Algal Research 51 (2020) 102030

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Potential of the marine diatom *Halamphora coffeaeformis* to simultaneously produce omega-3 fatty acids, chrysolaminarin and fucoxanthin in a raceway pond

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ARTICLE INFO

Keywords:
Seawater
Fertilizer
Marine diatom
Eicosapentaenoic fatty acid
Chrysolaminarin
Fucoxanthin

ABSTRACT

Omega-3 eicosapentaenoic fatty acid (C20:5; EPA), β-glucan and fucoxanthin have received widespread attention owing to their potential industrial applications; however, their availability is currently limited to unsustainable sources such as fish oils and macroalgae. Though some species of diatoms are also a natural source of these metabolites, progress needs to be made in reducing the cost barriers involved in their large-scale production. The aim of the present study was therefore to assess the potential of the marine diatom *Halamphora coffeaeformis* to simultaneously accumulate EPA, chrysolaminarin and fucoxanthin in a raceway pond sustained by fertilizer (Bayfolan®)-enriched seawater. The biomass production increased significantly along the experiment, the specific growth rate, biomass productivity and areal yield being 2.03 day^{-1}, 0.128 $g.L^{-}1.d^{-1}$ and 25.73 $g.m^{-2}.d^{-1}$, respectively. Proteins dominated in the first culture phase, while lipids, carbohydrates and pigments increased toward the end of the stationary phase. The harvested biomass presented 310 $mg.g^{-1}$ DW (dry weight) of total lipids containing high amounts of EPA (24% of total fatty acids), 114 $mg.g^{-1}$ DW of chrysolaminarin and 38 $mg.g^{-1}$ DW of fucoxanthin. These values compare favorably with those obtained from commercially used sources. The culture medium proposed represents a non-conventional, cost-effective resource allowing for sustained high biomass levels throughout 22 days, guaranteeing the accumulation of valuable metabolites. Furthermore, the robustness and auto-flocculation capacity of the species increase the chances of viable scalability. These findings indicate the potential of *H.coffeaeformis* as a high-value metabolite feedstock, focusing on sustainable bioprocesses.

1. Introduction

Diatoms are unicellular photosynthetic eukaryote microalgae belonging to the Bacillariophyceae class. They have been studied for more than a hundred years owing to their key role in oxygenizing the earth's atmosphere and as one of the ocean's most important sources of biomass [1]. These microalgae dominate in mixed and rich nutrient waters, and most species have an obligate requirement for silicon (Si) to form their cell walls. Although variations exist, many marine diatoms have a relatively balanced nitrogen (N):Si ratio within their biomass. Hence, changes in N and Si concentrations in the water will influence both the quantity (cell numbers and biomass) and quality (composition of this biomass) of a diatom population [2]. Regarding their biochemical composition, the chloroplasts are usually golden-brown, because the chlorophylls *a* and *c* are masked by the accessory pigment fucoxanthin. Chrysolaminarin is their main stored carbohydrate, a glucan that is

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https://doi.org/10.1016/j.algal.2020.102030
Received 18 May 2020; Received in revised form 27 July 2020; Accepted 29 July 2020

accumulated outside the chloroplast under normal growth. They also store carbon in the form of triglycerides (TAG) under adverse environmental conditions. The major fatty acids of diatoms include C16:0 (palmitic acid) and C16:1 (palmitoleic acid), and polyunsaturated fatty acids (PUFA) such as C20:5ω3 (eicosapentaenoic acid, EPA) and C20:4ω6 (arachidonic acid, ARA) [3].

Diatoms have gained widespread attention in recent decades owing to their enormous commercial and industrial potential [4]. Industrial diatom cultures have been developed for the aquaculture industry [4] and other relevant commercial applications of diatoms at the small and semi-industrial scale are being investigated. Some of the significant advances include: (1) frustule studies for novel applications in nanotechnology, drug delivery and material science [5]; (2) EPA, chrysolaminarin and fucoxanthin production for application in aquaculture, food and pharmaceutical areas [6–8]; and (3) TAG production for biodiesel and other valuable co-products such as exopolysaccharides (EPS) [9–12]. Diatoms could currently be used for the development of various types of biorefineries [13]. For example, the potential market based on the combined production of EPA and fucoxanthin from *P. tricornutum* in hybrid raceway ponds was estimated in the billion dollar range by Leu and Bousiba [14]. However, the global microalgal market is currently dominated by other non-diatom species such as the Cyanophyta *Spirulina* and the Chlorophyta *Chlorella*, *Dunaliella* and *Haematococcus* [13].

Maintaining high biomass production is an important strategy to guarantee the production of high-value metabolites in diatoms on a commercial scale [15,16]. For production to be profitable, the costs associated with the cultivation units (i.e., photobioreactors or raceway ponds) and resources (i.e., nutrient and water resources) have to be brought down. EPA, chrysolaminarin and fucoxanthin content in diatom cells are species and strain specific, and they are also strongly affected by culture conditions [3] such as light intensity, salinity, nutrient concentrations and the balance between N, phosphorus (P) and Si (N/P/Si) in the medium. The aim of the present study was therefore to evaluate the potential of the marine and benthic diatom *Halamphora coffeaeformis* to accumulate EPA, chrysolaminarin and fucoxanthin in a raceway pond sustained by fertilizer (Bayfolan®)-enriched seawater. Fertilizer was selected to maximize biomass production due to its richness in nitrogen, phosphorus, vitamins and micronutrients, and for being an economically feasible culture medium at large scale. In addition, the dissolved and internal nutrient kinetics were investigated to gain insight into the nutritional behaviour of this species in a non-conventional culture medium. Previous studies [11,12] have demonstrated the capacity of *H. coffeaeformis* to accumulate suitable TAG for biodiesel production under nutritional stress from a biorefinery approach. However, the potential of this species to simultaneously accumulate high-value compounds in a nutrient-rich raceway pond has not yet been studied and the studies that do exist on the simultaneous production of these valuable metabolites in diatoms grown in raceway ponds are of limited scope. The results of the present study are expected to contribute to the development of large-scale bioprocesses from marine benthic diatoms, focusing on sustainable high-value markets.

2. Material and methods

2.1. Algal strain and laboratory scale-up

Halamphora coffeaeformis (C. Agardh) Levkov was isolated from Bahía Blanca Estuary, Argentina, identified [11] and maintained in stock cultures in f/2 medium [17]. For small-scale culture (100 mL to 4 L), cells from stock cultures were collected by auto-floculation and the supernatant was removed. The cells were then transferred to different flasks containing the selected medium consisting of seawater enriched with fertilizer 0.28 $mL.L^{-1}$ Bayfolan® to obtain 470 μM nitrate (NO_3^-), 700 μM amonium (NH_4^+) and 100 μM phosphate (PO_4^{3-}, P). The silicate (SiO_4^{4-}; Si) was incorporated according to 5f medium (f medium [17] with a 5× concentration of Si) in order to obtain a ratio of Si:N ~ 1. TRIS buffer was incorporated according to f/2 [17]. At this stage of scale-up, aged seawater from Bahía Blanca Estuary with a salinity of 33‰ (ppt, parts per thousand) was filtered (0.45 μm Millipore) and autoclaved at 121 °C and 1 Atm for 20 min. Fertilizer, silicate and buffer were filtered (0.2 μm Millipore) independently and then incorporated into sterilized seawater. The highest growth of *H. coffeaeformis* was obtained with 0.28 $mL.L^{-1}$ Bayfolan®; growth was lower with concentrations of 0.1 $mL.L^{-1}$ and 0.38 $mL.L^{-1}$ of Bayfolan® (data not shown). Four-day cultures (approximately 0.5 × 10^6 $cells.mL^{-1}$) were used as inoculum at 10% volume in each scale-up step until reaching a 4 L cultivation flask, which was incubated for 7 days at 20 °C under continuous air bubbling (500–700 $cm^3.min^{-1}$) containing 0.30 $cm^3.min^{-1}$ of CO_2.

2.2. Scale-up of photobioreactor (PBR) to raceway pond

A cylindrical borosilicate 25 L photobioreactor (FIGMAY S.R.L, Córdoba, Argentina) was used to obtain a 20 L-inoculum for a 300 L raceway pond, for which the 4 L cultivation flask was inoculated in the PBR and the culture was carried out over 7 days in a room at 20 ± 2 °C with Bayfolan® medium (see Section 2.1). LED panels around the PBR provided 100 $\mu mol.m^{-2}.s^{-1}$ of light intensity under a cycle of 12:12 h light/darkness. The culture was supplied with 1% CO_2 in air during 2 h and maintained under stirring by means of central paddles at 30 rpm. These conditions were selected with the dual aim of avoiding a significant decrease in pH and maintaining a moderate mixing regimen, taking into account the marine and benthic habits of the studied strain, respectively.

Finally, a PVC raceway pond of 2 m length, 0.5 m width and 0.3 m height was used to obtain a biomass rich in high-value products. The 20 L- inoculum from the PBR was incorporated in the 300 L raceway pond and the culture was carried out over 22 days in a greenhouse with natural light and temperature. The initial culture volumen was 220 L (200 L seawater with 0.28 $mL.L^{-1}$ Bayfolan® + 20 L inoculum). The seawater was enriched with Bayfolan® fertilizer and silicate to obtain ratios of Si:N ~ 1 and N:P ~ 16 in accordance with the Redfield/Brzezinski Si:N:P ratios of 16:16:1 [2]. Buffer TRIS was not incorporated. The stirring was produced by acrylic paddle wheels and turbulence provided by air-bubbling enriched with 1% CO_2. At this stage of scale-up, filtered (1 μm Millipore) seawater (Bahía Blanca's Estuary, 33‰) was sterilized with 5% sodium hypochlorite and neutralized with 0.15 $g.L^{-1}$ sodium thiosulfate. Water temperature and pH were measured with pH meter (POCKET PRO pH TESTER, HACH), and salinity was measured daily in situ with a multiparameter digital meter CONSORT C562. The daily PAR was measured with a radiometer LI-COR model LI-192SB at different times of day (from 6:00 A.M to 20:00 P:M). The biomass was harvested by scraping with a shovel, since this species autoflocculate and forms a biofilm on the bottom.

2.3. Growth measurements and analytical methods

2.3.1. Growth rate and biomass determination

Samples were taken daily in triplicate for cell density and biomass determination. Cell density ($N°\ cell.mL^{-1}$) was determined by counting triplicate samples in a Sedgwick-Rafter chamber. The specific growth rate (μ) was calculated during the period of exponential growth by a least squares fit to a straight line of logarithmically transformed data, which were expressed as day^{-1}. For biomass, duplicate samples of 20 mL were filtred through pre-conditioned and pre-weighed Whatman GF/C filters (1.2 μm). The filters containing the samples were then washed three times with 10 mL distilled water and dried in an oven at 60 °C for 24 h, cooled in a desiccator and weighed until constant weight (DW).

2.3.2. Nutrient analyses

Samples of 20 mL were taken daily, filtered through Whatman GF/F filters (0.7 μm) and frozen at −80 °C until analysis. The filtrate was used for dissolved nutrient determination, and the filters containing the cellular pellet were used for internal nitrate analysis. Internal nitrate was determined after extraction with boiling, distilled and deionized water [11]. The procedure was complemented with an Omni Sonic Ruptor 400 cell disruptor, exposing the samples to pulses of 30% power for 3 min, using a 3 mm diameter tip. Nutrient concentrations were determined via colorimetric assays in order to measure phosphate (PO_4^{3-}); amonium (NH_4^+); nitrate (NO_3^-, dissolved and internal) and silicate (SiO_4^{4-}) [18]. 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 nutrients were referenced as μM and internal nitrate concentrations were normalized to cell number (pg.cell^{-1}). The standard ratios of 16Si:16 N:1P were used to evaluate the possible nutrient limitation. For this, dissolved inorganic nitrogen concentration (N) was calculated as the sum of dissolved NO_3^- and NH_4^+.

2.3.3. Protein, carbohydrate and lipid content quantification

Samples of 40 mL for biochemical composition were taken on selected days (0, 2, 5, 6, 7, 9, 11, 13, 15, 17, 22) to cover different growth phases. Samples in triplicate were filtered through Whatman GF/C filters (1.2 μm) and stored at −80 °C until analysis. Proteins and carbohydrates were then spectrophotometrically determined using the Coomassie Brilliant Blue dye method [19] and the phenol–sulfuric acid assay [20], respectively. These methods are explained in more detail in Popovich et al. [21]. Lipids were determined according to Weldy and Huesemann [22].

2.3.4. Fatty acid characterization

The fatty acid methyl ester (FAME) profile of total lipid from biomass harvested on day 22 was determined and analyzed by gas chromatography with an HP Agilent 4890D gas chromatograph according to Popovich et al. [23]. FAME identification was performed by comparison with standard certified material, Supelco FAME 10 mix 37 (Bellefonte, PA, USA), according to AOCS Official Method Ce1b-89. Four replicates of each FAME analysis were done.

2.3.5. Cell polysaccharide extraction and structural analysis

Polysaccharides from biomass harvested on day 22 were analized. Cellular polysaccharides were extracted from freeze-dried cells of *H. coffeaeformis* (9.4 g) using water at room temperature by magnetic stirring at 300 rpm for 2 h (product RTW). The supernatant was collected by centrifugation (10,000 ×*g* for 30 min), and the precipitate re-extracted with hot water at 100 °C (product W100) for 2 h. The extracts were dialyzed (72 h, 3500 MWCO; Spectrum laboratories, Pennsylvania, USA) and lyophilized (RTW: yield, 3.50 g; W100: yield, 1.52 g; Virtis Freezemobile 3, SP Scientific, Pennsylvania, USA).

For the structural analyses, W100 (22 mg) was methylated using powdered NaOH in dimethyl sulfoxide-iodomethane [24] containing 30 mg LiCl. The permethylated polysaccharide was extracted with chloroform (×4), the extracts were collected, and the solvent was evaporated under a current of dry air. All the chemicals were purchased from Sigma-Aldrich (USA).The yield after the second methylation step (W100-m) was 8.5 mg.

2.3.6. Monosaccharide composition

Monosaccharide composition was determined by gas-liquid chromatography (GLC) (Hewlett–Packard 5890A Gas Chromatograph, California, USA) after hydrolysis of the extraction products according to Daglio et al. [25]. When necessary, GLC–MS analyses were performed on a Shimadzu QP-5050A (Kyoto, Japan) apparatus working at 70 eV using the same column and conditions described by Daglio et al. [25], but with helium as a gas carrier at a total flow rate of 1 mL.min^{-1}; the injector temperature was 240 °C.

2.3.7. Nuclear magnetic resonance spectroscopy

W100 extract (20 mg) was dissolved in deuterated DMSO (0.5 mL) (Sigma-Aldrich, USA) using 5 mm tubes. Spectra were recorded at room temperature on a Bruker Avance II 500 MHz spectrometer (Karlsruhe, Germany). For the 500 MHz ^{1}H NMR experiment, the parameters were: spectral width of 7.5 kHz, 30° pulse, acquisition time of 4.4 s and relaxation delay of 1 s, for 64 scans. Signals were referenced to 2.50 ppm. The ^{1}H−^{13}C HSQC technique was supplied by the spectrometer manufacturer; spectra were recorded at room temperature and were obtained at a base frequency of 500 MHz for ^{1}H and 125 MHz for ^{13}C.

2.3.8. Fucoxanthin and chlorophyll a extraction and quantification

For fucoxanthin (Fx) and chlorophyll *a* (Chl *a*) determination, samples of 20 mL were taken on selected days (0, 2, 5, 6, 7, 9, 11, 13, 15, 17, 21 and 22) to cover different growth phases. Each sample was filtered through a Whatman GF/C filter (1.2 μm) and stored at −80 °C until analysis. The total carotenoid extraction procedure was optimized in line with an exhaustive bibliography search [6,26–28]. Filtered samples containing *H. coffeaeformis* cells in different culture stages were suspended in absolute ethanol (100% HPLC grade, Supelco, USA) for pigment extraction (ethanol:alga culture filtered volume = 1:6; v/v). The ethanol:biomass dry weight (DW) ratio was 5:1 v/w. The whole extraction process was performed in the dark to prevent pigment breakage. Each extraction system was mixed by vortex for 1 min and incubated in a brown tube with a magnetic stirrer at 40 °C for 2 h. Pigment ethanolic extracts were filtered through a 0.2-μm PTFE syringe filter and placed in brown 2-mL HPLC auto sampler vials with Teflon-coated lids. Samples were analyzed with a LS-MS - Thermo Scientific - UltiMate 3000 - MSQ PLUS HPLC system equipped with an AcclaimTM 120 - C18 reverse phase column (3 μm particle size, 2.1 mm × 150 mm; Dionex Bonded Silica Products) and a fixed wavelength UV detector. Fucoxanthin was monitored at an absorbance of 445 nm. For HPLC, a flow rate of 0.5 mL.min^{-1} of the mobile phase was controlled by binary pumps at 25 °C. The mobile phase consisted of acetonitrile (Supelco, USA) and Milli-Q water. Water was purified by using a Milli-Q (Millipore, Billerica, MA) system, giving a product with a resistivity of ~18.5 MΩ/cm. After loading the column with the pigment ethanolic extract, the mobile phase was programmed with an isocratic ratio 83:17 acetonitrile:water solution over 15 min. Total injection time was 15 min. Between each sample injection an absolute ethanol (100%) blank was run. Using fucoxanthin analytical standard (Supelco, USA), an external calibration curve was constructed; the peak area with a retention time of 4.566 min was employed for quantification. Each ethanol volumetric standard concentration, in the range of 4–100 μg.mL^{-1}, was determined by UV–Vis spectroscopy (Agilent Cary 60 UV–Vis spectrophotometer) with an E value (0.1%, 1 cm) of 160 L/g/cm at 449 nm [29]. Based on this information, the Fx volumetric concentration (mg.L^{-1}) and Fx content (mg.g^{-1} DW) in each sample were estimated.

Chl *a* content was determined by UV–Vis spectrophotometric assay using Agilent Cary 60 UV–Vis spectrophotometer. For construction of the calibration curve, several dilutions of purified Chl *a* solution (Sigma Aldrich, St. Louis, MO, USA) in acetone (HPLC grade) were prepared. Absorbance with an E value (0.1%, 1 cm) of 82 L.g^{-1}.cm^{-1} at 663 nm was used to calculate diluted concentrations in a range of 20–100 μg.mL^{-1}. The visible spectrum of pigment ethanolic extract in each culture stages was obtained by scanning from 300 to 800 nm. The Chl *a* volumetric concentration (mg.L^{-1}) in each sample was estimated by substrating Fx to the total concentration value calculated at 663 nm, which corresponded to Chl *a* + Fx concentration [28].

2.4. Statistical analysis

The results are expressed as mean values ± standard deviation

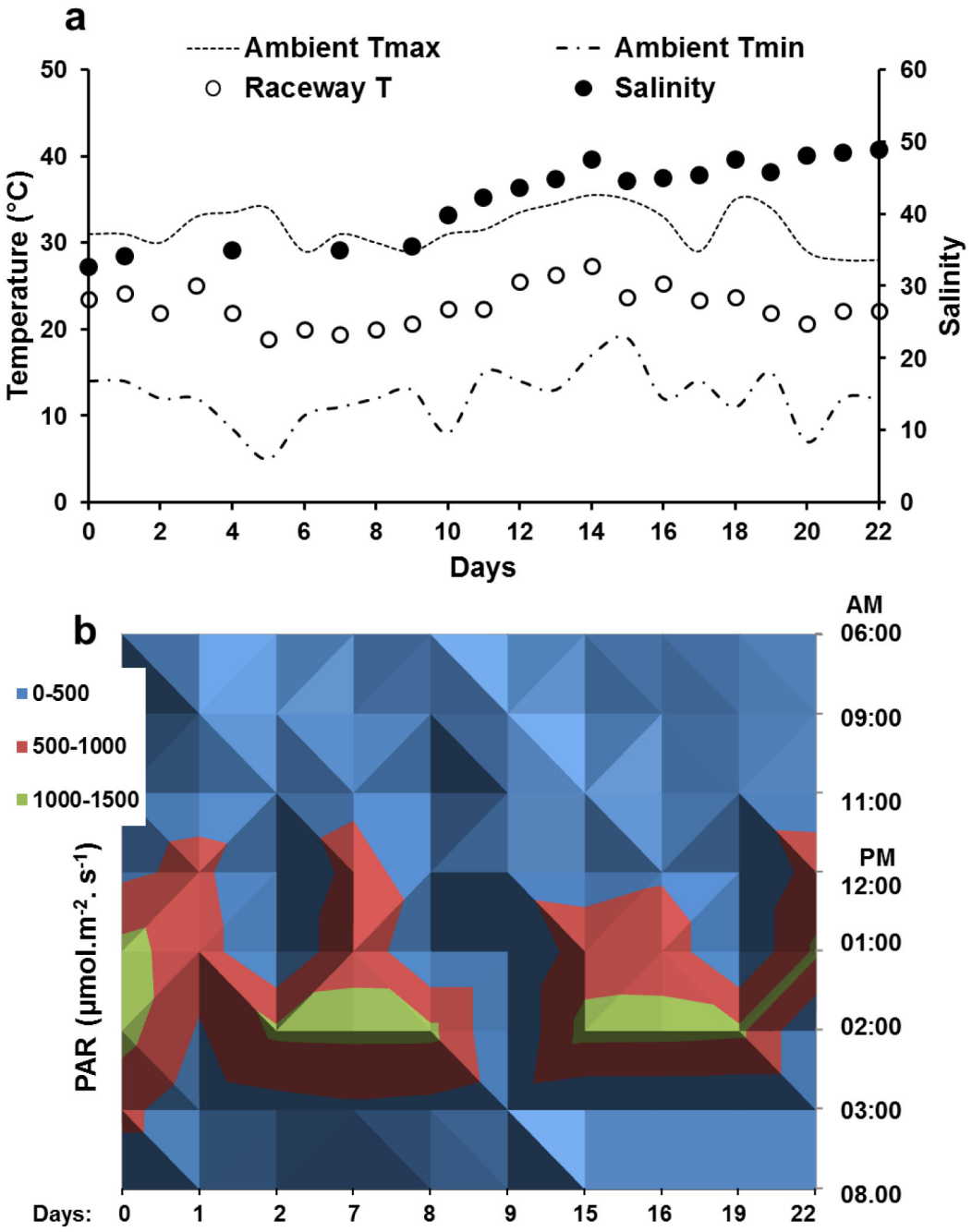

Fig. 1. *H. coffeaeformis'* environmental culture conditions. **(a)** Ambient temperature (maximum and minimum) (°C), raceway temperature (°C), and salinity ‰ (ppt, parts per thousand) throughout 22 days. **(b)** Incident PAR (µmol.m^{-2}.s^{-1}) at different daylight hours.

(SD). The differences in the mean values of the protein, carbohydrate and lipid contents ($n = 3$), as well as the fucoxanthin and chlorophyll volumetric concentration and content (n = 3) throughout the experiment were evaluated with a paired student's *t*-test ($\alpha = 0.05$). In all cases, comparisons that showed $p < 0.05$ were considered significant. Statistical analyses were performed using Infostat Software (version 2017.1.2).

3. Results and discussion

3.1. Environmental conditions and biomass production

The ambient temperature ranged from 5 °C to 35 °C and the water temperature varied between 18.8 °C and 27.3 °C (Fig. 1a), indicating the good thermal balance produced by the movement of the paddle wheels. The water temperature values were within the range of conditions used for culturing microalgae in raceway ponds [30]. The salinity was increased gradually from 32.7‰ to 35‰ until day 9, and then increased considerably up to 47.9‰ on day 22 (Fig. 1a). The studied strain remained productive throughout the culture, an advantageous feature for its growth in open pond systems, where salinity can increase over time due to evaporation. In addition, the culture was exposed to varying intensities of natural PAR throughout the day, with maximum values ranging from 1000 µmol.m^{-2}.s^{-1} to 1500 µmol.m^{-2}.s^{-1} between ~1:00 P.M. and ~2:30 P.M. (Fig. 1b). Increasing incident irradiance levels generally improve raceway productivity; however the rate of photosynthesis does not increase beyond a PAR value of about 100–200 µmol.m^{-2}.s^{-1} [30].

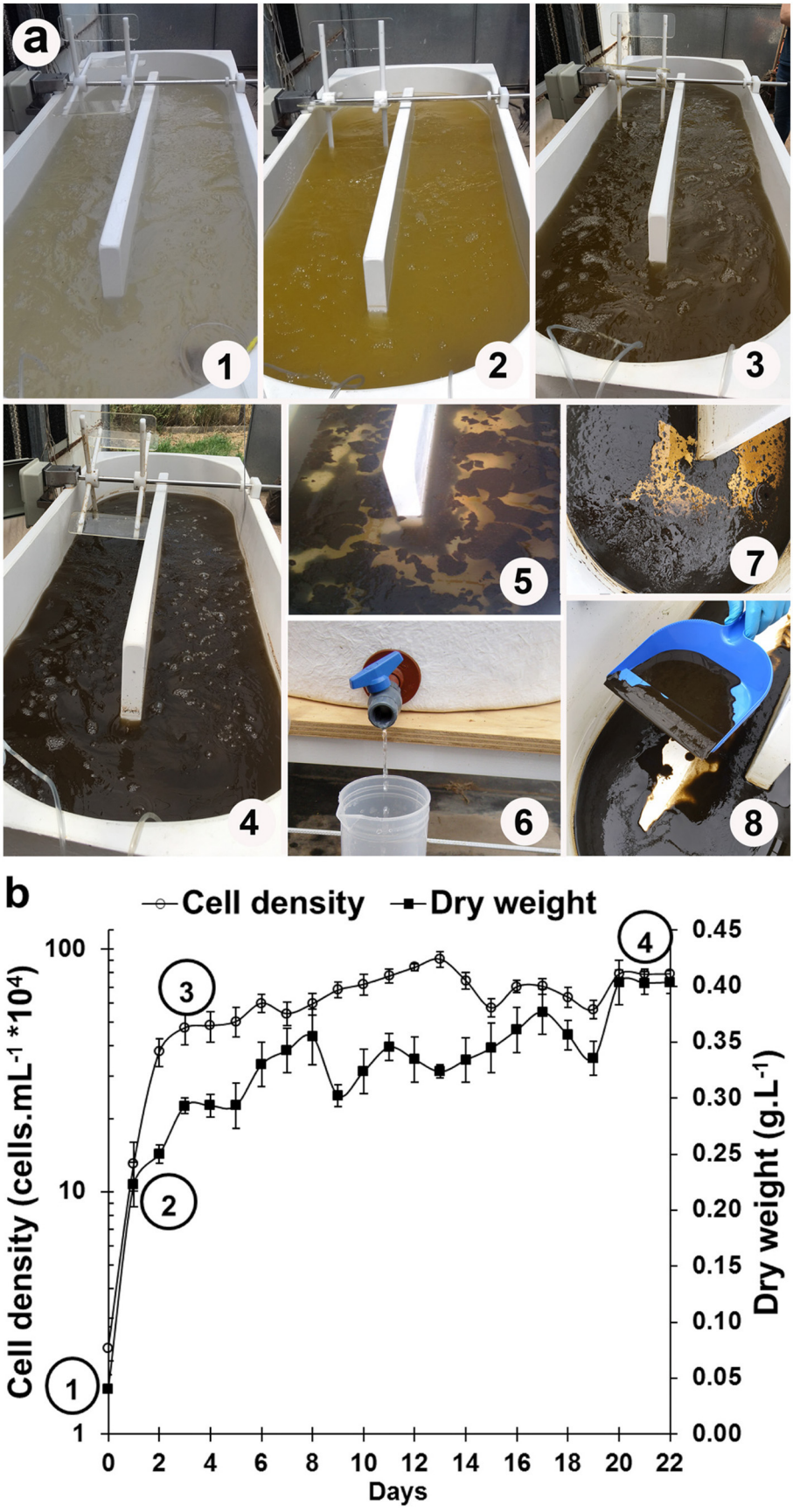

Fig. 2. *H. coffeaeformis* grown in a raceway pond throughout 22 days. **(a)** Several instances of *H.coffeaeformis* culture and harvesting: **(1)** day 0; **(2)** day 2; **(3)** day 4; **(4)** day 22; **(5)** biomass auto-flocculated with supernatant; **(6)** supernatant separation by a tap; **(7)** biofilm on the bottom without supernatant; **(8)** harvesting pellet by scraping. **(b)** Cell density and dry weight kinetics (inserted numbers match with the Fig. 2a ones). Data are expressed as mean ± standard deviation (SD), $n = 3$.

Several instances of *H.coffeaeformis* culture and harvesting in a raceway pond are shown in Fig. 2a.The cell density and dry weight (DW) kinetics of this species throughout 22 days of culture is presented in Fig. 2b (numbers 1–4 in Fig. 2a, belonging to different growth stages, match with Fig. 2b numbers). The exponential-growth phase ocurred from day 0 (Fig. 2a-1) to day 2 (Fig. 2a-2), followed by a stationary-growth phase from day 3 (Fig. 2a-3) to day 22 (Fig. 2a-4). The culture showed neither a lag phase nor a typical declination phase, an advantage at the production level. The specific growth rate (μ) was 2.03 day^{-1}, corresponding to a doubling time of 11.8 h. The initial cell density was 22 × 10^3 cells.mL^{-1}, reaching approximately one million cells.mL^{-1} at day 13. The biomass concentration presented a six-fold increase up to 0.3 g.L^{-1} during the exponential phase, with the maximum value of 0.43 g.L^{-1} at the end of the experiment. Biomass productivity during the exponential phase was 0.128 g.L^{-1}.d^{-1} and the areal yield was 25.73 g.m^{-2}.d^{-1}. One of the main goals of the current study was to evaluate the potential of fertilizer to maintain high biomass levels of *H. coffeaeformis* in a batch-cultivation system, as a strategy for stimulating the valuable metabolite production. For industrial purposes, culture media producing similar or higher biomass yields than conventional media (i.e. f/2 medium) signify a cost saving. The biomass yields obtained were higher than that reported in the literaure for the same strain grown in an indoor raceway pond with f/2 medium and artificial light [11], while the areal yield was similar to that observed in *Amphora* sp. MUR258 grown in an artificial 2f medium-based outdoor raceway pond [31]. This non-conventional culture medium therefore represents a highly promising resource for both increasing the growth rate of *H. coffeaeformis* and lowering the cost of large-scale biomass production.

For harvesting, the paddle wheels were stopped at day 22 and after 4 h the biomass auto-flocculated, settling to the bottom (Fig. 2a-5), thereby allowing a separation of microalgal cells (pellet) from the culture medium. The supernatant was removed under a tap (Fig. 2a-6) and the pellet (Fig. 2a-7) was harvested by scraping (Fig. 2a-8). *H. coffeaeformis* is a benthic diatom that grows naturally attached to the bottom of the water column; however, it grew successfully suspended in the culture medium under the mixing condition tested. Furthermore, its biomass auto-flocculated in a very short time by interruption of paddle wheels, without the requirement of chemicals or flocculants. Harvesting biomass by auto-flocculation requires less energy in large-scale systems [32]. Thus, the natural behaviour of *H. coffeaeformis* represents an advantage for downstream processing in raceway ponds since its harvesting does not require external energy.

3.2. Dissolved and intracelular nutrient kinetics

The dissolved and internal nutrient kinetics were investigated to evaluate the effect that a non-conventional culture medium can have on both biomass production and the biochemical composition of *H. coffeaeformis*. For this, dissolved nutrient (nitrate, ammonium, phosphate and silicate), internal nitrate (internal-NO_3^-) and both Si:N and N:P ratio kinetics in the raceway pond are shown in Fig. 3. The culture medium presented dissolved ammonium and nitrate as N source. The NH_4^+ level decreased 96% (from 700 μM) during the exponential growth phase, and then remained at a threshold value of ca. 20 μM throughout the remainder of the experiment (Fig. 3a). The NO_3^- level diminished 50% (from 466 μM), during the exponential growth phase and then decreased considerably from day 8 to day 15, reaching values close to the limiting value (average Ks [N] of 1.2 μM [33]). It is important to note that in the presence of two nitrogen sources, *H. coffeaeformis* showed a selective uptake of ammonium, though this nutrient never reached limiting values. Normally, NH_4^+ is preferentially taken up by diatoms if both nitrate and ammonium are available in abundance. Cells using NO_3^- must expend significant amounts of energy for reduction to NH_4^+ [34]. From the perspective of large-scale biomass production, the selective uptake of N observed in *H. coffeaeformis* represents a nutritional strategy that ensures a reserve of dissolved nitrogen for an extended period, consequently guaranteeing a prolonged stationary phase. Furthermore, some diatom species have the capacity to store significant amounts of NO_3^- internally [35], thus creating an additional N stock to maintain cell growth. *H. coffeaeformis* presented an internal NO_3^- pool that ranged from 0.2 pg.$cell^{-1}$ to 1.15 pg.$cell^{-1}$ (Fig. 3a). The N storage of *H. coffeaeformis* could therefore contribute to its growth under limiting values for external N. Phosphate reached limiting values (average Ks [P] of 1.2 μM [33]) from day 16 (Fig. 3b) and Si never reached limiting values (average Ks [Si] of 3.9 μM [33]) (Fig. 3c).

The N:P and Si:N ratio kinetics are shown in Fig. 3d. The initial N:P and Si:N ratios were ca. 11 and ca. 1, respectively, close to to the standard ratio of 16Si:16 N:1P ratios [2]. These ratios, reflected in the high value of μ (2.03 day^{-1}), indicate a favourable nutrient-sufficient scenario for setting up a diatom biomass production process. In fact, significant Si consumption was observed until day 5 (Si:N = 14) (Fig. 3d), indicating strong cell division and the formation of new valves in the daughter cells. However, the stationary phase started on day 3. This behaviour could be explained by the light limitation due to cell shading, which would not affect the incorporation of Si since the silicification process does not require concurrent photosynthesis [36]. In contrast, nitrogen metabolism is closely associated with carbon metabolism, and consequently with metabolite production [3]. Both Si:N and N:P ratios showed stronger nitrate uptake than Si and P between days 5 and 13, indicating a phase of nitrogen-dependent metabolite production (e.g. proteins). In the late stationary phase, the N:P ratio reached values > 100 from day 17 to day 22, indicating a stronger uptake of P than N. In addition, in this period the PO_4^{3-} reached limiting values. While this scenario would appear to indicate stressful growth conditions, the availability of ammonium, intracellular nitrate, and silicate may partially explain the lack of a declining growth phase. The findings indicate that the proposed culture medium shows a suitable Si:N:P balance, allowing for sustained biomass production of this species throughout 22 days, guaranteeing the accumulation of high-value metabolites.

3.3. Biochemical composition of H. coffeaeformis

Protein, carbohydrate and lipid volumetric concentration (mg.L^{-1}) and content (mg.g^{-1} DW) kinetics of *H. coffeaeformis* are shown in Fig. 4a and Fig. 4b, respectively. The culture showed a basal production (day 0) of 5.2 mg.L^{-1}, 10 mg.L^{-1} and 9 mg.L^{-1} of proteins, carbohidrates and lipids, respectively. Protein production increased 21-fold up to 107.7 mg.L^{-1} on day 13 ($p < 0.05$) and then decreased to 60 mg.L^{-1} at day 22. This increased protein production necessary for cell growth was favored by higher nitrogen concentrations; production decreased during the late stationary phase (from day 13) in line with the strong decrease in nitrate (Fig. 3a and Fig. 3d).

Lipid production increased 14-fold up to a maximum value of 125 mg.L^{-1} on day 22 ($p < 0.05$), the maximum content being 310 mg.g^{-1} DW (Fig. 4). Lipid accumulation in diatoms is triggered by stress conditions. Previous studies on *H. coffeaeformis* in f/2 reported significant lipid values, in particular TAG, under conditions of depleted dissolved and internal N, silicate and phosphate [11,12]. Although lipid content increased in the present study, N and Si availability did not reach the limiting levels needed to induce maximum lipid accumulation in *H. coffeaeformis*.

The fatty acid (FA) profile of total lipids (Table 1) on day 22 revealed that PUFAs were the dominant lipid class (36% of total fatty acids) of the harvested biomass, the most abundant among these (24.1%) being eicosapentaenoic acid (C20:5 ω-3, EPA) followed by arachidonic acid (C20:4 ω-6, ARA) (4.4%). The PUFA percentage was higher than that found for this strain grown on a f/2 medium-based indoor raceway pond under nutrient stress [11]. Both EPA and ARA are highly valuable to the aquaculture industry and at present, their main

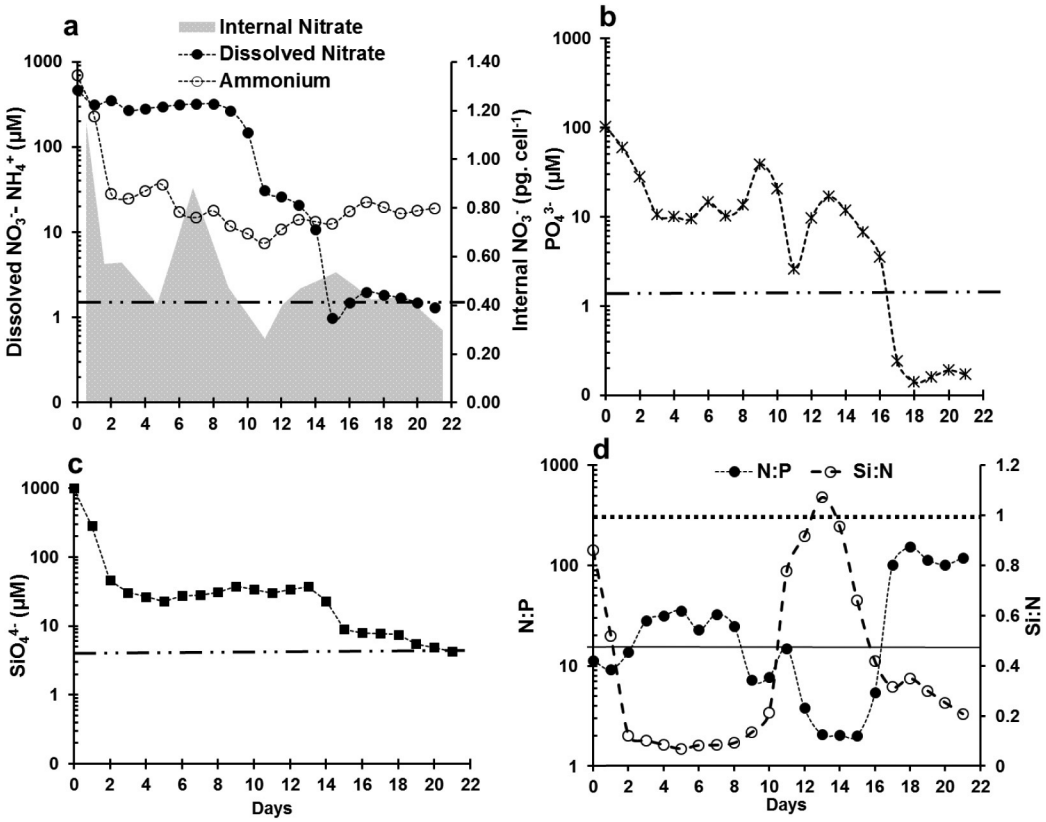

Fig. 3. *H. coffeaeformis*' nutrient kinetics in a raceway pond. **(a)** Dissolved nitrate and ammonium (µM), and internal nitrate (pg.cell^{-1}) kinetics; **(b)** dissolved phosphate (µM) kinetics; **(c)** dissolved silicate (µM) kinetics; **(d)** Si:N:P ratio kinetics. Data are expressed as mean, n = 3. Average limiting values of nutrients for diatom growth (N < 1.6 µM; P < 1.2 µM; Si < 3.9 µM) according to Sarthou et al. [33] are shown in Figs. a, b and c (dashed line). Non-limiting ratios are shown in Fig. d as N:P ~ 16 (straight line) and Si:N ~ 1 (dotted line) according to Redfield/Brzezinski ratios of 16Si:16 N:1P.

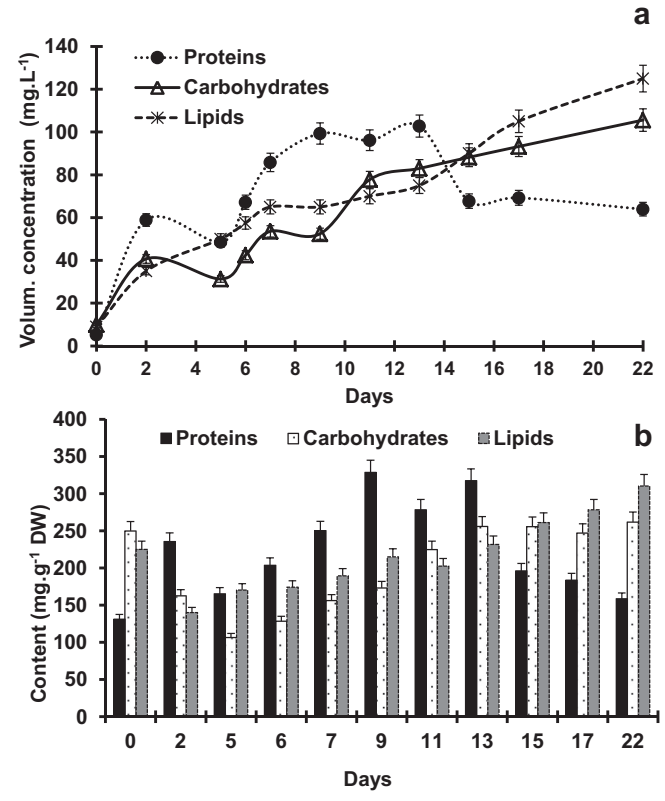

Fig. 4. *H. coffeaeformis*' valuable metabolites throughout 22 days of culture. (a) Kinetics of protein, carbohydrate and lipid volumetric concentration (mg.L^{-1}). (b) Kinetics of protein, carbohydrate and lipid content (mg.g^{-1} DW). Data are expressed as mean ± SD, n = 3.

Table 1
Main fatty acids (% of total fatty acids) from *H.coffeaeformis*' biomass on day 22; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Data are expressed as mean ± SD, n = 4.

Main FAMEs	% of total lipids
C14:0 (miristic)	9.3 ± 0.39
C16:0 (palmitic)	16.8 ± 1.72
C18:0 (stearic)	0.3 ± 0.08
C16:1 (palmitoleic)	25.9 ± 1.16
C18:1 ω-**9** (oleic)	6.4 ± 0.18
C18:2 ω-6c (linoleic)	2.7 ± 0.10
C18:3 ω-3 (linolenic) (ALA)	1.3 ± 0.07
C20:4 ω-6 (ARA)	4.4 ± 0.64
C20:5 ω-3 (EPA)	24.1 ± 0.78
C22:6 ω-3 (DHA)	0.7 ± 0.02
C20:2 ω-6	2.8 ± 0.13
ΣSFA	26.4 ± 0.70
ΣMUFA	32.3 ± 0.96
ΣPUFA	36 ± 0.45

commercial source is from fish oils [37]. Microalgal growth rate optimization has been indicated as the best way to improve EPA yields and consequently to produce cost-competitive microalgae [15]. The use of Bayfolan® fertilizer proved adequate for improving *H. coffeaeformis* growth and increasing its PUFA yield. Monounsaturated fatty acid (MUFA) reached 32%, palmitoleic acid (C16:1) being the most abundant (~26%), followed by oleic (C18:1 ω-9) acid (6.4%). According to the fatty acid profile, the ω-6:3 ratio of 0.4 was well below the suggested dietary ω-6:3 ratios (1:1 to 4:1) [38]. This strain could therefore be particularly suitable for products designed to balance diets with excess ω-6.

Carbohydrate production increased 10.5-fold up to 105.9 mg.L^{-1} on day 22 (p < 0.05), when total carbohydrate content was 250 mg.g^{-1} DW (Fig. 4). Hot water extraction from cells yielded a

Table 2
Composition of monosaccharide (mol %) produced by methylation and acid hydrolysis of the W100 extract from *H. coffeaeformis*. Data are expressed as media ± SD, n = 3.

Monosaccharide[a]	Deduced glycosidic linkage	W100-m
2,3,4,6-Me_4Glc	Terminal	14.0 ± 1.6
2,4,6-Me_3Glc	3-linked	73.4 ± 0.8
4,6-Me_2Glc	3-linked 2-substituted	3.3 ± 0.9
2,4-Me_2Glc	3-linked 6-substituted	4.9 ± 0.8
Glc		4.4 ± 0.3

[a] **2,4-Me_2Glc** = 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylglucitol; **4,6-Me_2Glc** = 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylglucitol; **2,4,6-Me_3Glc** = 1,3,5-tri-*O*-acetyl-2,4,6- tri-*O*-methylglucitol; **2,3,4,6-Me_4Glc** = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

product (W100) composed of monosacharides (mol %), with glucose as the main component (94.4 ± 0.3 mol%, *n* = 3) and smaller amounts of rhamnossa (1.7 ± 0.5 mol%, n = 3) and fucosa 2.4 ± 0.6 mol%, n = 3). Mannose and glactose presented trace values (< 1 mol%). Methylation analysis of W100 (W100-m) indicated the presence of a (1 → 3)-linked glucan with branching in C2 and C6 (Table 2). The ratio of tetramethylated terminal glucose residues (14.0%) to the rest of the glucose units (86.0%) showed that the glucan backbone was substituted every six glucose residues. The presence of a (1 → 3)- *β*-D-linked glucan suggests that this glucan derived from intracellular chrysolaminarin, as pointed out by Chiovitti et al. [39]. Chrysolaminarin from *H. coffeaeformis* presented branching in C2 and C6 as reported for analogous polymers in other diatom species [25,39,40]. A degree of branching (*DB*) of 0.16 for the stored glucan of *H. coffeaeformis* was estimated from methylation analysis [25]. A wide range of *DB*, from 0.015 to 0.39, has been reported [7,25,39]. Chrysolaminarin in diatoms is the primary carbon storage sink when nutrients are still available, whereas lipids, particularly TAG, predominate during high nutrient starvation [3]. In the present study, the chrysolaminarin content was 114 $mg.g^{-1}$ DW on day 22, representing 46% of total carbohydrate content.

The ^{13}CNMR spectrum of W100 showed distinct glucose-derived signals at 103.65, 73.23, 86.61, 68.83, 76.74, and 61.28 ppm assigned to C1, C3, C5, C2, C4, and C6, respectively (Table 3). To gain further insight into the derivatization reactions, 1H and 1H−13C HSQC NMR studies were performed. Substitution at the 6- position may be inferred from the 1H−13C HSQC results (Table 3). The C1/H1 of *β*-(1 → 6) was found at 104.24/4.37. Xia et al. [7] reported a similar structure in *Odontella aurita*, which presented a *β*-D-(1 → 3) (main chain) and *β*-D-(1 → 6) (branch chain)-linked glucan. These authors revealed that this chrysolaminarin presented strong antioxidant activity. The *β*-glucans (especially *β*-1,3/1,6-glucans) have also been introduced as additives to improve the health and performance in marine ranching of fish, shrimp and gastropods [41].

Table 3
^{1}H −^{13}C HSQC NMR spectral assignment of the W100 extract from *H. coffeaeformis*.

Position	Chemical shifts ^{13}C/^{1}H (ppm)
β-(1 → 3)[a]	
C1/H1	103.65/4.51
C2/H2	73.23/3.28
C3/H3	86.61/3.47
C4/H4	68.83/3.21
C5/H5	76.74/3.25
C6/H6	61.28/3.69, 3.43

[a] The C1/H1 of *β*-(1 → 6) was found at 104.24/4.37.

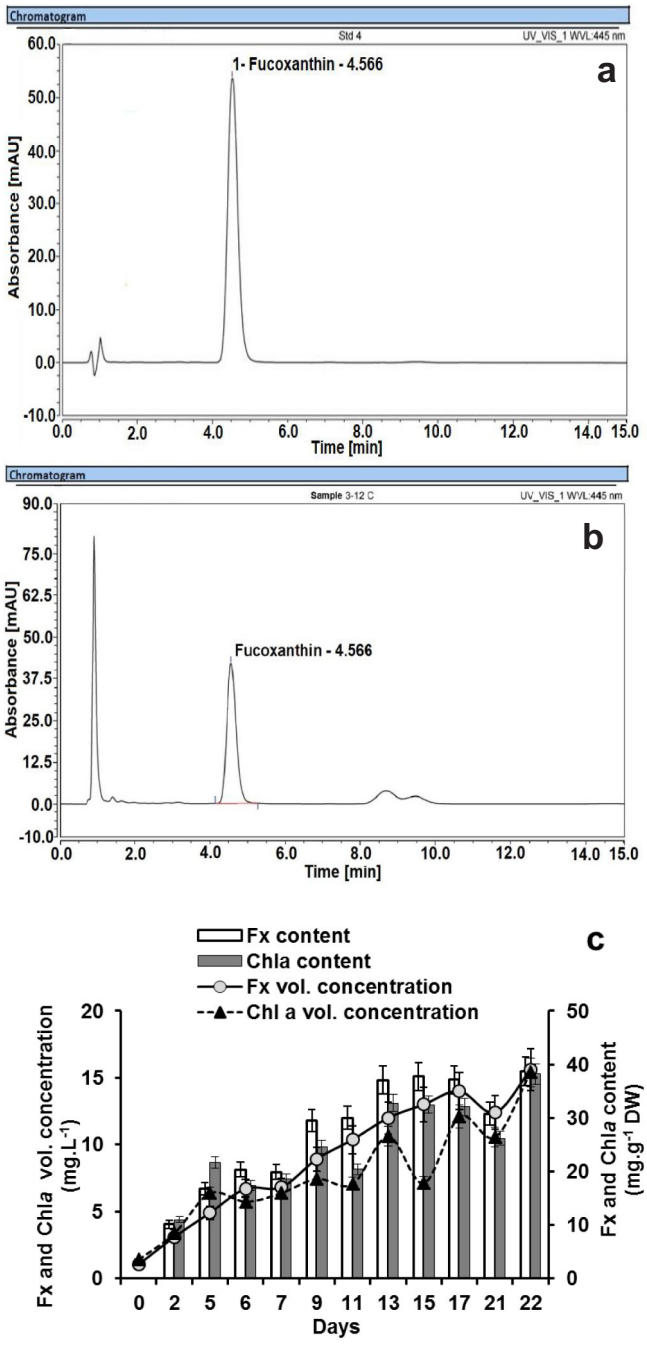

Fig. 5. *H. coffeaeformis'* pigments. (a) HPLC chromatogram of fucoxanthin (Fx) standard; (b) HPLC chromatogram of a crude extract; (c) Fx and Chl a volumetric concentration ($mg.L^{-1}$) and content ($mg.g^{-1}$ DW) kinetics. Data are expressed as mean ± relative standard deviation (RSD), n = 3. RSD was 2.1% according to HPLC analysis.

3.3.1. Fucoxanthin and chlorophyll a

Fucoxanthin (Fx), chlorophyll (Chl) *a*, Chl *c* and an apoprotein constitute the main light-harvesting fucoxanthin and chlorophyll *a*/*c*-complex of brown macroalgae, haptophytes and diatoms. This complex transfers the absorbed energy to the Chl *a*-reaction centre, which participates directly in photosynthesis [6]. Fx is the most important carotenoid produced by diatoms, and has been reported to be a strong antioxidant metabolite [6]. In the present study, the Fx volumetric concentration ($mg.L^{-1}$) of *H. coffeaeformis* was quantified by HPLC. Fx peaks from algal extracts were assigned by comparing their retention time with that of pure standard. The HPLC chromatogram of Fx

standard is shown in Fig. 5a, with a peak retention time of 4.566 min; Fig. 5b shows an Fx peak from an ethanolic extract with a retention time of 4.566 min. The Chl *a* volumetric concentration ($mg.L^{-1}$) in the same *H. coffeaeformis* crude extracts was quantified by UV–Vis spectroscopy. The volumetric concentration ($mg.L^{-1}$) and content ($mg.g^{-1}$ DW) kinetics of Fx and Chl *a* in *H. coffeaeformis* culture calculated from available HPLC and UV–Vis data are shown in Fig. 5c. Chl *a* and Fx production increased throughout the experience up to 15.4 $mg.L^{-1}$ and 15.6 $mg.L^{-1}$ on day 22, respectively; while the highest concentrations of Chl *a* (~26–38 $mg.g^{-1}$ DW) and Fx (~30–38 $mg.g^{-1}$ DW) were found from day 13 to day 22, respectively. Chl *a* and Fx production and content showed a significant increase between day 0 and day 22 ($p < 0.05$). The simultaneous increase of Chl *a* and Fx throughout the culture may be related to their similar physiological response to light and nutrient conditions. Fx content of *H. coffeaeformis* was higher than that reported for the related genus *Amphora* sp. (1.21 ± 0.18 $mg.g^{-1}$ of DW) [16], matched favorably with the Fx content of *Odontella aurita*, a high-yielding diatom (> 20 $mg.g^{-1}$ of DW) [6], and was at least thirty times higher than in brown macroalgae, the main commercial source of this pigment (e.g. 0.87 $mg.g^{-1}$ DW) [26]. Fucoxanthin content varies according to species and strains of the same species, and is affected by culture conditions such as biomass productivity [16], light intensity [42], nitrogen concentration and/or source and salinity [16]. For example, the supply of both nitrate and light promoted Fx accumulation in *Odontella aurita* [6] and *Cyclotella cryptica* [42]. High biomass productivity was also necessary to ensure high Fx production in diatoms [16]. In the present study, Fx production reached its maximum value in synchrony with maximum biomass production. It is important to note that neither silica nor nitrogen was found in growth-limiting concentrations during this period. The cells had an important intracellular nitrate reserve and Chl *a* showed no sign of a significant decline, suggesting that the culture remained active. On the other hand, the increase in salinity did not affect growth and may have favored Fx accumulation as has been proven in *Amphora* sp. and *Phaeodactylum tricornutum* [43,44].

Finally, the biochemical composition of *H. coffeaeformis* biomass harvested on day 22 presented the following content (% DW): 15% proteins, 31% total lipids rich in $\omega - 3$ PUFA, 26% carbohydrates (11.96% chrysolaminarin), 3.86% fucoxanthin and 3.81% chlorophyll *a*. The remaining content would include cell walls or frustules, which represent a significant fraction of *H. coffeaeformis* biomass (~20–25% DW), and present potential industrial applications [12]. Simultaneous accumulation of high-value metabolites was obtained under moderate N and Si limitation and P starvation, although continuous nutrient starvation could uncouple this relationship. This nutritional scenario must therefore be taken into account in programing the synchronized production of these valuable-biomolecules from *H. coffeaeformis*.

4. Conclusions

Value-added biocompound production is an attractive strategy that can efficiently contribute to the economic sustainability of the microalgal market. However, the scale of microalgal biomass production and harvesting costs are the major factors affecting economic investment in facility scale-up. In the present study, the optimal nutrient balance of the culture medium in combination with nutritional strategies (i.e. intracellular NO_3^- store; selective N-uptake) and salinity tolerance of *H. coffeaeformis*, represents a useful approach to guarantee both sustained biomass production and simultaneous accumulation of EPA, chrysolaminarin and fucoxanthin. The levels of these metabolites were relatively higher than those found in commercially used feedstocks. In addition, a cost-effective and environmental friendly bioprocess at prepilot scale has been established. In particular: a) the high *H. coffeaeformis* growth rate in a raceway pond indicates the optimum operational conditions and robustness of the species; b) the use of fertilizer (Bayfolan®)-enriched seawater represents a cost-effective culture medium; and c) the auto-flocculation capacity of this strain contributes to economic harvesting. The present findings open the way for further progress toward the profitable and safe exploitation of *H.coffeaeformis* for biotechnological purposes (e.g. novel aquafeed additives, and/or biorefinery focusing on high-value compounds). Moreover, the global scarcity of fresh water and conventional high-value feedstocks justifies the search for alternative sources, in particular those of marine origin.

CRediT authorship contribution statement

Cecilia A. Popovich: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Funding acquisition. **María B. Faraoni:** Formal analysis. **Alejandra Sequeira:** Formal analysis. **Yasmín Daglio:** Formal analysis. **Lucas A. Martín:** Conceptualization, Formal analysis. **Ana M. Martínez:** Formal analysis. **María C. Damiani:** Formal analysis. **María C. Matulewicz:** Formal analysis, Funding acquisition. **Patricia I. Leonardi:** Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration of competing interest

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.

Acknowledgments

This research was supported by grants from Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC) (Resolución N° 801/18) to CAP, Agencia Nacional de Promoción Científica y Tecnológica (PICT 2015-0800), Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET, PIP 112-2015 01-00510), Universidad Nacional del Sur (PGI 24/B246) to PIL and Universidad de Buenos Aires (UBACYT 2018-2021 GC20020170100292BA) to MCM. The technical assistance by Jorge Oyola is acknowledged. CAP and MBF are Research Members of CIC. LAM, MCM and PIL are Research Members of CONICET.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

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