whose roots absorb inorganic nutrients supplied by water irrigation [7–9]. This technique is exclusively used in greenhouse facilities, as controlled conditions are required [10]. Several advantages of this technique have been pointed out: i) facilities can be located on non-arable land, or even in urban areas, decreasing transportation costs [11]; ii) 50 % higher biomass productivity and higher growth rates compared to conventional agriculture [12]; iii) improved water usage, with a 70 to 80 % water reduction compared to traditional methods [13,14]; iv) application of methods for automatic control of both irrigation and fertilization [12] and v) reduced labor needs.

On the other hand, a primary concern of using this system is the generation and discharge of waters containing high nutrient levels, commonly referred to as drainwater (DWT), which represents the nutrient-rich water that plants do not utilize. In fact, it has been shown that DWT can contain 200–500 mg L^{-1} of nitrate and 40–200 mg L^{-1} of phosphate [15–18]. In most cases, the continuous reuse of DWT is unfeasible, either due to the accumulation of salts not assimilated by the plants, or the accumulation of inhibitory compounds released by the plants through their roots, or even due to the presence of plant pathogens [19,20]. Moreover, the direct discharge of these waters represents not only an environmental concern, increasing the likelihood of eutrophication [21] and groundwater pollution through leaching, but also legal implications, as there are limits for discharge (EU Directive 2024/3019). Although some conventional treatments can be implemented for DWT treatment, such as denitrification or artificial wetlands, these are often expensive and unsustainable in the long term [21,22]. Ultimately, the discharge of DWT results in a misallocation of nutrients, representing a monthly loss of financial resources per cultivation area [23]. Therefore, it is clear that there is a need for more environmentally friendly, cost-effective, and nature-based solutions, with a particular emphasis on nutrient recycling.

Microalgae are photosynthetic microorganisms able to grow in non-potable water that, through the process of photosynthesis, convert CO_2, light and inorganic nutrients into oxygen and biomass rich in proteins, lipids, carbohydrates and pigments [24,25], yielding multiple products with biotechnological applications [26]. Although high production costs remain a constraint in microalgae production, several strategies are being implemented to overcome this issue [27], such as utilizing less costly nutrient sources, including industrial effluents. Indeed, there is cumulative evidence of the ability of microalgae to bioremediate effluents from different sources [28–31] and, more recently, microalgae have also been used as a bioremediation tool for DWT [17,18,32,33], however still at small scale, ranging from 0.5 to 1000 L of treated DWT. Currently, microalgae cultivation is considered one of the most promising approaches for tertiary wastewater bioremediation, allowing the production of high-value products from a waste stream [34]. Few studies have addressed pilot or industrial scale bioremediation of DWT, while assessing biomass composition for potential valorization.

In this sense, the main objective of this work was to investigate the bioremediation capacity of selected microalgae strains cultivated in DWT from soilless farming, demonstrating the feasibility of a co-production system at larger scales, aligned with the circular bio-economy concept. For that, the present study was designed having in consideration three main factors: 1) microalgae selection, by screening of four microalgae strains, two well established species in bioremediation processes and two novel strains with capacity to produce eicosapentaenoic acid (EPA; C20-5*n*-3), targeting the valorization of the generated biomass; 2) scale of production, as previous studies on DWT treatment were mostly focused in laboratory scale, the present study aimed at demonstrating the process from laboratory, to pilot-, using flat panel photobioreactors (FP-PBR) and raceway (RW) ponds, and finally to industrial scale, treating up to 19-m^3 of DWT with microalgae, the largest volume reported so far to the best of the author's knowledge; and 3) a comprehensive biochemical characterization along the different trial scales and cultivation systems, supporting species selection and enhancing the potential biomass applications.

2. Material and methods

2.1. Strain selection and culture conditions

Four microalgae strains were selected for this study, namely *Nannochloropsis limnetica* SAG 18.99, *Raphidonema monicae* SAG 2030, previously known as *Koliella antarctica* [35], and two local isolates of *Chlorella* sp. and *Tetradesmus obliquus*, provided by Necton S.A. (Olhão, Portugal). Preliminary selection criteria were based on their potential applications for the agricultural sector (*Chlorella* sp. and *T. obliquus*; [36]) or for being sources of eicosapentaenoic acid (EPA; C20-5*n*-3), for potential applications in aquaculture [37,38], as is the case of freshwater microalga *N. limnetica* and *R. monicae* [37,38], with the latter being a psychrophilic (cold-adapted) marine strain with the ability to grow in freshwater, thus being a euryhaline microalga [39]. The scale-up of the cultures was performed under laboratory conditions, where colonies of these strains were transferred to 100-mL Erlenmeyer flasks, with a working volume of 50 mL, in freshwater supplemented with Nutribloom® Plus (NB+; Necton S.A., Olhão, Portugal) at a final nitrate concentration of 4 mM. The cultures were kept in an incubator with an orbital shaker (Jeiotech IST-3075R, Korea) under constant mixing (140 rpm), light intensity (30 µmol m^{-2} s^{-1}) and temperature (20 °C), except for *R. monicae*, which was kept at 15 °C.

2.2. Drainwater characterization

The DWT, derived from a soilless raspberry production facility, was provided by the commercial producer Hubel Verde (Olhão, Portugal). Nitrate concentration was determined by a modified protocol established by Armstrong [40], by adding 0.5 mL of supernatant to a solution of 0.2 mL of HCl (1 M) and 9.3 mL of distilled water. The absorbance of each solution was measured using a UVmini-1240 spectrophotometer (Shimadzu, Japan) at 220 and 275 nm. Nitrate concentrations were inferred from a pre-established calibration curve. Phosphate concentrations were determined using a Spectroquant® Phosphate Reagent Kit Test (Merck, Germany), following the manufacturer's protocol. The absorbance of the prepared solutions was then measured using a spectrophotometer at 880 nm, and phosphate concentrations were calculated based on a previously established calibration curve.

Elemental analysis of the DWT samples was carried out [41,42] using an Agilent Technologies 4200 microwave plasma-atomic emission spectroscopic (MP-AES) analyzer (CEM Corporation, USA), following Technical Note 5990-9005EN. Calibration curves were prepared using certified standard solutions (Agilent, USA), for each element analyzed (Ca, Fe, K, Mg, Na, P, Ag, Al, Ba, Be, Cd, Co, Cr, Cu, Mn, Ni, Pb, V and Zn). Before the MP-AES analysis, DWT samples were digested in 67 % HNO_3 using a Discover SPD 80ML-909,755 microwave digestion system (Agilent 4200 MP-AES, Australia). Each sample was analyzed in triplicate and, when necessary, diluted accordingly in 5 % HNO_3. To ensure analytical quality assessments, blank corrections were applied to each sample, and specific elements were adjusted accordingly. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated based on the measurement of the blank' replicates. A detailed composition of the drainwater of each trial is described in the supplementary data (Table S1, supplementary material).

In addition to the prior assessments, drainwater samples collected before and after microalgae industrial production were also evaluated for the presence of pesticides by a certified laboratory (Mérieux

NutriSciences, Portugal), using Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS). The presence of pesticides in the biomass after industrial production was also evaluated by the same laboratory, using Gas Chromatography - Tandem Mass Spectrometry (GC–MS/MS).

2.3. Lab-scale trials

Lab-scale experiments were conducted to compare the growth of the selected strains cultivated in freshwater supplemented with NB+ (control) and DWT media, under simulated summer and winter conditions. These experiments were conducted in an incubated shaker (Jeiotech ISS-7100R, Korea) under constant mixing (140 rpm), temperature, and light conditions (Fig. S1A, supplementary material), with 15 °C and 150 μmol m^{-2} s^{-1} for winter and 25 °C and 300 μmol m^{-2} s^{-1} for summer conditions. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; VWR, USA) was added to the culture media (freshwater and DWT; 25 mM) to maintain pH, which was then adjusted to 7.5 with NaOH (1 M). All material was priorly autoclaved (120 °C for 20 min), except for DWT, which was filtered at 0.22 μm using a sterile filtration system. Cultures were inoculated at an initial dry weight (DW) of 0.15 g L^{-1} in 100-mL Erlenmeyer flasks, with a working volume of 60 mL. After, NB+ was added to the control to a final nitrate concentration of 6 mM, as this was the nitrate concentration of DWT. The experiment was carried out in triplicate and lasted for 14 days, with evaporation adjusted daily. At the end, cultures were centrifuged at 5000 *g* for 5 min, and the recovered wet biomass was stored at −20 °C until further analysis.

2.4. Pilot-scale trials

At pilot scale, a comparative study was conducted between two cultivation systems, FP-PBR and RW (Fig. S1B and S1C, supplementary material), under outdoor conditions during the spring.

For *T. obliquus*, FP-PBR consisted of a 0.3-mm thick plastic bag with 5 m length, 1 m height, and 0.08 m width, with a working volume of 400 L, while for *R. monicae,* a FP-PBR with a working volume of 100 L was used (1.3 m length, 1 m height, and 0.08 m width). Each FP-PBR was equipped with an individual aeration system, filtered through a 0.22 μm PTFE filter, and a CO_2 injection pulse system, to ensure a maximum pH of 7.5 for both cultures. Additionally, the FP-PBR system was equipped with a water sprinkler system, which was activated when the culture temperature surpassed 30 °C for *T. obliquus* and 27 °C for *R. monicae*.

The RW used had a surface area of 3.0 m^2 and was composed of two channels (0.39 m wide each) of 3.21 m, and a paddle wheel located on one of the channels at 1.76 m from the bend. Herein, a water column of 10 cm (working volume of 300 L) was used. Moreover, RW were equipped with a dissolved O_2 sensor (SUP-DM2800, Supmea, China) and a pH and temperature probe (SUPPH5013A, Supmea, China), with a set-point of 7.5 for both species, which triggered an automatic CO_2 injection on demand.

Outdoor temperature and solar irradiance were recorded by a WatchDog WD-2700 meteorological station, which was coupled with a LICOR LI-190R-BL-15 quantum sensor installed in the facilities (Fig. S2, supplementary material).

Prior to cultivation, DWT was subjected to a chemical treatment with sodium hypochlorite (250 mg L^{-1}) to remove contaminants and further neutralized with sodium thiosulfate (300 g L^{-1}). Outdoor systems were inoculated with *T. obliquus* at an initial DW of 0.25 g L^{-1} and *R. monicae* at an initial DW of 0.35 g L^{-1}. Both species were grown in batch mode for 7 days in triplicate. To assess the daily variation of metabolites and nutrient removal, samples were collected daily at 9:00, 13:00, and 17:00, centrifuged (4000 *g*, 10 min), and the pellet and supernatant were stored at −20 °C until further analysis. The samples collected on day 0 and day 1 were considered as the samples for the lag phase of growth, the samples on day 3 and 4 considered as the logarithmic phase of growth (log) and the samples at day 6 and 7 as the late logarithmic (late log) phase of growth.

2.5. Bioremediation and biomass production at industrial scale

For biomass production at industrial-scale, 15 m^3 of DWT was transferred to a 19-m^3 TPBR at Necton's facilities (Fig. S1D, supplementary material). These TPBRs occupy an area of implementation of 364 m^2, with 52 L m^{-2} culture volume per m^2. They are divided into two areas, as described by Pereira et al. [43]. Temperature and pH were controlled with a probe connected to an online control system. A pH set-point of 7.5 was defined for each species, with automatic CO_2 injection on demand. A water sprinkling system controlled maximum temperature, activated at 30 °C for *T. obliquus* and 25 °C for *R. monicae*. Solar irradiance and temperature data were provided by the meteorological station described in the last section. Inside the TPBR, DWT was subjected to the same chemical treatment described above. Phosphates were added to DWT to ensure a 7:1 N:P ratio. *T. obliquus* and *R. monicae* were inoculated at an initial DW of 0.5 and 0.3 g L^{-1}, respectively. A sample of the culture medium was collected before and after biomass cultivation to assess nutrient removal and the presence of pesticides. The cultures were operated under a batch mode on a single replica ($n = 1$) with a duration of 8 days for *T. obliquus* and 18 days for *R. monicae*, harvested by centrifugation (GEA Wesfalia KB25, USA) when reaching stationary phase, and the biomass was stored at −20 °C.

2.6. Culture monitorization

For lab-scale experiments, microalgae growth was evaluated through optical density (OD) measurements using a Biotek Synergy4 microplate reader (Agilent, USA) at wavelength of 540 nm. The DW of each strain was assessed by filtration of 10 mL of culture through pre-weighed 0.7 μm fibreglass filters (VWR, USA) and oven-dried at 60 °C until constant weight [44]. The collected data allowed the establishment of a linear regression between OD and DW. For pilot- and industrial-scale trials, a UVmini-1240 spectrophotometer (Shimadzu, Japan) was used for OD measurements. Therefore, a new regression was established. Calibration curves are presented in Table S2, Supplementary material. Growth data of the pilot-scale trial was normalized to account for differences in the initial biomass concentration between cultures, and expressed as fold-change, using the following formula:

$$\text{Normalized growth} = \frac{Biomass_t}{Biomass_0} \tag{1}$$

where $Biomass_t$ represents the biomass concentration (g L^{-1}) at time *t*, and $Biomass_0$ represents the biomass concentration (g L^{-1}) at the initial time point ($t = 0$). Additionally, microscopic observations were performed daily to visually assess the cultures in terms of their morphology and the occurrence of biological contaminants. Nitrate and phosphate consumption were estimated using the methods described above.

Specific volumetric productivity (g L^{-1} d^{-1}) was calculated according to Eq. (2):

$$\text{Specific volumetric productivity}\left(\text{g L}^{-1}\ \text{d}^{-1}\right) = \frac{X_1 - X_2}{V \times (t_1 - t_2)} \tag{2}$$

where X_1 and X_2 represent biomass (g) at time t_1 and t_2 (days), and *V* represents the volume (L).

2.7. Biochemical characterization

Biomass collected during each trial was freeze-dried (Martin Christ Alpha 1–2 LDplus, Osterode am Harz, Germany) and stored at −20 °C until biochemical characterization.

2.7.1. Proximate composition

Elemental analysis of total nitrogen was performed using Vario EL III (Elementar Analysensysteme GmbH, Germany), according to the manufacturer's procedure. The total nitrogen content was multiplied by the conversion factor of 4.97 [45], yielding the total protein content. Total lipids were determined by gravimetry using a modified version of Bligh and Dyer [46], as described by Pereira et al. [47]. Ash content was also estimated by gravimetry, as 50 mg of freeze-dried biomass was burned in a furnace muffle (Sel horn R9-L, J.P. Selecta, Spain) for 8 h at 525 °C [48]. Carbohydrates were determined by difference, subtracting the sum of protein, lipid, and ash content from 100 %.

2.7.2. Fatty acids

The fatty acids profile was determined using a slightly modified version of Pereira et al. [49]. Approximately 20 mg of freeze-dried biomass and ~0.6 g of glass beads were resuspended in 1.5 mL of derivatization solution (methanol:acetyl chloride, 20:1, v/v) and disrupted using a Retsch MM 400 mixer mill (30 Hz for 3 min; Scansci, Portugal). Afterwards, *n*-hexane was added to the samples, and derivatization occurred at 70 °C for 60 min. The fatty acid methyl esters (FAME) were then extracted twice from the mixture using *n*-hexane, and the lipidic phase (hexane fraction) was recovered after centrifugation (2000 *g* for 5 min). Anhydrous sodium sulphate (VWR, USA) was added to remove residual water, and the extracts were dried under nitrogen flow. Afterwards, the dried extracts were resuspended in a known volume of gas chromatography (GC)-grade hexane, filtered, and an internal standard (Nonanoic acid C9:0, Sigma-Aldrich, Portugal) was added to each sample before being stored at −20 °C until Gas Chromatography - Mass Spectrometry (GC–MS) analysis. FAME profiles were analyzed by a Scion 456/GC Scion TQ MS (Bruker, USA) equipped with a 30-m ZB-5MS capillary column (30 × 0.25 mm of internal diameter with 0.25 μm film thickness; Phenomenex, USA). Helium was used as the carrier gas; elution was performed at a flow rate of 1 mL min^{-1}, and the injection temperature was set at 300 °C in splitless mode. The temperature program consisted of the following settings: 60 °C for 1 min; 30 °C min^{-1} to 120 °C; 4 °C min^{-1} to 250 °C; 20 °C min^{-1} to 300 °C; and hold for 4 min at 300 °C. For FAME identification and quantification, a Supelco® 37 component FAME Mix (Sigma-Aldrich, Portugal) was used as a standard for different calibration curves.

2.7.3. Amino acids

For the determination of the amino acids profile, approximately 10 mg of dried biomass were homogenized and hydrolyzed with HCl 6 N, at 121 °C for 72 h. After cooling, the extracts were concentrated to dryness in a speed vacuum system (Concentrator Plus; Eppendorf, Germany) and resuspended in 0.02 N HCl. Then, derivatization was performed according to the Waters AccQ-Tag™ procedure for hydrolysate amino acids, as described for high-performance liquid chromatography (HPLC). Amino acids quantification was performed by HPLC (Chromaster, Hitachi, VWR, USA) with a fluorescence detector (5440 FL detector, Hitachi, VWR, USA). Chromatographic conditions and gradient composition were set according to the certified Waters AccQ-Tag™ for hydrolysate amino acids.

2.7.4. Pigments

Extraction of pigments was achieved following Schüler et al. [50]. In sum, 20 mg of dried biomass was weighed and placed together with ~0.6 g of glass beads and one titanium bead. The samples were kept on ice and in the dark during the extraction procedure, and 1 mL of methanol with 0.03 % of butylhydroxytoluene (BHT; Honeywell, USA) was added. Cellular disruption was performed using a bead beater (Retsch MM 400 mixer mill, Scansci, Portugal) at 30 Hz for 3 min. Afterwards, samples were centrifuged at 6000 *g* for 4 min at 4 °C, and the supernatant was recovered into amber glass vials. This process was repeated until both the pellet and supernatant were colorless. Extracts were dried under a nitrogen flow, and the residue was resuspended in 1 mL of HPLC-grade methanol.

Chlorophylls were quantified spectrophotometrically at 653 nm and 666 nm, and the estimation of chlorophyll *a* and chlorophyll *b* extracted in methanol was determined following Eq. (3) and Eq. (4) of Lichtenthaler and Wellburn [51].

$$\text{Chl } a = 15.65A_{666} - 7.34A_{653} \tag{3}$$

$$\text{Chl } b = 27.05A_{653} - 11.21A_{666} \tag{4}$$

The carotenoids profile was determined by HPLC (Chromaster, Hitachi, VWR, USA) with a diode array detector (5430 DAD, Hitachi, VWR, USA), as described in Couso et al. [53] and Schüler et al. [52], with a Purospher® STAR RP-18 end-capped (250 × 2.1 mm, 5 μm; Merck, Germany) chromatographic column at 20 °C, in a flow rate of 1 mL min^{-1} for 40 min. The injection volume was set to 50 μL. Compound identification was performed at 450 nm, by comparison with commercial standards and its concentration was calculated through the respective calibration curve.

2.7.5. Phenolics

The extraction of phenolic metabolites was performed as described in the previous section for pigments. The dried extracts were then resuspended in methanol to a final concentration of 20 mg mL^{-1}. Likewise, the phenolic profile was determined by HPLC (Chromaster, Hitachi, VWR, USA) with a diode array detector (5430 DAD, Hitachi, VWR, USA), with slight modifications from Cardoso et al. [54]. The analysis was performed using a Surf C18 TriF 100A 5 μm 250 × 4.6 mm (Imchem, France) chromatographic column at 25 °C, and a flow rate of 0.5 mL min^{-1} for 60 min. The injection volume was set to 20 μL. Identification of compounds was performed at 280 nm by comparing the retention times of commercial standards, and their concentrations were determined using a calibration curve for each phenolic compound.

2.7.6. Phytohormones

Approximately 1 g of industrially produced dried biomass was sent to an external certified laboratory (Innoplant, Spain) for identification and quantification of 11 phytohormones by UHPLC analysis, namely 1-aminocyclopropane-1-carboxylic acid, cytokinins (trans-zeatin, zeatin riboside, isopentenyladenine), gibberellins (gibberellin A1, gibberellic acid, gibberellin A4), indoleacetic acid, abscisic acid, salicylic acid and jasmonic acid.

2.8. Statistical analysis

Data is presented as means ± standard deviation (SD). Statistical analyses were performed using R Studio software (version 2023.06.1). To analyze the data from both lab and pilot-scale trials, statistical significance was tested using analysis of variance (one-way ANOVA) and post-hoc Tukey HSD tests with a significance level set at 0.05. Principal components analysis (PCA) was applied in the pilot-scale trial to assess how the production system affects each species. Growth, nitrate removal, and biochemical composition were analyzed as dependent variables, with the production system and species as the independent variable.

3. Results and discussion

3.1. Strain selection based on lab-scale trials

The growth performance of the four selected strains was assessed over 14 days in batch mode at laboratory scale, simulating both summer and winter seasons (Fig. 1), coupled with the determination of protein content and fatty acids profiles (Table 1).

Under simulated summer conditions (Fig. 1A), *Chlorella* sp. and *T. obliquus* exhibited the highest biomass concentrations (0.72 and 0.78 g L^{-1}, respectively) and productivity (0.041–0.046 g L^{-1} d^{-1}; Table 1), followed by *N. limnetica*, independently of the culture medium. Yet, *T. obliquus* presented a higher protein content (40.66 %; Table 1) when grown in the DWT in comparison to the other two strains (around 36 % for *Chlorella* sp. and *N. limnetica;* Table 1). This was also reflected in the

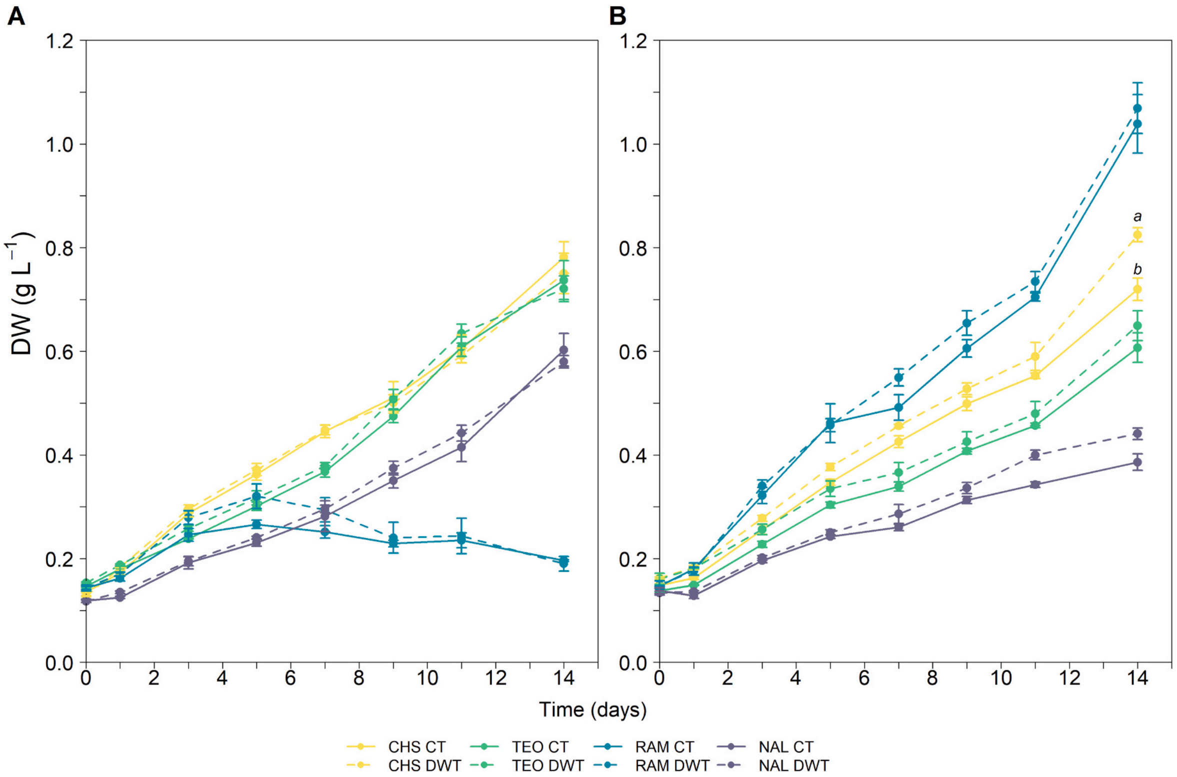

Fig. 1. Growth in biomass dry weight (DW; g L^{-1}) of *Chlorella* sp. (CHS), *Tetradesmus obliquus* (TEO), *Raphidonema monicae* (RAM) and *Nannochloropsis limnetica* (NAL) with freshwater and Nutribloom® Plus (CT; solid lines) and in drainwater (DWT; dotted lines) under simulated (A) summer conditions and (B) winter conditions, at laboratory scale and in a batch cultivation of 14 days ($n = 3$). Error bars represent standard deviation. Different letters indicate statistical differences ($p < 0.05$) between culture media.

polyunsaturated fatty acids (PUFA) contents, where *T. obliquus* and *Chlorella* sp. displayed PUFA concentrations above 50 % of total fatty acids (TFA; Table 1). However, EPA was only detected in *N. limnetica*, being significantly higher in DWT (21.5 ± 0.56 % of total fatty acids (TFA); Table 1) when compared to the control medium (16.12 % of TFA).

Simulated winter settings (Fig. 1B) impacted the growth and biochemical composition of the studied strains differently. *R. monicae* was able to reach its highest biomass concentration (1.16 g L^{-1}) and productivity (0.065 g L^{-1} d^{-1}; Table 1), followed by *Chlorella* sp., *T. obliquus* and *N. limnetica*. Interestingly, *Chlorella* sp. grew significantly better in DWT compared to the control medium, attaining a final concentration of 0.83 g L^{-1}, when compared to the control growth medium, i.e., NB+ (0.72 g L^{-1}; Fig. 1B). Still, the highest protein content was again observed in *T. obliquus* (45.5 %, Table 1), while the other strains presented similar protein content when grown in DWT, ranging from 37 to 38.8 %. Concerning PUFA contents, these were higher in *Chlorella* sp. (64 % of TFA) and in *R. monicae* (49.7 % of TFA) grown in DWT, while EPA was detected in *N. limnetica* (>20 % of TFA) but also in *R. monicae* (7–14 % of TFA, Table 1).

Overall, our results align with the available literature: *Chlorella* sp. and *T. obliquus* exhibited similar growth responses under both conditions, as expected, since these strains are known to withstand a wide range of temperatures, despite their optimal growth at around 30 °C [55–57], with similar protein content (40.7–45.5 %) reported in previous works [58]; *R. monicae* preferred lower light and temperature [37,39,59], whilst cell division has been reported as inhibited above 15 °C [60], resulting in higher protein content (~39 %), somewhat lower than those reported by Lima et al. [61], at 44.2 %. The present work also shows that, for *R. monicae,* higher PUFA (from 53.8 to 63.7 % of TFA) were recorded than previously estimated by Suzuki et al. (33.9–45.8 % of TFA) [59], with similar EPA content (6.7–13.8 % of TFA) as reported by Lima et al. [61], between 3.6 and 16.9 % of TFA and Fogliano et al. [37], between 4.5 and 6.8 % of TFA. Regarding *N. limnetica*, the best performance was achieved under simulated summer conditions, in alignment with previous reports indicating optimum growth at a temperature range of 22–24 °C, and a photon flux density ranging between 100 and 245 μmol m^{-2} s^{-1} [62,63]. Protein content of this species was similar to that previously reported of 47.2 % [63], while PUFA (37.8–58 % of TFA) and EPA (16.1–28 % of TFA) were higher than those reported by Couto et al. (20.3–41.7 % of PUFA and 14.3–33.1 % of EPA) [62].

Altogether, the lab-scale findings, particularly those obtained using DWT as a culture medium, guided the selection of two species for further trials: *T. obliquus* as the best-performing strain during summer, displaying enhanced growth, productivity, and protein content and *R. monicae* for winter conditions, coupling optimal growth, productivity, and PUFA contents, especially the high-value compound, EPA.

3.2. Pilot-scale production on drainwater

The two selected strains were scaled up, and their growth was compared when cultivated in two different systems under outdoor conditions during spring using FP-PBR and RW (Fig. 2, Fig. S2, supplementary material). *T. obliquus* presented an overall 3.8-fold increase in biomass, with a higher ($p < 0.05$) growth in the FP-PBR (productivity of 0.11 g L^{-1} d^{-1}) compared to the RW (productivity of 0.09 g L^{-1} d^{-1}). Banerjee and Ramaswamy [64] and Marsullo et al. [65] also demonstrated higher productivity when using a FP-PBR compared to a RW, primarily due to the former system's ability to receive more solar irradiance and tighter control over the growth conditions (e.g., temperature and pH). *R. monicae* showed a 1.7-fold increase in biomass in both systems (productivity of 0.05 g L^{-1} d^{-1} in both systems). The growth performance of *R. monicae* may have been affected by solar irradiance and

Table 1

Productivity ($g\ L^{-1}\ d^{-1}$), protein content (%), saturated fatty acids (SFA; %), monounsaturated fatty acids (MUFA; %), polyunsaturated fatty acids (PUFA; %) and eicosapentaenoic acid (EPA; %) of total fatty acids (TFA) content of *Chlorella* sp., *Tetradesmus obliquus*, *Raphidonema monicae* and *Nannochloropsis limnetica* grown with either freshwater with Nutribloom® Plus (CT) or drainwater (DWT) under summer and winter conditions at laboratory scale. Results are presented as mean ± standard deviation ($n = 3$). Different letters indicate statistical differences ($p < 0.05$) between culture media. N.a. – not analyzed; n.d. – not detected.

SUMMER		Productivity ($g\ L^{-1}\ d^{-1}$)	Protein (%)	∑ SFA (% TFA)	∑ MUFA (%TFA)	EPA (% TFA)	∑ PUFA (% TFA)
Chlorella sp.	CT	0.046 ± 0.00	34.05 ± 0.57^{b}	31.05 ± 0.90	9.98 ± 0.72	n.d.	58.96 ± 3.06
	DWT	0.044 ± 0.00	36.66 ± 0.78^{a}	31.51 ± 0.78	11.38 ± 0.70	n.d.	57.11 ± 5.19
Tetradesmus obliquus	CT	0.042 ± 0.00	41.10 ± 0.32	34.12 ± 0.83^{a}	31.29 ± 1.35^{a}	n.d.	34.59 ± 1.21^{b}
	DWT	0.041 ± 0.00	40.66 ± 0.24	28.93 ± 1.69^{b}	12.86 ± 1.10^{b}	n.d.	58.20 ± 2.75^{a}
Raphidonema monicae	CT	0.003 ± 0.00	n.a.	n.a.	n.a.	n.a.	n.a.
	DWT	0.003 ± 0.00	n.a.	n.a.	n.a.	n.a.	n.a.
Nannochloropsis limnetica	CT	0.035 ± 0.00	44.80 ± 1.06^{a}	23.78 ± 0.65^{b}	18.14 ± 1.34^{b}	16.12 ± 1.17^{b}	58.08 ± 5.14^{a}
	DWT	0.033 ± 0.00	36.34 ± 1.15^{b}	29.82 ± 0.64^{a}	32.72 ± 0.57^{a}	21.52 ± 0.59^{a}	37.84 ± 2.37^{b}
WINTER							
Chlorella sp.	CT	0.041 ± 0.00	38.98 ± 1.68	24.98 ± 0.73	9.98 ± 1.69	n.d.	65.03 ± 2.90
	DWT	0.047 ± 0.00	37.39 ± 0.56	26.85 ± 0.11	9.14 ± 0.63	n.d.	64.01 ± 0.74
Tetradesmus obliquus	CT	0.033 ± 0.00	45.39 ± 1.03	25.30 ± 1.37^{b}	10.05 ± 1.17	n.d.	64.66 ± 3.80^{a}
	DWT	0.035 ± 0.00	45.54 ± 0.33	39.50 ± 0.06^{a}	10.80 ± 0.52	n.d.	49.71 ± 0.45^{b}
Raphidonema monicae	CT	0.064 ± 0.00	41.88 ± 0.85^{a}	24.42 ± 1.37^{b}	11.84 ± 0.51	13.88 ± 1.25^{a}	63.74 ± 4.39^{a}
	DWT	0.066 ± 0.00	38.81 ± 1.19^{b}	36.41 ± 1.89^{a}	9.72 ± 0.67	6.65 ± 0.66^{b}	53.87 ± 3.12^{b}
Nannochloropsis limnetica	CT	0.018 ± 0.00	39.45 ± 0.83	25.69 ± 1.16	34.44 ± 0.65^{a}	28.08 ± 0.31^{a}	39.87 ± 1.16
	DWT	0.022 ± 0.00	37.00 ± 0.44	26.94 ± 1.90	32.13 ± 1.27^{b}	20.76 ± 0.43^{b}	40.94 ± 1.96

temperatures higher than expected at the time of the trial (spring; Fig. S2), with the culture's temperatures ranging from 16.5 to 26.6 °C. As previously discussed, this strain exhibits improved cellular performance at lower light intensities [39] and at temperatures below 15 °C [60].

In terms of nitrate removal, *T. obliquus* fully removed the nitrate present by the 5th day of cultivation, with no significant differences observed in either system, but with continued growth even after nitrate depletion. Previous studies have reported on nitrogen reserves in microalgae, that are used for the conversion into key enzymes or essential biomolecules, thus maintaining cell division even in nitrogen-free growth medium [66], which could explain the obtained results. In *R. monicae,* nitrate removal was slower, with a ~19 % removal on the FP-PBR and a ~56 % removal on the RW, by the end of the experiment, since growth performance of this species was impacted by the suboptimal growth conditions. The effect of suboptimal conditions was reported by Santos et al. [67], showing the impact on growth performance and, most likely, in the removal of nutrients, despite the presence of the latter in the culture medium. A higher rate of removal in the RW could also be related to denitrification processes through a synergistic interaction of *R. monicae* with associated heterotrophic bacteria, possibly due to a higher bacterial load [68,69], considering that these are open systems.

When analyzing *T. obliquus* and *R. monicae* in both systems over time, no significant differences were found regarding proximal composition's productivity in terms of growth phase or time of day (data not shown). However, significant differences were found in the proximal composition content of both species when the growth phase was considered (Fig. 3). The same pattern for protein content was observed in both species: higher content in cultures grown in the RW (~40 % in *T. obliquus* and ~ 35 % in *R. monicae*) compared to the FP-PBR (~35 % in *T. obliquus* and ~33 % in *R. monicae*; $p < 0.05$) during the late logarithmic (late log) phase of growth. The same pattern was observed for the ash content during the lag phase - a higher content in the cultures grown in the RW (~16 % in *T. obliquus* and ~12 % in *R. monicae*) compared to the cultures grown in the FP-PBR (~14 % in *T. obliquus* and ~8 % in *R. monicae; p* < 0.05). During the production of *R. monicae* (Fig. 3B)*,* there were no major differences regarding lipids, carbohydrates or ashes between systems, with only a slightly higher lipid content in the cultures grown in the FP-PBR (19 % of DW) during the lag phase. This result may be due to the slow growth of the culture, which was impacted by the high temperatures felt during spring (Fig. S2). However, such a trend was not found in the production of *T. obliquus* (Fig. 3A), as lipid content increased and ash content decreased over time, and were higher in cells grown in the RW (17.5–20.9 % of lipids and 7.2–9.89 % of ash; $p < 0.05$) compared to the microalgae cultivated in the FP-PBR in both log and late log phase of growth, ranging from 15.7 to 17.8 % of lipids and 5.1–7.7 % of ash. The observed lipid increase might be the result of a stressor applied to the culture, caused by nitrate depletion and the entry of the culture into the stationary phase, both of which lead to lipid accumulation [70]. Increased lipid content enhances the valorization of the biomass, enabling its application in the bioenergy sector [71]. The carbohydrate content presented an opposite trend, with higher concentrations in cultures grown in the FP-PBR, between 32.6 and 42.5 %, when compared with the cultures grown in the RW (between 25.1 and 31.1 %; $p < 0.05$).

In terms of fatty acids (Table 2), higher amounts of saturated (SFA) and monounsaturated (MUFA) fatty acids were found in *T. obliquus* grown in FP-PBR, whereas higher PUFA were found in *T. obliquus* grown in the RW, all during the late log phase of growth. SFA and MUFA usually accumulate in cells under adverse conditions [25,72]. Together with the significantly higher growth in the FP-PBR in *T. obliquus* (Fig. 2), these results suggest that the culture in this system entered nutrient stress conditions before the cultures in the RW. The PUFA content varied between 68.92 % and 77.26 % of TFA and between 74.73 % and 76.63 % of TFA for *T. obliquus* and *R. monicae*, respectively, in agreement with other reports [59,73]. *T. obliquus* grown in the RW presented a

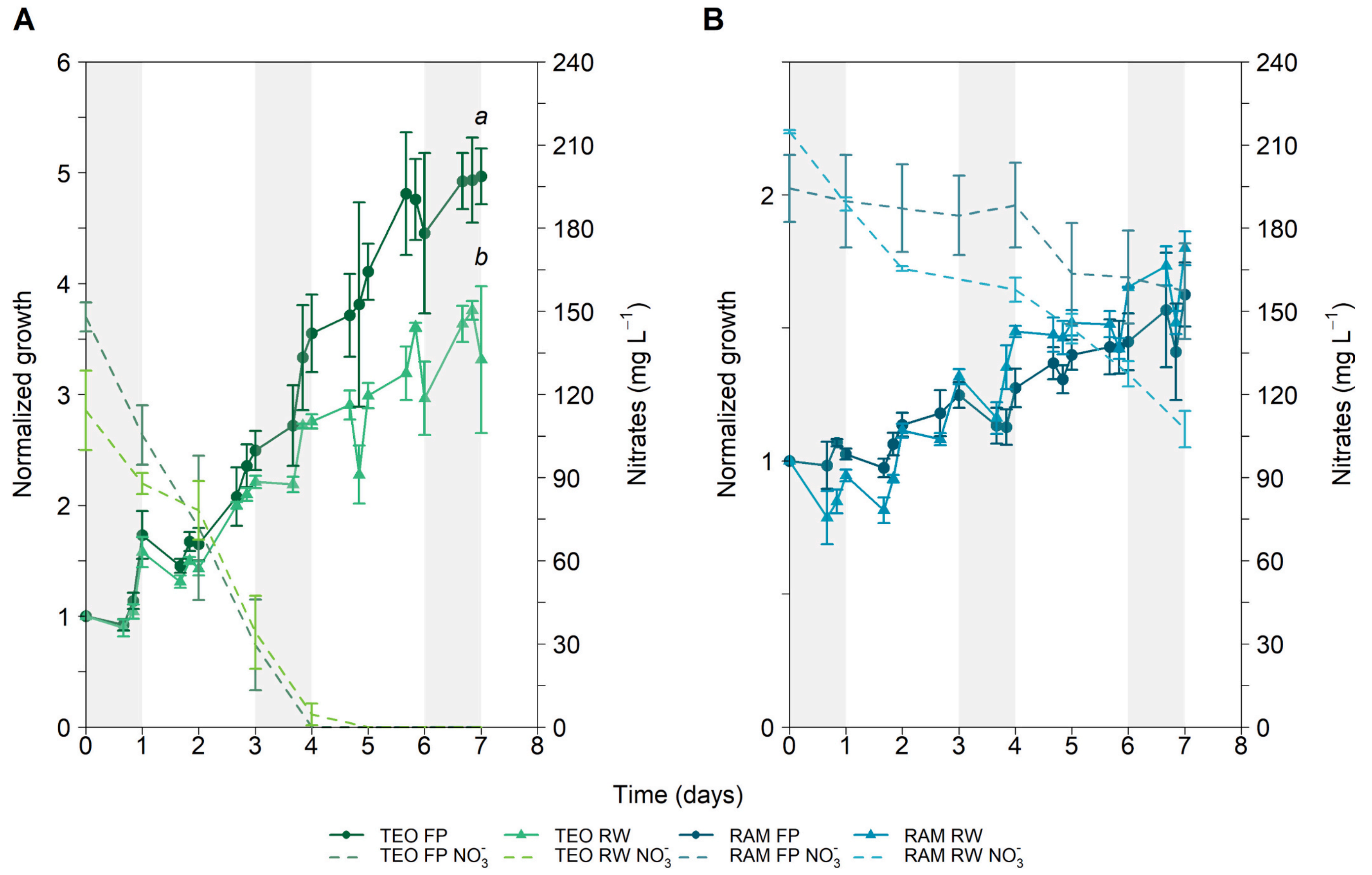

Fig. 2. Normalized growth performance of (A) *Tetradesmus obliquus* (TEO; green) and (B) *Raphidonema monicae* (RAM; blue) grown in drainwater during spring, in flat panels (circles) or raceways (triangles). Dashed lines represent nitrate concentration (mg L^{-1}) of each system ($n = 3$). Grey bars represent the samples considered for lag, logarithmic (log) and late logarithmic (late log) phases of growth. Error bars represent standard deviation. Different letters indicate statistical differences ($p < 0.05$) between the systems. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly higher PUFA content at 73.8 % of TFA, compared to the *T. obliquus*' cultures grown in the FP-PBR (64.75 % of TFA) at the late log phase of growth. PUFA are often part of structural lipids, indicating a shift to higher membrane fluidity under healthy growth conditions [25,70,74]. *R. monicae* displayed higher SFA contents in the RW during log and late log phases of growth, while higher MUFA levels were found in cells cultivated in the FP-PBR during the same phases. There were no significant differences in terms of total PUFA. Conversely, EPA contents were enhanced in *R. monicae* grown in the FP-PBR during the log phase of growth, representing 10.73 % of TFA, in comparison to 9.39 % produced in the RW ($p < 0.05$). Considering that this strain showed a lower nitrate removal ability, an adequate strategy could be to partially harvest the culture when necessary and recirculate the harvested water for continuous nutrient removal [75,76].

A principal components analysis (PCA) was conducted to better understand the relationships among variables for both species (*T. obliquus* and *R. monicae*) under the two production systems (RW and FP-PBR; Fig. S3, supplementary material). For *T. obliquus,* the PCA explained 80.3 % of the total variance (Fig. S3A, supplementary material), showing overlap between systems, indicating similar biochemical composition. Growth was more closely linked to the production in FP-PBR, whereas RW production favored higher fatty acid content, particularly PUFA. In contrast, *R. monicae* showed lower variance (65.8 %; Fig. S3B, supplementary material), but more pronounced system-dependent differences: FP-PBR production exhibited higher fatty acids (FA) content and, to a lower extent, higher nitrate removal, while RW samples presented higher ash and lower FA content. These results suggest that *R. monicae* seems more sensitive to production system' effects, supporting previous observations that biochemical responses are often species-specific and linked to differences in nutrient uptake and metabolic strategies [77,78]. When comparing both species within each system, *T. obliquus* displayed a variance of greater biochemical variability, characterized by protein, lipids, SFA and nitrate removal, whereas *R. monicae* had higher carbohydrate, ash and PUFA contents. This pattern was consistent across RW and FP-PBR production (Fig. S3C and Fig. S3D, supplementary material), suggesting once again that species-specific traits outweighed the production effects. Moreover, a positive correlation was observed between lipid content and nitrate removal (Fig. S3C and S3D, supplementary material), oriented in nearly the same direction and more pronounced for *T. obliquus*, suggesting that nitrate depletion promotes lipid accumulation, in line with previous reports [70]. Overall, the species' inherent characteristics outweighed the effects of production systems in shaping biochemical composition.

3.3. Bioremediation at industrial scale

As a proof of concept, the growth performance and nutrient removal ability of the two selected strains were also evaluated at an industrial scale, using a 19-m^3 TPBR (Fig. 4).

Industrial production of *T. obliquus* occurred in February 2022, lasting 8 days, while the production of *R. monicae* occurred in March 2022, spanning a total of 18 days of production, both in batch mode. Daily average maximum solar irradiance was similar for both strains, with an average of 1259 ± 47 μmol m^{-2} s^{-1} (minimum of 1162 and maximum 1314 μmol m^{-2} s^{-1}) during *T. obliquus* cultivation, compared to an average of 1174 ± 391 μmol m^{-2} s^{-1} (minimum of 213 and

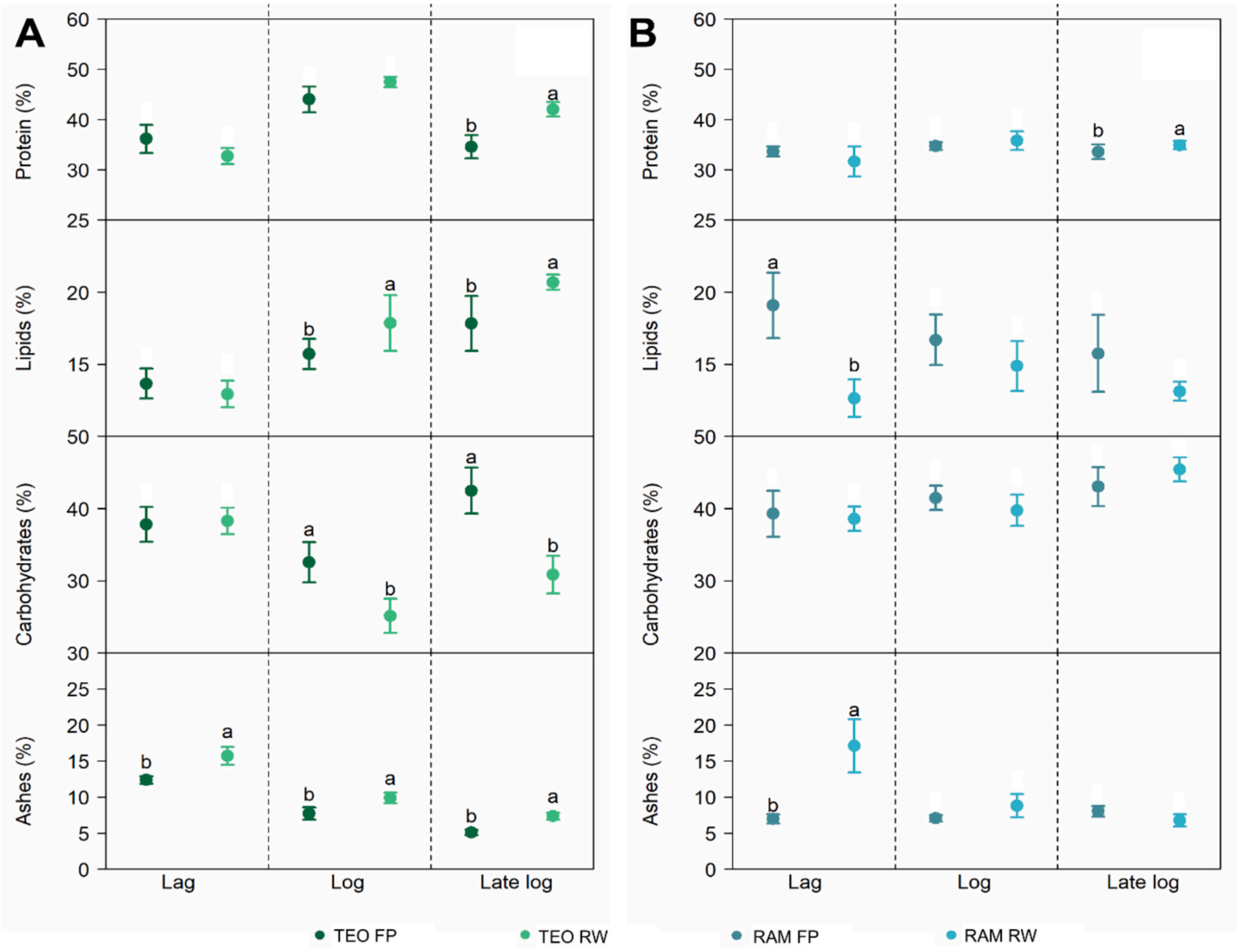

Fig. 3. Proximate composition (%) of *Tetradesmus obliquus* (A, green, TEO) and *Raphidonema monicae* (B, blue, RAM) grown in drainwater, in flat panels (FP, dark colors) and raceways (RW, light colors) during Lag, logarithmic (Log) and late logarithmic (Late log) phases of growth ($n = 3$). Error bars represent standard deviation. Different letters indicate statistical differences ($p < 0.05$) between the systems for each growth phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Fatty acids content (% of total fatty acids) of *Tetradesmus obliquus* and *Raphidonema monicae* during Lag, logarithmic (Log) and late logarithmic (Late log) phases of growth ($n = 3$), grown in raceways (RW) and flat-panels (FP-PBR). Different letters indicate statistical differences ($p < 0.05$) between the systems for each growth phase. SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; EPA – eicosapentaenoic acid.

Tetradesmus obliquus	RW			FP-PBR		
	Lag	Log	Late log	Lag	Log	Late log
∑ SFA	20.42 ± 0.63	14.94 ± 0.42	17.43 ± 0.32^{b}	19.72 ± 0.77	16.19 ± 0.89	21.83 ± 1.86^{a}
∑ MUFA	10.30 ± 0.92	7.79 ± 0.84	8.77 ± 0.91^{b}	11.48 ± 1.62	7.82 ± 0.88	13.42 ± 1.84^{a}
∑ PUFA	69.28 ± 2.31	77.26 ± 2.55	73.80 ± 2.19^{a}	68.92 ± 3.73	75.99 ± 4.02	64.75 ± 5.22^{b}
Raphidonema monicae						
∑ SFA	16.44 ± 0.95	18.37 ± 0.28^{a}	18.59 ± 0.22^{a}	17.33 ± 0.62	16.45 ± 0.52^{b}	16.71 ± 0.47^{b}
∑ MUFA	8.84 ± 0.40	5.32 ± 0.72^{b}	5.53 ± 0.27^{b}	7.69 ± 0.51	6.92 ± 0.52^{a}	7.31 ± 0.31^{a}
∑ PUFA	74.73 ± 1.15	76.31 ± 0.67	75.88 ± 0.33	74.98 ± 0.98	76.63 ± 1.02	75.98 ± 0.72
C20:5 (EPA)	9.65 ± 1.48	9.39 ± 0.26^{b}	10.18 ± 0.37	10.51 ± 0.39	10.73 ± 0.33^{a}	10.28 ± 0.39

maximum of 1722 μmol m^{-2} s^{-1}) during production of *R. monicae*. Mean ambient temperatures were also comparable: 15.8 ± 6.5 °C and 15.2 ± 3.3 °C for *T. obliquus* and *R. monicae,* respectively. However, minimum and maximum ambient temperatures varied between 4.7 and 30.5 °C for *T. obliquus* cultures and ranged between 7.7 and 24.3 °C for *R. monicae*.

In terms of growth performance, *T. obliquus* started with a biomass concentration of 0.50 g L^{-1}, reaching its maximum DW concentration on day 6 (1.88 g L^{-1}; Fig. 4). Harvesting took place on day 8 (1.79 g L^{-1}) at the stationary phase. On the other hand, *R. monicae* started with a biomass concentration of 0.30 g L^{-1} and reached the maximum DW

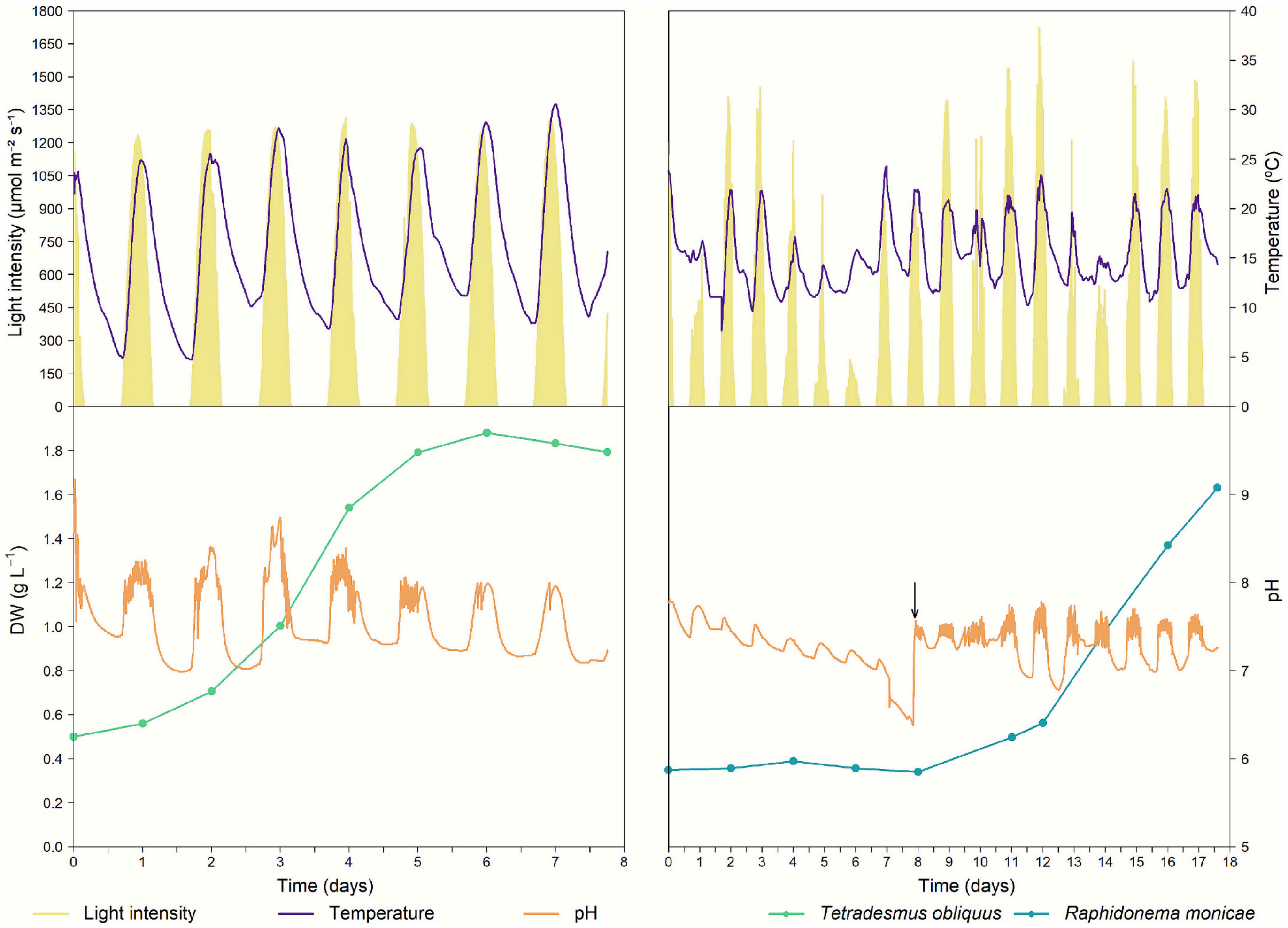

Fig. 4. Growth performance (DW g L^{-1}) of *Tetradesmus obliquus* (green line; left) and Raphidonema monicae (blue line; right) grown in 19-m^3 photobioreactors, using drainwater as culture medium ($n = 1$). In the production of both species, light intensity (µmol m^{-2} s^{-1}; yellow), temperature (°C; purple line) and pH (orange line) are present. The arrow indicates the addition of sodium bicarbonate for pH increase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentration of 1.63 g L^{-1} on day 18 of production, being harvested that day (Fig. 4), apparently still in the logarithmic growth phase. *R. monicae* presented an extended lag phase, with a continuous decline in pH. On the 8th day of production, 0.60 g L^{-1} of sodium bicarbonate was added to the culture to increase the pH and buffer capacity of the culture medium, bearing in mind that marine strains have a higher affinity for this form of carbon [79,80] and, consequently, an increase in growth performance was observed. *T. obliquus* reached a volumetric biomass productivity of 0.23 g L^{-1} d^{-1} (maximum daily productivity on day 15 of 0.54 g L^{-1} d^{-1}), while *R. monicae* reached a volumetric productivity of 0.13 g L^{-1} d^{-1} (maximum daily productivity on day 4 of 0.32 g L^{-1} d^{-1}) during the linear growth phase. The productivities obtained at industrial scale for both strains were higher than those obtained at lab- and pilot-scale trials. Similar volumetric productivities for *Scenedesmus* sp. were obtained in a 11.8 m^3 RW (19.1 g m^{-2} day^{-1}, estimated at 0.13 g L^{-1} d^{-1}) used in the bioremediation of urban wastewater [81]. Other volumetric productivities for *T. obliquus* and *Scenedesmus* sp. have been described in previous studies (0.08, 0.10 and 0.23 g L^{-1} d^{-1}) [82–84], however, with working volumes from 100 to 550 L. A similar volumetric productivity (0.08 g L^{-1} d^{-1}) was obtained in a cultivation of *Chlorella vulgaris* in 40.4 m^3 vertically-stacked TPBR [85], and a higher productivity in a 240-L thin-layer cascade system has also been described by Venancio et al. [86] (2.42 g L^{-1} d^{-1}). Regarding *R. monicae*, to the best of our knowledge, no studies has been performed under outdoor conditions; however, volumetric productivities at the laboratory scale ranged between 0.03 and 0.063 g L^{-1} d^{-1}, which is similar to the values obtained in the present work. In any case, when comparing this strain with *T. obliquus* or even other microalgal species grown in the same system [43], volumetric productivities were comparable (e.g., 0.11 g L^{-1} d^{-1} for *Phaeodactylum tricornutum* and 0.09 g L^{-1} d^{-1} for *Tisochrysis lutea*). From this production cycle, 19 kg DW and 23 kg DW of *T. obliquus* and *R. monicae* were obtained, respectively. To the best of the authors' knowledge, this is the first time that *R. monicae* has been produced on an industrial scale and used for bioremediation.

In terms of nutrient consumption, *T. obliquus* removed 99 % of nitrate (Table S1), similar to the trend described by Salazar et al. [73], also using DWT. In this case, a complete removal of phosphates and a removal above 50 % for Ca, Fe, Na, Cr, Pb and Zn were reported. Differently, *R. monicae* removed only 44 % of nitrate, a similar removal rate to that seen in the pilot-scale trials. However, this microalga also achieved complete removal of phosphates. Additionally, it had a removal efficiency above 50 % for Fe, K, Mg, Be, Ni, Pb, and V. The increase in Na observed was primarily related to the addition of sodium bicarbonate to the culture medium.

DWT samples before and after microalgae cultivation were analyzed for the presence of pesticides. The detected compounds before cultivation are known to be used as fungicides, mainly for disease prevention: dimethomorph, metalaxyl in both culture media, boscalid in DWT used for production of *T. obliquus*, and cyprodinil and fenhexamid in DWT for *R. monicae*. After cultivation, metalaxyl was completely removed from the growth medium by *T. obliquus*, while the concentration of boscalid and dimethomorph decreased by 65.6 and 53.3 %, respectively. In *R. monicae*, cyprodinil, fenhexamid and metalaxyl were removed entirely from the growth medium, while dimethomorph concentration decreased by 46.7 % (Table S3, supplementary material). Further analysis revealed that cyprodinil was the only fungicide detected in the biomass of *R. monicae* (0.056 ± 0.023 mg kg^{-1}; Table S4, supplementary material). This concentration represents 26.4 % of the cyprodinil detected in DWT prior to cultivation. Such content is low compared to a study by Wang et al. [87] on fungicide accumulation in strawberries, where the lowest cyprodinil concentration observed after foliar application was 0.88 mg kg^{-1} in the plants. The ability of *T. obliquus* to bioaccumulate and biodegrade pesticides has been previously recognized [88]. The results obtained suggest that cyprodinil was partially bioaccumulated in the biomass of *R. monicae*. In contrast, the other pesticides appeared to have been degraded during cultivation. Possible explanations for this range from biodegradation by microalgae or the accompanying microbiota to physical mechanisms such as photodegradation [89]. Further analysis of the biomass for the presence of heavy metals is required and should be considered in future work. The successful treatment of the DWT with microalgae results in two main products: clean water that can be reused by the soilless farming facility, and microalgal biomass that can be further exploited for further applications. A life cycle assessment (LCA) comparing conventional, i.e. RW, with novel microalgae production systems, i.e. TPBR, has identified TPBR as more demanding in comparison to RW. However, when combining renewable energy and nutrient sources from bioremediation of urban wastewater, the TPBR had an 80 % improvement in several impact categories of the LCA [90]. Despite the results obtained at industrial scale in the present study, future research should focus on a technoeconomic analysis or a LCA of the proposed process, to assess the benefits of using DWT as a nutrient source.

3.4. Biochemical valorization of the algal biomass industrially produced in TPBR

The proximal composition, including amino acids, fatty acids, phytohormones, pigments, and phenolic profiles, was determined in the freeze-dried biomass produced in the TPBR (Tables 3–6), aiming to valorize its biochemical components.

Comparing the proximal composition between the two strains, *R. monicae* had higher protein content (39.49 % of DW) compared to 32.28 % of DW in *T. obliquus*, while *T. obliquus* showed higher ash values: 18.90 % of DW, compared to 11.74 % of DW of *R. monicae* (Table 3). The higher protein content of *R. monicae* may be related to the fact that the culture was still in the exponential growth stage, when microalgae typically accumulate more protein [91], using the available nitrogen in the media for cell growth. The same applies to carbohydrates, with similar content between the two species (~35 % of DW), as they serve two main roles in cells: they are components of the cell wall and act as storage biomolecules [92]. Salazar et al. [73] observed a shift in protein and carbohydrate content in *T. obliquus* cultures during exponential growth phase in comparison with cultures at the stationary phase of growth. A decrease in protein and increase in carbohydrate contents were observed, a possible consequence of nutrient depletion, which might explain the lower protein content of *T. obliquus* in the present study, compared to *R. monicae*. Lipid content was similar in both species (~14 % of DW); however, as *T. obliquus* was at the stationary phase of growth, it is possible that lipid content would increase due to nitrogen limitation if production lasted longer, with accumulation as energy storage. Reports of lipid concentrations above 40 % are available for *T. obliquus* [93,94], and above 65 % for *T. bernardii* [95]. *R. monicae* lipid content was similar to that of *T. obliquus* and to those formerly described by Lima et al. [61]. Still, this concentration was achieved during the exponential phase and under nitrogen availability, hinting at the possibility of higher accumulation over time. It would also be interesting to understand whether this strain can accumulate triacylglycerols (TAG) during exponential growth without compromising

Table 3
Proximal composition (%) of *Tetradesmus obliquus* and *Raphidonema monicae*, grown in 19-m^3 tubular photobioreactors. Results are presented as mean ± standard deviation ($n = 3$).

	T. obliquus			*R. monicae*		
Protein	32.28	±	1.08	39.49	±	0.57
Lipid	14.02	±	0.52	14.19	±	0.27
Carbohydrate	34.80	±	0.97	34.58	±	0.96
Ash	18.90	±	0.46	11.74	±	0.58

Table 4
Amino acids concentration (mg g^{-1}) of *Tetradesmus obliquus* and *Raphidonema monicae*, grown in 19-m^3 tubular photobioreactors. Results are presented as mean ± standard deviation ($n = 3$). EAA - Essential amino acids; NEAA - Non-essential amino acids. Sum of EAA and NEAA are represented in bold.

	T. obliquus			*R. monicae*		
Essential amino acids (EAA)						
Histidine	4.18	±	0.08	8.35	±	0.21
Lysine	17.25	±	0.31	23.02	±	0.23
Threonine	19.48	±	0.34	23.08	±	0.36
Isoleucine	16.31	±	0.42	18.43	±	0.14
Leucine	32.29	±	0.69	33.98	±	1.19
Valine	22.13	±	0.43	23.83	±	0.20
Methionine	7.94	±	0.12	7.80	±	0.03
Phenylalanine	19.65	±	0.46	23.73	±	0.74
Total EAA	**139.22**	**±**	**2.84**	**162.23**	**±**	**3.10**
Non-essential amino acids (NEAA)						
Arginine	20.89	±	0.09	22.43	±	0.27
Cysteine	4.13	±	0.08	3.77	±	0.03
Tyrosine	12.03	±	0.16	12.71	±	0.22
Aspartic acid + Asparagine	30.77	±	0.73	38.60	±	0.56
Glutamic acid + Glutamine	38.58	±	0.58	41.50	±	0.70
Alanine	29.65	±	0.87	32.86	±	0.80
Glycine	22.20	±	0.46	25.32	±	0.37
Proline	11.73	±	0.10	21.92	±	0.22
Serine	15.59	±	0.31	18.76	±	0.31
Total NEAA	**185.57**	**±**	**3.39**	**217.87**	**±**	**3.48**

Table 5
Fatty acid profile (% of total fatty acids) of *Tetradesmus obliquus* and *Raphidonema monicae*, grown in 19-m^3 tubular photobioreactors. Results are presented as mean ± standard deviation ($n = 3$). Fatty acids with a concentration below 1 % were excluded from the table. SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; n.d. – not detected. Sum of SFA, MUFA and PUFA are written in bold.

%FA	*T. obliquus*			*R. monicae*		
C14:0	0.75	±	0.05	1.66	±	0.03
C16:0	17.01	±	0.32	12.34	±	0.28
C17:0	1.29	±	0.04	n.d.		
C18:0	1.72	±	0.13	0.32	±	0.05
∑ SFA	**21.58**	±	**0.58**	**14.87**	±	**0.39**
C16:1*n*-9	5.49	±	0.28	3.57	±	0.47
C18:1*n*-9	13.02	±	0.68	1.55	±	0.07
∑ MUFA	**19.08**	±	**0.99**	**6.93**	±	**0.57**
C16:2*n*-6	0.63	±	0.10	4.56	±	0.11
C16:3*n*-3	2.55	±	0.11	3.70	±	0.17
C16:4*n*-3	14.94	±	0.58	9.56	±	0.18
C18:2*n*-6	4.09	±	0.20	10.83	±	0.11
C18:3*n*-3	n.d.			30.00	±	0.24
C18:3*n*-6	31.06	±	0.35	0.95	±	0.21
C18:4*n*-3	6.06	±	0.38	3.64	±	0.22
C20:3*n*-6	n.d.			1.06	±	0.13
C20:4*n*-6	n.d.			3.73	±	0.07
C20:5*n*-3	n.d.			9.76	±	0.12
∑ PUFA	**59.33**	±	**1.71**	**78.20**	±	**1.61**

Table 6
Phytohormone content of *Tetradesmus obliquus* and *Raphidonema monicae*, grown in 19-m^3 tubular photobioreactors. Results are presented as mean ± standard deviation ($n = 3$). tZ – Trans-zeatin; iP – Isopentenyladenine; ACC - 1-aminocyclopropane-1-caroxylic acid; SA – Salicylic acid; ABA – Absisic acid; JA – Jasmonic acid; GA1 – Gibberellin A1; GA3 – Gibberellic acid; GA4 – Gibberellin A4; ZR – Zeatin riboside; IAA – Indole-3-acetic acid; Mel – Melatonin; n.d. – not detected.

		T. obliquus			*R. monicae*		
	tZ	37.01	±	0.97	39.52	±	2.38
μg g^{-1}	iP	0.35	±	0.05	12.65	±	1.12
	ACC	0.29	±	0.16	1.17	±	0.77
ng g^{-1}	SA	28.12	±	7.81	34.39	±	5.95
	ABA	103.90	±	4.62	118.39	±	8.73
	JA	411.92	±	20.47	723.24	±	64.12
	GA1	194.41	±	17.67	17.40	±	4.53
	GA3	1.63	±	1.05	0.18	±	0.02
	GA4	0.66	±	0.14	0.19	±	0.09
	ZR	n.d.			n.d.		
	IAA	1.22	±	0.78	22.84	±	5.14
	Mel	n.d.			n.d.		

biomass productivity when stressors are introduced during production. This has been described for *Fistulifera solaris*, which simultaneously accumulates TAGs and increases cell growth during the exponential phase [96]. Finally, *T. obliquus* presented a higher ash content in comparison to *R. monicae*, possibly related with the fact that *T. obliquus* has a more complex three-layer cell wall [97], thus retaining more inorganic material, while *R. monicae* has a simpler cell wall composed of glucan chains [98]. It is possible that the higher ash content could also be related with the accumulation of ions, including polyphosphates in specialized vacuoles from luxury uptake of phosphorus [99,100]. Nonetheless, an ash content ranging between 14 and 17 % is within observed values for freshwater microalgae [101].

Regarding amino acids profile, *R. monicae* presented higher concentrations of both essential (EAA; 162.23 ± 3.10 mg g^{-1}) and non-essential amino acids (NEAA; 217.87 ± 3.48 mg g^{-1}; Table 4), when compared to *T. obliquus* (EAA, 139.22 ± 2.84 mg g^{-1} and NEAA, 185.57 ± 3.39 mg g^{-1}). However, both species exhibited similar trends, i.e., the amino acids present in higher concentrations were the same in both species, namely leucine and glutamine, both of which are important primarily due to their antioxidant capacity [102]. These amino acids concentrations are somewhat higher than those reported by Pereira et al. [103] for *Tetraselmis striata* CTP4, by Santiago-Díaz et al. [104] for *Chloromonas* cf. *reticulata*, *Pseudopediastrum boryanum* and *Chloroidium saccharophilum,* and by Pinto et al. [105] for *C. vulgaris*, but lower than those reported by Tibbetts et al. [101] in *Chlorella* sp. and by Becker [106] in *T. obliquus*. Even so, the fact that microalgae exhibit the full spectrum of amino acids represents an advantage over other plant-based proteins commonly used for animal nutrition, which sometimes lack or are limited in certain amino acids, such as phenylalanine, methionine, and alanine [101,106].

The FA profile is presented in Table 5, showing that the profile of *T. obliquus* is mainly composed of γ-linolenic (C18:3*n*-6), palmitic (C16:0) and oleic (C18:1*n*-9) acids, showing a higher amount of SFA and MUFA (21.58 and 19.08 % of TFA, respectively) in comparison to *R. monicae* (14.87 and 6.93 % of TFA, respectively), while *R. monicae* is mainly composed of α-linolenic (C18:3*n*-3), palmitic and linoleic (C18:2*n*-6) acids, and a higher amount of PUFA (78.2 % of TFA) in comparison to *T. obliquus* (59.33 % of TFA). The concentrations of the PUFA γ-linolenic, and palmitidonic (C16:4*n*-3) represented 46 % of TFA in *T. obliquus,* while α-linolenic, linoleic acids and EPA represented 51 % of the TFA content of *R. monicae,* with EPA absent from *T. obliquus*. In freshwater species, such as *T. obliquus*, long-chain *n-3* fatty acids (e.g. EPA) are typically absent. An advantage of using *R. monicae* for bioremediation is the fact that this species is euryhaline and a producer of EPA, allowing the production of high-value compounds in a low-value medium (DWT). Together with docosahexaenoic acid (DHA), EPA represents an essential long-chain *n*-3 PUFA for both aquaculture and human nutrition, due to its important role in larval development and human health benefits [107,108]. However, the presence of linoleic and α-linolenic acids in the biomass is also an advantage, as these are precursors for long-chain PUFA [109].

Pigment profile of both species was evaluated, with β-carotene as the highest carotenoid present in both species (2.94 ± 0.15 and 3.04 ± 0.17 mg g^{-1} for *R. monicae* and *T. obliquus*, respectively), and a higher content of chlorophyll *a*, chlorophyll *b*, violaxanthin, astaxanthin, lutein and α-carotene in *R. monicae* compared to *T. obliquus* (Table S5, supplementary material). Considering the rising demand for novel sources of natural pigments, the presence of lutein and β-carotene represents an opportunity for this biomass [110]. Also, in terms of phenolics, the largest group of secondary metabolites in plants, gallic acid (ranging between 79 and 87 μg g^{-1}) and quercetin (ranging from 10 to 20 μg g^{-1}) were the most abundant in both species; catechin (2.17 μg g^{-1}) and flavone (2.89 μg g^{-1}) were found in higher concentrations in *R. monicae*, while quercetin was higher in *T. obliquus* (Table S6, supplementary material). Phenolic compounds have a wide range of functions, including responses to environmental stresses, improvement of nutrient uptake, and positive correlations with fungi and bacterial root colonizers, as well as serving as allelochemicals against other organisms [111,112].

Commonly found in higher plants, phytohormones are signaling molecules that take part in the growth and development of plants [113], and are mainly divided into 5 groups: gibberellins, auxins, cytokinins, ethylene, and abscisic acid. Therefore, to understand the full potential of the produced biomass, the phytohormone content was also determined. The phytohormones present in higher amounts were trans-zeatin (tZ), isopentenyladenine (iP) and 1-aminocyclopropane-1-caroxylic acid (ACC) (Table 6). *T. obliquus* presented a higher content in gibberellin A1 (GA1; 194 ng g^{-1}) and gibberellin A4 (GA4; 0.66 ng g^{-1}), while *R. monicae* had higher iP (12.65 μg g^{-1}), jasmonic acid (JA; 723 ng g^{-1}) and indole-3-acetic acid (IAA; 22.84 ng g^{-1}). The presence of gibberellins, auxins and cytokinins has been analyzed in 24 microalgae strains [114,115]. When comparing these reports with the present contents of GA1, tZ, and iP, they are present at increased concentrations in *T. obliquus* and *R. monicae*. In contrast, GA3 is present at similar amounts as reported, and GA4 is at much lower concentrations. The fact that the produced biomass contains these compounds opens up an opportunity for novel agricultural applications, such as plant biostimulants.

4. Conclusions

The current work validated the application of drainwater as a culture medium for microalgae production. To achieve this, two strains were selected for year-round production: *T. obliquus* for summer production and *R. monicae* for winter production. Pilot-scale trials comparing flat-panel photobioreactors and raceway ponds showcased the ability of *T. obliquus* to completely remove the nitrate in the medium in five days, while *R. monicae* exhibited a slower uptake rate, with 19 % nitrate removal efficiency in FP-PBR and 56 % nitrate removal efficiency in RW. However, the production systems showed little effect on shaping the biochemical composition of the biomass of both species. The concept was also successfully demonstrated on an industrial scale, being the first report on production of *R. monicae* at this scale, as well as being used for bioremediation of wastewater. *T. obliquus* exhibited a higher productivity (0.23 g L^{-1} d^{-1}) and nitrate removal rate (99 %) in comparison to *R. monicae*, with a productivity of 0.13 g L^{-1} d^{-1} and nitrate removal rate of 44 %. Despite showing a lower nitrate removal rate, *R. monicae* represents a source of high-value compounds, more specifically EPA, increasing the potential applications of the biomass. However, its performance might be further optimized in later trials, where strategies to handle the lower nitrate uptake can be tested, such as the implementation of a continuous system, with recirculation of water. The results obtained suggest that the selected microalgae strains are viable choices for the implementation of a microalgae co-production system with agricultural ventures. This will not only enhance water reuse but also produce biomass with different possible applications, which is in alignment with circular economy principles.

CRediT authorship contribution statement

Inês B. Maia: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bruno Pinto:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Mariana Carneiro:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Merve Konucu:** Writing – review & editing, Methodology, Investigation. **Peter Schulze:** Writing – review & editing, Methodology, Funding acquisition. **Tamára F. Santos:** Writing – review & editing, Visualization, Validation, Methodology, Data curation. **Monya M. Costa:** Writing – review & editing, Validation, Methodology, Data curation. **Alexandre M.C. Rodrigues:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition. **João Navalho:** Writing – review & editing, Resources, Funding acquisition. **Hugo Pereira:** Writing – review & editing, Writing – original draft, Validation, Methodology, Funding acquisition, Conceptualization. **João Varela:** Writing – review & editing, Writing – original draft, Validation, Methodology, Funding acquisition, Conceptualization.

Funding

This work was financially supported by FCT - Fundação para a Ciência e Tecnologia, I.P. by project reference and DOI identifier https://doi.org/10.54499/2021.06332.BD and https://doi.org/10.54499/2023.02764.BDANA PhD grants, https://doi.org/10.54499/UIDB/04326/2020, https://doi.org/10.54499/UIDP/04326/2020, and https://doi.org/10.54499/LA/P/0101/2020 grants, by EEA Grant ALGACYCLE-PT-INNOVATION-0023, by "Pacto da Bioeconomia azul" (Project No. C644915664-00000026) within the WP5 Algae Vertical, funded by Next Generation EU European Fund and the Portuguese Recovery and Resilience Plan (PRR) under the scope of the incentive line "Agendas for Business Innovation" through the funding scheme C5 - Capitalization and Business Innovation, and by the European Union (Grant agreement ID: 101060991, REALM).

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.

Acknowledgements

The authors would like to thank Necton S.A. and their members for helping in the pilot and industrial scale trials, the MarBiotech team at CCMAR for assisting in this work and to acknowledge Vera Gomes at CCMAR for the help with the biochemical characterization of the biomass.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104468.

Data availability

No data was used for the research described in the article.

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