Light regime, harvesting time and operation mode can optimize the productivity of nutritional protein in *Chlorella* and *Spirulina* biomass.

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Abstract

Microalgae have emerged as promising sustainable protein alternatives because of their high protein content and exceptional efficiency in nutrient utilization, land use, and freshwater usage. However, optimization of biomass and protein production in open raceway pond reactors, the most common reactors for large-scale production, remains largely unexplored. Additionally, little is known regarding the operational parameters necessary for essential amino acid (EAA)-rich protein production, which aligns with the nutritional requirements for human consumption. The influence of harvesting time, operation mode (batch and continuous reactor), and photoperiod (continuous and day-night illumination) were studied in 100-L raceway reactors by determining the biomass, protein, digestibility, and EAA productivity of *Limnospira indica* (previously *Arthrospira indica*) and *Chlorella vulgaris*. The continuous operation mode was also validated for *L. indica* in a closed 83-L photobioreactor. Harvesting time for optimal EEA-rich protein productivity did not necessarily occur when biomass productivity was optimal. The optimal protein and EAA productivities were 16 and 6.7 mg/L/d for *C. vulgaris*, and 14 and 2.8 mg/L/d for *L. indica*, respectively. In continuous operation mode, optimal protein and EAA productivities of 9.4 and 5.8 mg/L/d for *C. vulgaris* and 13 and 2.3 mg/L/d for *L. indica* were observed, but when a closed reactor was used, *L. indica* protein and EAA productivity ramped up to 300 and 33 mg/L/d, respectively. Continuous illumination increased the productivity of both microalgae species, especially EAA, which increased by up to 50%. The optimal EAA index for *C. vulgaris* (1.8) demonstrated its suitability as a human protein source, which was higher than that of *L. indica* (0.53), indicating strain dependency. This study provides a toolset to

determine a better production strategy for optimal productivity of high-quality protein in the industry.

Keywords

Microbial protein, respiration, photoautotrophic, diel variation, in-vitro digestibility

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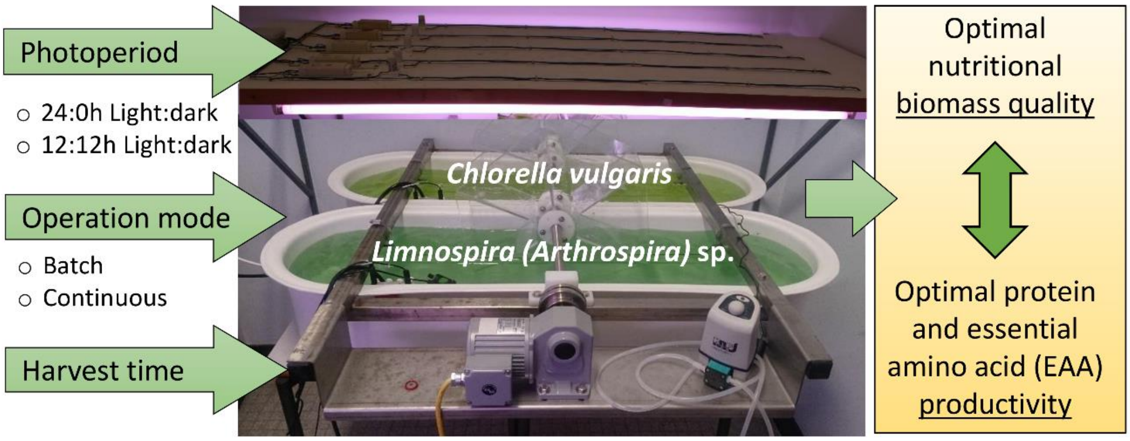

1. Introduction

The world population and standards of living are growing steadily. This reality urges us towards more sustainable protein production. Currently, conventional agriculture already demands 38% of the land surface and 70% of the global freshwater withdrawals, while related nutrient losses pose a major environmental burden, already falling outside safe planetary boundaries [1]. Microbial protein (MP), such as microalgae, could be a sustainable alternative to alleviate tensions on these resources due to their efficiency in the use of nutrients, land, and freshwater [2], [3]. Current protein productivities for microalgae containing 50% DW protein, cultivated in open raceway ponds, can range from 15 up to 90-ton protein/ha/yr [4] which is much higher than the conventional high-protein ingredient soybean (2 – 4ton protein/ha in 2020) [5]. In addition to its remarkable productivity, microalgal protein stands out for its exceptional protein quality. The Essential Amino Acid Index (EAAI), the geometrical mean of the ratio of all essential amino acids (EAA) in the evaluated protein relative to the human requirements, is usually higher than 1. This signifies that microalgal protein has the capacity to fulfill the human requirements of EAA [6]. Furthermore, microalgae are up to 100% efficient in nutrient use, thereby avoiding resource input and environmental pollution [7]. Additionally, when the microalgal growth medium is recycled, the water consumption is only 480 L/kg of produced protein [8]. This value is much lower than those of vegetables (26,000 L/kg protein) and beef protein (112,000 L/kg protein) [9]. Finally, microalgae contain several high-value compounds with additional benefits such as antioxidants and vitamins [10], [11].

Biomass of the cyanobacterium Spirulina (formally known as *Arthrospira* spp. and some species recently reclassified as *Limnospira* spp. [12]) and the green microalga *Chlorella* spp. have been commercially produced at large scale for food and feed applications since the early 1950s [13]. Currently, the estimated global production volumes of Chlorella and Spirulina are 6,600 and 12,000 tons of dry matter per year, respectively. However, the commercial products of these microalgae present a large variability in nutritional quality among species and strains, but also within the same strain [6]. Depending on cultivation parameters such as temperature, pH, nutrient concentrations, light quality, light intensity, and photoperiod, protein values between 7 and 70% dry weight (DW) were recorded for *C. vulgaris* and between 17 and 73% DW for *A. platensis* [6]. The same study observed a variability in protein quality, expressed as EAAI, of up to 37%. This observed variability in the nutritional value of commercial products is hypothesized to be a consequence of the predominance of open raceway pond for microalgae cultivation at industrial scale, which is often operated in batch, where the whole microalgae culture is harvested at mid to late logarithmic or stationary growth phases [14]. Such production systems are popular because of their relatively low investment costs and ease of operation [13]. However, a major drawback is the difficulty in controlling the operating conditions, such as biomass age (harvesting time) and light availability, at a constant value, which is likely the main contributor to the previously observed variability in the nutritional value of microalgae [6], [10], [15]. In contrast to batch cultivation, (semi-)continuous cultivation, i.e., (semi-)constant input of fresh medium and output of biomass, is hypothesized to offer a more constant biomass composition under steady-state conditions [16], [17]; however, no studies have verified this hypothesis for protein or EAA composition. Furthermore, because continuous cultivation results in a constant biomass concentration that can be controlled to allow for optimal light irradiation, higher productivities can be maintained [18].

To maintain a high biomass protein quality in batch or (semi-)continuous cultivation, the influence of growth parameters on biochemical biomass composition should be well understood. For protein content, the influence of cultivation parameters such as biomass age, photoperiod, light intensity, temperature, pH, and nutrient concentration was previously studied for Chlorella [19], [20], [21] and Spirulina species [22], [23], [24]. On protein quality (EAA profile), in contrast, an important knowledge gap exists regarding the influence of cultivation parameters. For Spirulina only the effect of nitrogen source [25], struvite as a phosphate source [26], temperature, and pH [27] were investigated before, while for *Chlorella* spp., only temperature [28], and phosphate and nitrogen sources [26] were tested. The effects of biomass age, photoperiod, light intensity, and operation mode (batch and (semi-)continuous) on protein quality are currently unknown, and no data exists on the influence on biomass digestibility.

Furthermore, most of the existing research has been performed in Erlenmeyer flasks or lab-scale photobioreactors (PBR), allowing optimal light distribution and mixing. In contrast, most large-scale microalgae production is performed in less expensive open pond raceway bioreactors that lack optimal light irradiation and distribution [29]. Therefore, the optimal operating parameters in industrial configurations in terms of biomass protein content and protein quality are still unknown.

This study aimed to define the optimal operational parameters, such as biomass age for harvesting, batch/continuous operation mode and photoperiod on a semi-technical scale, mainly using a 100 L open raceway reactor (and validation of some parameters in an 83 L closed photobioreactor for Spirulina), to boost the protein and EAA productivity of Chlorella and Spirulina species. Specifically, *Chlorella vulgaris* and *Limnospira indica* were selected due to its resource-efficient production and nutritionally appealing profile, and for their inclusion in projects to develop regenerative life support systems, such as the Micro-Ecological Life-Support System Alternative (MELiSSA) [7], [30].

The optimal biomass age (harvesting time) was determined in batch operation, and it was tested to see if this optimum could be maintained in continuous operation mode. Further, the effect of photoperiod was investigated under continuous illumination and in a light-dark regime. Nutritional quality was defined based on the biomass protein content, the rarely determined EAA profile, and biomass digestibility.

2. Materials and Methods

2.1. Microalgal strains

The eukaryotic *Chlorella vulgaris* strain SAG 211-11b (SAG Culture Collection of Algae, Göttingen University, Germany) and prokaryotic *Limnospira indica* strain PCC 8005 (SCK-CEN, Belgium) were studied under photoautotrophic growth conditions. It is important to note that *L. indica* PCC 8005 was previously known as *Arthrospira platensis* PCC 8005 [31]. The most recent denomination (*L. indica* PCC 8005) is used in this study. Both strains were pre-grown under their specific optimal conditions. *C. vulgaris* was cultivated in adapted Bold Basal Medium (BBM) and *L. indica* in adapted Zarrouk medium (see Supplementary Material).

2.2. Influence of harvesting time in batch operation

To determine the optimal harvesting time in relation to biomass, protein and EAA productivity, both microalga species were cultivated during 50 days in batch in two indoor lab-scale open raceway pond reactors, each presenting a volume of 100L and an illuminated area of 0.5 m² (µBio engineering, USA). A paddle wheel rotating at 8 rpm kept the cultures in suspension, while the culture depth was maintained at constant 0.20 m by manually making up for evaporation losses once per day. Both cultures were inoculated in the raceway ponds when they reached stationary phase (determined by measuring DW daily) to assure uniformity in the growth phase of the biomass. pH control was automated with adjustable set points using a Neptune Apex control system, using 100% CO_2 injection. Temperature was maintained constant (20ºC and 30ºC for *C. vulgaris* and *L. indica* respectively), and Nitrogen and Phosphorus measured regularly to control nutrients availability. Table 1 summarizes the applied reactor operation conditions, as well as the initial nutrient concentrations (detailed medium composition can be found in Supplementary Material).

2.3. Influence of continuous operation

To investigate whether the maximum protein and EAA productivity reached in batch mode could be maintained in continuous operation mode, a continuous experiment was performed for 30 days in the same raceway reactors and conditions as the batch operation mode (Table 1). The reactors were harvested daily and replenished with fresh medium. The dilution rate (or mean cell residence time) used in the reactors was selected after batch cultivation, which was estimated to retrieve the best protein and EAA productivity according to the batch cultivation results.

Raceway cultivation of *L. indica* in continuous operation resulted in contamination from grazing protozoa, and for this reason an indoor closed photobioreactor (PBR) experiment was performed with the same strain for 100 days (Table 1). In addition, the influence of a change in dilution rate was assessed. The applied dilution rates were all chosen to maintain a biomass density assuring exponential growth. The PBR was an 83 L continuous external loop gas-lift reactor with an illuminated volume of 55 L [32]. The illuminated parts of the reactor consist of two cylindrical 15 cm diameter sections and 1.5 m height, serving as riser and downcomer for the liquid circulation. These columns are connected in the upper and lower parts by curved stainless-steel parts, supporting the instrumentation and external jackets for water circulation for temperature control at 36°C. Illumination was provided by 350 halogen lamps (Sylvania, BAB 12V 20 W, Belgium). Similarly to the raceway reactor, PBR was harvested daily and replenished with fresh medium. The medium influent to the PBR was connected to the effluent of a nitrifying bioreactor with a co-culture of *Nitrosomonas europaea* and *Nitrobacter* *winogradskyi* for nitrogen supply. The applied medium was designed including all the components to meet the growth requirements of the three strains (Supplementary Material). Nitrogen was never limiting, with influent and effluent concentrations of 244 ± 51 mg N/L and 160 ± 29 mg N/L, respectively.

2.4. Influence of photoperiod and light intensity

The influence of changes in illumination on the biomass, protein and EAA productivity, as well as protein quality, was assessed. The influence of photoperiod was tested in batch in the raceway ponds, under a 12:12h light-dark illumination regime, with same conditions as in previous experiments (Table 1). In addition, the influence of a shift from light-dark regime to continuous light was researched by switching to continuous light after 21 days of light-dark illumination.

During the continuous PBR experiment with *L. indica* (see previous section), the influence of light intensity was assessed during each dilution rate phase (Table 1). The light intensity was set at 280±24 µmol/m²/s during equilibration of each dilution rate phase. At the end of each dilution rate phase, the light intensity was first decreased (short dark phase, followed by a 17±3% decrease in light intensity compared to equilibrium), after which a two-step increase in light intensity was induced, each step with a short peak to 970 µmol/m²/s followed by a light intensity 9±4% higher compared to the previous step. The short dark phase and peaks in light intensity were applied to quickly lower or increase microalgal photosynthesis, respectively.

2.5. Biomass growth and characterization

Growth was monitored daily by OD at 750 nm (UV - 2501 PC; SHIMADZU) and by biomass dry weight (DW). Biomass productivity (mg /L/d) during batch cultivation was calculated from its suspension concentration (mg/L) divided by the time of cultivation (days).and is given by following equation:

$P_{biomass}$ (mg/L/d) = $(X_s – X_0)/(t_s – t_0)$

with X_s and X_0 the biomass concentrations (mg/L) at the time of sampling (t_s) and initial (t_0) biomass concentration, respectively. Biomass productivity during continuous cultivation was calculated including the biomass that was already harvested (X_h) until t_s:

$P_{biomass}$ (mg/L/d) = $[(X_s – X_0) + X_h]/(t_s – t_0)$

Protein and EAA productivity were calculated by multiplying biomass productivity with their respective content at t_s.

Proteins were extracted from 5 mg of biomass with trichloroacetic acid according to Slocombe et al. [33] and adapted by Sui et al. [34]. Part of the extract was used to determine biomass protein content [35] and for essential amino acid (EAA) analysis. Prior to EAA analysis, the protein extracts were hydrolyzed with 6M HCl for 24 h at 110 °C in vacuum-sealed hydrolysis tubes (Wilmad Labglas). To avoid EAA oxidation, hydrolysis and subsequent acid evaporation were performed under alternating vacuum and nitrogen gas flushing. After evaporation and dissolution in 0.75 mM HCl, , EAA were derivatized with propyl chloroformate following the Phenomenex EZ:faast amino acid analysis procedure and analyzed using gas chromatography mass spectrometry (Agilent HP6890, USA; Agilent HP 5973 USA). Norvaline was used as an internal standard during EZ:faast sample preparation.

EAA data were normalized based on the WHO/FAO/UNU report of 2007 [36] established human reference pattern, with a value of 100 representing the best match between the sample EAA content and the consumer’s needs. The essential amino acid index (EAAI) was calculated according to the following equation [37]:

$$\mathrm{EAAI} = \sqrt[n]{\frac{\mathrm{aa}_1}{\mathrm{AA}_1} \times \frac{\mathrm{aa}_2}{\mathrm{AA}_2} \times \ldots \times \frac{\mathrm{aa}_n}{\mathrm{AA}_n}}$$

Here, aa_i represents the EAA content over total protein content (mg EAA/g protein) and AA_i represents the FAO/WHO/UNU established human reference content [36].

Human digestibility was determined in-vitro following the harmonized protocol of Minekus et al. [38]. Briefly, 5 mg of biomass were mixed with simulated gastric fluid (SGF) containing pepsin (2000 U/mL) and incubated for 2 h at 37 °C at 1200 rpm. Subsequently, simulated intestinal fluid (SIF) containing pancreatin (100 U trypsin activity/mL) and bile salt (10 mM) was added before the sample was incubated again for 2 h (37 °C and 1200 rpm). After centrifugation, the pellet was analyzed for Kjeldahl nitrogen (KjN) [39]. Digestibility was determined by subtracting KjN in the pellet after digestion (undigested fraction) from the KjN content of the sample before digestion.

Finally, reactor biomass suspension was regularly analyzed using light microscopy (Leica DM IL LED) and reactor ammonium and ortho-phosphate content was analyzed photospectrometrical (San++ Continuous Flow Analyzer). An independent sample t-test in SPSS statistics 24 was used to compare data at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Influence of harvesting time in raceway configuration

Biomass protein content is highly dependent on the growth phase (biomass age), influencing the optimal harvesting time (Figure 1 A, B). Under continuous illumination in batch, *C. vulgaris* presented an optimal protein content of 50 ± 1.7% DW during the early linear growth phase, after 9 days at a biomass density of 0.29 g/L, with a decrease towards the stationary phase (Figure 1 A).

A relatively constant EAA composition was observed for *C. vulgaris* with an EAAI of 1.2 ± 0.1 (Figure 1 A), with only a notable increase in lysine content of 57% towards the late linear growth phase (days 40 – 50; Supplementary material). These values were slightly higher than those observed in commercial *Chlorella vulgaris*, with an EAA content of 51 ± 1% compared to the commercial 47 ± 3% (AA-based). Comparing the EAA profile with commercial *Chlorella vulgaris* [6], an enrichment several EAA is observed, remarkably in tyrosine (40 ± 3 compared to 9.3 ± 5 mg tyr/g protein) and histidine (21 ± 3 compared to 9.0 ± 3 mg his/g protein). A decrease in methionine, however, was observed (10 ± 6 compared to 1.8 ± 2 mg met/g protein). Other authors also observed an increase of EAAI towards the stationary phase of the growth in other commercial microalgae species [34].

Biomass density at which the highest protein and EAA content was observed did not necessarily match the biomass density linked to the highest biomass, protein, or EAA productivity. *C. vulgaris* presented an optimal biomass productivity (48 mg DW/L/d or 53 ton/ha/yr) at a biomass density of 0.11 g/L at day 2 (Figure 1 A). However, more important are the maximum protein productivity (16 mg protein/L/d) and EAA productivity (6.7 mg EAA/L/d), reached after 9 days at a biomass density of 0.30 g biomass/L, indicating the best time for biomass harvesting. On the other hand, no trend between cultivation time and digestibility was observed for *C. vulgaris*, which ranged from 38 to 46% (Supplementary Material).

Batch cultivated *L. indica* under continuous light presented an optimal protein content (61 ± 1.0% DW) that was maintained for around 10 days at a biomass density between 0.16 g DW/L and 0.27 g DW/L (Figure 1 B). The following decrease in protein content was similar to that observed in *C. vulgaris*.

For *L. indica*, the variability in EAA was larger than that for *C. vulgaris*, with an average EAAI value of 0.37 ± 0.14. A higher quality was noted on days 30 – 50 (EAAI of c.a. 0.5). The maximum EAAI was observed when the protein content began to decrease. Regarding the EAA profile, the values obtained in this study, as well as the EAAI, were generally lower than those observed in other raceway reactors [17] and commercial Spirulina biomass [6]. Because nutrients were abundantly present, this observation suggests that reduced light availability induces an increase in protein quality. A possible explanation is the redistribution of cellular nitrogen induced by stress conditions. It is known that under nitrogen-sufficient conditions, algal cells assimilate and accumulate it in the form of various nitrogen-containing compounds (nitrate, ammonium, peptides, RNA, pigments, etc.) [19]. When environmental stress occurs, the cells degrade non-growth-related protein by autophagy [40], which can induce variations in the amino acid profile.

L. indica presented an optimal biomass productivity of 21 mg DW/L/d (15.3 ton/ha/yr) at day 8, while protein productivity reached a maximum of 12 mg protein/L/d after 9 days, indicating the optimal harvesting time at a biomass density of 0.19 g DW/L (Figure 1 B). Between days 30 and 50, protein quality peaked, leading to an optimal harvesting period for EAA productivity (1.1 mg EAA/L/d). However, the increase in EAA productivity from day 9 to day 30 was comparatively modest, registering 1.0 mg EAA/L/d after 9 days, when maximum protein productivity was achieved. Biomass productivities obtained are, however, relatively low compared with literature values of 58 – 109 ton/ha/yr (15.9 – 29.3 g/m^2/d), reported for Spirulina cultivated in an outdoor raceway operated in Spain [17].

On the other hand, biomass digestibility during the different growth phases presented values between 39% and 59% (see Supplementary Material). However, similar to *C. vulgaris*, no trend was observed between the cultivation time and biomass digestibility.

Finally, comparing the biomass quality of the *C. vulgaris* strain with that of the *L. indica* strain, the highest protein content was observed in *L. indica*. However, *C. vulgaris* was superior in terms of the protein quality. Radhakrishnan et al. [41] determined the amino acid composition of Spirulina and *C. vulgaris* in the same growth conditions and, in contrast to this study, results indicated relatively similar AA composition, which could indicate a significant effect of the studied species and specify strain in the final EAA results. Further research using different strains, should be conducted to clarify these differences.

3.2. Influence of operation mode

3.2.1. Continuous operation in raceway configuration

To investigate if the maximum protein and EAA productivity reached in batch could be maintained in continuous operation mode, *C. vulgaris* and *L. indica* were maintained at a biomass density of 0.27 ± 0.02 g DW/L (Figure 2 A) and 0.18 ± 0.01 g/L, respectively (Figure 2 B). These biomass densities provided the maximum protein productivity during the first batch experiment.

During continuous cultivation of *C. vulgaris*, protein content was maintained at a relatively constant 36 ± 2.2% DW (Figure 2 A). Unexpectedly, this value was lower than the optimum value under batch cultivation, although the light availability was higher at lower biomass densities. Light stress due to the slightly lower biomass densities (0.27 ± 0.02 g DW/L) compared to the optimal density in batch (0.30 g DW/L), could not have been the reason because light penetration depth is low at the applied light intensity (95 µmol photons/m²/s)[18]. Our observations correspond, however, with those of Matos et al. [15] of a decrease in protein content with increasing dilution rate, thus decreasing biomass density. In terms of protein quality, EAA composition was relatively constant for *C. vulgaris*, with an average EAAI value of 1.41 ± 0.17, which was slightly higher than the average EAAI during batch cultivation; however, this was not significant ($p > 0.05$). The largest variability in EAA content was observed for lysine, with values between 72 and 108 mg Lys/g protein on days 6 and 11, respectively (Supplementary Material).

Applying a dilution rate of 0.1 d^{-1} (aiming at a cell residence time of 10 days), a stable biomass, protein and EAA productivity (26 ± 0.8 mg DW/L/d, 9.4 ± 0.3 mg protein/L/d and 5.8 ± 0.1 mg EAA/L/d, respectively) could be maintained (Figure 2 A). These values were slightly lower than the expected maximum productivities observed during batch cultivation. Other authors observed a similar decrease in biomass productivity when switching from batch to continuous operation in both outdoor ponds [42] and PBR [14], [15].

L. indica in continuous cultivation presented a 16% higher protein content during the first 8 days (72 ± 5.6% DW) compared to the maximum observed during batch cultivation (Figure 2 B). Halfway through continuous cultivation, however, a sudden drop in the protein content was observed (43 ± 7.7% DW). During this period, microscopy revealed the presence of grazing protozoan contamination (Supplementary Material).

Similar to batch cultivation, *L. indica* presented a large variability for all EAA, with a similar average EAAI of 0.37 ± 0.12. Remarkably, the highest protein quality (EAAI of 0.51) was reached after 25 days, when the protein content was at its lowest value (32% DW). Similar to the protein quality increase of *C. vulgaris* under light limitation, a possible explanation could be the stress-related redistribution of nitrogenous compounds towards EAA-rich protein.

L. indica presented its highest protein productivity of 13 ± 0.6 mg protein/L/d during the first 8 days (Figure 2 B). During the last 12 days, protein productivity decreased simultaneously with protein content to 11 ± 0.9 mg protein/L/d. The overall maximum protein productivity was slightly lower than the observed optimal *C. vulgaris* protein productivity, and possible protozoan contamination (see Supplementary Material) could be the reason for the decrease in protein productivity [43]. The average EAA productivity was observed to be 2.3 ± 0.5 mg EAA/L/d, which was double as high compared to the maximum EAA productivity in batch cultivation mode.

3.2.2. Continuous operation in a closed PBR

Cultivation of *L. indica* in a closed PBR resulted with more success in a relatively stable protein content (68 ± 5.5% DW) and avoidance of protozoan contamination (Figure 3).

The amino acid composition was stable but rather low, with an average EAAI of 0.26 ± 0.03, comparable to the initial protein quality during continuous raceway cultivation. A possible reason for this is the abundant light availability in exponentially growing cultures, resulting in a protein pool containing a lower EAA content; however, no literature could be found studying the effect of light intensity on EAA. Considering dilution rate, biomass density decreased from 1.3 ± 0.16 g DW/L at a dilution rate of 0.24 d^{-1} to 0.9 ± 0.2 g DW/L at a dilution rate of 0.48 d^{-1}. Nonetheless, changing the dilution rates did, not significantly influence protein level ($p > 0.05$) and total EAA content. Mainly phenylalanine and tyrosine were influenced by a change in dilution rate (see Supplementary Material)

Furthermore, much higher average protein (300 ± 100 mg protein/L/d at a dilution rate of 0.48 d^{-1}) and EAA productivities (33 ± 14 mg EAA/L/d at a dilution rate of 0.48 d^{-1}) were reached (Figure 3). In closed PBR configurations, literature data reports optimal dilution rates between 0.12 and 0.3 d^{-1} for *A. platensis* resulting in biomass productivities between 104 and 1320 mg DW/L/d [44], [45]. With increasing dilution rates from 0.24 to 0.48 d^{-1}, the average biomass, protein and EAA productivity increased from 0.32 ± 0.04 g DW/L/d to 0.43 ± 0.10 g DW/L/d, from 0.20 ± 0.02 g protein/L/d to 0.30 ± 0.10 g protein/L/d and from 22 ± 1.5 mg EAA/L/d to 33 ± 14 mg EAA/L/d, respectively. However, these differences were not statistically significant ($p > 0.05$). Additionally, when the dilution rate increased, the average biomass protein content and quality remained relatively stable. A slightly lower EAAI is obtained in the PBR biomass (maximum of 0.31 obtained with 0.48 d^{-1} dilution rate) compared to the 0.37 ± 0.1 and the 0.36 ± 0.1 obtained in batch and continuous operation. Regarding the EAA profile, major differences are mainly observed in Leucine, showing a content of

25 ± 2 g leu/g compared to the 46 ± 14 g leu/g protein measured in the continuous open raceway configuration.

3.3. Influence of illumination

3.3.1. Photoperiod in raceway configuration

C. vulgaris presented a protein content of 38 ± 4.1% DW in a light-dark regime similar to batch growth under continuous light (Figure 4 A). The change in light regime after 21 days to continuous illumination, did not significantly influence protein content (40 ± 3.7% DW). In terms of EAA (45 ± 5%) content in biomass and EAAI (1.2 ± 0.1), the values were again similar to those under continuous illumination, and a slight increase was noticed between days 5 and 14. Changing to continuous illumination at day 21 resulted in higher EAA content (62% of protein) and protein quality (EAAI of 1.79), mainly due to an increase in lysine, valine, leucine, threonine, and (phenylalanine + tyrosine).

On the other hand, halving the illumination to a 12:12h light-dark cycle resulted in *C. vulgaris* biomass night loss and a decrease in biomass, protein and EAA productivity, compared to continuous illumination (Figure 4). Night biomass loss was observed during the first 17 days, with an average value of 12 ± 15% biomass loss compared to light phase biomass growth, resulting in average values for biomass and protein productivity of 17 ± 1.0 mg biomass/L/d and 6.4 ± 0.6 mg protein/L/d, respectively. The decrease was around 50% for maximum biomass productivity (from 48 mg DW/L/d to 19 mg DW/L/d), protein productivity (from 16 mg protein/L/d to 8 mg protein/L/d) and EAA productivity (from 6.7 mg EAA/L/d to 3.0 mg EAA/L/d), compared to continuous illumination. Finally, productivity was not influenced when the light regime was changed to continuous illumination.

To gain insight into the diurnal dynamics of protein and EAA productivity and EAAI, 3-hourly samples were analyzed on day 14 (Figure 4 B, D). *C. vulgaris* biomass increased from 0.25 to 0.3 g biomass/L during the light phase while protein content decreased from 33 ± 0.2% DW to 31 ± 1.1% DW (Figure 4 B). During the dark phase, the opposite effect was observed with a biomass decrease to 0.27 g biomass/L and protein content increase to 36 ± 1.0% DW. Protein productivity remained stable owing to the opposite trends of biomass and protein, while biomass productivity was optimum (19 mg biomass/L/d) in the evening. Changes in EAA during a light-dark cycle were remarkable, with a decrease in protein quality during the day from an EAAI of 1.44 to 1.36 and increase during the night to 1.46. Lysine, threonine, and phenylalanine + tyrosine were the most affected amino acids. Despite the occurrence of night biomass loss, the highest EAA content and quality were observed in the morning, indicating that this moment could be the best harvesting time.

L. indica presented an average protein content of 58 ± 5.5% DW during the 12:12h light-dark cycle, which decreased to 44 ± 3.6% DW after the change to continuous illumination (Figure 4 C). *L. indica* presented a similar EAA composition as observed during batch cultivation under continuous illumination when biomass density was between 0.1 and 0.3 g biomass/L (Figure 1 B). EAAI values increased steadily from 0.13 at day 10 to 0.39 at day 20, while the change from light-dark to continuous illumination did not affect EAA composition.

L. indica presented a night biomass loss during the first 10 days, with an average value of 22 ± 9.9% biomass loss compared to the light phase. This night biomass loss resulted in lower average biomass and protein productivities of 13 ± 1.5 mg biomass/L/d and 7.7 ± 0.9 mg protein/L/d, respectively (Figure 4 C). In contrast to *C. vulgaris*, the decreases in maximum biomass (from 21 mg DW/L/d to 16 mg DW/L/d), protein (from 12 mg protein/L/d to 9.3 mg protein/L/d) and EAA productivity (from 1.1 mg EAA/L/d to 1.0 mg EAA/L/d), compared to cultivation under continuous illumination, were less than 50%, meaning that light-dark cultivation has potential to be more energy efficient. This could be a consequence of faster growth during the light phase in a light-dark regime compared to continuous illumination, which was previously observed for *Dunaliella salina* [34]. After 10 days, some nights were registered with biomass loss, and some with slight biomass increase. This could be due to the higher biomass density (0.13 g/L at day 10) protecting the culture from substantial respiratory losses, due to lower light availability, as suggested by other authors in the literature [23], [46]

Zooming in to one light-dark cycle for *L. indica*, biomass increased from 0.18 to 0.206 g biomass/L during the light phase, while the protein content first increased from 60 ± 0.3% DW to 63 ± 1.3% DW after 8h, to fall back to 60 ± 0.02% DW at the end of the light phase (Figure 4 D). Similar to the protein productivity of *C. vulgaris*, a stable value was observed due to the opposite trends of biomass concentration and protein content. During the dark phase, a further increase in biomass was observed to 0.214 g biomass/L, whereas the protein content decreased to 56 ± 4.9% DW. This dark phase protein decrease is opposite to the trend observed in protein content in *C. vulgaris*, and also Torcillo et al. [46] noticed a different trend, with an average *A. platensis* protein content of 68% in the morning and 60% in the evening. Similar to *C. vulgaris*, however, was the observation of the highest protein quality in the morning with an EAAI of 0.35 and 0.42, compared to 0.20 in the evening.

3.3.2. Light intensity in PBR configuration

During the continuous PBR experiment with *L. indica*, light intensity was lowered at the end of a dilution rate, resulting in biomass and protein productivity decrease with 31 ± 21% and 15 ± 10%, respectively (Figure 3). The opposite was observed with a stepwise increase in light intensity. Furthermore, an increase in light intensity had a positive effect on protein content, which confirms the observations during batch cultivation. However, the total EAA content and protein quality, expressed as EAAI, remained relatively stable. A possible explanation is the abundant light availability, which keeps the cells in exponential growth and does not induce major changes in EAA composition. Phenylalanine and tyrosine were the most influenced EAA, with changes between 9 and 31%, between minimum and maximum values.

3.4. Overall optimal raceway protein production

In the presence of non-limiting nutrients, biomass and protein productivity are mainly functions of light availability, which is linked to biomass density (since light intensity remained constant). However, because protein quality (EEA content) also depends on this parameter, protein productivity should always be considered as complementary to quality. Furthermore, in this study, it was demonstrated that different microalgae display different protein contents and quality dynamics in relation to operational parameters. When determining the optimal cultivation strategy, the most suitable operational parameters are mainly determined by the species and strains used.

With respect to *C. vulgaris*, the highest protein and EAA productivities (16 and 6.7 mg/L/d) were achieved after 10 days of batch cultivation under continuous light. Continuous cultivation only resulted in a slightly lower EAA productivity but a higher biomass EAAI; however, possible contamination and the need for daily harvesting, which may increase labor costs, point to batch cultivation as an optimal strategy. If night illumination is an issue, the natural day-night illumination regime will decrease EAA productivity by approximately 50% (for 12:12 h light-dark). Despite the decrease in biomass during the dark phase, the loss in EAA productivity can be minimized by harvesting in the morning, because of the increased protein quality at the start of the light phase. Furthermore, switching from a light-dark regime to continuous illumination has the potential to increase protein quality in a short period, resulting in an increase of almost 50% in EAA productivity. The optimal protein and EAA productivity and EAAI achieved in the raceway reactor were significantly higher than those observed in commercial *C. vulgaris* products [6], [47]. Although further research is necessary to fully comprehend the effects of operational parameters on *C. vulgaris* biomass quality, optimized biomass age and operation mode under continuous illumination could potentially improve EEA-rich protein productivity for commercial microalgal products.

The most beneficial cultivation method for *L. indica* in terms of EAA productivity was continuous operation under continuous illumination. However, it is difficult to maintain algal cultures free from other species or grazing protozoa when operated in open systems. Even though Spirulina is considered a favorable species for production in open systems owing to its growth in brackish water at high pH [13], contamination was also observed in this study under indoor laboratory conditions. When *L. indica* was cultivated in batch mode, both under continuous light and light-dark regimes, the highest EAA productivity was determined by the trade-off between protein quantity and quality. The highest overall EAA productivity was observed when the biomass density reached 0.26-0.36 g DW/L. Similar to *Chlorella vulgaris*, if a light-dark regime was applied, harvesting in the morning was beneficial because of the significantly higher EAA-rich protein present in the biomass.

In general, the *Limnospira* strain used in this study presented low protein quality compared to other Spirulina values reported in the literature. Compared to the AA composition in the study by Radhakrishnan et al. [41], which presented an EAAI of 0.985, the highest observed quality in this study (EAAI of 0.531) was considerably lower. Other commercial species and strains of Spirulina, such as *A. platensis*, have also demonstrated superior protein and EAA productivities in open raceway reactors [6], [17], which suggests that the impact of operational parameters on protein and EAA is strain dependent. Therefore, further research should be conducted involving other commercially relevant Spirulina species.

Other parameters not addressed in this study could significantly affect EAA productivity. In both batch and continuous operation modes, a substantial amount of nitrogen remained in the effluent or at the end of the experiment, probably because of the slow growth rate of *L. indica*. This suggests that fine-tuning of the N content for a higher EAA-rich protein content should also be performed to optimize production. Reuse the effluent for new microalgae cultivation could also be applied in this case, being a strategy that can reduce the environmental impact of the process while boosting the growth of the microalgae [48]. However, the effect of effluent reuse on protein or EAA content has not yet been investigated. Furthermore, temperature fluctuations in open raceway reactors, even within greenhouse settings, can exceed 10 ºC between the day and night [17]. Moreover, temperature and photoperiod variations depend on the season and the geographical location of the reactor. Addressing the impact of these factors is crucial for production optimization, potentially reducing costs without compromising protein quality.

Finally, when comparing the performance of PBR and open pond raceway reactors, the first one showed much higher biomass, protein and EAA productivity. This reactor design allows for better controllability of the operational parameters (pH, dilution rate, optimal illumination, etc.), and several authors have observed increased microalgae growth in PBR compared to open raceway ponds [49]. In addition, PBR can prevent protozoan contamination, such as the observed in this study, which can have a positive influence on the performance of the reactor [29]. However, in this case, the higher investment required and elevated operation cost of closed photobioreactors hinder the implementation of this setup for commercial purposes [49], [50]. Therefore, scaling up the PBR implies greater costs, and raceway reactors are preferred due to their easier operation and cheap maintenance [29].

4. Conclusions

In this study, we observed that optimal protein and EAA productivity did not often occur simultaneously with optimal biomass productivity or protein content. Even though EAAI was higher in the biomass obtained during continuous operation mode, raceway reactor under batch operation mode resulted in higher productivities than the continuous one, observing better protein and EAA production with *Chlorella vulgaris* (16 and 6.7 mg/L/d) compared to *Limnospira indica* (14 and 2.8 mg/L/d). However, *L. indica* showed significantly improved productivity in a closed photobioreactor.

Furthermore, light availability has been demonstrated to be an important parameter for qualitative protein and EEA production. Photoperiod was demonstrated to have a significant influence, achieving a higher EAAI under continuous illumination (1.8) compared to the light-dark regime (1.3). In the light-dark regime, protein quality loss can be minimized by performing biomass harvesting in the morning. In addition, continuous illumination after the previous light-dark regime significantly improved EEA production and EAAI. The optimal EAAI for *C. vulgaris* was 1.8, which is consistent with human EAA requirements, whereas the tested *L. indica* strain reached an optimal EAAI value of only 0.53. This study provides novel insights to improve the productivity of high-quality EAA-rich protein in existing and new microalgal industrial production facilities.

Supplementary Material for this work can be found in the online version of the paper.

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Highlights

- Protein quality mainly depends on light availability and harvesting time
- Optimal protein quantity or quality does not guarantee optimal EAA productivity
- Continuous operation is favorable for EAA productivity in some strains
- Light/dark illumination does not necessarily imply a lower EAA productivity
- Under light/dark regime, EAA profile/productivity are optimal at light initiation

Figure 1

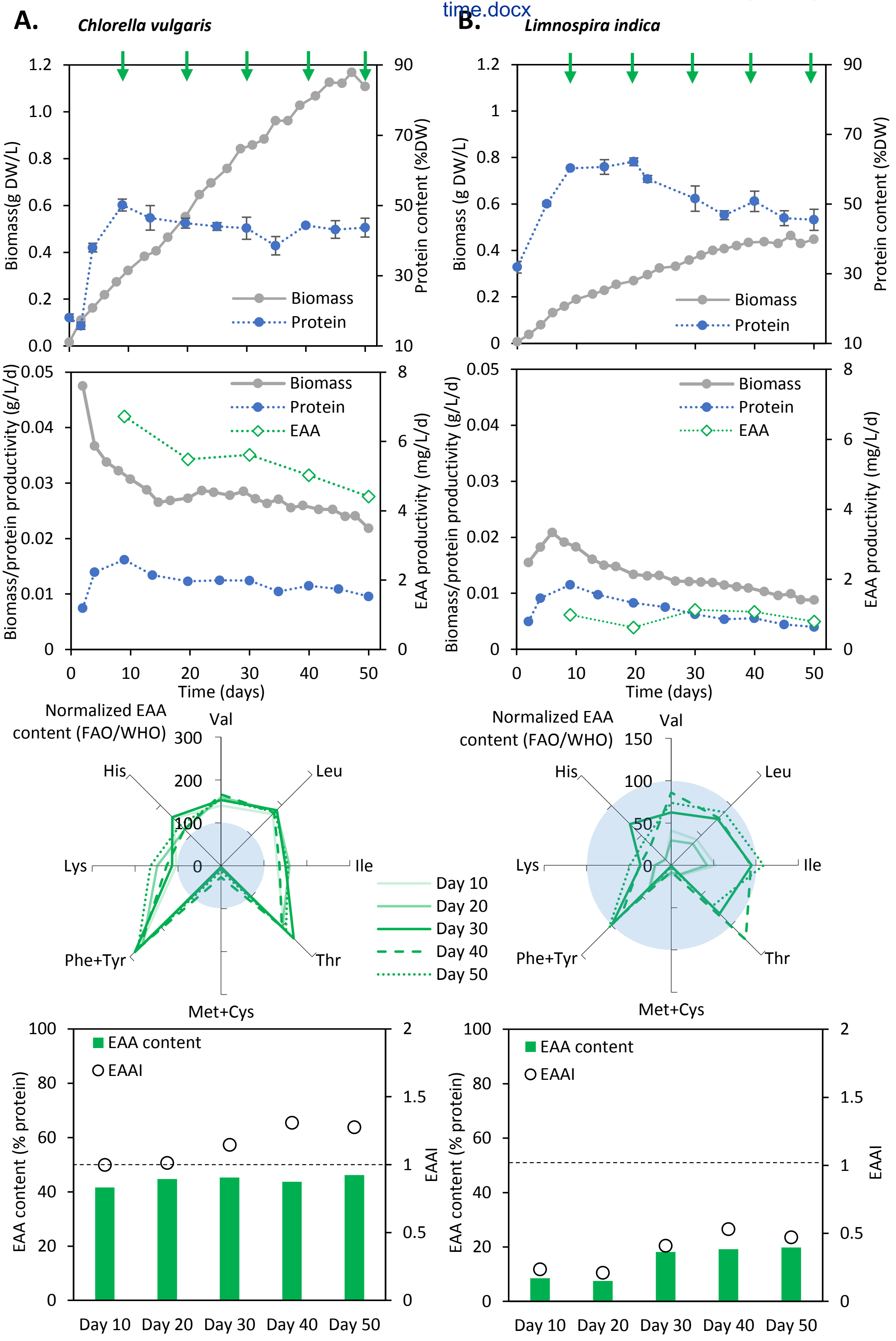

Figure 1. Influence of harvesting time in open raceways on biomass growth, protein content, normalized essential amino acid (EAA) composition, total EAA content, EAA index (EAAI) and biomass, protein and EAA productivity of *Chlorella vulgaris* (A) and *Limnospira indica* (B), under continuous illumination. Green arrows indicate the time at which the AA profile was

determined; blue circles indicate a value of 100 representing a perfect match with human requirements according to FAO/WHO.

Figure 2

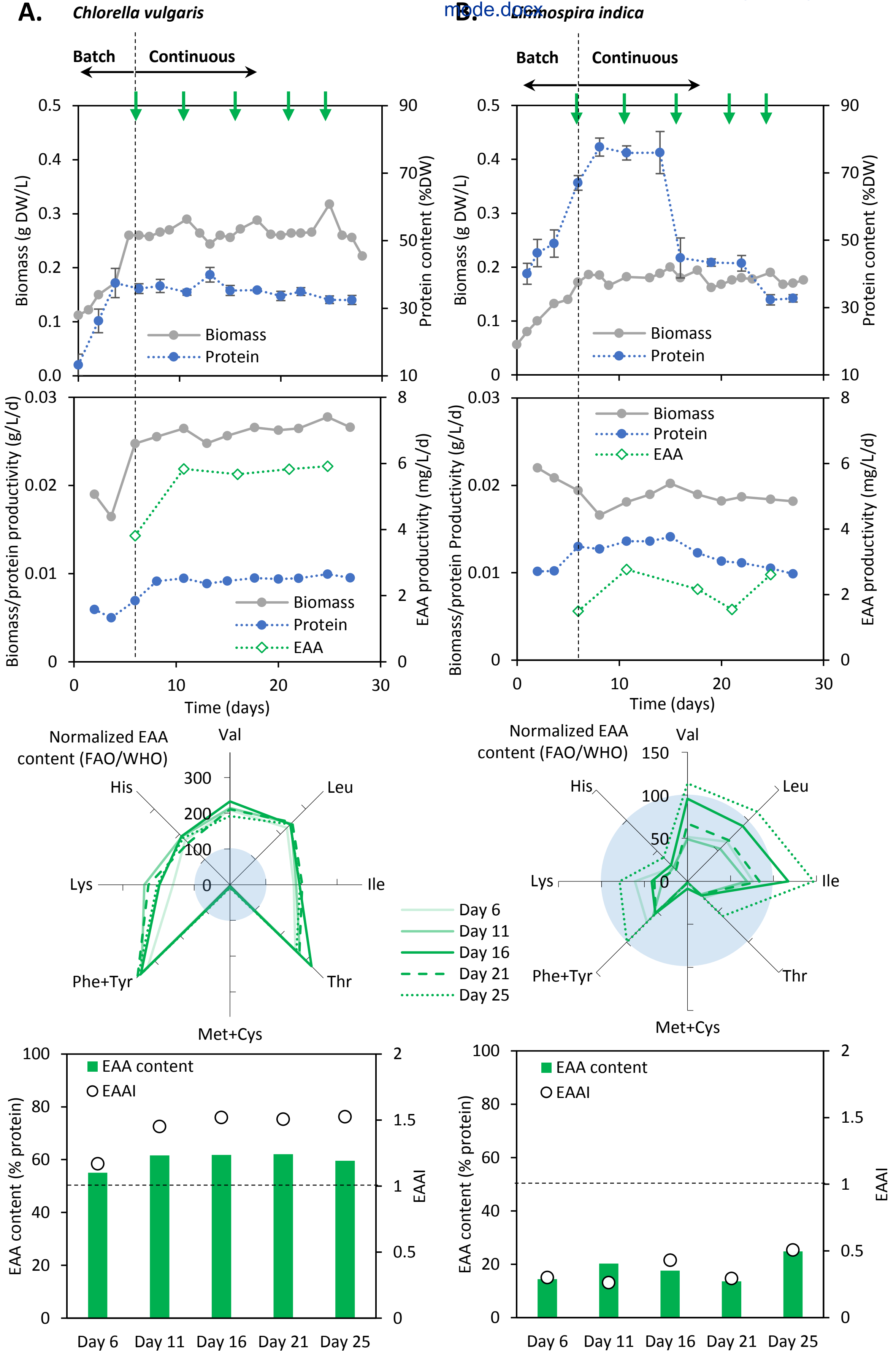

Figure 2. Influence of continuous operation mode in open raceways on biomass growth, protein content, essential amino acid (EAA) composition, total EAA content, EAA index (EAAI) and biomass, protein and EAA productivity of *C. vulgaris* (A) and *L. indica* (B), under continuous

illumination. Green arrows indicate the time at which the AA profile was determined; blue circles indicate a value of 100 representing a perfect match with human requirements according to FAO/WHO.

Figure 3

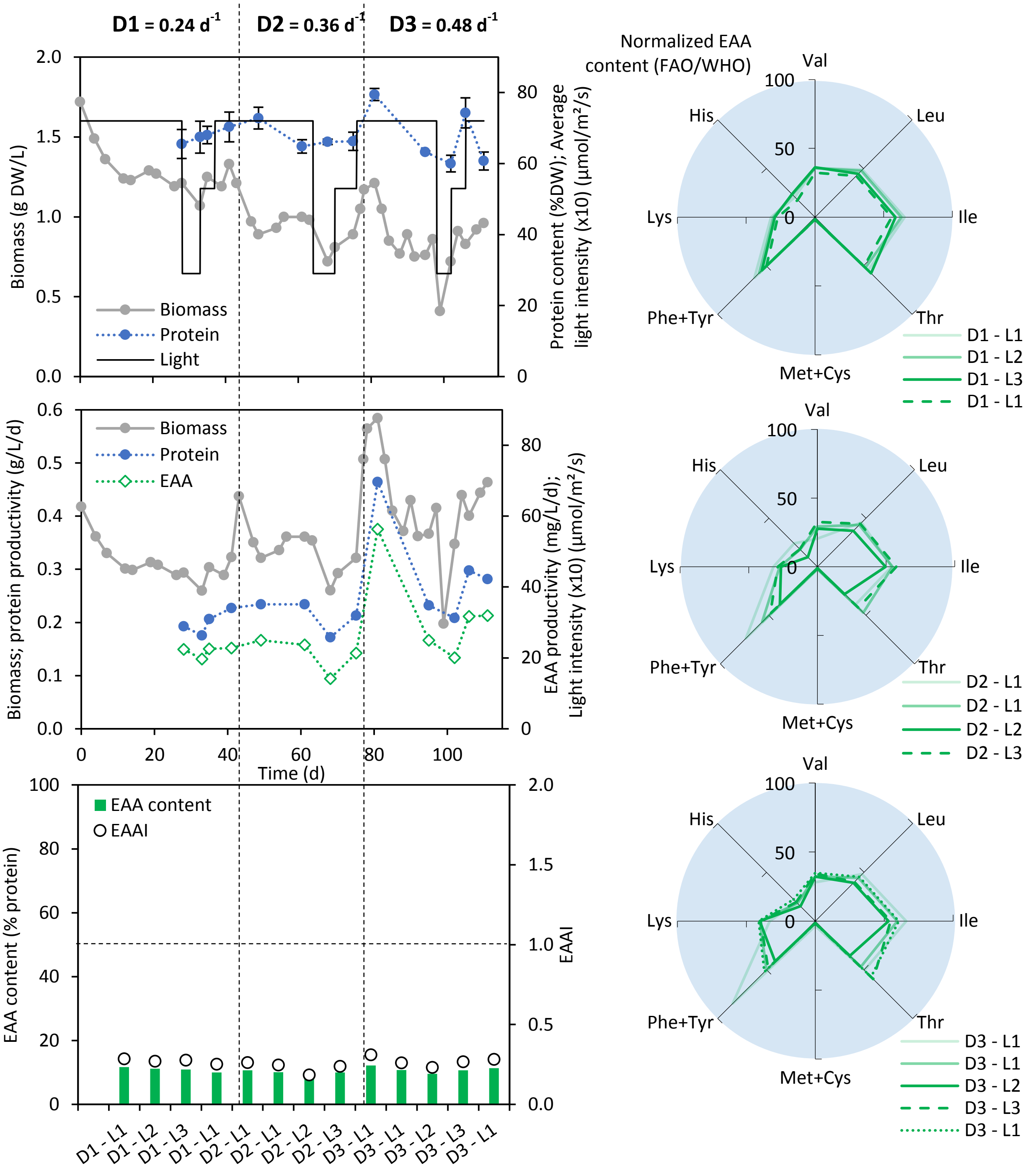

Figure 3. Influence of dilution rate (D1, D2 and D3) and average light intensity (L1, L2 and L3) in continuous operation in a closed PBR on biomass growth, protein content, essential amino acid (EAA) composition, total EAA content, EAA index (EAAI) and biomass, protein and EAA productivity of *L. indica* under continuous illumination. Blue circles indicate a value of 100 representing a perfect match with human requirements according to FAO/WHO.

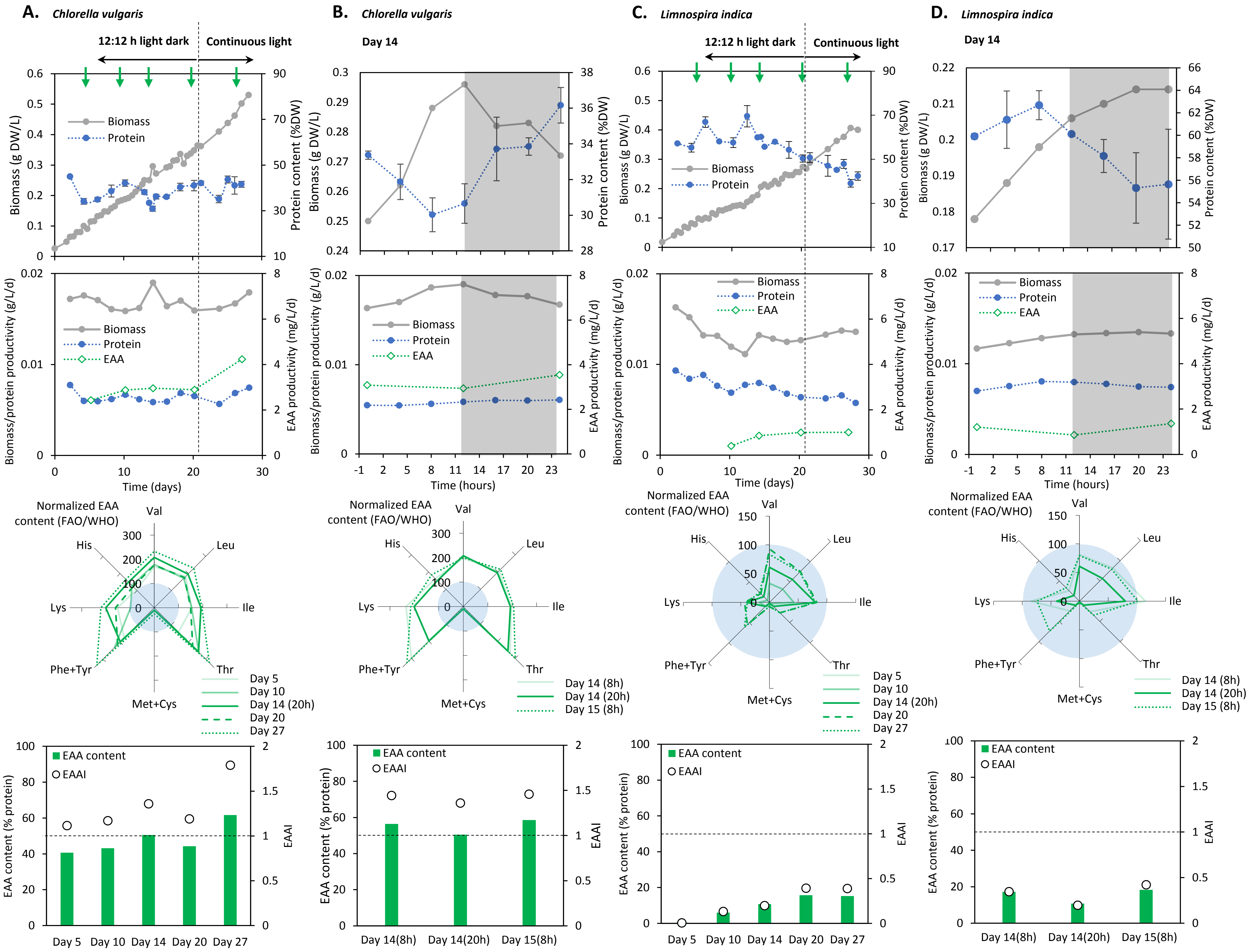

Figure 4. Effect of photoperiod in open raceways on biomass growth, protein content, essential amino acid (EAA) composition, total EAA content, EAA index (EAAI), biomass, protein, and EAA productivity of C. vulgaris (A, B) and L. indica (C, D) under continuous illumination. Panels (B) and (C) present the same parameters during 24 h of day 14. Red arrows indicate the samples for which the AA profile was determined; blue circles indicate a value of 100, representing a perfect match with human requirements according to FAO/WHO.

Table 1. Cultivation conditions applied during the different experiments performed in raceway ponds (*Chlorella vulgaris* and *Limnospira indica*) and in a closed photobioreactor (*Limnospira indica*).

Reactor	Raceway pond								Photobioreactor		
Experiment	**Influence harvesting time**		**Influence continuous operation**				**Influence photoperiod**		**Influence dilution rate/light intensity**		
Organism	*C. vulgaris*	*L. indica*	*C. vulgaris*		*L. indica*		*C. vulgaris*	*L. indica*	*L. indica*		
Operation mode	Batch	Batch	Batch	Continuous	Batch	Continuous	Batch	Batch	Continuous		
Dilution rate (d^{-1})	-	-	-	0.1[a]	-	0.1[a]	-	-	0.24	0.36	0.48
pH	7 ± 0.2	9.2 ± 0.2	7 ± 0.2		9.2 ± 0.2		7 ± 0.2	9.2 ± 0.2	8.5		
Temperature (°C)	20 ± 1	30 ± 1	20 ± 1		30 ± 1		20 ± 1	30 ± 1	36		
EC (mS/cm)	2.05	7.01	2.05	1.5	7.01	5.75	2.05	7.01	11.3		
Photoperiod (Light:Dark)	24:0 h	24:0 h	24:0 h		24:0 h		12:12 h[c]	12:12 h[c]	24:0 h		
Intensity (μmol/m²/s)	95[f]	95[f]	95[f]		95[f]		95[f]	95[f]	280/255/232[d]		
Initial density (g/L)	0.016	0.0073	0.112	0.26	0.056	0.172	0.02	0.02	1.72[h]	1.21[h]	1.05[h]
Initial OD (OD750)	0.08	0.04	0.50	1.1	0.13	0.37	0.12	0.05	1.72[h]	0.96[h]	0.94[h]
N-Source	$NaNO_3$	$NaNO_3$	$NaNO_3$		$NaNO_3$		$NaNO_3$	$NaNO_3$	$NaNO_3$		
N (mg/L)	206	411	206	103	411	205.5	206	411	244 ± 51		
P-Source	K_2HPO_4/ KH_2PO_4	K_2HPO_4	K_2HPO_4/KH_2PO_4		K_2HPO_4		K_2HPO_4/ KH_2PO_4	K_2HPO_4	Na_2HPO_4 / KH_2PO_4		
P (mg/L)	53	89	53	26.5	89	44.5	53	89	309.6		
C-Source	CO_2[b]	CO_2[b]	CO_2[b]		CO_2[b]		CO_2[b]	CO_2[b]	CO_2[e]		

'-': not applicable; EC: Electrical Conductivity

[a] dilution rate by daily harvesting of 10% of the reactor volume and refilling with BBM (*C. vulgaris*) or Zarrouk (*L. indica*) containing half of the initial concentration for N and P

[b] dosed as 100% CO_2 when the pH dropped below the set-point of 6.8 for *C. vulgaris* and 9 for *L. indica.*

[c] a switch to continuous light (24:0 h) was made after 21 days of light-dark operation.

[d] the 3 different light intensities were tested in each dilution rate. The 280 μmol/m²/s is the average light intensity (full spectrum) during largest part of each dilution rate phase (phase L1). At the end of this phase, light intensity was first decreased (short dark phase), after which it was increased in two steps (L2 and L3, with 232 and 252 μmol/m²/s respectively, see Figure 3).

[e] the reactor was aerated at 2.8 L/min with addition of 30 mL/min (1%) CO_2 to control the pH at 8.5.

[f] PAR light intensity.

[g] nitrogen dosed surpasses that of the original BBM recipe, aiming to enhance protein accumulation in *C. vulgaris* [42, 51]. Additionally, P content is increased to contribute to a more stable pH, particularly in the photobioreactor with *L. indica.*

[h] the three different dilution rate experiments took place in the same reactor and culture over 100 days; the initial density and OD shown are the ones in the reactor at the moment when a new dilution rate experiment started.