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PII: S2211-9264(23)00203-5

DOI: https://doi.org/10.1016/j.algal.2023.103170

Reference: ALGAL 103170

To appear in: *Algal Research*

Received date: 31 December 2022

Revised date: 4 May 2023

Accepted date: 6 June 2023

Please cite this article as: F.E. Navarro, P.I. Leonardi, M.C. Damiani, et al., Outdoor culture of Halamphora coffeaeformis in the semi-arid Pampa of Argentina: A comprehensive analysis of triacylglycerol production for biodiesel, *Algal Research* (2023), https://doi.org/10.1016/j.algal.2023.103170

Outdoor culture of *Halamphora coffeaeformis* in the semi-arid Pampa of Argentina: a comprehensive analysis of triacylglycerol production for biodiesel

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Keywords

Diatoms, bioenergy culture, raceway pond, seawater, biodiesel

Abstract

The feasibility of microalgal triacylglycerol (TAG) production for biodiesel remains constrained by high production costs under outdoor conditions. The aim of this study was to evaluate the capacity of an Argentinian strain of the diatom *Halamphora coffeaeformis*

grown in outdoor raceway ponds to produce TAG suitable for biodiesel. The strain was cultured in seawater enriched with a biofertilizer during summer and spring in the semi-arid Pampa of Argentina. The following variables were analysed: 1) growth rate; 2) TAG accumulation; 3) lipid content and quality; 4) dissolved nutrient and physicochemical properties; and 5) climatic conditions. Intracellular TAG accumulation was evaluated with fluorometry by Nile Red. The growth rate and biomass productivity of the species were similar in the two seasons, with approximate values of 1.35 div.day^{-1} and 61.25 kg.ha^{-1}.day^{-1}, respectively. Total lipid content increased about 4-fold with respect to *inocula* in the 2 cultures, reaching up to 44.7% ash free dry weight (AFDW) in summer and 36.4% AFDW in spring. However, lipid accumulation differed between seasons: neutral lipid accumulation began at 8 days of cultivation in summer and at 19 days in spring. Salinity was the main stressor that accelerated the TAG accumulation in the species, the nutrient levels did not reach limiting values. In spring, precipitation decreased salinity levels, reducing stress and delaying the onset of TAG accumulation. The biodiesel properties inferred from the fatty acid profile of the species meet international standards and their quality ensures good performance in cold climates. Furthermore, the reported lipid productivity of the species (~25.6 kg.ha^{-1}.day^{-1}) is higher than that of the soybean oil used commercially for biodiesel. Based on the results obtained, *H. coffeaeformis* has potential for exploitation as a renewable source of TAG for biodiesel production in arid and semi-arid environments with access to brackish or marine water.

1. Introduction

Fossil energy resource depletion, drought and malnutrition are among the main challenges facing humanity in ensuring a sustainable future for coming generations. In particular, the use of fossil fuel generates a large amount of greenhouse gas (GHG)

emissions (CO_2, CO, NO_x), accelerating global warming [1]. According to the United Nation Framework Convention on Climate Change and the Paris Climate Agreement, 196 countries have agreed to limit global warming to less than 2°C compared to pre-industrial levels [2]. It is therefore necessary to develop new energy sources to help complete the transition towards an energy matrix with zero emissions of CO_2 equivalent by 2050. Biodiesel is a renewable transportation fuel that can be produced by transesterification of any triacylglycerol (TAG) feedstock. Today, countries such as Austria, France, Germany, Italy, Malaysia, Spain, Sweden and the United States are pioneers in the testing, production and use of biodiesel in cars [3]. In 2020, the United States positioned itself as the main producer of biofuels worldwide, registering a production of approximately 600,000 barrels of oil equivalent per day [4]. At present, the main raw materials used for commercial biodiesel production are soybean, rapeseed, and palm oils [5]. In Brazil and Argentina, biodiesel is produced almost exclusively from soybean oil [6]. However, soybean crop yields are constrained by the availability of irrigation water and arable land; furthermore, soybean as a source of biofuel has been questioned in the context of the feed versus fuel debate and land-use issues [5]. To achieve environmental and economic sustainability, fuel production processes should not only use renewable energy sources but should also refrain from using fresh water. Some oleaginous microalgae (oil content > 20% dry weight) can accumulate high cellular neutral lipid content, particularly of TAG, the ideal raw material for biodiesel production by transesterification [7]. Microalgae farming can be developed in non-arable areas using seawater and/or urban and industrial effluents [8]. This constitutes an advantage in arid and semi-arid zones where freshwater resources are prioritized for human consumption. Additionally, microalgae appear to be superior in terms of biomass and lipid yield compared to soil-grown energy crops [9]. In order to benefit from the

advantages of microalgae as a source of energy, the sustainable commercialization of biodiesel from microalgal oil calls for more detailed data (stressor factors, biomass as well as lipid productivity and robust species, among others) on large-scale outdoor cultures.

Raceway ponds are a good option for large-scale microalgal farming since they require less capital investment, involve lower operational costs, utilize otherwise wasteland or barren land, and are easy to maintain [10]. However, it is important to consider that outdoor cultures in raceway ponds are directly affected by a variety of environmental factors such as air temperature, solar radiation, daylight hours and precipitation as well as water and land costs [11]. In terms of nutritional needs, microalgae obtain C, O, and H from water and air, while N and P are absorbed from the culture medium; their uptake is highly dependent on factors such as the specific species involved, nutrient ratios, temperature, salinity and pH [12]. Silicon is also an essential nutrient for diatom growth since it is involved in the formation of the cell wall or frustule. Diatoms can accumulate high TAG concentrations under silicon limitation, avoiding the deleterious effects on photosynthesis, gene expression and protein content associated with N limitation [13]. Furthermore, it is well known that TAG accumulation in diatoms growing in batch cultures under controlled environmental conditions occurs naturally in the stationary growth phase [14]. However, TAG accumulation in diatoms growing in open ponds is more susceptible to climatic conditions [15, 16]. Several studies have been published on outdoor diatom cultures for bioenergy purposes, for example on the diatoms *Fistulifera solaris* and *Mayamaea* sp. at Kitakyushu, Japan [15] and *Amphora* sp. at Perth, Australia [16]. In these studies, lipid production varied according to the selected strain, geographical location, climatic conditions (such as temperature, humidity, precipitation, and solar radiation), culture scale, *inoculum* production, and harvesting time, among

others. Since the development of outdoor diatom cultures is both site- and species-specific, more detailed studies are therefore required for achieving efficient production processes.

Neutral lipid production in microalgae has been associated with reduced cell growth, regardless of the conditions under which they are grown. Obtaining high levels of TAG-rich biomass therefore implies being alert to the point at which the imbalance in favour of lipid accumulation occurs, so that significant reductions in daily productivity can be avoided. For example, to monitor the photosynthetic performance or growth of microalgal mass cultures, the chlorophyll *a* (Chl-*a*) fluorescence assay has been widely used [17]; and to estimate the neutral lipid in microalgae, *i.e.* mainly intracellular TAG levels, the Nile Red (NR) fluorescence analysis [18]. In addition, the positive imbalance between NR and Chl-*a* fluorescence [19] or between total fatty acid and pigments [20] has been used for monitoring lipid accumulation in microalgal cultures exposed to stressful conditions.

Previously, we reported the good performance of the marine diatom *Halamphora coffeaeformis* isolated from the Bahía Blanca Estuary (Argentine South Atlantic coast) as feedstock for biodiesel [21, 22]. Its biomass can reach total lipid contents of up to 54.4 % ash free dry weight (AFDW), with a neutral lipid content of 34% AFDW. These studies were performed in laboratory cultures with f/2 (a conventional culture medium) under artificial light and controlled temperature [21, 22]. We also confirmed the robustness of the strain over a wide range of temperatures (5°C to 30 °C) and salinities (5‰ to 95‰) [23]. The maximum tolerance limits of the strain at laboratory scale were 35°C and at least 95‰. However, salinity values higher than 45‰ were found to be particularly stressful for its growth [23]. Hegel *et al*. [24] reported biodiesel production from this diatom oil by extraction with ethanol and subsequent supercritical ethanol

transesterification. In addition, the nuclear gene 18S rDNA and the chloroplast gene rbcL of the strain have been sequenced [25]. Nevertheless, despite all these advantages, there are no studies on the growth behaviour of *H. coffeaeformis* in outdoor cultures.

The main goal of this study was to assess the growth and TAG accumulation of the Argentinian strain *H. coffeaeformis* in outdoor raceway ponds. This information is crucial to provide input for large-scale bioenergy cultures. The species was grown in seawater enriched with Bayfolan® biofertilizer (Bayer CropScience) with the addition of silicon. Biofertilizer was selected as an economically feasible culture medium at large scale. The experiments were carried out in summer and spring when the mean temperatures of the region presented values between 14°C and 24°C; the wind speed was about 22 $km.h^{-1}$; and there were between 12.0 and 14.9 daylight hours [26]. In addition, the present study compares diatom oil yield and quality with that of a commercial biodiesel feedstock, such as soybean.

2. Material and methods

2.1 Algal strain and culture conditions

Halamphora coffeaeformis (C. Agardh) Lekov was isolated from Bahía Blanca Estuary (38° 45′ S, 62° 22′ W) [21]. The strain was first described by Martín *et al.* [21], and a detailed molecular and morphological characterization was provided by Sala *et al*. [25]. Unialgal non-axenic cultures were established in f/2 medium [22], prepared with sterile seawater from Bahía Blanca Estuary at 33‰ salinity, the typical level for this environment [28]. The strain is maintained in culture in our laboratory of CERZOS in Bahía Blanca, Argentina. Stock cultures are kept in 15 mL glass tubes in liquid culture without aeration at 15 °C ± 1 °C, 21 μmol photons.m^{-2}.s^{-1}, 33‰ salinity and 12:12 h of

light:darkness. Light is provided by cool white Phillips L-35 fluorescent lamps. A LI-COR model 192SB radiometer was used to measure photosynthetically active radiation (PAR).

2.2 Study area climate

A survey of the Bahía Blanca (38° 43′0 ″S 62° 16′ 0″ W) climate from 2010 to 2021 was carried out to select the most suitable seasons for developing outdoor cultures. The dataset used (air temperature, PAR, precipitation, and wind speed) pertains to the Meteorological Station located on the campus of the CERZOS [26].

2.3 Inoculum production

Halamphora coffeaeformis was grown exponentially in a cylindrical borosilicate 25 L photobioreactor (PBR) (FIGMAY S.R.L., Córdoba, Argentina) to obtain 12 L-*inocula* for raceway ponds with 158 L of culture medium. Aged, filtered (0.45 µm Millipore) and sterilized seawater was enriched with fertilizer 0.28 $ml.L^{-1}$ Bayfolan® 11-8-6 (N-P-K) (Bayer CropScience), which presents dissolved ammonium and nitrate as N source. $NaSiO_3$ was added as source of silicon (Si) (with non-limiting Si:N Redfield/Brzezinski ratio ~1 according to Sarthou *et al.* [29]). The cultures were carried out over 4 days in a room at 20° ± 2 °C. Lighting consisted of white LED panels around the PBR, providing 100 µmol photons.$m^{-2}.s^{-1}$ under a cycle of 12:12 h light:darkness. The cultures were supplied with 1% CO_2 in air during 2 h and maintained under continuous stirring by means of a central paddle system. The culture was harvested in the exponential growth phase in order to obtain photosynthetically active cells.

2.4 Outdoor cultures in raceway ponds

Laboratory studies indicate that *H. coffeaeformis* can grow well (≥ 0.8 $div.d^{-1}$) between 10°C and 30°C [23]. Thus, based on an evaluation of the climate over 12 years in the region (supplementary data), two outdoor culture experiments were performed as

follows: a) the first culture was carried out in the late summer of 2021 (summer-culture), and b) the second culture was carried out in the late spring of 2021 (spring-culture). PVC outdoor raceway ponds of ca. 0.96 m^2 (1.6 m length, 0.6 m width) and 0.3 m height were used to obtain biomass rich in TAG. The raceway ponds were located in the campus of the CERZOS. Cultures were carried out with seawater at 33‰ enriched with 0.28 $ml.L^{-1}$ Bayfolan® 11-8-6 (N-P-K) (Bayer CropScience) with the addition of $NaSiO_3$ (with a Si:N ratio of 1:7 in order to obtain deficient Si conditions according to Sarthout *et al*., [29]). At this stage of scale-up, seawater was aged, filtered (1 µm Millipore) and sterilized with 5% sodium hypochlorite, neutralized with 0.15 $g.L^{-1}$ sodium thiosulfate before adding Bayfolan® and $NaSiO_3$. The stirring was produced by a PVC paddle wheel attached to a rotator axle driven by an electric motor (37 W, 12 V and 5 A). For additional turbulence, compressed air enriched with CO_2 at 1% (v/v) was bubbled at the bottom of the pond through a perforated plastic tube. The pH values of the cultures oscillated between 7.8 and 10.1. Raceway ponds started at a culture depth of 0.2 m equivalent to a culture volume of 170 L, including 12 L of *inoculum*.

To determine the moment of optimal TAG production, thus indicating when to harvest, the duration of the experiments was fixed in accordance with a set ratio between Nile Red (NR) fluorescence and chlorophyll-*a* (Chl-*a*) fluorescence [19] (see section 2.5). According to unpublished data, when the NR:Chl-*a* values of *H. coffeaeformis* cultures increase to 40 or more, their lipid content equals or exceeds 20% of ash free dry weight (AFDW). This value was therefore used as a sign of the imbalance in favour of neutral lipid accumulation, indicating that the time was ripe for harvesting. At this point, the raceway pond paddle wheel was stopped and after about a few hours, *H. coffeaeformis* cells autoflocculated forming a biofilm at the bottom of the pond. The supernatant was removed through a tap on a side of the raceway pond and recovered for recycling. The

biofilm was then harvested by scraping with a shovel and scooping into plastic jars. The biomass was frozen at -80 ºC or lyophilized, depending on the analysis to be performed.

2.5 Chorophyll-a and Nile Red fluorescence intensity determination

2.5.1 Chlorophyll-a fluorescence intensity detection

Duplicate 5 ml samples were taken daily to determine Chl-*a* fluorescence intensity using a spectrofluorometer (Shimadzu RF-5301PC). The excitation wavelength was set at 430 nm and the emission wavelength was scanned from 600 to 750 nm (spectrum mode with excitation and emission slits set at 5 nm). The peak emission wavelength was selected at 680 ± 5 nm. Chl-*a* fluorescence intensity (Chl-*a* FI) was measured in arbitrary fluorescence units (au) and was used as an indicator of cell growth activity.

2.5.2 Nile Red fluorescence intensity detection

Nile Red (NR) fluorescence intensity kinetics was used as an indicator of neutral lipid accumulation. For this, 5 µL of NR [(9-diethylamino-5H-benzo (α) phenoxazine-5-one] in acetone (1 mg.ml^{-1}) were added daily to 5 ml samples in duplicate. The mixture was agitated vigorously in a vortex mixer. After 5 min, NR fluorescence intensity (NR-FI) was measured using a spectrofluorometer RF-5301PC Shimadzu at an excitation wavelength of 480 nm. Fluorescence peaks were detected in the emission spectrum between 450 and 750 nm. The main peaks were found at 577 ± 5 nm, which corresponds to the emission spectrum of neutral lipids [18]. The NR-FI was measured in arbitrary fluorescence units (au).

2.5.3 Nile Red and Chlorophyll-a ratio

The Nile Red and chlorophyll-*a* ratio (NR:Chl-*a*) was calculated as the value of NR-FI divided by the value of Chl-*a* FI [19] throughout both experiments. A threshold of NR:Chl-*a* ≥40 was used to determine the harvesting time.

2.6 Climate and physicochemical variable monitoring

Daily monitoring of climate and physicochemical variables of the cultures was carried out throughout summer-culture and spring-culture. Air temperature (°C) and precipitation (mm) data were collected at the Meteorological Station located on the campus of the CERZOS [26]. Incident PAR (μmol photons.m^{-2}.s^{-1}) was measured with a radiometer LICOR model LI-192SB. Water temperature (ºC) and salinity (‰) were measured *in situ* with a multiparameter digital meter CONSORT C562, depth was measured with a ruler fixed on the raceway pond and pH was measured *in situ* with a pH meter (POCKET PRO pH TESTER, HACH).

2.7 Growth rate and doubling time determinations

Cell density (cell.mL^{-1}) was determined daily by counting duplicate samples in a Sedgwick-Rafter chamber. The growth rate (*k*) (divisions per day; div d^{-1}) was estimated during the period of exponential growth by least squares fit to a straight line of the logarithmically (log 10) transformed data [30]. The slope of the line was multiplied by *3.322* in order to obtain the number of divisions per day (*k*) [30]. The doubling time (hours) was calculated as (24 hours.day^{-1})*(k)$^{-1}$. The calculations were made using Microsoft Excel 2019.

2.8 Dry weight and biomass productivity determination

Duplicate samples of 20 mL were filtered through pre-dried and pre-weighed glass-fibre filters (Whatman GF/F), washed with 10 mL distilled water to remove salts, dried in an oven at 60 ºC for 24 h, cooled in a desiccator and finally weighed. This process was repeated until a constant weight (DW) was achieved. To normalize the DW values, they were multiplied by the raceway pond volume at the sampling moment and divided by the raceway pond initial volume (170 L), thus avoiding DW overestimation due to evaporation. The samples were ashed in a muffle furnace at 450 ºC for 8 h, cooled in a vacuum desiccator, and weighed to obtain the AFDW.

Biomass (DW) productivity was calculated as volumetric biomass productivity (VBP) and areal biomass productivity (ABP) according to the following equations:

$$VBP_{DW}(g.L^{-1}.day^{-1}) = \frac{DW_{FE}\left(g.L^{-1}\right) - DW_0\left(g.L^{-1}\right)}{EGP(day)} \quad (1)$$

$$ABP_{DW}(kg.ha^{-1}.day^{-1}) = VBP_{DW}(g.L^{-1}.day^{-1}).ICV(L).\left(\frac{1}{area\ .m^2}\right).\left(\frac{kg}{000\ g}\right).\left(\frac{10000\ m^2}{ha}\right) \quad (2)$$

where, DW_0 is the dry weight on day 0; DW_{FE} is the dry weight on the final day of the exponential growth phase; EGP is the exponential growth phase duration. ABP_{DW} is equal to VBP multiplied by the initial culture volume (ICV), and divided by the culture area.

2.9 Dissolved nutrient analyses

For the determination of dissolved nutrient concentrations, 20 mL samples were taken daily from raceway ponds, filtered (Whatman GF/F filters) and frozen at −20 °C until analysis. Dissolved nutrient concentrations were determined via colorimetric assays in order to measure phosphate (PO_4^{3-}); ammonium (NH_4^+); nitrate (NO_3^-) and silicate (SiO_4^{4-}) [22]. Absorbances were measured with a Varian Cary 60 UV/Vis Spectrophotometer (Agilent, USA) at 543 nm for NO_3^-, 630 nm for NH_4^+, 885 nm for PO_4^{3-} and 810 nm for SiO_4^{4-}. Dissolved nutrient concentrations were referenced as μM.

2.10 Total lipid extraction

Once the outdoor cultures reached or exceeded a threshold of NR:Chl-*a* ratio ≥40, regardless of the experiment duration, the cultures were harvested and samples were

taken to determine total lipid (TL) content. Samples of 4 L were centrifuged (10 min at 3600 g) and the pellets were washed with 0.9% NaCl buffer (pH 7), centrifuged for 10 min at 800 g at 10 °C and kept at −80 °C. Total lipid extraction was carried out in a Soxhlet extractor to simulate industrial methods. Biomass samples of 3 g of dry biomass were crushed and placed in a cellulose cartridge. The extraction was performed during 12 h with ethanol. During this period, about 24 solvent evaporation/condensation/ percolation cycles per hour were observed in the extraction chamber. After extraction, the solvent was evaporated under reduced pressure and the lipid fraction was dried to constant weight in an oven at 60 ºC. Lipid content was determined gravimetrically, expressed as a percentage of dry weight (% DW) and converted to AFDW. All chemicals used were analytical grade. Lipid productivity was calculated as volumetric total lipid productivity (VP_{TL}) and areal total lipid productivity (AP_{TL}). Lipid productivities were calculated using the following equations:

$$VP_{TL}(g.L^{-1}.day^{-1}) = \frac{\text{FB-AFDW}\left(g.L^{-1}\right) \cdot \text{TL}\ (\%\ AFDW)}{\text{NLA}_t\ (day)} \quad (3)$$

$$AP_{TL}(k.ha^{-1}.day^{-1}) = VP_{TL}(g.L^{-1}.day^{-1}) \cdot ICV\ (L).\left(\frac{1}{area\ m^2}\right).\left(\frac{kg}{1.000\ g}\right).\left(\frac{10000\ m^2}{ha}\right) \quad (4)$$

where FB-AFDW is the final biomass ash free dry weight and NLA_t is the neutral lipid accumulation time calculated from the Nile Red kinetics [31]. Briefly, the NL accumulation time is the period during which the NR signals begin to increase significantly until harvesting. AP_{TL} is equal to VP_{TL} multiplied by the initial culture volume (ICV), and divided by the culture area.

2.11 Lipid fractionation

Lipid fractionation into neutral lipids (NL), glycolipids and phospholipids was performed using a silica Sep-Pack cartridge (SP) of 1000 mg (J. T. Baker Inc., Phillipsburg, N. J.), according to Popovich *et al*. [32]. After conditioning the silica cartridge with 30 ml of chloroform, the duplicate sample was loaded with 1 ml of chloroform/oil solution containing 20 mg of oil. For neutral lipid, glycolipid and phospholipid recovery, the following elutions were performed sequentially: (a) 15 mL of a chloroform/acetic acid (9:1, v/v) solution; (b) 20 mL of an acetone/methanol (9:1, v/v) solution and (e) 20 mL of methanol. Fractions were collected into a weighed conical vial, evaporated until dryness under a clean nitrogen stream and weighed. Glycolipids and phospholipids in the polar lipid (PL) fraction were collected and weighed together in the same vial. Two replicates of each lipid fraction were made.

2.12 Methyl ester derivation and fatty acid analysis

The fatty acid methyl ester (FAME) profile was determined by fatty acid derivation [32]. An aliquot of each lipid fraction (about 25–30 mg) was weighed in a hermetic flask, and 2 mL of KOH:methanol solution (10% w/v) was added while shaking vigorously. The air was purged under nitrogen stream and the flask was hermetically sealed. The flask with lipid-KOH:methanol solution was heated for 45 min in a water bath at 80 °C. The lower phase was treated with concentrated HCl, and the liberated fatty acids were extracted with two mL of petroleum ether in the upper phase. This step was repeated twice. The ether-lipid extract was dried under a nitrogen stream. An aliquot of 1.5 mL BF_3-methanol solution (10% v/v, Sigma Aldrich) was added and the samples were incubated at 80 °C for 30 min in a water bath. FAMEs were then extracted with petroleum ether (2 mL) and evaporated to dryness under a nitrogen stream. Finally, hexane (chromatography grade) was added to a final volume of 100 µL. FAMEs were analysed by gas chromatography with an HP Agilent 4890D gas

chromatograph, equipped with a flame-ionization detector at a temperature of 260 °C, a split/splitless injector (175 °C) and a capillary column SP–2560 (100 m, 0.25 mm and 0.2 μm; Supelco Inc., Bellefonte, PA). The carrier gas was high purity hydrogen at 18 cm s^{-1}. The GC oven was initially held at 140 °C for 5 min. The temperature was then increased at 4 °C.min^{-1} to 240 °C and held isothermal for 15 min. The detection limit of the chromatographic method was set at 0.01%. The HP 3398A GC Chemstation (Hewlett Packard 1998) was used for chromatographic analysis. FAME identification was performed by comparison with standard certified material, Supelco FAME 10 mix 37 (Bellefonte, USA), according to AOCS Official Method Ce 1b-89 [32]. Two replicates of each FAME analysis were carried out. All employed solvents were of analytical grade.

2.13 Biodiesel quality from fatty acid profiles

Biodiesel properties were determined based on the fatty acid profiles of neutral lipids. Average degree of unsaturation (ADU), iodine value (IV, g I_2/100 g FAME), cetane number (CN), cloud point (CP, ºC), specific gravity (SG, Kg/L), kinematic viscosity (KV, 40°C mm^2/s) and higher heating value (HHV, MJ/kg) were calculated according to Hoekman *et al.* [33]; long-chain saturated factor (LCSF) and cold filter plugging point (CFPP, ºC) were estimated according to Talebi *et al.* [34]. The respective equations were as follows:

$$ADU = \sum M\ Yi$$

$$IV = 74.373\ ADU + 12.71$$

$$CN = -6.6684\ ADU + 62.876$$

$$CP = -13.356\ ADU + 19.994$$

$$SG = 0.0055\ ADU + 0.8726$$

$$KV = -0.6316\ ADU + 5.2065$$

HHV= 1.7601 ADU + 38.534

LCSF = (0.1 x C16:0 + 0.5 x C18:0 + 1 x C20:0) + 1.5 x (C22:0 + 2 x C24:0).

CFPP= (3.1417 x LCSF) – 16.477

where Yi is the percentage fraction of each fatty acid (FA) component and M is the number of carbon-carbon double bounds in each fatty acid component.

2.14 Statistical Analysis

Infostat.3 software, one-way analysis of variance (ANOVA) and the LSD test were used for the statistical analysis. In all tests, the level of significance was $\alpha = 0.05$.

3. Results

3.1 Outdoor summer and spring cultures of H. coffeaeformis

3.1.1 Chl-a and NR kinetics, growth rate and biomass production

Figure 1 shows the time course of growth and neutral lipid (NL) accumulation of outdoor cultures of *H. coffeaeformis*. The growth rate and biomass productivity of the species were similar in the two experiments, although the NL accumulation started earlier in the summer-culture. During summer, Chl-*a* FI (Fig. 1a) increased from day 0 to day 10; while NR-FI increased linearly from day 8. In order to determine when the imbalance occurred in favour of TAG accumulation, the moment at which the NR:Chl-a ratio exceeded a threshold of 40 was selected. Thus, the harvest was carried out on day 13, when the NR:Chl-*a* ratio reached a value of 60 (Fig. 1a). The NL accumulation time was 6 days (from day 8 to day 13). During this 14-day period, the cell density of *H. coffeaeformis* (Fig. 1b) showed exponential growth from day 0 to day 3. The growth rate (k) was 1.39 div.day^{-1} corresponding to a doubling time of 17.3 hours. The biomass volumetric and areal productivities at the end of the exponential growth phase were ca. 0.039 g.L^{-1}.day $^{-1}$ and 68.25 kg.ha^{-1}.day^{-1}, respectively.

The spring-culture lasted longer than the summer-culture because the NL accumulation began later. During this season, the Chl-*a* FI (Fig. 1c) increased until day 3, remained in a stationary phase until day 12 and then decreased until day 27. The NR-FI increased significantly from day 19 and the NR:Chl-*a* ratio only exceeded the threshold of 40 on day 27, when the harvest was performed. Thus, the NL accumulation time was 9 days (from day 19 to day 27). During this 27-day period, the cell density (Fig. 1d) showed an exponential growth phase from day 0 to day 4. The value of *k* was 1.31 div.day^{-1} corresponding to a doubling time of 18.3 hours. The biomass volumetric and areal productivities at the end of the exponential growth phase were ca. 0.031 g.L^{-1}.day $^{-1}$ and 54.25 kg.ha^{-1}.day^{-1}, respectively. Neither of the seasons presented a lag phase or a typical declination phase in the cell density of the species (Figs. 1b and 1d), an advantage at the production level.

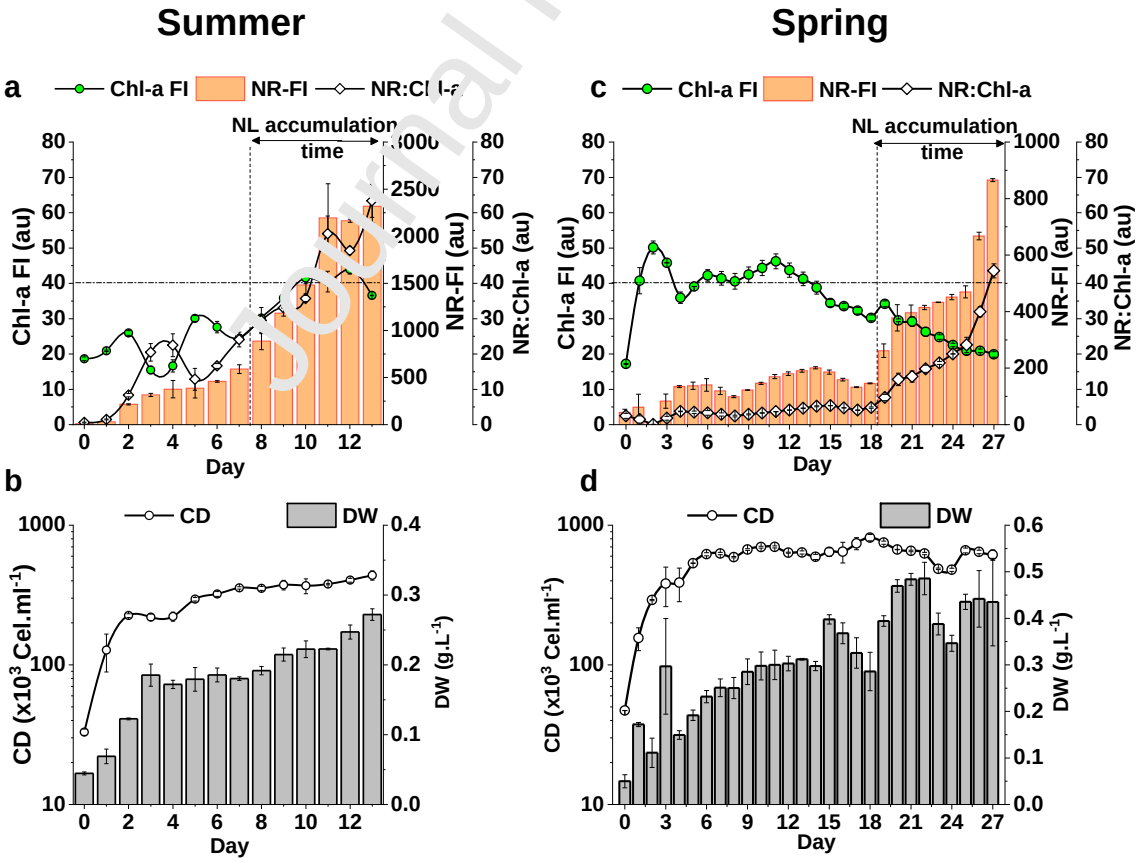

Fig. 1 Growth and neutral lipid accumulation of *H. coffeaeformis* outdoor cultures: (a and b) summer-culture, (c and d) spring-culture. (a and c) Kinetics of chlorophyll-*a* fluorescence intensity (Chl-*a* FI), Nile Red fluorescence intensity (NR-FI) and NR:Chl-*a* ratio. Fluorescence intensity expressed in arbitrary units (au). (b and d) Kinetics of cell density (CD) and biomass production (dry weight, DW). The vertical dashed line indicates the onset of the NL accumulation phase. The horizontal dashed line indicates when the NR:Chl-*a* ratio exceeds the threshold of 40. Data are expressed as the average ± standard deviation of 2 replicates (n=2).

3.1.2 Climate and physicochemical conditions during outdoor experiments

Figure 2 presents the physicochemical variables of the outdoor cultures of *H. coffeaeformis* in relation to the climate. The summer-culture was characterized by mean water temperatures from 13.3 ºC to 23.7 ºC (Fig. 2a), with maximum temperature values always below the maximum tolerance limit of the species (35.7°C) [23]. Mean daily PAR (Fig.2a) presented relatively constant values (~1300 µmol photons.m^{-2}.s^{-1}) until day 10 and then diminished down to 161 µmol photons.m^{-2}.s^{-1} reflecting cloudy days. The salinity (Fig. 2b) increased progressively from 32.1 ‰ to 48.2 ‰ reflecting the decrease in depth (from 13.2 cm to 13.4 cm) by water evaporation. Furthermore, salinity values were not significantly affected by the precipitation levels. The spring-culture was characterized by mean water temperatures (Fig. 2c) from 14.5 ºC to 26.3 ºC. The maximum water temperature values exceeded those corresponding to the maximum air temperature from day 21. These temperatures were higher than the maximum tolerance limit of the species (35.7°C) [23]. Mean daily PAR (Fig.2b) presented important variations throughout the experiment in correspondence with rainy days. The salinity (Fig. 2d) increased from 33.6 ‰ to 102.8 ‰ reflecting a further decrease in depth (from 19.7 cm to 5.4 cm). However, it is important to highlight that during the first 10 days of

the culture, salinity decreased to 26.4‰ and the culture did not show signs of stress compatible with the NR values (Fig.1c). In addition, the salinity values were higher than 40 ‰ from day 14 reaching 102.8 ‰ on the harvesting day.

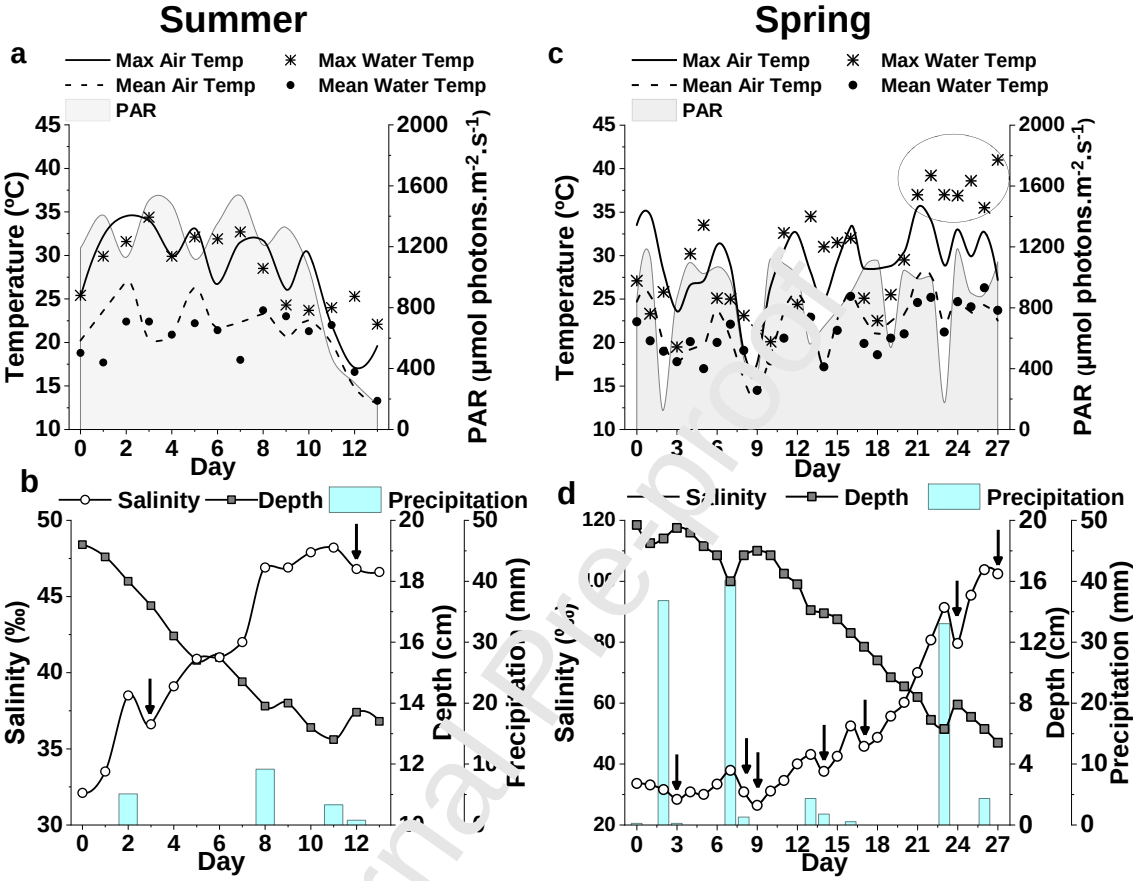

Fig 2. Climate and physicochemical variables of *H. coffeaeformis* outdoor cultures: (a and b) summer-culture, (c and d) spring-culture. (a and c) Air temperature (°C), water temperature (°C) and PAR (µmol photons.m^{-2}.s^{-1}). (b and d) Salinity (‰), depth (cm) and precipitation (mm). The circle in Figure c indicates water temperatures exceeding the maximum tolerance limit of the species. The arrows in Figures b and d indicate decrease in the salinity values after precipitation events.

3.2.3 Dissolved inorganic nutrient kinetics

Figure 3 shows the dissolved nutrient kinetics of *H. coffeaeformis* cultures. In the summer-culture, the NO_3^- level (Fig. 3a) decreased 54% throughout the experiment (from 469 µM to 214 µM). The NH_4^+ level (Fig. 3a) decreased 97% during the

exponential growth phase (from 693 µM to 14 µM), and then diminished gradually to 4 µM on harvesting day. The phosphate and silicate levels (Fig. 3b) decreased 87 % (from 131 to 16.5 µM) and 92% (from 184 µM to 13 µM), respectively, during the exponential growth phase, and then remained at a threshold level, with no evidence of recovery events. In the spring-culture, the NO_3^- level (Fig. 3c) presented relatively stable values until day18, after which they decreased 95% (from 377 µM to 19 µM) up to the day of harvesting. The NH_4^+ level (Fig. 3c) decreased 90% (from 984 µM to 99 µM) until day 18, and then diminished to 14.8 µM by harvesting day. Phosphate and silicates (Fig. 3d) presented erratic behaviour with a decreasing trend. Phosphate decreased 89% (from 129 to 14 µM) and silicates decreased 92% (from 170 to 13 µM) during the experiment. It should be noted that no nutrient was limiting for growth compared with the limiting values reported by Sarthou *et al.* [9] (Fig. 3).

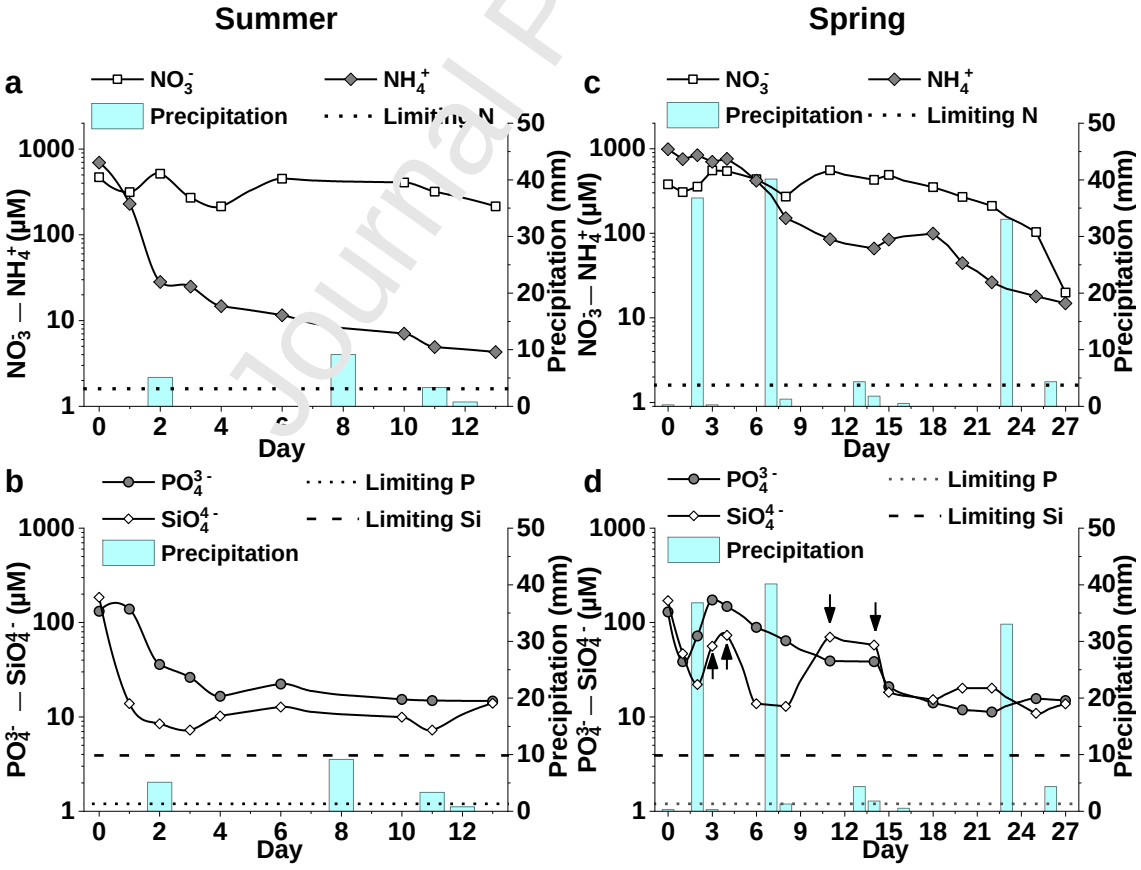

Fig 3. Nutrient kinetics of *H. coffeaeformis* outdoor cultures: (a and b) summer-culture, (c and d) spring-culture. (a and c) Dissolved nitrate (µM) and ammonium (µM). (b and d) Dissolved phosphate (µM) and silicate (µM). Average nutrient limiting values for diatom growth (N< 1.6±1.9 µM; P< 1.2 ±2.5 µM and Si<3.9±5.0 µM) according to Sarthou *et al*. [29] are shown (dashed lines). The arrows in Figure d indicate recovery of silicate levels after precipitation events. Data are expressed as the average ± standard deviation of 2 replicates (n=2).

3.2.4 Lipid quantity and quality

The total lipids (TL) of the *inocula* harvested from photobioreactors comprised 9.8 (± 0.1) % of AFDW. The cells inoculated in raceway ponds presented different lipid accumulation behaviours in accordance with the environmental scenario to which they were subjected (summer-culture or spring-culture). *H. coffeaeformis* cells produced biomass with the following TL contents: 44.4 (± 0.004) %AFDW in 14 days in the summer cultures (4-fold the inoculum TL value) and 37.5 (± 0.015) %AFDW in 27 days in the spring culture (3.75-fold the inoculum TL). The volumetric lipid productivities were 0.0153 $g.L^{-1}.day^{-1}$ in summer and 0.0148 $g.L^{-1}.day^{-1}$ in spring. The areal lipid productivities were 26.03 $kg.ha^{-1}.day^{-1}$ in summer and 25.22 $kg.ha^{-1}.day^{-1}$ in spring.

The lipid fraction values (neutral and polar lipids, NL and PL) of biomass harvested from outdoor cultures differed significantly from those of *inocula* ($F = 18.11$; $p < 0.01$) (Fig. 4a), with a marked increase in the NL:PL balance; however, no significant differences were observed in the changes measured for spring and summer (NL [summer-culture =72.3% of TL; spring-culture = 65.8% of TL] and PL [summer-culture =27.6% of TL; spring-culture = 34.2% of TL]). TAGs were the only source of fatty acids in the neutral lipids. Neither diacylglycerols nor monoacylglycerols were detected. With respect to fatty acid classes (Fig. 4b), both the summer and spring harvested

biomass showed a significant increase in the percentage of saturated fatty acids (SFA) (Fig. 4b) compared with the *inocula* (summer and spring cultures showed an increase in SFA of 14% and 21%, respectively). This can be explained by the increment of C14:0 (spring-culture) and C16:0 (summer and spring cultures) (Fig. 4c). The spring-culture presented the highest percentage of monounsaturated fatty acids (MUFA) with an increase of 26% over the *inoculum* (Fig. 4b). Both summer and spring cultures presented a significant increase in C16:1, whereas C18:1n9c was dominant in the *inocula* (Fig. 4c). Finally, the summer and spring cultures showed PUFA values significantly lower than the *inocula*, with a decrease of 18% and 54%, respectively (Fig. 4b), owing in particular to a decrease in C20:5n3 (EPA) (Fig. 4c).

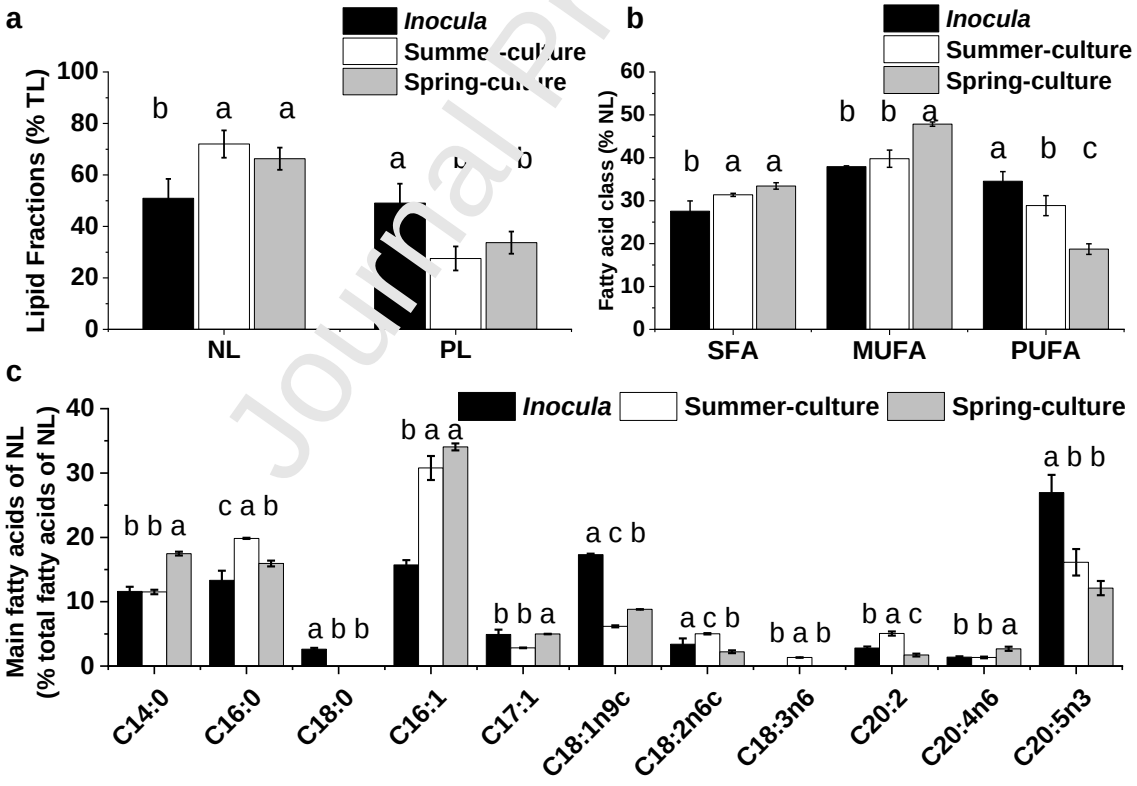

Fig 4. Lipid fractions and fatty acid profiles of *H. coffeaeformis* outdoor cultures. (a) Lipid fractions (NL, neutral lipids; PL, polar lipids) expressed as % of total lipids. Data

represented an average ± standard deviation of 2 replicates (n=2). (b) Fatty acid classes (SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, and PUFA, polyunsaturated fatty acids) expressed as % of neutral lipids. (c) Main fatty acids expressed as % of total fatty acids of NL. Data represented an average ± standard deviation of 4 replicates (n=4).

3.2.5 Biodiesel properties from the fatty acid profiles

Biodiesel properties of *H. coffeaeformis* and soybean as calculated from their fatty acid profiles can be observed in Table 1. The summer-culture showed: (a) higher degree of unsaturation (ADU); (b) higher HHV (higher heating value), (c) better KV (kinematic viscosity); (d) and better CFPP (cold filter plugging point) than the spring-culture. The spring-culture showed a higher cetane number (CN) and smaller iodine value (IV). The mean values of the biodiesel properties ADU, IV, CN, SG, KV, HHV of *H. coffeaeformis* were similar to those of soybean, except for LCSF and CFPP, which showed better values in the diatom at low temperatures.

	Haslea coffeaeformis oil			Soybean oil	
	[This study]			[1]	[35]
	Summer Mean (± SD) (n=4)	Spring Mean (± SD) (n=4)	Mean (± SD) (Summer and Spring)	Range (Multiple values)	Mean (± SD) (n=3)
ADU	1.50 (± 0.005)	1.27 (± 0.047)	1.38 (± 0.137)	1.46 - 1.62*	1.44 (± 0.020) *
IV (I_2/100g)	124.16 (± 6.34)	107.15 (± 3.504)	115.68 (± 10.184)	121.29 - 133.19*	119.74 (± 1.504) *
CN	52.88 (± 0.568)	54.40 (± 0.314)	53.64 (± 0.913)	48 - 56	53.28 (± 0.135) *
CP (°C)	-0.02 (± 1.139)	3.03 (± 0.629)	1.5 (± 1.829)	(-1.64) - 0.49*	0.77 (± 0.270) *
SG (Kg/L)	0.88 (±0.0004)	0.88 (± 0.0002)	0.88 (± 0.0007)	0.880-0.881*	0.88 (± 0.0001) *
KV (mm^2/s)	4.26 (± 0.054)	4.40 (± 0.029)	4.33 (± 0.086)	4.0 - 4.3	4.29 (± 0.013) *
HHV(MJ/Kg)	41.17 (± 0.150)	40.77 (± 0.083)	40.97 (± 0.241)	41.1 - 41.38*	41.07 (± 0.035) *
LCSF	1.98 (± 0.009)	1.59 (± 0.044)	1.79 (± 0.202)	4.6 - 6.2*	6.25 (± 1.034) *
CFPP (°C)	-10.21 (± 0.029)	-11.44 (± 0.139)	-10.82 (± 0.635)	(-2) - 3	3.20 (± 3.247) *

Table 1 Biodiesel properties estimated from the fatty acid profiles of *H. coffeaeformis*, cultured in outdoor *raceway* ponds with seawater, and soybean, cultured in arable land

with freshwater. Degree of unsaturation (ADU), iodine value (IV), cetane number (CN), cloud point (CP), specific gravity (SG), kinematic viscosity (KV), higher heating value (HHV), long chain saturated factor (LCSF) and cold filter plugging point (CFPP).
*These values were calculated from the fatty acid values reported in the cited articles.

4. Discussion

Climate conditions (solar radiation, temperature, precipitation and evaporation, storms, among others) and appropriate location (distance to water source, access to transportation and infrastructure, soils, among others) are decisive for constructing commercial-scale microalga cultures for biofuel. Bahía Blanca Estuary is a marine estuary located in the southern hemisphere in the semi-arid Pampa region of Argentina. The climate presents seasonal and daily variations typical of temperate regions, in general defined as environments with moderate precipitation distributed throughout the year or part of the year with sporadic drought, mild to hot summers, and cold winters [36]. The average annual precipitation in the zone studied between 2010 and 2021 was 568.7 mm, a typical value for semi-arid regions [37]. The most favourable seasons for outdoor cultures of *H. coffeaeformis* are summer and spring. During these periods, mean temperatures are expected to be moderate, ranging between 10°C and 30°C, without reaching mean temperatures above 30°C or below 5°C, and thus corresponding to the temperature range tolerated by the species [23]. This temperature range is higher than that of other diatoms grown at similar latitudes in outdoor cultures. For example, *Fistulifera solaris* and *Mayamaea* sp. presented growth ranges between 20°C and 35ºC and 10°C and 28ºC, respectively [15]. On the other hand, it is worth mentioning that in the laboratory, *H. coffeaeformis* presents the lowest growth rate (0.43 $div.d^{-1}$) at 5°C [23], so cultures grown in autumn and winter are expected to produce lower biomass

values. However, *H. coffeaeformis* has a higher tolerance to low temperatures than *Mayamea* sp. JPCCCTDA0820, which was selected as a promising candidate to be cultivated in winter for biofuel production [15]. Outdoor open pond systems are heated directly by sunlight and air temperature, depending on the thermal mass of the pond [11]. Thus, if the pond has a reduced volume due to evaporation, its temperature will be affected accordingly. This effect was observed in spring, when the culture reached a significant decrease in depth and consequently the water temperatures exceeded the maximum air temperature values (Fig. 2c). On the other hand, the summer-culture did not present such high water temperatures due to shorter duration, and consequently shorter evaporation time. It was possible to observe an inverse relationship between the depth and the salinity of the cultures, *i.e.* a reduction in depth due to evaporation generated an increase in salinity (Fig. 2b), while an increase in depth due to precipitation produced a reduction in salinity (Fig. 2d).

In view of the growing scarcity of freshwater for human consumption, the culture of eurytolerant species, especially using marine and brackish water, is presented as an interesting alternative for biodiesel production. The eurytolerant behaviour of *H. coffeaeformis* and the effect of salinity stress on its growth and neutral lipid accumulation have been demonstrated [23]. In summer-culture, the increase in salinity was sustained from day 0, reaching values of 47 ‰ in 8 days (Fig. 2b). In culture-spring, salinity decreased considerably on several occasions in line with levels of precipitation from 37 mm to 40 mm, so the sustained increase in salinity began on day 18 (Fig. 2d). In coincidence with these salinity trends, TAG accumulation began at 8 days and 19 days in summer and spring, respectively (Fig1. a and c). Salinity values of approximately 45 ‰ or more produced stress symptoms in the species, independently of the season. This observation corroborates the species behaviour under laboratory-

controlled conditions, where neutral lipid accumulation increased in cells adapted at 45‰ with respect to those growing at 20‰ [23]. Although the spring cultures exhibited extreme euryhalinity, growing well under saline to hypersaline conditions (three times that of seawater), its neutral lipid content did not exceed that achieved by the summer-cultures. Thus, other factors, such as nutrient availability, may influence the degree of TAG accumulation in *H. coffeaeformis* cultures.

In general, TAG accumulation in microalgae coincides with a decrease in growth, which may be due to cell aging or stress caused by nutrient deficiency [38, 39]. Diatoms alter their biosynthetic pathways for the production of neutral lipids in response to culture age [40], silicon deficiency [13, 14, 41], nitrogen depletion [13, 14, 42] and variations in medium salinity [43]. The summer-cultures showed faster TAG accumulation than the spring-cultures, though no cell aging or decay phase was observed. This may be due to the Bayfolan® composition, which contains two N sources - nitrate and ammonium- that promote cell growth. In the presence of two nitrogen sources, *H. coffeaeformis* showed a selective uptake of ammonium, regardless of the season of the year. Normally, NH_4^+ is preferentially taken up by diatoms if both nitrate and ammonium are available in abundance because cells using NO_3^- must expend significant amounts of energy for its reduction to NH_4^+ [44]. Furthermore, there is no energy cost for ammonium uptake since it accumulates spontaneously in acidic cellular compartments (e.g. chloroplast thylakoids) [45, 46]. Ammonium uptake was faster in summer than in spring cultures, without reaching limiting values. The use of ammonium by the species suggests that ammonium-rich wastewater could also be used to enrich seawater, as an alternative to biofertilizers. This modality is well received within circular economies, in order to bioremediate wastewater and recycles nutrients. Silicon limitation also induces TAG accumulation in diatoms [14, 32] by affecting the synthesis of the cell wall and reducing

therefore cell division. In the summer-culture, silicon decreased with growth and reached values close to the limiting values from day 3 (Fig. 3b). In the spring-culture there were several days of silica recovery events (Fig. 3d). Moreover, salinity has an effect on the dissolution of silicates and phosphates, higher salinities reducing their availability [47]. In the present study, the silicate recovery events observed in coincidence with the decrease in salinity due to precipitation could have generated relaxation of the stress and delay TAG accumulation in the studied species. Under this scenario, precipitation threshold values have a dual effect on *H. coffeaeformis* cultures supported by seawater, decreasing salinity, and increasing the availability of silicates [47]. This synergy could explain the delay in the accumulation of TAG in the spring-culture. The automated addition of seawater pulses could be a smart strategy to optimize *H. coffeaeformis* oil production by compensating for evaporative water loss, maintaining the buffering effect of water on temperature, reducing nutrient concentration by dilution, and increasing salinity. The species could be stressed in a more controlled way, avoiding biomass production collapse by culture overheating, leading to delays in oil production.

In the present study, the NR.Chl-*a* ratio constituted a useful and rapid index to determinate the trade-off between growth and neutral lipid accumulation in *H. coffeaeformis* cultures as well as to select the harvesting time. Summer and spring cultures showed practically the same growth pattern, with no lag phase, and presented an exponential growth phase of a few days, indicating the ability of the species to react to nutrient-rich waters. Considering the richness of nutrients in the culture medium, the early stationary phase could be due to a limitation of light caused by the shadow effect between the cells. Biomass production varied between 0.3 $g.L^{-1}$ and 0.45 $g.L^{-1}$ in summer and spring, respectively. These biomass yields were higher than indoor cultures

of the same strain growing with medium f/2 (0.22 - 0.23 $g.L^{-1}$) [17, 18] and similar or slightly higher than those obtained for other diatom bioenergy outdoor cultures, such as *Fistulifera solaris* and *Mayamaea* sp. JPCC CTDA0820 (0.18-0.33 $g.L^{-1}$ and 0.29 $g.L^{-1}$, respectively) [15, 48].

Neutral lipids of *H. coffeaeformis* outdoor cultures increased to the detriment of polar lipids, independently of the season. An improvement in TAG quantity and quality for biodiesel was detected in both cultures (Fig. 4a, b and c). Diatom fatty acid composition presented higher SFA (~32%) and MUFA (~44%) to the detriment of PUFA (~24%) (Fig. 4b). SFA was dominated by C14:0 and C16:0; MUFA by C16:1; and PUFA by EPA (Fig.4c). Soybean fatty acid composition appears to differ from that of *H. coffeaeformis*, showing a high percentage of PUFA (~60%), C18:2 (49-57%) and C18:3 (6-9%) being the main fatty acids [1]. MUFA presented lower values (~20%), C18:1 being the main constituent (18-26% of total fatty acids). Finally, SFA makes up ~20% of the fatty acids, with C16:0 (10-12%) and the C18:0 (3-5%) being the main ones [1, 35]. The suitability of lipids for biodiesel can be largely explained by their fatty acid profiles [33, 34]. The cetane number (CN) is a descriptor related to the ignition quality of a fuel in diesel engines. The higher the cetane number, the better the ignition quality of the fuel [1]. Oils with dominance of SFA and MUFA over PUFA confer a higher CN on biodiesel [1, 33]. For example, the predominant methyl esters in the neutral lipids of *H. coffeaeformis* growing in spring and summer were C16:0 (methyl palmitate) and C16:1 (methyl palmitoleate), which present CN of 85.9 and 56.59, respectively. For its part, the predominant methyl ester of soybean oil is C18:2 (methyl linoleate), which has a CN of 38.2. Furthermore, the melting point of methyl linoleate is not very different from that of methyl palmitoleate, although it does have slightly better kinematic viscosity at low temperatures, but poorer oxidative stability [1]. The IV, CN, KV and

SG calculated for *H. coffeaeformis* oils comply with the standards established by Europe (EN 14214) and the USA (ASTMD 6751-08). *H. coffeaeformis* showed similar biodiesel quality to soybean, with similar IV, CN, CP, SG, KV, and HHV values. However, the diatom oil presented better LCSF and CFPP values, giving them better properties at low temperatures especially in winter-prone environments [1]. Furthermore, *H. coffeaeformis* produces high amounts of fucoxanthin [49], which after being extracted could help as a natural and non-toxic antioxidant additive to improve the susceptibility of biodiesel to oxidative degradation.

Halamphora coffeaeformis lipid productivities were higher than those of other microalgae such as *Scenedesmus accuminatus* [50], *Chlorella sorokiniana* [51], *Chlorella minutissima* [52] and *Amphora coffeaeformis* RR03 [53], but similar to *Amphora sp*. MUR258 [16] and *Scenedesmus obliquus* (Turpin) Kützing GA 45 [54]. Argentinian soybean crops have an estimated annual biomass yield of 2,591 $kg.ha^{-1}$ and an annual oil yield of 471 – 487 $kg.ha^{-1}$ [55]. Projecting the daily lipid yield of *H. coffeaeformis* outdoor cultures to two months, an oil yield of 636 $kg.ha^{-1}$ was obtained. It is important to clarify that this projection was not annualized, since this would have required more detailed information on the behaviour of cultures of this species during the most unfavourable months. However, since *H. coffeaeformis* showed higher oil yield values than soybean, adding culture data in unfavourable months would only increase the difference in favour of the diatom. Soybean shows strong seasonality with a six-month cycle, the land being left fallow during the winter. In contrast, *H. coffeaeformis* can be cultivated during most of the year, as it can grow when the water temperature is about 5ºC or higher [23]. Additionally, bioenergy cultures from terrestrial plants, such as soybean or palm, generate direct and indirect land use changes. [5, 56]. These changes affect natural carbon sinks, such as forests, generating large

land-use related carbon emissions [5]. Moreover, if cultivation requires land use conversion from forest to cultivated land, the total GHG emissions exceed emission levels from fossil fuels [56].

5. Conclusion

The preferred conventional vegetable oils for biodiesel production are those that occur abundantly in the tested region. Similarly, the selection of fast-growing productive strains optimized in line with local weather conditions is of fundamental importance for the success of any microalgal mass culture for biodiesel production. This study demonstrates the capacity of the Argentinian strain *H. coffeaeformis* to produce TAG for biodiesel in outdoor raceway ponds sustained by seawater enriched with biofertilizers. This success is due in part to its robustness and adaptability to the natural environment. Salinity was the main stressor that accelerated TAG accumulation in the species, the nutrient levels did not reach limiting values. Precipitations above 40 mm relaxed the stressor effect and should be considered in outdoor cultures to anticipate TAG yield fluctuations. Since *H. coffeaeformis* is cosmopolitan, to standardize large-scale culture efforts worldwide it would be important to evaluate the behaviour of other strains of this species, particularly in different arid or semi-arid environments with access to seawater.

Acknowledgements

This study was funded by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT 2019-00348) and Universidad Nacional del Sur (PGI 24/B314) to CAP and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) de la

República Argentina (PIP 11220200100696CO) to PIL. The technical assistance by Jorge Oyola is acknowledged. We are also grateful to the Iberoamerican network 320RT0005-Renuwal-Cyted. CAP is a research member of Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), and PIL and LAM are research members of CONICET.

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Figure captions

Figure 1. Growth and neutral lipid accumulation of *H. coffeaeformis* outdoor cultures: (a and b) summer-culture, (c and d) spring-culture. (a and c) Kinetics of chlorophyll-*a* fluorescence intensity (Chl-*a* FI), Nile Red fluorescence intensity (NR-FI) and NR:Chl-*a* ratio. Fluorescence intensity expressed in arbitrary units (au). (b and d) Kinetics of cell density (CD) and biomass production (dry weight, DW). The vertical dashed line indicates the onset of the NL accumulation phase. The horizontal dashed line indicates when the NR:Chl-*a* ratio exceeds the threshold of 40. Data are expressed as the average ± standard deviation of 2 replicates (n=2).

Figure 2. Climate and physicochemical variables of *H. coffeaeformis* outdoor cultures: (a and b) summer-culture, (c and d) spring-culture. (a and c) Air temperature (°C), water temperature (°C) and PAR (µmol photons.m^{-2}.s^{-1}). (b and d) Salinity (‰), depth (cm) and precipitation (mm). The circle in Fig. c indicates water temperatures exceeding the maximum tolerance limit of the species. The arrows in Fig. b and d indicate decrease in the salinity values after precipitation events.

Figure 3. Nutrient kinetics of *H. coffeaeformis* outdoor cultures: (a and b) summer-culture, (c and d) spring-culture. (a and c) Dissolved nitrate (µM) and ammonium (µM). (b and d) Dissolved phosphate (µM) and silicate (µM). Average nutrient limiting values for diatom growth (N< 1.6±1.9 µM; P< 1.2 ±2.5 µM and Si<3.9±5.0 µM) according to Sarthou *et al*. [29] are shown (dashed lines). The arrows in Figure d indicate recovery of silicate levels after precipitation events. Data are expressed as the average ± standard deviation of 2 replicates (n=2).

Figure 4. Lipid fractions and fatty acid profiles of *H. coffeaeformis* outdoor cultures. (a) Lipid fractions (NL, neutral lipids; PL, polar lipids) expressed as % of total lipids. Data represented an average ± standard deviation of 2 replicates (n=2). (b) Fatty acid classes

(SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, and PUFA, polyunsaturated fatty acids) expressed as % of neutral lipids. (c) Main fatty acids expressed as % of total fatty acids of NL. Data represented an average ± standard deviation of 4 replicates (n=4).

Author statement

All authors have participated sufficiently in the conception and design of the work titled "Outdoor culture of *Halamphora coffeaeformis* in the semi-arid Pampa of Argentina: a comprehensive analysis of triacylglycerol production for biodiesel" or the analysis and interpretation of the data, as well as the writing of the manuscript, to take public responsibility for it.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract