Light regime and growth phase affect the microalgal production of protein quantity and quality with *Dunaliella salina*

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Abstract

The microalga *Dunaliella salina* has been widely studied for carotenogenesis, yet its protein production for human nutrition has rarely been reported. This study unveils the effects of growth phase and light regime on protein and essential amino acid (EAA) levels in *D. salina.* Cultivation under 24-h continuous light was compared to 12-h/12-h light/dark cycle. The essential amino acid index (EAAI) of *D. salina* showed accumulating trends up to 1.53 in the stationary phase, surpassing FAO/WHO standard for human nutrition. Light/dark conditions inferred a higher light-usage efficiency, yielding 5-97% higher protein and 18-28% higher EAA mass on light energy throughout the growth, accompanied by 138% faster growth during the light phase of the light/dark cycle, compared to continuous light. The findings revealed *D. salina* to be especially suitable for high-quality protein production, particularly grown under light/dark conditions, with nitrogen limitation as possible trigger, and harvested in the stationary phase.

Keywords

Single-cell protein; Microbial protein; Microalgae; Food; Photoperiod

1 Introduction

Novel protein sources are needed to satisfy the increasing demand of proteins for human consumption in the near future. In this context, microalgae, apart from bacteria, yeast and fungi, as a type of single-cell protein, are considered as potentially important contributors, whether produced on renewable or virgin materials, or on recovered resources (Verstraete et al., 2016). Since the early 1950s, microalgae have been explored as an alternative protein source, and their large-scale production has been successfully established since the 1980s (Vigani et al., 2015). From an economic point of view, open cultivation systems utilizing sunlight have been extensively used and preferred for commercial production (Vigani et al., 2015). By using natural light, microalgal cells are subject to a daily light/dark cycle, which will affect their growth rate and protein synthesis (de Winter et al., 2013). It is known that dark phase during cultivation can negatively impact biomass production due to respiratory loss (Edmundson and Huesemann, 2015). It has been reported that up to 35% of the biomass accumulated during the light phase can be lost through respiration during the dark phase (Torzillo et al., 1991). However, the rate and extent of biomass loss during the dark phase is dependent on the microalgal species and the specific cultivation conditions (Edmundson and Huesemann, 2015). Besides the biomass loss, light/dark regime also imposes changes in the cell's macromolecular biochemical composition, impacting for instance protein and carbohydrate content (Sukenik and Carmeli, 1990). Generally, energy storage compounds like carbohydrates build up during the light phase and decrease during the dark phase, for the usage of night

metabolism such as protein synthesis (Cuhel et al., 1984; de Winter et al., 2017, 2013; Han et al., 2013; Hidasi and Belay, 2018; Ogbonna and Tanaka, 1996; Torzillo et al., 1991). Even further, smaller molecules composing the macromolecules such as amino acids and fatty acids will be influenced as well. Consequently, it is important to determine the optimum harvesting time of biomass considering all variations introduced by different light regimes. Nevertheless, most studies mainly focused on variations of biochemical compositions between the light and dark phase, leaving a lack of knowledge on differences between a continuous light versus a light/dark regime.

Dunaliella salina is one of the most widely used species for commercial microalgae production, mainly due to its particularly high carotenogenesis, yielding β-carotene (Borowitzka, 2013). However, with reported protein content of over 57% (on dry weight basis), the potential of *D. salina* as protein source has hardly been investigated (Becker, 2007). Over the past 50 years, only a few studies mentioned and researched the protein synthesis within the *Dunaliella* genus, and information is far from complete (Sui and Vlaeminck, 2018). Apart from protein quantity, protein quality of microalgae based on essential amino acid (EAA) content substantially determines its true nutritional quality for food applications (Becker, 2007). As reported by several studies, the amino acids profile of *Dunaliella* spp. is comparable with commercial *Spirulina* and *Chlorella* products, matching perfectly the FAO/WHO reference for human requirements (Becker, 2007; Fabregas and Herrero, 1985; Gibbs and Duffus, 1976; Kent et al., 2015; WHO/FAO/UNU Expert Consultation, 2007). Nonetheless, these EAA profiles were obtained from a single growth

77 phase (mostly from the end of the exponential growth phase to stationary phase) and
78 cultivation conditions without internal comparisons, which makes the potential variations
79 of its EAA profile triggered by harvesting time or growth phases unclear. Specifically for *D.*
80 *salina*, no insights have been gained regarding the variations of biomass growth together
81 with protein quantity and quality under different light regimes.

82 In this study *D. salina* was cultivated in batch mode, both under 24-h continuous light and
83 12-h/12-h light/dark cycle to study the effect of different growth phases (e.g. exponential,
84 linear and stationary phase) on protein content and quality (as EAA content) variations.
85 Additionally, for the light/dark regime, diurnal and nocturnal changes of *D. salina* in terms
86 of biomass growth and protein synthesis were also studied. Based on the acquired
87 knowledge, the ultimate goal is to maximize protein production from *D. salina* with
88 optimized EAA profile at larger scale, by implementing the optimum light regime and
89 harvesting time.

90 **2 Materials and methods**

91 2.1 *Dunaliella* strain and cultivation conditions

92 *D. salina* SAG 184.80 was cultivated in 500mL Erlenmeyer flasks filled with 400mL
93 sterilized Modified Johnson's medium (Borowitzka, 1988) at 2M salinity provided by table
94 salt (Everyday, Colruyt Group, Belgium). The initial biomass concentration was set to an
95 optical density at 680 nm (OD_{680}) of ± 0.03. The culture flasks were kept on a magnetic
96 stirring plate (Thermo Scientific™ Cimarec™ i Poly 15) at 200 rpm in a temperature

97 controlled room at 20°C. Aeration was given by 0.2 µm filtered (Minisart® NML Syringe
98 Filter) air at a rate of 4.17 vvm from air pumps (TetraTech®, APS100). Light was provided
99 by fluorescent tubes (Sylvania F58W/GRO) at the intensity of 55 µmol/m^2/s. To provide
100 even light distribution, all flasks were randomized daily. The pH level was corrected daily
101 to 7.5 by 1M NaOH or 1M HCl. Two light regimes were applied, namely 24-h continuous
102 light regime and 12-h/12-h light/dark regime. Each light regime was conducted in
103 triplicate. Samples were collected every 12 hours during the experiment, data from day 4,
104 7, 10, 13, 16, 19, 24 and 28 were presented in the study. At the linear phase of the
105 microalgal growth, a 24-hour time series analysis was performed for both light regimes.
106 During this 24-hour time series analysis, samples were collected every 4 hours for
107 analyses. All the samples were analyzed freshly for OD_{680} and saved at -20°C for cell
108 number, protein and carbohydrate analyses at the end of the experiment. Cell integrity of
109 stored samples was checked by microscope analysis and cell size distribution, which
110 presented a nice bell-shaped normal distribution similar with well-maintained culture. A
111 neglected/damaged culture will show no pattern of size distribution (Ongena et al., 2010).

112 2.2 Biomass analyses and calculations

113 Based on OD_{680}, the ash-free dry weight (AFDW) of the biomass was estimated following a
114 calibration curve (R^2=0.99) obtained in advance:

115 $$AFDW\ (g/L) = 0.5069 \times OD_{680} - 0.0131$$

116 Presented AFDW data in Fig 1A were from day 0, 4, 7, 10, 13, 16, 19, 24 and 28 with
117 interval of 12 hours.
118 The maximum specific growth rate was calculated fitting the experimental data to the
119 Gompertz model (Gompertz, 1825) modified by Zwietering et al. (1990) in GraphPad
120 Prisma 5 software:

121 $$Ln\left(\frac{N_t}{N_0}\right) = Ln\left(\frac{N_m}{N_0}\right) \times exp[-\exp\frac{\mu_{max} \times e}{Ln\left(\frac{N_m}{N_0}\right)} \times (\lambda - t) + 1]$$

122 where N_t and N_0 are the biomass concentrations at time t and time 0. N_m is the maximum
123 biomass concentration (at stationary phase). μ_{max} is the maximum specific growth rate, λ
124 is the lag time and e (2.718) is the exponential constant. Cell number of the sample was
125 measured with Beckman Multisizer 3 Coulter Counter. Samples for protein and
126 carbohydrate measurement were analyzed directly without cell disruption due to the lack
127 of cell wall of *D. salina*. Samples for protein were from day 0, 4, 7, 10, 13, 16, 19, 24 and
128 28 and for carbohydrate were from day 7, 19 and 28. The protein and carbohydrate
129 content were determined using Markwell method, a modified Lowry method (Markwell et
130 al., 1978) and Dubois method (Dubois et al., 1956), respectively. Samples at day 7, day 10,
131 day 16 and day 28 from both light regimes were analyzed for EAA. Prior to essential amino
132 acid analysis, pelletized biomass (10min at 5000g) was hydrolyzed with 6M HCl for 24
133 hours at 110 °C, in vacuum-sealed hydrolysis tubes (Wilmad LabGlass). To remove all
134 oxygen, a vacuum was applied alternating with nitrogen gas flushing. After hydrolysis, the
135 samples were evaporated under vacuum conditions and re-dissolved in a 0.75 mM HCl

136 solution to end up with a pH between 3 and 5. Hydrolyzed samples were stored at -20°C
137 upon further use. Amino acids were derivatized with propyl chloroformate as described by
138 the EZ:faast amino acid analysis procedure (consisting of a solid phase extraction step,
139 derivatization and liquid/liquid extraction) (Phenomenex, 2003) and separated using gas
140 chromatography (Agilent HP 6890) and detected using mass spectrometry (Agilent HP
141 5973). Bovine Serum Albumin (BSA) was used as control from which the amino acid
142 recovery after hydrolysis was calculated. Norvaline was applied as internal standard
143 during EZ:faast sample preparation. Essential amino acid index (EAAI) was calculated
144 following equation:

145 $$EAAI = \sqrt[n]{\frac{aa1}{AA1} \times \frac{aa2}{AA2} \times \dots\dots \times \frac{aan}{AAn}}$$

146 using FAO/WHO established adult indispensable amino acid requirements as reference
147 (Oser, 1959; WHO/FAO/UNU Expert Consultation, 2007). Here, *aan* and *AAn* stand for the
148 content of one specific EAA relative to the total protein content (mg EAA/g protein) in the
149 sample and corresponding FAO/WHO reference, respectively. As indicated by EAAI, a
150 value of 1 or above refers to a matching quality, between 0.95 and 1 high quality, between
151 0.86 and 0.95 good quality, between 0.75 and 0.86 useful, and below 0.75 inadequate
152 (Zhang et al., 2009). As a reference, some conventional food products such as egg and
153 soybean have an EAAI of above 1 (Becker, 2007).
154 The biomass protein and carbohydrate contents were expressed as fractions of the
155 biomass (%AFDW). The suspension protein and carbohydrate contents (g/L) were the

156 results of multiplying the biomass concentration (g AFDW/L) with corresponding biomass
157 protein and carbohydrate contents (%AFDW). The biomass and protein productivity
158 (mg/L/d) were calculated as the biomass concentration (g AFDW/L) or the suspension
159 protein content (g/L) divided by the time period of cultivation (days) at each sampling
160 point. The biomass and protein yield on light energy (mg/mol photon) were calculated as
161 follows:

162 $$Y = \frac{C_t \times V}{\sum_{t=0}^{t=t} L_t \times A \times 3600 \times 24} \ (mg/mol\ photon)$$

163 where C_t is the biomass or protein concentration on day t (mg/L); V is the volume of the
164 reactor flask (L); L_t is the light input on day t (μmol/m^2/s) and A is the illuminated surface
165 area (m^2).
166 The cell number and cell volume changes were calculated as follows:

167 $$\%change = \frac{X_{t+12} - X_t}{X_t} \times 100\%$$

168 in the case of 24-h light regime, X_{t+12} is the cell number/volume measured 12 hours after
169 the cell number/volume measured at time t (X_t). In the case of 12-h/12-h light/dark
170 regime, X_{t+12} is the cell number/volume obtain after each light (dark) phase and X_t is the
171 cell number/volume obtained prior each light (dark) phase. Time t is every 12 hours.

172 2.3 Statistics
173 The experiment was performed in triplicate with results expressed as means ± standard
174 deviations in tables and figures. Independent sample t-test in SPSS statistics 24 was used

175 to compare data in Table 1. A significance level $p < 0.05$ was considered as statistically
176 different.

177 **3 Results and discussion**

178 3.1 Impact of light regime and growth phase on biomass level and protein quantity
179 Growth curves of *D. salina* under both light regime are shown in Fig. 1A. Microalgal
180 growth evidently benefited more from a longer lighting period, obtaining a maximum
181 specific growth rate of 0.45 d^{-1} and biomass concentration of 1.36 g AFDW/L, higher than
182 0.35 d^{-1} and 0.81 g AFDW/L obtained from light/dark regime. As shown in Fig. 1B, the
183 biomass protein content of both light regimes presented an increase-decrease pattern,
184 with a maximum around 80% AFDW reached during the exponential phase. The decrease
185 in protein content towards the stationary phase was 54% for continuous light regime and
186 32% for light/dark regime, respectively (Fig. 1B). This pattern has been described for other
187 microalgae such as *Chlorella* and *Scenedesmus*, but it has not been reported for *D. salina*
188 (Piorreck and Pohl, 1984). Between the two light regimes, no difference of biomass
189 protein content was observed until day 20, after which continuous light regime resulted in
190 more drastic decrease (Fig. 1B). At the end of the experiment (day 28), the biomass
191 protein content of the light/dark regime was 54% AFDW, 45% higher than in the
192 continuous light regime (Fig. 1B). These changes in protein level can be related to the
193 nitrogen availability, as nitrogen is a major protein-composing element. As reported,
194 microalgal biomass in the exponential phase is characterized by a higher protein content
195 with excess nitrogen availability for protein synthesis, while in the stationary phase the

196 protein content is lower due to insufficient nitrogen and consequently halted growth
197 (Uriarte et al., 1993). Although medium nitrogen levels were not monitored in this study,
198 based on the initial medium composition (0.14 gN/L) and the Redfield ratio (14.6% N in
199 biomass), nitrogen in the medium was depleted for the continuous light regime at
200 stationary phase, as 1.36 g AFDW/L would contain 0.19 gN/L, exceeding the nitrogen
201 supply from the medium. For the light/dark regime, 0.81 g AFDW/L would correspond to
202 0.11 gN/L, indicating nitrogen was still available in the medium. This was also confirmed
203 by the biomass carbohydrate content, as displayed in Fig. 2. On day 28, the biomass
204 carbohydrate content of *D. salina* grown under continuous light regime reached up to
205 30%, while in light/dark regime it only remained around 15%. When microalgae are
206 experiencing nutrient limitation or starvation, which often happens during the stationary
207 phase of a batch culture, their carbon-containing compounds such as carbohydrates will
208 be largely enhanced. This is mainly due to a switch of the metabolism of storage
209 compound from nitrogen to carbon pool (Pancha et al., 2014).

210 Regarding the suspension protein content, continuous light regime promoted its build-up
211 until the linear growth phase reaching a maximum of 0.62 g/L at day 16 and it declined to
212 0.49 g/L by 20% at stationary phase, day 28 (Fig. 1B). This is a result of both slower growth
213 and sharp reduction of biomass protein content, as can be seen in Fig. 1B. Conversely,
214 light/dark regime contributed to a steady accumulation of suspension protein, reaching
215 0.43 g/L in the stationary phase.

3.2 Impact of light regime and growth phase on protein quality

Apart from the protein quantity, protein quality as EAA content also varied during the different growth phases. From day 7 to day 28 ranging from the exponential phase to the stationary phase, both EAA content (excluding tryptophan) and EAA profile of *D. salina* from two light regimes presented an accumulating trend, with optimum EAA profile achieved in the stationary phase (Fig. 1E, Fig. 3). EAA content built up towards the stationary phase, reaching 44% and 30% of total protein for continuous light and light/dark regime, respectively (Fig. 1E). *D. salina* EAA content positively compares with the FAO/WHO reference, it is therefore clear that *D. salina* is capable of producing high-quality protein for human nutrition, regardless of the light regime applied. Since day 16, roughly around the linear growth phase, biomass from both light regimes presented an EAAI of useful and good quality, 0.90 for continuous light regime and 0.78 for light/dark regime (Fig. 1E). Further on day 28 in the stationary phase, both EAAI reached above 1 (1.53 for continuous light and 1.06 for light/dark regime), indicating a matching quality of EAA profile in the biomass (Fig. 1E). Moreover, the more EAAI above 1, the better quality of protein it stands for. For instance, if replacing food source with EAAI of 1 with microalgal biomass with EAAI of 1.53, 35% of biomass can be saved to still match the human requirement.

Regarding the content of the individual EAA, until day 16, they all showed a similar accumulating pattern reaching similar level regardless of light regimes (Fig. 3A, 3B). However from day 16 to day 28, all the individual EAA contents of continuous light regime

237 increased dramatically by 17-125%, where every EAA surpassed the level of human
238 requirement (Fig. 3C). Meanwhile for light/dark regime the increase was only 5-58% (Fig.
239 3C). The overall accumulating trend, especially sharper increase of EAA under continuous
240 light regime, seems to be related to the growth phases. Towards the stationary phase,
241 protein synthesis diminishes, and therefore the cells may attempt to preserve those
242 amino acids. Despite the complexity of protein synthesis, microalgae also rely on nitrogen
243 assimilation pathways, initiated by the nitrate reductase, which converts the nitrate
244 transported inside the cells into nitrite. Nitrite is then reduced to ammonia, which can be
245 assimilated into glutamate/glutamine via glutamine synthase and NADPH-dependent
246 glutamine:2-oxoglutarate aminotransferase (GS/GOGAT) pathway (Alipanah et al., 2015;
247 Halsey et al., 2011; Remmers et al., 2018; Sanz-Luque et al., 2015). As glutamate and
248 glutamine are the initial amino acids synthesized from nitrogen source, they play a crucial
249 role in the continuation of amino acids biosynthesis by providing the critical nitrogen entry
250 point (Guerra et al., 2013). For instance, glutamate provides the amino groups for other
251 amino acids and glutamine provides amide to various amino groups of other amino acids.
252 EAA and other more complex amino acid synthesis may depend on the availability of
253 glutamate/glutamine and their synthesis could essentially take longer. Nevertheless, the
254 dynamics of glutamate/glutamine content throughout the growth stage cannot be
255 predicted, as a simultaneous production and conversion pathway of glutamate/glutamine
256 is expected to happen. In this study, the glutamine content also presented an
257 accumulating trend throughout the growth phases, suggesting a possible preservation of

nitrogen content by the cells during stationary phase (data not shown). The sharp increase of EAA under continuous light regime at later growth phase suggests a major response of microalgal cells to preserve the cellular nitrogen capacity by activating e.g. nitrogen scavenging mechanisms involved in the acquisition, remobilization and redistribution of intracellular nitrogen (Alipanah et al., 2015; Halsey et al., 2011; Lv et al., 2017; Remmers et al., 2018; Zhang et al., 2016). These nitrogen related bio-pathways however can lead to different results depending on the microalgal species. It has also been suggested that the qualitative changes in amino acid content during low nitrogen availability may reflect changes in structural and metabolic proteins required for growth, rather than free amino acids, that are often more present during nitrogen abundance (Angell et al., 2014). The marine microalga *Isochrysis zhangjiangensis* performed similarly to this study, showing an increase in several amino acid and especially in phenylalanine content after nitrogen deprivation, which is possibly due to the nitrogen scavenging from other nitrogen-containing substances e.g. nucleotides and rubisco protein (Zhang et al., 2016). Higher proportions of alanine, serine, glycine, and the EAAs phenylalanine, threonine and valine were found in the green macroalga *Ulva ohnoi* with low nitrogen content (Angell et al., 2014). During the nitrogen starvation period an increased amino acid content, including all essential ones except histidine which was not measured, was also found in *Synechocystis* sp. (Kiyota et al., 2014). In addition to nitrogen limitation, it has been reported that phosphorus and sulfur limitation can also result in an increase of most EAA content in *D. salina* (Giordano et al., 2000; Lv et al., 2018, 2017). In contrast, *Dunaliella tertiolecta*

showed significant decrease of most EAA when shifted to nitrogen deprivation condition (Lee et al., 2014). Therefore, additionally to nutrient supply, the EAA profile in microalgae can also vary depending on the species. Nevertheless, the boost of EAA under continuous light regime can be considered a result of nutrient limitation rather than longer light regime, yet continuous lighting during nutrient limitation can also cause a more detrimental effect on several cellular pathways, including photosynthesis (Alipanah et al., 2015; Halsey et al., 2011; Remmers et al., 2018; Schmollinger et al., 2014; Zhang et al., 2016). From the aspect of human nutrition, several EAAs like valine, methionine, threonine and isoleucine are of hyper importance as they are necessary for the maintenance of inner nitrogen balance, without which pronounced symptoms such as poor appetite, extreme fatigue and high nervous irritability can be caused (Rose et al., 1951). From this study, these four EAAs have shown to be mostly boosted at the stationary phase under continuous light due to possible nitrogen limitation (Fig. 3C). Consequently, *D. salina* is not only capable of producing high-quality protein, but also highlights the hyper important EAAs for human nutrition. Besides in algal biomass, accumulation of EAA has been reported under several stresses in plants as well, especially accumulation of lysine, threonine, methionine and valine has been shown in plants during abiotic and light stress conditions (Galili et al., 2016; Obata and Fernie, 2012). In this study, these four amino acids have also shown the highest accumulation at later growth phase (Fig. 3C). Interestingly, the biosynthesis of methionine, lysine and threonine derive

299 from aspartate, which could suggest a biochemical activation of this pathway during later
300 stage of growth and/or abiotic stress.

301 These findings overall suggest the importance of adopting the suitable microalgal species,
302 understanding the biochemical pathways, and optimizing cultivation conditions.
303 Consequently, EAA content and profile of biomass can be improved to a larger extent,
304 presenting high-quality protein for human consumption.

305 3.3 Microalgal protein content dynamics in one diel cycle

306 To gain an in-depth knowledge on the behavior of *D. salina* during one diel cycle, a 24-
307 hour time series analysis on day 15 was performed for both light regimes (Fig. 4A, B). As
308 shown in Fig. 4A, during continuous light regime, biomass grew steadily over 24 hours
309 with 11.4% biomass increase at specific growth rate of 0.13 d^{-1}. During the light phase of
310 the light/dark regime, biomass showed 12.9% increase at specific growth rate of 0.31 d^{-1},
311 both higher than those under continuous light regime (Fig. 4B). Especially the specific
312 growth rate increased substantially by 138%, indicating a much faster growth. This was
313 also observed for most cultivation period, where the light-phase specific growth rate of
314 the light/dark regime presented a higher level than that of the continuous light regime,
315 especially during the exponential phase and early linear phase, roughly between day 8 and
316 day 16 (Fig. 4C). This might also indicate that the light/dark regime is better at maintaining
317 the high specific growth rate than continuous light regime. During the dark phase of the
318 diel cycle, the biomass concentration remained the same level with 1% difference, thus
319 was considered no change (Fig. 4B). Table 1 also summarized the biomass concentration

of both light phase and dark phase under light/dark regime from three different growth phases, which revealed no significant night biomass loss. This was supported by the dark-phase specific growth rate in Fig. 4C, which stayed constantly around zero. These suggest that *D. salina* can be a good microalgal species coping with night biomass losses, hence increase biomass productivity. Similar findings were observed in *Chlorella pyrenoidosa* by Ogbonna and Tanaka (1996) where the growth rate of the light phase in a light/dark regime was higher than that of continuous light regime. Nevertheless, the biomass concentration during the dark phase decreased (Ogbonna and Tanaka, 1996). Such changes of biomass during the dark phase is reported to be highly species-dependent and mediated by cultivation conditions (Edmundson and Huesemann, 2015; Han et al., 2013; Ogbonna and Tanaka, 1996). For instance, different growth phases prior the dark phase and the temperatures during the dark phase can result in 1-22% night biomass loss (Edmundson and Huesemann, 2015).

The diel cycle did not affect the biomass protein level, which remained around 65% over AFDW regardless of the light regimes (Fig. 4A, B). Furthermore, biomass protein content of *D. salina* after the dark phase also showed no significant difference compared with its light phase (Table 1). This showed that no protein loss occurred in *D. salina* during the dark phase at all stages of growth. This finding is partly in line with the present literature on many microalgal species, as some studies suggest that biomass protein content increases during the dark phase due to continuing protein synthesis (Cuhel et al., 1984; Hidasi and Belay, 2018; Ogbonna and Tanaka, 1996; Torzillo et al., 1991), while others also found no

341 effect of dark phase on the protein content (de Winter et al., 2017; Hidasi and Belay,
342 2018; Ogbonna and Tanaka, 1996). In our study, *D. salina* showed less susceptibility to
343 dark-phase cultivation and further demonstrated to be a robust species for microalgal
344 protein production.

345 Apart from the protein content, cell growth and cell division were also associated with the
346 light/dark regime. By analyzing the cell number and volume change following the above-
347 mentioned formula of *D. salina* from both light regimes, different behaviors were
348 noticeably observed (Fig. 5). During the growth under continuous light regime, both cell
349 number and volume increased steadily, resulting in an overall biomass accumulation (Fig.
350 5). Differently during the light/dark regime, the cell number increased mainly during the
351 dark phase and the cell volume changed predominantly during the light phase (Fig. 5). As
352 suggested by Cuhel et al. (1984), photosynthetic organisms commonly accumulate
353 sufficient amount of energy from the light phase for the night metabolism such as cell
354 division, thus despite the biomass growth halt or loss during the dark phase, cell division
355 still occurs (de Winter et al., 2013; Xu et al., 2016). Adversely, cell growth in diameter and
356 volume were primarily found during the light phase for both *D. salina* CCAP 19/30 and
357 *Neochloris oleoabundans* (de Winter et al., 2013; Xu et al., 2016). It is important to notice
358 that the night metabolism of microalgae can be dependent on a complex of factors like
359 prior light intensity and photoperiod before dark phase, nutrient status and the microalgal
360 species (Cuhel et al., 1984). Overall the results highlighted the intricate metabolism of

361 microalgal cells and how changes in cell characteristics may significantly affect the
362 biochemical and nutritional composition of these microorganisms.

363 3.4 Optimum lighting and timing for protein quantity and quality

364 When aiming at optimum high-quality protein production from *D. salina*, productivities of
365 biomass, protein and EAA are important parameters to interpret the overall performance.
366 Besides, their yields on light energy are also essential to estimate their light-usage
367 efficiency, thus energy input. As seen in Fig. 1C, biomass and protein productivities of both
368 light regimes showed increase-decrease patterns. The highest biomass productivity for
369 both light regimes was obtained during the linear growth phase (day 16), 60.6 mg/L/d for
370 continuous light regime and 35.4 mg/L/d for light/dark regime, respectively. The
371 respective 22% and 20% decline towards the stationary phase is mainly due to the halting
372 biomass growth towards the stationary growth phase. For an outdoor raceway pond
373 cultivating *Nannochloropsis gaditana* in Spain, biomass productivity as high as 190 mg/L/d
374 can be achieved high light intensity, temperature and CO_2 enrichment (San Pedro et al.,
375 2015). In accordance, it is foreseen that better lighting and extra inorganic carbon addition
376 can enhance productivities in our cultivation system. The highest protein productivity of
377 both light regimes was achieved during the exponential growth phase: 43.4 mg/L/d on day
378 10 for continuous light regime and 25.0 mg/L/d on day 7 for light/dark regime, while a
379 respective 59% and 39% reduction was observed towards the stationary phase (Fig. 1C).
380 This is likely due to the higher protein content present in the biomass during the
381 exponential phase (Fig. 1B). A similar trend has been observed for EAA productivity under

382 continuous light, with highest level of 10.1 mg/L/d reached on day 16, and decreased by
383 21% towards the stationary phase (Fig. 1F). EAA productivity under light/dark regime
384 however increased to the highest level of 4.8 mg/L/d during the stationary phase (Fig. 1F).

385 It is shown that biomass, protein and EAA all accumulated more during the continuous
386 light regime without considering the light energy input. However, providing artificial
387 illumination comes with both high cost and energy input (Blanken et al., 2013). As shown
388 in Fig. 1A, in the light/dark regime 50% less light was provided and biomass showed only
389 22% slower growth and 40% less biomass concentration compared with continuous light
390 regime. For light/dark regime, the maximum biomass yield on light of 0.76 mg/mol photon
391 was reached at the linear phase, the maximum protein yield on light of 0.54 mg/mol
392 photon was reached at the exponential phase, and the maximum EAA yield on light of 0.1
393 mg/mol photo was reached at the stationary phase (Fig. 1D and Fig. 1F). These values are
394 17%, 15% and 20% higher than those from continuous light regime, respectively. Clearly,
395 the energy from an extended lighting period was not efficiently used by the biomass,
396 resulting in lower yields on light energy. This is possibly due to the induction of photo-
397 inhibition by the excess light energy from continuous light to the microalgal
398 photosynthetic apparatus, leading to an inhibition of both biomass growth and protein
399 synthesis (Janssen, 2002). Consequently, continuous lighting is not suggested in practice
400 for microalgal cultivation despite higher protein quantity obtained.

401 Based on the findings, it is clear that light regime and growth phase play important roles in
402 the microalgal protein production process, determining intrinsic changes of protein

quantity and quality, and further affect their productivities. For the light regime, considering that lighting contributes significantly to the high energy consumption and cost, light/dark cycling is preferred for the higher light-usage efficiency, thus overall higher biomass, protein and EAA yields on light energy. For the growth phases under light/dark conditions, the stationary phase proves to be the optimum harvesting point where, despite lower biomass and protein productivities and yields on light energy, all EAA productivity, EAA yield on light energy and EAAI reached the maximum. To further boost the EAA quality and production, nitrogen limitation seems to be an effective way, as demonstrated from the findings under continuous light regime. Consequently, it is foreseen that having nitrogen limitation during the stationary phase of biomass growth under light/dark regime will be the most effective way to produce high-quality protein from *D. salina*. Further investigations should thus focus on understanding the effect of nitrogen limitation on the dynamics of EAA synthesis. Furthermore, to harvest the biomass before dark phase is preferred since no biomass and protein change was found after the dark phase, and 12-hour prolonged cultivation can be eliminated. To add on, freshly added biomass can directly benefit from the next light phase. Nevertheless, how EAA can vary during the dark phase needs to be studied.

As shown in this study, protein quantity and quality can be greatly affected by different operational conditions and growth phases, such evolution of variations can further give evidence for other types of single-cell protein studies at large.

4 Conclusions

D. salina can produce extremely high-quality protein for human nutrition at stationary phase, regardless of the light regime. The EAA content accumulated throughout the growth phases with an optimum achieved during the stationary phase, and may have been boosted by nitrogen limitation. Light/dark regime showed higher light-usage efficiency with no biomass and protein loss during the dark phase. This study highlights *D. salina* for high-quality protein production, and provides useful cultivation guidelines, including the application of light/dark cycling with nitrogen limitation during cultivation, and biomass harvest in the stationary phase to maximize the EAA production.

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Figure captions

Fig. 1. Impact of light regime (24-h light vs. 12-h/12-h light/dark) and growth phase on *Dunaliella salina:* (A) biomass concentration, (B) biomass and suspension protein content, (C) biomass and protein productivity, (D) biomass and protein yield on light energy, (E) essential amino acid index (EAAI) and EAA content and (F) EAA productivity and EAA yield on light energy. Cultivation occurred at 20°C, pH 7.5 and a light intensity of 55 µmol/m^2/s. Data are expressed as means ± standard deviation (n = 3)

Fig. 2. Impact of light regime (24-h light vs. 12-h/12-h light/dark) and growth phase on carbohydrate content of *Dunaliella salina.* Cultivation occurred at 20°C, pH 7.5 and a light intensity of 55 µmol/m^2/s. Data are expressed as means ± standard deviation (n = 3)

Fig. 3. Impact of light regime and growth phase on essential amino acid index (EAAI) of *Dunaliella salina:* (A) 24-h light, (B) 12-h/12-h light/dark and (C) individual EAA increase from day 16 to day 28

589 **Fig. 4.** Growth and biomass protein of *Dunaliella salina* during 24-hour impacted by (A) 24-
590 h light, (B) 12-h/12-h light/dark together with (C) specific growth rate impacted by light
591 regime. Cultivation occurred at 20°C, pH 7.5 and a light intensity of 55 µmol/m^2/s. Data
592 are expressed as means ± standard deviation (n = 3)

593 **Fig. 5.** Impact of light regime (24-h light vs. 12-h/12-h light/dark) on *Dunaliella salina:* (A)
594 cell number change and (B) cell volume change. Cultivation occurred at 20°C, pH 7.5 and a
595 light intensity of 55 µmol/m^2/s. Data are expressed as means ± standard deviation (n = 3)

596

Table 1 Light- and dark-phase biomass concentration and biomass protein content of *Dunaliella salina* in different growth phases under the 12-h/12-h light/dark regime.

	Phase in the light/dark regime	Biomass concentration (g AFDW/L)	Biomass protein (%AFDW)
Exponential phase (day 7)	Light	0.229 ± 0.003	82.0 ± 8.9
	Dark	0.232 ± 0.003	82.2 ± 4.9
Linear phase (day 15)	Light	0.532 ± 0.012	63.1 ± 2.7
	Dark	0.534 ± 0.009	64.6 ± 2.3
Stationary phase (day 28)	Light	0.801 ± 0.018	53.8 ± 3.7
	Dark	0.813 ± 0.014	51.2 ± 2.5

All parameters between light and dark phase had no significance difference ($p > 0.05$)

Fig. 1.

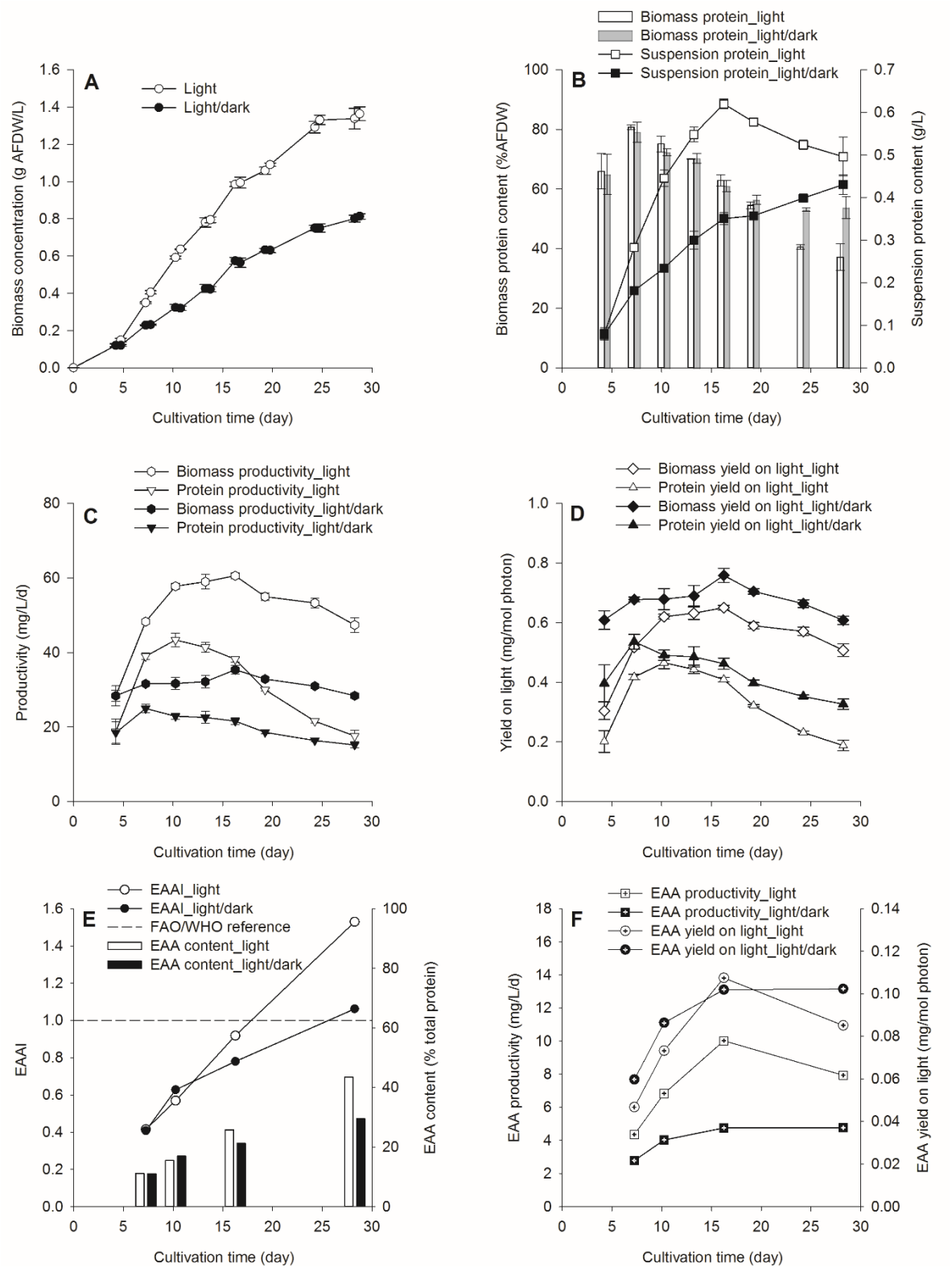

A
Light
Light/dark
Biomass concentration (g AFDW/L)
Cultivation time (day)
B
Biomass protein_light
Biomass protein_light/dark
Suspension protein_light
Suspension protein_light/dark
Biomass protein content (%AFDW)
Suspension protein content (g/L)
Cultivation time (day)
C
Biomass productivity_light
Protein productivity_light
Biomass productivity_light/dark
Protein productivity_light/dark
Productivity (mg/L/d)
Cultivation time (day)
D
Biomass yield on light_light
Protein yield on light_light
Biomass yield on light_light/dark
Protein yield on light_light/dark
Yield on light (mg/mol photon)
Cultivation time (day)
E
EAAI_light
EAAI_light/dark
FAO/WHO reference
EAA content_light
EAA content_light/dark
EAAI
EAA content (% total protein)
Cultivation time (day)
F
EAA productivity_light
EAA productivity_light/dark
EAA yield on light_light
EAA yield on light_light/dark
EAA productivity (mg/L/d)
EAA yield on light (mg/mol photon)
Cultivation time (day)

Fig. 2.

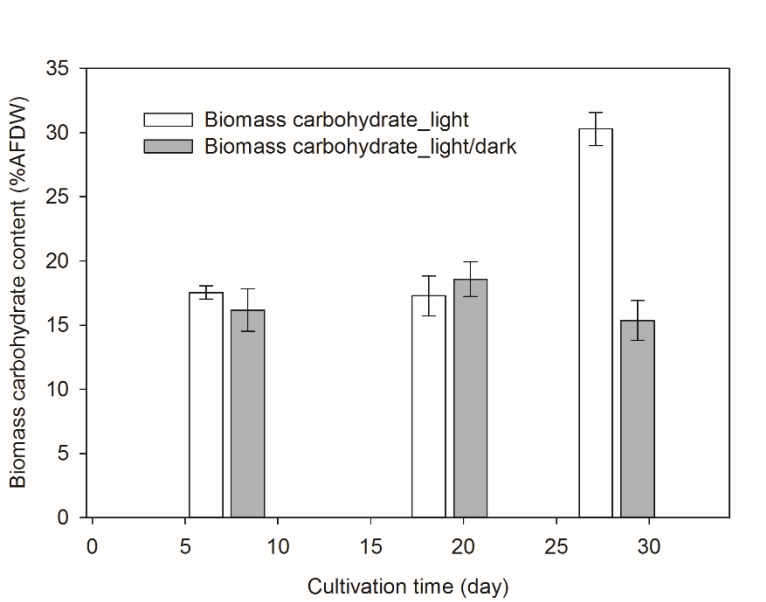

Biomass carbohydrate_light
Biomass carbohydrate_light/dark
Biomass carbohydrate content (%AFDW)
Cultivation time (day)
0
5
10
15
20
25
30
35

Fig. 3.

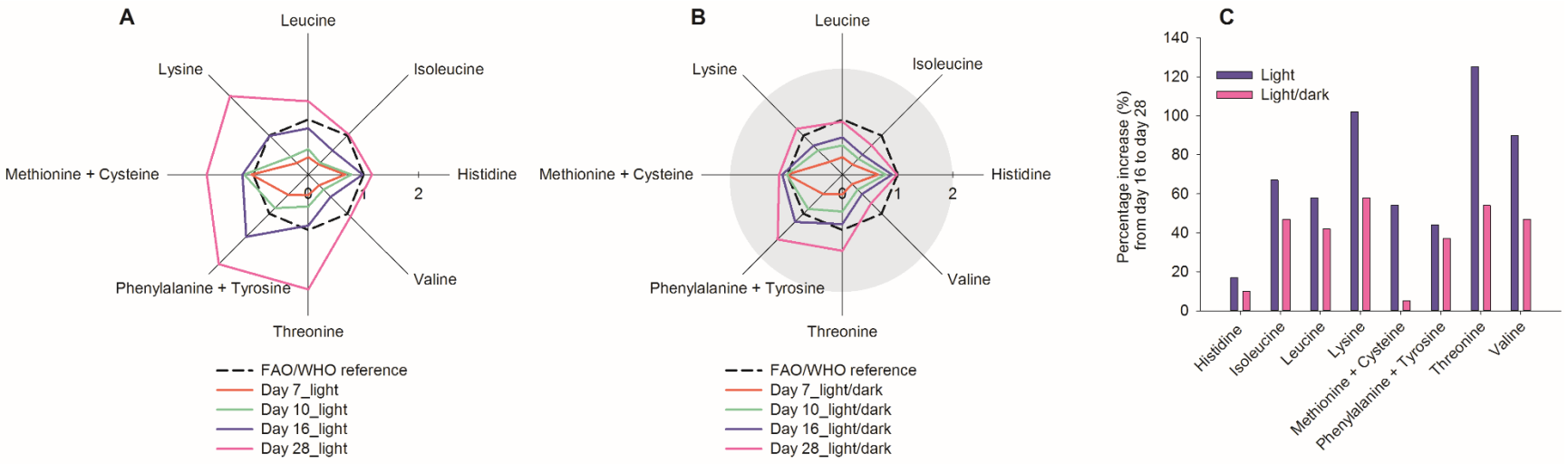

A
Leucine
Isoleucine
Histidine
Valine
Threonine
Phenylalanine + Tyrosine
Methionine + Cysteine
Lysine
0
1
2
FAO/WHO reference
Day 7_light
Day 10_light
Day 16_light
Day 28_light
B
Leucine
Isoleucine
Histidine
Valine
Threonine
Phenylalanine + Tyrosine
Methionine + Cysteine
Lysine
0
1
2
FAO/WHO reference
Day 7_light/dark
Day 10_light/dark
Day 16_light/dark
Day 28_light/dark
C
Percentage increase (%) from day 16 to day 28
140
120
100
80
60
40
20
0
Light
Light/dark
Histidine
Isoleucine
Leucine
Lysine
Methionine + Cysteine
Phenylalanine + Tyrosine
Threonine
Valine

Fig. 4.

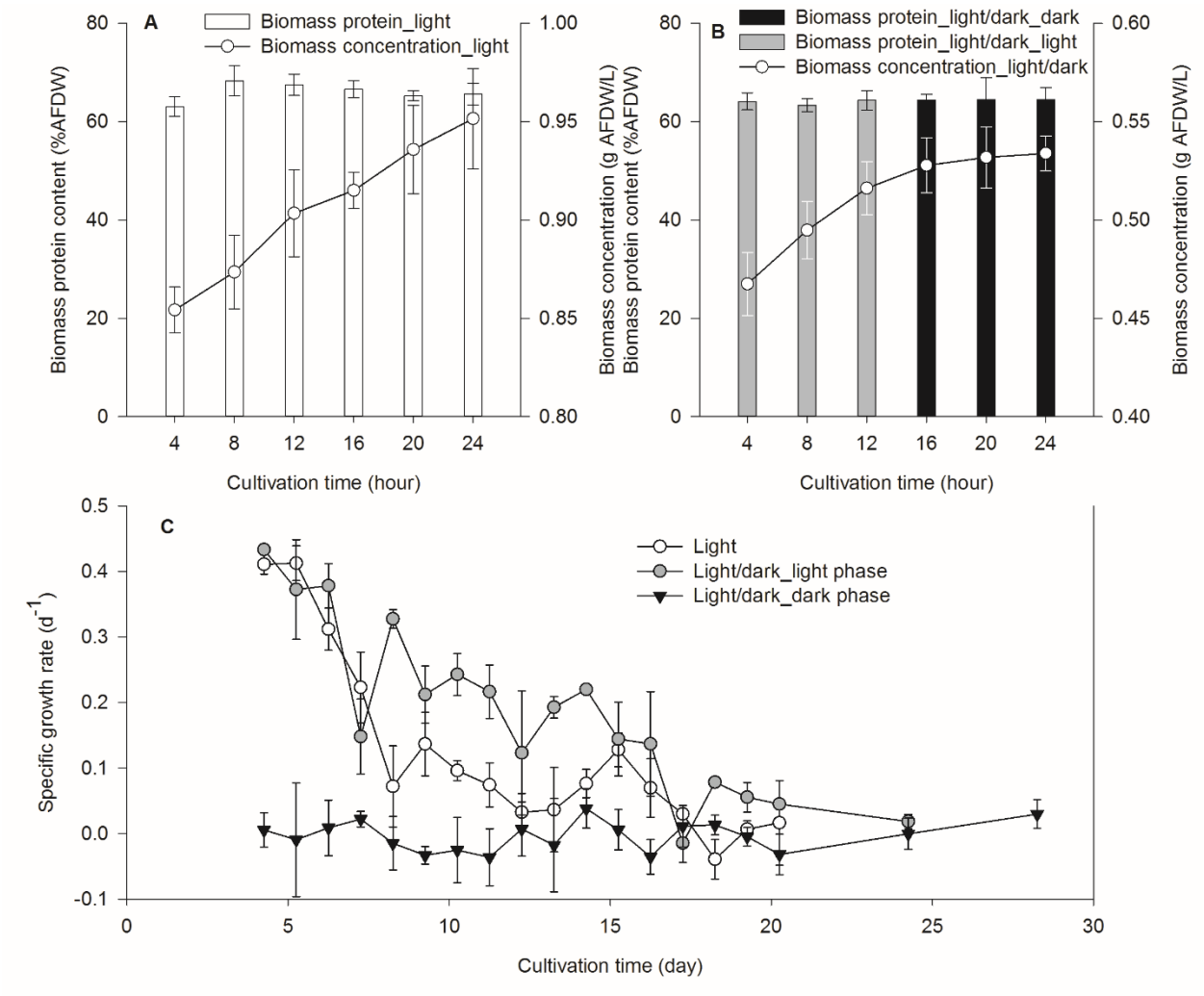
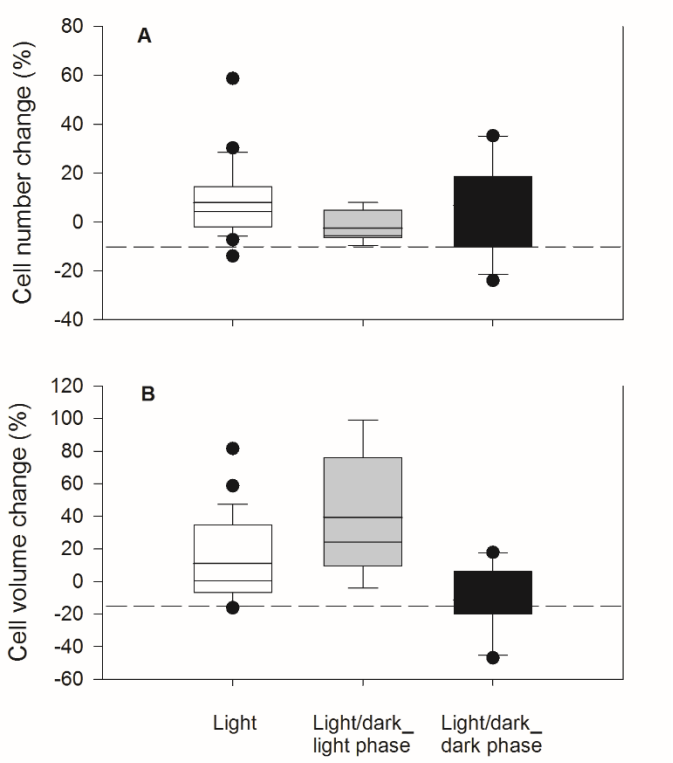

A
Biomass protein_light
Biomass concentration_light
Biomass protein content (%AFDW)
Cultivation time (hour)
B
Biomass protein_light/dark_dark
Biomass protein_light/dark_light
Biomass concentration_light/dark
Biomass concentration (g AFDW/L)
Biomass protein content (%AFDW)
Cultivation time (hour)
C
Light
Light/dark_light phase
Light/dark_dark phase
Specific growth rate (d-1)
Cultivation time (day)

Fig. 5.


A
Cell number change (%)
80
60
40
20
0
-20
-40
B
Cell volume change (%)
120
100
80
60
40
20
0
-20
-40
-60
Light
Light/dark_ light phase
Light/dark_ dark phase