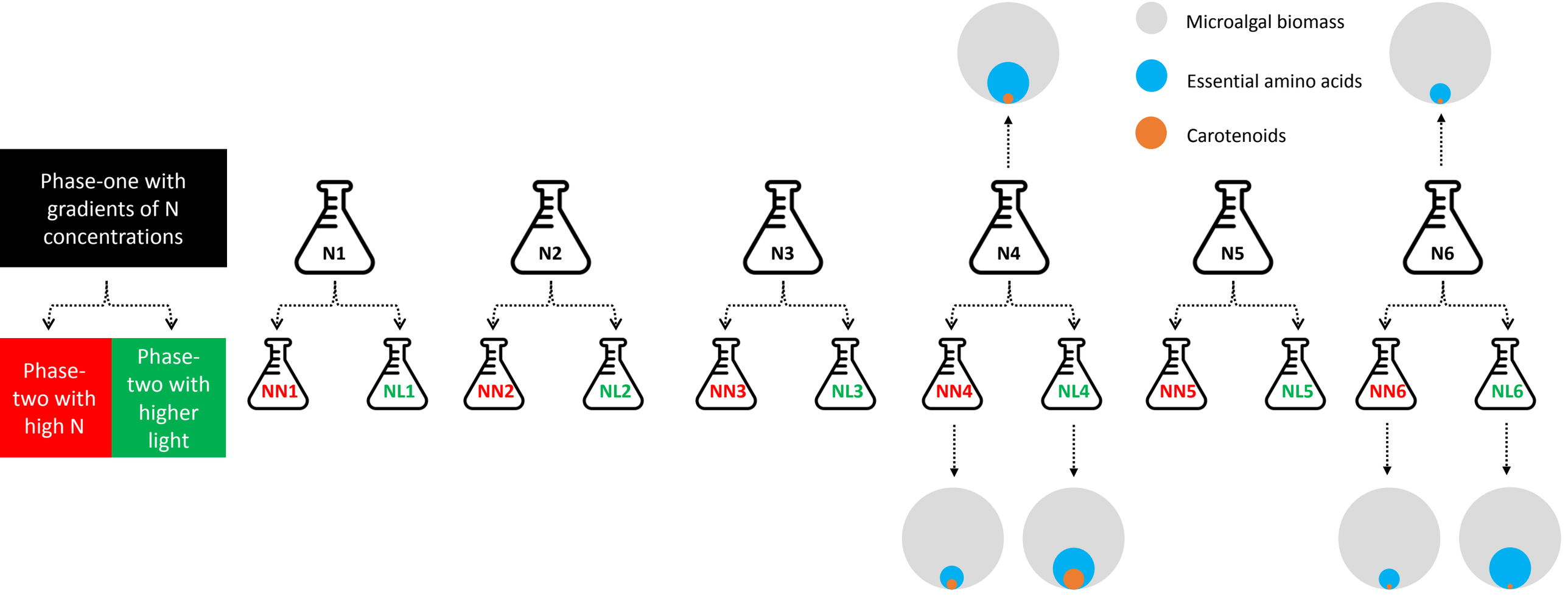
Phase-one with gradients of N concentrations
Phase-two with high N
Phase-two with higher light
N1
N2
N3
N4
N5
N6
NN1
NL1
NN2
NL2
NN3
NL3
NN4
NL4
NN5
NL5
NN6
NL6
Microalgal biomass
Essential amino acids
Carotenoids

Enhancement of co-production of nutritional protein and carotenoids in *Dunaliella salina* using a two-phase cultivation assisted by nitrogen level and light intensity

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Abstract

Microalga *Dunaliella salina* is known for its carotenogenesis. At the same time, it can also produce high-quality protein. The optimal conditions for *D. salina* to co-produce intracellular pools of both compounds, however, are yet unknown. This study investigated a two-phase cultivation strategy to optimize combined high-quality protein and carotenoid production of *D. salina*. In phase-one, a gradient of nitrogen concentrations was tested. In phase-two, effects of nitrogen pulse and high illumination were tested. Results reveal optimized protein quantity, quality (expressed as essential amino acid index EAAI) and carotenoids content in a two-phase cultivation, where short nitrogen starvation in phase-one was followed by high illumination during phase-two. Adopting this strategy, productivities of protein, EAA and carotenoids reached 22, 7 and 3 mg/L/d, respectively, with an EAAI of 1.1. The quality of this biomass surpasses FAO/WHO standard for human nutrition, and the observed level of β-carotene presents high antioxidant pro-vitamin A activity.

Key words

Single-cell protein; pigment; nitrogen limitation; food; microalgae

1. Introduction

The global population will reach 9.3 billion by 2050, with 6.4 billion of people in urban areas (Corcoran et al., 2010). The societal changes of both population and living standard are leading to 50% increase of protein demand, and even 82% and 102% increase of dairy and meat products by 2050, respectively (Boland et al., 2013). Along with protein shortage, the deficiency of functional nutrients in food, like β-carotene, are causing severe health problems for human. Specifically, β-carotene is essential for the human body due to its antioxidant pro-vitamin A activity, and insufficient uptake of β-carotene will lead to severe vitamin A deficiency, prompting human blindness and affecting immune response systems (Sommer, 2001). Currently, β-carotene and vitamin A deficiency have become a major public health concern in more than 70 countries (Sommer, 2001). To sustainably fulfill the protein gap for human consumption, novel protein sources such as microalgae are considered important contributions (Muys et al., 2019). Moreover, microalgae with elevated carotenogenesis can further increase nutritional quality by preventing vitamin A deficiency. Particularly, natural β-carotene found in microalgae, fruits and vegetables has the advantage of its mixed stereoisomers of all-*trans* and 9-*cis* β-carotene, which are more fat-soluble and less crystallizable than synthetic β-carotene (all-*trans* β-carotene) (Ben-Amotz, 1993). By consuming carotenoid-rich diet, potentially lower incidence of various kinds of cancer can be expected (Ben-Amotz, 1993)

Microalgal production is conventionally aiming at one specific target, such as biomass from *Chlorella*, protein from *Spirulina* and β-carotene from *Dunaliella* (Ben-Amotz, 1993).

Consequently, individual production lines are required to achieve production of multiple target compounds. If one microalgal species possesses the ability to optimally co-produce both high-quality protein as well as β-carotene at the same time, the efficiency in production of multiple high-value products can be substantially increased.

It is well established that the microalga *Dunaliella salina* is one of the best sources of natural β-carotene, which can contribute up to 14% of the cell dry weight (Aasen et al., 1969). Up to date, many studies have shown that stress conditions are major factors enhancing the accumulation of β-carotene in *D. salina*, such as high light intensity, high salinity, extreme temperatures and nitrogen (N) deficiency, as a protective mechanism to prevent cellular damages e.g. photo-damage (Lamers et al., 2008; Marín et al., 1998). While carotenogenesis by *D. salina* has been widely studied, its potential as protein source has drawn limited attention. In fact, *D. salina* can display high protein content (up to 80% of ash free dry weight), which is subjected to different cultivation conditions and growth phases (Sui and Vlaeminck, 2019). Furthermore, the essential amino acid (EAA) content of *D. salina* fulfills human requirement as indicated by FAO/WHO reference, which defines its high-quality protein profile (Becker, 2007; Sui et al., 2019). When comparing with other microalgae such as *Chlorella* and *Spirulina*, both protein and EAA content of *D. salina* have either comparable or even superior values (Becker, 2007; Muys et al., 2019; Sui et al., 2019). Based on the characteristics of *D. salina*, it strongly appears to be a valuable candidate as novel food source, containing both high-quality protein and β-carotene.

Based on both lab- and large-scale experience with cultivating *Dunaliella* for β-carotene production, a two-phase cultivation system was proven to be successful, and has been applied by commercial producers (Ben-Amotz, 1995). The concept of such two-phase systems is to increase microalgal biomass level at phase-one with optimum growth conditions, and to induce β-carotene production at phase-two with enhanced stress conditions, e.g. high light intensity and N deficiency (Ben-Amotz, 1995). Moreover, it has been reported that stress conditions can also contribute to the up-regulation of EAA levels in plants and microalgae (Galili et al., 2016; Obata and Fernie, 2012). Specifically, high light intensity and N deficiency have been shown to enhance the production of EAA, especially lysine, threonine, methionine, valine and isoleucine in microalgae (Kiyota et al., 2014; Zhang et al., 2016). For *D. salina*, there is no report on the regulation of EAA affected by cultivation conditions, and the combined production of two main nutritional compounds from *D. salina* has not been studied yet (Sui et al., 2019). Instead of a process targeting either β-carotene or protein production, a two-phase cultivation approach adopting sequential stress conditions may prove to be an effective way to boost both high-quality protein and β-carotene production from *D. salina*.

In this study, the impact of a gradient in N availability together with N pulses and high light intensities on the dynamics of biomass, protein, EAA and β-carotene production in *D. salina* have been explored over different growth phases in a two-phase cultivation approach. This study intends to demonstrate for the first time an optimized cultivation

condition and harvest regime for the maximum production of both EAA and β-carotene of *D. salina*.

2. Materials and methods

2.1 Microalgal strain, two-phase cultivation approach and cultivation conditions

D. salina CCAP 19/18 was purchased from Culture Collection of Algae and Protozoa (CCAP, Scotland, UK). Sterilized Modified Johnson's medium (Borowitzka, 1988) as standard medium for *D. salina* with different N levels was used for cultivation. Six treatments covering a gradient of N concentrations were tested in phase-one (N1 to N6; Table 1). When the algae reached stationary phase, each treatment was divided into two further conditions for phase-two: high N (NN1 to NN6) and high light intensity (NL1 to NL6; Table 1). All treatments were performed in triplicates for both phases. Triplicates in phase-two were derived from the pooled triplicates of corresponding treatments in phase-one. The initial biomass concentration in phase-one was around 40,000 cells/mL. Experiments were conducted in a water bath with a controlled temperature of 25°C. Continuous light was provided by fluorescent tubes at an incident irradiance of 70 µmol photons/m^2/s for standard conditions, and 110 µmol photons/m^2/s for high irradiance conditions (Philips TL-D 30W/33-640, the Netherlands). Mixing and aeration were given by a mixture of pre-humidified air and 2% CO_2. Although pH was not controlled, it was found stable (7.8-8) over the experiment due to the CO_2 addition (Table 1).

2.2 Sample analyses, calculations and statistics

Daily samples from all treatments were analyzed directly for cell number and cell volume, and preserved at -20°C for protein, carotenoids and EAA analyses at the end of the experiment. A Multisizer 3 Coulter Counter was used for both cell number and volume measurement. The protein content was determined following Markwell method, a modified Lowry method with bovine serum albumin as standard (Markwell et al., 1978). Total carotenoids content was measured according to Lichtenthaler, (1987) with 100% acetone extraction:

$$Chlorophyll\ a\ (mg/L) = 11.24 \times OD_{661.6} - 2.04 \times OD_{644.8}$$

$$Chlorophyll\ b\ (mg/L) = 20.13 \times OD_{644.8} - 4.19 \times OD_{661.6}$$

$$Total\ carotenoids\ (mg/L) = \frac{1000 \times OD_{470} - 1.90 \times Chlorophyll\ a - 63.14 \times Chlorophyll\ b}{214}$$

where $OD_{661.6}$, $OD_{644.8}$ and OD_{470} refer to the optical densities of the extracted supernatant measured at 661.6 nm, 644.8 nm and 470 nm, respectively.

To prepare for EAA analysis, samples were centrifuged (5,000 x *g*, 10 min), hydrolyzed (6M HCl, 110°C, 24 hours) with vacuum and evaporated, after which samples were re-dissolving in 0.75mM HCl and stored at -20°C before analysis. For EAA determination, the EZ:faast amino acids analysis procedure was adopted (Phenomenex, 2003), with separation using gas chromatography (Agilent HP 6890, USA) and detection using mass spectrometry (Agilent HP 5973, USA). The essential amino acids index (EAAI) was derived

based on the EAA content with FAO/WHO EAA requirements for human as reference (Oser, 1959; WHO/FAO/UNU Expert Consultation, 2007):

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

where *aan* and *AAn* are the EAA content over total protein content (mg EAA/g protein) in the sample and FAO/WHO reference, respectively. An EAAI value of ≥ 1, 0.95-1, 0.86-0.95, 0.75-0.86 and ≤ 0.75 indicates its superior quality, high quality, good quality, useful quality and inadequate quality, respectively (Kent et al., 2015). For comparison purposes, it has been calculated that the EAAI value of egg and soybean are 1.65 and 1.34 separately (Becker, 2007).

Total protein and carotenoids per liter of culture (mg/L) were defined as suspension protein and carotenoids content. The protein, carotenoid and EAA productivity (mg/L/d) were calculated from their suspension content (mg/L) divided by the time of cultivation (days) at each sampling point.

Samples at the end of the two phases were analyzed for nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) concentrations in the medium. As ammonium concentrations were not detected in any treatment, they were not reported in this study. Filtered samples were diluted with de-ionized water accordingly and a Seal QUAATRO Auto Analyzer (Seal Analytical Inc., the Netherlands) was used for determination following standard methods (APHA, 2012).

158 Multiple regression analysis in SPSS statistics 24 was used to compare data in Fig. 3. A
159 significance level $p < 0.05$ was considered as statistically different. All results were
160 expressed as means ± standard deviations in tables and figures (apart from EAA and EAA
161 derived parameters). The values stated in the main text were without standard deviation
162 for better readability.

163 **3. Results and discussion**

164 3.1 Biomass growth

165 Both the N level and light intensity greatly affected the microalgal growth. During phase-
166 one, the microalgal cells reached different concentrations at stationary growth phase from
167 N1 to N4, ranging from approximately 2×10^5 cells/mL to 2.5×10^6 cells/mL. This was mostly
168 contributed by the differences in initial N concentrations in the medium, which were
169 depleted at the end of phase-one (Table 2). From N4 to N6, the cell densities did not
170 change, despite different initial N concentrations (Fig 1A). Considering that there was still
171 N remaining in the medium after phase-one for N5 and N6, the cells in these treatments
172 were limited by another factor, while for N4 cells may have been co-limited. One likely
173 limiting factor can be phosphorus (P), as the residual P in the medium from N4 to N6 was
174 zero (Table 2). Besides, light limitation could occur as well, which will be discussed in more
175 detail later.

176 During phase-two, both higher N and higher light intensity had effect on biomass growth.
177 Specifically, from NN1 to NN3, cell numbers increased with extra N addition as a result

178 from their N starvation in phase-one. Differently, cell concentration from NN4 to NN6 did
179 not increase, and even slightly decreased after N addition, indicating that N was not the
180 limiting factor. Higher light intensity did not affect the cell concentration in NL1, most
181 likely due to the N scarcity reached during phase-one. For NL2 and NL3, higher light
182 intensity promoted cell growth, although N or P was limiting. It is possible that *D. saline* in
183 NL2 and NL3 might have stored both N and P inside the cell to be used for further growth
184 under higher light intensity (Dortch et al., 1984). From NL4 to NL6, higher light intensity
185 did not affect much or slightly lowered the biomass, which demonstrates that light is not
186 the limiting factor. At this point, P concentration in the medium is expected to be the main
187 limiting factor which prohibited the further growth of cells (Table 2). This finding is in line
188 with previous work, where higher light intensity could not further boost cell densities of
189 *Arthrospira platensis* when P was depleted (Markou et al., 2012).

190 Besides cell densities, also cell sizes were subject to changes in response to the N and NN
191 treatments (Fig. 1). An oscillation pattern was visible, indicating the variations of cell sizes
192 at different growth stages. This pattern might be related to cell division, where cells grew
193 exponentially in the exponential growth phase, and reached their maximum in stationary
194 phase. These findings suggest that microalgal cells start increasing in size until they reach
195 a critical point for cell division, which can be likely affected by external energy supply e.g.
196 light, temperature and nutrients (Zachleder et al., 2016). This explains the less
197 pronounced oscillation in NN5 and NN6 even when extra N was supplied, indicating again
198 that cells were P limited. Nonetheless in this study, higher light intensity does not seem to

result in distinct cell volume changes (Fig 1A). It has been shown that cell oscillation by lighting can be species-specific, where cell volume changes are not restricted to a fixed pattern (Agusti and Kalff, 1989).

3.2 Protein and carotenoids dynamics

During phase-one, all treatments from N1 to N6 showed increases of suspension protein along with cell growth, and the more initial N in the medium, the more suspension protein was reached at the end of phase-one, ranging from 17 mg/L in N1 to 597 mg/L in N6 (Fig 1B). The corresponding cellular protein content also reached the highest level in N6 (233 pg/cell) from N1 (83 pg/cell). Higher protein levels at increased N concentrations in suspension, cell, and biomass have all been reported for various species such as *Dunaliella tertiolecta*, *Scenedesmus* sp. LX1, and *Chlorella* sp. (Fabregas et al., 1989; Kiran et al., 2016; Zhuang et al., 2018), while N starvation is well known to reduce the protein content (Gao et al., 2018).

During the high-N treatments in phase-two, both suspension protein and cellular protein were significantly boosted from NN1 to NN4. Such rises were observed very shortly after N addition and continued until the end of phase-two (Fig 1B). The biggest increase of suspension protein occurred in NN1 with 3460% (from 17 mg/L to 599 mg/L), which was due to the extremely low protein and biomass concentration in N1. The highest suspension protein at the end of phase-two reached 902 mg/L in NN4. For cellular protein, similar results were found with 404% increase in NN1 reaching 419 pg/cell, which is comparable with NN2-NN6. When microalgae are supplied with excess of substrate after a

period of starvation, overcompensating mechanisms can occur, in which cells are triggered to uptake and store higher substrate amounts than necessary (Brown and Shilton, 2014). The boost of protein production in NN1-NN4 can likely be a result of these mechanisms. Another event that can occur when enough N is present in the culture is called “luxury uptake’’, which implies the natural uptake of resources beyond necessity without prior starvation. The increase in protein content of NN5-NN6 could be a result of this mechanism (Brown and Shilton, 2014). Both mechanisms linking to the survival of microalgae are commonly found and thoroughly described (Xie et al., 2017). Due to these two phenomena, protein or N content of many microalgal species such as *Chlorella vulgaris*, and also macroalgal species such as *Oedogonium* could be boosted with N addition (Cole et al., 2015; Dortch et al., 1982; Xie et al., 2017).

During the higher-light treatments in phase-two, however, both suspension and cellular protein level for NL1-NL4 did not substantially change, likely due to N depletion from the previous phase. In NL5 and NL6, however, where N was still present, suspension and cellular protein levels increased by up to 37% and 77%, respectively. Higher protein content induced by higher light intensity has been observed for *Chlorella vulgaris* and *Chlorella pyrenoidosa* (Chen et al., 2015; Ogbonna and Tanaka, 1996). However, other microalgal species could also present either decreased protein content or no change at all following higher light intensity, especially with low N availability (Chen et al., 2015; Markou et al., 2012). Thus, the results from both high-N and higher-light treatments suggest that N levels directly links to the total protein accumulation.

241 The dynamics of carotenoids generally showed the opposite pattern of protein. During
242 phase-one from N1 to N4, both suspension and cellular carotenoids accumulated, with
243 maximum 29 mg/L and 16 pg/cell reached during the stationary phases of N3 and N1,
244 respectively (Fig 1C). This trend is a consequence of N deficiency, which is one of the most
245 effective ways to induce β-carotene accumulation in *D. salina* (Lamers et al., 2012). The
246 cellular carotenoids showed no changes in N5 and N6, although a slight increase of
247 suspension carotenoids occurred, which was mainly due to an increase in biomass (Fig
248 1C). During the higher-N treatment in phase-two, all cellular carotenoids from NN1 to NN6
249 dropped to initial levels, around 4-8 pg/cell. Due to different biomass levels in this phase,
250 the suspension carotenoids either dropped or slightly increased, but remained in a similar
251 range from 9 to 22 pg/cell for all treatments (Fig 1C). During the higher-light treatment in
252 phase-two, both cellular and suspension carotenoids increased rapidly in NL1 to NL4. The
253 increases started shortly after the switch to higher light and continued till the end of the
254 experiment, with maximum 196% cellular carotenoids increase in NL4 and 262%
255 suspension carotenoids increase in NL2 (Fig 1C). Very little changes of carotenoids levels
256 were found in NN5, NL5, NN6 and NL6, primarily due to the presence of N, which suggest
257 N limitation to be the determining factor for effective carotenoids induction. When light
258 intensity is higher than needed for photosynthesis in *D. salina*, β-carotene is produced in
259 excess to overcome light stress and potential photo-oxidative damage, and it has been
260 well documented that high light intensity can significantly increase the production of β-
261 carotene in *D. salina* (Lamers et al., 2010; Raja et al., 2007). This is also one important

reason why large-scale cultivation of *D. salina* is located wherever high light intensity is expected (Ben-Amotz, 1993).

3.3 Protein and carotenoids productivities

In phase-one, protein productivities generally declined (N1 and N3), or rose and declined (N2, N4-N6) towards the stationary phase (Fig 1D). These results showed that higher protein productivity was obtained with high N availability. The highest protein productivities reached were 7, 28, 35, 50, 50 and 49 mg/L/d, for N1 to N6, respectively, and mostly occurred between exponential and linear growth phase. This is in agreement with earlier work on *D. salina* tested at different salinities, pH levels and light regimes (Sui et al., 2019; Sui and Vlaeminck, 2019). For treatments with N starvation at the end of phase-one, namely N1-N4, higher-N treatment in phase-two (NN1-NN4) significantly stimulated their protein productivity by 37-1024%. This again can be linked to N overcompensating mechanism. Differently, for N5 and N6, where N was still abundant after phase-one, protein productivity in phase-two (NN5-NN6) did not show notable trend changes but kept declining (Fig 1D). During the higher-light treatment in phase-two, all protein productivities continued to decrease, as a result from prolonged cultivation time and rather stable protein content (Fig 1B, 1D). Overall, these results show that when *D. salina* cells experience N starvation, extra N addition enhances protein productivity, likely due to overcompensating mechanisms. These findings can be used to optimize the design for harvesting strategy, which could increase biomass protein content. When abundant N is provided, the optimal harvesting point will be around exponential to linear growth

283 phase. This recommendation, however, only applies to maximize protein quantity without
284 considering its quality.

285 Differently from protein productivity, carotenoids productivities showed consistent
286 increase pattern during phase-one from N1 to N4, with a maximum of 3 mg/L/d reached
287 in N3 (Fig 1E). These high carotenoids productivities followed the pattern of their
288 suspension carotenoids contents, which depends on both biomass and cellular
289 carotenoids accumulation (Fig 1C). During higher-light treatment in phase-two, NL1 to NL4
290 all showed significant increases of carotenoids productivity, ranging from 15 to 107%. The
291 highest carotenoids productivity reached was 4 mg/L/d in NL3 (Fig 1E). In contrast, the
292 high-N treatments in phase-two contributed negatively to all carotenoids productivities
293 from NN1 to NN4, which decreased by 5 to 54% (Fig 1E). For the treatments of N5, NN5
294 and NL5, as well as N6, NN6 and NL6, low productivities without evident changes in
295 carotenoids were observed throughout the experiment, which was possibly due to the
296 presence of excess N. Generally, N starvation in *D. salina* enhances the accumulation of
297 carotenoids, which further increases with higher-light exposure. Thus, the combination of
298 N starvation and subsequent exposure to higher light can be a beneficial way to boost
299 carotenoids production.

300 3.4 EAA dynamics and productivities

301 A few samples have been selected for a more detailed analyses of EAA. They were the
302 intermediate treatment on the intersection of N and P limitation (N4) and the highest N
303 treatment (N6) as reference, together with the respective high nitrogen (NN4 and NN6)

304 and light (NL4 and NL6) treatments, both just after the transfer (start) and at the end of
305 the experiment (end). At the end of phase-one, N4 clearly showed higher levels of EAA
306 content, EAA productivity and EAAI relative to N6 (Fig 2A). The EAAI of N4 reached 1.3,
307 which is of superior quality for human consumption (threshold EAAI = 1), while the EAAI of
308 N6 was only 0.4, showing inadequate quality. Looking at individual EAA levels, all the EAA
309 in N4 were substantially present in higher amounts as compared to N6, exceeding
310 FAO/WHO reference except for valine (Fig 2B, 2C and Table 3). These findings indicate that
311 *D. salina* with EAAI of 1.3 is well suited to be incorporated into food which perfectly match
312 human requirement (EAAI = 1), and actually saving 23% of biomass, further increasing the
313 efficiency of food consumption. Protein and amino acid synthesis in microalgae naturally
314 relies on N assimilation pathways, where nitrate is transported inside the cell and
315 converted to nitrite by nitrate reductase. Ammonia is then obtained by further reduction
316 of nitrite and incorporated into glutamate/glutamine via glutamine synthase and NADPH-
317 dependent glutamine:2-oxoglutarate aminotransferase (GS/GOGAT) pathway (Alipanah et
318 al., 2015; Halsey et al., 2011; Remmers et al., 2018; Sanz-Luque et al., 2015).
319 Glutamate/glutamine sequentially provides the critical entry point of N into cellular
320 biochemicals, which can subsequently be used for synthesis of other EAAs (Guerra et al.,
321 2013). Although N availability associates closely with EAA production in microalgae, as
322 shown in this study and many others, a higher N level does not necessarily lead to higher
323 EAA production. When microalgal cells become N limited, mostly towards the stationary
324 growth phase, their major response is to preserve cellular N capacity via scavenging

mechanisms, by which EAA biosynthesis can still occur using intracellular N (Alipanah et al., 2015; Halsey et al., 2011; Lv et al., 2017; Remmers et al., 2018; Zhang et al., 2016). However, when N deprivation occurs in the long term, EAA synthesis is interrupted and results in sharp decreases of protein and amino acids (Kiyota et al., 2014; Van de Waal et al., 2010; Zhang et al., 2016).

Several studies have shown that N limitation or a short period of starvation can boost EAA production in microalgae and macroalgae. For instance, it has been shown that all EAA levels of *D. salina* SAG 184.80 were enhanced towards stationary growth phase, when N became limited and then shortly starved (Sui et al., 2019). *Synechocystis* sp. also exhibited rising levels of all EAA during short N starvation, while longer N starvation resulted in dropping of EAA levels (Kiyota et al., 2014). Similarly, short N starvation contributed to the production of several EAAs, especially phenylalanine, in marine microalga *Isochrysis zhangjiangensis*, which can be the consequence of N scavenging of e.g. nucleotides and rubisco protein. Nevertheless, significant decreased EAA levels were found after long-exposed N deficiency (Zhang et al., 2016). Besides, the macroalgae *Ulva ohnoi* was also shown to exhibit higher proportions of alanine, serine, glycine, and the EAAs phenylalanine, threonine and valine with low N concentration (Angell et al., 2014).

When extra N was supplied during phase-two, EAA content, EAA productivity and EAAI from NN4 (start) decreased sharply, resulting in inadequate protein quality (Fig 2A). A similar drop was also observed in NN6 (start). Regarding individual EAA levels, higher N addition also led to their reductions, where the overall level of EAA decreased

346 dramatically from N4 to NN4 (start and end) (Fig 2B and Table 3). Overall, these results
347 clearly demonstrate that short N starvation promotes EAA accumulation in *D. salina*, while
348 high N levels has a negative impact. At the end of phase-two after N addition, EAA
349 content, EAA productivity, and EAAI in NN4 (end) and NN5 (end) all increased by 14-28%,
350 3-14%, and 35-55%, respectively. This again indicates that cells tend to preserve EAAs
351 towards later growth phases to maintain the N capacity and cell functions, possibly due to
352 the drop of N concentration in the environment (Fig 2A, Table 2).

353 While the complex biosynthesis of EAAs following various pathways are associated with N
354 availability, it is not always easy to link them to other environmental conditions. Here,
355 higher light intensity in phase-two seemed to have differential effects on EAA production.
356 In the higher light treatment, NL4 (start and end) did not respond positively, resulting in
357 slight decreases of EAA content, EAA productivity, and EAAI (Fig 2A). Nonetheless, protein
358 quality of the biomass at this stage still remained superior for human consumption, as
359 indicated by EAAI and relatively high amounts of individual EAAs (Fig 2A, 2B and Table 3).
360 Although *D. salina* biomass seems to maintain protein quality during the higher-light
361 treatment, thus only slight differences between NL4 (start) and NL4 (end) in EAA content,
362 the EAA productivity in NL4 (end) decreased by 46%, which was mainly attributed to
363 growth stage (Fig 2A). In NL6 (start and end), higher light intensity evidently promoted the
364 accumulation of EAA in the biomass with higher EAA content (Fig 2A). Shortly after
365 introducing higher light in NL6 (start), EAA content, EAA productivity and EAAI increased
366 with 49, 25, and 30%, respectively. At the end of phase-two in NL6 (end), such increases

were even 232, 152, and 189%, reaching 36%, 14 mg/L/d and 1.2, respectively. Similarly, the individual EAA levels rose in NL6 from start to end (Fig 2C). The photo-acclimation response of microalgae towards excess light has been well-described, and upregulation of EAA during photo-acclimation was found in both microalgae and plants in response to high light exposure (Davis et al., 2013; Galili et al., 2016). Davis et al., (2013) suggest that the elevation of EAA could be due to an induction of *de novo* amino acid biosynthesis and/or protein catabolism from photodamage. Nevertheless, the fact that EAA content in NL4 (start and end) was not elevated might be related to their extreme N deficiency (Table 2). As stated, when N deprivation and high light were both applied to *Dunaliella tertioecta*, most amino acids were significantly decreased (Lee et al., 2014).

3.5 Optimized biomass with both high-quality protein and carotenoids

As the parameters indicating protein quantity, protein quality and carotenoid content varied substantially depending on the cultivating conditions, Fig 3 and Table 4 elucidate the effects of N and light intensity on the biomass quality of *D. salina*. Nitrogen concentration in the medium and light intensity, but particularly their interaction, significantly affected EAAI (Table 4; Fig 3B, 3C). These findings suggest that short N starvation and higher light intensity together enhance EAA production in *D. salina*, rather than either factor alone. Similar results were estimated for carotenoids content, where highest content was found with a combination of decreasing N and increasing light intensity, while only N starvation and not higher light showed also a significant separate effect (Table 4). It is noted however, that the correlation of light intensity to carotenoids

content was limited by the low sample sizes. When the effect of light intensity on carotenoid contents was analyzed from more samples obtained from this study, it indeed becomes evident that light intensity is correlated to carotenoid contents (Table 4; Fig 3G). Together, these results indicate that short N starvation and subsequent higher light intensity together are favored for carotenoids accumulation.

Overall, short N starvation together with higher light intensity are beneficial for the production of both EAA and carotenoids. Specifically, treatment NL4 (end) presented exceptional EAA enhancement and carotenoids accumulation in *D. salina*, indicating the possibility to produce microalgal biomass with both high protein quality and anti-oxidant strength. Consequently, *D. salina* demonstrated to be a valuable, and potentially unique species as novel protein source with high anti-oxidant activity for humans. Having been successfully applied in commercial production of *D. salina* using two-phase cultivation system, minor modifications can be applied to optimize biomass value, where short N starvation should be reached in the stationary phase in phase-one and a higher-light treatment should be applied in phase-two.

Broadly looking at the market of microalgal products, microalgae are mainly supplied as food supplement with high protein content and other nutritional values like vitamins. Specifically for *D. salina*, the global market is mainly for high-value food and feed supplement. To the authors' knowledge, there is no microalgal product in the food industry which provides both high-quality protein and carotenoids at the same time. The results from this study thus present a step further towards production of microalgae with

multiple high-value compounds, which largely improves the production efficiency. The biomass can therefore be a superior source for food supplement and food ingredient, either consumed next to food sources or be incorporated into food products.

Conclusions

Protein quality and carotenoids content of *D. salina* could be simultaneously enhanced applying a two-phase cultivation strategy. Short N starvation should be reached in the stationary phase in phase-one to upregulate EAA production, and phase-two should be given higher illumination to boost carotenoids production. The optimized cultivation conditions resulted in production of *D. salina* biomass with an EAAI of 1.1 and cellular carotenoids content of 24 pg/cell. This study reveals that *D. salina* is a valuable, and potentially unique species bringing together both high-quality protein and carotenoid, thus can be used as protein source with antioxidant pro-vitamin A effect for humans.

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Figure captions

Fig. 1. Effect of two-phase cultivation on *D. salina*: (A) cell number and cell volume, (B) suspension protein and cellular protein, (C) suspension carotenoids and cellular carotenoids, (D) protein productivity and (E) carotenoids productivity. Different N levels were applied in phase-one and both N addition and higher illumination were applied in phase-two. Cultivation occurred at 25°C and pH 7.8-8. Data are expressed as means ± standard deviation (n = 3)

Fig. 2. Effect of two-phase cultivation on *D. salina* at the end of each phase: (A) EAAI, EAA content and EAA productivity, (B) individual EAA level in treatment N4, NN4 (start and end) and NL4 (start and end) and (C) individual EAA level in treatment N6, NN6 (start and end) and NL6 (start and end). Different N levels were applied in phase-one and both N addition and higher illumination were applied in phase-two. Cultivation occurred at 25°C and pH 7.8-8. Data are expressed as means ± standard deviation (n = 3)

Fig. 3. N and light intensity on EAAI and carotenoids content in ten *D. salina* samples: (A) N and light intensity vs. EAAI, (B) N vs. EAAI, (C) light intensity vs. EAAI, (D) N and light intensity vs. carotenoids content, (E) N vs. carotenoids content and (F) light intensity vs. carotenoids content. Highlighted blue dots represent all conditions where an EAAI above 1 was obtained. The corresponding carotenoids content obtained from above conditions were highlighted as orange dots. Light intensity vs. carotenoids content from 24 *D. salina* samples was presented in (G).

Fig. 1.

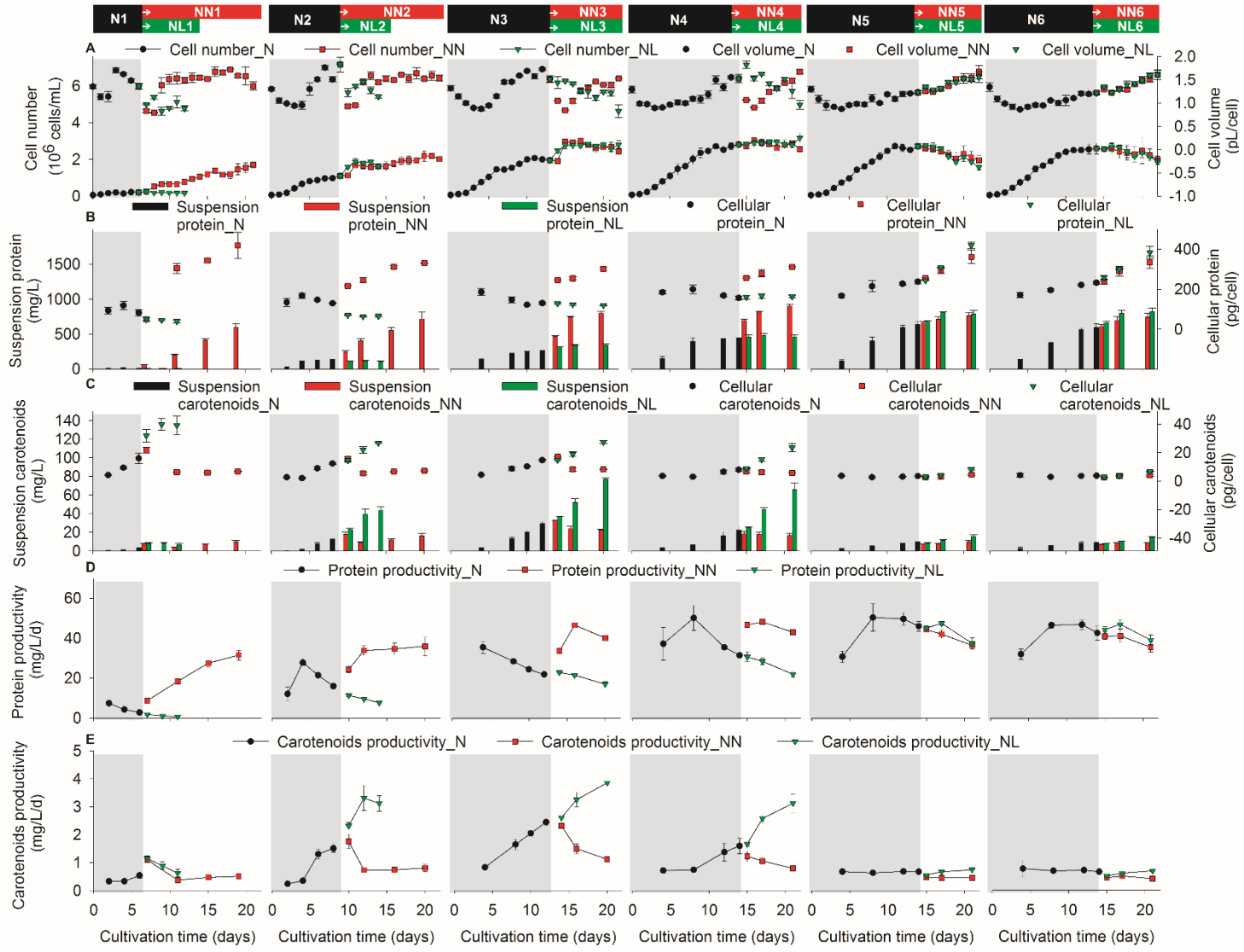
N1 → NN1 → NL1
N2 → NN2 → NL2
N3 → NN3 → NL3
N4 → NN4 → NL4
N5 → NN5 → NL5
N6 → NN6 → NL6
A
Cell number_N
Cell number_NN
Cell number_NL
Cell volume_N
Cell volume_NN
Cell volume_NL
Cell number (10^6 cells/mL)
Cell volume (pL/cell)
B
Suspension protein_N
Suspension protein_NN
Suspension protein_NL
Cellular protein_N
Cellular protein_NN
Cellular protein_NL
Suspension protein (mg/L)
Cellular protein (pg/cell)
C
Suspension carotenoids_N
Suspension carotenoids_NN
Suspension carotenoids_NL
Cellular carotenoids_N
Cellular carotenoids_NN
Cellular carotenoids_NL
Suspension carotenoids (mg/L)
Cellular carotenoids (pg/cell)
D
Protein productivity_N
Protein productivity_NN
Protein productivity_NL
Protein productivity (mg/L/d)
E
Carotenoids productivity_N
Carotenoids productivity_NN
Carotenoids productivity_NL
Carotenoids productivity (mg/L/d)
Cultivation time (days)

Fig. 2.

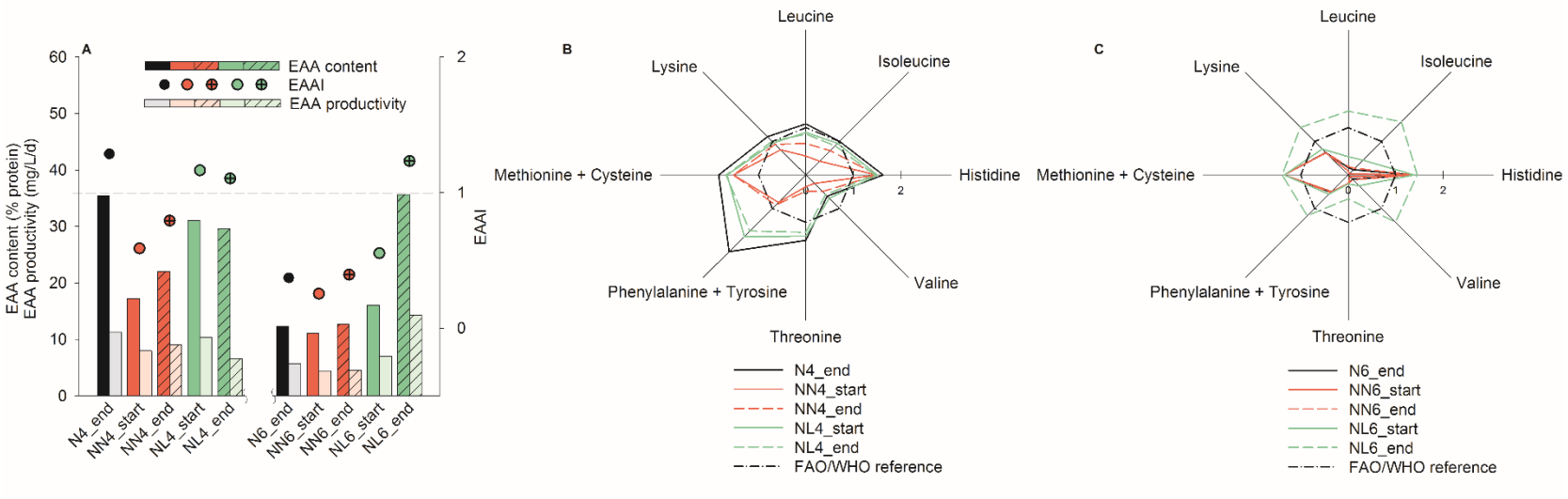
A
EAA content
EAAI
EAA productivity
EAA content (% protein)
EAA productivity (mg/L/d)
EAAI
N4_end
NN4_start
NN4_end
NL4_start
NL4_end
N6_end
NN6_start
NN6_end
NL6_start
NL6_end
B
Leucine
Isoleucine
Histidine
Valine
Threonine
Phenylalanine + Tyrosine
Methionine + Cysteine
Lysine
N4_end
NN4_start
NN4_end
NL4_start
NL4_end
FAO/WHO reference
C
N6_end
NN6_start
NN6_end
NL6_start
NL6_end
FAO/WHO reference

Fig. 3.

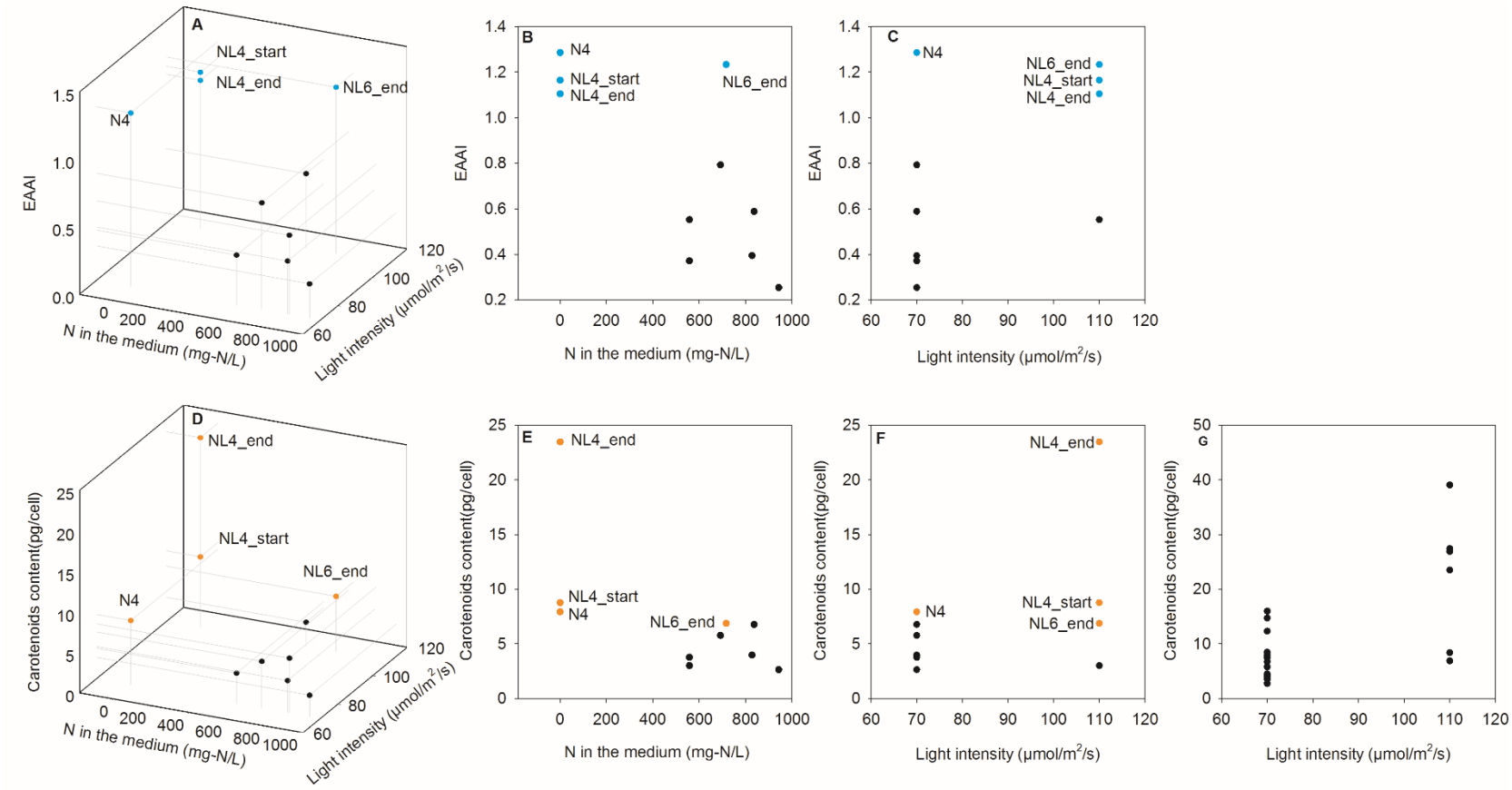
A
NL4_start
NL4_end
NL6_end
N4
EAAI
N in the medium (mg-N/L)
Light intensity (μmol/m2/s)
B
C
D
Carotenoids content(pg/cell)
E
F
G

Table 1 Experimental conditions of all treatments in phase-one and phase-two

Treatment	Phase-one						Phase-two (higher N)	Phase-two (higher light intensity)
	N1	N2	N3	N4	N5	N6	NN1-NN6	NL1-NL6
N concentration (mg-N/L)	1.4	11.2	22.4	44.8	179.2	716.8	932.8	n.a.
Light intensity (µmol/m^2/s)	70							110
Erlenmeyer flask volume (mL)	500						250	
Culture volume (mL)	400						200	
Temperature (°C)	25							
pH	7.8-8							
NaCl concentration (g/L)	117							
Aeration (L/h)	3.3							

n.a. not applicable

Table 2 Nitrate and phosphate concentration in the medium at the end of different treatments

	N1	**NN1**	**NL1**	**N2**	**NN2**	**NL2**	**N3**	**NN3**	**NL3**	**N4**	**NN4**	**NL4**	**N5**	**NN5**	**NL5**	**N6**	**NN6**	**NL6**
NO_3^-	0	1098	0	0	953	0	0	742	0	0	691	0	88	687	44	558	827	716
(mg-N/L)	(0)	(84)	(0)	(0)	(83)	(0)	(0)	(46)	(0)	(0)	(56)	(0)	(5)	(33)	(1)	(61)	(45)	(51)
PO_4^{3-}	7.2	0	7.1	3.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(mg-P/L)	(2.3)	(0)	(0.7)	(0.4)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)

Standard deviation is listed in brackets

Table 3 Individual and total amino acid content of selected treatments and FAO/WHO reference

mg/g protein	Histidine	Isoleucine	Leucine	Lysine	Methionine + Cysteine	Phenylalanine + Tyrosine	Threonine	Valine	Total EAA
N4_end	24.4	30.1	63.7	51.5	40.4	87.0	31.8	24.8	353.7
NN4_start	22.3	12.3	23.5	33.9	33.5	31.1	5.7	9.9	172.3
NN4_end	22.6	21.8	39.2	41.4	33.6	34.2	7.9	19.2	220.0
NL4_start	22.6	28.4	52.9	43.3	36.5	69.8	29.7	27.3	310.4
NL4_end	21.9	26.2	50.8	45.3	37.0	63.6	27.8	22.9	295.6
N6_end	20.7	4.7	9.2	30.2	29.7	19.6	4.2	4.9	123.2
NN6_start	20.7	1.1	4.9	29.8	29.7	18.7	4.1	2.1	111.1
NN6_end	20.7	6.0	10.6	30.1	29.8	19.8	4.0	5.6	126.6
NL6_start	20.9	13.2	22.8	34.8	29.7	21.9	4.4	12.5	160.2
NL6_end	21.8	47.6	79.6	64.0	30.6	46.3	11.6	55.0	356.5
FAO/WHO	15	30	59	45	22	38	23	39	271

Underlined values indicate levels above FAO/WHO reference.

Table 4 Correlation of N and light intensity on EAAI and carotenoids content from statistical results on data from Fig. 3.

Model	R^2	Significance	Corresponding figure
Dependent variable: EAAI Predictors: Nitrogen, Light[1]	0.582	0.047*	Fig. 3A
Dependent variable: EAAI Predictors: Nitrogen[1]	0.534	0.016*	Fig. 3B
Dependent variable: EAAI Predictors: Light[1]	0.421	0.042*	Fig. 3C
Dependent variable: Carotenoids content Predictors: Nitrogen, Light[1]	0.461	0.115	Fig. 3D
Dependent variable: Carotenoids content Predictors: Nitrogen[1]	0.426	0.041*	Fig. 3E
Dependent variable: Carotenoids content Predictors: Light[1]	0.211	0.182	Fig. 3F
Dependent variable: Carotenoids content Predictors: Light[2]	0.511	0.000*	Fig. 3G

*: significant difference ($p < 0.05$)

[1]: statistical analyses using 10 samples with EAA contents determined in this study.

[2]: statistical analysis using 24 samples with carotenoids content determined in this study