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Impact of temperature and growth phases on lipid composition and fatty acid profile of a thermophilic Bacillariophyta strain related to the genus *Halamphora* from north-eastern Tunisia

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Original Article

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Abstract

A thermo-tolerant diatom species has been isolated from Tunisian hot spring water (40°C). The isolated diatom has been molecularly identified and classified into the genus *Halamphora*. The growth kinetics, lipid content and distribution of fatty acids were assessed at 20 and 30°C temperature levels and constant irradiance in controlled batch cultures (11 days). *Halamphora* sp. showed better growth ($\mu = 0.53\ day^{-1}$) and a higher lipid yield (25% of the dry weight) at a higher temperature (30°C). Under the two temperatures tested, the highest lipid and fatty acid contents were mainly reached during the stationary growth phase. The fatty acid profile showed a significant content of two essential fatty acids, eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6), reaching ~15% and ~21% of the total fatty acids, respectively, at 20°C and 30°C. The distribution of the different components of the fatty acids showed that EPA and AA were mainly located in the neutral lipid fraction in the stationary phase.

Introduction

Over the last decade, there has been increasing demand for renewable and sustainable oil sources as biofuel or feedstock as well as discovering novel products for future biotechnological application which have opened the door for biotechnology research in the microalgal area. Many challenges are often presented especially in choosing the right alga with relevant properties for specific culture conditions and products (Jiang *et al.*, 2014). Discovering species that could combine fast growth and high lipid yields are an attractive target for biotechnology in the microalgal field.

Of great interest for bioprospecting are extreme environments, such as thermophilic hot springs. These kinds of harsh and adverse habitats are known to support a range of microorganisms including microalgae that may have already developed mechanisms for adaptation, including the ability to accumulate diverse chemicals as products that could promote their survival (Lim *et al.*, 2012). According to many authors (e.g. Richmond, 1986; Seckbach, 2007), organisms that can survive in thermal hot springs often have high optimal growth temperatures and usually can produce more lipids.

Oleaginous microalgae are commonly referred to as ones that can accumulate lipids at over 20% of their dry weight. Most of these belong to green algae (Chlorophyceae) and diatoms (Bacillariophyceae) (Pulz & Gross, 2004). Diatoms are considered to be highly diverse. It has been estimated that there are over 100,000 extant species of diatoms colonizing various environments (Stepanek *et al.*, 2016). Research on these aquatic organisms in Tunisia is scarce; we note in particular the study by Ghozzi *et al.* (2013), which consists of an inventory of thermophilic microalgae in geothermal waters. Other investigations tackled the potential use of diatom wild strains for biodiesel production (Chtourou *et al.*, 2015*a*, 2015*b*) and for phycoremediation (Dahmen-Ben Moussa *et al.*, 2018*a*). These same authors studied the effect of salinity on the increase of lipids, secondary metabolites and enzymatic activity in *Amphora subtropica* and *Dunaliella* sp. for biodiesel production (Dahmen-Ben Moussa *et al.*, 2018*b*).

While it is well documented that diatoms commonly acclimate better to low temperatures, as they are frequently described as cold-water flora (Anderson, 2000), some benthic ones, such as some species belonging to the genera *Pinnularia*, *Nitzschia* and *Amphora*, have shown be able to withstand extreme environments (Mannino, 2007; Covarrubias *et al.*, 2016). Diatoms are highly regarded for their versatile potential (Lebeau & Robert, 2003) in producing valuable and sustainable lipids with particularly valuable polyunsaturated fatty acids (PUFA), such as arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The importance of these fatty acids (FA) lies in their high

Table 1. Physicochemical composition (mg l^{-1}) of the hot water spring

HCO_3^-	SO_4^{2-}	Cl^-	NO_3^-	F	Na^+	Ca^{2+}	Mg^{2+}	K^+	PO_4^-
573.40	1625	5502	1.50	2.90	3250	840	299	100	324

potential as a raw material for pharmaceutical, cosmetic, chemical and nutraceutical (food) industries (Lebeau & Robert, 2003; Pulz & Gross, 2004), which could be suitable for large-scale biotechnology production (Fernández *et al.*, 2000).

This work was undertaken as part of an investigation of natural extreme hot springs in north-eastern Tunisia that are seldom explored, in order to isolate a new native species with potential for lipid production. The present work reports a first investigation of thermophilic diatom species isolated from mat communities from geothermal springs in the north of Tunisia, at water temperature of 40°C. The goal of this work is to improve the growth rate as much as possible and to push the metabolism to maximize lipid synthesis across a range of temperatures. In this study, we first identified the benthic diatom species *Halamphora* sp. Second, we evaluated the kinetics of growth rate and lipid content over a controlled laboratory variation of temperature (20°C and 30°C). Third, we evaluated the effect of temperature and growth phase on fatty acid profile and lipid class distribution, with a special emphasis on PUFA distribution within cells.

Materials and methods

Strain isolation

The native diatom strain was isolated from a thermal brackish spring in the north-eastern Tunisia (36°49′N 10°34′E), called 'Ain Fakroun'. Samples were taken from microbial mats naturally anchored to submerged stones at 40°C water temperature. Natural collected mats were first treated by filtration, centrifugation and dilution techniques according to standard microbiological protocols (Guillard, 2005). The strain was then purified from the mixed algal samples, proceeding by streaking water samples onto a series of agar plates prepared with spring water, previously filtered (0.45 µm) and autoclaved. This procedure was performed three times to ensure unialgal strain isolation. The purified strain was kept in an appropriate medium (see below), maintained at a temperature of 20°C and a luminosity of 75 µmol m^{-2} s^{-1}, and routinely checked by microscopic observation.

Molecular and genetic identification

DNA isolation: Prior to extraction of genomic DNA, diatom cells were harvested during the exponential growth phase, washed with 1‰ chloride solution, quick-frozen in liquid nitrogen and bead beating for 1–2 min with tungsten carbide beads from Qiagen. The extraction of DNA was carried out using the DNA easy ®Plant Mini Kit (50) Qiagen procedure according to the manufacturer's protocol. The final extract was checked with Nanodrop spectrophotometer (ThermoFisher Scientific) to ensure the purity of the final product. A ratio of 260/280 ≥1.8 was deemed to be of high quality and purity (Eland *et al.*, 2012).

Two primers were used to amplify the DNA template, Forward 18S-rRNA-1F (AACCTGGTTGATCCTGCCAGT) and Reverse 18S-rRNA-1528R (TGATCCTTCTGCAGGTTCACCTAC) (Bruder & Medlin, 2007). The PCR reaction was run on a thermocycler (Perkin Elmer thermal cycler model 480), using GoTaq® Flexi DNA Polymerase. A 25 µl final volume reaction mixture was composed respectively of 30 ng of DNA extract, 5 µl of each primer combined with 11 µl of PCR reagents: 5 µl of GoTaq Flexi buffer (Promega), 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTP, 0.25 µl GoTaq DNA polymerase. Negative control PCR was also performed using the same primers without DNA template. The PCR reaction programme was implemented as: initial denaturation step 2 min at 95°C, followed by 35 cycles at 95°C for 30 s, annealing step 50°C for 30 s ending with a final extension at 72°C for 2 min.

The final PCR product was separated using electrophoresis on 1% agarose gel and visualized with Red Safe. The final products with multiple bands were purified and excised from agarose gel to undergo a final cleaning step using QIA quick PCR Purification kit (50) (QIAGEN, Germany) according to the manufacturer's protocol. The Genomic DNA of the studied diatom was sequenced using a Perkin Elmer ABI_PRISM™ 3100 Genetic Analyzer.

A final sequence of 1700 pb was obtained. The DNA sequence data were compared with GenBank entries for classification, using BLAST online. Multiple alignments were achieved with ClustalX v.2.1. Phylogenetic trees were constructed with MEGA 5.05 software based on evolutionary distances that were calculated with a Neighbour-joining method with Maximum composite likelihood model (Tamura *et al.*, 2011).

Culture media

An artificial brackish water medium was developed for culturing the isolated benthic diatom. The medium was developed by dilution of the natural spring water. The final culture medium was prepared based on the initial chemical composition of the water spring (Table 1) and was equilibrated to get a final ratio proposed for freshwater microalgae of C:Si:N:P at 106:15:16:1 (Elser *et al.*, 2000); the media was amended by addition of NaCl, $NaHCO_3$, $NaNO_3$, $Na_2SiO_3{\cdot}9H_2O$, further addition of K_2HPO_4 was not required as it was already in excess (Table 1). The final concentrations of the different components were: $NaHCO_3$ at 9.5×10^{-3} mol l^{-1}, $NaNO_3$ at 1.4×10^{-3} mol l^{-1}, $Na_2S_iO_3{\cdot}9H_2O$ at 1.1×10^{-3} mol l^{-1}, the salinity (NaCl), maintained as in the original water source, at 165×10^{-3} mol l^{-1} (i.e. 9 g l^{-1}), 1 ml l^{-1} trace elements ($ZnCl_2$, $CoCl_2{\cdot}6H_2O$, $6(NH_4)$ $Mo_7O_{24}{\cdot}4H_2O$, $CuSO_4{\cdot}5H_2O$) and 1 ml l^{-1} vitamins (B_1/B_{12}) were added according to recipe for Conway medium (Walne, 1974). All nutrients were prepared as stock solutions and were added after sterilization. Initial pH was maintained at 6.6 for all experimental cultures.

Culture and growth conditions

At each tested condition, experimental batch cultures were acclimated for at least 2 generations by routine dilution every 5 days through using 20–30% of the total volume as inoculum. Cultivation in batch mode was done in triplicate and carried out in laboratory constructed photo-bioreactors consisting of 2-l sterilized flasks, containing 1.8 l medium and equipped with a device for aseptic removal of samples. Cultures were conducted at two temperatures, 20 and 30°C, under constant illumination of 75 µmol m^{-2} s^{-1}, provided by fluorescent tubes, and sparged continuously with air containing 1% CO_2 at a constant flow rate. The starting inocula were ~300 mg l^{-1} by dry weight (dw). Contamination controls were ensured by routine light microscopic observation.

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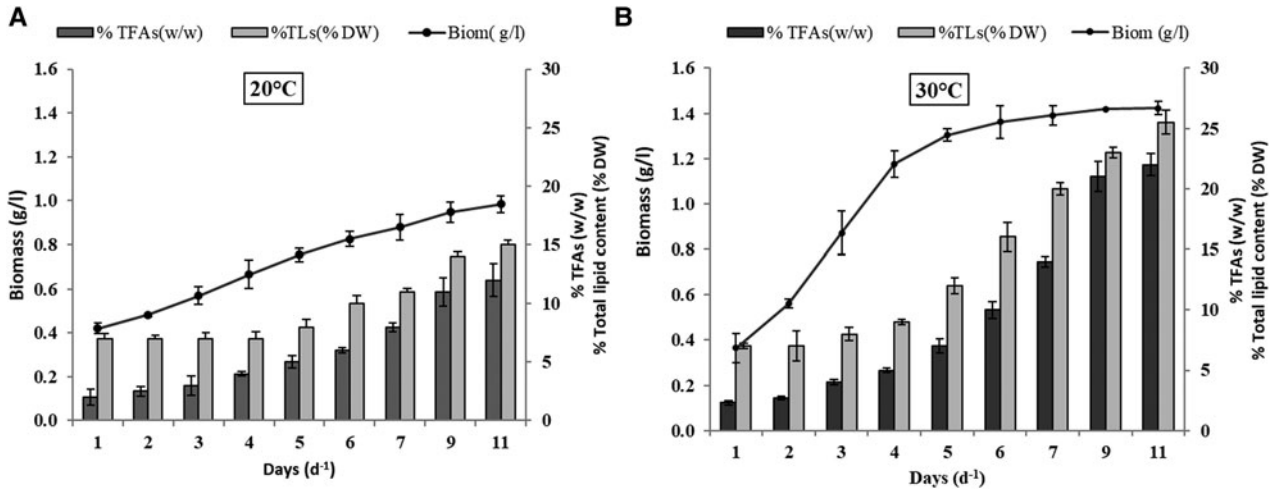

Fig. 1. Time course of biomass concentration (g l^{-1}), total lipids (TL, % dw), and total fatty acids (TFA, % w/w) in *Halamphora* sp. cultured at (a) 20°C and (b) 30°C.

Harvesting and dry weight

At the desired sampling culture times, the microalgae were harvested by centrifugation at 3500 rpm for 7 min. The resulting pellets were washed twice with isotonic ammonium formate using standard methods (Zhu & Lee, 1997; Moheimani *et al.*, 2013) to avoid cell disruption, and then freeze-dried. Samples were stored at −20°C until analysis. Dry weight was determined for each treatment gravimetrically. Samples (20 ml) (taken in triplicate) were removed daily from each culture. The fresh biomass was filtered onto a pre-weighed glass–fibre filter Whatman GF/C 47 mm, using a vacuum pump, then washed twice with isotonic ammonium formate, dried for 24 h at 105°C, and weighed to constant weight.

The instantaneous growth rate (GR) was calculated using a fitting program applied to the growth curve (calculated from the daily growth data) using the following formula: $GR = (dx/dt)$, where (x) is the biomass concentration and (t) is the time; unit of GR is g l^{-1} day^{-1}. The maximum specific growth rate (μ_{max}) was determined from the linear slope of the growth curve: $\mu = (\ln x_2 - \ln x_1)/(t_2 - t_1)$, where x_2 is the cell concentration at experimental time t_2 and x_1 the cell concentration at time t_1.

Fatty acid analysis

The FA composition of the different materials (microalgal biomass, lipid extract and lipid fractions) was analysed by gas chromatography (GC) of the methyl esters using heptadecanoic acid as the internal standard. The acyl lipid content was quantified by direct transesterification with acetyl chloride/methanol (1:20 v/v) following a standard method (Rodríguez-Ruiz *et al.*, 1998) to transform all FA into FA methyl esters (FAME) and then analysed by GC using an Agilent Technologies 6890 gas chromatograph (Avondale, PA, USA) provided with a capillary column of fused silica, Omegawax (0.25 mm × 30 m, 0.25-µm standard film, Supelco, USA) and a flame ionization detector (FID). Nitrogen was the carrier gas at a flow rate of 58.1 ml min^{-1} and a split ratio of 1:40. The injector and detector temperatures were set at 250 and 260°C, respectively. The oven temperature was initially set at 150°C for 3 min and then programmed to increase to 240°C at a rate of 7.5°C min^{-1} and set at 240°C for 12 min. Total lipids (TL) were extracted from 100 mg of lyophilized microalgal biomass.

The lipid extract was dried under a nitrogen stream and then re-solubilized in 2 ml of $CHCl_3$. The lipid extract was fractionated by column chromatography (CC) on a silica gel cartridge (Sep-Pak Classic, Waters) (Alonso *et al.*, 1998). Briefly, after cartridge equilibration with $CHCl_3$, the lipid extract was adsorbed into the silica gel cartridge and the lipid fractions were sequentially eluted with 30 ml of $CHCl_3$ (neutral lipids, NL), 30 ml of acetone and 20 ml of $CHCl_3$-MetOH (15%) (galactolipids or glycolipids) and then 30 ml of MetOH (phospholipids). Lipid fractions were dried in a G3 Heidolph rotary evaporator and re-solubilized in 2 mL $CHCl_3$. After the first GC analyses, we found that phospholipids were in minute quantities so then we combined glyco- and phospho-lipid fractions analysing both as a single fraction: polar lipids (PL).

Statistical analysis

Data were analysed with one-way analysis of variance (one-way ANOVA). Data showing significant differences ($P < 0.05$) were analysed by paired comparisons using Tukey's HSD test.

Equality of variance and normality of the data were assessed with Bartlett's test, and skewness and kurtosis, respectively.

Results

Strain identification

The final encoding sequence 18s rRNAs with 1700 bp length was submitted to GenBank with accession number kP057445. The sequence was most allied with the marine diatom species *Amphora caribaea* ($P = 0.005$) and *Amphora subtropica* ($P = 0.005$), and with hypersaline *Bacillariophyta* sp. 1 MAB 2013 ($P = 0.007$) which were recently transferred to the genus *Halamphora* according to the revision of Stepanek & Kociolek (2014). The isolated species in this study has thermophilic and brackish water origin (salt < 10) and could subsequently be considered as a species related to the genus *Halamphora* called *Halamphora* sp. (Supplementary Figure S1).

Growth kinetics, lipids and total fatty acid contents

Halamphora sp. cultured in batch system reached the maximum accumulation of dry biomass by the end of the experimental proposed culture period (11 days), where at 20°C the culture was entering into early stationary phase and at 30°C was well within stationary phase (Figure 1). Biomass concentrations were 1.46 ± 0.06 g l^{-1} and 1.0 ± 0.04 g l^{-1} at 30 and 20°C, respectively. The maximum growth rate was reached around the third day of culture for both tested temperature conditions. It was assessed

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Table 2. Fatty acid composition (mg g^{-1} dw) of biomass from *Halamphora* sp. cultured at two temperatures (20 and 30°C) and harvested at exponential and stationary phases

	20°C				30°C			
	Exponential		Stationary		Exponential		Stationary	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	6.53^{C}	0.000	7.93^{C}	0.432	10.78^{A}	0.234	18.04^{B}	1.548
UMCFA	7.81^{A}	5.016	6.63^{A}	5.763	8.05^{A}	4.258	5.28^{A}	7.468
16:0	6.76^{B}	0.025	11.33^{B}	0.378	17.59^{B}	0.697	48.03^{A}	12.322
16:1n7	19.14^{A}	7.272	19.12^{A}	1.030	22.15^{A}	2.619	28.69^{A}	6.170
20:4n6	5.22^{D}	0.546	15.36^{B}	4.064	11.04^{C}	0.073	23.49^{A}	2.164
20:5n3	3.43^{B}	2.409	10.83^{A}	4.592	11.62^{A}	0.601	14.60^{A}	2.921
C18[a]	4.45^{C}	1.160	5.25^{C}	3.733	13.82^{B}	1.722	23.19^{A}	12.705
C16:x[b]	28.25^{A}	11.009	25.88^{A}	6.122	23.50^{A}	10.461	21.00^{A}	14.491
TFA	81.58^{C}	5.418	102.33^{B}	11.325	118.55^{B}	15.359	182.32^{A}	9.772

Values are means and standard deviations (SD) of two cultures (N = 2). Different capital letters (A–D) indicate significant differences between groups at a confidence interval of 95%. UMCFA, unknown medium chain fatty acid. [a]Sum of all fatty acids with 18 carbons (18:0, 18:1n-9, 18:1n-7, 18:2n-6 and 18:3n-3); [b]Sum of remaining fatty acids with 16 carbons (16:1n-9, 16:2, 16:3 and 16:4).

to be 0.32 g l^{-1} day^{-1} at 30°C, which was near threefold higher than that attained at 20°C, 0.12 g l^{-1} day^{-1}. The specific growth rate (μ) was assessed to be 0.53 day^{-1} at 30°C and only 0.16 day^{-1} at 20°C, which provides doubling times of 1.2 days and 6.2 days, respectively.

Regardless of temperature, the total lipid content (TL), as percentage of dry weight (dw) biomass did not exceed 10–12% during the whole exponential growth phase (Figure 1). TL were significantly enhanced at both temperatures, when shifting to stationary phase, reaching around ~15% dw (150 mg g^{-1}) at 20°C ($P = 0.018$) (Figure 1A) and ~25% dw (255 mg g^{-1}) at 30°C ($P = 0.04$) (Figure 1B). Similarly, TFA (as % of w/w) increased significantly at the end of the culture time course (11th day) from ~12% (120 mg g^{-1}) at 20°C to ~22% (220 mg g^{-1}) at 30°C ($P = 0.001$), nearly a two-fold increase at the higher temperature (compare Figure 1A *vs* B), which led to a TFA value close to that of TL. As can be seen the process of lipid accumulation (as TL or TFA) is also favoured by elevated temperature. It was observed that young cells, from exponential phase, showed great differences between TL (80 mg g^{-1}) and TFA (40 mg g^{-1}) contents where TL was higher than TFA, while in old cells, from stationary phase, most of the TL (255 mg g^{-1}) content was explained by TFA (220 mg g^{-1}) content (Figure 1).

Fatty acid profile

FA composition of biomass was determined from cells harvested at exponential (3rd day) and stationary (11th day) phases from cultures at 20 and 30°C. Under all tested culture conditions, the overall FA profile of the *Halamphora* sp. biomass seemed consistent and was composed of around 17 different FA although only five of them, 14:0, 16:0, 16:1n-7, AA and EPA were always present in reliable amounts, contributing usually over 80% of TFA (Tables 2 & 3).

It was noticeable that two very long-chain PUFA such as EPA and AA were both present in substantial quantities (Table 2), generally well over 10% of TFA each (Table 3). Regarding dry weight data (Table 2), it was observed that the biomass of *Halamphora* sp. cultured at 30°C was a rich source of these PUFA since they accumulated, at stationary phase, 23 and 15 mg g^{-1} dw of AA and EPA, respectively (Table 2).

Fatty acid changes with culture conditions

A first common feature for all culture conditions was a trend to increase FA content (estimated as mg g^{-1} dw) with both temperature and age. With temperature the only exception was of the C16:x which slightly decreased from 28 mg g^{-1} at 20°C, to 24 mg g^{-1} at 30°C (Table 2). All remaining FAs showed a content increase at the higher temperature (Table 2) giving a maximum at stationary phase of 182 mg g^{-1} of TFA at 30°C *vs* 102 mg g^{-1} of TFA at 20°C. Something similar happened with culture age, where TFA increased from exponential to stationary phase when comparing cultures at the same temperature (Table 2). For example, at 20°C, TFA was 82 mg g^{-1} and 102 mg g^{-1}, at exponential and stationary phase, respectively.

This general increase was not equal for all FA. Substantial differences were observed in the proportions of some FA (Table 3). For instance, at 20°C and exponential phase 16:1n-7 was around 23% of TFA, down to 19% at 30°C (Table 3). Conversely, AA and EPA increased from 6 and 4% of TFA to 12 and 8% of TFA, respectively, when *Halamphora* sp. was cultured at 30°C (Table 3). Something similar happened with 16:0 that shifted from 8% at 20°C up to 26% at 30°C (Table 3). This suggests that 16:0 and 16:1n-7 played similar roles replacing one another depending on the temperature of culture.

We analysed two lipid fractions, NL and PL. They both followed the above-described trend of increasing with temperature, regardless of growth phase (Table 4). As a consequence, the quantity of PL and NL was always higher at 30°C than at 20°C (Table 4) indeed, at exponential phase, PL were 51 mg g^{-1} dw at 20°C and 83 mg g^{-1} dw at 30°C, while NL were 31 mg g^{-1} dw at 20°C and 35 mg g^{-1} dw at 30°C. Nevertheless, the relative proportions of NL and PL were equal, or roughly so, regardless of the temperature (Figure 2; Table 5). Conversely to the lack of changes in the proportions of lipid fractions with temperature, they showed a dramatic shift between culture phases: at exponential phase predominately PL (62–70%) *vs* NL (30–38%), and at stationary phase proportions were reversed with PL (33%) *vs* NL (67–75%) and in some cases over 90% (Figure 2; Table 5).

Discussion

According to the present work, *Halamphora* sp. showed the best specific growth rate ($\mu = 0.53$ day^{-1}) at a relatively elevated

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Table 3. Fatty acid composition (% of TFA) of biomass from *Halamphora* sp. cultured at two temperatures (20 and 30°C) and harvested at exponential and stationary phases

	20°C				30°C			
	Exponential		Stationary		Exponential		Stationary	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	8.0^{A}	0.53	7.8^{A}	0.79	9.2^{A}	1.39	9.9^{A}	1.38
UMCFA	9.4^{A}	5.52	6.1^{B}	5.76	6.6^{B}	2.74	3.0^{B}	4.26
16:0	8.3^{A}	0.52	11.2^{A}	1.35	15.0^{B}	2.53	26.1^{C}	10.48
16:1n7	23.2^{A}	7.37	18.8^{B}	1.26	18.7^{B}	0.21	15.8^{C}	4.23
20:4n6	6.4^{A}	0.24	15.2^{B}	4.57	9.4^{C}	1.16	12.9^{D}	0.50
20:5n3	4.1^{A}	2.68	10.4^{B}	2.51	9.8^{C}	0.77	8.0^{B}	1.18
C18[a]	5.4^{A}	1.06	5.4^{A}	3.83	11.8^{B}	2.99	12.6^{B}	6.30
C16:x[b]	35.1^{A}	15.83	24.1^{A}	4.51	19.4^{A}	6.31	11.8^{A}	8.58

Values are means and standard deviations (SD) of two cultures (N = 2). Different capital letters (A–D) indicate significant differences between groups at a confidence interval of 95%. UMCFA, unknown medium chain fatty acid. [a]Sum of all fatty acids with 18 carbons (18:0, 18:1n-9, 18:1n-7, 18:2n-6 and 18:3n-3); [b]Sum of remaining fatty acids with 16 carbons (16:1n-9, 16:2, 16:3 and 16:4).

temperature of 30°C. This growth rate decreased markedly at lower temperatures (20°C). According to Stepanek *et al.* (2016), common *Halamphora* species grew better at less than 25°C, which confirms the thermophilic nature of the present species, as could be expected from its ecological origin and supports the hypothesis that the species in question could be a new species. A morphological and molecular study is underway to confirm or invalidate this hypothesis. It is worthy to note that in all our experimental controlled laboratory cultures, growth rate decayed strongly when temperature exceeded 39°C (results not shown), which is substantially lower than that measured in the natural water spring from where it was isolated (40°C). Similar trends have been reported for other thermophilic microalgae investigated in laboratory cultures such as *Graesiella* sp. (Mezhoud *et al.*, 2014) and diatom species of the genus *Nitzschia*, *Pinnularia*, *Amphora* and *Stephanocyclus* (Covarrubias *et al.*, 2016) and might be attributed to the complex interrelation between different species in the mat community that could provide stratification with cooler microhabitat temperature niches, which facilitate the survival of moderately thermophilic species (Covarrubias *et al.*, 2016).

We have shown that the process of lipid accumulation in *Halamphora* sp. is favoured by temperature increase. In previous studies on diatom species (Rousch *et al.*, 2003; Liang *et al.*, 2005) and on several Chlorophyta species (Oliveira *et al.*, 1999) the increase of culture temperature was accompanied by an increase of TFA. However, other reports suggested the opposite trend (Thompson *et al.*, 1992; Renaud *et al.*, 1995), which makes it difficult to make generalizations about this process that could be considered as species-specific (Wah *et al.*, 2015). Furthermore, by comparing data from freshwater and marine species other studies concluded that temperature had a weak relationship to lipid amount in the algal communities and served only as an indirect indicator of the probable existence of unfavourable nutrient conditions (Wainman & Smith, 2003). Under both experimental temperatures in this work, the accumulation of lipids and TFAs was observed preferentially during the stationary growth phase. This trend has been commonly reported for many microalgae (Alonso *et al.*, 1998, 2000; Martín *et al.*, 2016) and was explained by the shifting of the carbon partitioning preferentially into the lipid pathways with depletion of nutrients in the culture media (Moll *et al.*, 2014). Therefore, the elevated lipid and FA content observed at 30°C compared with 20°C could be attributed to the higher growth rate that leads to substantial nutrient uptake and consequently to an accelerated depletion of some nutrients that precipitated the onset of stationary growth.

As we have noted, early cultures showed great differences between TL and TFA (TL >>> TFA) while old cultures showed quite similar values (Figure 1). Lipids are chemically very heterogeneous, while being defined simply by their insolubility in polar solvents and solubility in organic solvents. Under the lipid label there are numerous compounds having very different chemical compositions; for example, hydrocarbons, some photosynthetic pigments, isoprenoids (i.e. rubber), fats, oils, etc., are all lipids. Saponifiable lipids (acyl-lipids, i.e. FA-derived compounds) are by far the most abundant kind, making up most of the cell membranes. Even some of the most abundant energy-storing molecules are acyl-lipids (e.g. triacylglycerols). So, the lipid extract (total lipids, TL) always are a complex mix of different lipids. As we demonstrated, early cultures contain a significant amount of non-saponifiable lipids which seem to progressively disappear with culture age in favour of acyl-lipids. As we have commented above, on the last day of the 30°C experimental culture, TL were ~35% of dw and TFAs were ~22%, that is, non-saponifiable lipids are only ~3%. This suggests that ageing determined the conversion of non-acyl-lipids into acyl-lipids as well as increasing these lipids (most of them triacylglycerols). Again, the process is faster at 30°C than at 20°C.

Halamphora sp. produced AA and EPA in substantial quantities. This is a major feature of this species because to date, within microalgae, this dual biosynthetic capability is unusual, suggesting again the hypothesis that this diatom is a new species. There are many microalgae producing omega-3 PUFA but omega-6 PUFA are infrequent and AA is rarely found with the exception of freshwater microalgae, *Parietochloris incisa*, which accumulated AA well over 40% of TFA (Bigogno *et al.*, 2002). It is commonly known that diatoms often produce PUFA mainly in the form of EPA, compared with AA which often seems scarce (Yongmanitchai & Ward, 1991). Indeed, according to previous works on diatoms, a limited number of species are capable of producing small amounts of AA (Napolitano *et al.*, 1990), which are of the order of 6% of TFA in *Asterionella japonica* and not exceeding 1% of TFA in *Skeletonema costatum* (Chuecas & Riley, 1969). The dual ability, to produce both EPA and AA, is very exceptional because to date only red algae (e.g. *Porphyridium cruentum*) have shown this ability (Alonso *et al.*,

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Table 4. Fatty acid composition (mg g^{-1} dw) of lipid fractions from *Halamphora* sp. cultured at two temperatures (20 and 30°C) and harvested at exponential and stationary phases

	20°C								30°C							
	Exponential				Stationary				Exponential				Stationary			
	NL		PL		NL		PL		NL		PL		NL		PL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	1.85^{D}	2.610	4.69^{BCD}	2.610	4.77^{BCD}	0.550	3.16^{CD}	0.576	1.60^{D}	2.266	9.17^{AB}	2.032	10.60^{A}	4.896	7.44^{ABC}	3.348
UMCFA	4.56^{A}	3.524	3.25^{A}	1.492	4.54^{A}	3.934	2.09^{A}	1.843	5.21^{A}	2.987	2.84^{A}	1.270	3.47^{A}	4.911	1.81^{A}	2.557
16:0	1.87^{C}	2.642	4.89^{C}	2.667	6.74^{C}	0.907	4.59^{C}	0.632	4.14^{C}	2.091	13.45^{B}	1.394	34.62^{A}	15.393	13.41^{B}	6.266
16:1n7	7.51^{B}	0.171	11.63^{A}	7.443	12.59^{A}	0.870	6.54^{B}	1.512	7.55^{B}	0.261	14.61^{A}	2.880	16.14^{A}	12.108	12.55^{A}	5.939
20:4n6	1.21^{D}	1.705	4.01^{BCD}	2.251	10.27^{AB}	3.823	5.09^{BCD}	1.327	1.79^{CD}	2.532	9.25^{ABC}	2.605	13.12^{A}	5.993	10.37^{AB}	8.157
20:5n3	0.86^{B}	1.218	2.56^{B}	3.627	7.34^{A}	1.638	3.49^{B}	3.035	1.69^{B}	2.395	9.92^{A}	2.996	9.78^{A}	3.898	4.82^{AB}	6.819
C18[a]	1.99^{C}	1.138	2.46^{C}	0.022	4.59^{C}	2.597	0.66^{C}	1.137	3.27^{C}	0.132	10.55^{B}	1.590	21.75^{A}	14.747	1.44^{C}	2.042
C16:x[b]	11.14^{B}	3.762	17.10^{A}	7.247	17.32^{A}	3.071	8.56^{B}	3.121	10.08^{B}	4.554	13.42^{A}	5.907	13.08^{A}	12.248	7.91^{B}	2.244
TFA	30.98^{D}	7.447	50.60^{CD}	12.865	68.15^{BC}	1.806	34.18^{D}	10.389	35.33^{D}	2.135	83.22^{B}	17.494	122.5^{A}	13.913	59.75^{BC}	23.685

Values are means and standard deviations (SD) of two cultures ($N = 2$). Different capital letters (A–D) indicate significant differences between groups at a confidence interval of 95%.
UMCFA, unknown medium chain fatty acid. [a]Sum of all fatty acids with 18 carbons (18:0, 18:1n-9, 18:1n-7, 18:2n-6 and 18:3n-3); [b]Sum of remaining fatty acids with 16 carbons (16:1n-9, 16:2, 16:3 and 16:4).

1998; Khozin-Goldberg *et al.*, 2011). The high AA (15–21%) content of *Halamphora* sp. under both temperature conditions suggests this species is a natural resource for AA and, eventually, EPA. Nevertheless, considering the high and unexplored biodiversity existing in the diatoms (estimated to be near 100,000 species) it can be speculated that other diatoms, as research advances, could show a similar or enhanced ability.

Within the general trend of FA increase, we observed some exceptions. 16:1n-7 decreased when temperature increased while conversely 16:0 increased at higher temperature. This suggests that 16:0 and 16:1n-7 played similar roles as components of membrane lipids, replacing one with the other depending on the temperature of culture in the usual process of membrane fluidity adaptation to temperature (increasing saturated FA with increasing temperature) (Chen, 2012).

In contrast, the quantities of lipid fractions (NL and PL) changed with temperature while their proportions remained similar (Figure 2), i.e. temperature did not affect the proportions of these lipid fractions. Conversely, there was a dramatic change in the proportions of both lipid fractions with the culture phase: PL >>> NL at the exponential phase and Pl <<< NL at stationary phase (Figure 2). This changing pattern is common between exponential and stationary cultures of microalgae: NL increased and PL decreased with culture ageing (Alonso *et al.*, 1998, 2000 and references therein). Beyond the fact that PL was at a higher proportion at exponential phase, it can be noted that at 30°C the proportion of PL is higher than at 20°C (70% *vs* 62%) (Table 5). This means that at exponential phase the cells cultured at 30°C contained more membranes (more chloroplasts), i.e. they were more active. This suggests again that *Halamphora* sp. is naturally a thermophilic species.

The distribution of FAs between both lipid fractions mostly followed the above pattern suggesting that FAs were being transferred from PL to NL as the culture aged. For instance, at 30°C at exponential phase, over 84% of AA and EPA was located in PL but at stationary phase, 56% of AA and 87% of EPA was then located in NL (Table 5). Although the values showed some variation among FA distribution (Table 5) it seemed the transferring process worked similarly for all FA because all were around roughly similar values (~67% on NL) (Table 5). FA location seemed to be dependent on growth phase and independent of temperature because the above trend was observed at both temperatures (Table 5). Sometimes it has been suggested that PUFA (i.e. AA and EPA) were mainly located in PL because it was supposed that its major role is as membrane-lipid components. However, there has been evidence of accumulation in NL (e.g. TAG) in a few microalgae (Alonso *et al.*, 1998, 2000; Bigogno *et al.*, 2002) as we are reporting in this work. As has been noted, this fact conflicts with the usual view of TAG as a simple static storage lipid, suggesting a more dynamic role of TAG as a source of PUFA to re-tailor membranes allowing a fast recovery to environmental challenges (Bigogno *et al.*, 2002). This kind of information may be useful for the choice of a procedure for extraction and purification of AA or EPA.

Conclusion

The studied diatom species, *Halamphora* sp., isolated from an extreme water temperature was shown to be more active at 30°C than at 20°C and with fast growth, showing high productivity and elevated TFA content.

This diatom is naturally able to produce substantial amounts of two very valuable FA, AA and EPA. This makes it worthwhile to investigate the biotechnological possibilities of its use as producer for several oil-based bio-products that offer broad scope for aquaculture, food and pharmaceutical application.

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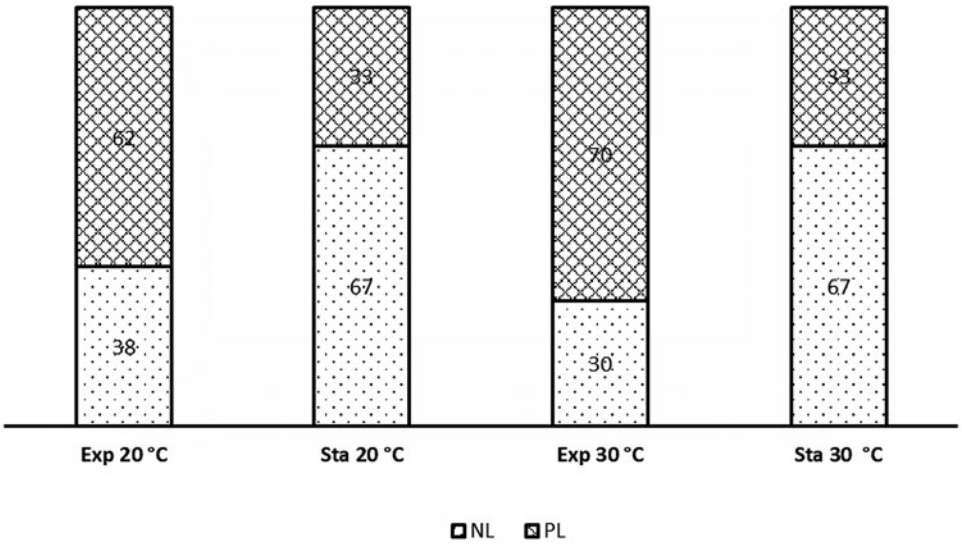

Fig. 2. Changes in neutral (NL) and polar lipid (PL) fractions, as percentages of total fatty acids, in *Halamphora* sp. cultured at two temperatures (20 and 30°C) and harvested at two growth phases (exponential and stationary).

Table 5. Distribution of FA (% of TFA of the lipid fraction) between the two lipid fractions (NL and PL) analysed from *Halamphora* sp. cultured at two temperatures (20 and 30°C) and harvested at exponential and stationary phases

	20°C				30°C			
	Exponential		Stationary		Exponential		Stationary	
	NL	PL	NL	PL	NL	PL	NL	PL
14:0	28^{BC}	72^{A}	60^{A}	40^{B}	15^{C}	85^{A}	59^{A}	41^{B}
UMCFA	58^{A}	42^{B}	68^{A}	32^{B}	65^{A}	35^{B}	66^{A}	34^{B}
16:0	28^{B}	72^{A}	59^{A}	41^{B}	24^{B}	76^{A}	72^{A}	28^{B}
16:1n7	39^{B}	61^{A}	66^{A}	34^{B}	34^{B}	66^{A}	56^{A}	44^{B}
20:4n6	23^{C}	77^{A}	67^{A}	33^{B}	16^{C}	84^{A}	56^{AB}	44^{B}
20:5n3	25^{B}	75^{A}	68^{A}	32^{C}	15^{C}	85^{A}	67^{A}	33^{C}
C18[a]	45^{C}	55^{B}	87^{A}	13^{D}	24^{D}	76^{B}	94^{A}	6^{D}
C16:x[b]	39^{B}	61^{A}	67^{A}	33^{B}	43^{B}	57^{A}	62^{A}	38^{B}
TFA	38^{B}	62^{A}	67^{A}	33^{B}	30^{B}	70^{A}	67^{A}	33^{B}

Different capital letters (A–D) indicate significant differences between groups at a confidence interval of 95%. UMCFA, unknown medium chain fatty acid; [a]Sum of all fatty acids with 18 carbons (18:0, 18:1n-9, 18:1n-7, 18:2n-6 and 18:3n-3); [b]Sum of remaining fatty acids with 16 carbons (16:1n-9, 16:2, 16:3 and 16:4).

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S002531542000048X

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