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Mixotrophic production of polyunsaturated fatty acids and carotenoids by the microalga *Nannochloropsis gaditana*

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Abstract

Microalgae are potential sources of high-value lipids and colorants for use in foods, cosmetics, and other applications. Biomass and metabolite productivities of photoautotrophic algae cultures are low because of limited availability of light. Therefore, mixotrophic cultures were investigated in parallel with photoautotrophic controls. In mixotrophy, some of the energy and carbon are supplied in the form of dissolved organic substrates in addition to inorganic carbon and light being available. The aim was to compare productivities of biomass, fatty acids, and carotenoid pigments in outdoor and indoor mixotrophic and photoautotrophic batch and continuous cultures. The edible and safe marine microalga *Nannochloropsis gaditana* was used in these studies. The alga could be grown mixotrophically using glucose and glycerol, but not acetate. Optimal concentrations of the organic carbon sources were 5 g L^{-1} for glucose and 1 g L^{-1} for glycerol. Mixotrophy substantially increased the biomass concentration and productivity relative to photoautotrophy. The maximum biomass productivity in mixotrophic batch cultures using glucose or glycerol was identical at 170 mg L^{-1} day^{-1}, being 30% greater than control cultures. In continuous outdoor culture with glucose (5 g L^{-1}) mixotrophy at 12 °C, the total carotenoids in the biomass were 83% higher compared to photoautotrophic control biomass, and the eicosapentaenoic acid (EPA) productivity was 2.2-fold higher relative to controls. The maximum EPA productivity was 11 mg L^{-1} day^{-1}. Glucose mixotrophy increased the total lipids content in the biomass by 34% relative to photoautotrophic operation.

Keywords Eustigmatophyceae · *Nannochloropsis gaditana* · Mixotrophic growth · Polyunsaturated fatty acids · Carotenoids · Eicosapentaenoic acid

Introduction

The marine green microalga *Nannochloropsis gaditana* has been extensively studied for lipid production in photoautotrophic cultures (Ren et al. 2013; San Pedro et al. 2014; Camacho-Rodríguez et al. 2014, 2015a, 2015b; Hallenbeck et al. 2015; Pedersen et al. 2018; Sung et al. 2018) both in closed outdoor photobioreactors (Camacho-Rodríguez et al. 2014; San Pedro et al. 2014, 2016; Ledda et al. 2015; Riveros et al. 2018; Moraes et al. 2019) and in open raceway ponds (Ledda et al. 2015; San Pedro et al. 2015; López et al. 2019). Inorganic nutrients from wastewater have been used to produce the alga outdoors (Ledda et al. 2015).

In photoautotrophic cultures not limited by the supply of inorganic carbon, *N. gaditana* can accumulate more than 48% of its dry mass as lipids in nitrogen limiting conditions (Pedersen et al. 2018). This enhanced lipid accumulation under nitrogen limitation (Simionato et al. 2013; Pedersen et al. 2018) is consistent with the predictions of the photoautotrophic metabolic models of the alga (Shah et al. 2017). The main fatty acids accumulated comprise of C16:0 (palmitic acid) and C16:1 (palmitoleic acid) (Pedersen et al. 2018). In addition, *N. gaditana* is a good photoautotrophic producer of eicosapentaenoic acid (C20:5n3, EPA) (Camacho-Rodríguez et al. 2014; Janssen et al. 2019), a high-value fatty acid with numerous health benefits (Dyall 2015). *Nannochloropsis gaditana* also produces carotenoids such as violaxanthin and vaucheraxanthin (Simionato et al. 2013). Both light intensity

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and temperature have been shown to affect production of some carotenoid pigments in *N. gaditana* during photoautotrophy (Lubián and Montero 1998). Other applications of the photoautotrophically grown *N. gaditana* include its use as a well-established aquaculture feed (Ferreira et al. 2009; Riveros et al. 2018). As an emerging application, extracts of *N. gaditana* may be useful in cosmetics in view of their skin protection activity against induced oxidative stress (Letsiou et al. 2017). The genome of the alga has been fully sequenced (Schwartz et al. 2018).

Photoautotrophic growth relies exclusively on inorganic carbon (e.g., bicarbonate, CO_2) with the energy being obtained from light (Chojnacka and Marquez-Rocha 2004). Many microalgae are capable of internalizing and metabolizing an organic carbon source (Neilson and Lewin 1974; Day et al. 1991; Chojnacka and Marquez-Rocha 2004; Fang et al. 2004; Das et al. 2011; Perez-Garcia et al. 2011; Cheirsilp and Torpee 2012; Sforza et al. 2012; Cerón-García et al. 2013; Shene et al. 2016a; Bouyam et al. 2017). Such algae may be grown heterotrophically in the dark using the relevant organic carbon source for energy and carbon (Chojnacka and Marquez-Rocha 2004). Heterotrophic growth requires oxygen (Bouyam et al. 2017). In contrast to the above two modes of growth, mixotrophic growth uses both inorganic and organic carbon and light. Photosynthesis occurs together with uptake and catabolism of the organic carbon source (Chojnacka and Marquez-Rocha 2004; Perez-Garcia et al. 2011). Mixotrophic growth may or may not require oxygen, as oxygen released through photosynthesis may be sufficient for mixotrophy although the rate of consumption of the organic carbon source may be limited in the absence of an external supply of oxygen. A dense culture of a microalga growing photoautotrophically has a limited productivity as a significant volume of the culture is always in the dark (Chisti 2013a, 2013b). No photosynthesis occurs in the dark and cells consume stored organic carbon through respiration (Chisti 2013a). Productivity of mixotrophic cultures can be as high as the productivity of a heterotrophic dark culture, but rapid periodic exposure of the cells to light in a turbulent culture ensures that metabolites associated with photosynthesis (e.g., many pigments) continue to be produced. Cells subjected to dark heterotrophic growth may lack some of the metabolites they would produce under light.

Although many microalgae have been grown heterotrophically and mixotrophically (Neilson and Lewin 1974; Day et al. 1991; Fang et al. 2004; Cerón-García et al. 2006, 2013; Das et al. 2011; Perez-Garcia et al. 2011; Cheirsilp and Torpee 2012; Sforza et al. 2012; Bouyam et al. 2017), no specific information appears to exist on mixotrophic production of *N. gaditana*. How the production of EPA and carotenoids may be impacted by mixotrophy is unknown. In a few cases, unidentified strains of *Nannochloropsis* have been shown to grow mixotrophically on glucose (Fang et al. 2004; Cheirsilp and Torpee 2012) and ethanol (Fang et al. 2004). The present work is focused on mixotrophic culture of *N. gaditana* to evaluate production of lipids, EPA, and carotenoids in batch and continuous-culture operations in comparison with photoautotrophic controls.

Materials and methods

Microorganism and indoor cultures

Nannochloropsis gaditana strain B-3 was obtained from the Marine Culture Collection of the Institute of Marine Sciences of Andalucía (CSIC, Cádiz, Spain). Stock cultures were maintained photoautotrophically in 1 L Erlenmeyer flasks containing 700 mL of culture. The inoculum was grown aseptically at 23 °C, under a constant incident irradiance of 100 μmol photons m^{-2} s^{-1}. The flasks were continuously bubbled with air at 350 mL min^{-1}. No carbon dioxide was added. The culture medium comprised of filter-sterilized Mediterranean Sea water formulated with the following agricultural grade inorganic components (g L^{-1}): KNO_3 1.0, $NaH_2PO_4{\cdot}H_2O$ 2.0×10^{-2}, $C_6H_5FeO_7{\cdot}H_2O$ 15×10^{-3}, $Na_2MoO_4{\cdot}2H_2O$ 5.0×10^{-4}, $MnCl_2{\cdot}4H_2O$ 4.0×10^{-4}, $ZnCl_2$ 8.0×10^{-4}, $CoCl_2{\cdot}6H_2O$ 9.0×10^{-4}, $CuSO_4{\cdot}5H_2O$ 8.0×10^{-5}, EDTA 0.015, thiamine 16.0×10^{-4}, biotin 6.0×10^{-5}, and cyanocobalamin 2.0×10^{-5}. This medium had been earlier established to be optimal for photoautotrophic production of the algal biomass (Camacho-Rodríguez et al. 2015a).

For indoor cultures, the alga was grown in 500-mL Erlenmeyer flasks containing 250 mL of the culture medium. The above specified medium was sterilized (126 °C, 20 min) and cooled. Separately, filter-sterilized (0.2-μm pore size membrane filter) organic carbon sources (sodium acetate, MIXACE; glucose, MIXGLU; glycerol, MIXGLYC) were added to the required concentration in mixotrophic operations. Only a single organic carbon substrate was used in a given culture. The sterile medium was inoculated with cells in the linear growth phase. A sufficient quantity of inoculum was added to obtain an initial dry biomass concentration of 0.2 g L^{-1}. Shake flasks were agitated at 150 rpm on an orbital shaker (Infors AG, Switzerland). The flasks were continuously illuminated at 190 μmol photons m^{-2} s^{-1} (Philips TLD 36 W/54 fluorescent lamp) measured at the surface of the flasks. (The light level used for growing the alga was of course higher than the level reported in an earlier paragraph for culture maintenance, as growth is not sought during maintenance.) The initial pH was set at 8.0 and the incubation temperature was 23 ± 0.5 °C. In batch cultures, the initial concentration of the organic carbon substrates varied between 1 and

15 g L^{-1}. Photoautotrophic control cultures (PHOTO; no organic carbon source) were run in parallel with each mixotrophic culture.

All cultures were grown in triplicate. Triplicate samples were taken from each of the culture vessels, when required. Data are average values ± standard deviation of nine experimental measurements at any instance.

Outdoor cultures

Nannochloropsis gaditana was grown in six identical outdoor flat-panel photobioreactors. Each reactor consisted of a disposable polyethylene bag held within a steel cage, as previously described (San Pedro et al. 2016). The larger flat face of each reactor was 1.7 m tall and 2.5 m wide. The distance between the larger parallel flat faces was 0.09 m. The reactors were located in Almería, Spain (36° 46′ 00″ N, 2° 30′ 00″ W).

The reactors were aerated using a sparger located at the bottom. The air flow rate was 0.09 vvm (FR4L72BVBN flow meters, Key Instruments, USA). The pH was controlled by injecting pure CO_2 whenever the pH rose above the set point value (pH = 8) and until the set point was regained. The injection rate was 0.008 vvm. A combined temperature-pH probe (5342 pH electrode, Crison Instruments S.A., Spain) was located near the top of the reactor. A thermoelectric pyranometer connected to an AC-420 adapter (LP-02, Geónica S.A., Spain) was used to monitor the photosynthetically active incident solar radiation at the location of the reactors (San Pedro et al. 2016). The cultures were continuously fed with fresh medium during daylight hours at a dilution rate of 0.2 day^{-1}. The feeding ceased each night.

The experiments were conducted over a period of 1 year. The temperature was monitored, but not controlled. The data were grouped based on the average daily temperature in the autumn and winter growing seasons. The average culture temperature in autumn was 21 °C and in winter it was 12 °C. The mean values of the average incident irradiance in autumn and winter were 580 and 421 μmol photons m^{-2} s^{-1}, respectively. A steady state was taken to exist if four consecutive daily measurements of the biomass concentration were within ± 5% of the averaged value. In all experiments, the culture samples were collected at steady state. The biomass was recovered by centrifugation at 9000×*g* for 5 min. The biomass was washed with 0.5 M aqueous ammonium bicarbonate solution (Camacho-Rodríguez et al. 2013) and freeze-dried (Telstar Cryodos 50, Telstar, Spain) for biochemical analyses.

Six replicate bioreactors were run in parallel. Once a steady state had been attained based on the criterion specified earlier in this section, three replicate samples were taken during the last 3 days of each steady state, from each bioreactor. The data shown are average values ± standard deviation of 54 measurements.

Analytical procedures

The biomass concentration was estimated by measuring the spectrophotometric absorbance at 540 nm of a suitably diluted culture sample and periodic corroboration with dry-weight determinations (Camacho-Rodríguez et al. 2013). Occasionally, unidentified invasive species appeared at low levels inside the reactors and, therefore, weekly microscopic (Nikon Eclipse E200, Nikon Europe, The Netherlands) observations were made to ensure that *N. gaditana* always represented more than 95% of the cells. If excessive contamination was detected, the plastic bag was replaced and the culture was started afresh. The physiological health of the algal cells was routinely checked by chlorophyll fluorescence measurements (AquaPen-C, AP-C 100 fluorimeter, Photon Systems Instruments, Czech Republic) to ensure that the ratio of variable fluorescence (F_v) to maximum fluorescence (F_m) was > 0.8 for unstressed cells. The cells were dark adapted for 30 min prior to the measurements. Inorganic nitrate and phosphate were measured to ensure a sufficiency of these key nutrients.

The fatty acids in freeze-dried biomass samples were transesterified in situ in accordance with a published protocol (Rodríguez-Ruiz et al. 1998) and quantified by gas chromatography (Agilent Technologies 6890 N Series Gas Chromatograph, USA). The carotenoids were determined using a high-performance liquid chromatograph (Shimadzu SPDM10AV, Shimadzu Corp, Japan) equipped with a photodiode array detector, as previously explained (Cerón-García et al. 2018).

Analyses were performed on triplicate sample taken over the last 3 days of a steady state operation. Therefore, the results represent average values ± standard deviation of at least nine replicate measurements.

Results and discussion

Indoor culture

Indoor batch cultures are useful for comparing the photoautotrophic and mixotrophic production because they can be better controlled relative to the naturally illuminated outdoor cultures. The mixotrophic biomass growth profiles of the alga on different substrates and different initial concentrations of the substrates are shown in Fig. 1 in comparison with photoautotrophic control cultures.

As shown in Fig. 1, in comparison with photoautotrophic controls, the biomass growth was nearly as rapid, and a higher final concentration of biomass was obtained during mixotrophy on glucose (Fig. 1a) and glycerol (Fig. 1b). Thus, the alga effectively took up and metabolized both glucose and glycerol. This was not the case when acetate was

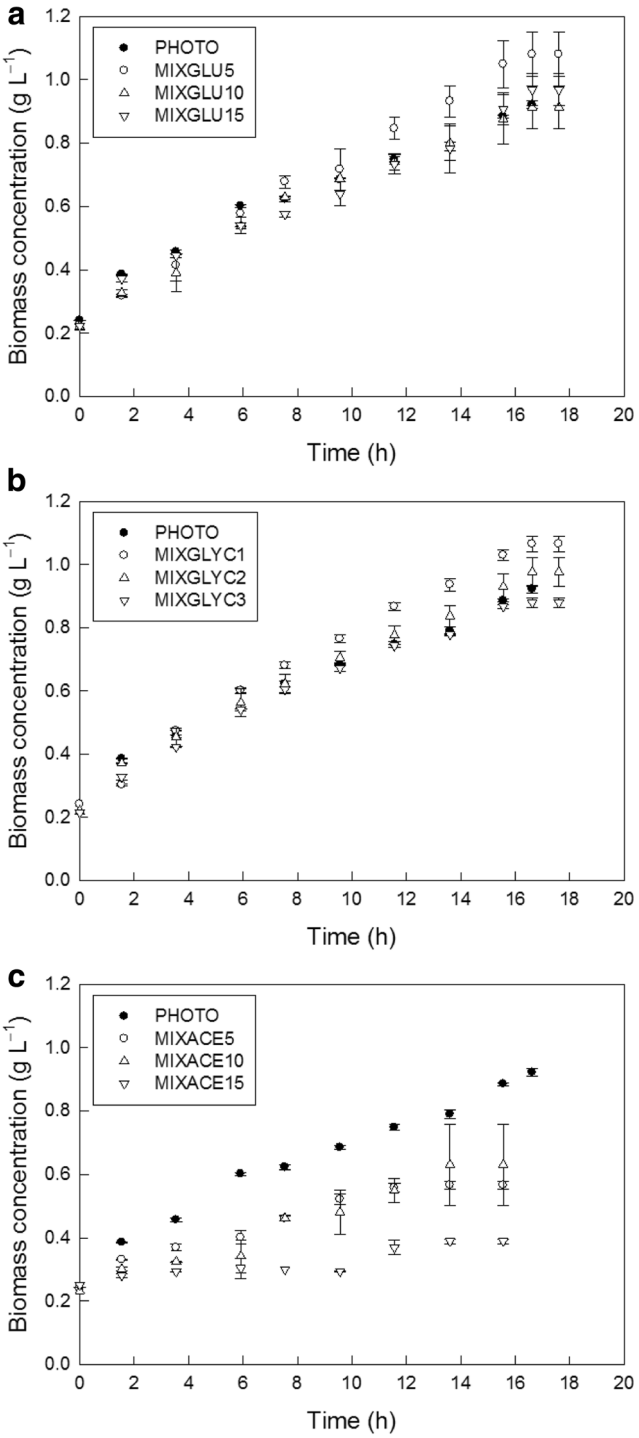

Fig. 1 Biomass production of *N. gaditana* in indoor batch cultures: comparison between photoautotrophic (PHOTO) and mixotrophic growth using different organic carbon sources (g L^{-1}). **a** 5.0, 10.0, and 15.0 of glucose (MIXGLU*n*). **b** 1.0, 2.0, and 3.0 of glycerol (MIXGLYC*n*). **c** 5.0, 10.0, and 15.0 of sodium acetate (MIXACE*n*). Data shown are average values and standard deviations of nine measurements (3 biological replicates × triplicate samples)

used (Fig. 1c). Compared with acetate-containing media, the photoautotrophic control culture always outperformed in terms of biomass growth (Fig. 1c). Although acetate is known to be readily metabolized by many microalgae (Chapman et al. 2015; Smith et al. 2015), this was not so with *N. gaditana*.

The main biomass growth and productivity indices calculated using the data in Fig. 1 are summarized in Table 1. The table reveals that compared with photoautotrophic control culture (PHOTO), the final biomass concentration in glucose and glycerol fed cultures (i.e., MIXGLU5–15, MIXGLYC1–2) was at least as high, or higher, with MIXGLYC3 being an exception. For glucose and glycerol, an increasing concentration of the carbon substrate in the range examined, tended to reduce the final biomass concentration. Thus, mixotrophy on glucose and glycerol was beneficial relative to control so long as the concentration of the organic carbon source was kept low. High concentrations of sugars (glucose) and sugar alcohols (glycerol) are known to suppress growth by reducing the water activity in a culture medium (Bouyam et al. 2017). In the media containing glucose at 5 g L^{-1} and glycerol at 1–3 g L^{-1}, the maximum specific growth rate was as high, or higher, than in the photoautotrophic control culture (Table 1). Thus, the ratio (μ_D) of the mixotrophic maximum specific growth rate to the photoautotrophic maximum specific growth rate exceeded 1 (Table 1).

In the concentration range of 1–3 g L^{-1}, glycerol was a superior organic carbon source compared with glucose at its best case concentration of 5 g L^{-1}. The carbon utilization efficiency of the alga was much higher with glycerol compared to glucose. This was because each mole of glucose provided 6 mol of organic carbon, but each mole of glycerol contained only 3 mol of carbon. Thus, at 1 g L^{-1}, glycerol provided an organic carbon molar amount that was only 19.6% compared to the carbon provided by glucose at a concentration of 5 g L^{-1}, but in terms of biomass production capabilities MIXGLYC1 was better than MIXGLU5 (Table 1).

No prior work appears to have been done on mixotrophic culture of *N. gaditana* per se. A few mixotrophic studies have been reported for unspecified *Nannochloropsis* strains (Fang et al. 2004; Cheirsilp and Torpee 2012). Using 30 mM glucose in 250-mL batch shake flask cultures, Fang et al. (2004) attained a dry biomass concentration of 550 mg L^{-1} in 8 days. This is equivalent to a biomass productivity of 0.07 mg L^{-1} day^{-1}, barely 41% of the productivity shown in Table 1 for MIXGLU5 which had a glucose concentration (= 27.7 mM) quite close to that used by Fang et al. (2004).

Outdoor culture

Although artificially illuminated indoor culture is suitable for small scale applications, it is generally too expensive for large volume production of low-cost algal biomass. Notwithstanding the good control of the culture environment that is possible indoors, outdoor culture relying on freely

Table 1 Indoor batch culture parameters

Culture type[a]	C_b (g L^{-1})	S_f (g L^{-1})	μ_{max} (day^{-1})	P_{max} (g L^{-1} day^{-1})	μ_D (−)	P_D (−)
PHOTO	0.92 ± 0.01	–	0.14 ± 0.00	0.13 ± 0.00	–	–
MIXGLU5	1.08 ± 0.07	1.42 ± 0.03	0.16 ± 0.00	0.17 ± 0.01	1.08	1.26
MIXGLU10	0.92 ± 0.07	3.45 ± 0.04	0.14 ± 0.00	0.13 ± 0.01	0.96	0.95
MIXGLU15	0.97 ± 0.05	4.65 ± 0.05	0.14 ± 0.01	0.13 ± 0.01	0.96	1.01
MIXGLYC1	1.07 ± 0.03	0.32 ± 0.06	0.16 ± 0.00	0.17 ± 0.01	1.11	1.29
MIXGLYC2	0.98 ± 0.05	0.59 ± 0.08	0.15 ± 0.02	0.15 ± 0.02	1.03	1.09
MIXGLYC3	0.88 ± 0.02	1.50 ± 0.07	0.15 ± 0.00	0.13 ± 0.00	1.04	0.99
MIXACE5	0.57 ± 0.01	ND	0.08 ± 0.01	0.05 ± 0.00	0.56	0.35
MIXACE10	0.64 ± 0.13	ND	0.06 ± 0.02	0.04 ± 0.02	0.58	0.39
MIXACE15	0.44 ± 0.05	ND	0.03 ± 001	0.01 ± 0.00	0.21	0.09

C_b, Final biomass concentration in the stationary phase; S_f, final substrate concentration in the stationary phase (ND, not determined because of the low biomass productivity with this substrate); μ_{max}, maximum specific growth rate; P_{max}, maximum biomass productivity; μ_D, dimensionless maximum specific growth rate (i.e., μ_{max} divided by the maximum specific growth rate for photoautotrophic control); P_D, maximum biomass productivity relative to photoautotrophic control in batch cultures

[a] PHOTO, photoautotrophic culture; MIXGLU5, mixotrophic glucose (5 g L^{-1}); MIXGLU10, mixotrophic glucose (10 g L^{-1}); MIXGLU15, mixotrophic glucose (15 g L^{-1}); MIXGLYC1, mixotrophic glycerol (1 g L^{-1}); MIXGLYC2, mixotrophic glycerol (2 g L^{-1}); MIXGLYC3, mixotrophic glycerol (3 g L^{-1}); MIXACE5, mixotrophic acetate (5 g L^{-1}); MIXACE10, mixotrophic acetate (10 g L^{-1}); MIXACE15, mixotrophic acetate (15 g L^{-1})

available sunlight for illumination is essential for developing a practicable production process. Thus, outdoor cultures were examined as discussed in this section. In view of the results of the previous section, sodium acetate was eliminated from outdoor studies as being a poor carbon substrate (Fig. 1c). In addition, only the media with glucose at a concentration of 5 g L^{-1} (i.e., MIXGLU5) and glycerol at a concentration of 1 g L^{-1} (i.e., MIXGLYC1) were evaluated outdoors because they had provided the highest biomass productivities indoors (Table 1).

In addition to biomass, the focus of outdoor studies was on production of total lipids including the high value fatty acid EPA. In large outdoor cultures, control of temperature is often impractical. Experiments were carried out in two different seasons with average daily uncontrolled culture temperatures of 12 °C (winter) and 21 °C (autumn). The culture media were MIXGLU5 and MIXGLYC1 with exactly the same other inorganic nutrients (Camacho-Rodríguez et al. 2015a) as used in the above noted indoor cultures. All outdoor operations were run as pseudo steady state continuous cultures. The key pseudo steady state parameters of the outdoor cultures in the two media and at two temperatures are shown in Table 2. The relevant data for the photoautotrophic control cultures are also shown (PHOTO, Table 2). For the two photoautotrophic control cultures, the biomass productivity values in Table 2 at both the temperatures were identical to those obtained independently by San Pedro et al. (2016) in exactly the same conditions, including the flat-panel photobioreactor culture system, the seasons, and the geographic location. This lent credibility to the reported data (Table 2) and its reproducibility, for a reliable comparison with the mixotrophic data. In PHOTO control cultures, the higher autumn mean temperature enhanced all production indices compared to the winter season (Table 2), as is typical for algal cultures.

Comparing the two MIXGLYC1 cultures to the two PHOTO controls at identical temperatures, the steady state biomass concentrations, the biomass productivities, and the lipid productivities were essentially unaffected by mixotrophy on glycerol (Table 2), but the mixotrophic cultures had distinctly higher EPA productivities (Table 2). In mixotrophic glycerol cultures, the productivities were positively correlated with the culture temperature (Table 2). A lack of impact of glycerol mixotrophy on biomass productivity in outdoor operations is in dramatic contrast to the observations discussed earlier for indoor cultures. The explanation for the difference is as follows: carbon dioxide was injected periodically in outdoor cultures, but was not used indoors. Carbon dioxide is taken up by microalgae in the form of bicarbonate (Shene et al. 2016b), and uptake of bicarbonate apparently interferes with and suppresses the uptake of glycerol. Hence, there is barely any uptake of glycerol if carbon dioxide is injected. This mutual antagonism between bicarbonate uptake and glycerol uptake has been reported earlier with several other microalgae (Sforza et al. 2012; Cerón-García et al. 2013).

The glucose-fed mixotrophic cultures (MIXGLU5, Table 2) performed substantially better than the corresponding control (PHOTO, Table 2) cultures and the glycerol-fed cultures (MIXGLYC1, Table 2). For example, at 21 °C: the steady state biomass concentration was 77% higher in MIXGLU5 relative to PHOTO (Table 2); the biomass

Table 2 Outdoor continuous culture parameters[a]

Culture type[b]	Temperature (°C)	Biomass concentration (g L^{-1})	Biomass productivity (g L^{-1} day^{-1})	EPA productivity (mg L^{-1} day^{-1})	Total lipid productivity (mg L^{-1} day^{-1})
PHOTO	21	0.71 ± 0.01	0.15 ± 0.01	7.05 ± 0.00	30.09 ± 1.04
PHOTO	12	0.52 ± 0.08	0.10 ± 0.03	5.01 ± 0.04	20.07 ± 0.55
MIXGLU5	21	1.26 ± 0.06	0.26 ± 0.04	9.10 ± 0.04	37.81 ± 0.70
MIXGLU5	12	0.97 ± 0.06	0.20 ± 0.01	11.02 ± 0.04	54.00 ± 0.88
MIXGLYC1	21	0.73 ± 0.06	0.15 ± 0.02	7.85 ± 0.03	29.00 ± 0.12
MIXGLYC1	12	0.57 ± 0.02	0.11 ± 0.01	5.50 ± 0.06	21.02 ± 1.14

The values shown are average of the last 3 days in steady state cultivation

[a] The dilution rate for all continuous cultures was 0.2 day^{-1}

[b] PHOTO, photoautotrophic culture; MIXGLU5, mixotrophic glucose (5 g L^{-1}); MIXGLYC1, mixotrophic glycerol (1 g L^{-1})

productivity was 73% higher; the EPA productivity was 29% higher; and the lipid productivity was 26% higher.

In the present work no attempt was made to use nutrient starvation to promote lipid accumulation in the biomass, although nitrogen starvation after growth has been shown to greatly enhance the lipid content of the biomass in many algae including photoautotrophically grown *N. gaditana* (Pedersen et al. 2018). Enhancement of lipid content of the biomass through limiting non-carbon inorganic nutrients such as nitrogen and phosphorous, has been demonstrated also in mixotrophic and heterotrophic cultures of some other microalgae (Bouyam et al. 2017) and has the potential to be used with *N. gaditana*.

The productivity of EPA was highest in the two glucose fed mixotrophic cultures (MIXGLU5, Table 2). In MIXGLU5 medium, the higher EPA productivity occurred at the lower temperature, but this situation was reversed in the control cultures (PHOTO, Table 2). Temperature has been found to both positively and negatively influence EPA production depending on the alga. Further studies of how temperature might influence EPA and lipid production in *N. gaditana* may be useful, but manipulating temperature in large scale outdoor culture is often prohibitively expensive.

Barely any published data exist for EPA productivity in mixotrophic growth of *N. gaditana*. For an unspecified *Nannochloropsis* strain grown mixotrophically on 30 mM glucose and the same concentration of ethanol in 8-day indoor batch cultures, Fang et al. (2004) reported a final EPA concentration of around 23 mg L^{-1} for both substrates. In an equivalent photoautotrophic batch culture, the final EPA concentration (= 21.9 mg L^{-1}) was nearly the same as in the mixotrophic cultures. Using the above referenced EPA concentration and the 8-day culture period (Fang et al. 2004), the EPA productivity of the mixotrophic cultures can be shown to be 2.9 mg L^{-1} day^{-1}. This is far lower than the values shown in Table 2. For example, the best case productivity with MIXGLU5 in Table 2 is 3.8-fold higher than observed by Fang et al. (2004) in a medium with a glucose concentration quite close to the MIXGLU5.

Mixotrophic production of EPA using glycerol has been reported in the related alga *Nannochloropsis oculata* (Shene et al. 2016a). The highest EPA concentration in a 10-day indoor batch culture was 27.5 ± 1.6 mg L^{-1}, corresponding to a productivity of around 2.8 mg L^{-1} day^{-1} in a glycerol containing mixotrophic operation (Shene et al. 2016a). This productivity was consistent with the values reported by Fang et al. (2004) in mixotrophic cultures on glucose and ethanol, but quite low compared to the data in Table 2.

Fatty acids and carotenoids

The fatty acids and carotenoid pigments are important potential products of microalgae culture. Therefore, these products were measured in the final biomass of the indoor batch cultures and the biomass harvested during steady state operations of continuous outdoor cultures at a dilution rate of 0.2 day^{-1}.

The fatty acid and carotenoid profiles of the biomass harvested from indoor batch operations are shown in Fig. 2. Data are shown for the photoautotrophic control cultures (PHOTO, Fig. 2) and the mixotrophic cultures MIXGLU5 and MIXGLYC1. The total fatty acid content of the biomass was around 11% (*w*/*w*) irrespective of whether photoautotrophic or mixotrophic operation was used (Fig. 2a). Thus, mixotrophy did not affect the total fatty acid content relative to control. These results are consistent with the data reported for an unspecified *Nannochloropsis* sp. where the total lipid content of the biomass was barely influenced by whether the alga was grown photoautotrophically or mixotrophically (Fang et al. 2004). The fatty acid composition of the lipids was

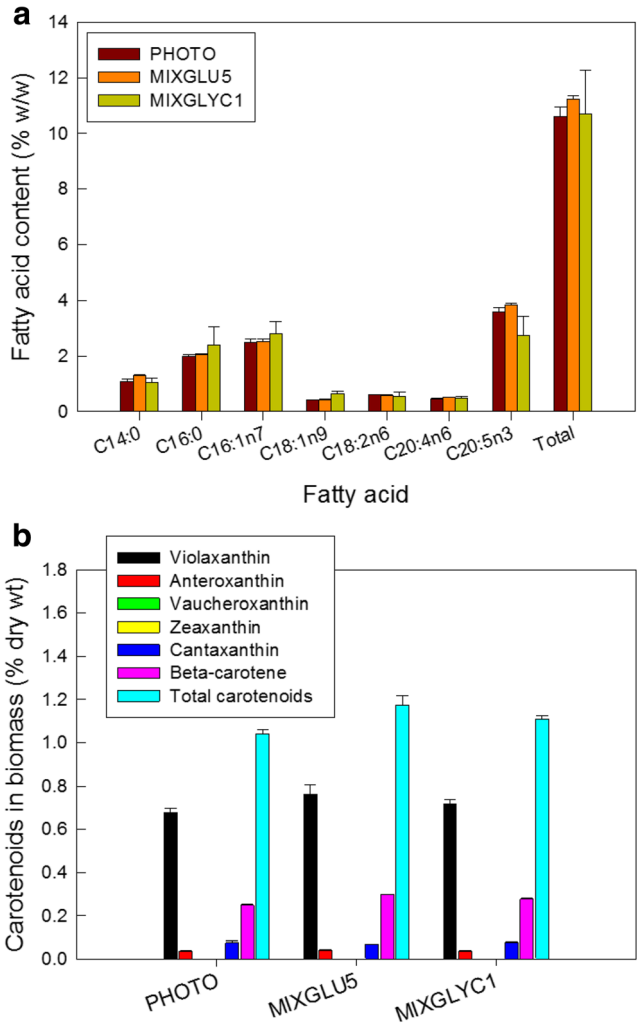

Fig. 2 Types of fatty acids (**a**) and carotenoids (**b**) in *N. gaditana* final biomass grown in indoor batch cultures at 23 ± 0.5 °C. PHOTO, photoautotrophic control; MIXGLU5, mixotrophic culture on glucose (5 g L^{-1}); and MIXGLYC1, mixotrophic culture on glycerol (1 g L^{-1}). (Fatty acids: myristic acid, C14:0; palmitic acid, C16:0; palmitoleic acid, C16:1n7; oleic acid, C18:1n9; linoleic acid, C18:2n6; α-linolenic acid, C18:3n3; arachidonic acid, C20:4n6; and eicosapentaenoic acid, C20:5n3.) Data shown are average values and standard deviations of nine measurements (3 biological replicates × triplicate samples)

not affected irrespective of whether the operation was mixotrophic or photoautotrophic (Fig. 2a). The three major fatty acids in the lipids were palmitic acid (C16:0), palmitoleic acid (C16:1), and EPA (C20:5n3) (Fig. 2a). The EPA content was nearly 30% of the total fatty acids.

The total levels of the various carotenoids in the biomass produced in indoor operations were similar irrespective of the culture mode (Fig. 2b). In heterotrophic cultures of some algae in the dark, synthesis of some light-induced pigments may be low, or absent, compared to biomass grown photoautotrophically (Li et al. 2015). In mixotrophic cultures, the light regime is the same as in the corresponding photoautotrophic control culture, and therefore the results in Fig. 2b are consistent with expectations.

The biomass produced in outdoor continuous cultures had all the same fatty acids (Fig. 3a) as the biomass grown in indoor batch operations (Fig. 2a), but also had measureable levels of α-linolenic acid (C18:3n3). These differences may have been due to several possible factors including differences in the light regimen (constant indoor illumination at 190 μmol photons m^{-2} s^{-1} versus cyclic natural illumination at 421 μmol photons m^{-2} s^{-1} for the data in Fig. 3), the prevailing temperature (23 ± 0.5 °C

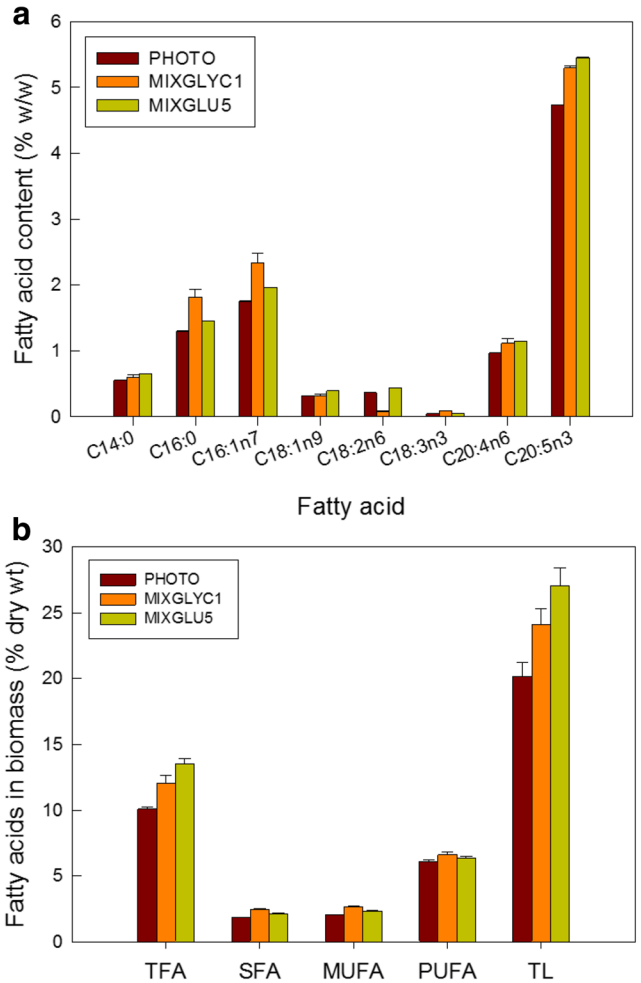

Fig. 3 Fatty acids profile (**a**) and classes of fatty acids (**b**) in *N. gaditana* biomass grown in outdoor continuous cultures at a dilution rate of 0.2 day^{-1} at 12 °C. PHOTO, photoautotrophic control; MIXGLYC1, mixotrophic culture on glycerol (1 g L^{-1}); and MIXGLU5, mixotrophic culture on glucose (5 g L^{-1}). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TL, total lipids; TFA, total fatty acids. (Fatty acids: myristic acid, C14:0; palmitic acid, C16:0; palmitoleic acid, C16:1n7; oleic acid, C18:1n9; linoleic acid, C18:2n6; α-linolenic acid, C18:3n3; arachidonic acid, C20:4n6; and eicosapentaenoic acid, C20:5n3.) Data shown are average values and standard deviations of 54 measurements (6 biological replicates × triplicate samples per day at steady state × 3 days during steady state)

indoors versus 12 °C for the data in Fig. 3), and the supply of inorganic carbon (supplied outdoors versus not supplied indoors). EPA (C20:5n3) was generally the predominant fatty acid, especially in the biomass produced in outdoor cultures (Fig. 3a). For both indoor batch and outdoor continuous cultures, whether the operation was photoautotrophic (PHOTO) or mixotrophic (MIXGLU5, MIXCLYC1) had relatively minor effect on the fatty acid composition of the lipids (Fig. 2a, Fig. 3a).

In the biomass produced outdoors, the average total fatty acid content (TFA, Fig. 3b) irrespective of the culture mode (PHOTO, MIXGLYC1, MIXGLU5) was 11.9%, or essentially the same as in the biomass produced indoors (Fig. 2a). The polyunsaturated fatty acids (PUFA, Fig. 3b) were the predominant class of fatty acids. The proportions of monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) in total fatty acids (TFA) were roughly equal (Fig. 3b).

The total lipid (TL) content of the biomass produced mixotrophically (i.e., MIXGLU5, MIXGLYC1 in Fig. 3b) were significantly greater relative to the control biomass (PHOTO, Fig. 3b). The average total lipid content of the biomass grown in the three modes (i.e., PHOTO, MIXGLU5, MIXGLYC1; Fig. 3b) was 24% (*w*/*w*) and the best case value for the mixotrophic culture MIXGLU5 (Fig. 3b) was 27.1%. Both, these were low compared to earlier reports for mixotrophically grown biomass of other *Nannochloropsis* algae (Fang et al. 2004; Shene et al. 2016a).

The total lipid level was low because the biomass was not starved of any nutrient. Lipid accumulation in microalgae generally occurs when nutrient supply limits growth. In carbon-sufficient but nitrogen-limited photoautotrophic cultures, *N. gaditana* has been shown to accumulate more than 48% of its dry mass as lipids (Pedersen et al. 2018). Similarly, the total lipid content of the dry biomass reported by Fang et al. (2004) were 38.7 ± 2.1% (*w*/*w*) in biomass produced mixotrophically on glucose (Fang et al. 2004) and 34.6 ± 0.6% in the biomass grown mixotrophically on ethanol. The total lipid content reported by Fang et al. (2004) for glucose mixotrophy was around 28% higher than our highest value for MIXGLU5 (Fig. 3b). The explanation lay in nitrogen starvation of the biomass towards the end of the batch culture in the work of Fang et al. (2004), but absence of starvation in the present work. The lowest measured nitrate concentration at steady state (dilution rate = 0.2 day^{-1}) was 248 mg L^{-1}, confirming an absence of nitrogen limitation.

The initial KNO_3 concentration used in our work was 1 g L^{-1}, corresponding to an initial nitrate concentration of 613 mg L^{-1}. In contrast, Fang et al. (2004) used the standard f/2 medium that has a nitrate concentration of only 54.7 mg L^{-1}. Data presented by Fang et al. (2004) do show the nitrate concentrations declining to less than 15 mg L^{-1} in the final 3 days of their mixotrophic cultures. Similarly, a high lipid content (> 30% by dry weight) was also reported in the final biomass of the related alga *N. oculata* grown mixotrophically using glycerol in a 10-day batch culture (Shene et al. 2016a). The explanation once again lay in the f/2 medium used (Shene et al. 2016a) compared to the high-nitrogen medium of the present work.

Based on the well-known principles of continuous culture (Bailey and Ollis 1986; Chisti 2010), in a steady-state operation with a stable concentration of the biomass, the dilution rate must equal the specific growth rate. The dilution rate used in the outdoor continuous culture studies was 0.2 day^{-1}. This value was higher than the highest value of the maximum specific growth rate observed in any indoor batch operation (Table 1), but the indoor operations had a much lower light level (= 190 μmol photons m^{-2} s^{-1}) compared to the lowest outdoor light level (= 421 μmol photons m^{-2} s^{-1} in autumn) and therefore a higher specific growth rate (= 0.2 day^{-1}) could be stably maintained outdoors.

The carotenoid profiles of the outdoor grown biomass are shown in Fig. 4. All the listed carotenoids were produced to a measureable level in all cultures irrespective of the mode of growth, but mixotrophy substantially enhanced the total carotenoids in the biomass compared to photoautotrophic control (Fig. 4). The total carotenoids in the biomass grown with glucose mixotrophy were 1.8-fold more than in the control biomass (PHOTO, Fig. 4). Similarly, with glycerol mixotrophy, the total carotenoids in the biomass were 1.5-fold greater relative to control. This suggests MIXGLU5 to be best for carotenoids production.

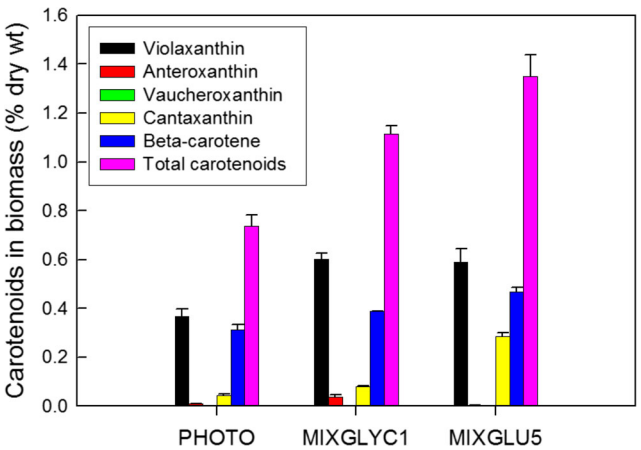

Fig. 4 Carotenoid profiles in the algal biomass grown in continuous cultures at a dilution rate of 0.2 day^{-1} at 12 °C. PHOTO, photoautotrophic control; MIXGLYC1, mixotrophic with glycerol (1 g L^{-1}); MIXGLU5, mixotrophic with glucose (5 g L^{-1}). Data shown are average values and standard deviations of 54 measurements (6 biological replicates × triplicate samples per day at steady state × 3 days during steady state)

Concluding remarks

Mixotrophic production of the biomass, lipids, and carotenoids of the microalga *N. gaditana* was shown to be possible using glucose or glycerol, but not acetate. The optimal concentrations of the organic carbon sources were 5 g L^{-1} for glucose and 1 g L^{-1} for glycerol. In the absence of any nutrient limitations in outdoor continuous cultures at a dilution rate of 0.2 day^{-1} during winter (12 °C average daily culture temperature), the total lipid productivity was 2.7-fold higher relative to photoautotrophic control when glucose (5 g L^{-1}) mixotrophy was used. For the same mixotrophic operation, the EPA productivity was 2.2-fold higher than controls. The total carotenoids in the mixotrophically grown biomass was 83% higher compared to control. Therefore, compared with photoautotrophy, mixotrophic operation on glucose is potentially useful for enhancing production of lipids, EPA, and carotenoids in *N. gaditana* cultures that are not nutrient limited.

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