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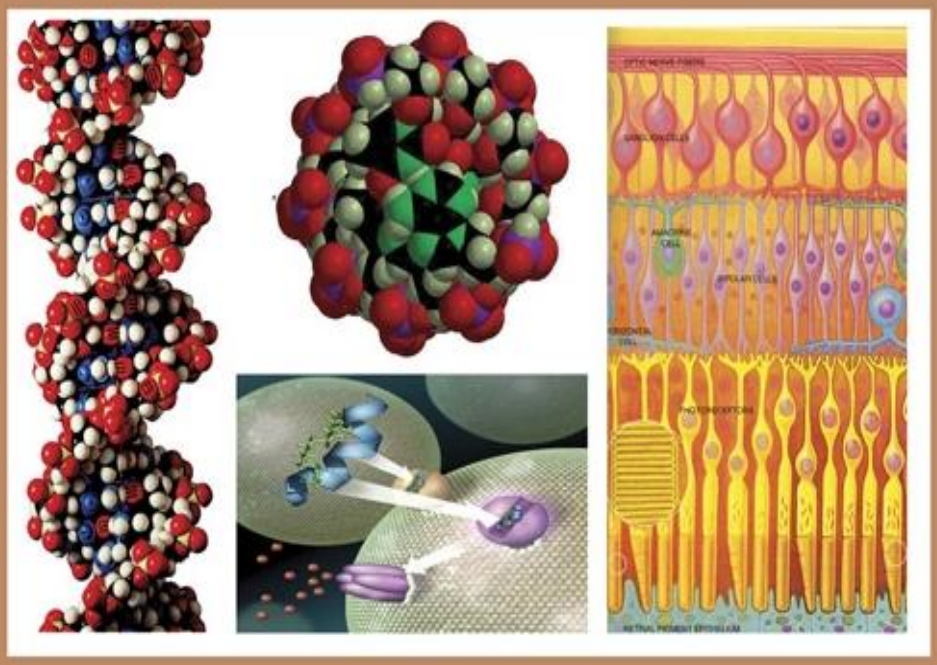

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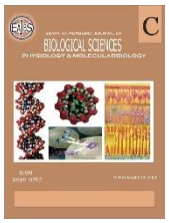

# Strategy for *Nannochloropsis gaditana* Culture Combining High Productivity and Lipid Content For Biofuel Production

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**ABSTRACT**

The aim of this work is to investigate the effects of the variation in the culture parameters on the production of lipids. We first determined the best conditions to promote biomass productivity. Then, we set up a culture system in two steps: the first step is to promote the growth of the algal biomass, via the use of a culture medium enriched in nutrients, in order to have a high concentration in the cell; and the second step consisting in stimulating the production of lipids, by inducing stress to the algal cells, via the substitution of essential nutrients.

Experimental results have shown that favouring growth kinetics is to the detriment of oil content. Indeed, a deficiency in essential nutrients (nitrogen and/or phosphorus) inhibits growth but favours lipid production. Lipid content tripled (51.33% dry weight) in *N. gaditana* grown in the total absence of nutrients (0 g/L nitrogen and phosphorus) compared to controls (12 g/L nitrogen and 5 g/L phosphorus). Nitrogen and phosphorus deficiency stimulates lipid accumulation but leads to a reduction in growth rate, which ultimately impairs lipid productivity. For better lipid productivity, a compromise must be found between growth and cellular lipid accumulation.

## INTRODUCTION

Microalgae are known to be able to accumulate significant amounts of lipids, up to 80% of their mass when grown under deficiency conditions. These by transesterification reaction with an alcohol lead to esters that can be used in combustion engines (Enamala *et al.*, 2018).

Lipids are present in microalgae in the form of triglycerides, which constitute an important energy reserve (Becker, 2007). Marine lipids can be classified into two different categories based on their polarity: on the one hand, neutral lipids including acylglycerols (monoglyceride or MAG, diglyceride or DAG, triglyceride or TAG) and free fatty acids and on the other hand, polar lipids which can be subdivided into two; glycolipids and phospholipids (Dejoye Tanzi, 2013). The latter two enter the composition of the cell walls of microalgae (Audo, 2013).

The production of biodiesel from microalgae gained global momentum following the first oil shock in the 1970s. During the 1990s, relatively low oil prices lead to a sharp slowdown in biodiesel research programs (Dejoye Tanzi, 2013). In the early 2000s, in the face of rising fuel prices and an oil shortage, algal fuel production is back on the agenda.

In addition to the productivity argument, microalgae have a major advantage over other solutions: non-competition with food crops (Dejoye Tanzi, 2013).

Microalgae are among the most promising raw materials for the development of 3rd generation biofuels. This alternative to fossil resources must be sustainable and viable ecologically but also economically. It is necessary to optimize the algal culture in order to produce the maximum of lipids, without however harming the development of the biomass.

## MATERIALS AND METHODS

### Microalgae Culture:

The strain of *Nannochloropsis gaditana* was provided by the company PBA-Partisano Biotech Algerie (Sidi Bel Abbes, West Algeria). The culture medium used was Guillard f/2 (Robert, 2005).

The experiments were performed in triplicate. The photobioreactor (PBR) used were column type with a useful volume of 1,5 liters. Agitation in the PBRs was provided by an air-bubbling system. Air was injected from the bottom of the reactor through a sterile filter (cellulose ester: 0.45 µm).

Illumination was provided by 4 neon white LED lights of 24 W.The incident light intensity provided by the lamps was 100 ± 5 $\mu mol^{-2}\ s^{-1}$ (measured using the Hansatech QRT1 Quantitherm photometer) with a photoperiod of 18:6 h light/dark cycle..

### Strategies and Experimental Designs:

To study the effect of nitrogen and phosphorus deficiency in the culture medium on the concentration of biomass and lipid rate produced, we performed a two-phase culture: a first phase of rapid growth in a Guillard f/2 medium followed by a second phase of lipid production in a medium containing different concentrations of nitrogen and phosphorus (Table 1**)**.

**Table 1.** Strategy for the combination of nitrogen and phosphorus deficiency

| Tests | N-$NO_3$ (g/L) | P-$PO_4$ (g/L) | Growing conditions |
|---|---|---|---|
| **T1** | 12 | 5 | • The rest of the components of the Guillard f/2 medium.<br>• Photoperiod: 18h/6h (illumination/darkness).<br>• Light intensity 190 ± 10 µmol-2 $s^{-1}$. |
| **T2** | 8 | 5 | |
| **T3** | 4 | 5 | |
| **T4** | 0 | 5 | |
| **T5** | 12 | 2,5 | |
| **T6** | 8 | 2,5 | |
| **T7** | 4 | 2,5 | |
| **T8** | 0 | 2,5 | |
| **T9** | 12 | 0 | |
| **T10** | 8 | 0 | |
| **T11** | 4 | 0 | |
| **T12** | 0 | 0 | |

### Determination of Growth Kinetic Parameters:

The specific growth rate (µ) ($d^{-1}$) was calculated using the following formula (Wen *et al.*, 2014):

$$\mu = \frac{Ln(X_{max}) - Ln(X_0)}{t_{max} - t_0} \qquad \text{(Eq.1)}$$

With $X_m$: maximum concentration that the system can achieve in batch (mg $SS.L^{-1}$), $X_0$:initial cell concentration (mg $SS.L^{-1}$)

The strain doubling time was ($T_d$) (d) was calculated by the equation proposed in the work of Madkour *et al.* (2012) :

$$T_d = \frac{Ln\ 2}{\mu} \qquad \text{(Eq.2)}$$

With µ: maximum specific growth rate ($d^{-1}$).

The reactor volumetric productivity (P) (mg. $L^{-1}.d^{-1}$) was calculated according to the following equation (Ruiz *et al.*, 2012):

$$P = \frac{X_{max} - X_0}{t_m - t_0} \qquad \text{(Eq.3)}$$

With $X_t$: biomass concentrations at the end of

the exponential phase, $X_0$: biomass concentrations at the beginning of the exponential phase, and $t_m$-$t_0$: duration of the exponential phase.

**Determination of Suspended Solids:**

Biomass concentration was measured gravimetrically as dry weight according to the standardized 2540-D method (APHA, AWWA, WEF, 1992).

**Determination of Lipid Content:**

The method of Bligh & Dyer (1959) was used for the determination of lipid content in biomass. It is considered the standard method for the determination of total lipids in biological tissues such as microorganisms. The lipid content was expressed as % of dry weight according to the following equation:

$$\textit{Lipid}\ (\%) = \frac{\textit{lipid}\ (mg)}{\textit{biomass}\ (mg)} \times 100 \qquad \text{(Eq.4)}$$

**Determination of Lipid Productivity:**

Lipid productivity (LP) expressed as mg lipid/L/d was determined according to the Eq. 5 (Praharyawan *et al.*, 2016):

$$LP = P \times LC \qquad \text{(Eq.5)}$$

With P: reactor volumetric productivity (mg SS/L/d), LC: lipid content of biomass (mg lipid/mg biomass).

**Statistical Analysis:**

The experiments were performed in triplicate. Statistical analysis consisted of a parametric ANOVA test (Tukey HSD) using MINITAB 18 software. Values of $P < 0.05$ were considered statistically significant.

## RESULTS AND DISCUSSION

**Growth Kinetic Parameters:**

The results shown in Figure 1 show that during the first phase of the experiment ($d_0$ - $d_5$), the growth starts with the acceleration phase (1 day), followed by an exponential growth phase. The biomass concentration at the end of the first phase reaches a value of 542 ± 29.5 mg SS/L. The growth kinetics during the second growth phase showed heterogeneous results depending on the composition of the culture media.

When the strains were transferred to Guillard f/2 culture medium poor or devoid of nitrogen, they required an adaptation time of 2 days (Fig. 1A). Microalgae transferred to the medium containing a nitrogen concentration of 12 and 8 g/L had a strong exponential growth to reach a maximum biomass concentration of 1340.2 ± 28 and 1163.7 ± 5.3 mg SS/L, respectively. However, when *N. gaditana* was grown in Guillard f/2 media devoid of nitrogen, its growth was very slow and reaches an $X_{max}$ biomass concentration of 719.3 ± 18.4 mg SS/L. The stationary phase was not reached until the end of the experiment (day 11) in all four groups of microalgae.

Figure 1B shows that after transferring the strains to the modified Guillard f/2 medium, they needed one day to adapt and resumed their exponential growth phase. This growth continued until day 8 in all four groups studied. In the strains grown in the medium containing a nitrogen concentration of 12 g/L, the stationary phase was reached after day 9. However, in strains grown in media containing a nitrogen concentration of 8, 4 and 0 g/L, growth reached the decline phase by day 10.

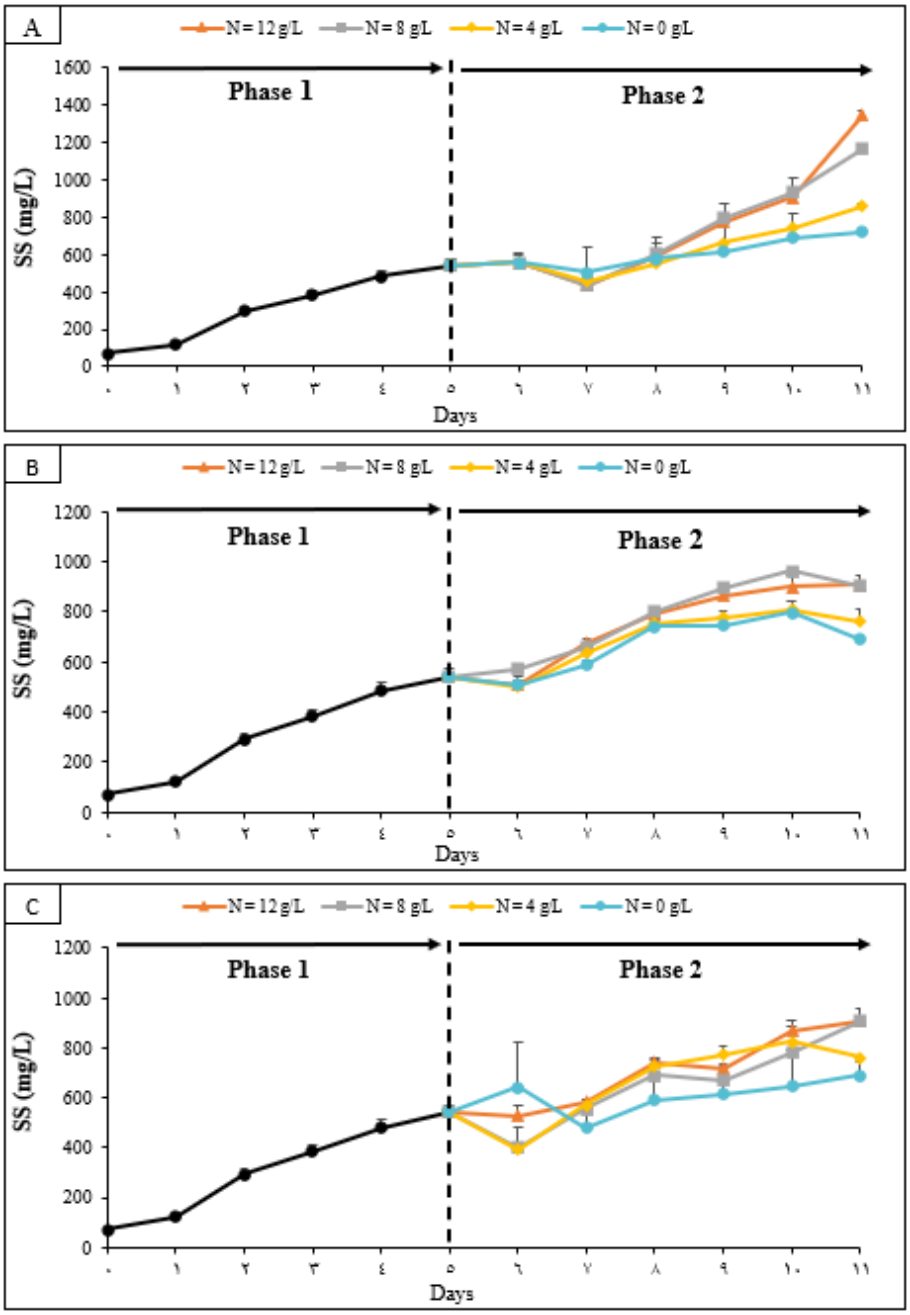


**Fig 1.** Effect of phosphorus P-$PO_4$ (A= 5 g/L; B= 2.5 g/L and C= 0 g/L) and nitrogen N-$NO_3$(12, 8, 4 and 0 g/L) deficiency in the culture medium on growth kinetics of *N. gaditana*.

For strains grown in phosphorus-free medium and at different nitrogen concentrations (Fig. 1C), we could see that the stationary phase was not reached until the end of the experiment (day 11) in strains grown in medium containing 12, 8, and 0 g/L nitrogen. Strains transferred to a completely nitrogen-free medium achieved the lowest biomass concentration $X_{max}$ (688.5 ± 4.9 mg SS/L).

Concerning the volumetric productivity (Tabel 2), it varies between 180.6 ± 8 and 24.6 ± 2.4 mg SS/L/d. Our results show that strains grown in a medium rich in nitrogen and phosphorus (T1) have volumetric productivity (180.6 ± 8 mg SS/L/d) significantly ($p<0.05$) higher than in strains grown in the absence of nutrients (T12) ( 24.6 ± 2.4 mg SS/L/d).

**Table 2.** Growth kinetic parameters in *N. gaditana* grown at different nitrogen and phosphorus concentrations.

| Tests | $N\text{-}NO_3$ (g/L) | $P\text{-}PO_4$ (g/L) | $X_m$ (mg SS/L) | μ ($d^{-1}$) | dt (d) | VP (mg SS/L/d) |
|---|---|---|---|---|---|---|
| **Phase 1** | | | | | | |
| | **12** | **5** | $541,6 \pm 29,5^{a}$ | $0,41 \pm 0,02^{a}$ | $1,7 \pm 0,1^{a}$ | $105,1 \pm 8,2^{ab}$ |
| **Phase 2** | | | | | | |
| **T1** | **12** | **5** | $1340,2 \pm 28^{b}$ | $0,22 \pm 0,01^{b}$ | $3,1 \pm 0,1^{ab}$ | $180,6 \pm 8^{c}$ |
| **T2** | **8** | **5** | $1163,7 \pm 5,3^{c}$ | $0,20 \pm 0,02^{bc}$ | $3,6 \pm 0,4^{abc}$ | $145,1 \pm 8,8^{ac}$ |
| **T3** | **4** | **5** | $857,0 \pm 14,1^{de}$ | $0,13 \pm 0,02^{cde}$ | $5,6 \pm 1^{abcd}$ | $79,9 \pm 11,7^{bd}$ |
| **T4** | **0** | **5** | $719,3 \pm 18,4^{f}$ | $0,07 \pm 0,001^{ef}$ | $9,8 \pm 0,2^{d}$ | $43,0 \pm 1,8^{de}$ |
| **T5** | **12** | **2,5** | $966,4 \pm 27,8^{dg}$ | $0,09 \pm 0,001^{def}$ | $7,7 \pm 0,1^{cd}$ | $91,8 \pm 4,9^{b}$ |
| **T6** | **8** | **2,5** | $1029,9 \pm 70,2^{g}$ | $0,11 \pm 0,02^{def}$ | $6,4 \pm 1,2^{bcd}$ | $91,2 \pm 11,4^{b}$ |
| **T7** | **4** | **2,5** | $909,1 \pm 39^{dg}$ | $0,09 \pm 0,01^{def}$ | $7,9 \pm 1,5^{cd}$ | $82 \pm 12,6^{bd}$ |
| **T8** | **0** | **2,5** | $861 \pm 5,6^{de}$ | $0,1 \pm 0,005^{def}$ | $7,3 \pm 0,4^{bcd}$ | $70 \pm 4,9^{bd}$ |
| **T9** | **12** | **0** | $906,2 \pm 18,4^{dg}$ | $0,12 \pm 0,1^{cde}$ | $6,2 \pm 1,9^{bcd}$ | $82,1 \pm 29,7^{bd}$ |
| **T10** | **8** | **0** | $906,2 \pm 37,2^{dg}$ | $0,16 \pm 0,01^{bcd}$ | $4,3 \pm 0,2^{abc}$ | $100,9 \pm 1,7^{ab}$ |
| **T11** | **4** | **0** | $762,6 \pm 45,1^{ef}$ | $0,13 \pm 0,02^{cde}$ | $5,3 \pm 0,7^{abcd}$ | $73,7 \pm 11,3^{bd}$ |
| **T12** | **0** | **0** | $688,5 \pm 4,9^{f}$ | $0,04 \pm 0,005^{f}$ | $17,7 \pm 2^{e}$ | $24,6 \pm 2,4^{e}$ |

Values marked by different indices indicate a significant difference ($P < 0.05$) according to Tukey's test.

Our results demonstrate that the final biomass concentration, specific growth rate, and volumetric productivity rate of *N. gaditana* decreased with decreasing phosphorus and nitrogen levels in the growing medium. Many of researches corroborate our results (Chen *et al.*, 2017). They observed that after carbon, nitrogen and phosphorus are quantitatively the most important elements in the natural phytoplankton biomass. Nitrogen and phosphorus are essential resources for all organisms; they are involved in the composition of essential molecules in life.

According to Martinez Sancho *et al.* (1997), the growth rate of the microalga *Scenedesmus* decreases by a factor of 2 in case of phosphorus deficiency. In *Chlorella vulgaris*, the same deficiency results in a specific decrease in the amount of intracellular inorganic polyphosphate (Villay, 2013). The affinity of microorganisms for phosphate varies depending on the species. It was 1-6 µM for species belonging to the genus *Scenedesmus*, and 4-5 µM for the genus *Chlorella* (Martinez Sancho *et al.*, 1997). Under conditions of phosphorus limitation (from 1 to 0.1 mg/L), the lipid content of *Scenedesmus sp*. increases by a factor of 2. This increase in lipid content was accompanied by a decrease in biomass production by the same factor (Xin *et al.*, 2010).

**Productivity and Lipid Content:**

The culture conditions of microalgae must be controlled in order to reach important growth kinetics and lipid contents. This aspect is essential to obtain interesting lipid productivity. We note that growth-promoting factors such as nitrate and phosphate limit the amount of oil in microalgae (Taleb, 2015). Indeed, our results show that lipid production is stimulated in microalgae that have been deficient in nitrogen and phosphorus.

During this experiment, we were able to obtain a maximum lipid concentration of 51.33% in microalgae grown in media lacking nitrogen and phosphorus (Fig. 2). This result is significantly higher than those reported by Enamala *et al.* (2018) in *Nannochlorpsis sp*. with lipid contents between 21 and 35%. It should be noted, however, that in terms of lipid productivity, culture media containing 0 g/L nitrogen and 2.5 g/L phosphorus are more interesting (34.34 mg/L/d) than the medium lacking nitrogen and phosphorus (12.68 mg/L/d).

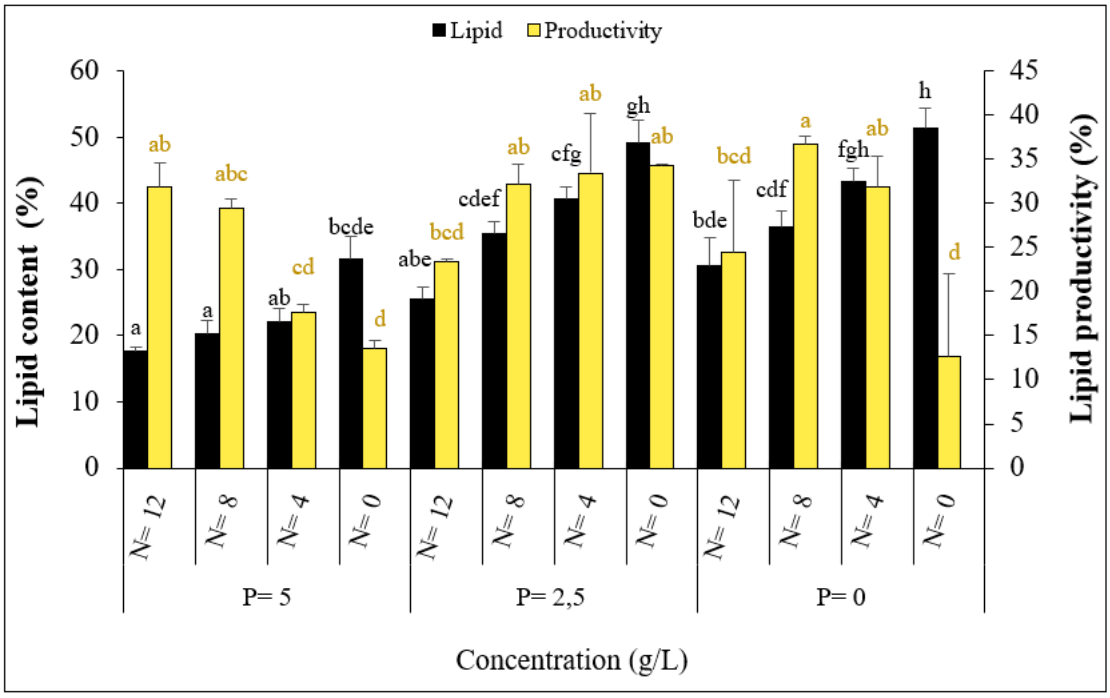


**Fig 2.** Effect of nitrogen (12, 8, 4 and 0 g/L N-$NO_3$) and phosphorus (5, 2.5 and 0 g/L P-$PO_4$) deficiency on lipid content and productivity in *N. gaditana*. (Histograms marked with different indices indicate a significant difference ($P < 0.05$) according to Tukey's test).

In the last decade, nitrogen limitation has been particularly studied in order to stimulate lipid synthesis in microalgae. Significant lipid productivities could be obtained for example in *Neochloris oleoabundans* (Li *et al.*, 2008), *Nannochloropsis sp.* (Pal *et al.*, 2011) and *Chlorella sp.* (Hsieh & Wu, 2009). Other authors report sensitive lipid accumulation in response to phosphorus limitation (Liang *et al.*, 2013). In the freshwater microalga *Monodus subterraneus*, phosphorus deficiency simultaneously leads to a reduction in cellular phospholipids (from 8.3 to 1.4%) and an accumulation of triglycerides (from 6.5 to 39.3%), relative to total lipids (Khozin-Goldberg & Cohen, 2011). Kilham *et al.* (1997) showed in *Ankistrodesmus falcatus* a significant increase in triglycerides, following the limitation of phosphorus supply, greater than that induced by the limitation of nitrogen supply.

**Conclusion**

In order to increase lipid accumulation in *N. gaditana*, we implemented a two-step culture strategy: a first step to promote algal biomass growth, via the use of a nutrient-enriched culture medium, and a second step to stimulate lipid production, via the substitution of essential nutrients. The experiment with the highest lipid content was Experiment 12 (Total nitrogen deficiency) with a content of 51.33%. This combination resulted in a threefold increase in lipid content compared to the control (Guillard f/2 medium). Our results showed that nitrogen and phosphorus deficiency stimulated lipid production. While nitrogen or phosphate limitation thus leads to a stimulation of lipid accumulation, it also leads to a reduction in growth rate, which ultimately affects lipid productivity. A trade-off between growth and lipid accumulation must be found.

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