

This item is the archived peer-reviewed author-version of:

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

Reference:

Sui Yixing, Muys Maarten, Vermeir Pieter, D' Adamo Sarah, Vlaeminck Siegfried.- Light regime and growth phase affect the microalgal production of protein quantity and quality with *Dunaliella salina*

Bioresource technology - ISSN 0960-8524 - 275(2019), p. 145-152

Full text (Publisher's DOI): <https://doi.org/10.1016/J.BIORTECH.2018.12.046>

To cite this reference: <https://hdl.handle.net/10067/1559810151162165141>

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

Yixing Sui¹, Maarten Muys¹, Pieter Vermeir², Sarah D'Adamo³ and Siegfried E. Vlaeminck^{1,*}

¹ Research Group of Sustainable Energy, Air and Water Technology, Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerpen, Belgium

² Laboratory of Chemical Analysis, Department of Green Chemistry and Technology, Gent University, Valentin Vaerwyckweg 1, 9000 Gent, Belgium

³ Bioprocess Engineering, Wageningen University & Research, PO Box 16, 6700 AA, Wageningen, The Netherlands

*: Corresponding author: siegfried.vlaeminck@uantwerpen.be

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

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

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

2 Materials and methods

2.1 *Dunaliella* strain and cultivation conditions

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

controlled room at 20°C. Aeration was given by 0.2 µm filtered (Minisart® NML Syringe Filter) air at a rate of 4.17 vvm from air pumps (TetraTech®, APS100). Light was provided by fluorescent tubes (Sylvania F58W/GRO) at the intensity of 55 µmol/m²/s. To provide even light distribution, all flasks were randomized daily. The pH level was corrected daily to 7.5 by 1M NaOH or 1M HCl. Two light regimes were applied, namely 24-h continuous light regime and 12-h/12-h light/dark regime. Each light regime was conducted in triplicate. Samples were collected every 12 hours during the experiment, data from day 4, 7, 10, 13, 16, 19, 24 and 28 were presented in the study. At the linear phase of the microalgal growth, a 24-hour time series analysis was performed for both light regimes. During this 24-hour time series analysis, samples were collected every 4 hours for analyses. All the samples were analyzed freshly for OD₆₈₀ and saved at -20°C for cell number, protein and carbohydrate analyses at the end of the experiment. Cell integrity of stored samples was checked by microscope analysis and cell size distribution, which presented a nice bell-shaped normal distribution similar with well-maintained culture. A neglected/damaged culture will show no pattern of size distribution (Ongena et al., 2010).

2.2 Biomass analyses and calculations

Based on OD₆₈₀, the ash-free dry weight (AFDW) of the biomass was estimated following a calibration curve ($R^2=0.99$) obtained in advance:

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

Presented AFDW data in Fig 1A were from day 0, 4, 7, 10, 13, 16, 19, 24 and 28 with interval of 12 hours.

The maximum specific growth rate was calculated fitting the experimental data to the Gompertz model (Gompertz, 1825) modified by Zwietering et al. (1990) in GraphPad Prisma 5 software:

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

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

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

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

using FAO/WHO established adult indispensable amino acid requirements as reference (Oser, 1959; WHO/FAO/UNU Expert Consultation, 2007). Here, aan and AAn stand for the content of one specific EAA relative to the total protein content (mg EAA/g protein) in the sample and corresponding FAO/WHO reference, respectively. As indicated by EAAI, a value of 1 or above refers to a matching quality, between 0.95 and 1 high quality, between 0.86 and 0.95 good quality, between 0.75 and 0.86 useful, and below 0.75 inadequate (Zhang et al., 2009). As a reference, some conventional food products such as egg and soybean have an EAAI of above 1 (Becker, 2007).

The biomass protein and carbohydrate contents were expressed as fractions of the biomass (%AFDW). The suspension protein and carbohydrate contents (g/L) were the

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

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

where C_t is the biomass or protein concentration on day t (mg/L); V is the volume of the reactor flask (L); L_t is the light input on day t ($\mu\text{mol}/\text{m}^2/\text{s}$) and A is the illuminated surface area (m^2).

The cell number and cell volume changes were calculated as follows:

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

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

2.3 Statistics

The experiment was performed in triplicate with results expressed as means \pm standard deviations in tables and figures. Independent sample t-test in SPSS statistics 24 was used

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

3 Results and discussion

3.1 Impact of light regime and growth phase on biomass level and protein quantity

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

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

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

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

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

These findings overall suggest the importance of adopting the suitable microalgal species, understanding the biochemical pathways, and optimizing cultivation conditions.

Consequently, EAA content and profile of biomass can be improved to a larger extent, presenting high-quality protein for human consumption.

3.3 Microalgal protein content dynamics in one diel cycle

To gain an in-depth knowledge on the behavior of *D. salina* during one diel cycle, a 24-hour time series analysis on day 15 was performed for both light regimes (Fig. 4A, B). As shown in Fig. 4A, during continuous light regime, biomass grew steadily over 24 hours with 11.4% biomass increase at specific growth rate of 0.13 d^{-1} . During the light phase of the light/dark regime, biomass showed 12.9% increase at specific growth rate of 0.31 d^{-1} , both higher than those under continuous light regime (Fig. 4B). Especially the specific growth rate increased substantially by 138%, indicating a much faster growth. This was also observed for most cultivation period, where the light-phase specific growth rate of the light/dark regime presented a higher level than that of the continuous light regime, especially during the exponential phase and early linear phase, roughly between day 8 and day 16 (Fig. 4C). This might also indicate that the light/dark regime is better at maintaining the high specific growth rate than continuous light regime. During the dark phase of the diel cycle, the biomass concentration remained the same level with 1% difference, thus 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

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

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

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

3.4 Optimum lighting and timing for protein quantity and quality

When aiming at optimum high-quality protein production from *D. salina*, productivities of biomass, protein and EAA are important parameters to interpret the overall performance. Besides, their yields on light energy are also essential to estimate their light-usage efficiency, thus energy input. As seen in Fig. 1C, biomass and protein productivities of both light regimes showed increase-decrease patterns. The highest biomass productivity for both light regimes was obtained during the linear growth phase (day 16), 60.6 mg/L/d for continuous light regime and 35.4 mg/L/d for light/dark regime, respectively. The respective 22% and 20% decline towards the stationary phase is mainly due to the halting biomass growth towards the stationary growth phase. For an outdoor raceway pond cultivating *Nannochloropsis gaditana* in Spain, biomass productivity as high as 190 mg/L/d can be achieved high light intensity, temperature and CO₂ enrichment (San Pedro et al., 2015). In accordance, it is foreseen that better lighting and extra inorganic carbon addition can enhance productivities in our cultivation system. The highest protein productivity of both light regimes was achieved during the exponential growth phase: 43.4 mg/L/d on day 10 for continuous light regime and 25.0 mg/L/d on day 7 for light/dark regime, while a respective 59% and 39% reduction was observed towards the stationary phase (Fig. 1C). This is likely due to the higher protein content present in the biomass during the exponential phase (Fig. 1B). A similar trend has been observed for EAA productivity under

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

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

Based on the findings, it is clear that light regime and growth phase play important roles in 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.

Acknowledgements

This work was supported by the China Scholarship Council (File No. 201507650015) and the MIP i-Cleantech Flanders (Milieu-innovatieplatform; Environment innovation platform) project Microbial Nutrients on Demand (MicroNOD). Prof. Ronny Blust and Karin Van den Bergh from Research Group of Systemic Physiological & Ecotoxicological Research (SPHERE) at University of Antwerp are acknowledged for the use of the Coulter counter.

References

1. Alipanah, L., Rohloff, J., Winge, P., Bones, A.M., Brembu, T., 2015. Whole-cell response to nitrogen deprivation in the diatom *Phaeodactylum tricornutum*. *J. Exp. Bot.* 66, 6281–6296.
2. Angell, A.R., Mata, L., de Nys, R., Paul, N.A., 2014. Variation in amino acid content

- and its relationship to nitrogen content and growth rate in *Ulva ohnoi* (Chlorophyta). *J. Phycol.* 50, 216–226.
3. Becker, E.W., 2007. Micro-algae as a source of protein. *Biotechnol. Adv.* 25, 207–210.
 4. Blanken, W., Cuaresma, M., Wijffels, R.H., Janssen, M., 2013. Cultivation of microalgae on artificial light comes at a cost. *Algal Res.* 2, 333–340.
 5. Borowitzka, M.A., 2013. High-value products from microalgae-their development and commercialisation. *J. Appl. Phycol.* 25, 743–756.
 6. Borowitzka, M.A., 1988. Algal growth media and sources of cultures, in: *Micro-Algal Biotechnology*. Cambridge University Press, Cambridge, pp. 456–465.
 7. Cuhel, R.L., Ortner, P.B., Lean, D.R.S., 1984. Night synthesis of protein by algae. *Limnol. Oceanogr.* 29, 731–744.
 8. de Winter, L., Cabanelas, I.T.D., Órfão, A.N., Vaessen, E., Martens, D.E., Wijffels, R.H., Barbosa, M.J., 2017. The influence of day length on circadian rhythms of *Neochloris oleoabundans*. *Algal Res.* 22, 31–38.
 9. de Winter, L., Klok, A.J., Cuaresma Franco, M., Barbosa, M.J., Wijffels, R.H., 2013. The synchronized cell cycle of *Neochloris oleoabundans* and its influence on biomass composition under constant light conditions. *Algal Res.* 2, 313–320.
 10. Dubois, M., Gilles, K. a., Hamilton, J.K., Rebers, P. a., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356.

11. Edmundson, S.J., Huesemann, M.H., 2015. The dark side of algae cultivation: Characterizing night biomass loss in three photosynthetic algae, *Chlorella sorokiniana*, *Nannochloropsis salina* and *Picochlorum* sp. *Algal Res.* 12, 470–476.
12. Fabregas, J., Herrero, C., 1985. Marine microalgae as a potential source of single cell protein (SCP). *Appl. Microbiol. Biotechnol.* 23, 110–113.
13. Galili, G., Amir, R., Fernie, A.R., 2016. The Regulation of Essential Amino Acid Synthesis and Accumulation in Plants. *Annu. Rev. Plant Biol.* 67, 153–178.
14. Gibbs, N., Duffus, C.M., 1976. Natural protoplast *Dunaliella* as a source of protein. *Appl. Environ. Microbiol.* 31, 602–604.
15. Giordano, M., Pezzoni, V., Hell, R., 2000. Strategies for the allocation of resources under sulfur limitation in the green alga *Dunaliella salina*. *Plant Physiol.* 124, 857–864.
16. Gompertz, B., 1825. On the nature of the function expressive of the law of human mortality, and on a new mode of determining the value of life contingencies. *Philos. Trans. R. Soc. London* 115, 513–583.
17. Guerra, L.T., Levitan, O., Frada, M.J., Sun, J.S., Falkowski, P.G., Dismukes, G.C., 2013. Regulatory branch points affecting protein and lipid biosynthesis in the diatom *phaeodactylum tricornutum*. *Biomass and Bioenergy* 59, 306–315.
18. Halsey, K.H., Milligan, A.J., Behrenfeld, M.J., 2011. Linking time-dependent carbon-fixation efficiencies in *Dunaliella Tertiolecta* (Chlorophyceae) to underlying metabolic pathways. *J. Phycol.* 47, 66–76.

19. Han, F., Wang, W., Li, Y., Shen, G., Wan, M., Wang, J., 2013. Changes of biomass, Lipid content and fatty acids composition under a light-dark cyclic culture of *Chlorella pyrenoidosa* in response to different temperature. *Bioresour. Technol.* 132, 182–189.
20. Hidasi, N., Belay, A., 2018. Diurnal variation of various culture and biochemical parameters of *Arthrospira platensis* in large-scale outdoor raceway ponds. *Algal Res.* 29, 121–129.
21. Janssen, M., 2002. Cultivation of microalgae: effect of light/dark cycles on biomass yield. PhD thesis. Wageningen University.
22. Kent, M., Welladsen, H.M., Mangott, A., Li, Y., 2015. Nutritional Evaluation of Australian Microalgae as Potential Human Health Supplements. *PLoS One* 10, e0118985.
23. Kiyota, H., Hirai, M., Ikeuchi, M., 2014. NblA1/A2-Dependent Homeostasis of Amino Acid Pools during Nitrogen Starvation in *Synechocystis* sp. PCC 6803. *Metabolites* 4, 517–531.
24. Lee, S.Y., Kim, S.H., Hyun, S.H., Suh, H.W., Hong, S.J., Cho, B.K., Lee, C.G., Lee, H., Choi, H.K., 2014. Fatty acids and global metabolites profiling of *Dunaliella tertiolecta* by shifting culture conditions to nitrate deficiency and high light at different growth phases. *Process Biochem.* 49, 996–1004.
25. Lv, H., Cui, X., Tan, Z., Jia, S., 2017. Analysis of metabolic responses of *Dunaliella salina* to phosphorus deprivation. *J. Appl. Phycol.* 29, 1251–1260.

26. Lv, H., Qiao, C., Zhong, C., Jia, S., 2018. Metabolic fingerprinting of *Dunaliella salina* cultured under sulfur deprivation conditions. *J. Appl. Phycol.* 30, 355–365.
27. Markwell, M.A.K., Haas, S.M., Bieber, L.L., Tolbert, N.E., 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87, 206–210.
28. Obata, T., Fernie, A.R., 2012. The use of metabolomics to dissect plant responses to abiotic stresses. *Cell. Mol. Life Sci.* 69, 3225–3243.
29. Ogbonna, J.C., Tanaka, H., 1996. Night biomass loss and changes in biochemical composition of cells during light/dark cyclic culture of *Chlorella pyrenoidosa*. *J. Ferment. Bioeng.* 82, 558–564.
30. Ongena, K., Das, C., Smith, J.L., Gil, S., Johnston, G., 2010. Determining Cell Number During Cell Culture using the Scepter Cell Counter. *J. Vis. Exp.* 2204.
31. Oser, B.L., 1959. An Integrated Essential Amino Acid Index for Predicting the Biological Value of Proteins, Protein and Amino Acid Nutrition. Academic Press, Inc.
32. Pancha, I., Chokshi, K., George, B., Ghosh, T., Paliwal, C., Maurya, R., Mishra, S., 2014. Nitrogen stress triggered biochemical and morphological changes in the microalgae *Scenedesmus* sp. CCNM 1077. *Bioresour. Technol.* 156, 146–154.
33. Phenomenex, 2003. Phenomenex EZ:faast amino acid analysis. Phenomenex, 411 Madrid Avenue, Torrance, CA 90501–1430, USA.
34. Piorreck, M., Pohl, P., 1984. Formation of biomass, total protein, chlorophylls, lipids and fatty acids in green and blue-green algae during one growth phase.

- Phytochemistry 23, 217–223.
35. Remmers, I.M., D'Adamo, S., Martens, D.E., de Vos, R.C.H., Mumm, R., America, A.H.P., Cordewener, J.H.G., Bakker, L. V., Peters, S.A., Wijffels, R.H., Lamers, P.P., 2018. Orchestration of transcriptome, proteome and metabolome in the diatom *Phaeodactylum tricornutum* during nitrogen limitation. *Algal Res.* 35, 33–49.
36. Rose, W.C., Haines, W.J., Warner, D.T., 1951. The amino acid requirements of man. III. The role of isoleucine: additional evidence concerning histidine. *J. Biol. Chem.* 193, 605–612.
37. San Pedro, A., González-López, C.V., Ación, F.G., Molina-Grima, E., 2015. Outdoor pilot production of *Nannochloropsis gaditana*: Influence of culture parameters and lipid production rates in raceway ponds. *Algal Res.* 8, 205–213.
38. Sanz-Luque, E., Chamizo-Ampudia, A., Llamas, A., Galvan, A., Fernandez, E., 2015. Understanding nitrate assimilation and its regulation in microalgae. *Front. Plant Sci.* 6, 899.
39. Schmollinger, S., Muhlhaus, T., Boyle, N.R., Blaby, I.K., Casero, D., Mettler, T., Moseley, J.L., Kropat, J., Sommer, F., Strenkert, D., Hemme, D., Pellegrini, M., Grossman, A.R., Stitt, M., Schroda, M., Merchant, S.S., 2014. Nitrogen-Sparing Mechanisms in *Chlamydomonas* Affect the Transcriptome, the Proteome, and Photosynthetic Metabolism. *Plant Cell* 26, 1410–1435.
40. Sui, Y., Vlaeminck, S.E., 2018. Effects of salinity, pH and growth phase on the protein productivity by *Dunaliella salina*. *J. Chem. Technol. Biotechnol.* In press.

41. Sukenik, A., Carmeli, Y., 1990. Lipid synthesis and fatty acid composition in *Nannochloropsis* sp. (Eustigmatophyceae) grown in a light-dark cycle. *J. Phycol.* 26, 463–469.
42. Torzillo, G., Sacchi, A., Materassi, R., Richmond, A., 1991. Effect of temperature on yield and night biomass loss in *Spirulina platensis* grown outdoors in tubular photobioreactors. *J. Appl. Phycol.* 3, 103–109.
43. Uriarte, I., Farías, A., Hawkins, A.J.S., Bayne, B.L., 1993. Cell characteristics and biochemical composition of *Dunaliella primolecta* Butcher conditioned at different concentrations of dissolved nitrogen. *J. Appl. Phycol.* 5, 447–453.
44. Verstraete, W., Clauwaert, P., Vlaeminck, S.E., 2016. Used water and nutrients: Recovery perspectives in a “panta rhei” context. *Bioresour. Technol.* 215, 199–208.
45. Vigani, M., Parisi, C., Rodríguez-Cerezo, E., Barbosa, M.J., Sijtsma, L., Ploeg, M., Enzing, C., 2015. Food and feed products from micro-algae: Market opportunities and challenges for the EU. *Trends Food Sci. Technol.* 42, 81–92.
46. WHO/FAO/UNU Expert Consultation, 2007. Protein and amino acid requirements in human nutrition. *World Health Organ. Tech. Rep. Ser.* 1–265.
47. Xu, Y., Ibrahim, I.M., Harvey, P.J., 2016. The influence of photoperiod and light intensity on the growth and photosynthesis of *Dunaliella salina* (chlorophyta) CCAP 19/30. *Plant Physiol. Biochem.* 106, 305–315.
48. Zhang, T., Chi, Z., Sheng, J., 2009. A highly thermosensitive and permeable mutant of the marine yeast *Cryptococcus aureus* G7a potentially useful for single-cell

protein production and its nutritive components. *Mar. Biotechnol.* 11, 280–286.

49. Zhang, Y., Liu, Y., Cao, X., Gao, P., Liu, X., Wang, X., Zhang, J., Zhou, J., Xue, S., Xu, G., Tian, J., 2016. Free amino acids and small molecular acids profiling of marine microalga *Isochrysis zhangjiangensis* under nitrogen deficiency. *Algal Res.* 13, 207–217.
50. Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Van't Riet, K., 1990. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* 56, 1875–1881.

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 $\mu\text{mol}/\text{m}^2/\text{s}$. Data are expressed as means \pm 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 $\mu\text{mol}/\text{m}^2/\text{s}$. Data are expressed as means \pm 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

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

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

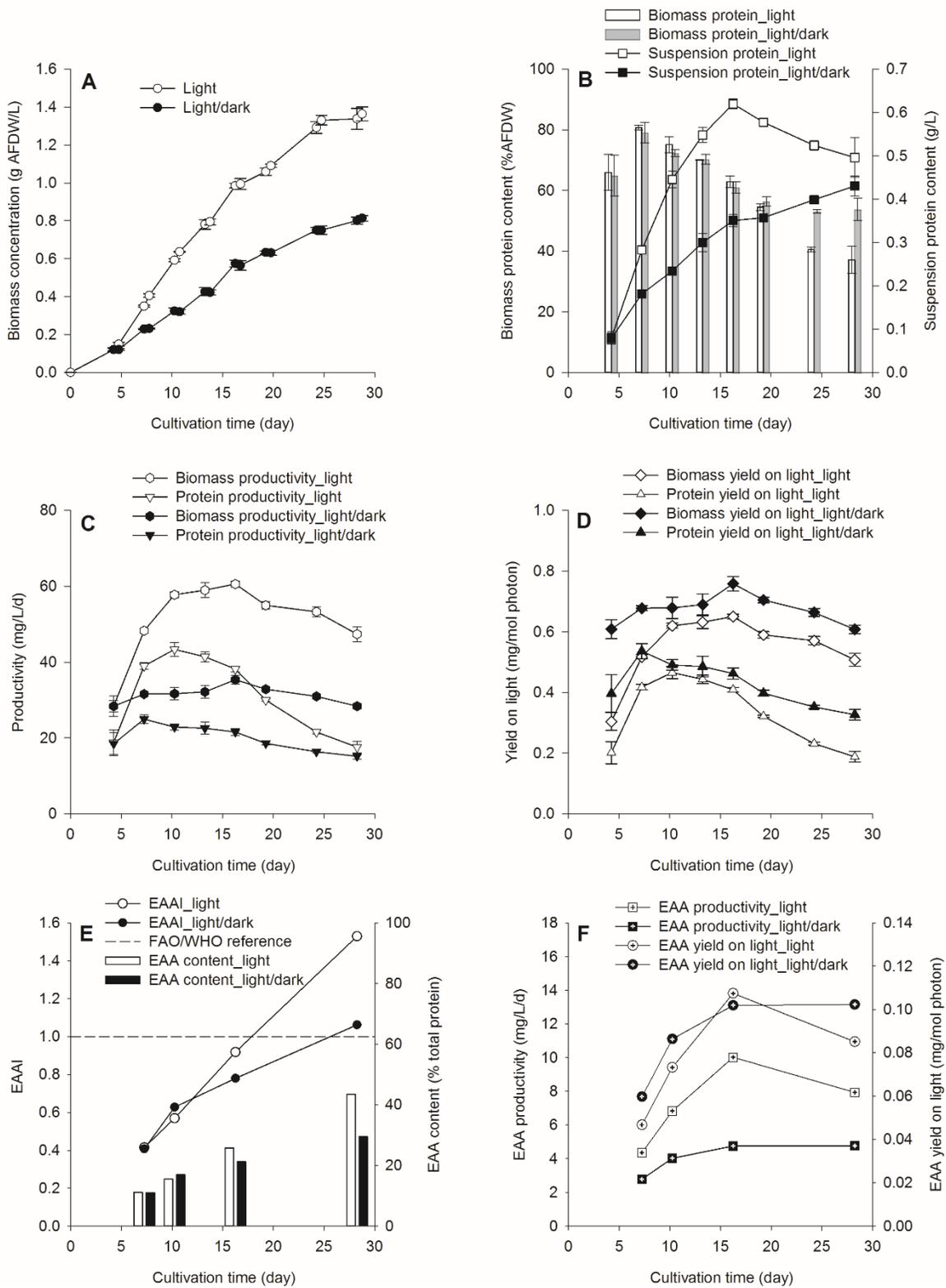


Fig. 2.

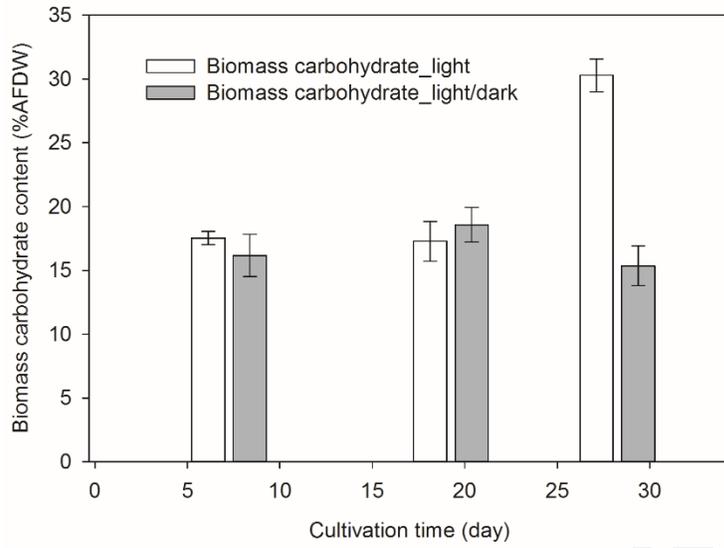


Fig. 3.

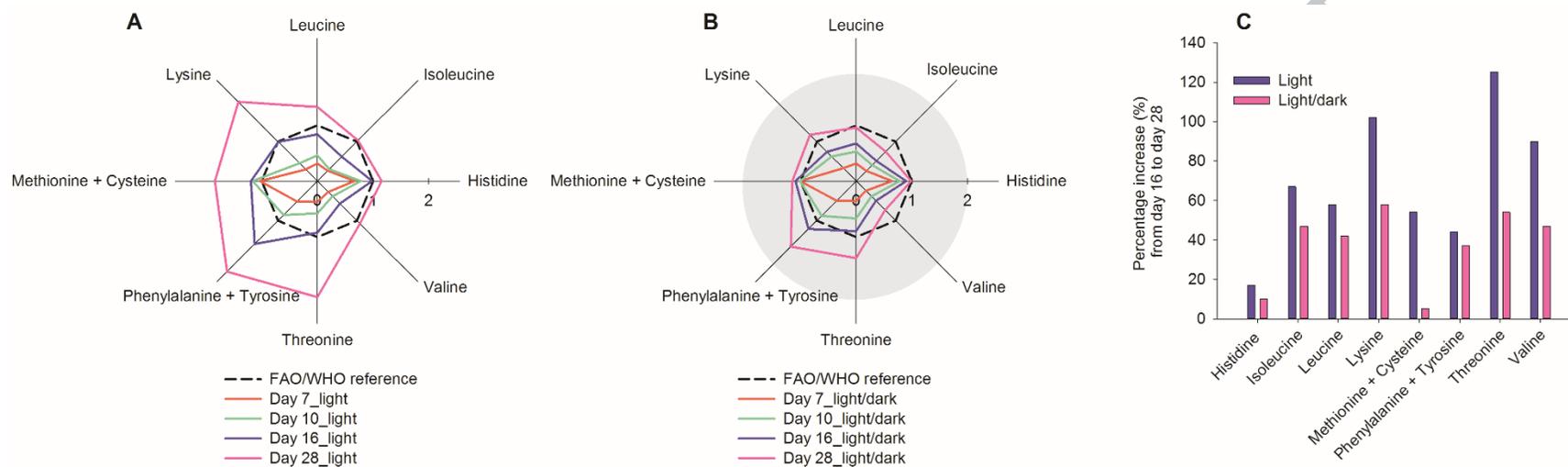
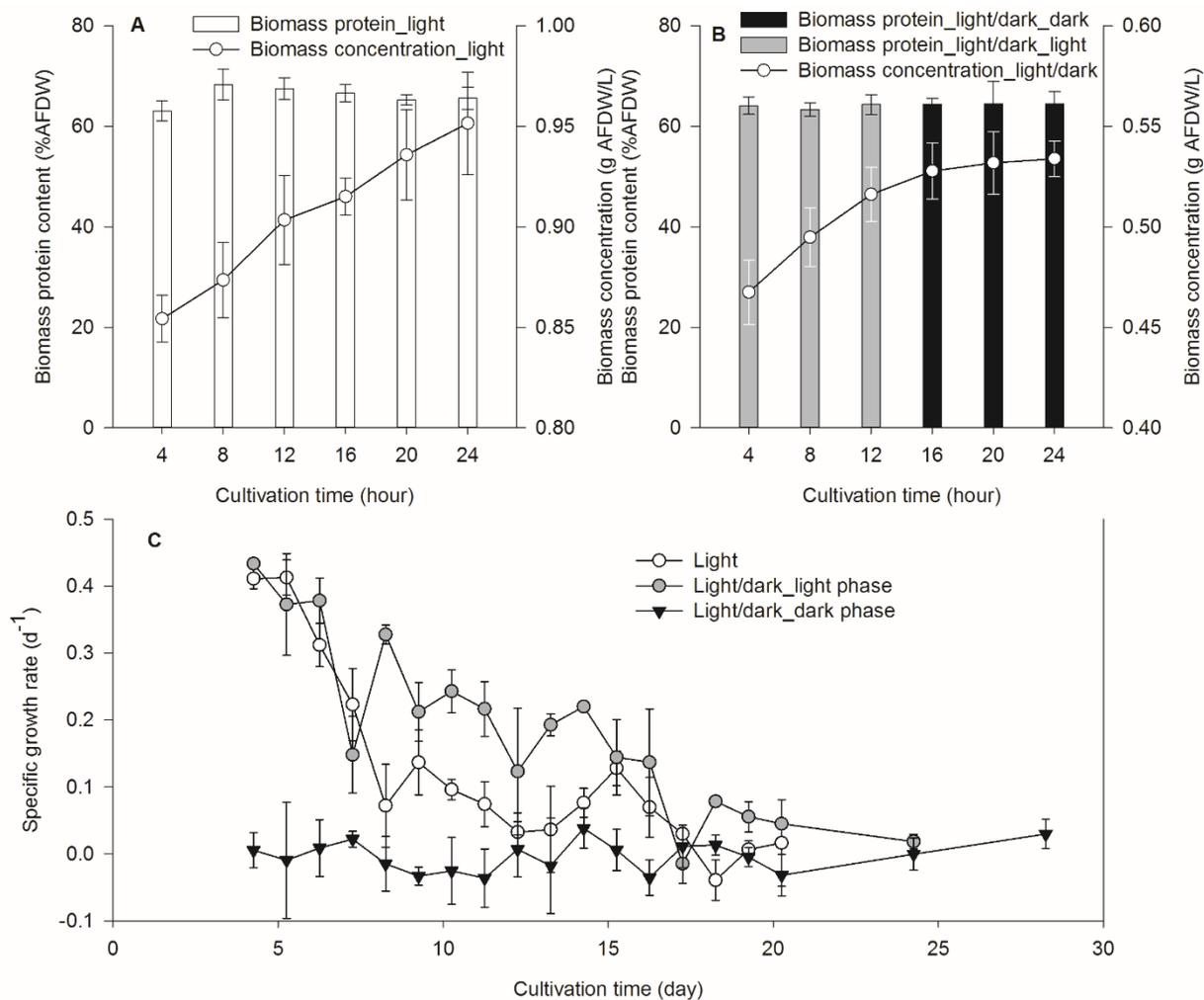
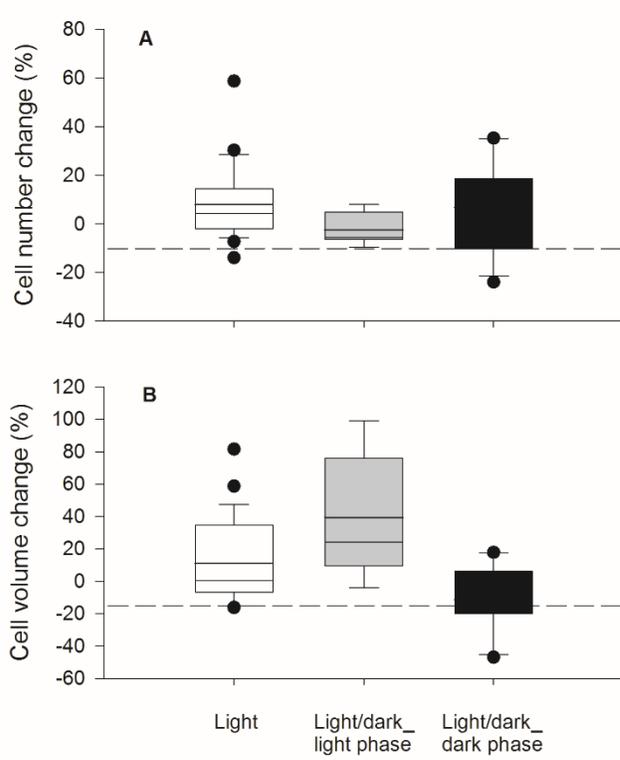


Fig. 4.



ACCE

Fig. 5.



ACCEPTED

MANUSCRIPT

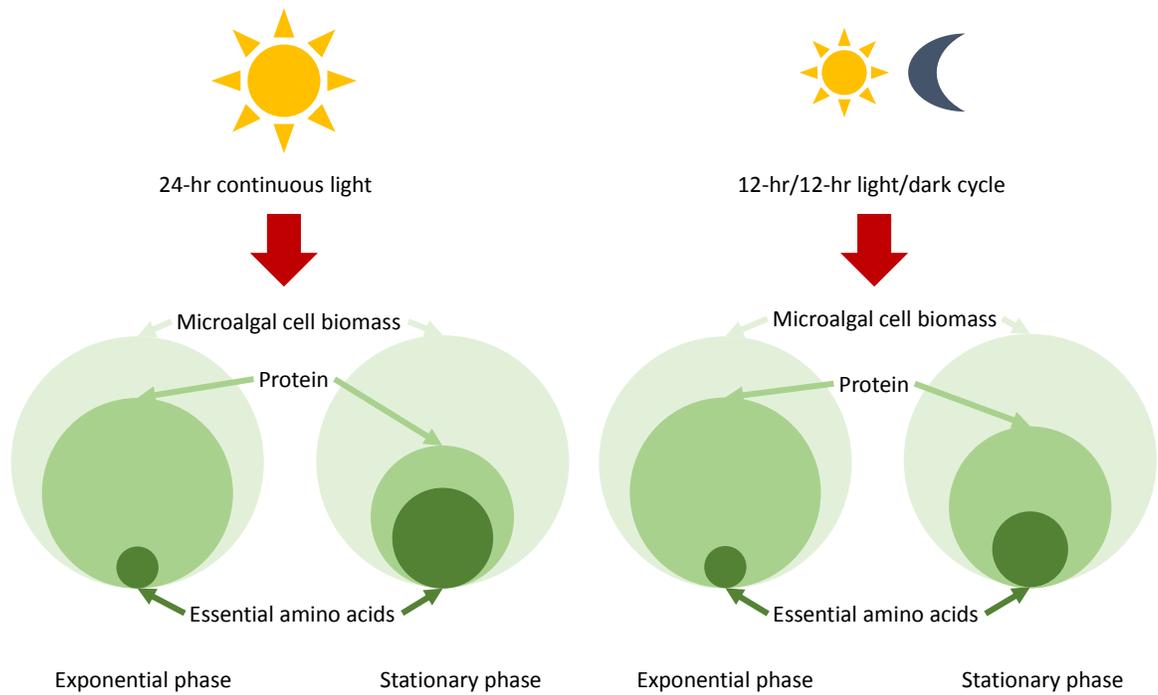
Table 1 Light- and dark-phase biomass concentration and biomass protein content of *Dunaliella salina* in different growth phases under the 12-h/12-h light/dark regime.

	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 ($p > 0.05$)

ACCEPTED MANUSCRIPT

Graphical abstract



ACCEPTED

Highlights

- *D. salina* can produce protein at high quantity and quality for human nutrition
- The essential amino acid index (EAAI) revealed potential for high-quality protein
- EAAI values presented an accumulating trend throughout the growth phases
- The highest EAA values were obtained in the stationary phase
- Light/dark provided a higher light-usage efficiency than continuous light

ACCEPTED MANUSCRIPT