

Identification and Fatty Acid Composition of Coccolithophore and Diatom Species Isolated from Aegean Sea

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Abstract

Diatoms are an important group of phytoplankton and widely distribute in the world seas. Diatoms have also attracted attention because of their siliceous cell walls. In this research, 6 pennates samples and 1 coccolithophore were collected from 4 different stations of Aegean Sea. *Ochrosphaera* sp. (JQ809711)-Izmir Bay and *Phaeodactylum tricornutum* (JQ886461)-Seferihisar; *Cylindrotheca closterium* (JQ886456)-Urla; *Amphora* cf. *capitellata* (JQ886459)-Izmir Bay; *Nitzschia communis* (JQ886460)-Urkmez; *Nitzschia* sp. (JQ886457)-Seferihisar; *Nitzschia thermalis* (JQ886458)-Seferihisar were isolated and identified by molecular and morphological methods. 18S rDNA sequences were amplified by PCR and sequence analyses were applied for molecular characterization of the species. These strains were cultured in F/2 medium under the temperature of 22 ± 2 °C at the light intensities ($40 \mu\text{E m}^{-2}\text{s}^{-1}$) for 22 days. Growth rates of diatom cells existed from 0.05 to 0.249 day^{-1} and doubling time 13.86 and 2.78 day. Also, lipid content of *C. closterium* and *Ochrosphaera* sp. were highest concentration compared to that of the other diatoms. The dominant fatty acid of *P. tricornutum* and *Nitzschia* sp. were palmitoleic acid (C16:1) which constituted 38% and 30% of total fatty acids, respectively.

Keywords: Coccolithophore and diatoms, PCR, growth rate, fatty acid, lipid

1. Introduction

The great majority of phytoplankton is eukaryotic cells that spend their life stages in the oceans and lakes (1). The main components of phytoplankton are diatoms (Bacillariophyceae), dinoflagellates (Dinophyceae), coccolithophores (Prymnesiophyceae) and some flagellates (Chlorophyceae, Rhaphidophyceae, Cryptophyceae, Dictyochophyceae). Diatoms represent about 25% of the flora in the world. These microscopic unicellular microalgae are important biomass and oxygen producers that can be found in all aquatic ecosystems (2). Environmental culture conditions can change the growth and physiology of microalgal cultures (3). Various morphological characters have been used for the classification of diatoms. It is being recommended to use the classical and molecular methods together to identify diatoms, since the cell morphologically differentiates in different environmental conditions and developmental life stages. Thus, the use of a single method is considered as unreliable for correct taxonomic identification. The lacks of genetic and morphological data have been resolved by employing molecular techniques (e.g. PCR, DNA sequencing) for proper identification and comparisons (4). Generally, nuclear-encoded small ribosomal subunit (18S) and large ribosomal subunit (28S) rRNA genes, the plastid-encoded large subunit of rubisco (*rbcL*), mitochondrial cytochrome oxidase 1 (*coxI*), and the internal transcribed spacer (ITS) regions are used in taxonomic and diagnostic signatures of microorganisms, including diatoms (5, 6, 7). Up till now, only few DNA sequences (e.g. 18S, 28S, mitochondrial genes) from the diatoms have been revealed in the public database (NCBI-GenBank). Owing to a shortage of both the genetic and morphological data, diatom identities sometimes remain undefined in different

environments. Diatoms are strong candidate in the biofuel industry due to their high growth rates, high diversity and nutrient use efficiency (8). Two diatom strains (*Nitzschia* sp.) have important characteristics for large-scale cultivation, fast growth rates and high lipid production for biofuel production (9). For biofuel industry, sixty percent of the selected strains were diatoms, which were chosen based on criteria such as high growth rates and lipid yields, tolerance of harsh environmental conditions, and performance in large-scale outdoor cultures (biodiesel production and feed aquaculture). The aim of this study was to determine the growth rates and fatty acid profiles of isolated and identified 6 pennates diatoms and 1 coccolithophore that were cultured in F/2 medium under the temperature of 22 ± 2 °C at the light intensities ($40 \mu\text{E m}^{-2}\text{s}^{-1}$) for 22 days.

2. Material and Method

2.1. Field study

Water samples were collected from the seaside of Aegean Sea by plankton net (mesh size $20 \mu\text{m}$). The samples were placed on ice in a cool container, and the organism arrived to the lab in 48 hours. Clean water polyethylene bottles were used in order to store the strains. The details of the field study of the collected species are given in Table 1.

2.2. Culture of coccolithophore and diatoms

The isolations of the strains were done using serial dilution and the streaking plate method (10). Cultures were initiated from these samples using an F/2 medium (11). The sample (1 mL) was inoculated into 9 mL sterilized F/2 medium in 15 mL tube. All isolates were grown under a light with photon flux densities $30 \mu\text{E m}^{-2}\text{s}^{-1}$ at 22 ± 2 °C. Diatom cells was transferred to fresh medium several times and then grown on F/2 agar plates (prepared from liquid media) for one to three weeks. A small number of diatom cells were transferred from these plates to liquid medium. The isolates were incubated at 22 ± 2 °C at the light intensity of $40 \mu\text{E m}^{-2}\text{s}^{-1}$ in 250 mL flasks for 14 days. The isolated species were joined to Ege University Microalga Culture Collection (EGEMACC) (<http://www.egemacc.com/en/index.php>). Stock cultures were monoalgal (non-axenic) and cultivated in F/2 medium at 22 ± 2 °C under continuous illumination ($40 \mu\text{E m}^{-2}\text{s}^{-1}$) in 1L sterile bottle for 22 days. Samples were taken at indicated times, and the optical densities of the cultures were measured by using a spectrophotometer (Ultraspec 1100 pro, UK) at 555 nm for *Ochrosphaera* sp., *Nitzschia thermalis*, *Nitzschia communis*, *Phaeodactylum tricorutum*; at 505 nm for *Amphora* cf. *capitellata*; at 550 nm for *Cylindrotheca closterium* and *Nitzschia* sp. The specific growth rate (μ) of the cells was calculated from the initial logarithmic phase of growth for at least 48 h, as $\mu = (\ln C_2 - \ln C_1)/dt$, where C_2 is the final cell concentration, C_1 is the initial cell concentration and dt is the time required for the increase in concentration from C_1 to C_2 . Doubling time (DT) was also calculated as $DT = \ln 2/\mu$.

2.3. Morphological identification

Cell morphologies were clarified with the microscopes. For identification and morphological investigations of the cultures, phase-contrast, light and scanning electron microscopy (SEM) images were used. Living cells as well as cleaned frustules were examined and photographed by bright field microscopy using a Leica DMIL fluorescent microscope (Leica, Germany) with 63x achromatic objective lens. To remove all organic material, the cells were bleached with NaOCl for overnight. Then methanol was added until its color turned to light yellow. The liquid was discarded and the frustules were washed 5 times with distilled water. The cleaned frustules were stored in distilled water. Finally, species were coated with gold for 2 min, and then examined by using FEI Quanta-250 FEG scanning

electron microscope (FEI Company, Czech Republic). SEM analysis at 5 kV was done on samples with the working distances of 9 mm for *Ochrosphaera* sp., *Cylindrotheca closterium* and 6.5 mm for the other species.

2.4. Molecular identification

A total of 40 mL cultures at logarithmic growth phase were harvested by centrifugation at 3500 rpm for 5 min. The concentrated cells were transferred to 1.5 mL micro tubes were stored at -20°C until DNA extraction.

Genomic DNA was extracted from the cell pellets by using the ZR Fungal/Bacterial DNA Kits (Zymo Research, USA) and stored at -20°C . For the PCR amplification, we used PCR primers that targeted to bind nuclear 18S rDNA (AT18F, 5'-YACCTGGTTGATCCTGCCAGTAG-3' and AT18R, 5'-GCTTGATCCTTCTGCAGGTTTACC-3') (4). PCR reaction was performed with 94°C for 2 min, following 35 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 5 min.

Resulting PCR products were electrophoresed in a 1.0% agarose gel, stained with SYBR, and visualized by ultraviolet transillumination. In the amplification step, dye terminator sequencing was done using the primers and the nucleotide sequences were determined by genetic analyzer (Applied Biosystems 3130XL with 16-capillary array).

2.5. Total lipid content and fatty acid composition

Lipids were extracted from lyophilized algal biomass by a modified BLIGH&DYER method (12). Freeze-dried cells (100 ± 0.5 mg) were weighed accurately, placed into a 15 mL centrifuge tube and sonicated by a sonicator (Bandelin Sonoplus UW 2070, Germany) operated at a constant frequency of 20 kHz with a 45 % power, at 0.9 sec intervals for 5 min in 15 mL falcon tubes in an ice-water bath to avoid the increase of temperature that could affect the cell viability and the product quality. For extraction, 3 mL chloroform/methanol (2:1) containing 1.0 mg/mL nonadecanoic acid (19:0) and 0.5 mg/mL butylated hydroxytoluene (BHT) was used, and the tube was shaken gently overnight at room temperature. After centrifugation at 6000 rpm for 5 min and then the supernatant was filtrated by Minisart SRP 25 (Syringe filter, hydrophobic), the extracted oil was stored at 4°C until analysis. The extract was evaporated in a water bath (30°C) using a rotary evaporator (Stuart, RE300, UK) to remove solvents. The final lipid concentration was determined gravimetrically.

Fatty acids were analyzed by gas chromatography (GC) after direct transmethylation with hydrochloric acid in methanol (13). The fatty acid methyl esters were extracted with n-hexane and analyzed by Agilent 7890 gas chromatography equipped with a flame-ionization detector and a Supelco sp-2380 capillary column ($60\text{ m}\times 250\ \mu\text{m}\times 0.2\ \mu\text{m}$) with helium as a carrier gas at a flow rate of 0.7 mL/min. One microliter sample was injected in the split (20:1) injection mode. The inlet and detector temperatures were 260°C , and the oven temperature was programmed at an initial temperature of 100°C , then increased at $10^{\circ}\text{C}/\text{min}$ interval to 250°C , and held there for 3 min. Fatty acid methyl esters were identified by chromatographic comparison with authentic standards (Sigma Chemical Co., St. Louis, MO).

3. Results and Discussion

3.1. Morphological identification

Identification of diatoms is routinely based on the morphology of their silica valves. These features lead to identification errors and different researchers may identify the same individual as different species (14). Six commonly found pennate diatoms of *Phaeodactylum tricorutum* Bohlin 1897, *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C. Lewin 1964, *Amphora* cf. *capitellata* J. Frenguelli, *Nitzschia communis* Rabenhorst 1860, *Nitzschia*

sp. Hassall 1845, *Nitzschia thermalis* (Ehrenberg) Auerswald in Rabenhorst 1861 and one coccolithophore *Ochrosphaera* sp. Schussnig 1930 were examined by using light microscopy (LM) and electron microscopy (SEM) images isolated from Aegean Sea. Species were identified based on some specific features examined in different phases. In a series of articles had dealt with the living cells in different phases, the aim of this study was to provide detailed descriptions of diatoms in the exponential phases by using LM and SEM. *Ochrosphaera* is the most frequently observed coccolithophorid in littoral zone samples from diverse locations in the North Atlantic, Indian Oceans, the Mediterranean Sea and Japanese (15). Under the light microscopy; the diameter of cells were measured as 10-12.5 μm (total range of 6–12 μm) and the cells were covered by a single layer of tremaliths and vase-shaped in section. Each cell had two thin parietal, often bilobed, golden-yellow chloroplasts (figure 2). As a result of the observation of electron microscopy; *Ochrosphaera* sp. (EGEMACC57) was considerable variability in coccolith morphology (figure 1). The species of *Cylindrotheca* were characterized by having cylindrical frustules which were fusiform. They were needle-like with a swollen center. The most common species that identified in this genus was *Cylindrotheca closterium* (figure 1, 2). *C. closterium* (EGEMACC45) was found in Urla, Aegean Sea. The length of the cells varied from 250 μm to 500 μm and the width ranged from 2.5 μm to 8 μm . *C. closterium* was considerable the weakly silicified valves by SEM (figure 1).

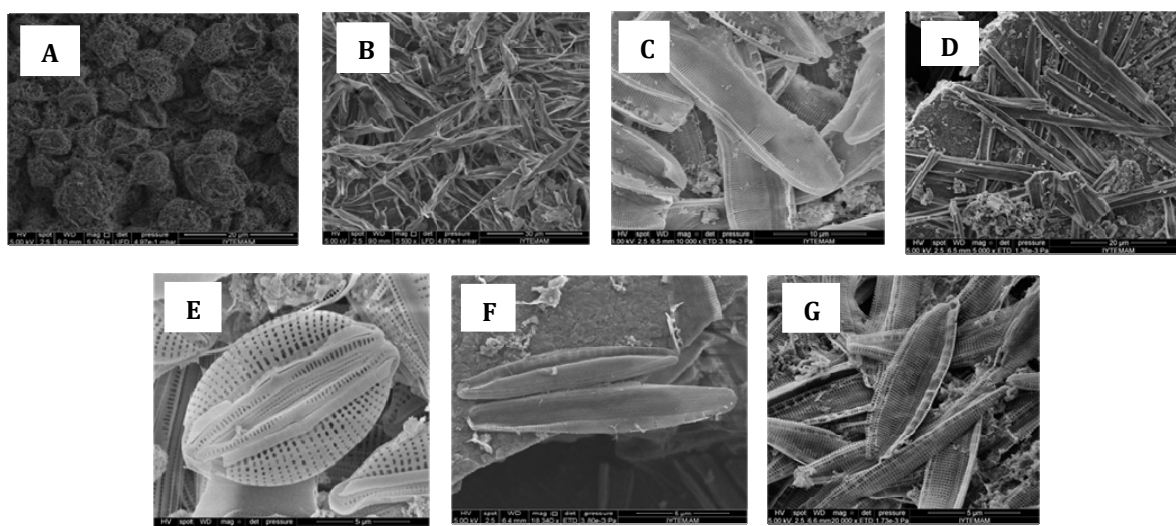


Figure 1. Identification of coccolithophore and diatoms with SEM image (10000x); A-*Ochrosphaera* sp., B-C- *closterium*; C-*Nitzschia* sp., D-*N. thermalis*,E-*A. cf. capitellata*,F-*P. tricorutum*, G-*N. communis*

Nitzschia was the other genus of diatom that most commonly found in Turkey water. Three species of *Nitzschia*, *Nitzschia* sp., *N. thermalis* and *N. communis* were screened in Urkmez, Seferihisar-Aegean Sea. *Nitzschia* could be often found in form of chain or as free living cells. According to ROUND & al. (16), the fibulae of *Nitzschia* could even extend across the valve and the raphe was usually observed near the proximal margin of the valve. The length of the *Nitzschia* sp. cells varied from 30 μm to 32.5 μm and the width ranged from 7 μm to 10 μm measured by light microscopy. Each cell had two thin parietal, golden-yellow chloroplasts (figure 2). The length of *N. thermalis* (EGEMACC56) was measured between the ranges of 28 to 50 μm and the width varied from 2.5 to 5 μm while the length of *N. communis* (EGEMACC48) ranged from 10 to 20 μm and the width varied from 2.5 to 5 μm (figure 2). The samples were initially observed under the bright field microscopy (figure 2), and the

taxon immediately appeared to belong to the genus *Amphora*. Key characteristics visible with light and scanning electron microscopies were included frustular shape, arched valves, raphe structure, and multiple bands between the 2 valves of each frustule (17). *Phaeodactylum tricornutum* (EGEMACC-71) was extended to a length of approximately 35µm.

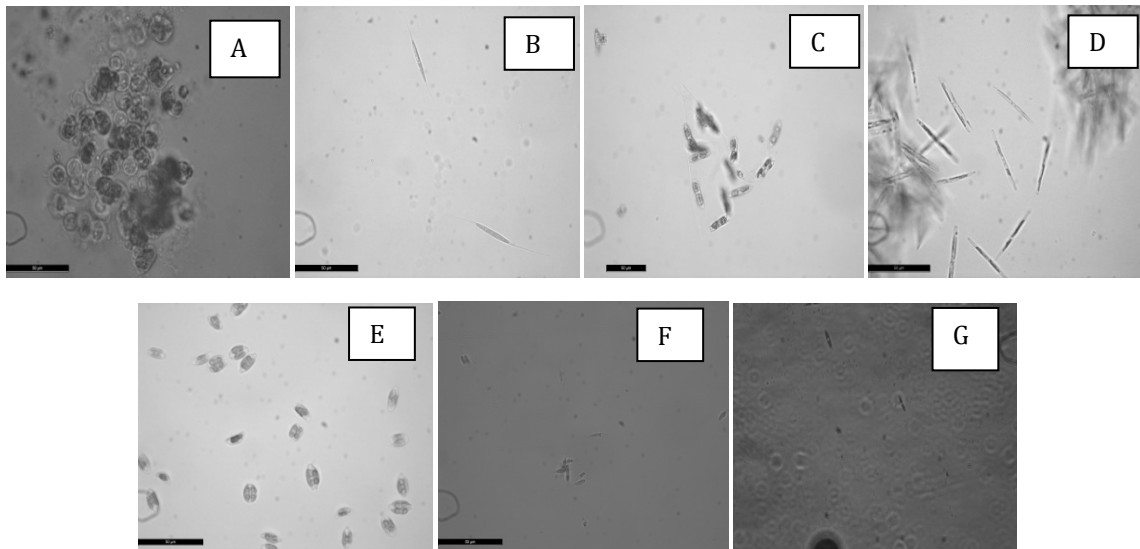


Figure 2. Light microscopy observation (63xmagnification) for samples; A - *Ochrosphaera* sp., B-C. *closterium*, C-*Nitzschia* sp., D-*N. thermalis*, E-*A. cf. capitellata*, F-*P. tricornutum*, G-*N. communis*

3.2. Molecular identification

Species identities and taxonomic systematic of the diatoms can also be revealed by using molecular comparisons (4, 18). Molecular techniques that included phylogenetic assessments commonly relied on DNA sequence information of ribosomal DNA (rDNA). These genetic groups could be examined for morphological characters that could be used to more easily identify these diatoms objectively. Molecular data were presented in figure 3. Nuclear encoded rDNA sequences for all taxa were approximately 1000 nucleotides in length. Editing and counting assembly of rDNA sequence fragments were carried out with Applied Biosystems 3130XL. The coding 18S rDNA genes were submitted to the NCBI database for verification. The sequences determined and had been deposited in the GenBank with the accession numbers as seen in table 1.

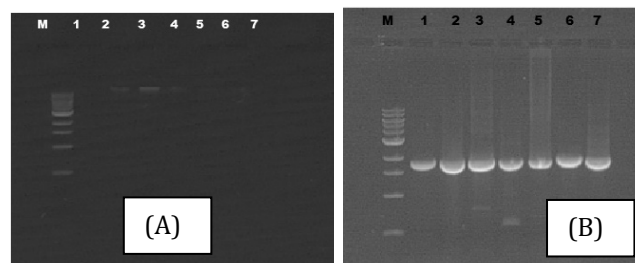


Figure 3. DNA amplification of the 18S rDNA (A) and PCR amplification of the 18s rDNA(B); Line M: Marker (1 kb DNA ladder BioLabs, England), A- Line 1: DNA isolation of *Ochrosphaera* sp., Line 2: *C. closterium*, Line 3: *Nitzschia* sp., Line 4: *N. thermalis*, Line 5 *A. cf. capitellata*, Line 6: *P. tricornutum*, Line 7: *N. communis*, B-Line 1: PCR production of *Ochrosphaera* sp., Line 2: *C. closterium*, Line 3: *Nitzschiasp.*, Line 4: *N. thermalis*, Line 5: *A. cf. capitellata*, Line 6: *P. tricornutum*, Line 7: *N. communis*

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Table 1. Details of the field study for the collection of coccolithophore and diatoms

Collection conditions	<i>Ochrosphaera</i> sp.	<i>P. tricorutum</i>	<i>C. closterium</i>	<i>Amphora</i> cf. <i>capitellata</i>	<i>N. communis</i>	<i>Nitzschia</i> sp.	<i>N. thermalis</i>
Kingdom	Chromista	Chromista	Chromista	Chromista	Chromista	Chromista	Chromista
Phylum	Haptophyta	Ochrophyta	Ochrophyta	Ochrophyta	Ochrophyta	Ochrophyta	Ochrophyta
Class	Coccolithophyceae	Bacillariophyceae	Bacillariophyceae	Bacillariophyceae	Bacillariophyceae	Bacillariophyceae	Bacillariophyceae
Station Name	Izmir Bay	Sigacik-Mavi Teos	Urla-Gelinkaya	Izmir Bay	Sigacik-Mavi Teos	Sigacik-Teos	Sigacik-Teos
Latitude	38.3255	38.1829	38.1916	38.3255	38.1829	38.1829	38.1829
Longitude	26.6535	26.7718	26.7737	26.6535	26.7718	26.7718	26.7718
Average Depth (m)	2	1	1	2	1	1	1
Relative humidity (%)	70	70	70	70	70	70	70
Salinity (‰)	39	39	39	39	39	39	39
Water Temperature (°C)	24	23.6	24	24	23.6	22.8	22.8
Weather	Sunny	Sunny	Sunny	Sunny	Sunny	Sunny	Sunny
Accession Number	JQ809711	JQ886461	JQ886456	JQ886459	JQ886460	JQ886457	JQ886458

3.3. Growth conditions

There were many reports for the growth rate of cultivated sea water phytoplankton species isolated from coastal and oceanic habits (19, 20). The cell size of marine phytoplankton was the most important factor for the growth rate due to the relationship between the morphological and physiological properties (21). In this study, the specific growth rates of *Ochrosphaera* sp. and *Amphora* cf. *capitellata* were determined with the lowest values of 0.08 d⁻¹ and 0.05 d⁻¹, respectively. As expected, the growth rate decreased with decreasing the cell size. The maximum specific growth rates of 0.249 day⁻¹ and 0.209 day⁻¹, which corresponded to the doubling times of 2.78 day and 3.32 day, were obtained for the cultivation of *P. tricorutum* and *C. closterium*, respectively (table 2). In other studies, the obtained maximum exponential specific growth rates (μ_{max}) for diatoms under the conditions of saturating light and nutrient sufficiency, ranged from 0.2 d⁻¹ to 3.3 d⁻¹ (22).

Table 2. Specific growth rates and doubling times and optical densities of coccolithophore and diatoms.

Species	Specific growth rate (μ , day ⁻¹)	Doubling Time (DT, day)	OD
<i>Ochrosphaera</i> sp.	0.08	8.66	0.440±0.01
<i>Amphora</i> cf. <i>capitellata</i>	0.05	13.86	0.170±0.02
<i>Cylindrotheca closterium</i>	0.209	3.32	0.970± 0.04
<i>Nitzschia</i> sp.	0.181	3.83	0.536± 0.02
<i>Nitzschia thermalis</i>	0.098	7.07	0.148± 0.06
<i>Nitzschia communis</i>	0.128	5.42	0.058± 0.01
<i>Phaeodactylum tricorutum</i>	0.249	2.78	0.907± 0.03

3.4. Total lipid content and fatty acid composition

Saturated and mono-unsaturated fatty acids are predominant in most algae examined. Specifically, the major fatty acids are myristic acid (C14:0), palmitic acid (C16:0) and palmitoleic acid (C16:1) in the Bacillariophyceae (9, 23). A wide variety of neutral lipid composition had been identified in diatoms, however, there were seven species, which were enriched in C14:0 and C16:0, known as the most common fatty acids contained in biofuel (table 3). Diatom fatty acids were among the most highly enriched in C14 chain lengths

compared with other classes of algae, especially chlorophytes (24). As reported by HU& al. (23) the percentage of C14:0 of the total fatty acids composition for diatoms ranged from 4 to 32 %. As shown in table 3, C16 fatty acids were highly represented in species and C16:0 and C16:1 combined could represent 50-60 % of the total fatty acids. On the other hand, C18 lengths were poorly represented in species. In table 3, coccolithophore and diatoms were rarely enriched for eicosapentaenoic acid (EPA; C20:5) but for *N. communis*, C20:5 was the dominant fatty acid. Table 3 was shown that docosahexaenoic acid (DHA; C22:6) levels in diatoms were less than EPA. Generally, shorter chain lengths were desirable to improve cold-flow properties and oxidative stability of biofuels and saturated fatty acids were more desirable because they increased the ignition quality of the fuel (23). In this study, the total lipid contents ranged from 24% to 42% of dry biomass weight for all species. Maximum lipid content of 42% was obtained with the dominant fatty acid of C16:1 (29 % of the total fatty acids) for *C. closterium*.

Table 3. Lipid content and fatty acid profile of coccolithophore and diatoms

Sample	Total lipid % w/w	Total fattyacids (%)											
		C14:0	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:1	C20:5	C22:6
<i>Ochrosphaera</i> sp.	41	10.2	14.3	23.0	2.0	2.0	3.0	2.0	1.0	-	2.5	11.0	-
<i>P.</i> <i>tricornutum</i>	33	8.5	23.7	38.0	-	1.0	5.0	1.5	1.5	-	2.0	11.0	-
<i>C. closterium</i>	42	13.0	23.0	29.0	3.5	2.0	4.0	1.0	2.0	-	3.0	5.0	-
<i>Amphora</i> cf. <i>capitellata</i>	24	10.6	23.0	23.0	6.2	2.9	2.6	2.4	-	2.6	6.0	14.0	-
<i>N. communis</i>	32	13.7	12.5	15.1	2.1	5.0	6.5	2.1	-	-	-	18.3	-
<i>Nitzschia</i> sp.	31	10.0	13.3	30.0	3.0	2.0	6.0	2.0	2.0	-	7.0	14.0	3.0
<i>N. thermalis</i>	32	12.0	16.5	22.0	6.5	2.5	3.0	1.5	-	-	8.0	10.0	2.0

Abbreviations: C14:0 (Myristic acid); C16:0 (Palmitic acid); C16:1 (Palmitoleic acid); C17:1 (cis-10-Heptadecenoic acid); C18:0 (Stearic acid); C18:1 (Oleic acid); C18: 2 (Linoleic acid); C18:3 (cis-6, 9, 12-Linolenic acid); C20:0 (Arachidic acid); C22:1 (Erucic Acid); C20:5 (cis-5,8,11,14,17-Eicosapentaenoic acid); C22:6 (cis-4,7,10,13,16,19-Docosahexaenoic acid)

4. Conclusions

Locally isolated six pennates diatoms and one coccolithophore were identified both by morphological and molecular techniques. The maximum specific growth rates of 0.249 day⁻¹ and 0.209 day⁻¹, which corresponded to the doubling times of 2.78 day and 3.32 day, were obtained for the cultivation of *P. tricornutum* and *C. closterium*, respectively. Furthermore, maximum lipid content of 42% was obtained with the dominant fatty acid of C16:1 (29 % of the total fatty acids) for *C. closterium*. The noteworthy finding is that the lipid content increases with increasing the growth rate and the cell size for diatoms.

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