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Assessment of the lipid production potential of six benthic diatom species grown in airlift photobioreactors

Eva Cointet[1] · Elise Séverin[1] · Aurélie Couzinet-Mossion[1] · Vona Méléder[1] · Olivier Gonçalves[2] · Gaëtane Wielgosz-Collin[1]

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Abstract

In recent years diatoms have emerged as a major algal source for the production of bioactive compounds. Marine diatoms grow quickly and can store high amount of lipids. Unfortunately, they are little studied and underexploited resources. The current work deals with an original and rarely investigated source of diatoms: intertidal mudflats. It aims to evaluate the lipid production potential of some strains of benthic diatom species, isolated and hosted in the Nantes Culture Collection (NCC) when cultivated in an airlift photobioreactor. Six strains known for their high biomass and/or lipid productivity: *Amphora* sp. (NCC169), *Entomoneis paludosa* (NCC18.2), *Nitzschia alexandrina* (NCC33), *Nitzschia* sp. (NCC109), *Opephora* sp. (NCC366), and *Staurosira* sp. (NCC182) were cultivated in airlift photobioreactors for the first time. Their lipid class composition, fatty acid, and sterol distribution were studied. Total lipid production varied from 11.4 (*Amphora* sp.) to 41%DW (*Staurosira* sp.). Neutral lipid amounts varied from 23 (*Amphora* sp.) to 76% (*Staurosira* sp.) of total lipids (%TL). Glycolipids ranged from 18 (*Staurosira* sp.) to 59%TL (*Opephora* sp.) and phospholipids accounted for 6 (*Staurosira* sp.) to 26%TL (*Amphora* sp.). Some qualitative and quantitative differences were identified in both fatty acid and sterol composition in the different strains analyzed. *Staurosira* sp. seems to be the most promising species in terms of lipid production and most particularly in triacylglycerol production. *Entomoneis paludosa* produced phytosterols and eicosapentaenoic acid (EPA), compounds with potential for application in the pharmaceutical sector. *Nitzschia alexandrina* produced squalene and low levels of saturated fatty acids which could both be interesting in the nutraceutical industry as antioxidants.

Keywords Benthic diatoms · Nantes Culture Collection · Bioactive lipid diversity · Airlift photobioreactor · Fatty acids · Eicosapentaenoic acid

✉ Gaëtane Wielgosz-Collin
Gaetane.Wielgosz-Collin@univ-nantes.fr

Eva Cointet
evacointet@gmail.com

Elise Séverin
elise.severin7@gmail.com

Aurélie Couzinet-Mossion
Aurelie.couzinet-mossion@univ-nantes.fr

Vona Méléder
vona.meleder@univ-nantes.fr

Olivier Gonçalves
olivier.goncalves@univ-nantes.fr

[1] Université de Nantes, Laboratoire Mer Molécules Santé, EA 2160, BP 92208, F-44322 Nantes, France

[2] Université de Nantes, GEPEA, UMR CNRS-6144, Bât. CRTT, 37 Boulevard de l'Université, BP406, F-44602 Saint-Nazaire Cedex, France

Introduction

Marine diatoms are able to store high amounts of lipids and grow quickly (Niu et al. 2013). Their lipids are mainly composed of a neutral fraction with traces of sterols and polar lipids (Yi et al. 2017). Neutral lipids constitute the reserve fraction, with triacylglycerol (TAG) accounting for more than 60% of total lipids (Artamonova et al. 2017). The polyunsaturated fatty acids (PUFA) of marine diatoms are mainly composed of eicosapentaenoic acid (EPA, 20:5 n-3) (Chew et al. 2017), but some species also have been found to contain docosahexaenoic acid (DHA, 22:6 n-3) (Dunstan et al. 1993). The biosynthesis of lipids varies between different diatom species, their growth stages, and their environment (Chuecas and Riley 1969; Chen 2012). Previous studies (Dunstan et al. 1993; Niu et al. 2013; Artamonova et al. 2017; Chew et al. 2017; Yi et al. 2017) have demonstrated their ability for lipid production, more specifically for the

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PUFA fraction (DHA and EPA), recognized for its broad-spectrum bioactivities (anti-carcinogen, immune modulator, anti-diabetic, anti-obesity, anti-thrombotic and anti-atherogenic) (Nagao and Yanagita 2005).

The use of diatoms in biotechnology is currently under development. To date, only a few diatom species have been grown and used for industrial purposes because of the lack of cultivation systems available for their exploitation. The need for high production and the use of monoalgal crops has led to the establishment of experimental units for tubular and tubular, closed-circuit systems for seaweed cultivation known as photobioreactors. The escalating development of these systems was reinforced in the late 1980s following a general interest in the development of commercial-sized photobioreactors (Pulz 2001; Tredici 2004; Krichnavaruk et al. 2007). Operating characteristics of these systems include the ability to control important parameters such as temperature, CO_2 content, hydrodynamics, and contamination (Pulz 2001). Some planktonic diatom species are used in the aquaculture market, for example, *Chaetoceros calcitrans* (Krichnavaruk et al. 2005, 2007), *Skeletonema costatum* (Granum and Myklestad 2002), and *Phaeodactylum tricornutum* (Fernández et al. 2000; Molina et al. 2000) have been successfully cultured in these systems. However, regarding benthic diatoms, one of the main features of their growth is their capacity to form biofilms on the substrate. The life habits, typical to these diatoms, make them difficult to cultivate in traditionally used systems, oriented towards maintaining algae in suspension. Unlike most commercially produced microalgae (*Chlorella* spp., *Spirulina* spp., *Dunaliella* salina) that develop under very selective conditions, with little contamination by other microalgae or protozoa, most diatoms have no such selective advantage and need to be cultivated in closed systems to avoid high levels of contamination (Borowitzka 2013). Photobioreactors can be placed indoors or outdoors and are composed of bags, tubular reactors, or flat reactors. Because of the photoautotrophic status of the majority of diatoms, microalgal cultures suffer from limited light diffusion. The use of an airlift photobioreactor counteracts this problem with a circulatory flow in the system which helps prevent cell precipitation and enhances light utilization efficiency (Monkonsit et al. 2011). In tubular or flat photobioreactors, the scattering of light inside the reactor is more efficient, the medium is homogeneous, gas exchange is more significant and temperature is controlled, making it possible to obtain higher biomass, and thus reduce harvesting costs.

In a previous study (Cointet et al. 2019a), 76 strains of benthic diatom species, isolated from intertidal mudflats of the Pays de la Loire area (Atlantic coast, France) and hosted in the Nantes Culture Collection (NCC), were cultivated in Erlenmeyer flasks. Among them, six strains were selected due to their promising lipid production using HTSXT-FTIR analysis. These strains are *Amphora* sp. (NCC169) (order Thalassiophysales), *Entomoneis paludosa* (NCC18.2) (Surirellates), *Nitzschia alexandrina* (NCC33) and *Nitzschia* sp. (NCC109) (Bacillariales), *Opephora* sp. (NCC366), and *Staurosira* sp. (NCC182) (Fragilariales). The lipid composition of *Opephora* sp. has never yet been described. Large-scale cultivation of *Staurosira* sp. has already been carried out in a photobioreactor, but only the lipid rate was studied (Huntley et al. 2015). Regarding the other species, no data are available for fatty acid composition after growth in a photobioreactor.

The purpose of the present work is to study the lipid production potential of the six selected strains using controlled growth conditions in airlift photobioreactors (PBR). The culture conditions for light and nutrients were chosen based on our previous results (Cointet et al. 2019a, 2019b), namely, continuous light of 127 μmol photons.m^{-2}.s^{-1} and no nutrient limitation. Also, the lipid classes, fatty acids, and unsaponifiables will be described for the six strains to envisage large-scale production for different fields of application.

Materials and methods

Airlift photobioreactor description

The airlift photobioreactor (PBR) used was a flat-panel airlift PBR (Fig. 1). The light supplying device was placed in front of the PBR perpendicular to its optical surface. The light source was a LED panel (Effilux, Orsay, France) placed at the same height as and parallel to the front side of the PBR. Air was injected at the bottom for culture mixing. The PBR consisted of three parts: the central part where air was injected (riser) and two lateral parts for culture recirculation (downcomer). This ensured good mixing conditions and prevented cell sedimentation. PBR volume was 1 L with a depth of culture Lz = 30 mm (perpendicular to the optical surface). The illuminated surface to volume ratio of the reactor was equal to 33.3 m^{-1}. The PBR was built in transparent polymethyl methacrylate (PMMA) with the exception of the rear panel which was made of stainless steel for reactor cooling by ambient air blowing (fan).

The PBR was equipped with a complete loop of sensors and automation for microalgal culture, namely temperature, pH, and gas injections (CO_2 and air). The pH was regulated by automatic injections of CO_2 and the temperature by ambient air blowing. The PBR was sterilized 30 min prior to all experiments with a 5 mM peroxyacetic acid solution. Batch cultures were produced in chemostat mode under continuous light illumination. The incident light flux (qo) or photons flux density (PFD) was measured in the 400–700-nm wavelength range (photosynthetically active radiation, PAR) for different distances between the PBR and tubes using a flat cosine quantum sensor (Li-190SA, Li-COR, USA). The incident light flux was obtained by averaging sensor measurements for 12

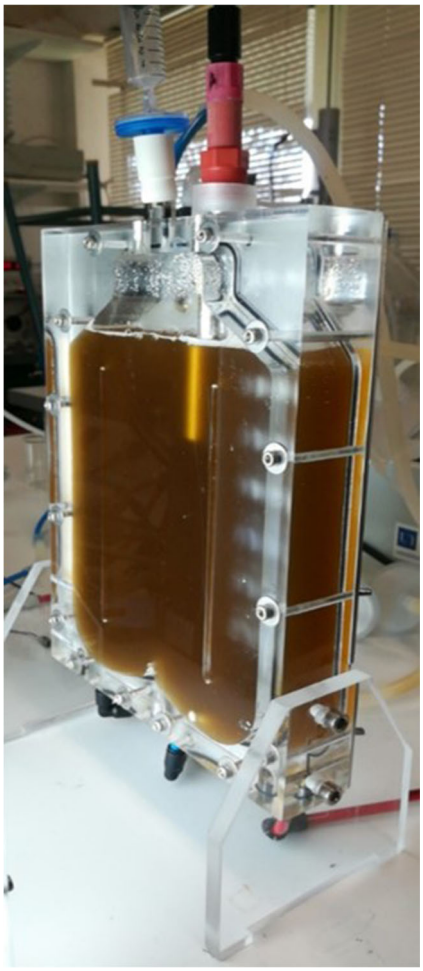

Fig. 1 PBR containing *Entomoneis paludosa* culture

different locations on the PBR surface. A variation of less than 10% was observed revealing homogeneous illumination of the PBR's optical surface.

Culture conditions

The six benthic strains, *Amphora* sp. NCC169, *Entomoneis paludosa* NCC18.2, *Nitzschia alexandrina* NCC33, *Nitzschia* sp. NCC109, *Opephora* sp. NCC366, and *Staurosira* sp. NCC182, obtained from the NCC, were grown in the airlift PBR described above.

Each strain was grown using an enriched natural seawater medium (F/2 medium) (Guillard 1975), filtered (0.2 μm) to avoid nutrient precipitation during the sterilization step. Fifty milliliters of culture stock were treated with 2 mL of an antibiotic and antimycotic solution (Sigma-Aldrich) for 48 h, followed by re-suspension in 150 mL of sterile culture solution to ensure the absence of bacteria and/or protozoa during culture growth. Stock cultures were maintained in 250 mL Erlenmeyer flasks filled with 150 mL of the medium at 20 °C, under continuous light of 127 μmol photons.m^{-2}.s^{-1}. Inoculation in the PBR was carried out when the optical density of the starter (250 mL reconcentrated before inoculation) at 680 nm was at least 0.05. During PBR growth, culture conditions were pH = 7.8 and T = 20 °C, continuous qo = 127 μmol photons.m^{-2}.s^{-1} without nutrient limitation. This was controlled daily by measuring the remaining nitrate and phosphate concentrations using ionic chromatography (Online resource - Table S1) and by addition of 21 mg.L^{-1} of silica (Na_2SiO_3, $5H_2O$) and 200 mg.L^{-1} of sodium bicarbonate ($NaHCO_3$).

Analytical methods

Growth

Every day, 2 mL culture samples were fixed with Lugol and counted ($n \geq 300$) using a Neubauer hemocytometer and an optical microscope (Olympus CH40, ×400). As in Cointet et al. (2019a), maximum growth rates (μ_{max} in day^{-1}) were determined by fitting growth kinetic data with a Gompertz model using Matlab software (Eq. 1):

$$f(x) = A \times e^{-e\left(\mu\max \times \frac{e^1}{A} \times (\lambda - x) + 1\right)} \quad (1)$$

with A, the maximum cell concentration in the natural logarithm of the biomass; μ_{max}, the maximum growth rate (day^{-1}); and λ, the latency (days).

Growth was also monitored by measuring the optical density (OD) daily at 680 nm using a spectrophotometer (JASCO V-630).

Culture in the PBR was stopped and cells were harvested when phosphate and nitrate concentrations dropped to 62 mg.L^{-1} and 224 mg.L^{-1}, respectively. This precaution avoided nutrient limitation and thus the shift from growth conditions into limited-growth conditions (Nghiem Xuan et al. 2020).

Total dry weight biomass

At the end of the culture, the total remaining volume in the PBR was harvested and filtered on previously weighted filters (Whatman GF/F, 47 mm diameter, 0.7 μM pore). Filters containing cells were washed using 10 mL ammonium formate (68 g.L^{-1}) to remove any salt (Lukavský 2000). Wet filters were frozen at –80 °C and freeze-dried under vacuum for 24 h in order to obtain the total dry biomass (dry weight—DW in g.L^{-1}).

Total lipid extraction

The freeze-dried biomass was divided into three parts. All of the following experiments were performed separately on each of the three parts in order to obtain technical replicates and to determine standard deviation. Total lipids were extracted using a modified method of Bligh and Dyer (1959). For each

group, two macerations were carried out in Erlenmeyer flasks using an orbital shaker (Edmund Bühler GmbH, SM-30) at room temperature, with 100 mL of solvent per gram of biomass (dichloromethane (CH_2Cl_2)/methanol (MeOH) 1:1 (v/v) for 24 h. Mixtures were then filtered to obtain the delipidified biomass. Organic phases were then washed by adding 40% of the volume of 0.9% KCl solution. Organic phases were combined, dried over anhydrous Na_2SO_4 and then evaporated to dryness under N_2 to obtain the crude lipid extract (CLE) and estimated total lipid percentage (TL) (Eq. 2)

$$TL = \frac{CLE}{DW} \times 100 \quad (2)$$

Lipid analyses

Lipid class fractionation

Lipid classes were separated by open silica gel column chromatography. The column size and amount of silica used (60 Å, 35–75 μm) were adjusted to obtain a final ratio of 20 g of silica for 1 g of CLE. Lipids were eluted using CH_2Cl_2 for neutral lipids (NL), acetone for glycolipids (GL), and MeOH for phospholipids (PL) as successive mobile phases.

Thin-layer chromatography analysis

Thin-layer chromatography (TLC) was performed using a silica plate on an aluminum support (20 cm × 20 cm, 0.2 mm, AlugramSil G/UV_{254}, Macherey-Nagel). Fractions were applied (10 μL with a concentration of 1 mg.mL^{-1}) to the TLC plate with standardized micropipettes for elution. Mobile phases, controls, and revealers were adapted according to sample structure and polarity (Table 1).

Fatty acid and sterol analyses

Fatty acids and unsaponifiable fractions (sterols, hydrocarbons…) were analyzed as described previously (Kendel et al. 2013). Briefly, CLE was saponified with 2 M ethanolic potassium hydroxide (1.5 h at 80 °C under reflux). The unsaponifiable fraction was acetylated using acetic anhydride and pyridine, giving a mixture containing sterol acetates. Fatty acid methyl esters (FAME) were obtained by methylation of the free fatty acids (FFA) (40 min at 80 °C, under reflux in 6% hydrochloric MeOH). FAME were then converted into *N*-Acyl pyrrolidines (NAP) (60 min at 80 °C under reflux in a pyrrolidine/acetic acid mixture 5:1 v/v). FAME, NAP, free, and acetylated sterols were then analyzed by gas chromatography coupled with mass spectrometry (GC-MS).

Gas chromatography coupled with mass spectrometry

Samples (1 mg.mL^{-1} in CH_2Cl_2) were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) (Hewlett Packard HP 7890-GC system/HP 5975C – 70 ev), equipped with a HP-5^{MS} column (30 m × 0.25 mm × 0.25 μm, Sigma-Aldrich). Injector and detector temperatures were set at 250 and 280 °C, respectively. Helium carrier gas had a flow rate of 1 mL.min^{-1}. For FAME analyses, the oven temperature was programmed at 170°C for 4 min, then increased by 3 °C min^{-1} up to 300°C for 10 min (cycle = 57.33 min); for NAP, the oven was programmed at 200 °C for 4 min, increased by 3 °C.min^{-1} up to 310°C for 20 min (cycle = 60.67 min); for sterols and sterol acetates, 200 °C for 4 min, increased by 3 °C.min^{-1} up to 310°C, for 25 min (cycle = 61.67 min). The injected volume was 1 μL in splitless mode and the solvent delay was 4 min. To identify and quantify the FAME, sterols, and sterol acetates, identification was confirmed by comparing mass spectra and retention data with those previously reported and with those obtained from commercial standards. The proportion of sterols can be expressed both as a percentage of the unsaponifiable fraction and as a percentage of the total lipid content (%TL) following Equation 3:

$$\text{Sterols } (\%TL) = \%\text{total sterols} \times \frac{\text{unsaponifiable weight}}{\text{CLE weight}} \quad (3)$$

Results

Growth kinetics

The growth curves for the six strains of benthic diatom species grown in the airlift PBR are shown in Fig. 2. *Nitzschia alexandrina*, *E. paludosa*, *Nitzschia* sp., and *Staurosira* sp.

Table 1 Thin-layer chromatography (TLC): mobile phases, controls, and revealers used according to fractions analyzed

Fractions analyzed	Mobile phase	Controls	Revealers
Neutral lipids	Hexane/diethyl ether/acetic acid (50/50/0.75 v/v/v)	Sesame oil Cholesterol/cholesterol acetate	UV/vanillin
Glycolipids (according to polarity)	CH_2Cl_2/MeOH (90/10 v/v) CH_2Cl_2/MeOH (80/20 v/v)	Spinach	Vanillin/orcinol

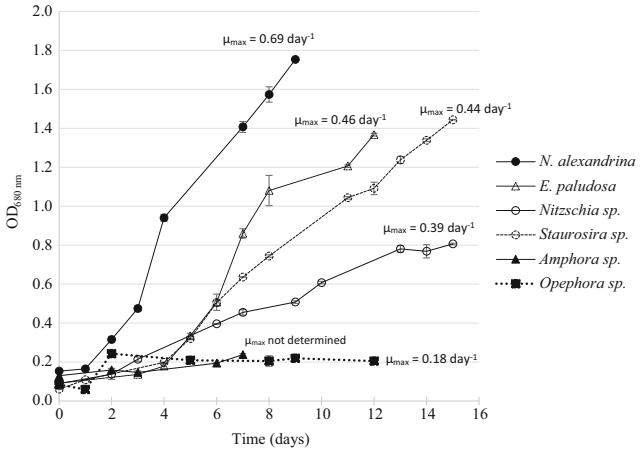

Fig. 2 Growth curves for six strains of benthic diatom species grown in the airlift PBR (average ± SD, n = 3, technical replicates). μ_{max} is specified above each curve.

have shown a significant growth, contrary to *Amphora* sp. and *Opephora* sp.

Nitzschia alexandrina presented the highest μmax while *Opephora* sp. presented the lowest. For *Amphora* sp., it was not possible to determine μmax since the strain did not grow. Details for growth parameters, biomass, and productivity are given in supplementary files (Online resource – Table S2).

Lipid analyses

Total lipid (TL) and lipid class fractionation are presented in Table 2. *Staurosira* sp. had the highest lipid content with 40.92 ± 0.58% DW, followed by *Nitzschia* sp. (20.84 ± 0.53% DW); the other species had a lipid content below 16% DW.

Staurosira sp. was the only diatom to produce predominantly neutral lipids, NL (>76%). *Nitzschia* sp. and *E. paludosa* produced NL in lower proportions at 59.11 ± 0.59% and 46.70 ± 2.22% respectively. The other species produced mainly glycolipids, GL. TLC analysis of the fractions allowed determination of their composition by comparison with the controls. It was possible to estimate the different proportions of each lipid class by gravimetry (Online resource - Table S3). Phospholipids, PL, classes were not characterized because they were too difficult to observe. With regards to NL, triacylglycerol (TAG) was mainly present in *Staurosira* sp. (>70%). Free fatty acids (FFA) represented more than 11% in *E. paludosa* and *N. alexandrina*. In the GL fractions, pigments and Monogalactosyldiacylglycerols (MGDG) were above 30% in *Amphora* sp. and *Opephora* sp. Digalactosyldiacylglycerols (DGDG) were higher than 16% in *E. paludosa*, *N. alexandrina*, and *Opephora* sp. Sulfoquinovosyl diacylglycerols (SQDG) were over 10% in *Amphora* sp. PL were present to a lesser extent in *Staurosira* sp. (6%) but represented more than 15% in *N. alexandrina* and *Amphora* sp.

Analyses of unsaponifiable fraction by GC-MS

CLE composition was obtained after saponification which allowed the separation of the unsaponifiables (free and acetylated for GC-MS analysis) from the FA (derivatives in FAME and NAP for GC-MS analysis).

CLE composition was variable according to the species studied. CLE of *E. paludosa* and *Amphora* sp. were composed of more than 20% unsaponifiable content (20.7% and 25.4% respectively). Conversely, levels of unsaponifiable content were low for *Nitzschia* sp. (6.4%). CLE of the other three species were composed of 12 to 14% unsaponifiable content.

As shown in Table 3, the composition of the unsaponifiable fractions differed according to species. Indeed, phytol was present in all six species in different amounts and 10 different sterols were identified (structures are given in Figure S1 – Online resource).

The highest amount of phytol was obtained from *Opephora* sp. and *Nitzschia* sp. (40.42 ± 0.22% and 26.57 ± 0.14% of the unsaponifiable fraction, respectively) (Table 3). The amount of phytol for the other species was under 20%. Not all species studied produced hydrocarbons (HC). The highest HC amount was for *Amphora* sp. (16.12 ± 0.18%) and *Opephora* sp. (18.21 ± 0.33%). Among these HC, squalene was only quantifiable in *N. alexandrina* and represented 1.91 ± 0.13% of the unsaponifiable fraction. The amount of sterols in each species was related to the unsaponifiable fraction.

Table 2 Total lipid, TL (average ± SD, n = 3, technical replicates, % dry weight (DW)) and lipid class fractionation (average ± SD, n = 3, technical replicates, %CLE) for the six strains of benthic diatom species

Strains	Total lipid, TL (% DW)	Neutral lipids, NL (%CLE)	Glycolipids, GL (%CLE)	Phospholipids, PL (%CLE)
N. alexandrina	15.75 ± 0.41	39.18 ± 1.30	45.12 ± 2.87	15.70 ± 0.18
E. paludosa	12.34 ± 0.43	46.70 ± 2.22	38.45 ± 0.51	14.85 ± 0.33
Nitzschia sp.	20.84 ± 0.53	59.11 ± 0.59	30.78 ± 0.51	10.11 ± 0.41
Staurosira sp.	40.92 ± 0.58	76.40 ± 0.43	17.61 ± 0.19	5.99 ± 0.37
Amphora sp.	11.35 ± 0.39	22.55 ± 1.21	50.98 ± 0.41	26.47 ± 1.08
Opephora sp.	14.32 ± 0.36	28.98 ± 0.92	58.52 ± 0.83	12.50 ± 0.61

Table 3 Unsaponifiable composition (average ± SD, $n = 3$, technical replicates, % unsaponifiable fraction) of the six strains of benthic diatom species

Compounds	% (% unsaponifiable fraction)					
	N. alexandrina	*E. paludosa*	*Nitzschia* sp.	*Staurosira* sp.	*Amphora* sp.	*Opephora* sp.
Phytol	9.09 ± 0.21	4.33 ± 0.13	26.57 ± 0.14	14.99 ± 0.24	18.95 ± 0.11	40.42 ± 0.22
Hydrocarbons	4.68 ± 0.18	1.11 ± 0.06	3.51 ± 0.12		16.12 ± 0.18	18.21 ± 0.33
With squalene	1.91 ± 0.13	tr	tr		tr	
Sterols						
Cholesta-5-en-3β-ol	23.61 ± 0.08				tr	
Cholesta-5,22-dien-3β-ol	30.79 ± 0.19			45.83 ± 0.29		
24-Methylcholest-5-en-3β-ol	3.91 ± 0.11	2.23 ± 0.29	10.73 ± 0.09			2.21 ± 0.13
24-Methylcholesta-5,22*E*-dien-3β-ol	12.48 ± 0.31	tr		34.56 ± 0.18	tr	
24-Methylcholesta-5,24(28)-dien-3β-ol	15.41 ± 0.24					
24-Ethylcholest-5-en-3β-ol		61.16 ± 0.21	59.18 ± 0.34			
24-Ethylcholesta-5,22*E*-dien-3β-ol		28.68 ± 0.29		4.62 ± 0.11	62.16 ± 0.34	7.15 ± 0.11
24-Ethylcholest-7,22*E*-en-3β-ol						16.25 ± 0.18
24-Ethylcholesta-5,22-dien-3-one						13.58 ± 0.24
24-Ethylcholesta-3,5-dien-7-one		2.27 ± 0.11				
Σ sterols	86.20 ± 0.93	94.34 ± 0.90	69.91 ± 0.43	85.01 ± 0.58	62.16 ± 0.34	39.19 ± 0.66

Several strain profiles emerged according to the variety of sterols present (Table 3). In fact, the strains with low variability in sterol composition corresponded to the species *Amphora* sp. containing only one sterol, 24-ethylcholesta-5,22*E*-dien-3β-ol at 62.16 ± 0.34% of the unsaponifiable fraction (15.8% TL), *Nitzschia* sp. containing only two sterols, 24-Ethylcholest-5-en-3β-ol at 59.18 ± 0.34% (3.8 % TL) and 24-methylcholest-5-en-3β-ol at 10.73 ± 0.09% (0.7% TL;), *Staurosira* sp. containing three different sterols, among which the cholesta-5,22-dien-3β-ol at 45.83 ± 0.29% (5.5% TL) and the 24-methylcholesta-5,22*E*-dien-3β-ol at 34.56 ± 0.18% (4.2%TL). Three strains showed a profile with four or more different sterols. 24-Ethylcholest-5-en-3β-ol (61.16 ± 0.21%; 12.6% of TL), 24-ethylcholesta-5,22*E*-dien-3β-ol (28.68 ± 0.29%), 24-methylcholest-5-en-3β-ol (2.23 ± 0.29%), and a ketone (2.27 ± 0.11%) were quantified in *E. paludosa*. The highest diversity was observed for *N. alexandrina* with six different sterols, the majority being cholesta-5,22-dien-3β-ol at 30.79 ± 0.19% (4.31% TL). 24-Ethylcholest-7,22*E*-en-3β-ol was the most abundant sterol in *Opephora* sp. at 16.25 ± 0.18% (2% TL). The two strains with the lowest amounts of total sterols within CLE were *Opephora* sp. (5.21% TL) and *Nitzschia* sp. (4.5% TL).

Total fatty acid composition of the six strains of benthic diatom species

Fifteen FA were identified in the six strains, the percentages for which are presented in Table 4. Variability in the quality of FA was observed, as the different proportions of saturated and unsaturated FA present in each strain. All of them produced more unsaturated than saturated FA, this asymmetry was more pronounced for *N. alexandrina*, *Opephora* sp., and *Staurosira* sp. in particular. Strains of species *Nitzschia* sp., *Opephora* sp. and *Staurosira* sp. produced more than 50% MUFA. Two strains produced more than 18% PUFA, corresponding to *E. paludosa* and *N. alexandrina*. Major FA present in the six strains were palmitoleic acid (9–16:1), followed by palmitic acid (16:0) and myristic acid (14:0). Palmitoleic acid was produced less by *E. paludosa* compared to the other species. However, *E. paludosa* produced a greater amount of myristic acid (14:0) and eicosapentaenoic acid (EPA, 20:5*n*-3) than did the other species. Moreover, it was the only one to produce nervonic acid (15–24:1). Of the 15 FA characterized, 11 were common to the six strains. The 6,9,12–16:3 acid was produced by three strains only (corresponding to *Amphora* sp., *Nitzschia* sp. and *Staurosira* sp. species), while the 6,9–16:2 acid was produced by the other three strains (corresponding to *E. paludosa*, *N. alexandrina*, and *Opephora* sp. species). 6,9,12,15–18:4 acid was produced by all, except the two strains belonging to the genus *Nitzschia*.

Discussion

Growth rate, biomass production, and lipid content Growth rate, biomass production, and lipid content were dependent not only on the species but also on the culture methods used. In a previous study (Cointet et al. 2019a), all six species showed higher μmax values when grown in batch cultures using 250-mL Erlenmeyer flasks filled with 150 mL of F/2 medium, enriched with silica, and stirred once a day for 2 min.

Table 4 Fatty acid composition (average ± SD, $n = 3$, technical replicates, % total FA) of the six strains of benthic diatom species

Fatty acid	% FA (% total FA)					
	N. alexandrina	*E. paludosa*	*Nitzschia* sp.	*Staurosira* sp.	*Amphora* sp.	*Opephora* sp.
Saturated FA						
14:0	3.85 ± 0.02	18.90 ± 0.01	2.99 ± 0.01	3.66 ± 0.01	12.42 ± 0.02	1.93 ±0.03
15:0	2.12 ± 0.02	0.72 ± 0.02	0.82 ± 0.02	tra	2.40 ± 0.04	tra
16:0	19.93 ± 0.18	22.43 ± 0.02	32.24 ± 0.12	29.45 ± 0.21	24.92 ± 0.06	23.53 ± 0.08
18:0	0.82 ± 0.03	0.49 ± 0.03	0.57 ± 0.02	0.60 ± 0.03	0.95 ± 0.01	0.94 ± 0.02
Σ SFA	**26.72 ± 0.25**	**42.53 ± 0.08**	**36.62 ± 0.17**	**33.71 ± 0.25**	**40.69 ± 0.13**	**26.39 ± 0.12**
Monounsaturated FA						
9–16:1	44.77 ± 0.08	28.33 ± 0.17	45.25 ± 0.32	51.83 ± 0.29	36.30 ± 0.12	50.23 ± 0.09
9–18:1	0.93 ± 0.02	1.06 ± 0.04	3.47 ± 0.04	0.99 ± 0.02	1.24 ± 0.02	0.73 ± 0.01
11–18:1	2.00 ± 0.01	1.26 ± 0.03	2.27 ± 0.01	1.36 ± 0.01	1.93 ± 0.01	3.07± 0.04
15–24:1	-	1.13 ± 0.02	-	-	-b	-
Σ MUFA	**47.70 ± 0.10**	**31.77 ± 0.25**	**50.98 ± 0.37**	**54.18 ± 0.32**	**39.47 ± 0.15**	**54.02 ± 0.14**
Polyunsaturated FA						
6,9–16:2	6.56 ± 0.02	2.93 ± 0.01	-	-	-	1.52 ± 0.03
6,9,12–16:3	-	-	1.32 ± 0.03	0.65 ± 0.02	4.52 ± 0.02	-
6,9,12–18:3	1.82 ± 0.02	0.81 ± 0.02	1.82 ± 0.01	0.89 ± 0.02	0.55 ± 0.02	Tr
6,9,12,15–18:4	-	2.36 ± 0.01	-	0.53 ± 0.01	1.25 ± 0.01	1.52 ± 0.03
9,12-18:2	tr[1]	1.83 ± 0.03	1.75 ± 0.01	Tr	1.18 ± 0.02	0.72 ± 0.01
5,8,11,14–20:4	4.84 ± 0.03	tr[1]	0.57 ± 0.02	0.89 ± 0.01	0.97 ± 0.03	2.67± 0.04
5,8,11,14,17–20:5	5.51 ± 0.04	12.56 ± 0.04	2.75 ± 0.04	5.50 ± 0.02	7.98 ± 0.23	7.08 ± 0.02
Σ PUFA	**18.73 ± 0.11**	**20.49 ± 0.11**	**8.20 ± 0.11**	**8.45 ± 0.08**	**16.44 ± 0.32**	**13.52 ± 0.13**
Otherc	6.85 ± 0.46[4]	5.24 ± 0.44	4.20 ± 0.65	3.66 ± 0.65	3.40 ± 0.60	6.07 ± 0.39

[a] *Tr*, trace amounts < 0.5%

[b] Not detected

[c] other FA include *i*-15:0, *ai*-15:0, 9-17:1, 20:0, 20:3, 20:4, 22:0, 22:6(*n*-3), 24:0, 26:0

In fact, the two species that were unable to grow in the airlift PBR in the present study (i.e., *Opephora* sp. and *Amphora* sp.) showed significant growth in batch culture (Cointet et al. 2019a). Despite higher growth rates in batch culture, total lipid and biomass production were higher in the airlift PBR, due to the fact that the cultures were not nutrient-limited. However, the airlift design should not be the best for cultivating benthic species. This is supported by Silva-Aciares and Riquelme (2008) who compared the growth of six diatom species (including *Amphora* and *Nitzschia* genera) with two different airlift PBRs. Variations in biomass production were observed between the six species, and also for each species cultivated in two different airlift PBRs. Some adhesive species, such as those from the genus *Amphora*, produced more biomass in a system with a rough PVC surface (known as PBB "bristles photobioreactor") rather than in a conventional system in constant movement (1.28 $g.L^{-1}$ *vs* 1.08 $g.L^{-1}$). However, the genus *Nitzschia* produced more biomass in an airlift PBR without a rough PVC plate (2.26 *vs* 2.02 $g.L^{-1}$) and tolerated suspension in the airlift very well. This variability in biomass production, relating to diatom characteristics, was also observed in the present study for *Amphora* sp. and *Opephora* sp. which showed the lowest biomass yield (0.23 $g.L^{-1}$ and 0.29 $g.L^{-1}$ – Table S2 – Online resource). Scanning electron microscopy culture analysis showed that these species develop in clusters and, therefore, their growth must have been disturbed by the continuous movement in the airlift PBR due to the injection of air. The use of an airlift PBR with PVC allowing for the adhesion of cells could be tested to optimize biomass production for these two diatom species.

Total lipid content obtained for all six species from the airlift PBR cultures differs from our previous study (Cointet et al. 2019a) (Fig. 3).

Comparison of the lipid content of the six diatom species grown in the airlift PBR (20 °C, stirring of the culture medium and pH control) and in Erlenmeyer flasks (16 °C, without stirring and no pH adjustment) (previous study) shows that two of the six species (*E. paludosa* and *Staurosira* sp.) have higher lipid content under controlled conditions. This is particularly noteworthy for *Staurosira* sp. (40.9%). Both *Amphora* sp. and *Nitzschia* sp. produced fewer lipids in the airlift PBR. These results can be explained by the increase of

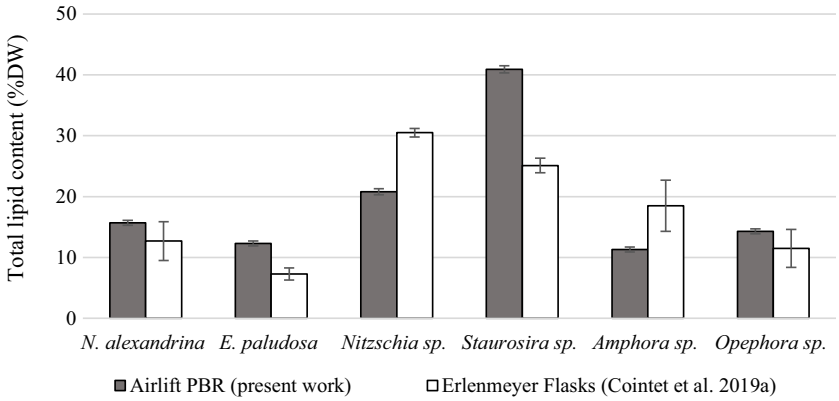

Fig. 3 Total lipid content (average ± SD, $n = 3$, technical replicates, % DW) in the airlift PBR and in Erlenmeyer flasks (Cointet et al. 2019a) for the six species studied

20 °C of the temperature parameter in the present study versus 16 °C in our previous study. As shown by de Castro and Garcia (2005), a temperature increase of five degrees can increase lipid content. As demonstrated by Chen (2012), culture conditions impact lipid production. The authors compared the lipid production of 10 diatom genera including *Amphora* and *Nitzschia*, both included in the present study. Their cultures were grown in a greenhouse or incubators in both summer and winter. Summer temperatures ranged from 25 to 34.5 °C and winter temperatures, from 14 to 22.5 °C. Light intensity ranged from 47 to 969 μmol.photons.m^{-2}.s^{-1} in the summer greenhouse and from 13 to 360 μmol photons.m^{-2}.s^{-1} in the winter greenhouse. The temperature in the incubator was 24°C, with a light intensity of 122 μmol photons.m^{-2}.s^{-1} in a 12:12 light-dark cycle. Lipid content variation was observed for the different species studied depending on the culture conditions. As an example, the genus *Amphora* had a higher lipid content in the greenhouse in summer than in winter (45% *vs.* 33%) and an intermediate lipid level (39%) when grown in an incubator. Conversely, the *Nitzschia* genus produced more lipids when grown in the greenhouse in winter than in summer (38% *vs.* 33%) but produced more lipids yet in the incubator (42%). These results are not in agreement with our own, which showed that these two species seem to produce more lipids under 16 °C. However, in the Chen (2012) study, the temperature and light variations in the greenhouse may explain this difference. Moreover, the application of a light-dark cycle may improve lipid production for these species. The incubator temperature was higher than that in the airlift PBR which may also explain why these species produced more lipids in the Chen study than in the present study.

Increases in biomass and lipid production can be explained by cell stress induced by CO_2 (Medina et al. 1998), temperature (Dunstan et al. 1993), the culture medium used, and/or nutrient composition (Dunstan et al. 1993; Schnurr and Allen 2015) and also by the achievement of a late exponential phase (Lebeau and Robert 2003; Sharma et al. 2012). In the present study, *Staurosira* sp. was the only species to produce a total lipid content higher than 35% which is close to literature for this genus (25-32%DW) (Huntley et al. 2015). According to our findings, the most interesting species in terms of biomass production and lipid content are *Staurosira* sp. (40.9%), *Nitzschia* sp. (20.8%), and *N. alexandrina* (15.7%).

Lipid class analyses It has been reported that GL are predominantly produced during the exponential phase and TAG during the stationary phase (Bergé and Barnathan 2005). The large amount of GL produced in *Opephora* sp. can be explained by the microscopic cell structure. As this species needs to grow in clusters, the continuous movement created by the airlift PBR is sure to have disrupted biomass production. We can therefore assume that the growth phase took longer, and the stationary phase may never have been reached, resulting in a higher amount of GL (58.5%) and lower lipid storage (NL) (29.0%). This assumption is confirmed by the growth curve. In the single publication dealing with lipid production in *Staurosira* sp. (Huntley et al. 2015), the authors demonstrated that *Staurosira* sp. could be grown on a large scale for valorization as biodiesel since this species presents a high lipid content (25–32%DW), a finding in accordance with our own. Biodiesel is, in fact, produced by TAG transesterification with MeOH and is a substitute for petroleum diesel. Biofuels are composed of a larger amount of MUFA than PUFA since biodiesel-saturated FA have better oxidative stability but poorer flow properties, while PUFA have better flow properties but are sensitive to oxidation. MUFA are therefore the most suitable FA for biodiesel (Sabia et al. 2018). The present work showed a significant production of TAG (71%) with less than 9% PUFA for the *Staurosira* sp., demonstrating that it is therefore an excellent candidate for the valorization of its lipids in biodiesel. This high TAG content could also be interesting for the cosmetology market. FA are found in different proportions in polar lipids. As described in the literature, GL and PL contained in algae, have anti-inflammatory, anti-cancer, and antimicrobial activity (Plouguerné et al. 2013; da Costa et al. 2017). It would be interesting to investigate the different GL and PL cellular models in the six species, according to their quantity and their

structural profile, in order to define their potential biological activities.

Unsaponifiable analyses Unsaponifiable analyses (phytol, HC, sterols) of the six diatom species showed a composition of 4.3 to 40.4% phytol. This molecule is a degradation product of chlorophyll (Ponomarenko et al. 2004) and is also present as a free molecule, playing a role in diatom photosynthesis and photoprotection (Stonik and Stonik 2015). Chlorophyll concentration varies according to the species, which explains phytol content variability (Massé et al. 2004; Zapata et al. 2011; Yao et al. 2015). The unsaponifiable fraction was also composed of HC, found in low quantities (1–4% TL). These molecules are commonly found in diatoms (Nichols et al. 1988; Volkman and Hallegraeff 1988; Grossi et al. 2004; Yao et al. 2015) and their presence may be explained by certain FA decarboxylations (Volkman et al. 1994). *Nitzschia alexandrina* was the only species to produce detectable amounts of squalene, which is a sterol precursor (0.7% TL). This molecule is of interest in cosmetology but production would have to be optimized since other microalgae, such as *Botryococcus braunii*, produce it in far higher quantities (14% DW) (Achitouv et al. 2004).

Sterols are found in all organisms (Blunt et al. 2011), including macro and microalgae (Thompson Jr 1996; Hamed et al. 2015). They are present in all diatoms. According to the literature, the most common sterols found in diatoms belong to the Δ5 series (Stonik and Stonik 2015), which is consistent with our findings (Table 3).

Sterols in diatoms are described as being mostly composed of 28 carbons but certain phylogenetic groups synthetize 27-, 29-, or 30-carbon sterols (Volkman 2016), which is in accordance with the present study and consistent with the biosynthetic pathway. Sterol classification is, therefore, in some cases, an aid to the phylogenetic classification of diatoms (Stonik and Stonik 2015). A study conducted by Rampen et al. (2010) on 106 diatom strains highlighted the presence of 44 sterols and 2 steroidal ketones. The 9 sterols characterized in the GC-MS analyses are found among the 44 sterols described. Sterol quantity and quality can be variable between two species belonging to the same genus (Rampen et al. 2010; Geng et al. 2017) as seen for *N. alexandrina* and *Nitzschia* sp. in the present study. Other studies have described the presence (in variable concentrations) or absence of a given kind of sterol according to the species studied (Nichols et al. 1988; Gladu et al. 1991; Barrett et al. 1995; Ponomarenko et al. 2004; Nappo et al. 2009; Sharma et al. 2012).

Sterol proportions found in the Rampen et al. (2010) study for *E. paludosa* (previously *Amphiprora paludosa*) differ in our findings. Indeed, they found that 24-methylcholesta-5,22*E*-dien-3β-ol represented 27% of total sterols while we found only trace amounts. Moreover, 24-methylcholest-5-en-3β-ol and 24-ethylcholesta-5,22*E*-dien-3β-ol were found in higher concentrations (14% and 55% respectively) in Rampen et al.'s work than in our own (2.2% and 28.7% of total sterols, respectively). Conversely, Rampen et al. (2010) found lower concentrations of 24-ethylcholest-5-en-3β-ol than we did (4% *vs.* 61.1%). In the present study, *Amphora* sp. was composed of almost 100% 24-ethylcholesta-5,22*E*-dien-3β-ol versus 88 to 96% and 4 to 9% of 24-ethylcholest-5-en-3β-ol recorded in the literature (Gladu et al. 1991; Barrett et al. 1995; Rampen et al. 2010). These differences can be explained by the fact that within the same genus, species produce sterols in different ways (Volkman and Hallegraeff 1988). The *E. paludosa* species produced more sterols than the others (19.5% TLC).

Fatty acid analyses According to the literature, in diatoms, saturated FA proportions range from 0.2 to 35% of total FA according to the species (Dunstan et al. 1993; Nichols et al. 1993; Viso and Marty 1993; Medina et al. 1998; Yao et al. 2014; Sabia et al. 2018). Diatoms are described as rich in myristic acid (14:0) (4–32%) and produce less stearic acid (18:0) (Hildebrand et al. 2012). They produce more PUFA with 16 and 18 carbons and more EPA compared to green algae (Thompson Jr 1996). These findings are in agreement with our own. A FA analysis of 17 diatom species (including 6 *Nitzschia* species and 2 *Amphora* species) (Levitan et al. 2014) has made it possible to bring to light the predominant FA within. Palmitic and palmitoleic acids as well as EPA were those predominantly found in the 17 species. FA found to a lesser extent were myristic, 6,9-hexadecadienoic, linoleic, oleic, vaccinic, and 6,9,12 hexadecatrienoic acids. Once more, these results are consistent with our own. Some species of the *Nitzschia* genus produce less EPA than others, which could explain the difference between *N. alexandrina* (5.5%) and *Nitzschia* sp. (2.8%) (Levitan et al. 2014). All six species in the present study had proportions of unsaturated FA higher than 50%, which remains consistent with the study of Dunstan et al. (1993) which investigated FA content in 14 diatom species. However, the quantities found for these FA differed. Proportions of saturated FA were higher for the six species studied here: 26.4 to 42.5% *vs.* 17.5 to 36.6% in the Dunstan et al. study. This is also true of the MUFA (31.8–54.2% *vs.* 20.6–27.2%). FA proportions are thus independent of the species. Regarding, PUFA and more particularly EPA, the species studied here produced between 2.8 (*Nitzschia* sp.) and 12.6% (*E. paludosa*) EPA. The latter species contained more EPA than do Atlantic salmon (6.5%) or *Chlorella vulgaris* (0.46%), although still slightly less than the brown alga *Laminaria* sp. (16.2%) (Hamed et al. 2015).

Conclusion

Six strains of benthic diatom species were grown in an airlift PBR and their precise lipid composition was determined.

Results showed that in terms of biomass productivity, *N. alexandrina*, *Staurosira* sp., and *E. paludosa* are the most promising species. However, in terms of lipid content, *Staurosira* sp., *Nitzschia* sp., and *N. alexandrina* are the most productive. In terms of biochemical diversity, the species which produced interesting chemical compounds were not easily grown in the airlift PBR. Indeed, despite interesting sterol production for *Amphora* sp. and interesting quantities of GL for *Opephora* sp., they both produced little biomass and lipids in the airlift PBR. In order to produce harvestable quantities of these compounds, different photobioreactor designs, such as flat panel PBR, must be tested. However, the culture method applied in this study did demonstrate that three of the six species can be easily grown in this system to produce different valuable compounds such as sterol and EPA (*Entomoneis paludosa*); squalene and a few SFA (*Nitzschia alexandrina*) as well as TAG with a large proportion of MUFA (*Staurosira* sp.). These molecules can be harvested for different industrial purposes, particularly human nutrition, aquaculture, and cosmetology.

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Author contribution Eva Cointet and Elise Séverin conducted experiments. Eva Cointet, Elise Séverin, Aurélie Couzinet-Mossion, Vona Méléder, Olivier Gonçalves, and Gaëtane Wielgosz-Collin analyzed and interpreted the data. Vona Méléder, Olivier Gonçalves, and Gaëtane Wielgosz-Collin designed and supervised the research. All the authors drafted the work and/or revised it critically and approved the final version of the manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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