JKAU: Mar. Sci., **Vol. 34** No. 2, pp: 55-65 (2024 A.D.)
DOI :10.4197/Mar.34-2.5

Growth Rate, Lipid and Fatty Acids Content in Some Marine and Freshwater Diatoms

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Abstract. Diatoms are photosynthetic organisms that can produce lipids in large amounts within a short time. Their biomass can be processed into biofuels and other valuable commercial products. In this research, six diatom species were isolated from Malaysian water (seawater and freshwater) and grown under the same conditions to observe differences in their lipids and fatty acids composition. The results showed significant differences in total lipid contents between freshwater and marine species. Where *Sellaphora pupula*, *Nitzschia palea*, *and Craticula cuspidata* (Freshwater species) had a relatively constant percentage of lipid (13 to 16.1 % of dw) while *Nitzschia* sp. *Chaetoceros calcitrans*, and *Nitzschia sigma* (marine species) had high lipid contents (>18 %). Twenty-eight of different fatty acids were detected in six diatoms studied. Palmitoleic acid-C16:1 (29.9 to 39.5%) and Palmitic acid-C16:0 (15.6 to 33.2%) was predominant in most of the six diatoms. In addition, ω-3 and ω-6 PUFA showed a high percentage in some marine and freshwater species (*Nitzschia* sp. and *C. cuspidata*), which suggested both species had a good quality of polyunsaturated fatty acid and could be used as food sources in aquaculture or in other useful applications.

Keywords: Microalgae, Malaysia, diatoms, Growth rates, lipid, Fatty acids.

1. Introduction

Diatoms are unicellular organisms that have the ability to conduct photosynthesis, with characteristic silica cell walls. There are more than 80,000 strains of diatoms ranging in size between 4 and 200 µm (Sheehan *et al*., 1998; Levitan *et al*., 2014), and they constitute one of the bigger groups of eukaryotic phytoplankton in marine water but also found in freshwater. Their growth depends on the availability of light, nutrients (N, P, and Si), dissolved carbon dioxide, and trace metals (De Baar *et al*., 1999). Diatoms have pulled expanding consideration for their potential to produce various bioactive compounds and fine chemicals for industrial applications (Vinayak *et al*., 2015). For example, diatoms are rich in pigments such as carotenoids that are broadly connected to nourishment supplements and feeds, pharmaceutical ingredients, and beauty care products (Vilchez *et al*., 2011; Fu et al., 2015). In addition, diatoms can produce lipids in large amounts within a short time and the average lipid content under normal conditions could reach 25% of dry weight (Levitan *et al*., 2014). While lipid content may increase considerably (doubles) when the cells are subjected to unfavorable culture conditions, such as light intensity, salinity, temperature, nutrient starvation, and carbon dioxide (Qin, 2005; Hu *et al*., 2008; Chiu et al., 2009; Widjaja *et al*.,

2009). Moreover, the main fatty acid content in diatoms is (14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n-7), DHA, and EPA (Ying *et al*., 2000; Jiang et al., 2016) and these fatty acids play important roles in biofuel production as wile as human and animal health. In literature, much attention has been given to the comparison of lipid and fatty acid contents as individual species under different environmental conditions, for example, genus *Nitzschia* spp and *Chetoceros* spp (Raghavan *et al*., 2008; Griffiths and Harrison, 2009; Abdel-Hamid et al., 2013; Scholz and Liebezeit, 2013; Chagoya *et al*., 2014; Jiang et al., 2014; Fuad *et al*., 2015). However, few studies have been published on the comparison of lipid and fatty acids content as a group of marine and freshwater microalgae. In this research, six diatom species were isolated from Malaysian water (seawater and freshwater) and grown under the same conditions to observe differences in their lipid and fatty acid composition.

2. Materials and Methods

1.1 Isolation and identification of microalgae 2.972528, 101.782167

Freshwater diatoms (*Sellaphora pupula*, *Nitzschia palea*, *Craticula cuspidata*) were isolated from the freshwater pond near the Melor Park in Kajang, Selangor, Malaysia (2°58'21.1"N101°46'55.8"E) and marine diatoms (*Nitzschia sigma*, *Nitzschia* sp.) were isolated from Pulau Pangkor coast, Malaysia (4°14'10.5"N 100°32'40.8"E). *Chaetoceros calcitrans* (UPMC-A0010) was obtained from the Microalgae Culture Collection of Marine Biotechnology Laboratory, Institute of Bioscience, University Putra Malaysia. Two techniques were used to obtain pure microalgal strains: streak plate and micropipette (Andersen, 2005). Pure microalgal strains were incubated under controlled conditions (light/dark cycle 12/12 hour with photon densities between 60 to 75 µmol photons m^{-2} s^{-1} at 25°C) and used as pure algae stock. All isolated species were identified by field emission scanning electron microscopy (FESEM) after removing organic material from the frustule and dehydrated as described in Jiang *et al*. (2015). The key characteristics of diatom species were visible by scanning electron microscopy including frustule shape, arched valves, and raphe structure.

2.2 Culture and Determination of Growth Rates

Three replicates from each species were grown in 500 ml Erlenmeyer flasks containing 300 ml sterilized medium. Wright Chu (WC) medium (Guillard and Lorenzen, 1972) was used to culture freshwater diatoms with slight modification to culture marine diatoms (Sodium Chloride was added to the WC medium to change salinity to 30 ‰). All cultures were incubated under light: dark cycle (12/12 hour) with photon densities between 100 and 125 µmol m^{-2} s^{-1} for 24 days at 25°C (Figure 1), pH was not controlled but ranged from 7.5 to 8.5. Spectrophotometer (UV-VIS 1601, Shimadzu, Japan) at 750 nm was used to determine cell density every two days and, the growth was expressed as specific growth rate (per day) of the exponential growth phase using the equation (Nunez and Quigg, 2016).

$$\mu = 1/T\ (\ln N_{t+1} - \ln N_t)$$

Where N_t is the optical density at the start; N_{t+1} is the optical density at the end, and T is the number of days between two measurements. In addition, Doublings time (days) of microalgae was calculated once the specific growth rate (µ) was known.

$$\text{Doubling time (days)} = \frac{0.6931}{\mu}$$

2.3 Lipid Extraction

Total lipid was assessed by using the colorimetric method (Marsh and Weinstein,

1966). Once the cultures reached the stationary phase, duplicate samples of each culture were harvested by centrifugation (at 3000g for 5 min) and then dried by freezing- dry at -50°C under vacuum for 48 hours. Five milligrams of Freeze-dried cells was homogenized in the test tube with 1 ml of the chloroform-methanol mixture (a ratio 1:2 v/v). Glass beads were added to the sample and vortexed for 3 minutes. The sample was placed into a water bath for 3 h at 60 °C, then centrifuged at 3000 rpm for 5h and, the supernatant was transferred into a new test tube. The pellet was re-extracted by adding 3.5 ml of chloroform-methanol mixture. To separation of lipids from the supernatant 3 ml of chloroform-water mixture (a ratio 1:1 v/v) was added. Lipid (non-polar phase) was transferred by glass Pasteur pipette to a rotavapor flask and, dried in a rotary evaporator, then re-suspended in 1 ml of chloroform. Different concentrations of standard lipid solution (0.05, 0.2, 0.4, 0.6, 0.8, and 1.0 µg/ml) were prepared from stock solution (0.3 mg/ml-1 in chloroform). The Sample (200µm), blank (200µm of chloroform), and standard solutions (200µm) were evaporated by putting them in a dryer under vacuum. Two milliliter of H_2SO_4 was added and vortexed, then the sample was heated using a thermos block at 200 °C for 15 min. After cooling at room temperature, 3 ml of distilled water was added and mixed well by inversion. Finally, a Spectrophotometric (at 375 nm) was used to quantify lipids from calibration curves of each sample with different concentrations of Standard Lipid Solution using a polynomial line. The lipid content was calculated as the % dry weight of biomass.

2.4 Fatty acid Extraction and Transesterification

Fatty acid methyl esters (FAMEs) were extracted from diatoms according to Abdulkadir and Tsuchiya (2008). About 300mg ±1 of the sample was dissolved in 4 ml of hexane in the test tube, 2 ml of 14% BF3 in methanol was added. The empty part of the test tube was filled with nitrogen gas and placed into a water bath at 100 C^0 for 120 min with vortexing every 5 min. After cooling in an ice bath, 1 ml of hexane and 2 ml of MilliQ water were added, vortexed for 1 minute, and centrifuged for 3 min at 2500 rpm. Two phases were formed. The upper phase containing the hexane and free fatty acid methyl esters (FAMEs) was used for fatty acid analysis.

2.5 Fatty Acids Analysis

Fatty acids were analysed using Gas Chromatography-Mass spectrometry (GCMs). One microliter (µL) of sample was run by an auto-sampler injector (Agilent Technologies 7693) and an HP88 column (100 m × 0.250 mm internal diameter, 0.25 µm film thickness, J&W Scientific, Agilent Technologies, USA). Helium was used as the carrier gas at a flow rate 1ml/min. The injector temperature was operated at 250 ◦C. The oven temperature was programmed at 150 °C for 5 min, at a heating rate of 4 °C/min, and to 240 °C for 15 min, at a heating rate of 4 °C/min. Run Time 42.5. The FAMEs were identified by comparison retention times and mass spectra of authentic standards and available spectra in mass spectral libraries, and analysed with the software MSD (Agilent Technologies 5975 C triple axis detector). Total fatty acid (TFA) content was determined as the sum of all FAMEs in the sample and individual fatty acids (FA) are expressed as percent of total fatty acid.

2.6 Statistical Analysis

Shapiro-Wilk test was used to check normality. Differences in lipid and fatty acids were performed using one-way ANOVA. Significant differences among the different species were determined using Tukey's post-hoc test at a 0.05 level of probability. All statistical analysis was done using IMB SPSS statistics 22.

3. Results and Discussion

3.1 Identification of Diatoms

Scanning electron microscope (SEM) for six diatoms studied are shown in figure 2. SEM allowed identification of the six diatoms as *Sellaphora pupula*, *Nitzschia palea*, *Craticula cuspidata*, *Nitzschia sigma*, *Nitzschia* sp. and *Chaetoceros calcitrans*.

3.2 Growth Rate

As shown in Figure 3, the exponential growth phase of freshwater diatom was seen from day 0 to 12 and the stationary phase from day 12 to 24, while the exponential growth phase of marine diatom was seen from day 0 to 8 and the stationary phase from day 8 to 24.

Specific growths of diatoms are shown in Table 1. *C. calcitrans*, *Nitzschia* sp., and *N. sigma* (marine diatom) had higher growth rates (μ), 0.33 to 0.55 per day and, doubling time from 1.2 to 2.1 days. While *S. pupula*, *N. palea*, and *C. cuspidata* (freshwater diatoms) grew slower with growth rates 0.14 to 0.25 per day and doubling time of 2.7 to 4.9 days. The difference between species in growth rate is normal because each species has a different growth rate. In the present investigation, the growth rate of *C. calcitrans* was similar as reported by Miller *et al*. (2012), but *N. palea* was less than the growth as reported by Abdel-Hamid *et al*. (2013). In contrast, *Nitzschia* sp. in this study had higher growth than another species of *Nitzschia* studied by (Chagoya et al., 2014; Jiang et al., 2014; Demirel *et al*., 2016). This difference between studies on the same type may be due to differences in culture conditions. For both *C. cuspidata* and *N. sigma* there was no data reported about the growth rate in previous studies.

3.3 Total Lipid Contents

The total lipid of six diatoms studied shone in Table 2. There was a significant difference ($P<0.05$) in the total lipid production between marine and freshwater diatoms. The highest lipid content was observed in *Nitzschia* sp. 28%, followed by *C. calcitrans* 19% and *N. sigma* 18.7% dw (marine diatoms). On the other hand, *S. pupula*, *C. cuspidata* and *N. palea* (freshwater species) had lipid contents of less than 16.5% dw). Rodolfi *et al*. (2009) reported similar results and found that marine micro-algae species including diatoms had higher lipid contents than freshwater species. As individual species, *Nitzschia* sp. recorded the highest percentage of lipid contents (28.0% dw), followed by *C. calcitrans* (19.2% dw), *N. sigma* (18.7% dw), *N. palea* (16.1% dw) and *S. pupula* (16% dw). These values were different from *Nitzschia* sp. (31% dw) as reported by Demirel et al. (2016), *C. calcitrans* (23.0% dw) as reported by Velasco et al. (2016) and *N. palea* (20.1% dw) as reported by Abdel-Hamid et al. (2013) and *S. pupula* (19.52% dw) as reported by Moreno *et al*. (2013). However, the accumulation of lipids in microalgae cells is dependent on many factors such as culture nutrients, and physical condition (Banerjee et al., 2011; Sharma *et al*., 2012; Gifuni et al., 2019; Udayan *et al*., 2022).

3.4 Fatty Acid Composition

Table 3 shows the fatty acid (FA) composition during the stationary phases of growth. A total of 28 different FA was detected in six diatoms isolated. Where Freshwater diatoms had 26 FA compared to marine species (22 FA). Fatty acids C13:0 and C20:1 was found only in *N. palea*, while C10:0, C20:2, and C22:2 was found only in *C. calcitrans, S. pupula*. and *C. cuspidata*, respectively. According to Levitan *et al*. (2014), diatoms predominantly produce 13–21-carbon FA.

In this study, Palmitoleic acid C16:1 and Palmitic acid C16:0 was predominant in both marine and freshwater diatom, together constitute up to 60%. As individual species, all species had similar percentage of C16:1 (29.9

to 34%) except *S. pupula* had the highest percentage (39 %). The presence of palmitoleic acid (C16:1) is the most favourable for biodiesel production (Durrett *et al*., 2008). In addition, high proportions of C20:5n-3 (EPA) were found in *C. cuspidata* 25.5%, and *Nitzschia* sp. 15.7%, which suggested these species as a healthy food additive in the aquaculture industry. In general, Bacillariophyceae strains including diatoms have usually high proportions of C20:5n-3 (Kates and Volcani, 1966; Ackman *et al.*, 1968; Dunstan et al., 1993; Renaud *et al*., 1994; Brown *et al*., 1997).

The fatty acid pattern can be divided into groups based on its saturation; namely saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA). In previous studies, SFA was dominant in most diatoms compared to MUFA and PUFA (Pratiwi *et al*., 2009; Prartono et al., 2013). In the present study, SFA was also dominant fatty acid in all species except *C. cuspidata* and *Nitzschia* sp. PUFA were dominant in their TFA. However, the FA content of microalgae depends not only on culture conditions (including the composition of the medium, aeration, light intensity, temperature, and age of culture) but also depends on the strains (Stonik & Stonik, 2015; Jiang *et al*., 2016) and habitats (Peltomaa *et al*. 2019).

Fig. 1. Diatom culture in growth chambers with control temperature and light.

Table 1. Growth rate of Marine and Freshwater diatoms.

Habitat	Strains	Growth rate (μ per day)	Doubling Time (days)
Freshwater diatoms	*S. pupula*	0.21± 0.02	3.3 ± 0.20
	N. palea	0.25± 0.01	2.7 ± 0.01
	C. cuspidata	0.14± 0.03	4.9 ± 1.00
Marine diatoms	*N. sigma*	0.33± 0.01	2.1 ± 0.01
	Nitzschia sp.	0.51± 0.01	1.3 ± 0.03
	C. calcitrans	0.55± 0.01	1.2 ± 0.03

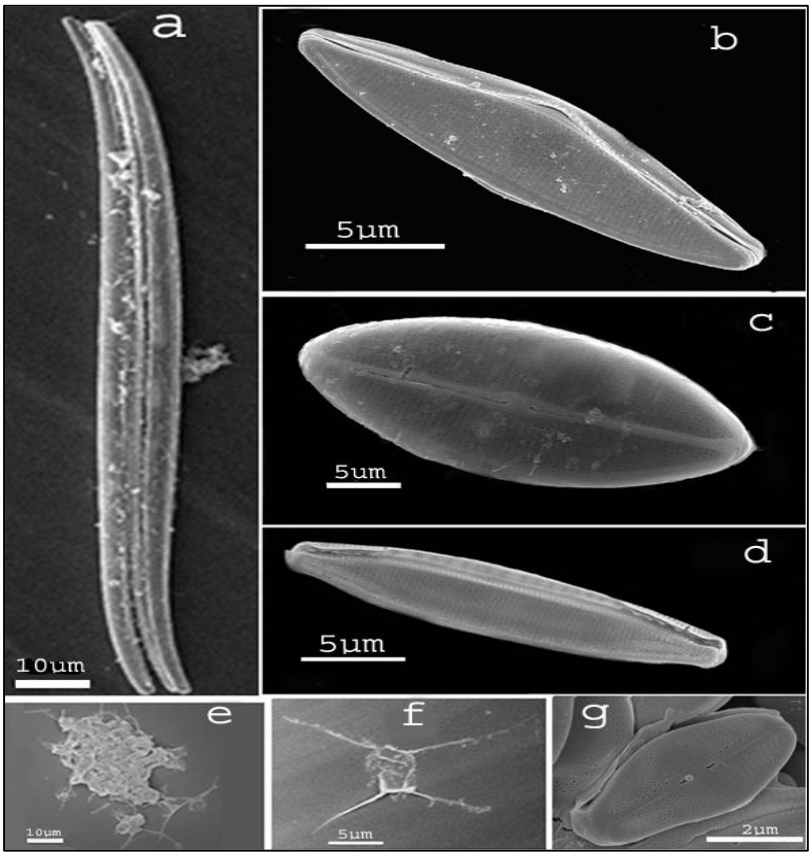

Fig. 2. Scanning electron microscopy (SEM) of the six diatoms studied. (a) *N. sigma*. (b) *Nitzschia* sp.

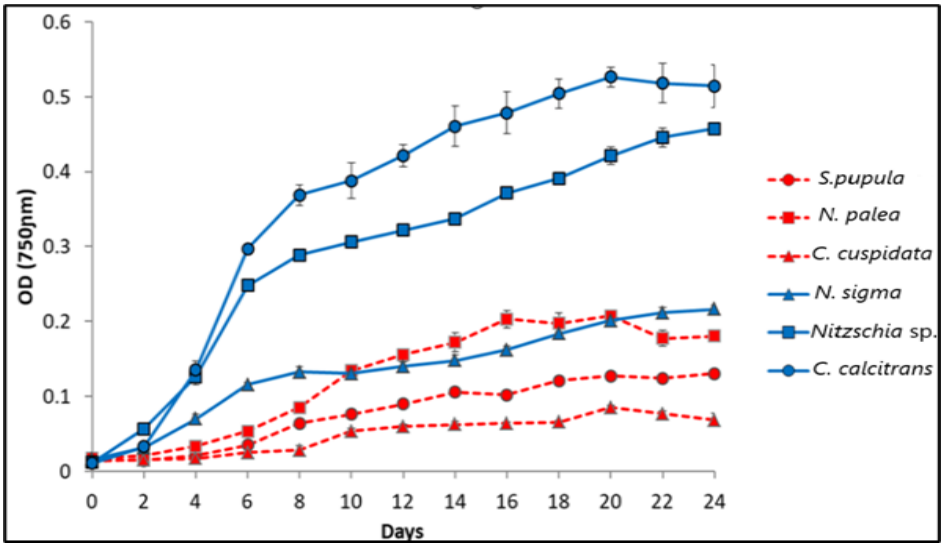

Fig. 3. Growth curves of six diatoms grown under similar conditions.

Marine diatoms (blue lines), Freshwater diatoms (red lines).

Table 2. Total lipid contents (% dw) in diatoms.

Habitat	Species	Lipid	mean
Freshwater Diatoms	*S. pupula*.	16.0 ± 1.0^{c}	15^{a}
	N. palea	16.1 ± 0.3^{c}	
	C. cuspidata	13.0 ± 1.0^{d}	
Marine Diatoms	*N. sigma*	18.7 ± 0.5^{b}	22^{b}
	Nitzschia sp.	28.0 ± 1.4^{a}	
	C. calcitrans	19.2 ± 1.5^{b}	

Different superscript letters in the same column indicate significant differences ($p < 0.05$) in lipid contents.

Table 3. Fatty acids composition of six marine and freshwater Diatoms (as % of total fatty acids).

Fatty acid	Freshwater Diatoms			Marine Diatoms		
	S. pupula	*N. palea*	*C. cuspidata*	*N. sigma*	*Nitzschia* sp.	*C. calcitrans*
Saturated Fatty Acid (SFA)						
C6:0	0.04	ND	ND	ND	ND	0.5
C10:0	ND	ND	ND	ND	ND	0.14
C12:0	0.04	ND	0.34	ND	ND	0.15
C 13:0	ND	0.12	ND	ND	ND	ND
C14:0	9.25	6.72	7.79	12.2	3.49	13.2
C16:0	33.2	26.2	15.6	29.2	27.9	33.7
C 17:0	0.05	0.29	ND	ND	ND	0.52
C 18:0	ND	7.14	3.34	1.19	ND	ND
C 20:0	1.11	ND	ND	ND	ND	ND
C 24:0	0.3	0.56	ND	0.54	ND	0.18
Monounsaturated Fatty Acid (MUFA)						
C 14:1 cis - ^{9}	0.12	0.12	ND	ND	ND	ND
C 15:1 cis - ^{10}	ND	ND	0.3	0.18	ND	ND
C16:1 cis-^{9}	39.5	34.2	32.3	29.9	30.4	31
C18:1 cis-^{9}	2.15	2.42	1.83	5.4	2.02	1.19
C18:1 trans-^{9}	ND	1.3	ND	2.06	1.46	0.97
C20:1 cis-^{11}	ND	0.65	ND	ND	ND	ND
C 24:1 cis - ^{15}	ND	1	ND	0.19	ND	ND
Polyunsaturated Fatty Acid (PUFA)						
C 18:2n-6 trans, trans -^{9,12}	0.94	0.5	5.01	1.5	ND	ND
C18:2n-6 cis, cis-^{9,12}	ND	0.5	ND	ND	1.92	0.45
C18:3n-3 (ALA)	5.04	1.23	1.94	3.66	8.66	2.2
C18:3n-6 cis-^{6,9,12}	0.67	1.18	1.63	0.07	2.04	1.17
C 20:2n-6 cis - ^{11,14}	0.14	ND	ND	ND	ND	ND
C 20:3n-6 cis - ^{8,11,14}	0.23	0.39	2.12	0	0.33	0.86
C 20:3n-3 cis-^{11,14,17}	ND	ND	ND	ND	ND	1.95
C 20:4n-6 (ARA)	7.11	2.96	1.58	0.59	4.8	2.52
C20:5n-3 (EPA)	0.13	11.6	25.5	12.6	15.7	7.62
C 22:2n-6 all cis -^{13,16}	ND	ND	0.18	ND	ND	ND
C22:6n-3 (DHA)	ND	0.81	0.47	0.62	1.28	1.6
SUM (SFA)	44	41	27.1	43.2	31.4	48.4
SUM (MUFA)	41.8	39.7	34.4	37.7	33.9	33.2
SUM (PUFA)	14.3	19.2	38.5	19.1	34.7	18.4
Sum ω-3(PUFA)	5.17	13.6	28	16.9	25.6	13.4
Sum ω-6(PUFA)	9.09	5.55	10.5	2.16	9.09	5
Total number fatty acid	17	20	15	15	12	18
	26			22		
ND: Non detected						

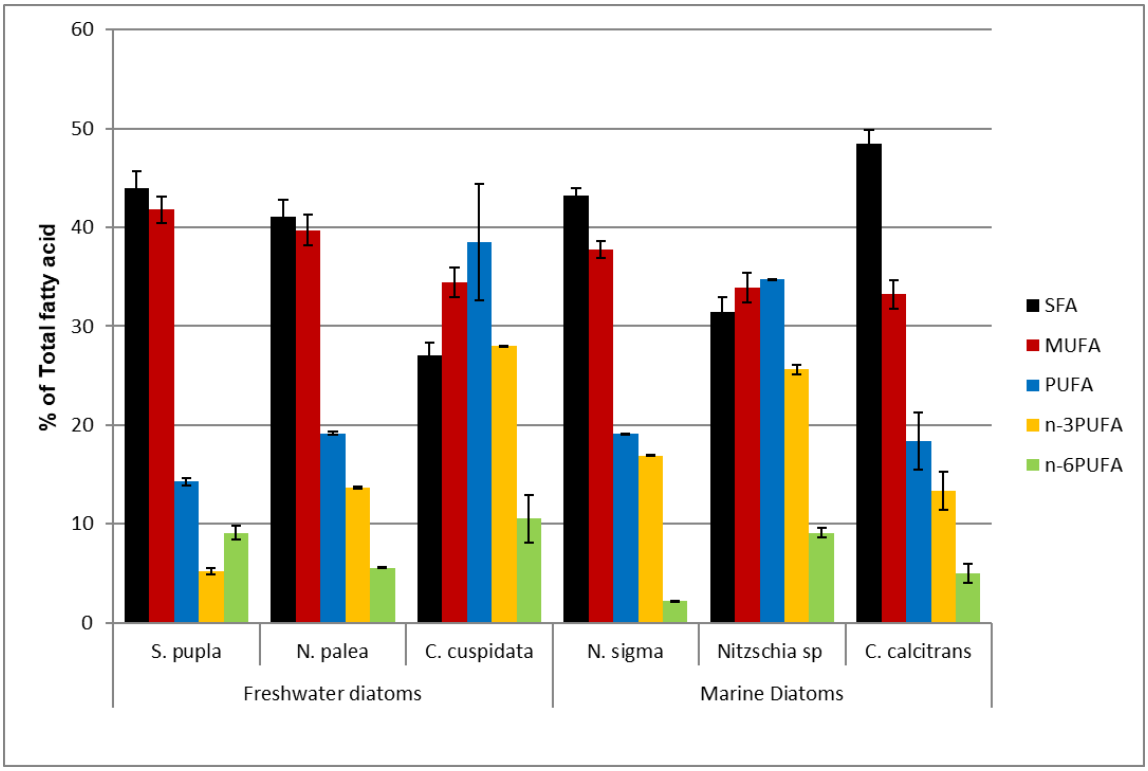

Fig. 4. SFA, MUFA, PUFA, ω-3PUFA and ω-6PUFA content of marine and freshwater Diatoms.

4. Conclusions

In conclusion, this study demonstrates the total lipids of marine diatoms (C. calcitrans, *Nitzschia* sp., and N. sigma) were significantly higher ($p < 0.05$) than freshwater diatoms (*S. pupula*, *N. palea*, *C. cuspidata*). As for quality, both marine and freshwater species had a good amount of long-chain polyunsaturated fatty acids (ω-3 and ω-6 PUFA). The lipids and fatty acids found in diatoms are vital for their survival and hold significant potential for human health and various industries such as biodiesel production. The diverse composition and functions of diatom lipids, including their role in cell structure, energy storage, and omega-3 fatty acid production, make them a fascinating subject of study. As we continue to explore the untapped potential of diatom, we may unlock innovative solutions to address environmental and health challenges.

Acknowledgements

This work was supported by the FRGS/1/2012/GS05/UPM/01/1 (Code: 02-01-13-1224FR) and the SATREPS-COSMOS Matching Fund and from the Ministry of Higher Education (MOHE).

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معدل النمو ومحتوى الدهون والأحماض الدهنية في بعض الدياتومات البحرية والعذبة

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المستخلص. الدياتومات هي كائنات حية ضوئية يمكنها إنتاج الدهون بكميات كبيرة في غضون فترة زمنية قصيرة. يمكن معالجة كتلتها الحيوية وتحويلها إلى وقود حيوي ومنتجات تجارية قيمة أخرى. في هذا البحث، تم عزل ستة أنواع من الدياتوم من المياه الماليزية (مياه البحر والمياه العذبة) ونمت تحت نفس الظروف لمراقبة الاختلافات في تركيب الدهون والأحماض الدهنية. أظهرت النتائج اختلافات كبيرة في محتوى الدهون الكلي بين أنواع المياه العذبة والبحرية. حيث كان لدى *Sellaphora pupula* و*Nitzschia palea* و*Craticula cuspidata* (أنواع المياه العذبة) نسبة ثابتة نسبيًا من الدهون (١٣ إلى ١٦,١٪ من الوزن الجاف) بينما كان لدى *Nitzschia* sp. *Chaetoceros calcitrans* و*Nitzschia sigma* (الأنواع البحرية) محتوى دهني مرتفع (> ١٨٪). تم الكشف عن ثمانية وعشرين من الأحماض الدهنية المختلفة في ستة أنواع من الدياتومات المدروسة. كان حمض البالميتوليك−C16: 1 (٢٩,٩ إلى ٣٩,٥٪) وحمض البالمتيك−C16: 0 (١٥,٦ إلى ٣٣,٢٪) هو السائد في معظم الدياتومات الستة. بالإضافة إلى ذلك، أظهرت الأحماض الدهنية غير المشبعة المتعددة ω-3 وω-6 نسبة عالية في بعض الأنواع البحرية والمياه العذبة (Nitzschia sp. و C. cuspidata)، مما يشير إلى أن كلا النوعين يتمتعان بجودة جيدة من الأحماض الدهنية المتعددة غير المشبعة ويمكن استخدامهما كمصدر للغذاء في تربية الأحياء المائية أو في تطبيقات مفيدة أخرى.

الكلمات المفتاحية: الطحالب الدقيقة، ماليزيا، الدياتومات، معدلات النمو، الدهون، الأحماض الدهنية.