



# Mixotrophic growth of a highly acidic habitat microalga for production of valuable fatty acids

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## Abstract

Photoautotrophic algal cultivation is challenging due to the shadowing effect produced by an increase in the number of cells; under such circumstances, mixotrophic growth might be an efficient alternative. We grew cultures of the autochthonous acidotolerant microalga *Elliptochloris* sp. on crude technical glycerin or glucose bubbled with either only air or air containing 2.5 % (v/v) CO<sub>2</sub>. We found that CO<sub>2</sub> strongly influenced the production of *Elliptochloris* sp., as higher growth occurred in mixotrophy with CO<sub>2</sub>-enriched air compared to that with only air. Mixotrophy with CO<sub>2</sub>-enriched air allowed to reach higher biomass productivities and facilitated an increase in the relative abundance of saturated fatty acids. Nevertheless, mixotrophy with only air resulted in an increase of both saturated and unsaturated fatty acids, but biomass productivities were lower. These results, along with the limited biological contamination facilitated by low pH, suggest that this microalga might be attractive for large-scale production within the circular economy model.

**Keywords** Acidotolerant microalga · Chlorophyta · Mixotrophy · Lipids · Fatty acids · Linolenic acid

## Introduction

The current energy model based on oil and other fossil fuels is nearing its end. Such an energy model strongly affects the environment due to the emission of CO<sub>2</sub> into the atmosphere, which significantly contributes to climate change (González et al. 2009). Thus, sustainable energy sources that have a lesser impact on the climate need to be identified. Advanced biofuel is an alternative fuel that has attracted the attention of researchers around the world (Ethier et al. 2011). The production of algal biofuels might be an effective alternative

since algae can fix CO<sub>2</sub> with the energy that they obtain during photosynthesis. This process can not only reduce CO<sub>2</sub> (Cheirsilp and Torpee 2012) but also convert it into fuel-related biomolecules, including lipids, mainly in the form of fatty acids, and carbohydrates from which different biofuels, such as bioethanol, biobutanol, and biohydrogen, can be obtained using biotechnology (Markou et al. 2012).

Despite the above-mentioned advantages of photoautotrophic growth, microalgal biomass and lipid production might be limited by the reduction in light availability. That is, during the production of microalgae at high cell densities, the shadowing effect among cells might lead to growth photolimitation (Vaquero et al. 2014). As an alternative to photoautotrophic growth, some microalgal species can use organic carbon sources (OCS) and perform mixotrophic or heterotrophic growth. The fixing of CO<sub>2</sub> while assimilating OCS is defined as mixotrophic growth. The process yields metabolic energy as OCS can directly follow a carbon-oxidation metabolic route. This process decreases the relevance of energy production by light-dependent growth, which is limited by the lower availability of light in dense algal cultures (D'Imporzano et al. 2017).

Several microalgal species can grow mixotrophically, including *Chlorella*, *Chlorococcum*, *Haematococcus*,

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*Nannochloropsis*, *Navicula*, *Phaeodactylum*, and *Nitzschia* (Perez-Garcia and Bashan 2015). The most commonly used organic carbon sources during mixotrophic growth at the lab scale are glucose and acetate (Paranjape et al. 2016). However, these carbon sources are not suitable for industrial-scale production as they are expensive (Li et al. 2007). Glycerol, a by-product of biodiesel production, can be obtained at a lower price (Vivek et al. 2017). Thus, the large-scale production of microalgae on glycerol can reduce production costs while increasing the value of glycerol through its conversion into oil as a raw material for advanced biofuel (Da Silva et al. 2009).

The use of an extra OCS supports activities in the cell related to metabolic energy production through the oxidation of the carbon source. Additionally, using OCS promotes fatty acid biosynthesis in the chloroplast. Glycerol can be transported to the cytosol by passive diffusion and then be phosphorylated and converted into glycerol-3-phosphate (G3P), which is involved in triacylglyceride (TAG) synthesis. Also, glucose is phosphorylated to glucose-6-phosphate (G6P), which is converted first into fructose-6-phosphate (F6P) and subsequently into G3P (Chen et al. 2017).

Bacterial contamination is a serious concern when microalgae are cultivated in a medium containing OCS, as microalgae have a growth rate one order of magnitude lower than their competitors (Abiusi et al. 2022). This is why the interest in extremophilic microalgae has increased in the last few years. Extreme conditions are not suitable for all microorganisms, allowing only the growth of the extremophilic microorganisms that can cope with these harsh conditions. Based on these facts, the autochthonous acidophilic microalga *Elliptochloris* sp. isolated from Tinto River (Huelva) was selected for this study. The relevance of *Elliptochloris* sp. arises from its ability to grow in extreme acidic environment as fast as other non-extremophilic microalgae which grow at a near-neutral pH. Published information on the growth of *Elliptochloris* strains under controlled conditions is limited. However, the concentrations of *Elliptochloris* sp. (0.17–0.25 g L<sup>-1</sup>, unpublished data) are in the range of those obtained by Chunzhuk et al. (2023) for a non-extremophilic *Elliptochloris subsphaerica* and other non-extremophilic microalgae (0.12–0.37 g L<sup>-1</sup>).

In this study, the production of the acidotolerant microalga *Elliptochloris* sp. in photoautotrophic and mixotrophic conditions was analyzed. Studies performed by Cuaresma et al. (2011) on an acidotolerant microalga revealed that glucose was the only organic carbon source which enabled reaching biomass concentration values similar to those obtained in photoautotrophic cultures; based on that, glucose was selected as carbon source for this study. In addition, technical glycerol was selected as a carbon source to promote the circular economy, i.e., the insertion of an industrial by-product in the metabolic pathway of the microalga. The supplemented glycerol was obtained in the process of

advanced biodiesel production. This way, the added value of glycerol could be enhanced due to its application in the production of valuable microalgal biomolecules, such as fatty acids; for instance, glycerol might be re-used as an organic carbon source for microalgal biofuel production. The selected carbon sources were supplemented at 5 mM, based on studies conducted by Martínez et al. (1997) and Chojnacka and Noworyta (2004), who reported a glucose concentration of 3–5 mM to be the carbon saturation limit for the growth of microalgae in batch culture.

This study provides insights into the growth and production of lipids by a microalgal strain recently isolated from a highly acidic environment. Our findings provide information on biotechnological strategies related to the production and application of extremophiles.

## Materials and methods

### Biological material and standard culture conditions

The microalgal species used in this study was isolated from the Tinto River in the north of the Huelva province (site GPS coordinates: 37.72326, -6.55081). Samples taken from the location were transferred to a sterile K9 culture medium (Silverman and Lundgren 1959) prepared on agar in Petri dishes. Green spots were transferred from the agar plates to the K9 liquid medium. Liquid cultures were checked periodically in an optical microscope (CX40, Olympus, UK). Based on morphological observations, only one microalga species was found in the taken samples and subsequently DNA was extracted from cell pellets in the liquid culture for identification. The isolated *Elliptochloris* sp. was cultivated at pH 2.5 in sterile K9 medium originally used for bacteria and modified for acidophilic microalgae. Changes to K9 medium included modification of nitrogen source, replacing ammonia by nitrate, and Hutner solution (Hutner et al. 1950) was added to satisfy trace elements needs. Trace element Hutner solution was prepared as described in Garbayo et al. (2012). K9 was prepared according to the following composition: 3.95 g K<sub>2</sub>SO<sub>4</sub>, 0.1 g KCl, 0.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.41 g MgCl<sub>2</sub>, 2.29 g KNO<sub>3</sub>, 0.01 g CaCl<sub>2</sub>, and 5 mL of Hutner traces; distilled water was added to make the final volume 1 L and pH was set at 2.5 by the addition of 20 % (v/v) HCl. The strain can be obtained from the culture collection of the Microalgal Biotechnology Unit, Central Research Services (CIDERTA) of the University of Huelva, Spain, by direct request to the authors (cvilchez@uhu.es).

### DNA extraction, PCR amplification, and sequencing

Cell samples from a liquid culture were harvested by centrifugation, 5 replicates for DNA extraction have been

used. Genomic DNA extraction was done by using the Quick-DNA Fecal/Soil Microbe MiniPrep kit (Zymo Research). The protocol was adapted by disrupting the cells in a cell mill for 30 s at 30 Hz twice using glass beads tubes. The supernatant obtained was used for DNA purification, which was performed according to standard procedures. A sequence of the 18S rDNA was amplified using a pair of conserved, universal primers for eukaryotes: NS1 (forward primer) and NS2 (reverse primer) (Hillis and Dixon 1991). PCR was run by using iTaq Universal SYBR BIORAD (Bio-Rad Laboratories, Inc.). The PCR product was purified using the E.Z.N.A. Cycle Pure Kit purchased from OMEGA Bio-Tek (USA). Sanger sequencing was performed by Secugen S.L. (Madrid, Spain). The sequence was analyzed using the BlastN software against the non-redundant database available in GenBank (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) with a cutoff of  $1 \times 10^{-5}$ .

### Phylogenetic analysis

The nucleotide sequence of the 18S rDNA of the acidophilic strain was aligned with the sequences of *Elliptochloris* sp. The phylogenetic tree was deduced from all available positions of the 18S rDNA gene sequence by Neighbor-Joining methods (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1,000 replicates (Felsenstein 1985) represented the evolutionary history of the analyzed taxa. Evolutionary distances were calculated using the Jukes-Cantor method (Jukes and Cantor 1969) and were in units of the number of base substitutions per site. A total of 255 positions were identified in the final dataset. All phylogenetic analyses were performed using the MEGA 11 program (Tamura et al. 2021).

### Photoautotrophic and mixotrophic culture conditions

Photoautotrophic and mixotrophic cultures were grown in batch cultivation mode for 16 days and were prepared from a repeated-batch culture in the middle of the exponential growth phase ( $OD_{750}$  value around 5). Cultures of 400 mL were prepared in 500 mL round bottles using K9 medium at a pH of 2.5, at an initial cell concentration measured as  $OD_{750}$  of approximately 0.7. pH of the cultures was checked daily and adjusted with 20 % (v/v) HCl to 2.5. The cultures were established in a culture room at  $25 \pm 2$  °C with continuous mixing by magnetic stirring and at a continuous light intensity of  $150 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  for 24 h under white light fluorescent tubes. The photoautotrophic and mixotrophic cultures were bubbled with either air alone or air containing 2.5 % (v/v)  $\text{CO}_2$ . A flowmeter was connected to the air and  $\text{CO}_2$  pipes to adjust the proportion of both gases bubbled into the cultures. Only

those cultures supplemented with  $\text{CO}_2$ -enriched air (2.5 % v/v) and without additional carbon sources were called “photoautotrophic cultures” in this study. The organic carbon sources selected for mixotrophic cultivation were glucose (D (+)-Glucose, anhydrous compound, VWR) and glycerin at a concentration of 5 mM. In this study, residual technical glycerin from biodiesel production was used. Two types of residual technical glycerin were provided to the cultures. Purity of both technical glycerin sources is around 80 % in glycerol and their MONG (matter organic non-glycerol) is in the range 1–2 %. One of them was obtained from vegetal residues while the other was obtained from used cooking oil residues (UCO). Both glycerin types were supplied by Bio-Oils Huelva S.L.U., a biodiesel production company located in Palos de la Frontera (Huelva, Spain). Three replicates of each culture condition were performed.

### Growth and photosynthetic viability

Growth was determined by measuring the optical density (OD) at 750 nm of 1 mL culture samples in a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, USA) equipped with temperature control system adjusted to 25 °C. The  $OD_{750}$  data provided the information of culture turbidity, which is proportional to the amount of biomass present in the culture (Shuler and Kargi 2005). Additionally, dry weight of biomass was analyzed gravimetrically in an analytical balance, model CP225D (Sartorius, Germany). Culture samples (2 mL) were filtered through 0.7  $\mu\text{m}$  filters, (MFV5 model, Anovia, Spain), the filters were dried for 24 h at 100°C and the remaining dry residue was further weighed.

Photosynthetic viability was determined based on the measurement of chlorophyll fluorescence, the maximum quantum yield ( $F_v/F_m$ ) of Photosystem II (PSII), following published methods (Schreiber 2004). Quantum yield (Qy) was measured in an AquaPEN AP-C 100 portable pulse amplitude-modulated (PAM) fluorimeter (Photon Systems Instruments, Czech Republic), at 620 nm of actinic light. Each culture sample (1 mL) was pre-incubated in the dark for 15 min and subsequently placed in the measurement chamber of the AquaPEN fluorimeter.  $F_v$  represents the minimum fluorescence recorded after exposing cells to a non-actinic beam and acclimating them in the dark for 15 min, while  $F_m$  represents the maximum fluorescence recorded in cells after exposing them to a brief but saturating pulse of actinic light.

### Lipids and fatty acids analysis

Lipid content of the microalgal biomass was measured in lyophilized biomass samples as described by Südfeld et al. (2021). Briefly, 10 mg samples were extracted with chloroform:methanol (2:1, v/v). The extraction process

was followed by solvent evaporation using a N<sub>2</sub> stream. Subsequently, lipid content was determined gravimetrically.

Fatty acid analysis was performed according to the following procedure (Robles et al. 2023). Once the acylglycerides were extracted, the corresponding fatty acids methyl-ester (FAME) were obtained through acid catalysis-mediated transesterification. The mixture was heated at 85 °C for 1 h, then cooled and washed with hexane and water. The FAMES were separated by centrifugation (1,000 xg, 10 min). FAMES were separated and analyzed in a gas chromatograph system equipped with flame ionization detector (model 7890A, Agilent, USA). A 1- $\mu$ L sample was injected into a silica capillary column (30 m, 0.32 mm id and 0.25- $\mu$ m film thickness). Helium was used as carrier gas. The applied flow rate was 1.5 mL min<sup>-1</sup> and the split ratio 20:1. The injector and detector temperature values were 100 °C and 220 °C, respectively. The programmed oven temperature raised from 80 to 140 °C at 5 °C min<sup>-1</sup>, followed by increase to 170 °C at 4 °C min<sup>-1</sup> and then maintained for 2 min at 170 °C. Temperature was then increased to 190 °C at 1 °C min<sup>-1</sup> and maintained for 2 min. The final temperature in the oven was 210 °C. Each one of the FAME peaks was identified by comparing retention times with those of mixed fatty acids standards (FAMES Mix C4-C24 Supelco Analytical). Concentrations of FAMES (ppm) were quantified by comparing their peak areas with those obtained from the standards of known concentration (Sigma Aldrich, USA). Fatty acid composition was calculated as percentage of the total fatty acids in the volume of hexane. For quantification, tripentadecanoin was used as internal standard (Sigma Aldrich).

## Statistical analysis

Unless otherwise stated, all data are presented as the mean of three replicates and the standard deviations are shown in the corresponding figures and tables. The data from the different treatments were evaluated by constructing univariate statistical models and performing the analysis of variance (ANOVA) (confidence of 95 % was considered

for determining statistical significance). All analyses were performed using the Minitab 21 software (Additional data in Online Resource 2).

## Results

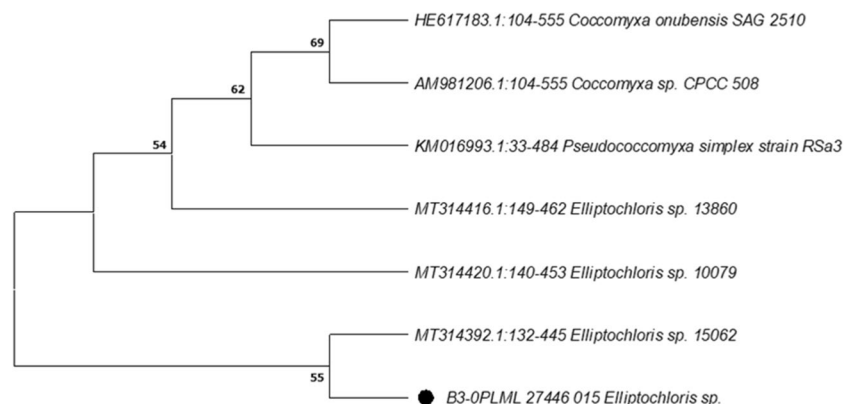
### Identification and phylogenetic approach of *Elliptochloris* sp.

The microalga used in this study was isolated following the procedure described in the Materials and Methods section. BLAST analysis of 18S rDNA sequences was performed to produce significant alignments (query cover, 99 %) with the sequences obtained from the isolated extremophilic microalga. The results indicated the presence of significant homology of >80 % with 17 sequences; 15 of them (approx. 90 %) belonged to the genus *Elliptochloris*, and only two belonged to the genus *Chloroidium*. The microscope images of the algae in culture collections showed robust morphological similarities with the isolated *Elliptochloris* species from Tinto River (Supplementary figure 1). Based on the identification approach, we placed the isolated species into the genus *Elliptochloris*. A phylogenetic analysis showed that the isolated species could be included in the green algal class Trebouxiophyceae. In the 18S rDNA tree (Fig. 1), *Elliptochloris* sp. was placed close to *Coccomyxa onubensis* and *Pseudococcomyxa simplex*, which also belong to the Trebouxiophyceae.

### Effect of the carbon source on the growth and photosynthetic viability of *Elliptochloris* sp.

In photoautotrophic cultures, an increase in the number of cells leads to a shadowing effect among cells, which limits growth due to a reduction in available light (Richmond 2004). The use of an organic carbon source (OCS) can satisfy the energy requirements for growth; thus, photosynthetically

**Fig. 1** Molecular phylogeny of *Elliptochloris* sp. based on 18S rDNA gene sequence comparisons of the genus *Elliptochloris*. More details in Materials and Methods section



produced energy demand can decrease significantly. Based on this, mixotrophic growth of the acidotolerant microalga *Elliptochloris* sp. bubbled with either air alone or air containing 2.5 % (v/v) CO<sub>2</sub> was analyzed and compared to photoautotrophic growth.

The time-course evolution of the growth of *Elliptochloris* sp. as a function of the carbon source is shown in Fig. 2. The growth curves obtained for cultures of *Elliptochloris* sp. were completed in about 16 days of growth and showed a typical linear growth rather than the typical sigmoidal growth pattern with exponential and stationary phases. The growth curves obtained for mixotrophic cultivation bubbled with CO<sub>2</sub>-enriched air (Fig. 2a) showed less steep trends than those obtained for the photoautotrophic culture. Conversely, the curves obtained from cultures subjected to mixotrophic cultivation bubbled with air alone (Fig. 2b) showed steeper trends than those obtained for the culture grown only on air. However, the growth rates obtained under these conditions were approximately 80 % lower than those obtained for the photoautotrophic culture bubbled with CO<sub>2</sub>-enriched air.

As reported above, the use of an organic carbon source can satisfy the energy requirements for growth; thus, photosynthetically produced energy demand can decrease significantly. Based on that, the effect of the carbon source on photosynthetic efficiency is shown in Fig. 3, based on the Quantum yield (Qy) data and the intracellular chlorophyll content of *Elliptochloris* sp. Cultures grown mixotrophically (Fig. 3a and b) showed a decrease in the Qy values till 0.4–0.5 during the first few days of growth, i.e., up to the middle of the linear phase, day 4 of cultivation. After that, all mixotrophic cultures showed higher Qy values of about 0.6. Additionally, the intracellular content of chlorophyll in cultures grown mixotrophically was lower than that in the photoautotrophic culture. Two linear trends with different slopes were obtained. (i) The cultures grown mixotrophically and bubbled with CO<sub>2</sub>-enriched air (Fig. 3c) showed a linear trend in the chlorophyll content during the first 3–4 days. This was followed by a considerable increase in the slope

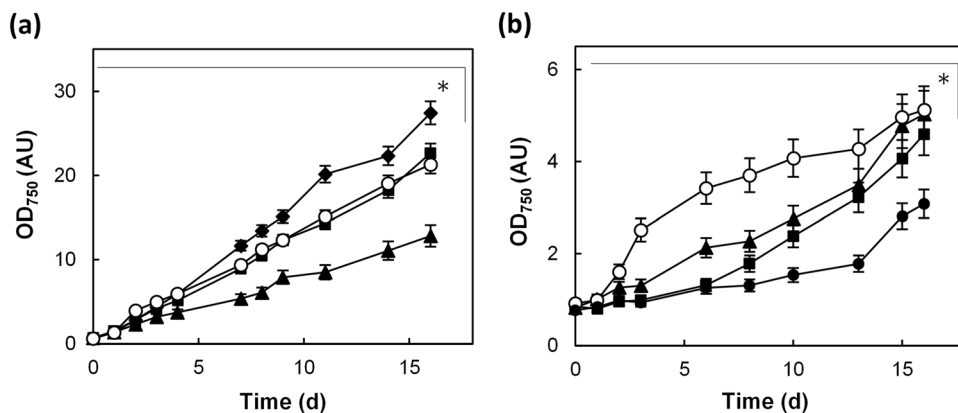
approximately by the middle of the linear growth phase, day 9–11 of cultivation. (ii) The cultures grown mixotrophically and bubbled with air alone (Fig. 3d) presented the highest intracellular content of chlorophyll at the beginning of the linear growth phase, day 6–8 of cultivation. This was followed by a slight decrease and stabilization of the chlorophyll content.

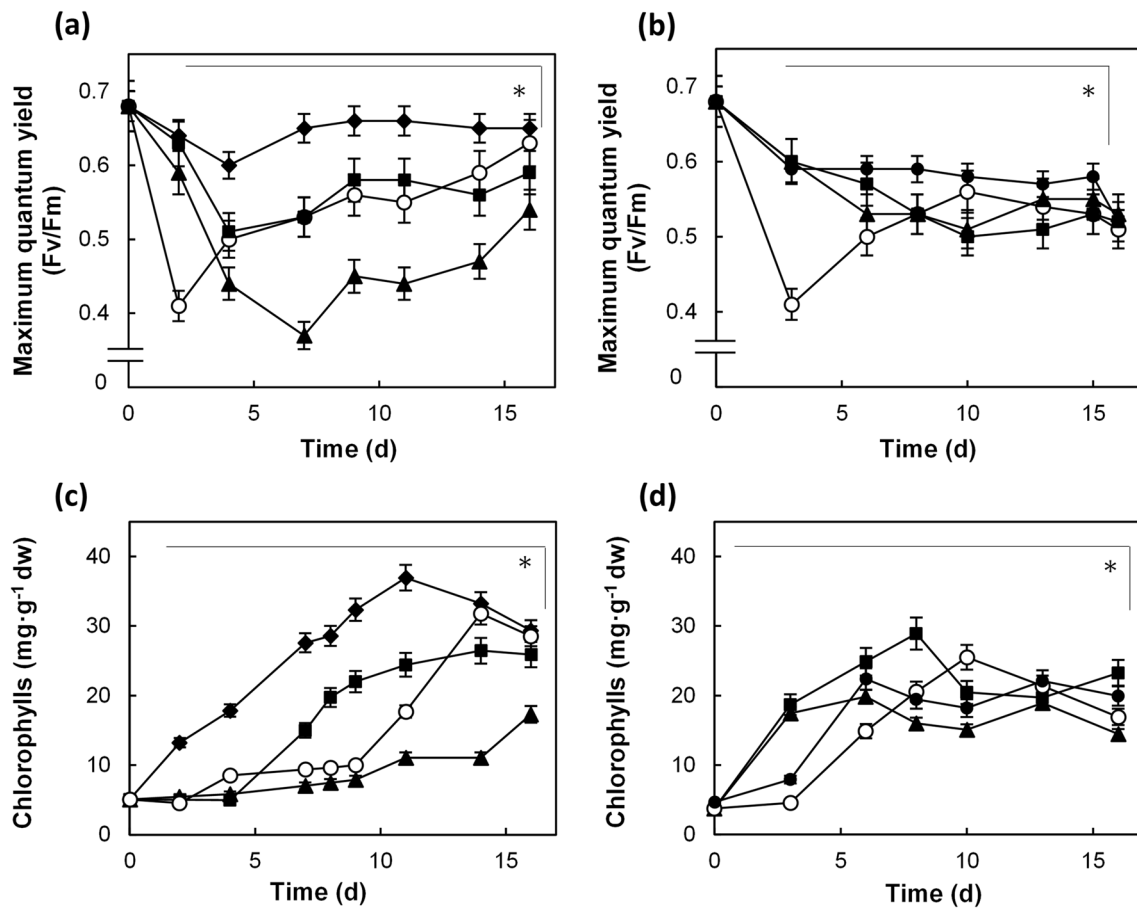
### Effect of the carbon source on the lipid production of *Elliptochloris* sp.

We investigated the effect of using additional OCS –glycerin or glucose– bubbled with either air alone or air containing 2.5 % (v/v) CO<sub>2</sub> on the production of lipids and fatty acids in *Elliptochloris* sp.

The evolution of intracellular lipids and fatty acids as a function of the carbon source for cultures of *Elliptochloris* sp. is shown in Fig. 4. The intracellular lipid content in cultures grown mixotrophically and bubbled with CO<sub>2</sub>-enriched air (Fig. 4a) remained within a range of about 20 to 30 % of the total biomass and varying within this range along the culture incubation time. These variations (from 20 to 30 %, dry weight basis) are significant and could be related with metabolic adjustments in response to variations in key growth parameters along the batch-mode incubation, e.g. light availability due to increased cell density and, decreased availability of key nutrients by the end of the curve (Alishah et al. 2019). A similar pattern was recorded for mixotrophically grown cultures bubbled with air alone (Fig. 4b), except for the culture grown with technical glycerin obtained from UCO residues; this culture showed an increase in intracellular lipid content of about 40 % during the first few days of growth, day 6–8 of cultivation, followed by a decrease in the lipid content to initial levels in the later stages obtaining, approximately 22 % dw. Under this condition, the highest content of intracellular lipids was obtained by day 8, approximately, 30 % dw, 48 % higher than that recorded in the photoautotrophic culture.

**Fig. 2** Growth curves of the acidotolerant microalga *Elliptochloris* sp. as a function of the carbon source in cultures bubbled with CO<sub>2</sub> (a) or air alone (b). Symbols represent: Photoautotrophic condition (-◆-), technical glycerin from vegetal residues (-■-), and from UCO residues (-▲-), glucose (-○-) and air (-●-). (\*) Represents the significant differences of all treatments with respect to the photoautotrophic culture with 95 % confidence





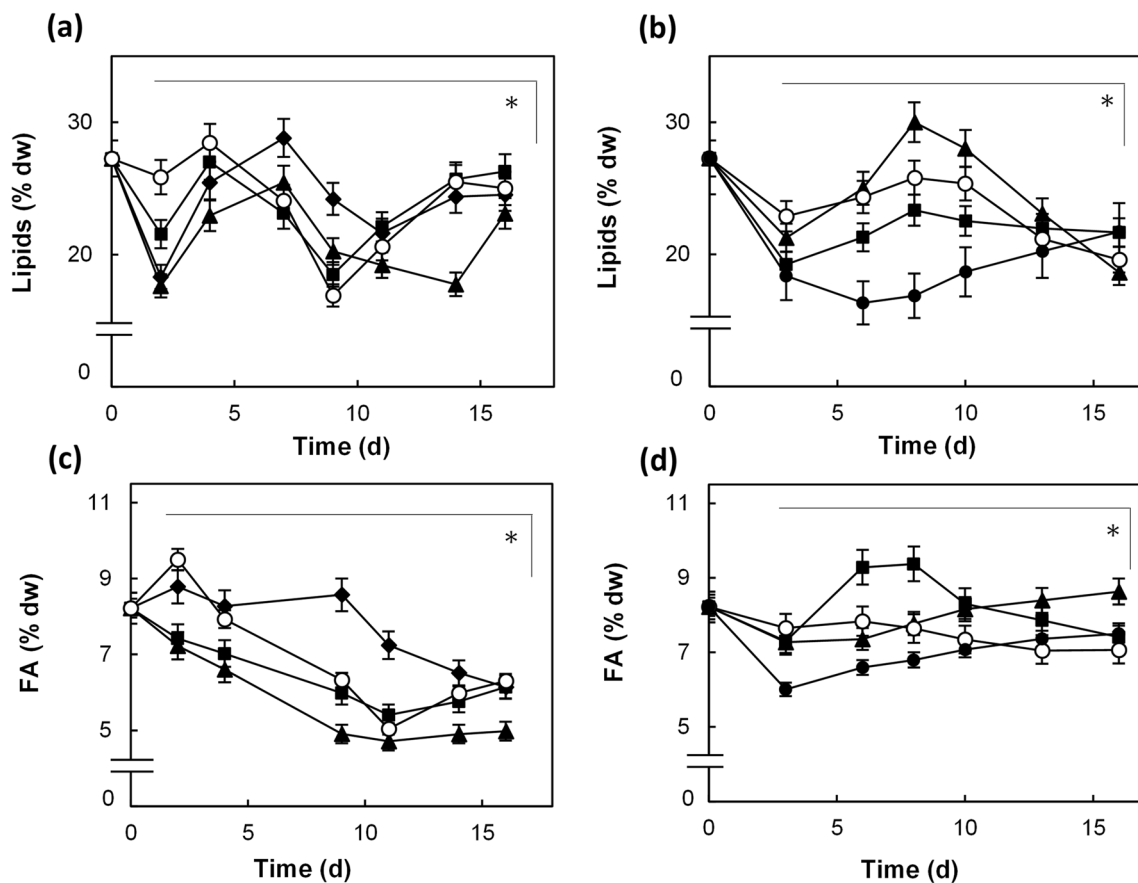
**Fig. 3** Photosynthetic efficiency (a–b) and intracellular content of chlorophylls produced (c–d) for the microalga *Elliptochloris* sp as a function of the carbon source in cultures bubbled with CO<sub>2</sub> (a, c) or air alone (b, d). Symbols represent: Photoautotrophic condition (–◆–),

technical glycerin from vegetal residues (–■–) and from UCO residues (–▲–), glucose (–○–) and air (–●–). (\*) Represents the significant differences of all treatments with respect to the photoautotrophic culture with 95 % confidence

The intracellular content of fatty acids showed a different trend depending on whether the cultures were grown mixotrophically bubbled with air alone or CO<sub>2</sub>-enriched air. Cultures grown mixotrophically with CO<sub>2</sub>-enriched air (Fig. 4c) showed a decrease in the intracellular fatty acid concentration of about 25–40 % until, approximately, day 10 of cell culture; after that day the fatty acid content remained unchanged, approximately, 6 % dw. In contrast, the intracellular fatty acid content of cultures grown mixotrophically bubbled with air alone (Fig. 4d) increased moderately after the first days of cultivation, day 3; and the highest data, approximately 9.3 % dw, 26 % higher than that obtained for the photoautotrophic culture, were obtained from the culture grown with technical glycerin obtained from vegetal residues.

The composition of specific fatty acids of biomass samples from the different cultures was also analyzed (Fig. 5). The content of saturated fatty acids increased about 20 % in the mixotrophic cultures bubbled with CO<sub>2</sub>-enriched air (Fig. 5a) until day 4, obtaining

values of, approximately, 2.3 % dw. Then, the content of saturated fatty acids decreased considerably about 30–40 %. The intracellular content of unsaturated fatty acids (Fig. 5c) decreased about 30–50 % till day 9 of cultivation, and then, their content remained unchanged, approximately 3.36 % dw. The changes in the content of these compounds in mixotrophic cultures bubbled with air alone were different. In general, the content of saturated fatty acids (Fig. 5b) increased moderately about 10–20 %, obtaining the highest value, approximately 2.5 % dw, on day 8 from the culture grown with the technical glycerin from vegetal residues. However, the content of unsaturated fatty acids remained constant throughout the culture in almost all conditions (Fig. 5d), approximately, 4.5 % dw. In contrast, in photoautotrophic cultures, the content of unsaturated fatty acids remained stable (roughly 5 % dw) during the first few days of growth till day 9 of cultivation, where the content decreased about 30 % and was lower than that recorded in mixotrophic cultures.

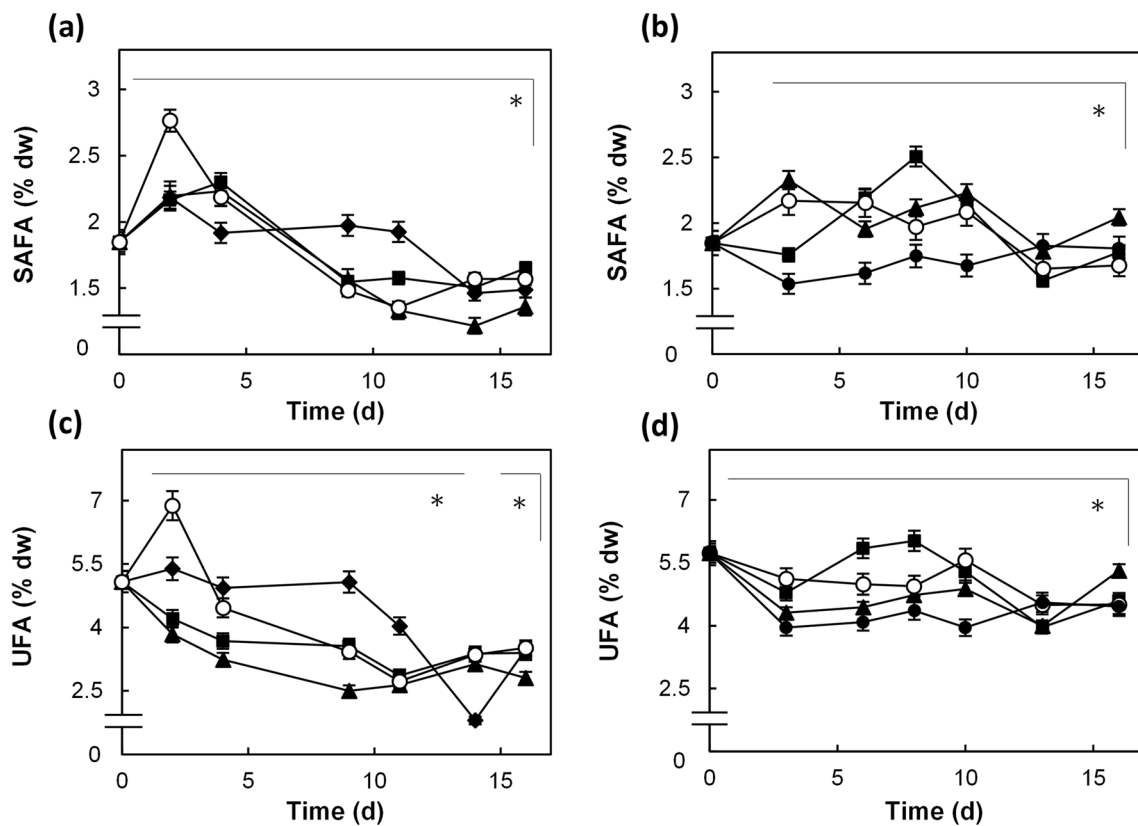


**Fig. 4** Intracellular content of lipids and fatty acids produced for the microalga *Elliptochloris* sp. as a function of the carbon source in cultures bubbled with CO<sub>2</sub> (a, c) or air alone (b, d). Symbols represent: Photoautotrophic condition (-◆-), technical glycerin from vegetal

residues (-▲-) and from UCO residues (-▲-), glucose (-○-) and air (-●-). (\*) Represents the significant differences of all treatments with respect to the photoautotrophic culture with 95 % confidence

The overall composition of fatty acids is shown in Table S1. The Table compares all growth conditions tested regardless cultivation time; thus, only highest data obtained from each cultivation are presented in the table. Data exceeding those obtained for the control culture are presented in bold. According to the table, an increase of about 20–60 % C18:0 is obtained in mixotrophic cultures regardless the cultures being bubbled with either only air or air containing 2.5 % (v/v) CO<sub>2</sub>. Saturated fatty acids are used to obtain advanced biofuels (e.g. hydrogenated vegetable oils), while fatty acids from the omega-3 and omega-6 families are considered essential to human health. Accordingly, Table S1 also shows how the content of C18:2 (linoleic acid, omega 6) in the culture grown in glucose and bubbled with air alone was 28 % higher than that in the photoautotrophic culture, 3.23 % dw, and the content of C18:3 (linolenic acid, omega 3) in the culture grown in technical glycerin from vegetal residues and bubbled with air alone was 24 % higher than that in the photoautotrophic culture, 3.03 % dw. Thus, the variation in the C18:3/C18:2

(omega 3/omega 6) ratio across time depending on the carbon source used for cultures of *Elliptochloris* sp. is shown in Fig. 6. The photoautotrophic culture treated with 2.5 % v/v CO<sub>2</sub> showed a considerable increase of about 35 % in the intracellular content of C18:2 over time, this being higher on day 9 of cultivation, approximately, 2.12 % dw, whereas the content of C18:3 remained constant over time of, approximately, 1.18 % dw. However, when the cultures were grown mixotrophically and bubbled with CO<sub>2</sub>-enriched air (Fig. 6a-b), a slight increase in the intracellular concentration of C18:2 of about 15 % and a decrease of about 50 % in the concentration of C18:3 was observed, indicating a decrease in the C18:3/C18:2 ratio (supplementary figure 2). In contrast, cultures grown mixotrophically and bubbled with air alone (Fig. 6c-d) showed a substantial decrease of about 35 % in the intracellular content of C18:2 and an increase of about 20 % in the content of C18:3, indicating an increase in the C18:3/C18:2 ratio. In addition, routine analysis of protein and carbohydrate content of control culture samples showed values of roughly 42–45 % (dw basis) for protein and



**Fig. 5** Time-course evolution of saturated (SAFA) and unsaturated (UFA) fatty acids content produced by the microalga *Elliptochloris* sp. as a function of the carbon source in cultures bubbled with CO<sub>2</sub> (a, c) or air alone (b, d). Symbols represent: Photoautotrophic condi-

tion (○), technical glycerin from vegetal residues (■), UCO residues (▲), glucose (□) and air (●). (\*) Represents the significant differences of all treatments with respect to the photoautotrophic culture with 95 % confidence

19–21 % (dw basis) for carbohydrates, in the range of other microalgae spp. (Richmond 2004).

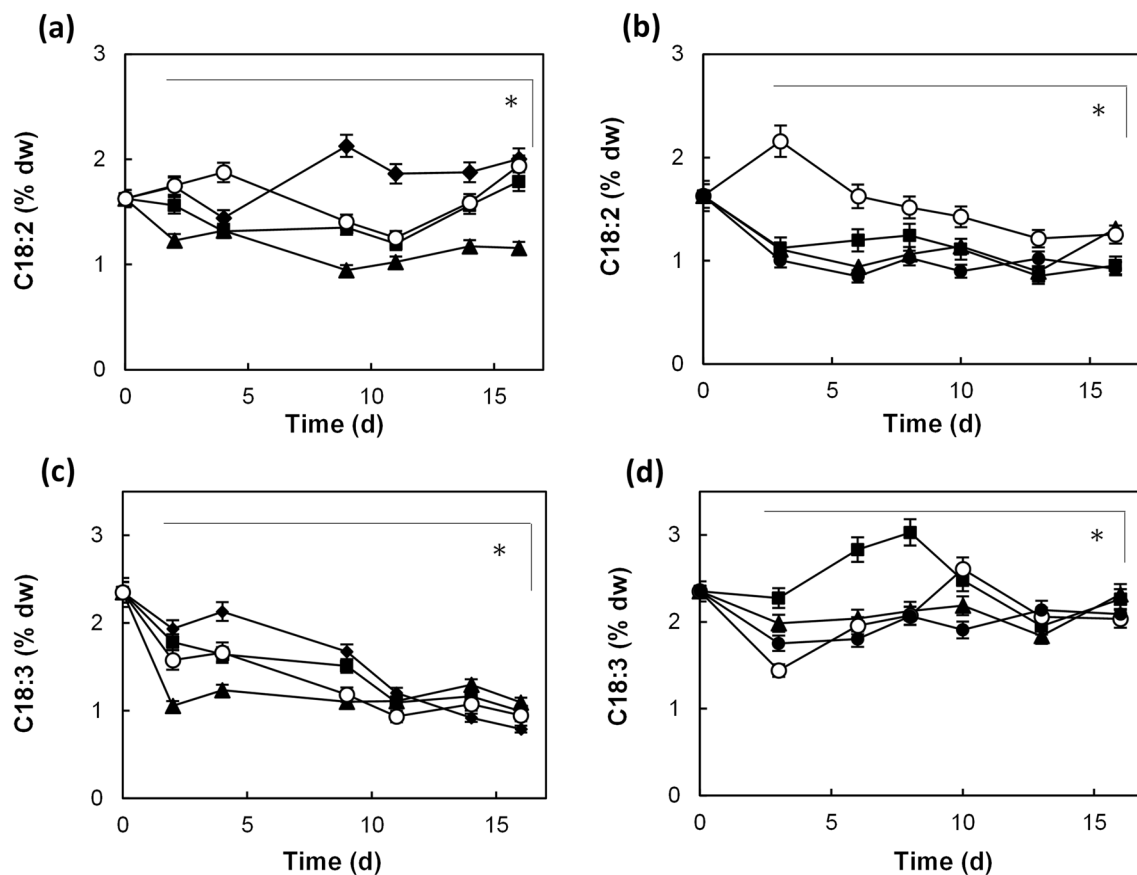
## Discussion

### Identification and phylogenetic approach of *Elliptochloris* sp.

The strain used in this study was isolated from Tinto River. The so-called “red river” flows along 100 km from the Iberian Pyritic Belt down to the Atlantic Ocean and is one of the most extensive examples of extreme acidic environments, exhibiting low pH values in the range of 1.7 to 3.1 and presence of cations in solution, of which Fe (II) and (III) and Cu (II) have a relevant quantitative presence (Fernández-Remolar et al. 2003; Amils et al. 2014). In spite of these extreme conditions, this river has been reported to contain a wide biodiversity, including many eukaryotic organisms (algae, fungi, and protists) as the main contributors to the biomass production in the river (López-Archilla et al. 2001; Garbayo et al. 2008). It has been shown

that the extremely acidic conditions of the Tinto River basin are not just the product of 5000 years of mining activity in the area but the consequence of an active underground bioreactor that obtains its energy from the oxidation of sulfur minerals of the Iberian Pyrite Belt (Amils et al. 2014). The bio-oxidation of pyrite produces a sulfate-enriched acidic solution (pH roughly between 1 and 3) that prevents ferric iron precipitation (Fernández-Remolar et al. 2003), contrary to what occurs in neutral-pH conditions. Several photosynthetic microalgae species adapted to the low pH and the oxidative stress produced by the dissolved metal ions in the river, in addition to strong metal tolerance (Garbayo et al. 2012). This is the case of *Coccomyxa onubensis* (Fuentes et al. 2016). The *Elliptochloris* sp. strain used in this study should be subjected to further characterization studies to define their tolerance features to the extreme conditions of its natural habitat.

*Elliptochloris* chlorophyte genus is similar to *Chlorella* and is closely related to the genus *Coccomyxa* (Darienko et al. 2016). Members of this group have spherical and elliptical cells, possess bilobed chloroplasts with or without pyrenoid, and produce two types of auto-spores, which are



**Fig. 6** Time-course evolution of linoleic acid (C18:2) and linolenic acid (C18:3) produced by the microalga *Elliptochloris* sp. as a function of the carbon source in cultures bubbled with CO<sub>2</sub> (a, c) or air alone (b, d). Symbols represent: Photoautotrophic condition (-◆-),

technical glycerin from vegetal residues (-■-), glucose (-○-), and air (-●-). (\*) Represents the significant differences of all treatments with respect to the photoautotrophic culture with 95 % confidence

used for a unique type of reproduction (Eliáš et al. 2008; Gustavs et al. 2011; Darienko et al. 2016). This genus was first described by Tschermak-Woess (1988) based on the species *E. bilobata*. Species belonging to this genus are widely distributed throughout the world. They are found in terrestrial habitats in free-living form or as a photobiont with lichens (Darienko et al. 2016). Members of the genus *Elliptochloris* are difficult to identify because of their flexible phenotype and lack of characteristic features. According to Gustavs et al. (2011), algae belonging to this group contain high concentrations of ribitol, a type of polyol, which plays a protective role and helps these microorganisms adapt to extreme environments. Our experience with *Elliptochloris* sp. cultures allowed us to state that this strain can grow at around neutral pH, thus it behaves as an acidotolerant strain. Previous experiments (data not published) of growth at pH between 2.5 to 7 showed slight differences in biomass productivity as a function of the pH value. Nevertheless, the highest productivities were obtained at pH 4. Microscopic observations revealed increased presence of a fungus at around neutral pH. Further communities' abundance

analysis would be required for understanding any eventual relationship between the strain of this study and any other associated community.

As this study intended to grow *Elliptochloris* sp. in a majoritarian form, a pH of 2.5 was selected as it allows obtaining moderate productivities whilst growth of any associated and non-associated microorganism is neglected. Because of this species ability to grow under acidic conditions which limits the growth of opportunist heterotrophic organisms in the presence of an organic carbon source, we selected it to conduct this study.

### Effect of the carbon source on the growth and photosynthetic viability of *Elliptochloris* sp.

As observed in Fig. 2, *Elliptochloris* sp. growth curve shows a typical linear growth shape rather than the typical sigmoidal growth pattern with exponential and stationary phases. Biomass concentration was not low enough to obtain a clear and visible adaptation phase and the duration of this experiment was not long enough to obtain a stationary phase.

The observed linear growth, rather than exponential growth, is the result of each cell having fewer photons per unit of time as long as growth progresses. The reason is that the light intensity provided to the cultures does not vary throughout growth, while there is an increase in the number of cells in the culture. In this way, a shadowing effect of some cells on others occurs, limiting the availability of light for each cell (Richmond 2004). The lower availability of light per unit of time results in a lower activity of photosynthesis, therefore a lower rate of production of NADPH and ATP, necessary for cell growth. However, this lower light bioavailability does not seem to affect growth significantly. The reason is that the growth rates of *Elliptochloris* sp., around  $0.8 \text{ g L}^{-1} \text{ day}^{-1}$  (data not published), are in the range of those recorded by Chunzhuk et al. (2023) for a non-extremophilic *Elliptochloris subsphaerica* and other non-extremophilic microalgae (Richmond 2004). *Elliptochloris* spp. have not been proved to grow heterotrophically. However, microalgal blooms have been described to occur at a depth of 11 m below the acidic lakes in the Pyritic Belt of Huelva (Spain) surface (Sánchez-España et al. 2020) where only PAR radiations of low wavelengths are available to the algal cells. Thus, this natural behavior is coherent with the linear growth phase obtained for *Elliptochloris* sp. regardless light intensity availability; thus, *Elliptochloris* sp. might be an adequate candidate to grow heterotrophically.

The results in Fig. 2 showed that *Elliptochloris* sp. could assimilate an OCS. This was inferred by comparing the growth patterns of mixotrophic cultures supplemented with an OCS and bubbled with air (Fig. 2) to those of cultures supplied only with air. Our findings indicated that  $\text{CO}_2$  strongly influenced the growth rate of *Elliptochloris* sp. Thus, the growth patterns obtained when supplementing the cultures with an OCS can be improved greatly by supplementing the cultures with  $\text{CO}_2$ . However, the maximum growth rate was recorded in cultures grown under photoautotrophic conditions. This finding showed that adding an OCS to cultures growing under conditions of standard light intensity and  $\text{CO}_2$  concentration could partly slow down the growth of *Elliptochloris* sp.  $\text{CO}_2$  is a quite soluble gas in water. When dissolved in water, it produces carbonic acid ( $\text{H}_2\text{CO}_3$ ), a weak acid which at around neutral pH dissociates into bicarbonate and protons; thus, causing the acidification of the water (Thomas et al. 2022). In this sense, constant bubbling with  $\text{CO}_2$  causes pH to drop in neutral media and the supply in large microalgal cultures is usually controlled to keep pH at optimal values. However,  $\text{pK}_{\text{a}1}$  of carbonic acid is 6.35 (Martinez-Hincapie et al. 2021); therefore, bubbling  $\text{CO}_2$  at an acidic pH of 2.5, as it is the case of our study, will have no influence on the medium pH. Based on that, the alkalization of pH that generally occurs when growing photosynthetic microorganisms was counteracted in this study by the addition of 20 % (v/v) HCl, in order to maintain

pH levels around 2.5-3.5. In addition, growth at highly acidic pH becomes advantageous for getting robust cultures that are free from biological contaminants, as only acidophilic strains might compete with the targeted strain (e.g. *Elliptochloris* sp. in this study). In fact, the referred eventual advantage was already shown for *Coccomyxa onubensis*, a highly acidic environment strain isolated by our team from Tinto river, when grown under non-sterile conditions in an outdoor tubular PBR (Fuentes et al. 2020).

Mixotrophic growth is a commonly used strategy to counteract the slow growth in photoautotrophic cultures because of the limited availability of light. However, adding an extra OCS affects the growth of some species negatively, probably because assimilating the extra carbon source might lead to temporary metabolic imbalances, resulting in the partial inhibition of growth (Paranjape et al. 2016). This might occur due to the combination of two biochemical events, including slow uptake rates and/or assimilation of the OCS and low photosynthetic activity (Markou et al. 2021).

In this sense, the effect of the carbon source on photosynthetic efficiency is shown in Fig. 3, based on the quantum yield (Qy) data and the intracellular chlorophyll content of *Elliptochloris* sp. Generally, an increase in  $\text{CO}_2$  concentrations increases the photosynthesis rate in microalgae (Martinez and Orus 1991), which leads to a greater production of carbohydrates and biomass. This finding matched the increase in the growth and photosynthetic efficiency of the photoautotrophic cultures when compared with the culture supplied with only air (Fig. 2 and 3). The PSII photosynthetic efficiency of healthy cultures should be around 0.6 – 0.7, which generally indicates that the culture is not under stress and is viable (Maxwell and Johnson 2000; Zijffers et al. 2010). Some of the Qy values obtained in mixotrophic cultures were significantly below 0.6, even when supplemented with  $\text{CO}_2$  which, as reported above, led to an increase in the photosynthesis rates. This finding could be justified by the lower photosynthesis-related energy needs for nutrient assimilatory reduction, as most of the anabolic energy needs can be obtained by oxidizing the OCS added to the culture medium (Castillo et al. 2021). These processes can lead to the partial inhibition (or downregulation) of the light energy bioconversion cell machinery, which might be indicated by a decrease in the photosynthetic efficiency of PSII. The above-mentioned changes should result in a lower Quantum yield, which matches the findings of our study. As the OCS was only supplied at the beginning of the experiment, the increase in the Qy values for mixotrophic cultures from the middle of the linear growth phase occurred probably because of the lower bioavailability of the OCS.

The time-course evolution of the chlorophyll content of the cells grown under mixotrophic conditions (Fig. 3c and d) was consistent with the results of photosynthetic efficiency discussed above. Generally, the demand of cells for

photosynthetic energy is low under mixotrophic conditions, which could be the reason for the lower chlorophyll content recorded. Particularly, in the mixotrophic cultures bubbled with CO<sub>2</sub>-enriched air, the chlorophyll content was roughly constant during the first few days of incubation. A large part of the metabolic energy necessary to satisfy the energy needs of cells can be obtained through the oxidation of OCS, based on its major bioavailability, as shown by the Q<sub>y</sub> data in Fig. 3a. From the middle of the linear growth phase, the increase in the chlorophyll content was consistent with the lower bioavailability of the OCS, which led to the subsequent increase in the energy produced by photosynthesis.

Similar downregulation events occurred in mixotrophic cultures bubbled with air alone. However, the highest content of chlorophyll was found during the first few days of growth when the biomass concentration was low (Fig. 2b). This was consistent with the lower shadowing effects among cells, which induced the cells to experience slight photoinhibition. Thus, the increase in the chlorophyll content in the first few days increased turbidity and decreased damage due to photoinhibition (Xu et al. 2020).

Our results showed that when an extra OCS is available, cells have low requirements for photosynthetic pigments. These results matched the findings of Kong et al. (2020), who suggested that under mixotrophic conditions, microalgae can obtain energy by oxidizing organic compounds; thus, algal growth is less dependent on light availability. Deng et al. (2019) studied *Chlorella kessleri* cultures by supplying them with glucose and showed that increasing the irradiance of the incident light resulted in maladjustments in glucose assimilation and decreased the chlorophyll content in cells. Their findings suggested that light intensity can inhibit mixotrophic growth. Similarly, Markou et al. (2021) stated that the low growth rates obtained in the mixotrophic cultures of *A. protothecoides* were probably due to growth inhibition by light. Consequently, the growth limitation and the decrease in the photosynthetic efficiency found in mixotrophic cultures of *Elliptochloris* sp. might be due to the selected light irradiance effect in the presence of an organic carbon source.

### Effect of the carbon source on the lipid production of *Elliptochloris* sp.

As shown above, for cultures supplemented with an OCS part of the light energy fixed through photosynthesis in the form of NAD(P)H may not be utilized for the assimilation of nutrients (Fig. 3); under such conditions, this excess chemical energy might be oxidized by reactive oxygen species (ROS) formed in the chloroplast (Hasanuzzaman et al. 2020). One of the metabolic capacities of microalgae to dissipate excess reducing power, i.e., chemical energy from the photosynthetic fixation of light energy, is to divert

excess NAD(P)H to the synthesis of triacylglycerides (TAG); using this strategy, they can store carbon and energy in the chemical form of fatty acids (Schüler et al. 2017). Additionally, part of the acetyl-CoA derived from the oxidative degradation of the OCS can be diverted to the production of fatty acids using excess NAD(P)H. The use of excess NAD(P)H might help in maintaining the balance between the reduced and oxidized forms of this coenzyme (NAD(P)H/NAD<sup>+</sup>) in cells.

Based on this fact, the excess energy from light and organic carbon provided to the cultures in mixotrophic cultures led to the overproduction of NADPH. This stimulated the production of ROS and increased oxidative stress. Our results showed that the mixotrophic cultures bubbled with CO<sub>2</sub>-enriched air had lower photosynthetic efficiency (Fig. 3a), which decreased the production of NADPH. The decrease in the intracellular fatty acid content in mixotrophic cultures bubbled with CO<sub>2</sub>-enriched air could be justified by a lower production of NADPH to balance the NADPH and NADP<sup>+</sup> levels in the cells. Conversely, in mixotrophic cultures bubbled with air alone, a part of the photosynthetic energy produced could not be utilized for fixing CO<sub>2</sub>. This led to an excess production of NADPH for the available CO<sub>2</sub>. The excess chemical energy can be stored in the form of fatty acids (Fig. 4b) to maintain the balance between NADPH and NADP<sup>+</sup> levels in the cells.

The ratio of the saturated to unsaturated fatty acid content was constant throughout the experiment and independent of the carbon source used (supplementary figure 3). Thus, the addition of CO<sub>2</sub> did not affect the ratio of total saturated to unsaturated fatty acids. Then, by analyzing the total content of saturated and unsaturated fatty acids, we found that the air-bubbled mixotrophic cultures generally produced greater intracellular content of both saturated and unsaturated fatty acids than photoautotrophic cultures. This was shown in Fig. 5 and supplementary figure 4. Supplementary figure 4 resumes the average relative variation with respect to photoautotrophic cultures of total saturated (black bar) and unsaturated (white bar) fatty acid content throughout the cultivation period in any of the mixotrophic conditions tested. The figure shows that the use of OCS and only air enhanced the accumulation of fatty acids (dry biomass basis).

Additionally, the changes described suggested that the behavior of mixotrophic cultures of *Elliptochloris* sp. under high CO<sub>2</sub> throughout the growth phase was similar to that of cultures exposed to oxidative stress, irrespective of whether OCS was added. We found that the content of unsaturated fatty acids decreased. This might occur partly because of the involvement of unsaturated fatty acids in the neutralization of ROS, which lead to a decrease in the level of unsaturation due to lipid peroxidation. This led to the production of conjugated dienes, lipids with peroxy radicals, and hydroperoxides (Smirnov 2000). The slight increase in saturated fatty

acid content in these cultures was consistent with a greater expression of reducing power dissipation mechanisms, which helped maintain the NAD(P)H/NAD(P)<sup>+</sup> ratio in the cells and decrease oxidative stress (Schüler et al. 2017).

In relation with the latter, the results obtained in Fig. 6 suggested that low CO<sub>2</sub> availability (growth in air alone) influenced the changes in the content and structure of fatty acids, which eventually affected the permeability of the lipid membrane favoring the absorption of CO<sub>2</sub>. Our arguments matched the findings of Uprety et al. (2010). They found that the conversion of linoleic to linolenic acid requires an abundance of O<sub>2</sub> in cells. This condition was not feasible for mixotrophic cultures bubbled with CO<sub>2</sub>-enriched air, as the CO<sub>2</sub>/O<sub>2</sub> ratio was high. Therefore, the higher proportion of oxygen in mixotrophic cultures bubbled with air alone partly explained the higher conversion of linoleic into linolenic acid. This finding also explained the increase in the C18:3/C18:2 ratio in the mixotrophic culture bubbled with CO<sub>2</sub>-enriched air.

The arguments presented above were consistent with the variation in the photochemical efficiency of *Elliptochloris* sp. (Fig. 3) recorded in mixotrophic cultures. The lower rates of photosynthetically produced energy recorded were consistent with a reported mechanism to reduce the chlorophyll content, which is an effective strategy to avoid unnecessary and excess absorption of photons (Xu et al. 2020).

The growth of aerobic opportunist heterotrophs is limited under the extreme pH conditions in which *Elliptochloris* sp. thrives. For these reasons, the acidotolerant microalga is a promising candidate as a novel bioresource for the production of microalgal biomass within the circular economic model. Regarding costs and sustainability, the value of crude technical glycerin might increase when it is used as a carbon source for synthesizing fatty acids (raw material for biodiesel production). We found similar results when glycerin was added as a carbon source to cultures of the acidotolerant autochthonous microalga *Elliptochloris* sp.

## Conclusions

The use of CO<sub>2</sub> along with technical glycerin affects growth and fatty acid production in the autochthonous acidotolerant microalga *Elliptochloris* sp. We suggest two cultivation strategies for the effective production of *Elliptochloris* sp. First, mixotrophy in CO<sub>2</sub>-enriched air allows higher biomass productivity, but metabolic imbalances can decrease the content of fatty acids and increase the relative abundance of saturated fatty acids. Second, mixotrophy in air alone can increase the content of both saturated and unsaturated fatty acids but biomass productivity decreases. These strategies

can further determine the application of *Elliptochloris* sp. for biofuel or production of food supplements.

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**Author contribution** María Robles and Carol Ostojic contributed equally to this work. Conceptualization, María Robles, Virginia Obregón and Carlos Vélchez; investigation, María Robles, Carol Ostojic, Juan Luis Fuentes and Alejandra Bartolomé; writing—original draft preparation, María Robles, Carol Ostojic and Carlos Vélchez; methodology, María Robles, Carol Ostojic, Mari Carmen Ruiz-Domínguez, María Cuaresma and Carlos Gonzalo; supervision, Carlos Vélchez. All authors have read and agreed to the published version of the manuscript.

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**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** The authors declare no competing interests.

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