

ARTICLE

Effect of nitrate and irradiance on fatty acid production in microalgae cultivated for feeding larvae and broodstock conditioning in batch culture

Efectos de nitrato e irradiación en la producción de ácidos grasos en microalgas destinadas a la alimentación de larvas y reproductores, en cultivo experimental cerrado

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Resumen.- El objetivo de este estudio fue comparar las condiciones para producir altos niveles de AGPI, EPA y DHA en microalgas cultivadas para alimentar las etapas larvianas de los bivalvos. Se evaluó por cromatografía de gas la concentración de ácidos grasos poliinsaturados en tres especies de microalgas, *Isochrysis galbana* Parke (T-ISO), *Chaetoceros gracilis* Pantocsek y *C. constrictus* Gran, con el fin de ser usadas como fuente de alimento de larvas y adultos de bivalvos en cultivo. *Isochrysis galbana* produce mayor concentración de ácido linolénico y ácido docosahexaenoico que ácido eicosapentaenoico en condiciones limitantes de nitrato, sin embargo estos ácidos grasos se incrementan notablemente con el exceso de N-nitrato, por encima del contenido del medio F/2. Por otra parte, la baja intensidad lumínica y la concentración normal de nitratos en los cultivos de la especie *Chaetoceros gracilis* producen mayor contenido de ácido eicosapentaenoico (EPA). En el caso de *Chaetoceros constrictus*, la microalga sintetiza mayor concentración de ácidos grasos en condiciones de alta intensidad lumínica y bajas concentraciones de nitrato.

Palabras clave: Microalgas, ácidos grasos, ácido eicosapentaenoico

Abstract.- The aim of this study was to compare conditions for the production of high levels of PUFA, EPA and DHA in cultured microalgae for larval bivalve feeding stages. It assessed fatty acid composition by gas chromatography for *Isochrysis galbana* Parke (T-ISO) and 2 species of *Chaetoceros*, *C. gracilis* Pantocsek and *C. constrictus* Gran. Species were studied in batch cultures, at the beginning of stationary growth phase with different irradiance and 2 levels of nitrogen content for *Chaetoceros* sp., and constant irradiance with 5 concentrations of sodium nitrate for *I. galbana* in an F/2 medium. *Isochrysis galbana* produced higher levels of linolenic acid and docosahexaenoic acid than eicosapentaenoic acid (EPA) at low N-nitrate concentrations; however, fatty acids increased notably with surplus N-nitrate, above F/2 medium content. Low irradiance and normal concentration of nitrates in the batches of *Chaetoceros gracilis* species produced a greater EPA content. *C. constrictus*, a diatom isolated from phytoplankton samples, presented higher levels of EPA and DHA at low levels of nitrogen and low irradiance.

Key words: Microalgae, fatty acids, eicosapentaenoic acid

INTRODUCTION

Microalgae play an important role in aquaculture as feed for larvae and adult conditioning for marine invertebrate species; nutritional value is related to biochemical composition, and broodstock adult conditioning is especially important for the following developmental stages. When dietary nutritional value is high in polyunsaturated fatty acids (PUFAs), successful oyster spatfalls are produced (Fakhri & Christianus 2018). Oyster larvae accumulate lipid reserves, and larvae with higher reserves are more

competent at settling than those with lower reserves (Liang *et al.* 2006). Marine microalgae are an important feed source in aquaculture, being used in hatcheries to feed bivalves such as *Argopecten purpuratus* (Cerpa *et al.* 2003) as well as fish (Hamre *et al.* 2013). Long-chain polyunsaturated fatty acids (LC-PUFAs), namely eicosapentaenoic acid (20:5 n-3, EPA), docosahexaenoic acid (22:6 n-3, DHA) and arachidonic acid (20:4 n-6, ARA), are considered essential fatty acids (EFAs) in teleosts for both natural environments (Castro *et al.* 2010) and hatchery conditions,

as a source of metabolic energy and cell membrane structure (Bonacic *et al.* 2016). As metabolism regulators, long chain polyunsaturated fatty acid derivatives, such as leukotriene, act in extracellular or intracellular ligands as transcription factors that control gene expression (Tocher 2015, Bonacic *et al.* 2016).

Lipid composition varies according to irradiance, culture medium nutrient composition for microalgae and species, and it is usually necessary to mix different species to offer balanced nutrition for larvae (Cerpa *et al.* 2003, Hemaiswarya *et al.* 2011, Guevara *et al.* 2016). The most important aspect of lipids in animal nutrition is the content and proportion of certain fatty acids. In particular, some polyunsaturated fatty acids (PUFAs) synthesized by algae are essential for the growth and development of marine fish, shrimp and mollusk larvae (Adarme-Vega *et al.* 2012). The presence of long unsaturated fatty acids is important when selecting microalgae for aquaculture processes, and different species of microalgae have different metabolic ability for optimal synthesis. Unsaturated long chain fatty acids are synthesized by elongases and desaturases from palmitic, stearic or oleic acid in plastids and rough endoplasmic reticulum (Mühlroth *et al.* 2013). Meanwhile, in plastids, membrane structural lipid synthesis occurs in the rough endoplasmic reticulum, along with those that store energy, such as triacylglycerols (TAG) (Bellou *et al.* 2014).

Nitrogen availability affects the synthesis and accumulation of metabolites such as lipids, pigments and cell components (Lourenço *et al.* 2002). It is possible to change the lipid composition of microalgae, varying culture conditions near to metabolic stress (Gordillo *et al.* 1998, Yu *et al.* 2011, Benavente-Valdés *et al.* 2016, Minhas *et al.* 2016). Among different culture medium components, the source and concentration of nitrogen can induce significant changes in growth and biochemical composition, particularly with respect to the lipid composition (Madkour *et al.* 2012, Zhang *et al.* 2013, Kim *et al.* 2016). Therefore, it was tested different culture conditions that could increase unsaturated fatty acids in 2 species, *Isochrysis galbana*, *Chaetoceros gracilis*, as well as a wild microalga, *Chaetoceros constrictus*, under batch conditions. The aim of this study was to compare conditions for producing high levels of PUFA, EPA and DHA to improve growth efficiency for larval bivalve feeding stages in hatchery only changing culture conditions in microalgae production (temperature and irradiance) according to nutrient necessity of larval stage.

MATERIALS AND METHODS

The experimental design was performed on 3 species, 2 of which are traditionally used for feeding in bivalve hatcheries, *e.g.*, *Argopecten purpuratus* (Cerpa *et al.* 2003): *Isochrysis galbana* (T-ISO) and tropical *Chaetoceros gracilis*. *Chaetoceros constrictus* was isolated from wild phytoplankton samples.

Isochrysis galbana (T-ISO) (Haptophyta) was isolated from 50 L pure mass culture containers for massive bivalves feeding in hatchery cultures. An experimental culture was made in 3 bags of 0.3 mm thick polyethylene, in a volume of 20 L of sea water filtered and sterilized with UV light and 5 ppm sodium hypochlorite for 30 min, to standardize fatty acid and protein extraction. We added F/2 culture medium containing 75 mg L⁻¹ of sodium nitrate. It was inoculated with 2 L of microalga, under constant aeration conditions, with a photoperiod of 12:12, at a temperature of 20 ± 1 °C and photonic flow of 60 to 70 μE m⁻²s⁻¹. The microalgae were harvested at the end of the exponential phase (6-7 days)/beginning of the stationary phase (Roopnarain *et al.* 2015).

EXPERIMENTAL DESIGN

One hundred mL of culture at exponential growth phase, with an initial concentration of 75,000 cells mL⁻¹ was transferred to 5 L bottles, with modified F/2 or F/2 culture medium. Samples of T-ISO were grown under 5 different concentrations of sodium nitrate (20, 40, 75, 160, 320 mg L⁻¹ in duplicate). The culture conditions were photoperiods of 16:8, with constant aeration and photonic flow from 60 to 70 μEm⁻²s⁻¹, with vertical fluorescent tubes, at 20-23 °C.

Cell growth was quantified with a Neubauer counting chambers with samples stained with lugol according to Ramírez (1982).

Conditions for growing *C. gracilis* Pantocsek (Bacillariophyta), a microalga strain commonly used for feeding bivalves at Universidad de Concepción hatchery (Cerpa *et al.* 2003). From a culture bag, it was obtained 50 mL of *C. gracilis* culture, during the stationary growth phase at 200 μEm⁻² s⁻¹, 20 °C, and put in a 5 L flask at 40 μEm⁻² s⁻¹, with 16:8, at 15 °C for acclimatization, for 4 days. Then, we added it to 12 flasks of 350 mL, provided with 200 mL of F/2 by an air pump.

Wild *Chaetoceros constrictus* was isolated from phytoplankton samples obtained at Coliumo Bay (Dichato, 36°32'S, 72°57'W), with a phytoplankton net of 32 μm mesh, identified and kept under constant photonic flux of 60 μEm⁻² s⁻¹, at 18 °C. Acclimatization of the cultures was performed at 30 μEm⁻² s⁻¹, with a photoperiod of 16:8, at 15 °C. Constant air pumping was maintained for a further 2 weeks. Massive cultures were settled 2 weeks later, in 5 L glass bottles.

Twelve 250 mL flasks were distributed in groupings of two. Groups of 4 flasks were cultured at the same light intensity: 30, 60 or 100 $\mu\text{Em}^{-2}\text{s}^{-1}$, provided with fluorescence tubes. In each group, 2 of 4 flasks were used for culture at high sodium nitrate (NaNO_3) concentration (75 mg L^{-1}), which is the normal concentration of F/2 medium culture, or low concentration (37.5 mg L^{-1}), at 15-16 °C. In total, there were 6 different growing conditions for the 2 species of *Chaetoceros* sp. (Table 1).

MASS CULTURE

For both *Chaetoceros* species, bulk cultures were prepared in 5 L sterilized bottles with F/2 culture medium. A 200 mL culture sample in its exponential phase was inoculated. The harvest was performed at the beginning of the algae's stationary phase (15 days in *C. gracilis*, 13 days in *C. constrictus*). Cultures of *C. gracilis* were centrifuged at 3500 rpm for 10 min. Cultures of *C. constrictus* were settled for 4 h, and the culture medium sucked out. The pellets were centrifuged at 4500 rpm for 15 min. The pellets obtained were frozen at -4 °C and lyophilized for 24 h in LABCONCO Lyphlock 4.5 lyophilizer at -50 °C and 0.05 mBar pressures to a constant weight.

Microalgae growth was calculated based on daily sampling in triplicates.

MICROALGAE FATTY ACID PRODUCTION

Total lipids were extracted according to Folch *et al.* (1957), and determined gravimetrically after solvent evaporation. 100 to 250 mg of lyophilized sample was extracted with 30 mL chloroform:methanol at 2:1 for 30 min, sonicated, as recommended by Ryckebosch *et al.* (2013, 2014a,b). Aliquots of the final lipid extracts were deposited in chloroform, and used to prepare the fatty acid methyl esters (FAME) with 12% BF_3 (Metcalf *et al.* 1966). GC-FI-547 was used for gas chromatography analysis with a flame ionization detector at 5 m x 0.25 mm ID capillary column (20 m in length). The temperature for this programmed operation was initially 50 °C, increasing to 150 °C at 8 °C min^{-1} , then to 250 °C at 10 °C min^{-1} , and maintained at constant temperature for an additional 10 min. Injector temperature was 250 °C, and detector temperature was 300 °C (El Menyawi *et al.* 2000, Cerpa *et al.* 2003). Nitrogen gas flow was 1 mL min^{-1} . Standard mixtures of fatty acid methyl esters (Supelco) were used to obtain relative retention times and identify fatty acids in the samples by comparison. Relative fatty acid levels were calculated as ng of fatty acid per g of dry sample, according to peak area compared to the standard for capric acid C10 (Santos *et al.* 2012). To determine saturated fatty acids, a standard was used with a mixture of fatty acids from C14 to C20; C 20:5 (n-3) eicosapentaenoic acid was determined with a standard at a concentration of 5 mg.

Table 1. Experimental design for *Chaetoceros* sp. irradiance (3 levels, as indicated in the second column) and sodium nitrate concentration (2 levels, as indicated in the fourth column) / Diseño experimental para intensidad lumínica en *Chaetoceros* sp. (3 niveles, como se indica en la segunda columna) y concentración de nitrato de sodio (2 niveles, como se indica en la cuarta columna)

	Treatment	Irradiance $\mu\text{Em}^{-2}\text{s}^{-1}$		Concentration of $[\text{NaNO}_3]$ in the culture media mg L^{-1}	
1	Control	60	Normal (M)*	75	Normal (A)**
2	Experimental	60	Normal (M)***	37.5	Stress (B)
3	Experimental	100	High (A)	75	Normal (A)
4	Experimental	100	High (A)	37.5	Stress (B)
5	Experimental	30	Low (B)	75	Normal (A)
6	Experimental	30	Low (B)	37.5	Stress (B)

(*) Letters between parentheses indicate a symbol for graphics

(**) Normal means sodium nitrate concentration in F/2 culture media

(***) Normal irradiance is the habitual light intensity used in the hatchery cultivation *Chaetoceros* sp.

MICROALGAE PROTEIN PRODUCTION

Total proteins were determined using the method in Lowry *et al.* (1951), using 300 µg mL⁻¹ albumin solution as standard.

STATISTICAL ANALYSIS

Principal component analysis (PCA) was performed with the InfoStat program (Di Rienzo *et al.* 2015) for comparison of fatty acids profile in both species of *Chaetoceros*. The Pearson coefficient was also calculated to understand the possible relationship in metabolic pathways.

RESULTS

ISOCHRYSIS GALBANA GROWTH

Growth curves for different culture conditions of microalgae *Isochrysis galbana* (T-ISO) were obtained. In normal conditions, at a concentration of 75 mg L⁻¹ sodium nitrate, an exponential cell growth was observed up to day 7 of culture, increasing to a maximum value of 12.81*10⁶ cells mL⁻¹. At deficient concentrations of sodium nitrate, (20 and 40 mg L⁻¹), cell growth reached a maximum of 8.5 to 8.88*10⁶ cells mL⁻¹, on day 7 of culture. Under higher sodium nitrate conditions, cell number responses at 160 mg L⁻¹, reaching 14.26*10⁶ cells mL⁻¹ from day 7 to day 14. At 320 mg L⁻¹ concentration, the number of cells increased from 11.45*10⁶ cells mL⁻¹ per day to 13.36*10⁶ cells mL⁻¹ per day by day 14. Figure 1 shows biomass (wet and dry weight) and cell density in the culture. Growth rate at harvesting time (day 7) was between 0.026 and 0.028 h⁻¹.

LIPID AND FATTY ACID CONTENTS OF ISOCHRYSIS

Figure 2 shows the fatty acid profiles obtained with different concentrations of sodium nitrate contained in the F/2 culture medium. Fatty acids content increased at low concentrations of sodium nitrate and even more at high concentrations, especially at 160 mg L⁻¹ sodium nitrate, reaching values of 63 mg g⁻¹ lyophilized PUFA C18>2.

Lipid content increased in relation to dry weight both at low and high nitrate concentrations with respect to the normal medium (75 mg L⁻¹ sodium nitrate), as shown in Figure 2. Saturated (42%) and monounsaturated fatty acid (40%) contents were higher at low concentrations (20 mg L⁻¹ of sodium nitrate). With high concentrations of sodium nitrate, monounsaturated fatty acids (MUFA) were not detected; saturated fatty acids were 29% and PUFAs were 71%, of which the most important fatty acids were 18:> 2 and 22: 6ω3. The ratio of saturated fatty acids to total lipids was greatest for the lowest nitrate concentration (20 mg L⁻¹), reaching 42%, which decreased to 39% (at 40 mg L⁻¹), 35% (at 75 mg L⁻¹), and 29% (at 160 and 320 mg L⁻¹).

Protein concentration gradually increased as nitrate availability increased in the culture medium (Fig. 2). Protein content increased from 23% (at 20 mg L⁻¹) to 56% lyophilized weight (at 320 mg L⁻¹) in relation to nitrate concentration in the culture medium.

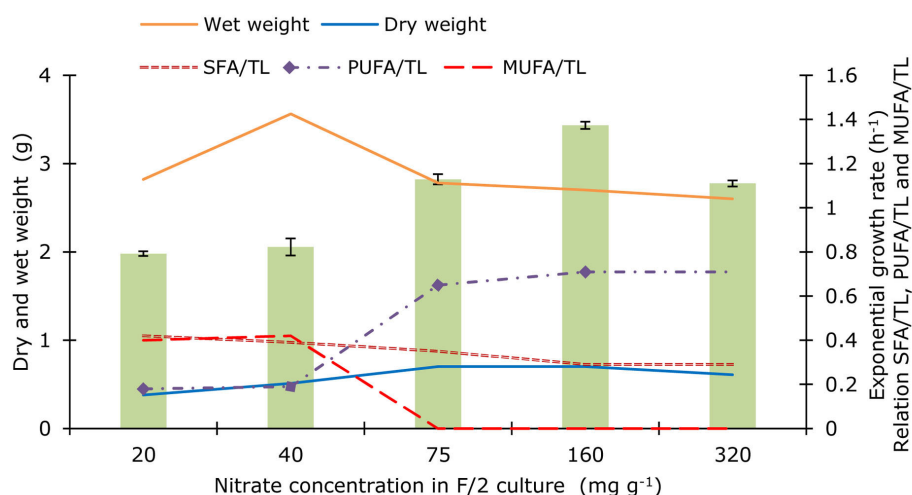


Figure 1. Biomass, number cells mL⁻¹, and relation SFA/TL, PUFA/TL and MUFA/TL at the finishing of exponential growth rate in *Isochrysis galbana* (T-ISO) (7th day of cultivation) / Biomasa, número de células mL⁻¹ y relación SFA/TL, PUFA/TL y MUFA/TL al finalizar la tasa de crecimiento exponencial en *Isochrysis galbana* (T-ISO) (7^o día de cultivo)

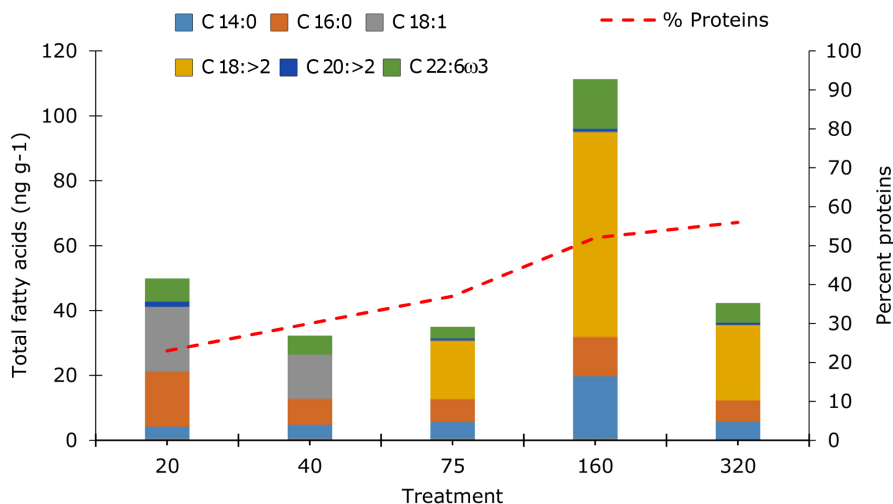


Figure 2. Distribution of total fatty acids and percent of total protein content for each nitrate concentration in *Isochrysis galbana* (T-ISO) in the culture medium: It is indicated in each bar, the fatty acid composition for each treatment / Distribución de ácidos grasos totales y porcentaje del contenido de proteína total para cada concentración de nitrato en *Isochrysis galbana* (T-ISO) en el medio de cultivo: En cada barra se indica la composición de ácidos grasos para cada tratamiento

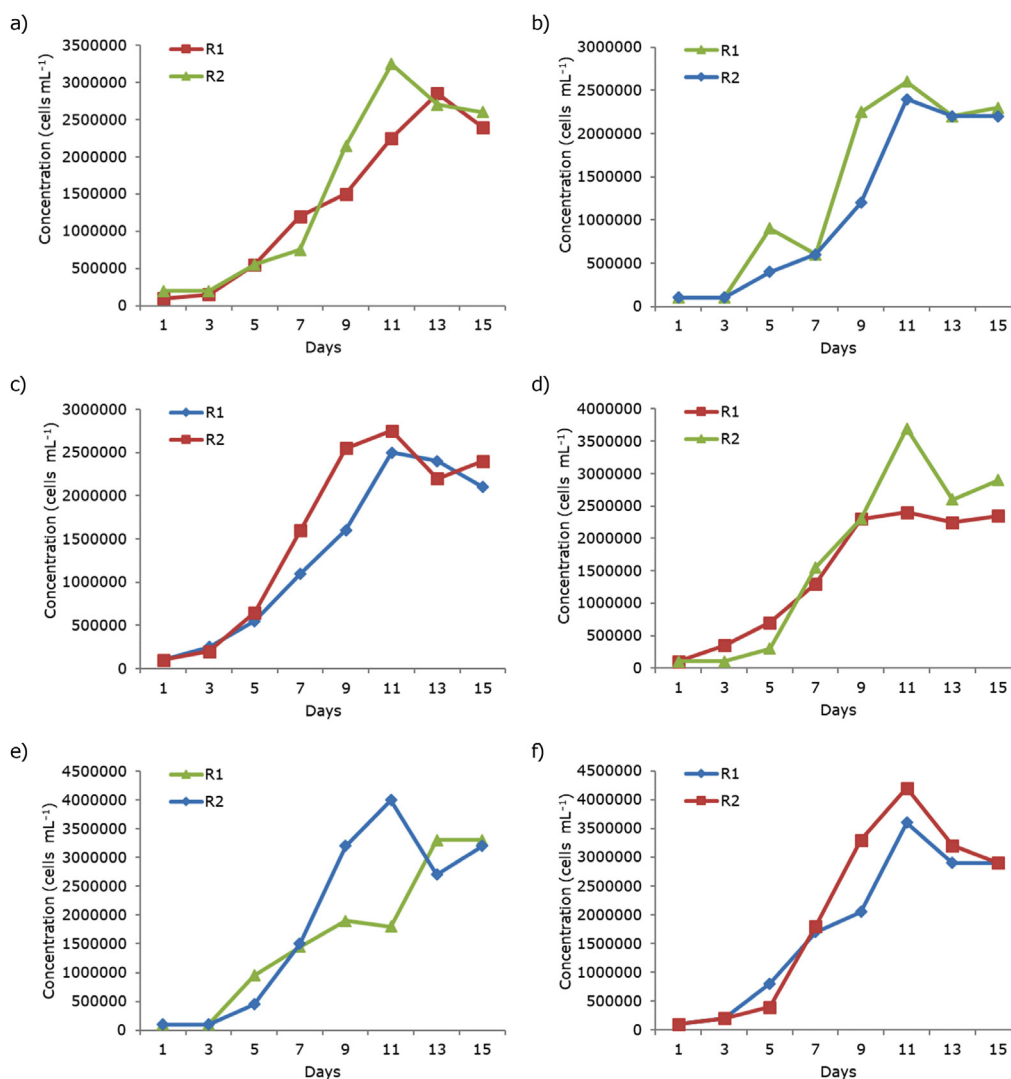


Figure 3. Growth curves of *Chaetoceros gracilis* cultures obtained from two replicates (R1, R2) at different experimental conditions of irradiance and sodium nitrate concentrations. Irradiance $30 \mu\text{E m}^{-2} \text{s}^{-1}$ (a, b); $60 \mu\text{E m}^{-2} \text{s}^{-1}$ (c, d); $100 \mu\text{E m}^{-2} \text{s}^{-1}$ (e, f). Sodium nitrate concentration 75 mg mL^{-1} (a, c, e); 37.5 mg mL^{-1} (b, d, f) / Curvas de crecimiento de cultivos de *Chaetoceros gracilis* obtenidas de dos réplicas (R1, R2) en diferentes condiciones experimentales de intensidad lumínica $30 \mu\text{E m}^{-2} \text{s}^{-1}$ (a, b); $60 \mu\text{E m}^{-2} \text{s}^{-1}$ (c, d); $100 \mu\text{E m}^{-2} \text{s}^{-1}$ (e, f). Concentración de nitrato de sodio de 75 mg mL^{-1} (a, c, e); $37,5 \text{ mg mL}^{-1}$ (b, d, f)

CHAETOCEROS GRACILIS GROWTH

During the exponential growth phase, we observed that cultures at low light intensity grew slower than those subjected to high intensity, regardless of nitrate concentration in the culture medium (Fig. 3a, c, e). During the final part of the exponential phase, culture growth rate tended to stabilize. On the eleventh day of culture, the stationary phase of growth started, with concentrations ranging from $2.5 \cdot 10^6$ cells mL^{-1} . Cultures maintained at $100 \mu\text{E m}^{-2} \text{s}^{-1}$ reached greater cell density (Fig. 3e, f). There were no statistically significant differences ($P < 0.05$) in culture growth rates for the two nitrate concentration levels (Fig. 3 a-f).

CHAETOCEROS CONSTRICTUS GROWTH

During the exponential phase, cultures maintained at $30 \mu\text{E m}^{-2} \text{s}^{-1}$ presented a growth rate lower than $100 \mu\text{E m}^{-2} \text{s}^{-1}$ (Fig. 4a-d). There were no significant differences between nitrate concentration levels for the same light intensity. The highest density reached was $62 \cdot 10^4$ cells mL^{-1} under high irradiance and normal nitrate levels (Fig. 4c).

EFFECT OF SPECIES ON LIPID PRODUCTION IN CHAETOCEROS SPP.

Both species of *Chaetoceros*, in all conditions tested, produced on average, high proportions of the monounsaturated fatty acids C 16:1, followed by C 14:00, and then C 20:6.

C. gracilis produced more unsaturated fatty acids than *C. constrictus*, as the ratio of unsaturated/saturated fatty acids showed in Figure 5.

Total content of the main fatty acid groups for *C. gracilis* are shown in Figure 6 and *C. constrictus* in Figure 7. ANOVA of unsaturated C 18 (MUFA+PUFA) indicated that factor irradiance was statistically significant ($P = 0.0055$), as well as irradiance*nitrate ($P = 0.0189$), irradiance*sp ($P = 0.0005$), nitrate*sp ($P = 0.0405$). ANOVA of unsaturated C 22 (PUFA) only presented statistically significant difference ($P = 0.0516$) when compared to irradiance*nitrate. In *C. gracilis*, the predominance of SFA is observed only when culture conditions were at normal irradiance. High irradiance allowed the accumulation of both unsaturated C 20 and C 22, with culture at normal nitrate F/2 (Fig. 6).

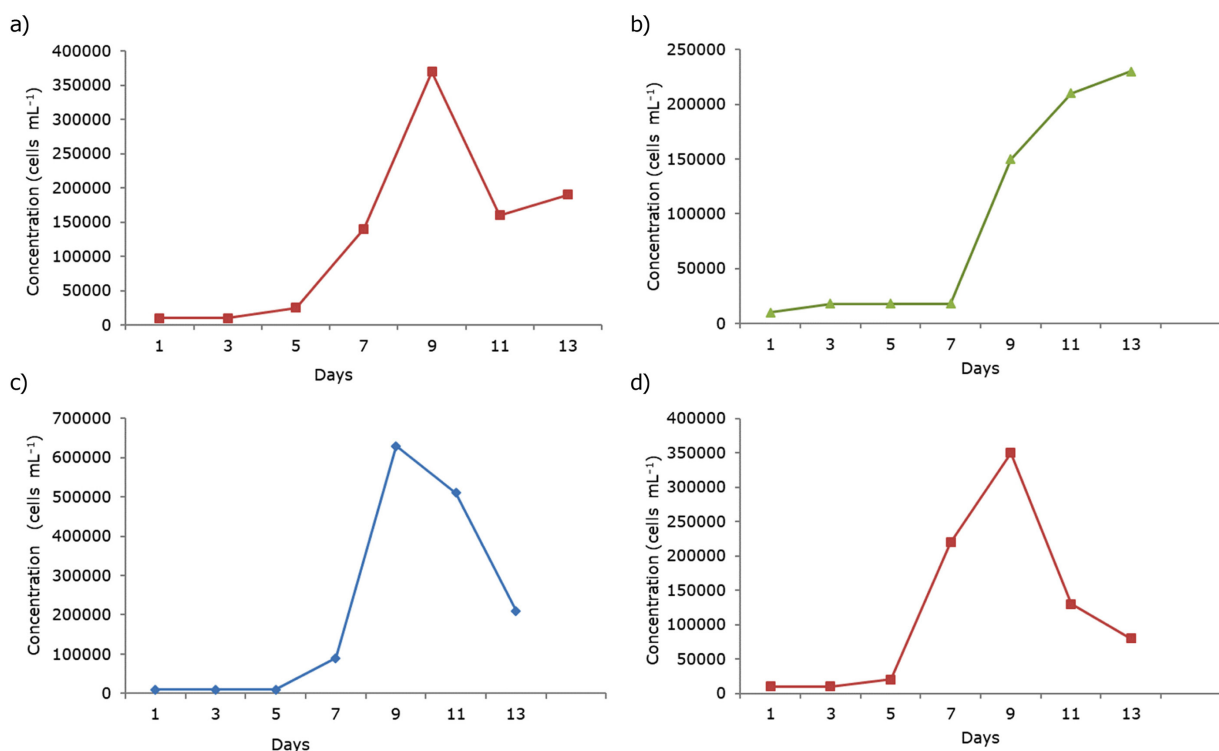


Figure 4. Growth curves of *Chaetoceros constrictus* cultures obtained at different experimental conditions of irradiance and sodium nitrate concentrations. a) Irradiance $30 \mu\text{E m}^{-2} \text{s}^{-1}$ (a, b); $100 \mu\text{E m}^{-2} \text{s}^{-1}$ (c, d). Sodium nitrate concentration 75 mg mL^{-1} (a, c); 37.5 mg mL^{-1} (b, d) / Curvas de crecimiento de cultivos de *Chaetoceros constrictus* obtenidas en diferentes condiciones experimentales de intensidad luminica y concentración de nitrato de sodio. Intensidad luminica $30 \mu\text{E m}^{-2} \text{s}^{-1}$ (a, b); $100 \mu\text{E m}^{-2} \text{s}^{-1}$ (c, d). Concentración de nitrato de sodio de 75 mg mL^{-1} (a, c); $37,5 \text{ mg mL}^{-1}$ (b, d)

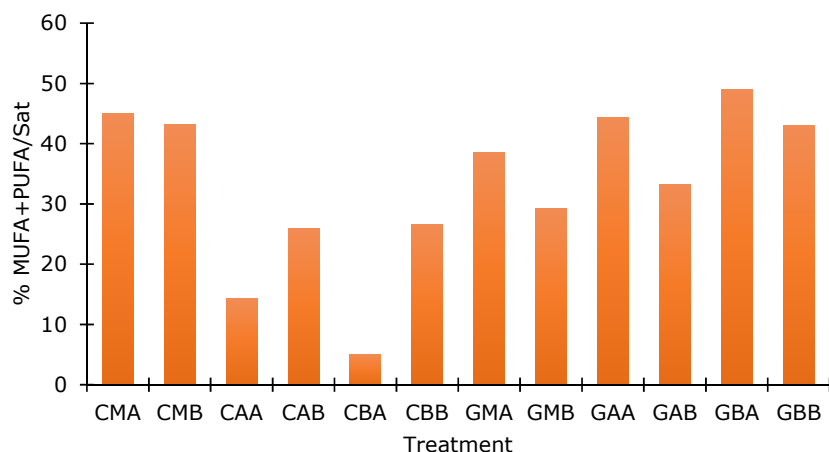


Figure 5. Mean Percentage MUFA+PUFA/Saturated fatty acids in *Chaetoceros constrictus* (CMA, CMB, CAA, CAB, CBA, CBB) and *Chaetoceros gracilis* (GMA, GMB, GAA, GAB, GBA, GBB) for each treatment according to Table 1 / Promedio del porcentaje de MUFA+PUFA/Acidos grasos saturados en *Chaetoceros constrictus* (CMA, CMB, CAA, CAB, CBA, CBB) y *Chaetoceros gracilis* (GMA, GMB, GAA, GAB, GBA, GBB) para cada tratamiento según la Tabla 1

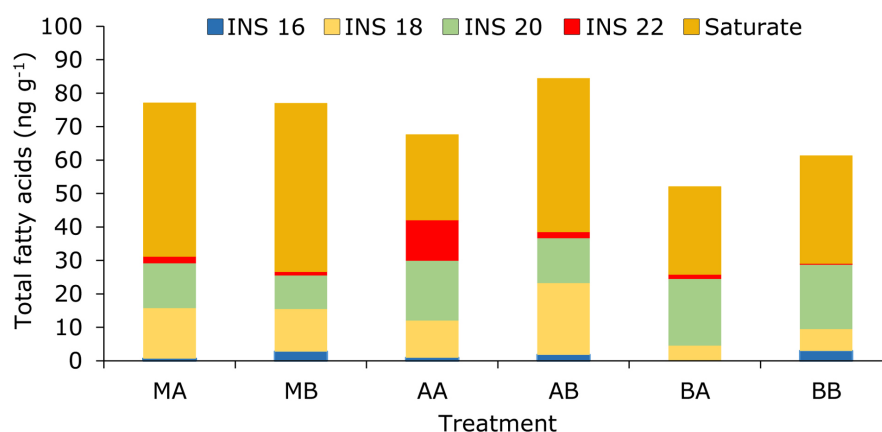


Figure 6. Distribution of main groups of fatty acid content in each treatment, in *Chaetoceros gracilis*. Unsaturated fatty acids (MUFA+PUFA) C16, C18, C20, C22 and SFA (saturated fatty acids C14, C16, C18, C20, C22). MUFA: monounsaturated fatty acid. PUFA: polyunsaturated fatty acids, chains of fatty acids with two or more double bond between carbons. MA: moderate or normal irradiance, and normal or high sodium nitrate in F/2 culture medium, MB: moderate or normal irradiance, and low sodium nitrate in F/2 culture medium, AA: high irradiance, and normal or high sodium nitrate in F/2 culture medium, AB: moderate or normal irradiance, and low sodium nitrate in F/2 culture medium, BA: low irradiance, and normal or high sodium nitrate in F/2 culture medium, BB: low irradiance and low sodium nitrate in F/2 culture medium / Distribución de los principales grupos de contenido de ácidos grasos en cada tratamiento, en *Chaetoceros gracilis*. Ácidos grasos insaturados (MUFA + PUFA) C16, C18, C20, C22 y SFA (ácidos grasos saturados C14, C16, C18, C20, C22). MUFA: ácido graso monoinsaturado. PUFA: ácidos grasos poliinsaturados, cadenas de ácidos grasos con dos o más enlaces dobles entre el carbono. MA: intensidad lumínica normal o moderada y concentración normal o alta de nitrato de sodio en el medio F/2, MB: intensidad lumínica normal o moderada y concentración baja de nitrato de sodio en el medio F/2, AA: intensidad lumínica alta y concentración normal o alta de nitrato de sodio en el medio F/2, AB: intensidad lumínica normal o moderada y concentración baja de nitrato de sodio en el medio F/2, BA: intensidad lumínica baja y concentración normal o alta de nitrato de sodio en el medio F/2, BB: intensidad lumínica baja y concentración baja de nitrato de sodio en el medio F/2

In general, *C. constrictus* presented a predominance of saturated fatty acids (SFA) over unsaturated (Fig. 7).

The production of C 16:3 fatty acids was higher in *C. constrictus* than *C. gracilis* for both low and high nitrate concentrations, with statistical significance ($P < 0.0001$).

According to the results, there are statistically significant differences between fatty acid production levels when comparing C 16:3 fatty acids, with higher concentrations observed in *C. constrictus* than *C. gracilis* ($P = 0.0063$). The

contrary occurs with C 18:1; *C. gracilis* produced higher fatty acid concentrations in high nitrate concentration ($P = 0.0043$) (Fig. 8a, b). Unsaturated C 18 was higher in *C. gracilis* than *C. constrictus* (statistically significant, $P < 0.0001$).

In relation to the low, medium and high irradiance levels tested, the values observed in *C. constrictus* were higher than in *C. gracilis* for C 16:3 fatty acids, as in the case of nitrate concentration. C 18:1 and C 20:5 values were higher

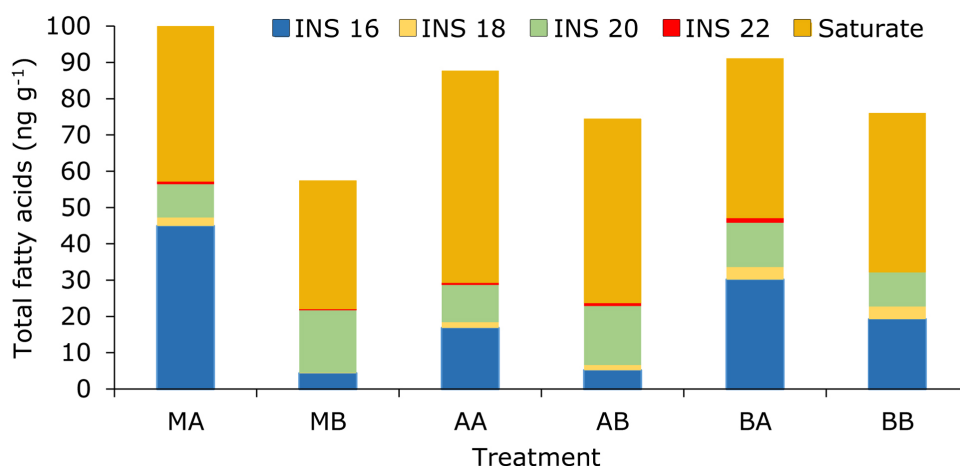


Figure 7. Distribution of main groups of fatty acid content in each treatment, in *Chaetoceros constrictus*. Unsaturated fatty acids (MUFA+PUFA) C16, C18, C20, C22 and SFA (saturated fatty acids C14, C16, C18, C20, C22). Abbreviations according to Figure 6 / Distribución del contenido de los principales grupos de ácidos grasos en cada tratamiento, en *Chaetoceros constrictus*. Ácidos grasos insaturados (MUFA + PUFA) C16, C18, C20, C22 y SFA (ácidos grasos saturados C14, C16, C18, C20, C22). Abreviaturas según la Figura 6

in *C. gracilis* than *C. constrictus*. In *C. constrictus*, fatty acid concentration increased with greater irradiance, whereas in *C. gracilis* it tended to decrease.

Fatty acid distribution identified in each treatment for both species of *Chaetoceros* is presented in Figure 8a, b, where one can see the differences in fatty acid composition for each treatment, as well as differences between species.

FATTY ACIDS PRODUCTION OF *CHAETOCEROS GRACILIS*

With irradiance and nitrate concentrations, *C. gracilis* produced similar concentrations of saturated and unsaturated fatty acids of C16 (C16:2), C18 (C18:2) and C20 (C20:5), as well as reduced concentrations of unsaturated C22 (C22:6) fatty acids (Fig. 8a).

Under stress conditions (low nitrate concentration) at normal irradiance, total lipids and saturated fatty acids increased proportionally, maintaining the concentration of unsaturated C16 (C16:1, C16:2, C16:3), and decreasing fatty acids C18 (C18:1, C18:3), C20 (C20:5) and C22 (C22:6). C14 and C16 saturated fatty acids increased their concentration. Although absent under normal conditions, C18 stearic acid accumulated under stress conditions.

At high intensity light conditions, the difference in total lipid concentrations between normal and stress conditions was less than at normal light intensities. Under normal nitrate conditions, unsaturated fatty acids C16 (C16:1, C16:3, C16:2), C18 (C18:1, C18:3), C20 (C20:5) and especially C22 (C22:5, C22:6) increased, yet total saturated fatty acid concentrations remained stable. Myristic acid levels increased, palmitic acid decreased, and stearic acid

appeared. Due to low nitrate concentrations, under stress conditions, myristic, palmitic, stearic and saturated fatty acids increased. As well as arachidic and behenic fatty acids and C18 unsaturated fatty acids (C18:2 fatty acid appeared); C16 unsaturated fatty acids decreased drastically (C16:1 and C16:2 appeared, yet C16:3 disappeared), along with C20 and unsaturated C22 (C22:5 disappeared, C22:6 decreased) (Figure 8a).

At low light intensity, total lipid production is almost equal, yet slightly lower, at normal nitrate concentrations. Under normal nitrate conditions there was less saturated fatty acids and unsaturated C18 production (under normal nitrate conditions oleic acid is produced and under stress conditions it decreases and linolenic appears), but increased unsaturated C22 (C22:6) than in stress conditions. Specifically, in normal nitrate conditions, there is higher myristic and less palmitic, stearic and behenic production. The main difference is in palmitic acid production. Total C20 unsaturated fatty acids remained unchanged; C20:5 is produced under normal conditions, which decreases under stress conditions, generating C20:4. Under stress conditions, palmitoleic acid decreases slightly.

FATTY ACIDS PRODUCTION OF *CHAETOCEROS CONSTRICTUS*

Under normal irradiance, normal nitrate concentration, and stress conditions, there were similar total lipid concentrations (Fig. 8b). Under normal conditions, there were equivalent unsaturated C16 concentrations (with predominance of C16:1 and lower concentration of C16:3). Total saturated fatty acids (with similar concentrations of

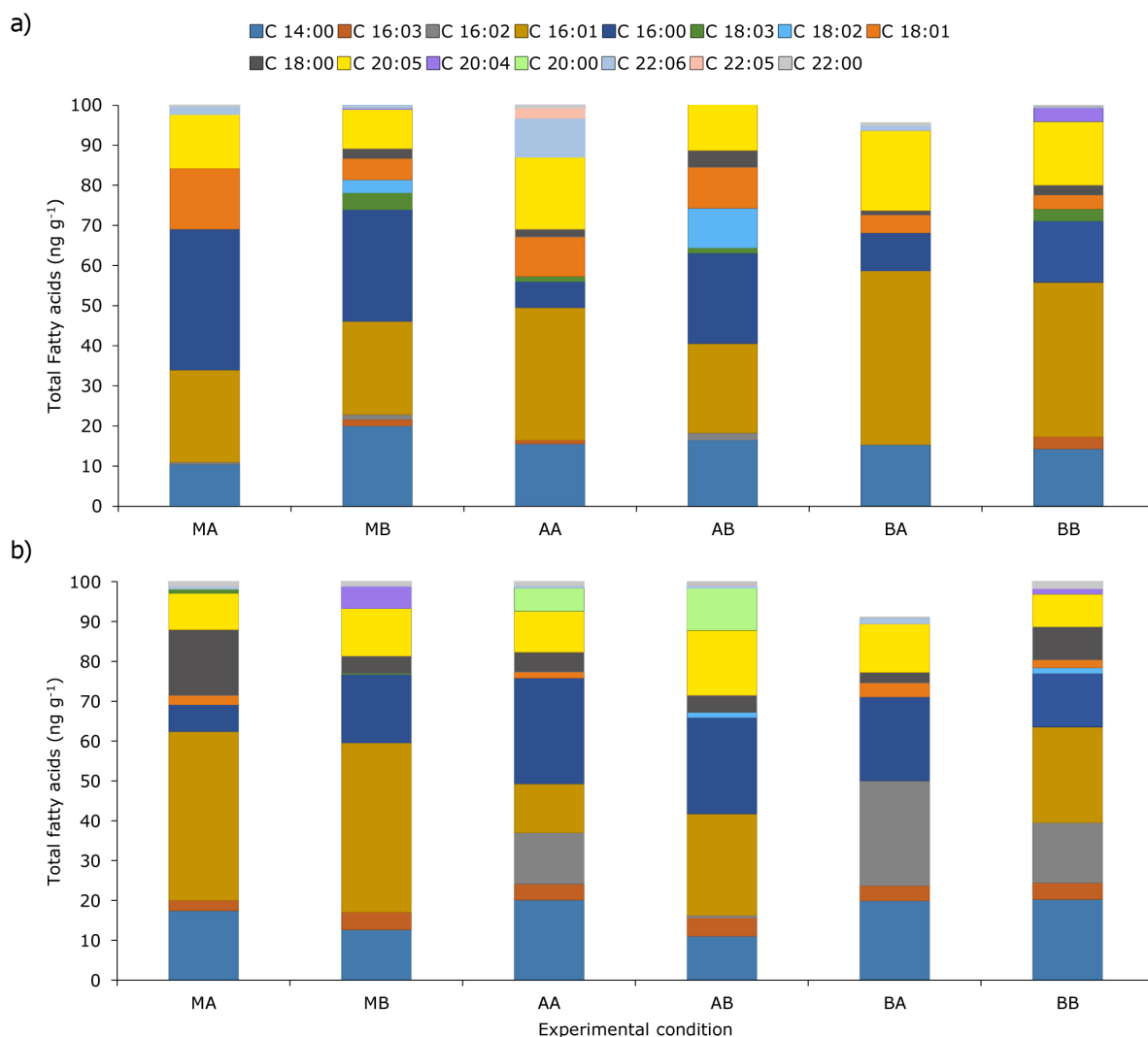


Figure 8. Distribution of fatty acid content in each treatment, identify each fatty acid with carbon number and the number of double bond in the molecule in: a) *Chaetoceros gracilis* and b) *Chaetoceros constrictus*. Abbreviations according to Figure 6 / Distribución del contenido de ácidos grasos en cada tratamiento, identifica cada ácido graso con el número de carbonos y el número de dobles enlaces en la molécula en: a) *Chaetoceros gracilis* y b) *Chaetoceros constrictus*. Abreviaturas según la Figura 6

myristic and stearic acid) were increased. Less palmitic and much less arachidic and behenic acids were observed, as well as reduced unsaturated C18 (C18:1), C20 (C20:5), and C22 (C22:6) concentrations.

Under stress conditions, we observed differences in unsaturated C16 (similar C16:1 and higher C16:3 concentrations than normal nitrate conditions) and C20 (higher C20:5 concentration than in normal conditions; C20:4 appeared), furthermore there were no unsaturated C18 and C22 fatty acids. The myristic and stearic saturated fatty acids decreased, C22 levels were maintained and palmitic acid concentration increased (Fig. 8b).

In conditions of high irradiance in *C. constrictus*, there was a greater diversity of fatty acids present, with total lipid concentrations similar to normal irradiance, with saturated fatty acids predominating over unsaturated fatty acids; the

latter increased under stress conditions. At normal nitrate concentrations, myristic and palmitic acids were present, which decrease their concentration under stress conditions, and arachidic acid is increased. Stearic acid concentration remained the same under both conditions. Unsaturated C16:1 increased in stress conditions, C16:2 decreased and C16:3 remained the same. Unsaturated C18:1 appeared under normal conditions and C18:2 under stress conditions; C20:5 increased under stress conditions and traces of C22:5 were observed.

With low irradiance and normal nitrate concentrations, there was some decrease in total lipids; however, under stress conditions total lipids were higher and very similar to those obtained in the previous conditions. Saturated fatty acids under normal conditions were similar to those obtained under stress conditions. Myristic acid maintained its levels in both conditions. Palmitic acid decreased under

stress conditions, whereas stearic acid and behenic acid increased. Unsaturated C16 (C16:3 and C16:2 decreased; C16:1 concentration was the highest) was higher than C18 (in normal conditions C18:1 only decreased under stress conditions and C18:2 appeared in equal concentration) and C20:5, which is higher under normal conditions. Unsaturated C22:6 were very low and practically did not appear under stress conditions, being replaced by C20:4.

FATTY ACIDS PRODUCTION OF *CHAETOCEROS GRACILIS* AND *CHAETOCEROS CONSTRICTUS*

In *C. gracilis* there was no C18:0 produced under conditions of moderate illumination and high nitrate concentration, while in *C. constrictus* the range was 5.93-26.9 ng g⁻¹. In *C. constrictus* there was no C18:1 produced under conditions of high and moderate irradiance and low nitrate concentrations, while in *C. gracilis* the range was 0-10.62 and 0-20.55 ng g⁻¹, respectively. In *C. gracilis* there was no C16:3 produced in high nitrate concentrations and low to moderate irradiance,

nor in low nitrate concentration with high irradiance, whereas in *C. gracilis* the range was 0.36-7.25, 2.31-2.68, and 4.4-4.86 ng g⁻¹, respectively. In *C. constrictus* there was no C22:6 produced, with low irradiance and low nitrate concentration, while in *C. gracilis* the range was 0-0.51 ng g⁻¹. The production of polyunsaturated fatty acids is favoured by high irradiance in *C. constrictus* and *C. gracilis*, and when low irradiance and low sodium nitrate concentration were combined in *C. gracilis* (Fig. 5).

The overall statistical analysis of the combined effect of irradiance and sodium nitrate concentration on fatty acid concentration produced species-specific responses.

While in *C. gracilis* significant positive or negative correlations occur between fatty acids of lower carbon numbers (myristic acid) and those with higher carbon numbers, in *C. constrictus* myristic acid present no correlation with other fatty acids, acids of C:16 or C:18 (Fig. 9a, b).

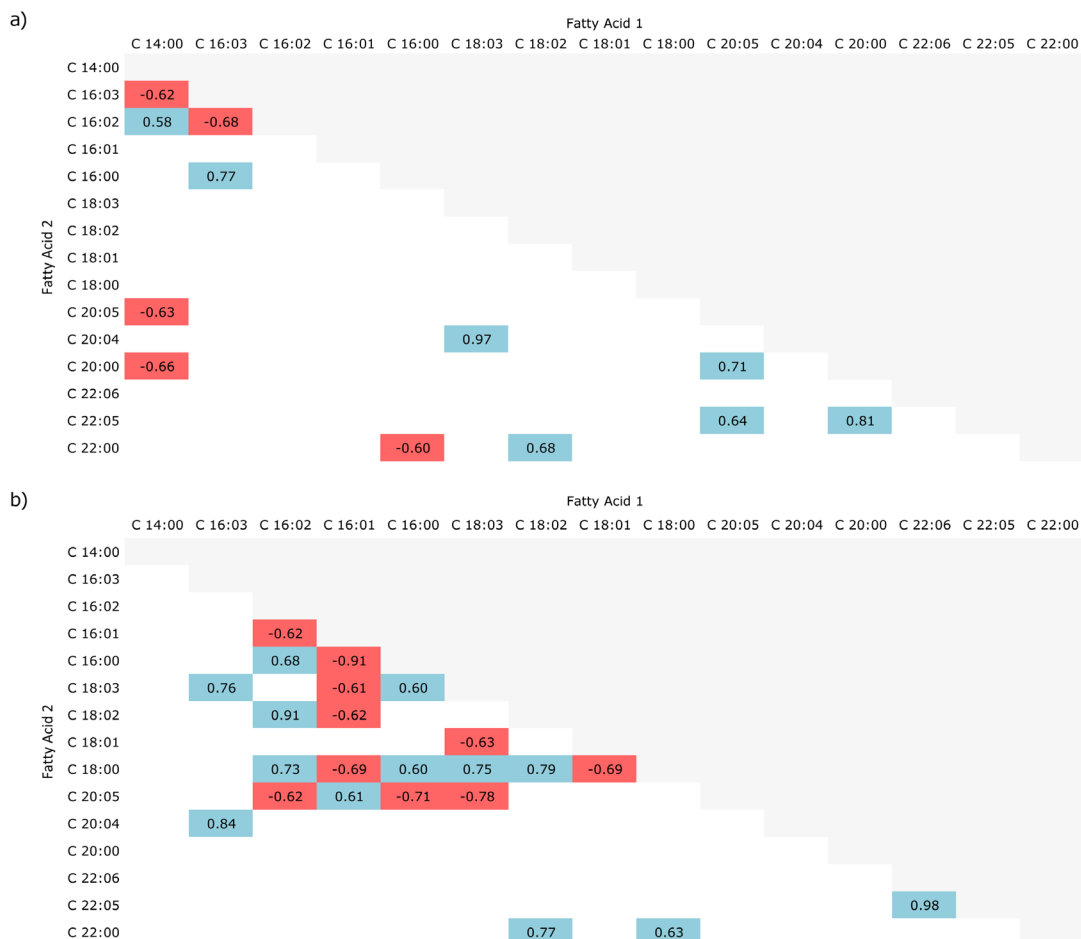


Figure 9. Matrix showing Pearson Correlation Coefficient significant statistically between fatty acids pairs, both in the experimental control in: a) *Chaetoceros gracilis* and b) *Chaetoceros constrictus*. Positive coefficients between pairs are in blue and the negative coefficients in red / Matriz que muestra el coeficiente de correlación de Pearson estadísticamente significativo entre los pares de ácidos grasos, tanto en el control experimental como en: a) *Chaetoceros gracilis* y b) *Chaetoceros constrictus*. Los coeficientes positivos entre pares están en azul y los negativos en rojo

Principal component analysis revealed that the metabolic pathway responses to different irradiance conditions and nitrate concentration levels were different for PUFA, MUFA, SAT, EPA and DHA production (Fig. 10; Table 2). The best correlation that explains CP1, with 49% variance in Figure 10, is shown by unsaturated C20 (Table 2). In Figure 10, EPA, presented the best correlation with CP1.

During correlation analysis between fatty acid concentrations in the different test conditions, there are positive correlations between pairs of fatty acids, which implies a metabolic pathway whereby increases in one of the fatty acids engender increases in the other, or vice versa when the correlation is negative (Figs. 9a, b).

Table 2. Correlation between original variables and the first two principal components in PCA analysis shown in Figure 6 / Correlación entre las variables originales y los dos primeros componentes principales en el análisis de PCA mostrados en la Figura 6

Lipid content	Variables label	PCA1	PCA2
Unsaturated C16	Ins 16	-0.7501	-0.3311
Unsaturated C18	Ins 18	0.3652	0.8758
Unsaturated C20	Ins 20	0.8522	-0.3809
Unsaturated C22	Ins 22	0.6562	0.1683
Saturated FA	Saturate	-0.7879	0.4494
EPA	C 20:05	0.848	-0.286
DHA	C 22:06	0.729	0.008

Cophenetic correlation= 0.937

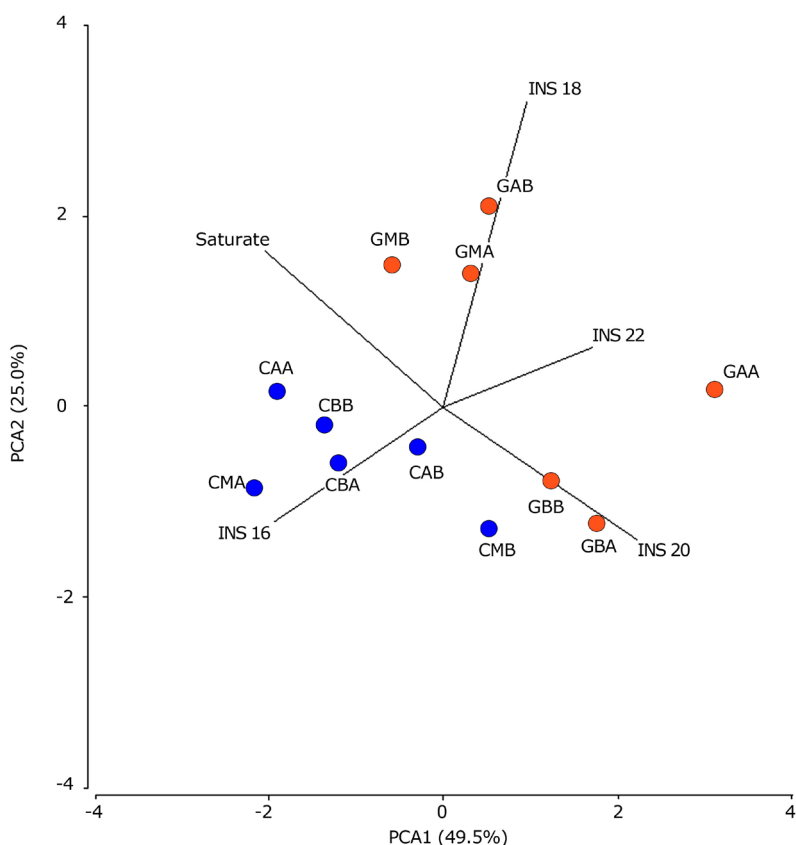


Figure 10. Vector diagram indicating the loading on PCA1 and PCA2 of biochemical composition of *Chaetoceros constrictus* (blue dot) and *Chaetoceros gracilis* (orange dot) for each treatment. Cophenetic correlation index= 0.94 / Diagrama vectorial que indica la carga en PCA1 y PCA2 de la composición bioquímica de *Chaetoceros constrictus* (punto azul) y *Chaetoceros gracilis* (punto naranja) para cada tratamiento. Índice de correlación cofenética= 0,94

EICOSAPENTAENOIC ACID PRODUCTION

Eicosapentaenoic acid production in both species of *Chaetoceros* is presented in Figure 11a for *C. gracilis* and Figure 11b for *C. constrictus*. In the same graph, one can observe the biomass (wet and dry weight) for each treatment. In *C. gracilis*, both high and low irradiance and normal nitrate concentrations produced higher levels of EPA than normal irradiance and nitrate levels. In *C. constrictus*, high irradiance and low nitrate concentration (stress) produced higher EPA concentration than the control.

DISCUSSION

Photosynthetic organisms can produce the total polyunsaturated fatty acids that they need for living (Guschina & Harwood 2009). Heterotrophs can get it from food, their different metabolic pathways can modify fatty acids by desaturases and elongases, given rise to a diversity of new compounds necessary for growth (Brett & Müller-Navarra 1997, Fang *et al.* 2004, Ho *et al.* 2007, Monroig *et al.* 2013), and therefore they are essential nutrients for aquaculture. Colombo *et al.* (2018), in a recent review, concluded that omega n-3 long chain PUFA content is

dependent on taxonomy (Galloway & Winder 2015) and latitude. Low temperatures engender increased unsaturated fatty acid content to improve cell membrane fluidity (Sasaki *et al.* 2006, Bell *et al.* 1986) and maintain cellular homeostasis. According to Cohen *et al.* (2000), PUFA are rapidly mobilized from triacylglycerides that function as a depot to respond to low temperature stress and high pressure, as demonstrated by Fang *et al.* (2004). Also in ovigerous females of crustacean *Pleuroncodes monodon* was found that the fatty acids composition varies according seasonally (Bascur *et al.* 2017).

Isochrysis galbana produced higher levels of linolenic acid and docosahexaenoic acid than eicosapentaenoic acid (EPA) at low N-nitrate concentration however, fatty acids increased notably with surplus N-nitrate, above F/2 medium content. Besides, *Isochrysis zhangjiangensis* can accumulate lipids under nitrogen-repletion conditions and carbohydrates under nitrogen-depletion conditions (Feng *et al.* 2011). Regarding protein content, an increase was found in relation to high nitrate content in the medium. These findings are in accordance with those reported for diatoms by De La Peña *et al.* (2007) and Yodsuwan *et al.* (2017)

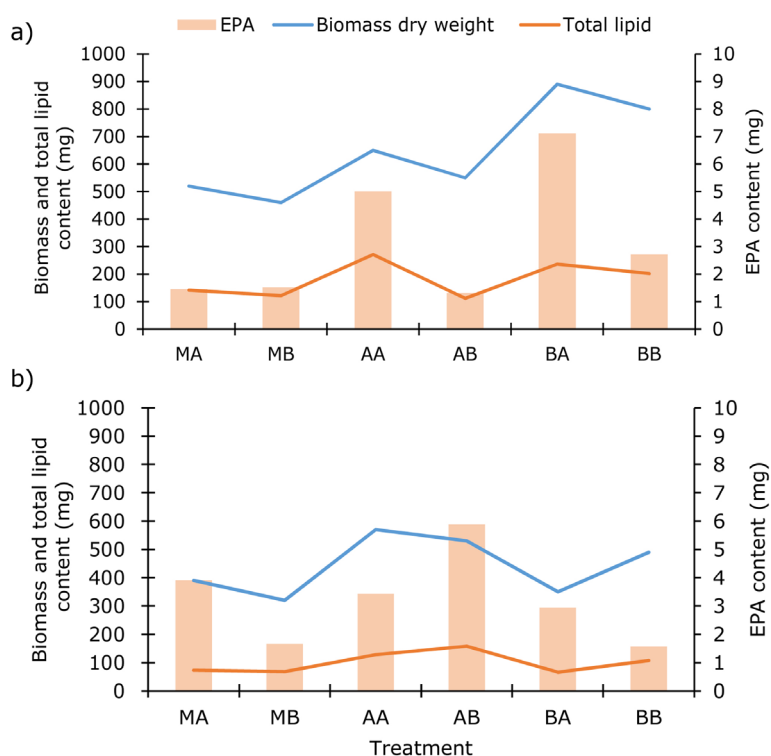


Figure 11. Total lipid content, dry weight of biomass and production of eicosapentaenoic acid in each treatment in: a) *Chaetoceros gracilis* and b) *Chaetoceros constrictus* / Total de lípidos, peso seco de la biomasa y producción de ácido eicosapentaenoico en cada tratamiento en: a) *Chaetoceros gracilis* y b) *Chaetoceros constrictus*

and for *Chlorella vulgaris* (Chen *et al.* 2015). Caporgno & Mathys (2018) suggest that microalgae are a new source of proteins for feeding. Moreover, Sukenik & Livne (1991) found in *Isochrysis galbana*, that limited nitrogen in the culture medium correlated with acetyl CoA carboxylase and malonyl CoA synthesis, which are necessary for fatty acid production.

As average irradiance increases, saturated and monounsaturated fatty acid percentages decreased, increasing the proportion of EPA. By taking into account the different relations between EPA content and irradiance, EPA productivity variation over the year can be simulated as a function of average and external irradiance. Irradiance was selected according to previous studies, which concluded that low intensity favors PUFA production (Grima *et al.* 1992, Solovchenko *et al.* 2008, Khoeyi *et al.* 2012).

In this study, the increase in DHA concentration observed under 160 mg L⁻¹ sodium nitrate concentration, could be explained by Huerlimann *et al.* (2014), who found that the plastidial acetyl CoA carboxylase enzyme is stimulated in *I. aff. galbana* (during the logarithmic phase) in cultures with sodium nitrate overload. This was not observed for *Chaetoceros* species, where the main concentrations were under limited nitrate conditions, corroborating that this is a species-specific response (Huerlimann *et al.* 2014).

In the present study, there is some evidence of specific differences in the results. According to Napolitano *et al.* (1997), high C14:0, C16:4 and C20:5 concentrations are due to a diatom in the phytoplankton. Mortensen *et al.* (1988) found the same results in *Chaetoceros gracilis* at temperature of 18 and 20°C in batch cultures. In both *Chaetoceros* species, we observed correlations between fatty acid pairs; these results suggest that there are different metabolic pathways for the factors (irradiance and nitrate) tested. It bears noting that our experiments were performed at lower temperatures than those reported in the literature (Renaud *et al.* 2002, van Wagenen *et al.* 2012, Miller *et al.* 2014).

In addition to acclimatization, *C. constrictus* was isolated from environmental samples taken in Coliumo Bay (Dichato); *Chaetoceros* spp. are an important quantitative component in phytoplankton composition, especially during spring (August to November) (Rivera 1969) in an upwelling event (González *et al.* 1987).

Thus, whereas in *C. gracilis* myristic acid (C14:0) is negatively correlated with hexadecatrienoic acid (C16:3), eicosanoic acid (C20:0), eicosapentaenoic acid (C20:5) and docosapentaenoic acid (C22:5), in *C. constrictus* palmitic acid (C16:0) is negatively correlated with palmitoleic acid (C16:1), oleic acid (C18:1) and eicosapentaenoic acid (C20:5), yet positively correlated with stearic acid (C18:0), hexadecadienoic acid and linolenic acid (C18:3).

High irradiation conditions favored saturated and unsaturated C16 and saturated and monounsaturated C18 fatty acid production, a metabolic pathway which occurs specifically in the chloroplast; these fatty acids can be stored in adverse growth conditions (Hu *et al.* 2008).

Variations in irradiance will affect the chloroplast metabolism, and therefore fatty acid biosynthesis in the prokaryotic chloroplast pathway (Mühlroth *et al.* 2013). Therefore, it can exclude docosapentaenoic acid, linolenic acid, eicosanoic acid, and eicosapentaenoic acid, which come from the endoplasmic reticulum, and were synthesized due to the stress caused by low nitrate availability in the medium (Hu *et al.* 2008).

In this study, we found that saturated fatty acid concentrations increased considerably in *C. constrictus* with high irradiation, although they were slightly lower in the case of limited nitrate in the culture medium when compared to normal nitrate concentrations. In normal nitrate conditions, twice as much myristic fatty acid content was produced than in reduced nitrate concentrations. PUFA+MUFA production at low irradiance was lower than normal irradiance. Some species, such as *Chlorella vulgaris*, *Chromochloris zofingiensis*, *Ettlia oleoabundans*, and *Tetrademus obliquus*, can accumulate approximately 35% of their dry weight as triacylglycerols in poor nitrogen conditions (Breuer *et al.* 2012), palmitic acid and oleic acid predominating. Similarly to Breuer *et al.* (2012), recorded growth rate and biomass were highest for *C. constrictus* at high irradiation conditions. Hu *et al.* (2008) found that many microalgae could accumulate large quantities of lipids as TAGs under environmental stress conditions (*e.g.* nitrogen starvation). They explained that TAGs are energy and carbon storage compounds when photosynthesis exceeds growth requirements (Hu *et al.* 2008). EPA is mainly obtained from fish oil, but microalgae such *C. constrictus* could also be a good source – with high irradiation and low nitrate concentrations in the F/2 medium – of oils with a simpler composition than fish oil, as demonstrated with *Phaeodactylum tricornutum* (Domergue *et al.* 2003, Haro *et al.* 2017).

The findings presented in this paper answered the hypothesis, which regarded the culture conditions that engender increased unsaturated fatty acids in *Isochrysis galbana*, *Chaetoceros gracilis* and *C. constrictus* in batch conditions. Different profiles of fatty acids were obtained at different irradiance and nitrate concentration, both in *Chaetoceros* sp. and *Isochrysis galbana* given the opportunity to a hatchery manager to bring the best mix for feeding. Cerpa *et al.* (2003) reported on a mix of microalgae for *Argopecten purpuratus* broodstock conditioning with 4 treatments in a hatchery and at the sea as control. On the other hand, larval bivalve feeding composed of *Chaetoceros calcitrans* and *Isochrysis galbana* (1:1) would achieve

successful metamorphosis due to a balanced diet based on fatty acid composition (Farias *et al.* 2003, Rico-Villa *et al.* 2006, Pettersen *et al.* 2010, Ragg *et al.* 2010). It was found that *Isochrysis galbana* and *Chaetoceros* spp. fatty acids profiles vary in relation to number of carbons of fatty acids (Napolitano *et al.* 1990). In this study it was shown that depends on nitrate availability and irradiance the same species could vary the concentration of some components. The results presented herein provide support to take some microalgae culture conditions into account, and optimize nutrition for both larvae and broodstock. For *I. galbana* especially, the high nitrate (160 mg L⁻¹), protein and PUFA culture is highly recommended for broodstock conditioning, since Cerpa *et al.* (2003) found reduced high molecular weight polypeptide content in the gonad of *Argopecten purpuratus* specimens raised in hatcheries compared to ocean-bred (Velasco *et al.* 2016).

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