Modulated stress to balance *Nannochloropsis oculata* growth and eicosapentaenoic acid production

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Key points

- Temperature stress (10 ºC) presented the highest impact increasing EPA content 158%
- Lower light intensity stress was able to increase EPA to 126% more
- EPA increased in individual cell contents simultaneous with biomass increase

Abstract

Two environmental parameters, temperature and light intensity, were independently used as stress modulators to enhance eicosapentaenoic acid (EPA) production by the microalga *Nannochloropsis oculata*, without hindering biomass production. A sinusoidal approach was used, as environmental conditions were alternated between optimum and stress status in multi-day cycles. Low temperatures (5 and 10 ºC) and light intensities (30 and 50 µmol photons/m^2/s) were tested. Results revealed that the modulated stress approach used was able to avoid decreases in biomass production. Temperature stress (10 ºC) presented the highest impact, increasing EPA content to 12.8 mg_{EPA}/L, 158% more than the amount obtained in optimum (non-modulated) growth conditions at that point in time, while the lower light intensity stress was able to increase to 126% more. It is important to point out that in both cases increases in EPA amounts resulted from increased content in each individual cell and not just from increased biomass contents.

Keywords: Abiotic factors, microalgae, temperature, light intensity, omega-3 polyunsaturated fatty acids

Introduction

Microalgae have long been known to be a source of several bioactive compounds, which confers them the potential to be used in several distinct applications: they can be directly applied as single cells in animal feed and human nutrition, or used as sources of compounds for such different industries as health products and biodiesel. Those compounds include, among others, carbohydrates, vitamins, proteins, pigments and lipids (de Souza et al. 2019).

Polyunsaturated fatty acids (PUFA), and more specifically, omega-3 polyunsaturated fatty acids (n3-PUFA), are some of the important lipids found in several microalgal species (Ma et al. 2016b; Santos-Sánchez et al. 2016; Paliwal et al. 2017). Among them, eicosapentaenoic acid (EPA, C20:5 n3) and docosahexaenoic acid (DHA, C22:6 n3) have been shown to present beneficial effects on human health, such as positive effects on brain development and vision health, depression, dementia, anti-inflammatory activity, and prevention of cardiovascular diseases (Ma et al., 2016; Peltomaa et al., 2018).

Richness in EPA, particularly important in cardiovascular protection, is an important trait of *Nannochloropsis* strains (Zanella and Vianello 2020). Indeed, *N. oculata* was found to present an EPA content of 49% of total fatty acids (Martins et al. 2013). Furthermore, this microalga is also rich in proteins, pigments and other PUFA, among other bioactive compounds, supporting its use in feed and food applications (Sukarni et al. 2014; Zanella and Vianello 2020).

Several environmental conditions (physical and chemical parameters) can impact microalgae metabolism and, consequently, alter its cellular composition. Chemical parameters include nutrient deficiency, salinity, minerals deficiency and pH (Chen et al. 2011; Solovchenko 2012; Paliwal et al. 2017), whereas physical parameters can include light intensity, light quality and temperature (Kurpan Nogueira et al. 2015; Sirisuk et al. 2018). All of these abiotic parameters have already been extensively manipulated in order to increase biomass production and/or enhance production of selected metabolites, namely, lipids (Paliwal et al. 2017; Alishah Aratboni et al. 2019). Indeed, PUFA, and EPA in particular, have been the subject of several studies in which environmental factors were modulated to enhance their production by microalgae, specifically *Nannochloropsis* sp. Low salinity has been found to increase EPA contents in *N. oculata* (Gu et al. 2012)

and *N. oceanica* (Chen et al. 2013; Pal et al. 2013), whereas Manisali et al. (2019) were able to increase phospholipids (which together with glycolipids are the main lipid classes that contain EPA and other PUFA) content by cultivating *N. oculata* in a phosphate-rich medium. Light intensity has also been explored, and low light intensities were reported to relate with higher EPA and other PUFA contents (Ma et al., 2016; Mitra et al., 2015b; Xiao et al., 2015). As EPA, among other PUFA, are major components of thylakoid membranes, their increase can be related to an increase in total thylakoid membranes (Mitra et al. 2015). Schulze et al. (2016) showed that light quality can also impact fatty acids profile, with LED405 originating the highest EPA and other PUFA contents in *N. oculata*, whereas UV-C radiation was able to produce a two-fold increase in total EPA content in *Nannochloropsis* sp. (Sharma and Schenk 2015). Low temperature promotes a decrease in cell membrane fluidity, leading microalgae to react by increasing EPA and PUFA amounts in membranes, aiming to increase fluidity (Aussant et al. 2018; Willette et al. 2018; Gachelin et al. 2021; Huang and Cheung 2021). Combinations of stresses such as salinity, high light and nitrogen starvation, have also been explored with positive results (Solovchenko et al. 2014), although their manipulation and control is a more complex procedure.

However, when stresses are applied, microalgae metabolism shifts to an increased production of lipids and growth metabolism usually slows down, leading to lower growth rates (Chen et al. 2011; Paliwal et al. 2017; Yuan et al. 2019; Poh et al. 2020). To overcome this problem, a mechanism/strategy often employed is a two-stage approach, which consists in initially increasing biomass by providing the microalgae with optimum growth conditions and, after a significant amount of biomass is achieved, start a second stage, in which stress conditions are applied so that the metabolic pathways are shifted to increase lipid production, or accumulation (Ho et al. 2014; Mitra et al. 2015). Despite its

wide application in industry, this approach is time consuming, which indirectly leads to economic losses.

Taking into account the previous considerations, the aim of this research work was to use two independent environmental factors (temperature and light intensity) to induce a modulated stress in *N. oculata* cultures during cell growth, in order to increase the amount of EPA produced without significantly decreasing biomass productivity. This would result in faster production of EPA, as when the growth phase finished, the cells would already have increased EPA content.

Temperature and light intensity were selected as variable factors, since manipulation and modulation of such conditions are simple, allowing for variations throughout growth to be easily performed, not requiring manipulation of the cultures and monitorization of chemical parameters. Biomass growth, EPA content and fatty acids profile were assessed.

Materials and methods

Microalga and growth conditions

Nannochloropsis oculata CCAP 849/1 was originated from Culture Collection of Algae and Protozoa (CCAP; SAMS Ltd., Scottish Marine Institute, Oban, Scotland, United Kingdom) and grown in modified artificial seawater medium (ASW) (Darley and Volcani 1969).

During experimental work, microalga was grown in 250 mL Erlenmeyer flasks, with 100 mL working volume. Inoculum was prepared at initial cell concentration of ca. 6-8.5×10^6 cell/mL, with cells in exponential growth phase. The culture was then incubated according to the temperature or light intensity conditions being tested (Fig. 1a-d and Fig. 3a-d, respectively). At each sampling point, before the environmental condition was changed, three flasks were collected and analyzed independently.

Stress modulation

Stress modulation was performed by changing the environmental condition to be tested (temperature or light intensity) throughout the growth curve in multi-day cycles (Fig. 1); for each environmental condition tested/changed the other was kept at optimum value. Both environmental conditions were tested in ranges between optimal and below-optimal values. A culture grown at optimum conditions (25 ºC and 75 µmol photons/m^2/s) was used as control.

Throughout the text, cultures/curves pertaining to temperature experiments were identified with the prefix T- and the ones related to light intensity with L-, for a better understanding.

Growth monitoring

Biomass

Biomass was assessed by cell numbers (Neubauer improved chamber, 0.1 mm depth, Hirschmann, Darmstadt, Germany) and ash-free dry weight (AFDW). For the latter, pre-weighed and pre-combusted glass-GF/C Whatman filters were utilized to filter growth culture, thereby retaining the biomass, which was then (together with the filter) washed with ammonium bicarbonate 0.5 M (Sigma-Aldrich, St. Louis, MO, USA) before combusting at 550 ºC, for 6 hours.

EPA and fatty acid profiles

EPA and remaining fatty acid profiles were determined by gas chromatography after previous derivatization. Biomass was freeze-dried before transesterification, which was performed according to the method described by Fontes et al. (2018), with slight modifications. Briefly, 50 mg of freeze-dried biomass were added to 200 µL of glyceryl

tritridecanoate (1.5 mg/mL) and 800 µL of hexane (Hxn). Then, 2.26 mL of methanol (MeOH) and 240 µL of sodium methoxide (5.4M) in MeOH were added, and the mixture was heated at 80 ºC for 10 min. After a rapid cooling (ice), 1.25 mL of dimethylformamide and 1.25 mL of sulphuric acid (3M) in MeOH were added, the mixture was vortexed, heated at 60 ºC for 30 min, and cooled again with ice. Finally, 1 mL of Hxn was added to the mixture, which was vortexed and centrifuged at 1,250 *g* and 18 ºC, for 5 min. The upper layer, containing the fatty acid methyl esters (FAME), was collected.

FAME were analyzed in a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector and a BPX70 capillary column (60m x 0.25 mm x 0.25 µm, SGE Europe, Courtaboeuf, France). Analysis conditions were as follows: injector (split 25:1) and detector temperatures were 250 ºC and 275 ºC, respectively; carrier gas was hydrogen (20.5 psi) and the oven temperature program started at 60 ºC (hold 5 min), raised 15 ºC/min to 165 ºC (hold 1 min), and finally 2 ºC/min to 225 ºC (hold 2 min). Supelco 37 (Sigma-Aldrich, St. Louis, MO, USA) and CRM-164 (Fedelco, Madrid, Spain) were used for identification of fatty acids. GLC-Nestlé36 (Elysian, Minnesota, USA) was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79 ng FA/mL; LOQ: 2.64 ng FA/mL).

Statistical analysis

Statistical analyses were performed using SPSS 20 software (SPSS, Chicago, IL, USA). When all assumptions were met, analysis of variance (ANOVA), with Tukey as post-hoc, was used to compare samples. When assumptions were not met, non-parametric Kruskal-Wallis and Mann-Whitney tests were used as substitute for ANOVA and for pair-wise comparisons, respectively. All tests were performed with 0.05 significance.

Results

Manipulation of environmental conditions in order to increase production of a specific metabolite is being used in microalgae production for a long time. Altering an abiotic factor can induce a stress which leads to a response reflected as a change in microalgal metabolism and, consequently, microalgal composition. Since cell growth is usually hindered when subjected to stress, in this study we explored an alternative approach, i.e. the possibility of subjecting *N. oculata* to modulated stress conditions throughout growth, in order to increase the production of n3-PUFA, more specifically EPA, without compromising cell growth.

Temperature

Temperature is known to be an environmental factor that has an impact in microalgae metabolism, and more specifically, in lipid metabolism. A decrease in temperature can lead to membrane rearrangement, as an adaptive response to maintain membrane fluidity, which results in increased amounts of unsaturated fatty acids, namely, PUFA (Solovchenko 2012; Paliwal et al. 2017; Gachelin et al. 2021; Huang and Cheung 2021).

Nannochloropsis oculata culture was grown under optimum temperature (25 °C) – control (Fig. 1a) – and under stress modulation, achieved by changing the temperature throughout growth, at different time points (Fig. 1b-d), always keeping light intensity constant (75 µmol photons/m^2/s). The temperatures used to stress the culture were 5 and 10 °C. In T-Str1 growth was extended to 19 days, as the last stress cycle ended at day 16 and growth at optimum temperature was the last step in all cultures (Fig. 1b).

Biomass

225 Regarding cell number (Fig. 1e), a sharp increase was registered when the second stress
226 was applied to T-Str2 and T-Str3 cultures (day 9), when compared with T-Str1 (day 11)
227 and T-Ctr; at the end of this stress cycle Str2 and T-Str3 cultures presented cell numbers
228 45% and 54% higher than T-Ctrl, respectively, and 54 and 41% higher than T-Str1, at
229 similar time points. As temperature was raised back to 25 °C growth rates slowed down,
230 yet by the end of the experiment (day 16) T-Str3 cell numbers ($1.16x10^8$ cells/mL)
231 remained the highest. Since T-Str1 underwent a further 3 days growth (19 days), it
232 registered the highest cell numbers ($1.21x10^8$ cells/mL); however, there was no
233 significant difference ($p > 0.05$) between the values observed in T-Str1 at 19 days and T-
234 Str3 at 16 days, pointing out that equivalent results could be obtained in a shorter period
235 of time.

236 Concerning AFDW, in T-Ctrl and T-Str1 (Fig. 1f) the behaviors previously
237 observed in cell numbers were mostly repeated, although with some changes, namely the
238 impact of the stress temperatures on T-Str1, noticed by the decrease in growth rate after
239 temperature decreases. However, the increase in growth rate registered after the second
240 stress, when temperature was raised to 25 °C, allowed to reach, at the end of growth,
241 similar values to those obtained in T-Ctrl ($p > 0.05$). Concerning T-Str2 and T-Str3, a
242 relatively constant increase in AFDW was observed, registering similar values between
243 cultures, and no relevant alterations being observed as result of exposure to stresses.
244

245 **EPA and fatty acids profiles**

246 Analysis of EPA contents was, as with biomass, presented in distinct parameters (fg/cell,
247 mg/g_{AFDW} and mg/L) since each one conveyed a different type of information about the
248 physiology of the culture and the cells: amount *per* cell (fg/cell) provides insights on
249 individual cell metabolism, while amount *per* weight (mg/g_{AFDW}) translates the

250 composition of the culture. Amount *per* volume (mg/L) is the most important expression
251 parameter concerning industry, since it provides total amount of compound that is
252 obtained in a specific reactor, although without information about how it was achieved,
253 i.e. if it is resultant from few cells with high EPA content, or originated by a high number
254 of cells with low EPA content.

255 Results pertaining to EPA at different growth temperatures are presented in Fig.
256 1g-i and show that temperature stress cycles had significant impact on EPA contents.
257 Culture growing at optimum temperature (T-Ctrl) increased EPA contents within the first
258 three days, and although it underwent some fluctuations onwards, at the end of the growth
259 experiment contents remained relatively stable, except for EPA content expressed as
260 mg/L (Fig. 1i) which, as a consequence of the biomass increase, also (generally) increased
261 with time. Concerning the T-Str experiments, temperature was able to significantly
262 increase EPA contents in all stressed cultures during the first stress cycle, although to a
263 lower extent in T-Str1. As such, at the end of the second stress cycle, EPA contents inT-
264 Str2 and T-Str3 were considerably higher than in T-Ctrl, and the extension of the second
265 stress for one more day in the case of T-Str3, in comparison with T-Str2, allowed to obtain
266 the highest contents, i.e. 46.9 mg_{EPA}/g_{AFDW} and 12.8 mg_{EPA}/L (Fig. 1h and i), 227 and
267 158%, respectively, higher than in T-Ctrl (Fig. 2b and c). Regarding fg_{EPA}/cell (Fig. 1g),
268 it was slightly lower than the registered after the first stress (161 and 166 fg_{EPA}/cell,
269 respectively), and represented a 122% increase, in comparison with T-Ctrl. At the end of
270 growth T-Str1 showed equivalent mg/g_{AFDW} and mg/L contents to T-Ctrl ($p > 0.05$) and,
271 in terms of fg/cell, T-Str1 EPA content was considerably lower ($p < 0.05$) than that of T-
272 Ctrl (68 and 105 fg_{EPA}/cell, respectively).

273 Fatty acids profiles throughout growth of the culture in which best results were
274 obtained (T-Str3) are presented in Table1, and showed that the most prevalent fatty acids

all throughout were C16:0 (palmitic acid), C16:1 c9 (palmitoleic acid) and C20:5 n3 (EPA), composing ca. 78% of total fatty acid content (maintained throughout growth).

Fatty acids profiles also showed that, when exposed to stress, cultures increased EPA and PUFA contents, while amounts of SFA decreased. This behavior can be observed as PUFA percentages are the highest at the end of the stress cycles (days 6 and 13), while SFA are the lowest. When stresses ceased and temperature was restored to optimum values, opposite behavior was observed.

Light intensity

Light intensity is also an environmental factor with considerable impact on microalgae cellular growth and metabolism, and several studies report an inverse relation between EPA and light irradiation (Paliwal et al., 2017).

As in temperature studies, a control culture was grown under optimum conditions (75 µmol photons/m^2/s) for 19 days (late exponential) (Fig. 3a). Cultures were stressed by decreasing light intensity for some period of time, at different time points, after which it was restored to optimum value. Two different light intensities were used, namely, 30 and 50 µmol photons/m^2/s, two stress cycles were performed, and growth at optimum conditions was the last step in all cultures (Fig. 3b-d). Equivalent to when temperature was used as stress factor, L-Str1 culture growth was extended to 19 days since the second stress cycle ended at 16 days.

Biomass

Regarding cell numbers (Fig. 3e) and as opposite to temperature stress, when only the growth in T-Str2 and T-Str3 curves was markedly superior to the one observed in T-Ctr and T-Str1, light intensity lowered the growth rates of the cultures exposed to stress. In

300 detail, it can be observed that while L-Str2 and L-Str3 showed decreased rates during
301 exposure to stress, followed by increases when optimum condition was reestablished,
302 allowing to reach similar values to L-Ctrl, L-Str1 presented values consistently lower.
303 However, as growth in L-Ctrl decreased in the final stages of the growth curve, at the end
304 of all growth curves no significant differences were observed as all presented similar
305 values, between 9.4 and 9.9x10^7 cells/mL.

306 Concerning AFDW (Fig. 3f), reduction of light intensity only impacted growth
307 (negatively) in L-Str1, as this curve was the one with lower values. Nevertheless, L-Str1
308 achieved at the end of the growth curve (19 days), similar values than L-Ctrl, (342 and
309 376 mg_{AFDW}/L, respectively). Regarding L-Str2 and L-Str3, AFDW steadily increased,
310 not showing differences ($p > 0.05$) between the cultures, and no significant variations in
311 growth rates when stresses were applied.

312

313 **EPA and fatty acids profiles**

314 Result presented in Fig. 3g-i showed that light stresses did have an impact in L-Str2 and
315 L-Str3 cultures, in which stronger responses to light intensity variations were registered:
316 the first light intensity stress (day 3) promoted intracellular increases in EPA, whereas the
317 second light stress (day 9) originated a slight decrease. When light intensity was again
318 raised to 75 µmol photons/s/m^2, a sharp increase was observed in both fg_{EPA}/cell and
319 mg_{EPA}/g_{AFDW} (Fig. 2g and h, respectively), resulting in the highest EPA contents. At the
320 end of the growth curve L-Str2 registered 140 fg_{EPA}/cell and 36.7 mg_{EPA}/g_{AFDW}, while L-
321 Str3 (which was subjected to one more day at stress conditions), reported 146 fg_{EPA}/cell
322 and 39.6 mg_{EPA}/g_{AFDW}, representing 134 and 68% increases comparatively with L-Ctrl
323 (Fig. 4a and b); again, both fg_{EPA}/cell values were not significantly different ($p > 0.05$).

Overall EPA contents (mg/L) (Fig. 3i), increased throughout growth in all cultures, and the highest contents were registered in L-Str2 and L-Str3 (14.0 and 15.0 mg_{EPA}/L, respectively ($p < 0.05$)). While L-Ctrl and L-Str1 cultures increased their contents as result of biomass increase, L-Str2 and L-Str3 also increased the contents of each individual cell, which resulted in a final concentration of over two times the amount registered in L-Ctrl culture (6.7 mg_{EPA}/L at 15 and 16 days). Indeed, the increase in L-Str3, represented 126%, in comparison with L-Ctrl (Fig. 4c).

Concerning fatty acids profiles throughout growth, results pertaining to L-Str3, which was the culture in which the best results (regarding EPA) were obtained, are presented in Table 2. Similarly to the found concerning temperature, culture was mainly composed of C16:0, C16:1 c9 and C20:5 n3, which represented c.a. 79% of total fatty acids. Composition of fatty acid fraction did not present relevant alterations as, despite variations in contents, percentages of individual fatty acids were similar throughout growth.

Discussion

Concerning growth temperature, biomass results revealed that modulated temperature stress did not significantly interfere with cell growth, as no considerable differences were found between T-Ctrl and the different T-Str curves at the end of the growth cycles, with an exception: in fact, the decrease in the stress extension period (from 5 to 3 or 4 d) that occurred in experiments T-Str1, T-Str2 and T-Str3, respectively, seems to promote an increase in cell numbers, not followed by concomitant increases in AFDW, suggesting that the latest stress conditions may enhance cell proliferation, although of smaller sized cells. Such reduction in cells size could be the result of alterations in cell composition.

348 The abovementioned results are not in accordance with several studies, which
349 document a decrease in growth rate (and concomitant lower biomass content) when
350 *Nannochloropsis* culture is exposed to low temperatures: this behavior was reported for
351 *N. gaditana* (Camacho-Rodríguez et al., 2015), *N. salina* (Hoffmann et al., 2010; Van
352 Wagenen et al., 2012) and *N. oculata* (Aussant et al., 2018; Converti et al., 2009).
353 However, the apparent discrepancy can be explained by the modulation hereby employed:
354 herein the temperature was not kept constant throughout growth, and the consecutive
355 cycles of temperature shift may have prevented the usually observed decline in biomass.
356 Moreover, we hypothesize that the previous exposure of the cells to the first stress may
357 have adapted cell (growth) metabolism to the lower temperature applied at that point
358 (namely, 10 ºC), which resulted in, when cells were later exposed to such temperature, an
359 increased growth rate, in comparison with Ctrl (which in that growth stage also increased
360 growth rate, although to a lower extent). Willette et al. (2018) also used reduced
361 temperature during sinusoidal temperature (12:12 h) and light (14:10 h) regimes and
362 concluded that losses in biomass productivity are lower when using sinusoidal
363 temperature control as compared with constant reduced cultivation temperature;
364 nevertheless, cold stress affects growth after 24h, with concomitant effects on biomass
365 productivity, that reached a 27.4% reduction at 5 ºC (when compared with growth at 25
366 ºC).

367 Fatty acids and EPA profiles are in accordance with the reported in literature for
368 *Nannochloropsis oculata* (Gu et al., 2012; X. N. Ma et al., 2016; Shene et al., 2016).
369 Regarding the stresses, the lower impact of the stresses in T-Str1 (when compared with
370 T-Str2 and T-Str3) could have been the result of the prolonged exposure to the stress
371 during the first stress cycle, as the culture was submitted to 10 °C for 5 consecutive days,
372 while its counterparts were only submitted to the corresponding stress for 3 days;

regarding the second stress cycle, in which no impact was observed, this could have also resulted from the temperature being too low, as temperature was lowered to 5 °C, besides the prolonged exposure. Willette et al. (2018) found that at temperatures lower than 25 °C EPA contents (mg/g_{AFDW}) were increased in *N. salina*, except at 5 °C, which presented similar content (31.68 and 29.96 mg_{EPA}/g_{AFDW} at 25 and 5 °C, respectively). Similarly, Huang and Cheung (2021) decreased *Porphyridium cruentum* culture temperature to 0 °C also for a period of 5 days, and found maintenance of EPA amount throughout. The fluctuations registered in T-Str2 and T-Str3 may be understood as a cellular response to temperature, with metabolism being shifted to production of EPA and PUFA when temperature decreased, to increase membrane fluidity (Aussant et al. 2018; Willette et al. 2018; Gachelin et al. 2021; Huang and Cheung 2021), and shifting back to the previous metabolic processes when temperature was restored to its optimum.

Based on results expressed as mg_{EPA}/L, which is the most important parameter for industry analysis and decision making, when EPA would be the goal, when temperature stresses were used as the mechanism to increase EPA content, the appropriate moment to collect biomass and proceed with the extraction process would be after the second stress cycle in T-Str3, as content was the highest.

Concerning light intensity as stress factor, the variations (decreases) observed in cell numbers when light intensity was decreased, allied with the fact that similar behaviors were not observed in AFDW, could be indicative of changes in cell composition.

Mitra et al. (2015b), studying *Nannochloropsis* sp. registered a slight decrease in biomass productivity when light intensity decreased from 100 to 30 µmol photons/m^2/s, while Simionato et al. (2011), varying light intensities from 5 to 2100 µE/m^2/s, only found lower growth rates of *N. gaditana* at 5, 1200 and 2100 µE/m^2/s. Therefore, our findings are in agreement with the results obtained in other studies, as when light intensities decreased,

biomass growth rates decreased or showed no relevant variation (compared to L-Ctrl growth rates), depending on the parameter. The fact that L-Str1 presented the highest decreases could indicate that exposures to the stresses were too long, as discussed for temperature stresses. The fact that different behaviors were registered when different parameters were concerned (e.g. cell numbers and AFDW) was also observed in the case of Ma et al. (2016) study, in which different increased percentages were observed when the parameter assessed was cell numbers or dry weight.

Regarding light intensity as the stress factor, an important issue to refer is that when the second time light stress was applied (light intensity decreased to 30 µmol photons/m^2/s), no relevant variations in EPA and fatty acids contents were registered. This could have resulted from the cells not being completely adapted to 75 µmol photons/m^2/s, as the culture had only been at optimum light intensity for 3 days, after being submitted to stress. When the second stress was applied cellular metabolism could have been in a "transient" state, and therefore not able to respond as it did to the first stress; such behaviour would be in accordance with Borowitzka (2018), that stated that cellular response is dependent of the physiological state of the microalgae. When the first stress was applied, although cultures were only growing for 3 days, inocula had been growing at the same conditions and, as such, metabolism would have been unchanged when growth curves were initiated.

From an industrial perspective, wishing to obtain the highest possible amounts of a determined metabolite, modulated light intensity stress was able to increase the final content of EPA by 126%. However, in this case, and unlike temperature stress, in which highest contents were achieved just after the second stress (day 12-13), it is necessary to extend the growth another 3 days to achieve the highest EPA content, as there are significant differences ($p < 0.05$) between its values at days 12-13 and day 16 (11.7 and

15.1 mg_{EPA}/L, respectively). As such, when comparing both stresses modulations, considering potential industrial application, it would be up to the interested industry to calculate the costs of each stress modulation, in order to determine which would be less expensive.

In conclusion, modulation of environmental stress through temperature or light intensity impacted cellular metabolism, resulting in increased production of EPA by *N. oculata*. Each environmental stress presented a different impact on biomass and EPA contents. Nevertheless, both stresses were able to, at the appropriate conditions, increase EPA content without significantly hindering biomass production. Noticeably, by alternating between optimum conditions and decreasing temperature to 10 °C, or light intensity to 30 µmol photons/s/m^2, during short periods throughout growth, final amounts of EPA *per* volume (L) obtained were 126% (concerning light intensity) or 158% (concerning temperature) higher, in comparison to non-modulated growth conditions, at the same time periods.

Disclosures and Declarations

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Funding This work was funded by National Funds through project Norte-01-0145-FEDER-000011-RL1–QUALIFOOD, FCT (Fundação para a Ciência e a Tecnologia) through projects UID/Multi/50016/2019 and SFRH/BD/105304/2014 and by FCT/MEC (PIDDAC) project reference IF/00588/2015.

448

449 **Conflict of interest** Sérgio Sousa declares that he has no conflict of interest. Ana C.
450 Freitas declares that she has no conflict of interest. Ana M. Gomes declares that she has
451 no conflict of interest. Ana P. Carvalho declares that she has no conflict of interest.

452

453 **Ethical approval** This article does not contain any studies with human participants or
454 animals performed by any of the authors.

455

456 **Authors' contributions** SS, ACF, AMG and APC conceived and designed the work.
457 SS conducted the experiments, analyzed data and prepared the original draft. ACF, AMG
458 and APC reviewed and edited the manuscript, acquired funding and resources, and
459 supervised the work.

460

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Figure captions

Fig. 1 Temperature profiles (a-d), biomass variation (e – cell numbers; f – AFDW) and evolution of EPA production (g – fg_{EPA}/cell; h – mg_{EPA}/g_{AFDW}; i – mg_{EPA}/L) throughout growth. Control (T-Ctrl – ●), stress 1 (T-Str1 –○), stress 2 (T-Str2 – □) and stress 3 (T-Str3 – Δ) (open symbols represent the points at which samples were analyzed, before the condition was changed)

Fig. 2 Percentual variation (Δ%) between EPA production in T-Str3 and T-Ctrl ($\Delta = (EPA_{T\text{-}Str3}/EPA_{T\text{-}Ctrl}) \times 100$) (a – fg_{EPA}/cell; b – mg_{EPA}/g_{AFDW}; c – mg_{EPA}/L) throughout growth. Dotted line (•••) indicates temperature.

598 **Fig. 3** Light intensity profiles (a-d), biomass variation (e – cell numbers; f – AFDW) and
599 evolution of EPA production (g – fg_{EPA}/cell; h – mg_{EPA}/g_{AFDW}; i – mg_{EPA}/L) throughout
600 growth. Control (L-Ctrl – ●), stress 1 (L-Str1 –○), stress 2 (L-Str2 – □) and stress 3 (L-
601 Str3 – Δ) (open symbols represent the points at which samples were analyzed, before the
602 condition was changed)
603
604 **Fig. 4** Percentual variation (Δ%) between EPA production in L-Str3 and L-Ctrl (Δ =
605 ($EPA_{L\text{-}Str3}/EPA_{L\text{-}Ctrl}$) x 100) (a – fg_{EPA}/cell; b – mg_{EPA}/g_{AFDW}; c – mg_{EPA}/L) throughout
606 growth. Dotted line (···) indicates light intensity
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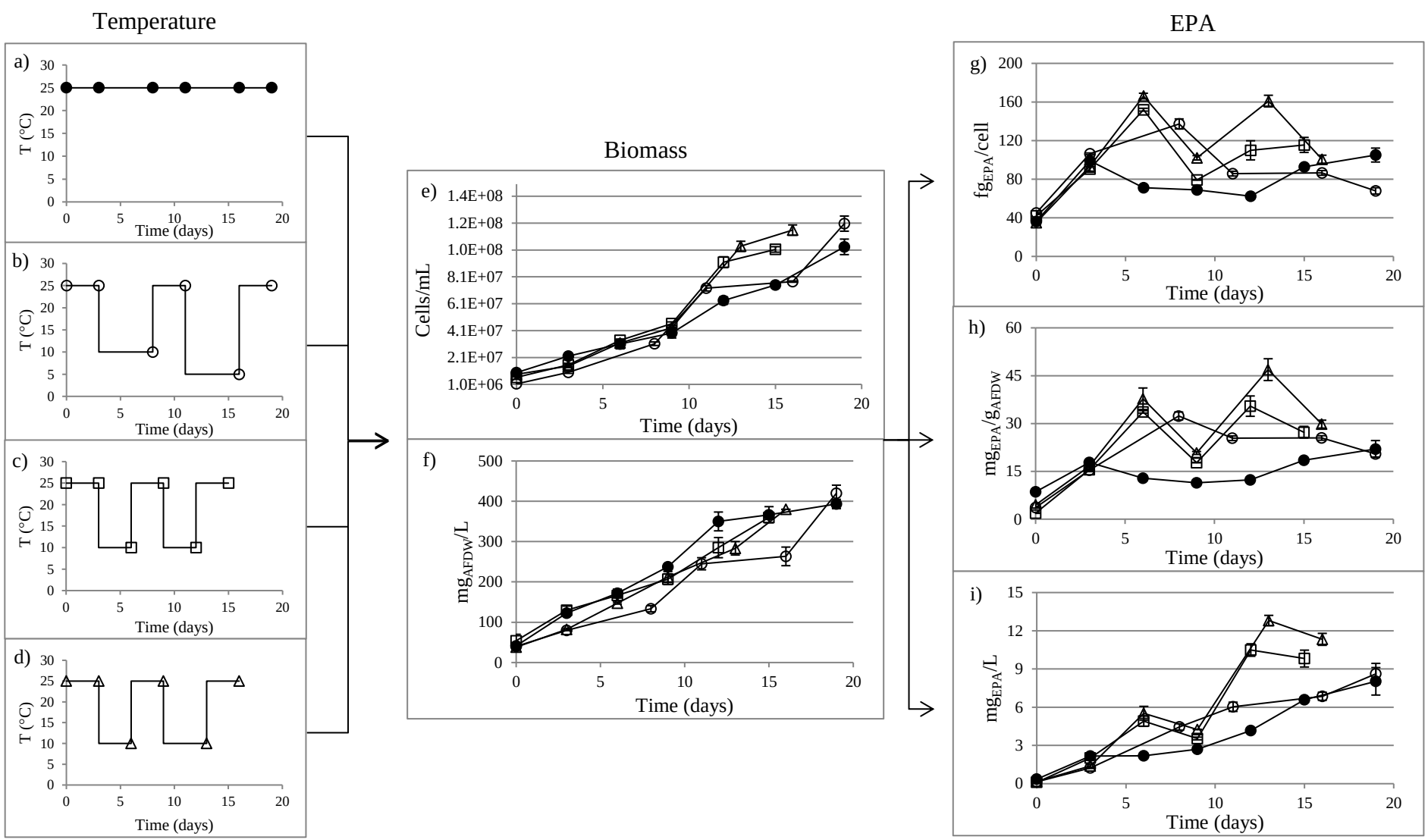
Temperature
a)
b)
c)
d)
T (°C)
Time (days)
Biomass
e)
Cells/mL
f)
mg$_{AFDW}$/L
EPA
g)
fg$_{EPA}$/cell
h)
mg$_{EPA}$/g$_{AFDW}$
i)
mg$_{EPA}$/L

628 **Fig. 1**

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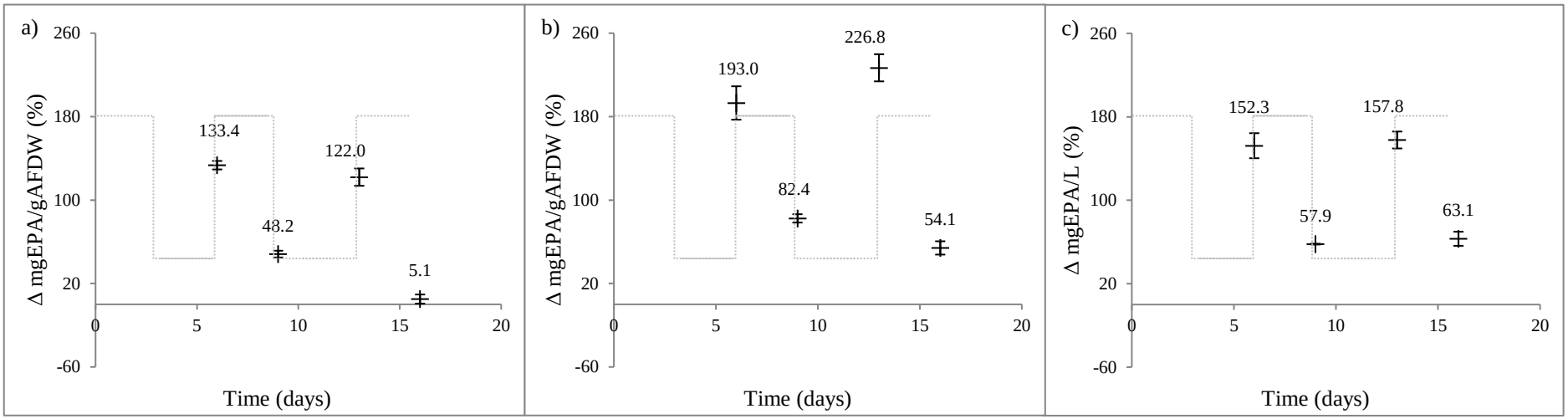

642 **Fig. 2**

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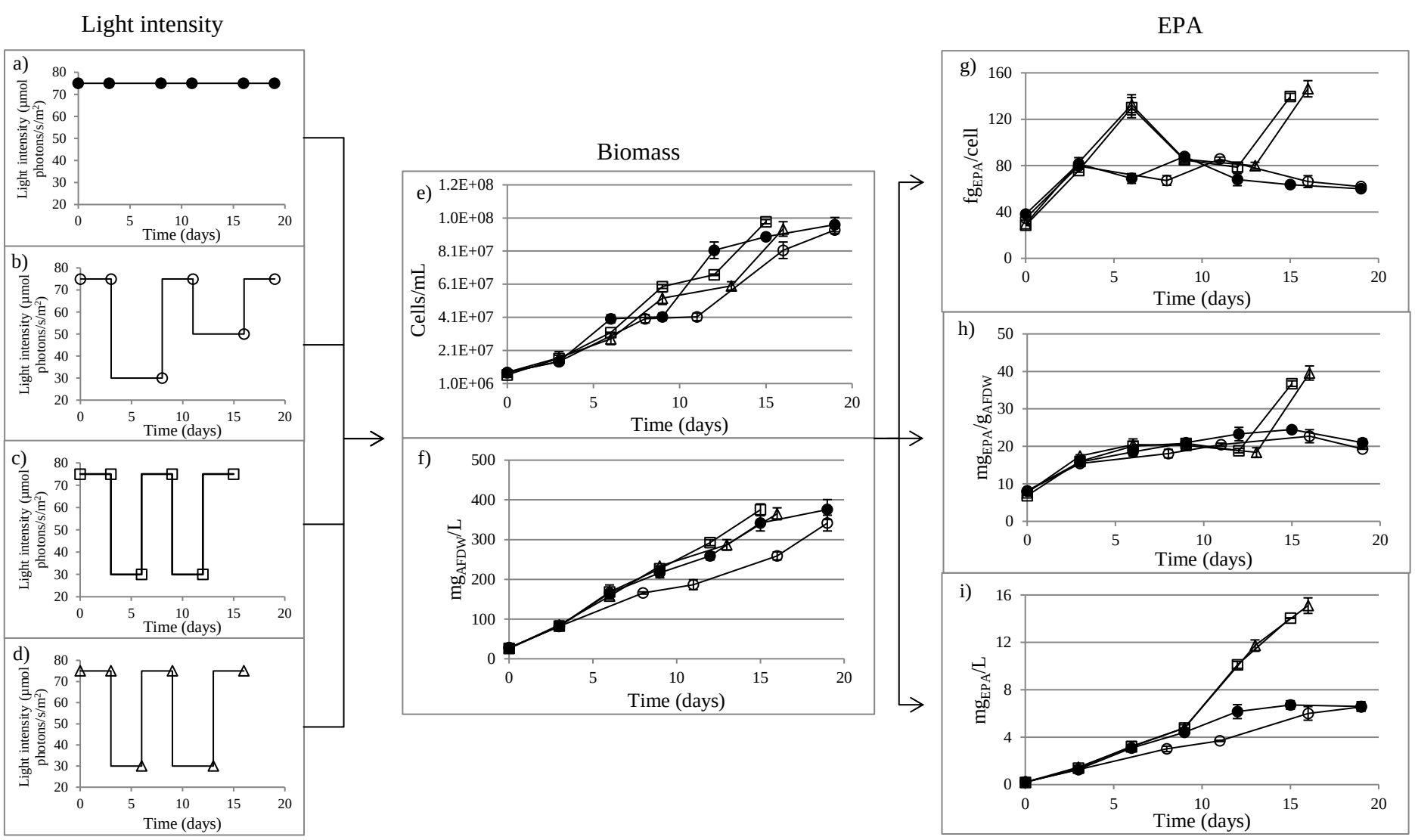
Light intensity
a)
b)
c)
d)
Light intensity (µmol photons/s/m²)
Time (days)
Biomass
e)
Cells/mL
f)
mg_AFDW/L
EPA
g)
fg_EPA/cell
h)
mg_EPA/g_AFDW
i)
mg_EPA/L

Fig. 3

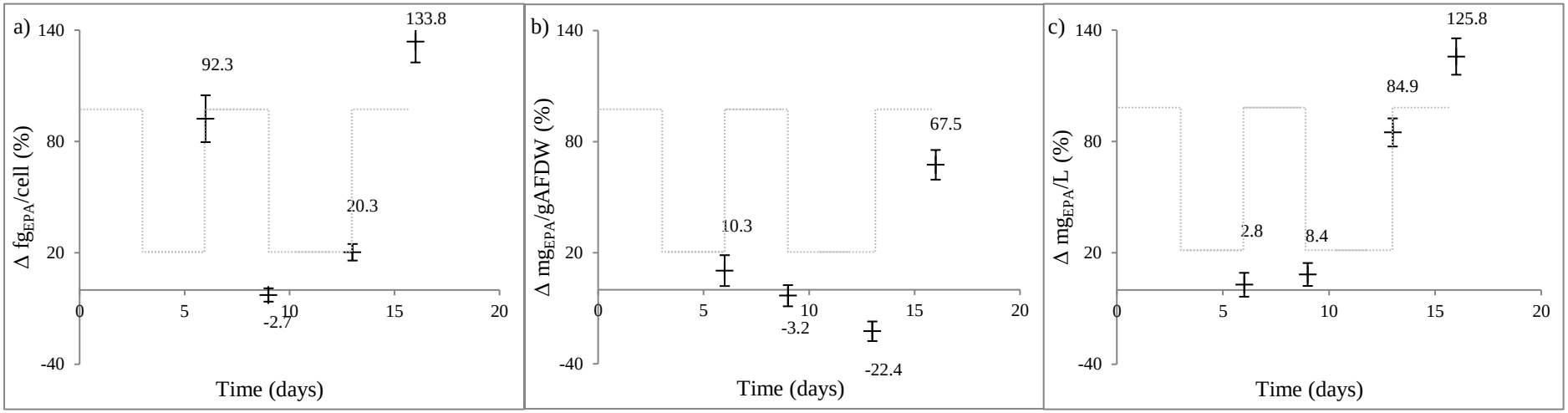
a)
b)
c)
Δ fg$_{EPA}$/cell (%)
Δ mg$_{EPA}$/gAFDW (%)
Δ mg$_{EPA}$/L (%)
Time (days)
92.3
133.8
20.3
-2.7
10.3
67.5
-3.2
-22.4
2.8
8.4
84.9
125.8

Fig. 4

676 **Table 1**

677 Fatty acids profiles of T-Str3 culture throughout growth (% of total fatty acids)

Fatty acid	day					
	0	3	6	9	13	16
C12	0.2±0.1	0.2±0.1	0.2±0.1	0.4±0.1	0.2±0.1	0.3±0.1
C14	4.0±0.3	3.8±0.1	3.6±0.1	4.7±0.1	3.9±0.1	4.6±0.1
C15	0.9±0.1	0.7±0.1	0.5±0.1	0.7±0.1	0.5±0.1	0.8±0.1
C15:1	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1	n.d.	n.d.
C16	34.4±0.1	35.2±0.5	25.1±0.4	35.1±0.4	22.7±1.1	33.6±0.5
C16:1 c9	32.0±0.2	28.4±0.1	30.2±0.1	29.4±0.2	30.7±0.1	29.4±0.2
C17	0.5±0.1	0.6±0.1	0.5±0.1	0.5±0.1	0.6±0.1	0.4±0.1
C17:1 c10	0.3±0.1	0.2±0.1	0.4±0.1	0.3±0.1	0.5±0.1	0.4±0.1
C18i	n.d.	n.d.	0.2±0.1	0.1±0.1	0.2±0.1	0.1±0.1
C18	0.9±0.1	1.1±0.1	0.6±0.1	0.8±0.1	0.5±0.1	0.7±0.1
C18:1 c9	3.3±0.1	5.0±0.1	6.0±0.1	4.2±0.1	5.5±0.2	4.5±0.1
C18:1 c11	1.1±0.1	1.5±0.1	1.2±0.1	1.1±0.1	1.1±0.1	1.0±0.1
C18:2 c9c12	6.4±0.1	4.0±0.1	3.5±0.1	4.1±0.1	3.5±0.1	4.9±0.1
γ C18:3 c6c9c12	0.1±0.1	0.2±0.1	0.5±0.1	0.1±0.1	0.8±0.1	0.1±0.1
C18:3 c9c12c15	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
C20:2 c11c14	0.3±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.1±0.1	0.2±0.1
C20:3 n6	1.3±0.2	1.7±0.2	1.8±0.2	1.4±0.2	1.6±0.2	1.1±0.1
C20:4 n6	2.5±0.1	3.6±0.2	4.6±0.1	3.8±0.1	5.0±0.1	3.7±0.1
C20:5 n3 (EPA)	11.3±0.3	13.3±0.5	20.6±0.4	13.1±0.3	22.4±1.7	14.1±0.3
SUM	100	100	100	100	100	100
∑ SFA	40.9±0.4	41.5±0.7	30.5±0.4	42.1±0.4	28.4±1.2	40.4±0.4
∑ MUFA	37.0±0.1	35.2±0.1	38.0±0.1	35.0±0.2	37.9±0.5	35.3±0.1
∑ PUFA	22.1±0.5	23.2±0.5	31.4±0.4	22.8±0.2	33.5±1.7	24.2±0.3

678 *n.d. – not detected*

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689 **Table 2**

690 Fatty acids profiles of L-Str3 culture throughout growth (% of total fatty acids)

Fatty acid	day					
	0	**3**	**6**	**9**	**13**	**16**
C12	0.2±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.4±0.1	0.3±0.1
C14	4.3±0.1	3.8±0.1	4.6±0.7	4.8±0.1	4.8±0.1	4.7±0.1
C15	1.1±0.1	0.7±0.1	0.6±0.1	0.7±0.1	0.7±0.1	0.8±0.1
C15:1	0.3±0.1	0.2±0.1	0.1±0.1	n.d.	n.d.	n.d.
C16	33.9±0.3	37.0±0.1	35.6±1.3	36.0±0.1	35.4±1.6	34.6±0.1
C16:1 c9	32.5±0.1	29.1±0.1	28.6±0.5	29.1±1.5	30.9±0.9	31.5±0.4
C17	0.5±0.1	0.6±0.1	0.5±0.1	0.5±0.1	0.4±0.1	0.4±0.1
C17:1 c10	0.3±0.1	0.2±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1
C18i	n.d.	0.1±0.1	n.d.	0.1±0.1	0.1±0.1	0.1±0.1
C18	1.0±0.1	1.2±0.1	0.9±0.2	0.8±0.1	0.7±0.1	0.7±0.2
C18:1 c9	3.4±0.1	4.1±0.1	4.1±0.1	3.8±0.6	3.2±0.1	3.2±0.4
C18:1 c11	1.3±0.1	1.5±0.1	1.1±0.3	1.0±0.1	0.9±0.1	0.8±0.2
C18:2 c9c12	6.3±0.1	3.4±0.1	4.1±0.7	4.2±0.1	4.8±1.1	5.7±0.2
γ C18:3 c6c9c12	n.d.	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
C18:3 c9c12c15	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.2±0.1	0.1±0.1
C20:2 c11c14	0.4±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.3±0.1
C20:3 n6	1.1±0.1	1.6±0.1	1.4±0.1	1.4±0.4	1.1±0.2	1.1±0.1
C20:4 n6	2.3±0.1	3.5±0.1	3.8±0.2	3.5±0.4	3.0±0.2	2.8±0.1
C20:5 n3 (EPA)	10.9±0.1	12.5±0.1	13.5±0.9	13.2±0.5	12.6±0.2	12.5±0.4
SUM	100.0	100.0	100.0	100.0	100.0	100.0
∑ SFA	41.0±0.2	43.4±0.1	42.4±0.9	43.0±0.5	42.5±1.6	41.5±0.3
∑ MUFA	37.7±0.2	35.1±0.1	34.2±0.8	34.2±0.8	35.4±1.0	35.9±0.3
∑ PUFA	21.2±0.3	21.5±0.1	23.3±1.7	22.8±1.3	22.0±0.6	22.5±0.6

691 *n.d. – not detected*