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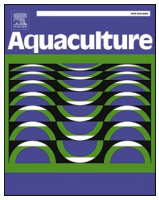

Revalorization of microalgae cultivation effluent: a circular strategy for long-term sustainable production of *Tetraselmis striata*☆

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ABSTRACT

Energy and fertilizers constitute the main economic and environmental costs for microalgae cultivation. This study aims to optimize the production of *Tetraselmis striata* by recirculating the whole untreated culture medium. Firstly, *T. striata* was cultivated inside a greenhouse ($_{IN}$) to assess process viability. Subsequently, 10-m^3 cultures were operated outside ($_{OUT}$) semicontinuously to assess the effect of medium recirculation on biomass productivity, composition and quality. Additionally, *T. striata* was cultivated under nutrient depletion to identify changes in biomass composition according to nutrient status. Medium was successfully recirculated for two months. Productivities were similar in all conditions ($_{OUT}$ recirculation: 0.097 ± 0.024 g/L/day; 43.7 t/ha/year). Biomass protein and carbohydrate content did not differ between fresh (F) and recirculated (R) medium, whereas higher protein and lower carbohydrate levels were obtained $_{OUT}$ than $_{IN}$ (%AFDW protein: F_{IN} 46.1 ± 0.5, R_{IN} 47.6 ± 1.4, F_{OUT} 53.6 ± 1.5, R_{OUT} 54.3 ± 1.5; carbohydrates: F_{IN} 43.2 ± 0.5, R_{IN} 41.1 ± 2.5, F_{OUT} 35.9 ± 2.9, R_{OUT} 34.8 ± 2.4). Lipid and ash content were similar across conditions. Heavy metals and microbiological analyses complied with EU feed and food standards. Recirculation reduced energy for cultivation by 7.5 % and effluent volume by 84 %, saving 3 tons/year of nitrate and 1 ton/year of phosphate for a 10-ha facility. A final effluent with a limited load in nutrient was generated obtaining a protein-rich biomass apt for feed, while low-nitrogen biomass suitable for biofuel was obtained under nutrient depletion. Thus, medium revalorization enables a sustainable strategy for *T. striata* cultivation, reducing costs and impacts while providing high-value biomass for multiple industrial applications within a circular economy framework.

1. Introduction

The mass cultivation of marine microalgae offers a sustainable alternative to agriculture of traditional crops and animal aquaculture, providing a reliable way to produce high-value biomass and a variety of compounds with applications across the food, feed, agricultural and pharmaceutical industries among many others (Bibi et al., 2017; Barkia et al., 2019; Puri et al., 2022). Additionally, large-scale microalgae production can be established on non-arable land and use widely available, less expensive alternative water resources to freshwater such as municipal wastewater, brackish water or seawater, not competing with agriculture (White and Ryan, 2015; Morillas-España et al., 2021). Furthermore, microalgae cultivation is also a CO_2 capture and utilization strategy, as microalgae can incorporate CO_2 from flue gas for their growth reducing greenhouse gas emissions (Lage et al., 2018; Pereira et al., 2018). Despite the significant advancements in knowledge and technology regarding outdoor mass microalgae production, its profitability remains still constrained due to the high costs associated with cultivation and downstream processing (Acién et al., 2014; Pereira Da Silva and Ribeiro, 2019; Masojídek et al., 2023; Novoveská et al., 2023). Energy, freshwater and fertilizer consumption represent the most relevant economic costs for companies. Moreover, inefficient resource

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utilization poses considerable environmental challenges. For instance, excessive residual nutrients in effluents can lead to eutrophication (Nixon, 1995; Diaz and Rosenberg, 2008), while elevated energy consumption increases the carbon footprint, especially when the production process is not powered by renewable energy sources. Therefore, there is an urgent need to develop strategies that optimize the use of local resources, reduce production costs and promote the sustainable development of marine algae aquaculture. At the same time, it is crucial to implement innovative technologies based on eco-friendly practices to minimize environmental impacts as this sector continues to grow.

Laboratory-scale studies on recycling culture medium have already shown how microalgae can grow effectively in recycled media over multiple cycles. Often, this leads to comparable or even enhanced biomass productivity compared to fresh media, depending on the species selected and other various factors (Loftus and Johnson, 2017; Wang et al., 2018; Dzuman et al., 2022). Moreover, among the strategies for optimizing energy, water and nutrient use, culture medium recycling is commonly applied in the industrial cultivation of species resistant to biological contamination. This approach is particularly effective for species with larger cell size, which enables effective dead-end filtration for biomass harvesting; e.g., *Arthrospira platensis* (Belay, 2013; Guidi et al., 2021). However, few studies have addressed the reuse of the supernatant resulting from centrifugation of large-scale outdoor microalgae cultures with successful or contradictory results (White and Ryan, 2015; Lu et al., 2020; Schädler et al., 2020). Additionally, most attempts involve costly treatment steps of the supernatant (e.g., ultrafiltration) to prevent early drops in culture productivity and biomass quality (Fon Sing et al., 2014; Shahid et al., 2019; Fret et al., 2020; Dias et al., 2025).

Fast-growing chlorophytes of the genus *Tetraselmis* are versatile microorganisms able to assimilate nutrients from urban wastewater, anaerobically digested piggery effluent, and fish farm effluents (Mehariya et al., 2024). They can also use carbon from untreated industrial flue gas (Moheimani, 2016). Due to its high nutritional quality (up to 36 % proteins and 15 % lipids) with a composition rich in omega-3 fatty acids such as alpha-linolenic acid (ALA, 18:3n-3) and eicosapentaenoic acid (EPA, 20:5n-3) (Kim et al., 2016; Pereira et al., 2018; Gojkovic et al., 2021), *Tetraselmis* is widely cultivated worldwide and used as feed in aquaculture (Rahman et al., 2017). Its biomass is also used for nutraceutical and cosmetic applications due to its production of pigments like violaxanthin, lutein and β-carotene (Schüler et al., 2021). Interestingly, previous studies have shown that the biochemical composition of marine chlorophytes is strongly dependent on nutrient availability (Hsieh and Wu, 2009; Kim et al., 2016). This highlights that biomass levels of desired compounds for specific applications, such as high protein content for feed (Patrinou et al., 2022) or low nitrogen content for biocrude production (Eboibi et al., 2015), can be modulated according to a proper nutrient supply strategy in the culture medium.

The Canary Islands are a strategic location for outdoor microalgae mass production as mild and stable temperatures with no marked seasonality, together with high solar irradiation, allow obtaining elevated and consistent productivities all year long (Gojkovic et al., 2021; Guidi et al., 2021). Moreover, there are large areas of infertile soil anthropogenically modified by mining activity and high quality seawater for cultivation. In addition, their strategic geographical position between three continents, make the Canary Islands ideal candidates for the industrial development of the microalgae production sector. *Tetraselmis* sp. is allowed for cultivation in the Archipelago according to the Regional Plan for Aquaculture (Gobierno de Canarias, 2018). A recent study carried out at pilot scale in an operational environment pointed out the biotechnological potential of a Canarian strain of *T. striata* for large-scale production in the Canary Islands thanks to its high resistance to biological contaminations (microzooplankton and other photoautotrophs), and its high and stable productivities (38.8 t/ha/year for a 8000-L outdoor raceway pond (RW); Gojkovic et al., 2021). However, to the best of our knowledge, no studies exist so far focusing on the optimization of mass production strategies of *T. striata* through the reduction of economic and environmental costs.

The main goal of this study was to evaluate, for the first time, the technical feasibility of recirculating the untreated supernatant obtained after culture harvesting by centrifugation, as a viable strategy for the reduction of energy and nutrient use in the large-scale outdoor production of *T. striata*. For this purpose, *T. striata* was scaled up in RWs up to a volume of 10,000 L and cultivated semicontinuously. The supernatant was recirculated repeatedly in order to reduce the energy consumption associated to the pumping of fresh seawater, as well as to reduce effluent volume and nutrient loss. Biomass productivity, biochemical composition and microbiological quality of the biomass obtained by using fresh seawater medium versus recirculated supernatant were compared. Additionally, to explore the possibility of minimizing the residual nutrient content in the exhausted medium destined for discharge during the cultivation process of *T. striata*, 250-L RW cultures were gradually induced to nutrient starvation to assess changes in biomass biochemical composition as a function of culture nutrient status. Importantly, the cultivation scale and experimental conditions were selected to ensure that the results are directly translatable to commercial applications. Conducting the study at a demonstrative scale in an outdoor operational environment allowed to account for the influence of environmental factors (e.g., atmospheric dust and compensation of evaporation losses from the cultures) and exposure to biological contamination on the feasibility of medium recycling. Moreover, working with industrial-type infrastructure and equipment provided a realistic estimation of energy demands, while accounting for the actual effects of pumping and centrifugation, such as potential cell disruption by shear stress and the consequent release of cell debris and dissolved organic matter that may promote bacterial and microzooplankton growth (Qin et al., 2023).

2. Materials and methods

2.1. Strain isolation and maintenance

Tetraselmis striata strain BEA 1978B (formerly ITC-TETRA-03; Gojkovic et al., 2021); GenBank access ID: MT012288, https://www.ncbi.nlm.nih.gov/nuccore/MT012288, accessed on: 12 September 2024) was isolated from reverse-osmosis desalination brine samples collected from the industrial desalination plant "EDAM Sureste de Gran Canaria", next to the Instituto Tecnológico de Canarias (ITC) facilities in Pozo Izquierdo (Gran Canaria, Spain; 27°48′52" N, 15°25′25" W).

The strain was aseptically maintained and grown for indoor scale-up to a volume of 9 L, in the ITC culture chamber in natural seawater-based *f* medium (Guillard and Ryther, 1962) with some modifications, at 25 ± 1 °C temperature, 16:8 h light:dark photoperiod, and cool white light at photosynthetic photon flux density (PPFD) of 300 μmol photons/m^2/s. Agitation was provided with bubbled air mixed with 1 % CO_2.

Nutrient composition of the culture medium was (macronutrient in mg/L): $NaNO_3$, 150; $NaH_2PO_4{\cdot}H_2O$, 10; and (micronutrient in μg/L): $FeCl_3{\cdot}6H_2O$, 3150; $Na_2EDTA{\cdot}2H_2O$, 8.72; $CuSO_4{\cdot}5H_2O$, 9.8; $Na_2MoO_4{\cdot}2H_2O$, 63; $ZnSO_4{\cdot}7H_2O$, 22; $CoCl_2{\cdot}6H_2O$, 10; $MnCl_2{\cdot}4H_2O$, 180.

2.2. Outdoor cultivation experiments

The assays were performed at the ITC facilities located in Pozo Izquierdo. This semi-desertic area of the Southeast of Gran Canaria is characterized by a subtropical climate with year-round sunny conditions, day length > 10 h, warm temperatures (17–24 °C daily average temperatures) and limited rainfall (<100 mm average annual precipitation). Average daily global horizontal solar irradiation is 5.7 Wh/m^2/day (2.1 kWh/m^2/year), ranging between 3.2 and 7.8 Wh/m^2/day in December and June, respectively. The annual dominant wind direction is the NNE, blowing at peak intensities during the summer months (monthly average at 60 m height varies from 5.8 ± 2.8 m/s in January to

12.8 ± 3.2 m/s in July). Moreover, the SE wind *Calima* (Saharan dust events) can occasionally lead to high concentrations of dust particles in the atmosphere.

Average monthly values of outdoor air temperature, daily global horizontal solar irradiation (G_0) and wind speed at the cultivation site are shown in Table 1. It must be noted that compared with open-air conditions, there is an average attenuation of 28 % on G_0 in the greenhouse (Gojkovic et al., 2021). Also, wind is virtually absent inside the greenhouse, and avg. temperature, generally higher than outside during daytime, is prevented from surpassing 35 °C by means of fan extractors.

As a general procedure for all the experiments, a total of 36 L of chamber cultures (4 × 9 L) were used to inoculate 250-L fiberglass RWs (2.5 m^2 surface area) inside the greenhouse. Some of these RWs were subsequently used as inoculum for 1600-L fiberglass RW (16 m^2), whose culture was then used to inoculate 10,000-L concrete-PVC RWs (80 m^2), both inside and outside a greenhouse. Culture depth was 0.1 m in 250-L and 1600-L RWs, and 0.125 m in 10,000-L RWs. Mixing was provided by a paddlewheel. Cultures were initially filled with 1 μm, UV filtered natural seawater pumped from a borehole. The medium used for these cultures is described in section 2.1. To prevent contamination from grazers in the larger cultures, 1 mM of urea was added to the medium (Mendez and Uribe, 2012). Pure CO_2 was injected through a porous ceramic diffuser (0.5–1 L/min) to maintain pH between 7 and 9 during daytime (8 AM to 8 PM). The evaporation of water in the culture was compensated daily using 1 μm, UV filtered desalinated water (average Na and Mg content of 90 and 19 mg/L, respectively). All chemicals used in the medium preparation were of commercial grade and locally available, and were chosen based on the criteria of low metal content and high solubility.

2.2.1. Medium recirculation inside the greenhouse (Experiment-1)

This assay was performed in a 10,000-L RW for 81 days, from April 9 to July 13, 2021. It was conducted as a pilot study to assess the technical viability of medium recirculation and its effects on culture productivity, and biomass composition and quality, in an operational set-up under partially controlled and protected environmental conditions. The trial consisted of two cultivation cycles during which the culture volume was replenished after harvesting with fresh seawater medium (F_{IN}, fresh medium inside the greenhouse), followed by subsequent cycles of recirculation of the whole untreated supernatant resulting from the solid-liquid separation by centrifugation at harvesting (R_{IN}, recirculated medium inside the greenhouse). Cultures were operated in semi-continuous mode: 80 % of the culture volume (8000 L) was harvested weekly, maintaining the culture concentration above 0.3 g/L. Nutrients were replenished on each cycle to the initial concentration after measuring the residual concentration of macronutrients (N and P, see section 2.1) in the culture medium. Micronutrients were reintegrated proportionally to the nitrate assimilated by the alga and according to the relative concentration of the culture medium used (see section 2.1). The supernatant resulting from the F_{IN} treatment, was discarded as effluent through a filtering well. The methodology used for recirculating the medium was to transfer the supernatant resulting from harvesting into an empty reservoir RW and pumping it back into the culture at the end of the harvesting process. This was done in order to standardize the medium recirculation process after each harvest. There was no effluent resulting for this later procedure (see also forward section 2.6). Harvesting was performed with an industrial continuous-flow disc-stack centrifuge with automatic discharge (Alfalaval VPNX 510SFD-34G, Alfa Laval Iberia, S.A., Madrid, Spain), and the biomass was dried with a rotary atomizer spray dryer L-12 (Ohkawara Kakohki CO., LTD., Yokohama, Japan). The greenhouse (1500 m^2 surface) was made with high-transparent corrugated polycarbonate (Suntuf® Plus, Palram Industries Ltd., Ramat Yohanan, Israel).

Table 1
Average monthly values of outdoor air temperature, daily global horizontal solar irradiation (G_0) and wind speed at the cultivation site.

Month	Avg. temperature (°C)	Avg. G_0 (Wh/m^2/day)	Avg. wind speed (m/s)
Jan	17.0	3.6	5.8
Feb	16.9	4.5	7.3
Mar	17.8	5.8	8.5
Apr	18.6	6.3	6.9
May	19.8	7.2	8.1
Jun	21.5	7.8	8.2
Jul	23.1	7.8	12.8
Aug	23.7	7.2	11.1
Sep	23.5	6.1	7.8
Oct	22.6	4.7	5.7
Nov	20.4	3.7	5.8
Dec	18.2	3.2	6.6

2.2.2. Medium recirculation outside the greenhouse (Experiment-2)

The assay was conducted for 48 days (from July 14 to August 31, 2021) in two simultaneous 10,000-L cultures to assess the effect of medium recirculation on culture productivity, biomass composition and quality under the open-air conditions of the Southeast of Gran Canaria. Both cultures were operated in semicontinuous mode with 80 % of culture volume (8000-L) harvested weekly. Taking advantage of the higher light availability outside the greenhouse, cultures were maintained at a concentration above 0.6 g/L, as a preventive strategy to limit the appearance of phototrophic contaminants (Guidi et al., 2021), such as diatoms, which had appeared in Experiment-1 (see sections 3.1 and 4.1). In one RW, the culture volume was replenished with fresh seawater medium (F_{OUT}, fresh media outside the greenhouse), whereas in the other one the volume was reestablished with the whole untreated supernatant resulting from its harvesting (R_{OUT}, recirculated medium outside the greenhouse). Nutrient replenishment and the methodology for medium recirculation, effluent disposal, biomass harvesting, and drying were as described in Experiment-1. The results obtained for F_{OUT} and R_{OUT} were compared with those obtained for F_{IN} and R_{IN} in order to detect possible differences that could result from the higher exposition to light and dust of the outside cultures.

2.2.3. Cultivation under nutrient-replete and nutrient-deplete conditions (Experiment-3)

The assay was conducted to explore the possibility of minimizing the residual nutrient content in the exhausted medium destined for discharge and lasted 16 days (December 14–30, 2020). *T. striata* cultures where gradually induced to nutrient starvation to identify possible changes in biomass composition as a function of culture nutrient status. In order to compare biomass composition in nutrient-replete vs nutrient-deplete conditions, two 250-L RWs in the greenhouse were each inoculated with 36 L of *T. striata* indoor cultures. Cultures were: i) set up simultaneously imposing the same nutrient protocol (section 2.1); ii) grown until nitrate in the medium was completely depleted; and iii) let grow in nitrate depleted conditions for further 9 days. The residual macronutrient (NO_3^- and PO_4^{3-}) content in the culture medium was monitored every two days. In order to analyze the biomass composition, 20 % of the cultivation volume (i.e., 50 L per RW) was harvested with a continuous-flow disc-stack centrifuge with manual discharge (GEA Westfalia Separator OTC3–02-137, Houma, LA, USA). Harvesting and biomass sampling took place at different times corresponding to the different culture nutrient status: nutrient-replete conditions on days 2 (75 mg/L of residual nitrate and 2 mg/L of residual phosphate) and 4 (10 mg/L of residual nitrate and 2 mg/L of residual phosphate); nutrient-deplete conditions on days 14 and 16 of the assay (0 mg/L of residual nitrate and 0 mg/L of residual phosphate). Biomass samples were kept frozen at −20 °C until analysis.

2.3. Daily measurements of the outdoor cultures

Culture parameters including temperature, pH, and salinity were

measured. Additionally, culture concentration was determined by measuring the optical density at 750 nm wavelength (OD_{750nm}) with a HACH Lange DR3900 UV/visible spectrophotometer (Hach Company, Loveland, CO, USA). Biomass concentration in the culture (C_x) overtime during the upscale process was also determined by measuring the dry weight as described by (Gojkovic et al., 2014). These measurements were then used to create a correlation curve with the optical density against culture dry weight in g/L both inside ($C_x = 1.10 \cdot OD + 0.02$, $r^2 = 0.999$) and outside the greenhouse ($C_x = 1.12 \cdot OD + 0.01$, $r^2 = 0.991$), in order to estimate C_x of the cultures during the experiment. Cultures were observed under the light microscope using a Leica DMi1 microscope (Leica Microsystems, Wetzlar, Germany) at 40× magnification, and the maximum photosynthetic quantum yield of the photosystem II (max. QY) was determined by chlorophyll fluorescence measurement with the AquaPen fluorimeter (Photon Systems Instruments, Brno, Czech Republic). Dissolved nitrate and phosphate concentration in the culture media were measured every two days and after each harvesting event with QUANTOFIX® test strips (Macherey-Nagel, Düren, Germany; gradation: 0, 10, 25, 50, 100, 250 and 500 mg/L for NO_3^-; 0, 3, 10, 25, 50 and 100 mg/L for PO_4^{3-}; instrumental measuring range: 10–500 mg/L for NO_3^- and 3–100 mg/L for PO_4^{3-}). Culture samples for measurements were taken on working days in front of the paddlewheel and placed in 50-mL sterile Falcon tubes for transport to the laboratory. One sample per RW was taken at around 9:00 AM, and an additional sample was collected after harvesting on harvesting days.

2.4. Biomass biochemical analysis

Biomass analyses for Experiment-1 and -2 were performed on spray dried *T. striata* biomass. The analysis carried out on biomass samples harvested at cycles 1, 2, 4, 6, 8, 10 and 12 of the Experiment-1, and on biomass samples collected during cultivation cycles 1, 3 and 5 of Experiment-2. For Experiment-3, frozen algal paste harvested on days 2, 4, 14 and 16 of the cultivation was used. Proximate composition of the algal biomass was assessed following standard procedures (Latimer, 2023). Protein content (N × 6.25) was measured using the Kjeldahl method, while lipid content was determined according to Folch et al. (1957). For moisture determination, samples were dried in an oven at 105 °C until constant weight was reached, and the successive combustion in a muffle furnace of the dried samples at 550 °C for 12 h was applied to establish ash content. Quantification of total carbohydrates resulted from subtracting proteins, lipids, ash, and moisture from the total algal biomass (James, 1995).

For the analysis of the fatty acid composition, equal-weight aliquots of biomass samples collected during cultivation cycles 1, 3 and 5 of Experiment-2 were pooled. Fatty acid methyl esters (FAME) were obtained by acid-catalyzed transmethylation of 1 mg of total lipid extract over 16 h at 50 °C. FAME purification was performed using thin-layer chromatography (TLC; Christie and Han, 2012) with a solvent mixture of hexane/diethyl ether/acetic acid (90:10:1, *v*/v). The purified FAMEs were subsequently separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, MA, USA). The gas chromatograph was equipped with an on-column injector, a flame ionization detector, and a fused silica capillary column, Supelcowax TM 10 (30 m × 0.32 mm I.D. x 0.25 μm; Sigma-Aldrich Co., St. Louis, Missouri, USA). Helium served as the carrier gas, with temperature programming set to 50–150 °C at 40 °C/min, then from 150 to 200 °C at 2 °C/min, to 214 °C at 1 °C/min, and finally to 230 °C at 40 °C/min, that was maintained for 5 min. When necessary, identification of individual FAME was confirmed via GC–MS chromatography (DSQ II, Thermo Fisher Scientific Inc.).

Inductively coupled plasma optical emission spectrometry (ICP-OES; AVIO 500, Perkin Elmer Inc., Waltham, MA, USA) after acid digestion of the dried biomass in a microwave digestion system (Ethos Easy, Milestone Srl, Bergamo, Italy) was used to determine mineral composition. The sample analyzed was cycle 5 from *T. striata* biomass cultivated outside (F_{OUT} and R_{OUT}).

The microbiological quality of vacuum packaged samples form cycles 1, 2, 4, 6, 8, 10 and 12 of the Experiment-1 and cycles 1, 3 and 5 of Experiment-2 were assessed by counting the total aerobic mesophilic flora counts (UNE EN ISO 4833-2 at 30 °C), yeasts and molds (ISO 21527), Enterobacteriaceae (ISO 21528-2), total coliforms (NFV 08–050 at 30 °C), *Escherichia coli* (ISO 16649-2), *Staphylococcus* spp. (ISO 6888-2 at 37 °C), *Clostridium perfrigens* (ISO 7937), and by detection of *Salmonella* spp. (ISO 6579) (EC 2073/2005).

2.5. Statistical analysis

Statistical analysis was conducted using Past4.17 software (Paleontological Statistics Software Package for Education and Data Analysis) (Hammer et al., 2001). Differences in culture parameters, biomass productivity, proximate composition and microbiological quality under the various conditions assayed were evaluated as follows: the Shapiro-Wilk test assessed data normality, and Levene's test evaluated variance homogeneity. Group comparisons were performed using: i) ANOVA followed by Tukey's pairwise test for data with normal distribution and homoscedasticity, or ii) the Kruskal-Wallis test for equal medians, followed by Dunn's post-hoc test with Bonferroni adjustment when normality and homoscedasticity were not met. For the biomass mineral and fatty acid content in Experiment-2, where comparisons were restricted to two groups (F_{OUT} vs R_{OUT}), the *t*-test was applied after that data normality and variance homogeneity was confirmed. Statistical significance was set at $p < 0.05$ for all analyses.

2.6. Calculations on energy savings and effluent reduction

Based on the differences between R and F processes (depicted in Fig. 1), the advantage of applying R over F strategy was quantified owed to: i) energy savings on pumping and on the whole biomass cultivation process; ii) effluent volume reduction; and iii) the amount of nitrate and phosphate saved and prevented from being discharged. Calculations have been performed in a case scenario of a 10-ha production facility (assuming 80 % productive surface, with a culture depth of 0.125 m) on a yearly basis (360-days operation). Briefly:

- both F and R conditions require culture RWs initially filled with 10,000 L of natural seawater (including the seawater volume for the inoculum scale up), which is ensured by a submersible pump installed inside a borehole with an electric power consumption (EPC) of 0.25 kWh/m^3;
- once a week, harvesting is performed for both F and R conditions: a pump with 0.07 kWh/m^3 EPC is used to bring 8000 L of the culture (80 % of the volume, see section 2.2.2) to the centrifuge. Energy consumption for centrifugation was the same for F and R (EPC: 1.85 kWh/m^3);
- the supernatant resulting from centrifugation (8000 L for each condition) is directly pumped to the filtration well for its discharge as an effluent (pump EPC: 0.07 kWh/m^3) for the F condition, whereas no pumping is needed to transfer the supernatant into the reservoir RW for the R condition;
- for the F condition, the 8000 L volume replenishment of the culture RW is carried out with natural seawater pumped from the borehole (EPC: 0.25 kWh/m^3), whereas for the R condition, supernatant is pumped back to the culture RW from the reservoir RW (EPC: 0.07 kWh/m^3);
- the cultures under both F and R conditions need to be fully renewed periodically from a new inoculum, mainly due to the occurrence of biological contaminations, either when *T. striata* cells were observed to be actively grazed (Experiment-1) or when the number of other phototrophs exceeded 15 % of total cell abundance in at least one of the cultures (F_{OUT}, Experiment-2). This implies the harvesting of the whole culture, the discharge of the whole supernatant to the filtration well (EPC: 0.07 kWh/m^3) and pumping of 10,000 L of seawater from the borehole (EPC: 0.25 kWh/m^3). Based on culture performance in

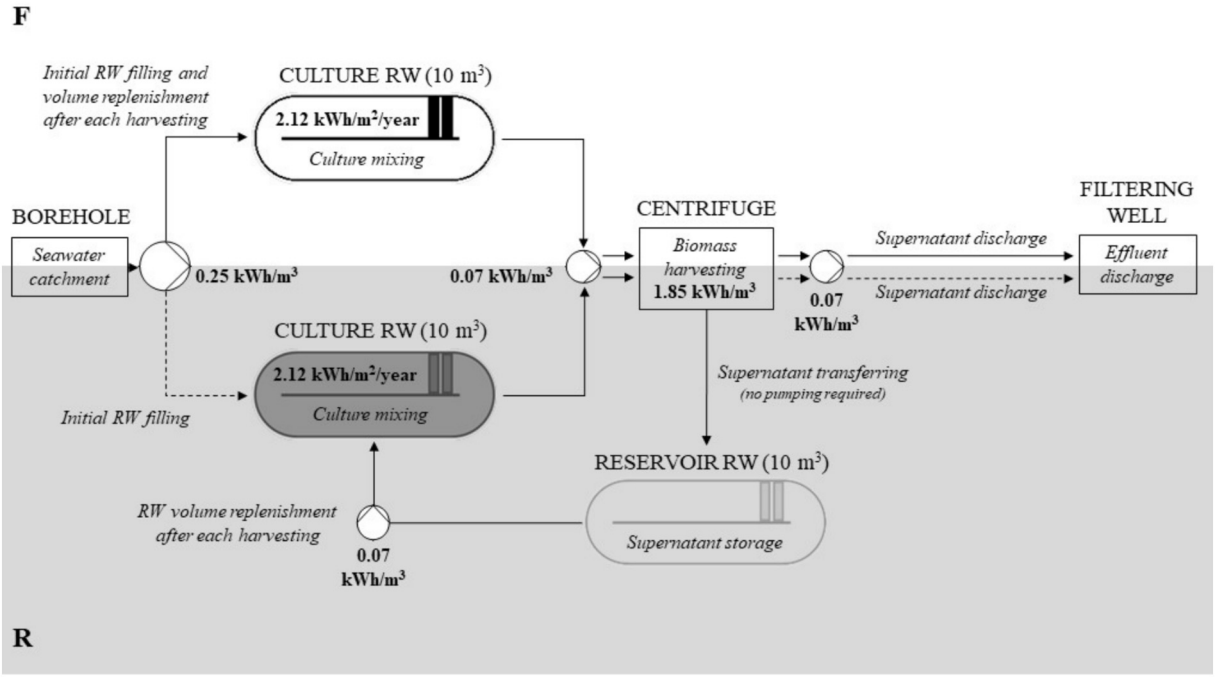

Fig. 1. Simplified diagram of the *T. striata* biomass cultivation process in the condition using fresh seawater media (F, white upper panel) and in the condition using recirculated medium (R, grey lower panel). Main infrastructure and equipment are detailed in capital letters; process steps are in italic; electric consumption of the paddlewheel, centrifuge and pumps involved in the process are marked in bold. Solid arrows indicate process steps which are performed at the beginning or at the end of each semicontinuous cultivation cycle. Dotted arrows indicate process steps which are only performed in R cultures when medium recirculation is not possible anymore; at that point, the supernatant is fully discharged and the culture has to be started again with a new inoculum and a complete refill with fresh seawater.

Experiment-1 and 2, it was assumed that both semicontinuous cultivations –with fresh medium and with full culture medium recirculation– would last for at least two months. After this period, the whole culture volume would be discharged and renovated with fresh seawater medium and a new inoculum.

- culture mixing with paddlewheels is the same for F and R. EPC was set at 2.12 kWh/m^2/year. This value was estimated by multiplying an average power requirement of 0.245 W/m^2 (calculated as the mean of the values reported by Weissman and Goebel, 1987 [0.25 W/m^2] and Lundquist et al., 2010 [0.24 W/m^2]) by 24 h and 360 days, assuming continuous paddlewheel operation.

3. Results

3.1. Medium recirculation assay inside the greenhouse (Experiment-1)

A total of 12 harvests in 81 days were performed for the 10,000-L semicontinuous culture RW (Fig. 2A). There were no significant differences in the culture parameters between the tested conditions for each cycle of F_{IN} and R_{IN}, except for average pH which was slightly lower (<10 %) in R_{IN} compared to F_{IN} (F_{IN}: 8.41 ± 0.54; R_{IN}: 7.75 ± 0.21, $p < 0.05$) (Table 2). Unidentified diatoms and euplotid ciliates appeared from cycle 8 (day 48, cycle 6 of R_{IN}). Nevertheless, abundance of the phototrophs remained below 7 % of the total microalgae cells throughout the experiment, while the protozoans were not able to ingest *T. striata* cells. Lastly, rotifers appeared abundantly at the end of the last cycle (day 81, cycle 12, cycle 10 of R_{IN}) and were observed to actively graze on *T. striata* cells.

3.2. Medium recirculation assay outside the greenhouse (Experiment-2)

A total of 7 harvests were performed throughout the experiment (Fig. 2B). Similar to the results obtained in the greenhouse assay, culture parameters including average culture concentrations (C_x Start and C_x End), and consequently, productivity, did not significantly differ between F_{OUT} and R_{OUT} conditions (Table 2). Unidentified diatoms quite similar to those observed in the Experiment-1 appeared in both F and R cultures from cycle 2 (day 12). However, while under F_{OUT} condition these phototrophs initially accounted for ~8 % of the total microalgae cells, and progressively increased to 18 % in the final cultivation cycle, they remained below 4 % throughout the entire R_{OUT} condition. Euplotid ciliates, comparable to those observed in Experiment-1, appeared from cycle 4 (day 26) under both F and R conditions and, once again, were not observed actively ingesting *T. striata* cells. Interestingly, no rotifers were found under any condition outside the greenhouse.

No significant differences for temperature and salinity were detected when comparing fresh and recirculated media conditions inside and outside (F_{IN}, R_{IN}, F_{OUT} and R_{OUT}) (Table 2). However, average pH was significantly higher in F_{IN} cultures with respect to the other conditions ($p < 0.05$). The max. QY was significantly higher in the cultures inside than in those outside, while the average C_x Start and C_x End presented the opposite trend ($p < 0.05$). Average P_{vol} was similar across all conditions (Table 2 and Fig. 3). Specifically, for R_{OUT}, P_{vol} was 0.097 ± 0.024 g DW/L/day corresponding to an areal productivity of 12.13 ± 3.00 g DW/m^2/day. This productivity value can be extrapolated to a year-round operation for 1 ha (360 days × 10,000 m^2 of open RW surface) giving a productivity of 43.7 t/ha/year.

3.3. Biomass composition and quality (experiments 1–2)

The average composition of *T. striata* biomass on an ash- and water-free basis was 51.1 ± 3.6 % of proteins (46.1–54.3 %), 38.0 ± 3.9 % of carbohydrates (34.8–43.2 %) and 10.9 ± 2.1 % of lipids (10.5–11.3 %) (Fig. 4), corresponding to 36.3 % proteins, 27.0 % carbohydrates, 7.7 % lipids and 29.0 % ash on a water-free basis. Both F and R conditions presented similar protein, carbohydrate and lipid content inside and outside the greenhouse ($p > 0.05$). In contrast, protein content was 14 % and 12 % higher in conditions outside compared to the inside ones, while carbohydrate content was 17 % and 15 % lower ($p < 0.05$). Lipid

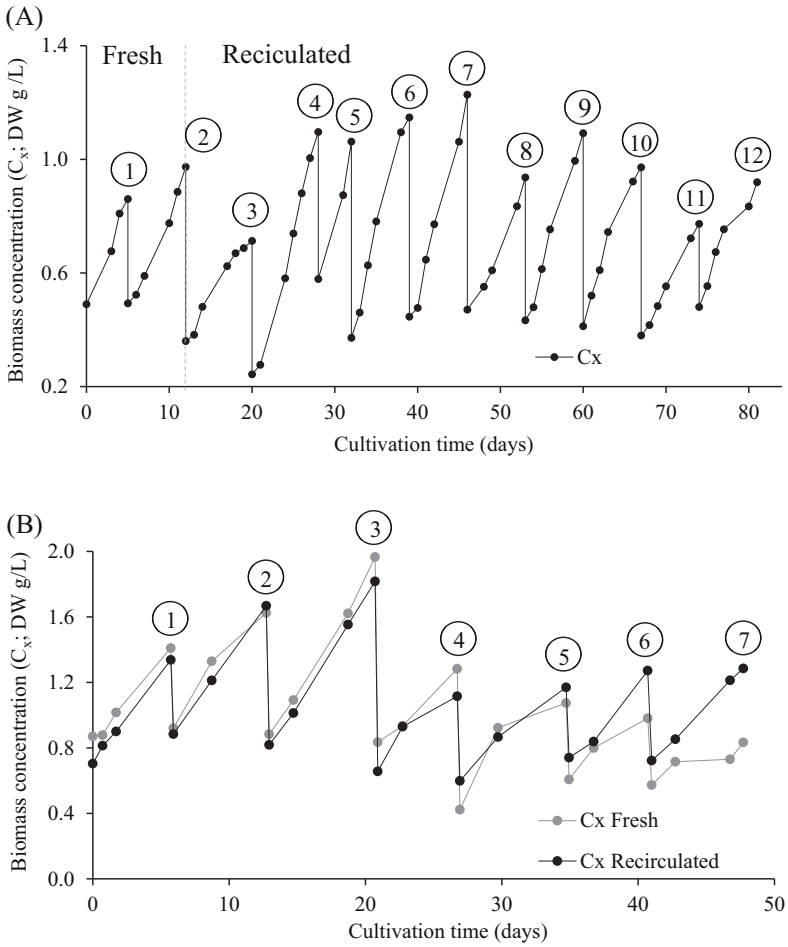

Fig. 2. (A) Biomass concentration (C_x; DW g/L) of the *T. striata* culture over the time-course of semicontinuous cultivation in 10,000-L raceway pond inside the greenhouse during the experimental phases Fresh (F, April 9–21, 2021) and Recirculated (R, April 21 to July 13, 2021) of Experiment-1. The vertical dashed line indicates the end of the F phase and the beginning of the R phase. (B) Biomass concentration (C_x; DW g/L) of the simultaneous 10,000-L cultures of *T. striata* established outside in raceway ponds during Experiment-2 (July 14 to August 31, 2021).

levels did not significantly vary between F_{IN} and F_{OUT} or R_{IN} and R_{OUT}. The ash content in *T. striata* biomass remained unchanged across experimental conditions ($p > 0.05$), with an average value of 29.0 ± 3.0 % DW.

Table 3 presents the main fatty acid profile of *T. striata* biomass cultivated outside. Notably, ALA was almost two-fold higher in R_{OUT} (10.9 ± 1.1 % of total fatty acids) than in F_{OUT} (6.0 ± 0.2 %), Conversely, EPA content was similar under both experimental conditions, with an average value of 3.5 ± 0.5 %, while DHA was present in very low amounts in both conditions (<0.5 %), being significantly higher in F_{OUT} (0.3 ± 0.0 %) than in R_{OUT} (0.2 ± 0.0 %). Overall, R_{OUT} cultures presented higher total omega-3 fatty acid levels and a higher omega-3 to omega-6 ratio compared to F_{OUT} ($p < 0.05$).

The mineral profile of *T. striata* harvested at cycle 5 is shown in Table 4. Na and Ca were the most abundant minerals, accounting together for more than 3.5 % of the dried biomass, followed by K and Mg (aprox. 1.5 % DW). Na and Mg were 33 and 12 % higher in R_{OUT} with respect to F_{OUT} ($p < 0.05$). As regards to trace elements, Fe was significantly lower, while Cu and Zn were significantly higher in R_{OUT} than in F_{OUT} ($p < 0.05$). Within heavy metals, Pb was detected in similar concentrations in both samples (5.47 and 5.79 mg/kg), while Cd, Mo and Hg were not detected in any condition. Se and Cr where only detected in R_{OUT} and F_{OUT}, respectively. Finally, As was four-fold higher in R_{OUT} than in F_{OUT} ($p < 0.05$).

As it is shown in Table 5, neither the total aerobic mesophilic flora (2.0 to 3.4 × 10^3 cfu/g) nor yeast and molds, and bacterial pathogens (i.e., total coliforms, *Escherichia coli*, *Enterobacteriaceae*, *Staphylococcus* spp., *Salmonella* spp. and *Clostridium perfringens*) significantly varied among the four experimental conditions.

3.4. Cultivation under nutrient-replete and nutrient-deplete conditions (Experiment-3)

Culture salinity, temperature, pH, and max. QY values remained stable across the experiment (32.4–52.0 g/L, 16.6–25.4 °C, 6.9–9.9 and 0.69–0.80, respectively). Concentrations of dissolved nitrate and phosphate in the culture medium at the beginning of the experiment were 137.5 ± 53.0 mg/L and 70 ± 0.0 mg/L, respectively. No phototrophs or grazers appeared in any of the cultures along the experiment. The overall culture growth rate and P_{vol} were 0.084 ± 0.003 1/day and 0.095 ± 0.006 g DW/L/day, respectively. *T. striata* growth was characterized by a logistic growth pattern (Fig. 5): the exponential growth phase occurred under nutrient-replete condition in the culture medium (days 0–7; growth rate: 0.153 ± 0.006 1/day; P_{vol}: 0.143 ± 0.010 g DW/L/day), followed by a stationary phase occurring under nutrient-deplete condition (days 7–16; growth rate: 0.032 ± 0.010 day^{-1}; P_{vol}: 0.057 ± 0.018 g DW/L/day). Nitrate and phosphate were completely depleted in the culture media within the first 7 days of cultivation, concomitantly with the end of the exponential growth phase.

There was a 3-fold significant reduction in the protein content (53.2

Table 2
Culture temperature, pH, salinity, max. QY, culture concentration at the beginning (C_x Start), and at the end (C_x End), and volumetric productivity (P_{vol}) for each semicontinuous cycle of *T. striata* in 10,000-L raceway ponds with fresh (F) and recirculated (R) media inside ($_{IN}$) and outside ($_{OUT}$) the greenhouse. Data are presented as mean ± standard deviation. [a, b] Letters indicate significant differences between average values of F_{IN}, R_{IN}, F_{OUT} and R_{OUT} for each parameter ($p < 0.05$).

	Condition	Cycle	Time (days)	T (°C)	pH	Salinity (g/L)	Max. QY	Cx Start (g/L DW)	Cx End (g/L DW)	Pvol (g/L/Day)
Experiment 1	F_{IN}	1	0–5	22.6 ± 1.2	8.27 ± 0.29	39.4 ± 1.8	0.79 ± 0.03	0.490 ± 0.008	0.861 ± 0.016	0.073 ± 0.001
		2	5–12	23.3 ± 2.9	8.55 ± 0.79	39.2 ± 1.8	0.76 ± 0.03	0.493 ± 0.002	0.973 ± 0.003	0.068 ± 0.001
	R_{IN}	3	12–20	23.7 ± 1.2	7.82 ± 0.48	40.6 ± 1.6	0.76 ± 0.02	0.360 ± 0.006	0.713 ± 0.006	0.041 ± 0.000
		4	20–28	22.9 ± 2.1	7.54 ± 0.36	43.2 ± 2.3	0.78 ± 0.03	0.243 ± 0.002	1.097 ± 0.004	0.107 ± 0.001
		5	28–32	24.9 ± 4.3	7.80 ± 0.12	41.9 ± 3.1	0.79 ± 0.02	0.579 ± 0.003	1.097 ± 0.009	0.121 ± 0.002
		6	32–39	23.1 ± 2.4	7.70 ± 0.40	40.4 ± 2.4	0.76 ± 0.03	0.372 ± 0.001	1.148 ± 0.014	0.111 ± 0.002
		7	39–46	22.2 ± 1.5	7.35 ± 0.23	40.2 ± 2.2	0.81 ± 0.01	0.446 ± 0.003	1.228 ± 0.004	0.112 ± 0.001
		8	46–53	23.9 ± 1.7	7.30 ± 0.06	40.5 ± 1.8	0.81 ± 0.01	0.471 ± 0.003	0.937 ± 0.007	0.067 ± 0.001
		9	53–60	23.1 ± 1.8	7.72 ± 0.07	40.9 ± 2.1	0.78 ± 0.02	0.434 ± 0.005	1.093 ± 0.018	0.094 ± 0.002
		10	60–67	23.5 ± 1.4	7.91 ± 0.12	40.7 ± 1.7	0.76 ± 0.01	0.413 ± 0.003	0.972 ± 0.019	0.080 ± 0.003
		11	67–74	23.4 ± 1.7	8.22 ± 0.11	41.9 ± 1.7	0.74 ± 0.03	0.380 ± 0.008	0.773 ± 0.002	0.056 ± 0.001
		12	74–81	23.9 ± 1.9	8.16 ± 0.11	43.7 ± 1.9	0.77 ± 0.01	0.480 ± 0.006	0.920 ± 0.033	0.063 ± 0.004
	Avg. F_{IN}	1–2	0–12	22.9 ± 2.1[a]	8.41 ± 0.54[a]	39.3 ± 1.8[a]	0.78 ± 0.03[a]	0.492 ± 0.005[a]	0.917 ± 0.009[a]	0.070 ± 0.004[a]
	Avg. R_{IN}	3–12	12–81	23.4 ± 2.0[a]	7.75 ± 0.21[b]	41.4 ± 2.1[a]	0.78 ± 0.02[a]	0.418 ± 0.004[a]	0.994 ± 0.012[a]	0.085 ± 0.028[a]
Experiment 2	F_{OUT}	1	0–6	22.6 ± 2.7	7.65 ± 0.21	45.7 ± 5.8	0.75 ± 0.01	0.870 ± 0.006	1.410 ± 0.008	0.085 ± 0.001
		2	6–13	21.9 ± 2.1	7.62 ± 0.29	45.7 ± 3.8	0.75 ± 0.02	0.921 ± 0.007	1.625 ± 0.005	0.103 ± 0.002
		3	13–21	21.6 ± 2.3	7.64 ± 0.26	37.1 ± 5.5	0.77 ± 0.01	0.884 ± 0.001	1.966 ± 0.007	0.122 ± 0.001
		4	21–27	22.4 ± 1.5	7.73 ± 0.13	40.8 ± 5.3	0.72 ± 0.04	0.835 ± 0.004	1.283 ± 0.008	0.071 ± 0.002
		5	27–35	23.3 ± 1.1	7.67 ± 0.15	39.4 ± 4.2	0.73 ± 0.01	0.423 ± 0.001	1.073 ± 0.001	0.075 ± 0.000
		6	35–41	21.6 ± 0.8	7.61 ± 0.11	41.9 ± 4.5	0.70 ± 0.05	0.607 ± 0.008	0.981 ± 0.001	0.066 ± 0.001
		7	41–48	23.4 ± 2.6	7.72 ± 0.34	41.5 ± 4.0	0.72 ± 0.03	0.574 ± 0.002	0.834 ± 0.002	0.037 ± 0.000
	R_{OUT}	1	0–6	22.5 ± 2.3	7.76 ± 0.26	41.9 ± 5.9	0.74 ± 0.02	0.704 ± 0.002	1.337 ± 0.002	0.111 ± 0.001
		2	6–13	22.1 ± 2.4	7.99 ± 0.21	46.5 ± 7.2	0.73 ± 0.02	0.884 ± 0.023	1.668 ± 0.005	0.115 ± 0.004
		3	13–21	22.2 ± 2.4	7.80 ± 0.19	40.9 ± 9.7	0.75 ± 0.02	0.818 ± 0.024	1.817 ± 0.018	0.129 ± 0.005
		4	21–27	22.7 ± 1.8	7.77 ± 0.09	40.4 ± 8.4	0.69 ± 0.03	0.657 ± 0.003	1.116 ± 0.001	0.069 ± 0.001
		5	27–35	23.5 ± 1.2	7.79 ± 0.03	37.8 ± 5.5	0.77 ± 0.02	0.598 ± 0.005	1.170 ± 0.002	0.062 ± 0.001
		6	35–41	23.0 ± 2.5	7.67 ± 0.24	44.3 ± 3.9	0.75 ± 0.01	0.740 ± 0.002	1.272 ± 0.016	0.109 ± 0.003
		7	41–48	23.7 ± 2.5	7.82 ± 0.19	42.3 ± 3.4	0.75 ± 0.02	0.722 ± 0.003	1.285 ± 0.007	0.085 ± 0.001
	Avg. F_{OUT}	1–7	0–48	22.4 ± 1.9[a]	7.66 ± 0.21[b]	41.7 ± 4.7[a]	0.73 ± 0.02[b]	0.730 ± 0.004[b]	1.310 ± 0.005[b]	0.080 ± 0.026[a]
	Avg. R_{OUT}	1–7	0–48	22.8 ± 2.1[a]	7.80 ± 0.17[b]	42.0 ± 6.3[a]	0.74 ± 0.02[b]	0.732 ± 0.009[b]	1.381 ± 0.007[b]	0.097 ± 0.024[a]

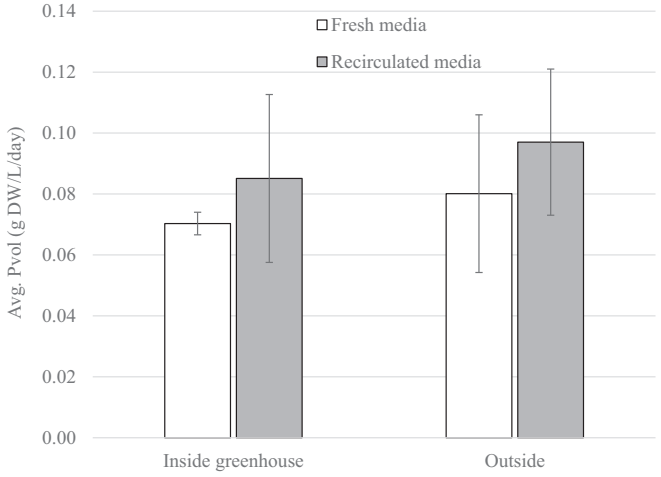

Fig. 3. Average volumetric productivity (Avg. P_{vol}; g DW/L/day) of each condition of Experiment-1 and -2. White bars indicate condition with fresh media (F) and grey bars indicate condition with recirculated media (R). No significant differences were observed between the experimental conditions ($p > 0.05$).

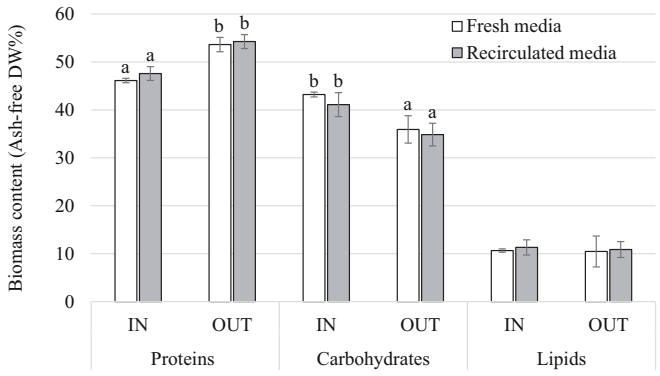

Fig. 4. Ash free and water free biochemical composition of the biomass (proteins, carbohydrates and lipids) for samples from each condition. IN stands for cultivations inside the greenhouse (Experiment-1) and OUT for cultivations outside the greenhouse (Experiment-2). White bars indicate condition with fresh media and grey bars indicate condition with recirculated media. The data shown correspond to analysis carried out on biomass samples harvested at cycles 1, 2, 4, 6, 8, 10 and 12 of the Experiment-1 and cycles 1, 3 and 5 of Experiment-2. [a, b] Letters above the bars indicate significant differences among experimental conditions for each biochemical component ($p < 0.05$), being [a] the lowest value and [b] the highest value for each comparison.

vs 16.5 % ash-free dry weight, AFDW) between samples under nutrient-replete (days 2 and 4) and nutrient-deplete conditions (days 14 and 16) ($p < 0.05$). Furthermore, protein levels at day 4 were significantly lower than at day 2 (<9.2 %; $p < 0.05$) (Fig. 6). On the other hand, carbohydrate and lipid content were respectively 3.4- and 1.6-fold higher under nutrient-deplete than under nutrient-replete conditions ($p < 0.05$). Ash content was ~16 % lower in days 14 (17.7 ± 0.8 % DW) and 16 (17.9 ± 0.6 % DW) with respect to days 2 (21.5 ± 0.7 % DW) and 4 (21.0 ± 1.3 % DW; $p < 0.05$).

3.5. Energy savings and effluent volume reduction

From the results obtained in terms of the occurrence and extent of biological contaminations, two months was established as the maximum culture duration before entirely renewing both F and R cultures with new inoculum and fresh seawater.

The difference in energy consumption between F and R strategies lies fundamentally on the need to pump natural seawater from the borehole

Table 3
Main polyunsaturated fatty acid profile (percentage of total fatty acids) of *T. striata* biomass harvests from cycles 1, 3 and 5 under F and R outside conditions. Data are presented as mean ± standard deviation ($n = 3$). Significant differences between conditions are indicated with an asterisk (*) ($p < 0.05$).

Fatty Acid	F_{OUT} (% FA)	R_{OUT} (% FA)
Alpha-Linolenic acid (ALA;18:3n-3)	6.0 ± 0.2	10.9 ± 1.1*
Eicosapentaenoic acid (EPA; 20:5n-3)	3.4 ± 0.4	3.6 ± 0.6
Docosahexaenoic acid (DHA; 22:6n-3)	0.3 ± 0.0	0.2 ± 0.0*
Total Omega-3	11.3 ± 0.5	17.7 ± 2.1*
Linoleic acid (LA; 18:2n-6)	2.8 ± 0.1	3.8 ± 0.4*
Total Omega-6	5.1 ± 0.3	5.9 ± 0.8
Omega-3/Omega-6	2.2 ± 0.1	3.0 ± 0.1*

Table 4
Minerals (mg/100 g), trace elements (mg/100 g) and heavy metals (mg/kg) content in *T. striata* biomass cultivated outside (F_{OUT} and R_{OUT}, Experiment 2; cycle 5). Data are presented as mean ± standard deviation of 3 technical replicates. Significant differences between conditions are indicated with an asterisk (*) ($p < 0.05$). nd: not detected.

Elements		F_{OUT}	R_{OUT}
Minerals (mg/100 g)	K	964 ± 89	1048 ± 45
	Na	2017 ± 93	2685 ± 64*
	Mg	590 ± 40	663 ± 20*
	Ca	1675 ± 124	1740 ± 46
	Fe	147 ± 11	109 ± 6*
Trace elements (mg/100 g)	Mn	12.0 ± 1.0	12.8 ± 0.5
	Cu	1.7 ± 0.2	2.4 ± 0.1*
	Zn	6.0 ± 0.2	7.1 ± 0.3*
	Se	nd	0.17 ± 0.11
	Cr	0.030 ± 0.004	nd
	Pb	5.79 ± 0.65	5.47 ± 0.30
Heavy metals (mg/kg)	Cd	nd	nd
	Mo	nd	nd
	As	0.12 ± 0.02	0.43 ± 0.06*
	Hg	nd	nd

Table 5
Microbiological analysis of total aerobic mesophilic bacteria, pathogenic bacteria and yeasts and molds, in *T. striata* biomass. The data correspond to the average values of biomass harvested on cycles 1, 2, 4, 6, 8, 10 and 12 of the condition inside and cycles 1, 3 and 5 of the F and R conditions outside. [a] limit value: 10 cfu/g; [b] limit value: 300 cfu/g; [c] limit value: absence in 25 g (EU Regulation 142/2011 and BOE 300/1988). nd: not detected.

Microbiological parameter	F_{IN}	R_{IN}	F_{OUT}	R_{OUT}
Total aerobic mesophilic flora (cfu/g)	2.0 ± 1.3 × 10^3	2.6 ± 2.4 × 10^3	2.8 ± 2.3 × 10^3	3.4 ± 1.7 × 10^3
Total coliforms (cfu/g)	< 10	< 10	< 10	< 10
Yeasts and molds (cfu/g)	< 50	< 50	< 50	< 50
Escherichia coli (cfu/g)[a]	< 10	< 10	< 10	< 10
Enterobacteriaceae (cfu/g)[b]	< 50	< 50	< 50	< 50
Staphylococcus spp. (cfu/g)	< 50	< 50	< 50	< 50
Salmonella spp. (Abs/25 g)[c]	nd	nd	nd	nd
Clostridium perfringens (cfu/g)	< 10	< 10	< 10	< 10

(Fig. 1). In fact, while under the R condition fresh seawater from the borehole is only pumped (EPC: 0.25 kWh/m^3) once every two months for culture renewal starting from a new inoculum, under the F strategy, fresh seawater needs to be additionally pumped every time the culture needs to be replenished after harvesting (80 % of the culture volume weekly). This would result in a total of 77.5 MWh of energy saved per year in a 10-ha production facility when applying the R rather than the F strategy, corresponding to a 51 % reduction on the EPC associated to the pumping and to a 7.5 % reduction on the EPC associated to the whole biomass cultivation process of *T. striata*.

Likewise, the R strategy generated an effluent only once every two months, whereas the F strategy produced an additional effluent corresponding to the 80 % of the culture volume, every week at harvest (Fig. 1). This would result in a reduction of 324,000 m^3 of the effluent generated per year in a 10-ha production facility, equivalent to an 84 % decrease of effluent discharged during the biomass cultivation of *T. striata*.

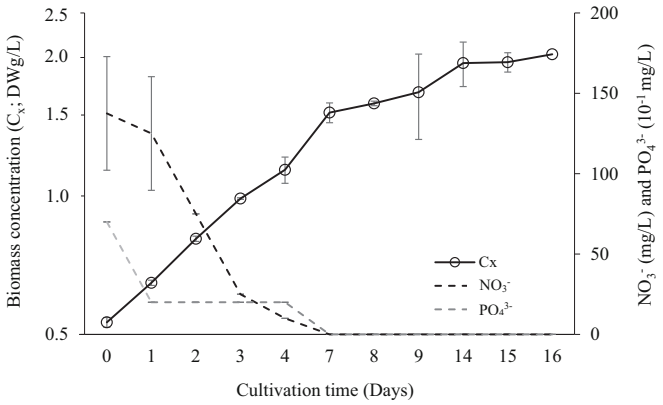

Fig. 5. Biomass (C_x), nitrate (NO_3^-) and phosphate (PO_4^{3-}) concentration over time during Experiment-3. Biomass concentration values are represented in a logarithmic scale. Phosphate values were multiplied by 10 to facilitate graphical representation on the same scale as nitrate values.

According to the results obtained in the Experiment-3, harvesting the culture at day 2 with the maximum biomass protein content (53.2 ± 2.5 % AFDW) would generate an effluent with 75 mg/L of nitrate, which would not be allowed with the current legal limits for effluent discharge to the environment in the Canary Islands (Decreto 174/1994). In contrast, the effluent resulting from the cultivation of *T. striata* at day 4 with a biomass protein content of 48.3 ± 1.5 % AFDW (<9.2 % lower than at day 2) would contain a maximum of 10 mg/L of nitrate (2.3 mg/L of N) and 3 mg/L of phosphate (1 mg/L of P) (see section 2.3 for the range of measurement of the nutrients used in this study), which is four- and ten-fold less N and P, respectively, than the allowed effluent discharge limits (Decreto 174/1994). Based on these residual concentrations of nitrate (≤ 10 mg/L) and phosphate (≤ 3 mg/L) in the effluent of nutrient-replete cultures, we estimated that 3.2 t of nitrate (6 %) and 1 t of phosphate (31 %) could be saved and prevented from being discharged by recirculating the culture medium during semicontinuos cultivation of *T. striata* on a yearly basis in a 10-ha production facility at a cultivation depth of 0.125 m, assuming 80 % productive areal surface.

4. Discussion

4.1. Medium recirculation under inside and outside conditions

Average productivity did not differ between F and R cultures in both Experiment-1 inside (F_{IN} and R_{IN}) and Experiment-2 outside (F_{OUT} and R_{OUT}) under similar environmental conditions (T, pH, salinity), indicating that *T. striata* growth, in terms of biomass concentration, is not affected by medium recirculation in either partially protected or open-air conditions in Southeast Gran Canaria. The average outside areal productivity with medium recirculation during two months (R_{OUT} 12.13 g/m^2/day) is higher than values reported in other locations for the same species cultivated in smaller open RW ponds, where biomass output is normally larger (Borowitzka, 2005). For instance, a productivity of 9.5 g/m^2/day was obtained in India in 10 m^2 RWs with a culture depth of 0.150 m (Boopathy et al., 2020), while *Tetraselmis* sp. cultivated in western Australia in 1 m^2 RWs yielded 8.3 g/m^2/day (Fon-Sing and Borowitzka, 2016). Nevertheless, it is important to note that experiments in the present study were conducted during summer, when productivity is generally the highest due to high solar irradiation. When extrapolating the R_{OUT} productivity to a year-round operation for 1-ha

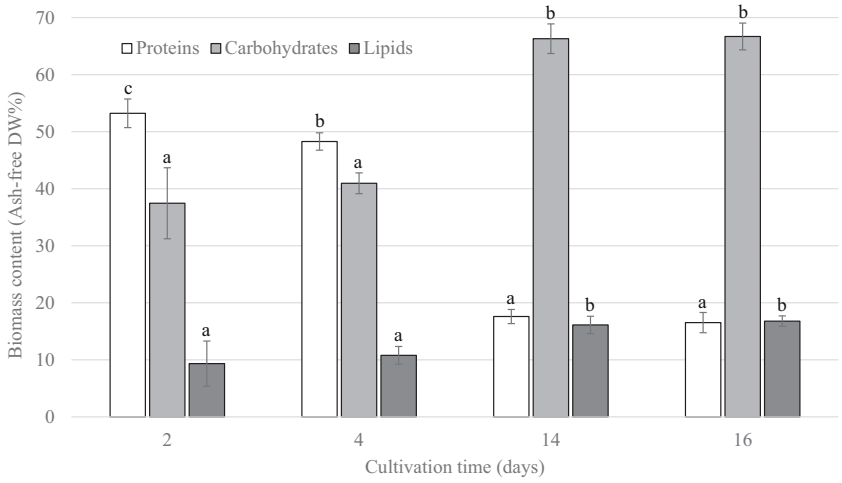

Fig. 6. Ash-and water-free biochemical biomass composition at different sampling times during Experiment-3, before (day 2 and 4) and after (day 14 and 16) complete nitrate depletion. [a, b, c] Letters indicate significant differences between the four different sampling times for each biochemical component ($p < 0.05$), being [a] the lowest value and [c] the highest value for each comparison.

facility (360 days × 10,000 m^2 of open RW surface), an estimated annual biomass of 43.7 t/ha/year is obtained. This biomass output is comparable to the areal productivity previously reported for the same microalgae strain in the same geographic area, season and RW size (38.8 t/ha/year, 300 days × 10,000 m^2 of open RW surface) (Gojkovic et al., 2021), emphasizing the consistency of this strain and its reliability for large-scale biomass production in the Canary Islands.

The average 28 % attenuation of sunlight irradiation by the greenhouse roof, combined with the lower overall solar irradiation during Experiment-1 compared to Experiment-2 likely explains the significantly higher maximum photosynthetic quantum yield (max. QY) of the photosystem II in the greenhouse culture with respect to the outside culture, as a consequence of cells exposure to lower light intensity (Tredici, 2010). However, there were no significant differences in productivity between both conditions, despite outside set-up being exposed to more sunlight. This could be explained by the significantly higher outside than inside culture densities along the semicontinuous cycles. Although this strategy could have partially limited the peak productivity outdoors, culture operation at higher biomass concentrations largely reduce energy use for harvesting per unit of centrifuged culture volume. It also reduces the risk and extent of biological contaminations (Belay, 2013; Guidi et al., 2021), which is known to constitute an economic challenge to commercial algal cultivation (Carney and Lane, 2014). The low extent of biological contaminations in these experiments allowed maintaining the semicontinuous cultivation for 2 months (12 weekly harvests in Experiment-1 and 7 weekly harvests in Experiment-2) without the need to promptly interrupt the culture with salvage harvesting. These results are comparable to those by Pereira et al. (2018), who grew semicontinuously *Tetraselmis* sp. for 60 days in a 100-m^3 closed PBR outdoor, and confirm that *T. striata* BEA 1978B is a consistent, contamination resistant strain, which is a key prerequisite for successful microalgae production outdoor (Gojkovic et al., 2021).

Interestingly, the abundance of undesired phototrophs in F_{OUT} increased progressively reaching up to 18 % of the total microalgae cells during the final cultivation cycle, whereas it remained below 4 % in R_{OUT}. This suggests that *T. striata* may release certain compounds that accumulate in the recirculated medium at concentrations able to affect the setting of other phototrophs in the culture without negatively affecting *T. striata* productivity or max. QY. In this context, recycled media from microalgae cultures are known to carry —apart from cell debris and dissolved organic matter (DOM) potentially acting as promoters of bacterial and microzooplankton growth (Lu et al., 2020)— secondary metabolites such as humic acid, free fatty acids and polyunsaturated aldehydes that may have autoinhibitory effects (Arora et al., 2023). In addition, microalgae are able to secrete a large variety of metabolites to the external environment that have inhibitory effects over other populations (competitors, grazers; Borowitzka, 2016). The production and release of autoinhibitors and allelochemicals is linked to precise adaptive strategies including control of population density and competition for resources (Gross et al., 2012; Venuleo et al., 2017) which are very likely to emerge also in mass cultures. Further analyses are needed to clarify this matter.

The addition of urea in the culture medium as a preventive measure against protozoan grazers (Mendez and Uribe, 2012; Gojkovic et al., 2021) may have been helpful to delay their appearance both by directly acting on the grazers or being used as nitrogen source by *T. striata*, as suggested by Gojkovic et al. (2021). The protective effect of urea against grazing would likely emerge especially in R cultures, where urea concentration (not measured in the effluent and fully reintroduced in the recirculated medium at each cultivation cycle) would build up. Previous studies pointed out the mixotrophic and heterotrophic growth of *Tetraselmis* spp. (Lu et al., 2017; Lari et al., 2019), demonstrating their ability to readily assimilate nitrogen sources such as urea (Kim et al., 2016) and other organic nutrients from urban wastewater, digested piggery and fish farm effluents (Mehariya et al., 2024). Although organic carbon was not measured in the current study, its accumulation is likely to have taken place in the recirculated cultures of *T. striata* from the partial decomposition of residual biomass such as lysates and cell debris from the microalgae cells that undergoes industrial centrifugation. Further studies are currently being carried out to gain insight into the metabolic growth strategies of *T. striata* BEA 1978B and its potential ability to assimilate organic substances present in the culture medium. This capability would open the way to its cultivation in carbon-rich saline effluents, producing high-quality, low-cost biomass for zero-net carbon emission applications (e.g., bioenergy and biomaterials), while simultaneously revalorizing and bioremediating residual effluents abundant in arid and semiarid coastal areas.

4.2. Biomass composition and quality

The proximate composition of *T. striata* BEA 1978B was not affected by medium recirculation. The average proximate composition of the microalgae biomass (36.3 % proteins, 27.0 % carbohydrates, 7.7 % lipids and 29.0 % ash), being consistent with the values reported by Gojkovic et al. (2021) for the same strain cultivated in the same location and RW systems, and with the lipid content reported by Pereira et al. (2018). Notably, higher protein and lower carbohydrate content were registered for both F and R conditions outside compared to inside

conditions. As culture temperature, pH and salinity were similar inside and outside, and light availability likely had only a limited impact on biomass composition as productivities were not different, potential external inputs of nutrients may have occurred in the outside cultures. Actually, Experiment-2 was carried out during the windiest months of July and August (avg. wind speed: 12.8 and 11.1 m/s, respectively), when outside RWs could be exposed to relevant amounts of dust carried by the wind (Gojkovic et al., 2021; Guidi et al., 2021). Previous studies indicate that Saharan aerosol inputs to the Canary Current during *Calima* episodes are among the highest worldwide (Gelado-Caballero, 2015), and act as precursors to phytoplankton blooms in oligotrophic oceanic areas, primarily due to their contribution of nitrogen, phosphorus, iron, and magnesium (Gallisai et al., 2012; Morales-Baquero et al., 2013), as well as other elements limiting photosynthesis and nitrogen fixation (Baker et al., 2003; Ohde and Siegel, 2010; Gallisai et al., 2012). Some studies indicate that *Calima* events can supply up to 2 mg NO_3/m^2/d and the air masses originating from the Sahara can reach phosphorus and sulphate concentrations on the order of 90 and 300 ng/m^3, respectively (Morales-Baquero et al., 2013). A more detailed analysis of the amount and composition of the dust entering the outdoor RW is needed to confirm this hypothesis and would be helpful to optimize nutrient supply strategy in *T. striata* cultures according to the external amount of nutrient inputs.

The 25 % lower ash content in the microalgal dry biomass observed in the present study compared to the value reported by Gojkovic et al. (2021) could be due to the higher biomass concentration at harvest (average: 1.2 g DW/L vs 0.7 g DW/L), which could have ultimately resulted in a less liquid algal paste with a lower residual content of the saline culture medium (Lu et al., 2022). This finding points out that culture operation at higher biomass concentration will not only reduce the energy use for harvesting and the risk of biological contaminations, but can also lead to a lower biomass ash content without the need to apply additional washing steps after centrifugation, largely increasing the biomass quality for several applications (Liu, 2017). Taking into account a mean dust accumulation rate of 218.4 mg/m^2/day for the Island of Gran Canaria (Menéndez et al., 2007), the ash content was expected to be more elevated outside than inside the greenhouse. However, it would be possible that most of the dust would either dissolve in the liquid culture medium or settle to the bottom of the raceway not being incorporated into the biomass.

Delving deeper into the mineral content of the biomass at cycle 5 outdoor, the accumulation of Na and Mg in R_{OUT} with respect to F_{OUT} (33 and 12 % higher, respectively) could be partially due to the desalinated freshwater used to compensate evaporation along the semi-continuous cycles. In fact, taking into account a daily evaporation rate of 6 mm (Vonshak, 1997), and the average Na and Mg content in the freshwater (see section 2.2), an input of 151 mg/L of Na (90 × 35 × 0,006 × 1 × 1000 / 125) and 32 mg/L of Mg (19 × 35 × 0,006 × 1 × 1000 / 125) would be expected after 35 days of cultivation. External inputs of minerals through dust (Gelado-Caballero et al., 2012), freshwater for evaporation and build-up due to nutrient replenishment (see section 2.2.1) may also explain the higher concentration of Cu and Zn in R_{OUT} with respect to F_{OUT}. Contrarily, Fe was lower in R_{OUT} with respect to F_{OUT} suggesting that this micronutrient could have been consumed at a higher rate than it was replenished, or partially precipitated in R_{OUT} due to the higher concentrations of Na and Mg (Guidi et al., 2021).

Low heavy metal levels were detected under all tested conditions. Pb, As, Cd and Hg were quite below the limits set by the EU regulations for undesirable substances in food and animal feed (EC 32/2002 and EC 1869/2019), and also for heavy metals in food supplements (EC 629/2008). A high microbiological quality was also maintained under all conditions, with total aerobic bacteria in the range of 10^3 cfu/g, and yeast and molds, and bacterial pathogens absent or below the detection limit, meeting the European and Spanish food safety standards for both animal and human consumption regulations for feed products (EC 142/2011; EC 2073/2005; BOE 300/1988). These results pave the way for *T. striata* dried biomass to be presented as a novel foodstuff (EC 258/1997). To the best of our knowledge these are the first data on heavy metal content and microbiological quality of *T. striata* cultivated in open RWs outdoor with medium recirculation, indicating that this process does not affect the standards of biomass quality for feed and future food applications.

Under our experimental conditions, *T. striata* was rich in essential omega-3 polyunsaturated fatty acids (PUFAs), including ALA and EPA, which are highly relevant to be used as feed in marine aquaculture (El-Sheekh et al., 2015). In contrast, DHA was found at very low levels (<0.5 % of total fatty acids). This finding is consistent with previous studies stating that *Tetraselmis* species typically produce ALA and EPA, but not DHA, unlike other marine microalgae such as *Schizochytrium* or *Crypthecodinium* (Robert et al., 2001; Gojkovic et al., 2021; Schüler et al., 2021; Conde et al., 2023). While ALA and EPA levels match those obtained by Gojkovic et al. (2021), the DHA content here is lower than the 3.1 % of total fatty acids reported by these authors. EPA and DHA contents can vary widely depending on environmental and culture conditions (Sang et al., 2012; El-Sheekh et al., 2015; Sajjadi et al., 2018). Although the outside cultures were conducted in a similar season and using the same RW systems, the higher biomass concentration maintained in the present study may have influenced light and/or nutrients availability. The average n-3/n-6 ratio of 2.6, is within the 2–3 range previously reported for *Tetraselmis* sp. (Molina et al., 1991), and in the desirable ratio of 2–4 for farmed fish (Sargent et al., 1999). On the other hand, the consumption of n-6 fatty acids in Western diets often surpasses that of n-3 fatty acids, contributing to the rise in diet-related chronic diseases (Zárate et al., 2017). The maintenance of a balanced omega-3/omega-6 ratio, ideally close to 1:4, is crucial for regulating homeostasis and mitigating pathological effects linked to imbalances between these fatty acids (Mariamenatu and Abdu, 2021). The higher PUFA proportion in the recirculated medium (except for DHA, which remained low in both conditions) may be attributed to the nutrient accumulation in the culture, likely originated from external sources such as atmospheric dust, sea aerosol, and freshwater used to compensate for evaporation (Sajjadi et al., 2018). Moreover, as mentioned earlier, it is reasonable to hypothesize that *T. striata* may grow mixotrophically under R_{OUT} conditions, assimilating organic carbon accumulated in the recirculated medium. Although the role of the organic carbon sources in the biosynthetic pathways of PUFA in microalgae is not yet fully understood (Castillo et al., 2021), several studies have reported increased production of n-3 and n-6 fatty acids when microalgae are cultivated in mixotrophic compared to autotrophic conditions (Menegol et al., 2019; Přibyl and Cepák, 2019).

4.3. Two-phase cultivation process for low-nitrogen, carbon-rich biomass production

Nutrient availability clearly influenced biomass biochemical composition: protein levels decreased 3-fold, while carbohydrates and lipids increased 3.4- and 1.6-fold, respectively, as nutrients became depleted. This is consistent with many previous findings for *Tetraselmis* sp. and other marine microalgae in N-deficient cultures (Kim et al., 2016; Gojkovic et al., 2020; Yaakob et al., 2021) as carbon allocation inside the cells is directed towards the synthesis of C-rich compounds at the same time as C:N ratio increase (Palmucci et al., 2011; Yaakob et al., 2021; Şîrîn and Serdar, 2023). Interestingly, in Experiment-3, ash content was lower on days 14 and 16 than on days 2 and 4, possibly due to the fact that cultures were harvested at higher biomass concentrations (average: 2.0 g DW/L days 14 and 16 vs 1.0 g DW/L days 2 and 4), resulting in more dense algal paste with a lower residual content of the saline culture medium (Lu et al., 2022). Results of Experiment-3 also revealed that nutrient abundance in the culture medium is required to obtain the maximum biomass protein content, and that the residual nutrient concentration in the effluent under this condition could easily overcome the local legal limits for water discharges to the environment

(Decreto 174/1994). Therefore, recirculating the culture medium represents a remarkable and somehow necessary improvement in the cultivation process of *T. striata* for feed application, as it allows obtaining high biomass protein content, complying with regulations, and prevents a significant loss of nutrients in the effluent resulting in economic and environmental costs. Moreover, delaying the harvesting a couple of days in the last cycle of semicontinuous cultivation with medium recirculation, would result in a biomass with less than 10 % reduced protein content, and in an effluent with nitrate and phosphate content largely below the limit for discharge (in this study, at least 4- and 10-fold less N and P, respectively, than the allowed effluent discharge limits in the Canary Islands; Decreto 174/1994).

According to the findings of Experiment-3, *T. striata* could be cultivated in a two-phase process for the production of low-nitrogen, carbon-rich biomass suitable for industrial applications as biofuel production, among others (Eboibi et al., 2015). The first phase would take place under nutrient-replete condition, to reach high biomass concentration at high culture productivity, while the second phase, characterized by a slower growth rate, would take place under nutrient starvation to enforce the cells using up their internal nitrogen reserves and synthesize C-rich compounds (Kim et al., 2016; Yaakob et al., 2021; Liang et al., 2023). Cultivation under nutrient-deplete conditions will also result in a negligible nutrient load in the effluent, facilitating compliance with local regulations for water discharges and further reducing its environmental impact in the aquatic ecosystem (Nixon, 1995; Diaz and Rosenberg, 2008). The evaluation of carbon-rich *T. striata* biomass production in a single-phase step, in the attempt to increase the overall productivity of the biomass cultivation process, warrants future investigation.

4.4. Implications and perspectives of effluent revalorization

In the Canary Islands, freshwater production is limited by the available desalination plants, and reverse-osmosis technology has the drawback of requiring chemicals for periodic membrane cleaning and generating brine as a by-product, with a significant environmental impact on the marine ecosystem (Sirota et al., 2024). In addition, the average energy consumption to produce desalinated freshwater has been estimated in 3.91 kWh/m^3 (Gobierno de Canarias, 2022. Consejería de Politica Territorial Cohesión Territorial y Aguas, 2024), in comparison to the 0.25 kWh/m^3 needed in this study to pump seawater from the borehole for the cultivation of the marine microalga *T. striata*. Moreover, the production of *T. striata* biomass under the culture medium recirculation strategy allows a 51 % energy saving associated with pumping, corresponding to a 7.5 % on the whole biomass cultivation process. Most notably, medium recirculation allows an 84 % reduction in effluent discharge volume, together with a yearly saving of at least 3 tons of nitrate and 1 ton of phosphate in a 10-ha facility at a cultivation depth of 0.125 m, assuming 80 % productive areal surface. Therefore, supernatant recycling reduces not only the need of pumping new seawater, but also of importing fertilizers while increasing the economic viability of the process (Shahid et al., 2019). Actually, Fret et al. (2020) found that recirculating the medium in *Nannochloropsis* sp. and *Tisochrysis lutea* cultures resulted in an average reduction in nutrient expenditure (68 % or 9 €/kg dry biomass). A 25 % reduction in nitrogen and 12.5 % reduction in phosphorus was achieved operating 1 m^3 photobioreactor with *Chlorella sorokiniana* under medium recirculation for one year (Daiek et al., 2022), while a life cycle assessment (LCA) study found that nutrient usage decreased 55 % in culture of *Desmodesmus subspicatus* (Pereira Da Silva and Ribeiro, 2019). Beyond the economic benefits, this would also reduce the dependency of the Canary Islands on fertilizer supply, which is a strategic issue for an outermost region of Europe. It is important to stress that the supernatant used in our study was untreated unlike it has been performed in other medium recirculation studies, which apply electro-flocculation, microfiltration and other treatments that add additional costs to the process (Fon Sing et al., 2014; Shahid et al., 2019; Fret et al., 2020). While a detailed cost analysis of capital and operational expenditures is beyond the goal of this study, the consistent reduction in the effluent volume generated by the recirculation strategy, is expected to largely reduce costs associated with effluent management such as: i) effluent discharge fees per unit of volume; ii) costs in equipment for effluent treatment required to comply with local regulations and meet required standards prior to discharge; iii) cost for construction and maintenance of large-size infrastructure (e.g., pipelines, wells, pumps) for effluent disposal if a greater effluent volume is generated. Moreover it would make possible to implement large-scale facilities in contexts where effluent discharge volumes represent a bottleneck or restriction, as is sometimes the case in the Canary Islands due to land constraints and rigorous regulations aimed at preserving the local environment.

5. Conclusion

Although in theory the recycling of culture medium may last for an entire production season, in practice it is limited to only a few cultivation cycles due to organic matter accumulation, contaminants, atmospheric dust, and nutrient build-up from replenishment and evaporation compensation. In this study, *Tetraselmis striata* BEA 1978B was grown semicontinuously at a demonstrative scale in an outdoor operational environment for two months, repeatedly recirculating the entire untreated supernatant obtained after biomass harvesting. No significant differences in biomass productivity, composition and quality with respect to the control with fresh seawater medium were observed, demonstrating for the first time the technical feasibility of recirculating the supernatant resulting from the harvesting process by centrifugation without additional treatments, at least for this microalgal strain when cultivated under the open-air conditions of the southeast Gran Canaria. The recirculation of the culture medium allows a 7.5 % energy savings in the whole biomass cultivation process (51 % in the pumping), and a 84 % reduction in effluent discharge volume, together with a yearly saving of at least 3 tons of nitrate and 1 ton of phosphate for a 10-ha facility, emerging as a viable, circular, long-term strategy to reduce the economic and environmental costs of large-scale outdoor cultivation of this microalgae. A final effluent with a limited load in nutrient can be generated while obtaining a protein-rich biomass suitable for feed and food applications, while depletion of nutrient allows to generate a low-nitrogen biomass suitable for biofuel production. Taken together, these results pave the way for the implementation of an innovative clean and resource-efficient approach for the production of high-value biomass for feed and bioenergy purposes under a framework of circular economy and efficient water management in arid and semiarid coastal areas.

CRediT authorship contribution statement

Begoña Bustamante: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Monserrat Alemán:** Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Diana B. Reis:** Writing – original draft, Resources, Investigation. **José A. Pérez:** Writing – review & editing, Resources, Investigation. **Marianna Venuleo:** Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Juan Luis Gómez-Pinchetti:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Eduardo Portillo:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Flavio Guidi:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Ethical approval

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.743297.

Data availability

The raw data supporting the findings of this study are provided as supplementary material in this submission.

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