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Variation in growth, fucoxanthin, fatty acids profile and lipid content of marine diatoms *Nitzschia* sp. and *Nanofrustulum shiloi* in response to nitrogen and iron

Mustafa Suha Sahin[a,1], Mahammed Ilyas Khazi[a,*,1], Zeliha Demirel[b], Meltem Conk Dalay[b]

[a] Ege University, Graduate School of Natural and Applied Sciences, Department of Biotechnology, Izmir 35040, Turkey
[b] Ege University, Faculty of Engineering, Department of Bioengineering, Izmir 35040, Turkey

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ABSTRACT

The diatoms have numerous industrial and pharmaceutical applications, especially for carotenoids and lipid production. Here, the influence of different nitrogen (N) and iron concentrations on growth, fucoxanthin, lipid accumulation and fatty acids profile of two marine diatoms *Nitzschia* sp. and *Nanofrustulum shiloi* were investigated. Both diatoms displayed relatively high growth rate and biomass concentration under N-rich conditions. Iron-rich strategy for lipid production dramatically increased the lipid content (37.7 ± 0.77% in *Nitzschia* sp. and 37.44 ± 0.54% in *N. shiloi*). N-deficiency strategy had less impact on lipid accumulation in *N. shiloi* (28.06 ± 0.54%), while, it led to a significant increase of total lipid in *Nitzschia* sp. (37.7 ± 0.77%). N- and Iron-rich conditions led to a significant increase in fucoxanthin content of both diatoms as measured by HPLC-DAD and vice versa. The data suggested that marine diatoms *Nitzschia* sp. and *N. shiloi* could be potential candidates for fucoxanthin and lipids production.

1. Introduction

Diatoms are one of the most distinctive groups of eukaryotic unicellular algae, characterized by the presence of glassy cell walls composed of silicon dioxide, occurs in various habitats like marine, brackish, freshwaters as well as in soil. They are usually estimated to contribute at least 20% of the global primary productivity (in terms of carbon fixation) and biogeochemical cycling of important nutrients (Brembu et al., 2017). Microalgae and cyanobacteria have proven to be an attractive and potentially cost-effective production platform for high-value compounds because of their fast growth, simple and inexpensive media requirements, and some species have the ability to accumulate high levels of primary and secondary carotenoids along with lipids in their biomass (Begum et al., 2016; Benavente-Valdés et al., 2016; Borowitzka, 2013). Among the xanthophylls, fucoxanthin is one of the most prominent light-harvesting carotenoid in diatoms and brown macroalgae, exhibit a characteristic brown color. Fucoxanthin binds to proteins and chlorophyll-a pigment, and forms fucoxanthin-chlorophyll-protein complexes (FCP) in the thylakoid membranes of chloroplasts, that are functionally related to the light-harvesting complex of diatoms and brown algae (Veith et al., 2009). Despite its functional food applications, fucoxanthin also possesses numerous biological activities like anticancer, anti-obese, antioxidant, anti-inflammatory, antidiabetic, antiangiogenic and antimalarial activities (Bozarth et al., 2009; Peng et al., 2011; Shannon and Abu-Ghannam, 2017). Currently, fucoxanthin is mainly produced from brown macroalgae such as *Eisenia bicyclis*, *Laminaria japonica*, and *Undaria pinnatifida*. However, fucoxanthin production using brown macroalgae is economically not feasible for commercial development due to low fucoxanthin content (Ishika et al., 2017; Xia et al., 2013). Compared to macroalgae, microalgae (diatoms) are usually rich in fucoxanthin under controlled conditions and considered as a good candidate for the mass production of fucoxanthin for commercial purposes (Kim et al., 2012).

The production of large amounts of high-value compounds and lipids by microalgae especially diatoms depends on the species and growth conditions. Under optimal growth conditions, diatoms produce a low concentration of high-value compounds which is economically not feasible for large-scale production. However, their production can be enhanced by manipulating the culture conditions and genetic manipulation (Begum et al., 2016; Benavente-Valdés et al., 2016). For instance, many studies have been documented that nutrient-stress, high-light intensity and high salt concentration induce the production

* Corresponding author.
E-mail address: khazi.ilyas@gmail.com (M.I. Khazi).
[1] Equal author contribution.

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of lipids and carotenoids such as lutein, astaxanthin, and β-carotene as a response to unfavorable growth conditions (Chrismadha and Borowitzka, 1994; Gong and Bassi, 2016; Minhas et al., 2016). Among all macro and micronutrients, nitrogen, phosphorus, silicon, and iron are an important factor that regulates growth, pigments (chlorophyll and carotenoids) and lipid accumulation in diatoms (Fu et al., 2015; Kosakowska et al., 2004; Valenzuela et al., 2013). In this study, we examined the effect of nitrogen and iron on morphological and biochemical characteristics in two marine diatoms *Nitzschia* sp. and *Nanofrustulum shiloi* of Aegean Sea, Turkey.

2. Material and methods

2.1. Strain, inoculum preparation

The diatoms *Nitzschia* sp. (EGEMACC 49) and *Nanofrustulum shiloi* (EGEMACC 47) were obtained from Ege University, Microalgae Culture Collection, Izmir, Turkey (http://www.egemacc.com/en/index.php). The two diatom strains were initially isolated and deposited in EGEMACC. The diatom *Nitzschia* sp. cultured in f/2 (Guillard, 1975) and *N. shiloi* in BG11 medium (Stanier et al., 1971) made with artificial seawater and the cultures were maintained at 20 ± 2 °C under continuous illumination of 40 μmol photons m^{-2} s^{-1} in 250 mL Erlenmeyer flasks. Artificial seawater used in this study was prepared by dissolving 25 g L^{-1} sea salt (Marinium reaf sea salt) in deionized water (Khazi et al., 2018).

Cultures were grown in 500 mL Erlenmeyer flasks containing 200 mL of both (f/2 for *Nitzschia* sp. and BG11 for *N. shiloi*) on a rotary shaker at 120 rpm, 22 ± 2 °C, and continuous illumination of 40 μmol photons m^{-2} s^{-1}. Cells were harvested during the exponential growth phase; centrifuged at 4500 ×*g* for 5 min and the pelleted cells were washed with phosphate buffer saline (pH 7.4) to remove salts. The recovered cells were then re-inoculated in nitrogen and iron-deficient f/2 and BG11 medium and used as inoculum at 10% (v/v) concentration for further experiments.

2.2. Experimental design

The experiments were carried out with varied nitrogen and iron concentrations in f/2 and BG11 culture media formulated in artificial seawater. Standard f/2 and BG11 media were used as a control. N-deficient and Iron-deficient media were prepared by omitting the addition of nitrogen and iron from the standard f/2 and BG11 formulations. Medium containing 2 g L^{-1} nitrate ($NaNO_3$) correspond to the "N-rich" and medium containing 0.0158 g L^{-1} iron ($FeCl_36H_2O$ and Ferric ammonium citrate) correspond to the "Iron-rich" formulations of standard f/2 and BG11. The concentrations of nitrogen and iron used in the media are described in Table 1. A set of three 2 L sterile bottles which containing 1.8 L of respective medium were inoculated with 10% (v/v) inoculum were prepared and cultivated under the light intensity of 300 μmol photons m^{-2} s^{-1} (LED CATA, Cool white), the temperature of 22 ± 2 °C, and with the aeration of 2 L min^{-1}. The growth was monitored by counting cells at 2-day intervals over the period of 14 days. After 14 days biomass was harvested and used for fucoxanthin, total lipid content and fatty acids analysis.

2.3. Specific growth rate and biomass calculation

The specific growth rate (μ) and doubling time (t_d) was determined at the exponential growth phase of cultures and calculated according to standard formula

$$\mu = \frac{\ln (X_2) - \ln (X_1)}{(t_2 - t_1)} t_d = \frac{\ln 2}{\mu}$$

Where X_1 is the initial cell number at time t_1 and X_2 is the cell number at time t_2.

Dry weight was determined by filtering a 5 mL culture sample through pre-weighed GF/C filter (Whatman, UK), then washed with distilled water and dried at 65 °C to a constant weight.

2.4. Measurement of total lipid content

Lipids were extracted from lyophilized diatoms biomass by a modified method of Bligh and Dyer (Bligh and Dyer, 1959). Freeze-dried cells (0.1 g) were weighed accurately into a 15.0 mL centrifuge tube and disrupted with an ultrasonic cell crusher (9 cycles, 90% power for 1 min). Extraction was carried out using 4 mL of chloroform and methanol at a proportion of 2:1 (v/v) containing 0.5 mg mL^{-1} Butylated hydroxytoluene (BHT). The tubes were shaken gently for 12 h at room temperature, then centrifuged at 6000 *xg* for 15 min and the supernatant containing the extracted oil was filtered through 0.45 μm PTFE disk filters and added the water, then the upper phase was separated and the extract was evaporated using a rotary evaporator (Stuart, RE300, UK) to remove solvents. To calculate lipid content the following formula was used.

$$X(\%) = \frac{X(T)}{Y} \mathrm{X100}$$

Where, *X* (%) is the percent lipid, *X* (*T*) is total lipid, Y is total biomass.

2.5. Fatty acids analysis by Gas Chromatography (GC)

Fatty acids were analyzed by GC (Typical Agilent 6890 GC Gas Chromatograph FID System) using the Turkish standard methods TS EN ISO 12966–2:2011 and TS EN ISO 15304 described previously (Demirel et al., 2015).

2.6. Fucoxanthin quantification by HPLC

The biomass (0.05 g) was extracted with 10.0 mL of methanol containing 0.010% (w/v) pyrogallol. The mixture was placed in an ultrasonic bath (Hydra Ultrasonic) for extraction. After the ultrasonic extraction, the solution was centrifuged at 6000 rpm for 10 min. The supernatant was filtered through 0.45 μm PTFE disk filters and fucoxanthin was analyzed by HPLC-DAD (Thermo Scientific Ultimate 3000, Diode array detector) using YMC Carotenoid column (C30, 5 mm, 250 × 4.6 mm I.D.). The column temperature was set at 25.0 °C. For the fucoxanthin assay, Methanol: Methyl tert-butyl ether: water (70:25:5)

Table 1
Concentrations of nitrogen and iron used in the medium.

	Concentrations in the standard medium		N-rich	N-deficient	Iron-rich	Iron-deficient
	$NaNO_3$ g L^{-1}	Iron g L^{-1}	($NaNO_3$ g L^{-1})	($NaNO_3$ g L^{-1})	(Iron g L^{-1})	(Iron g L^{-1})
f/2	0.075	0.00315 ($FeCl_36H_2O$)	2	0	0.0158 ($FeCl_36H_2O$)	0
BG11	1.5	0.006 (Ferric ammonium citrate)	2	0	0.0158 (Ferric ammonium citrate)	0

was used as the mobile phase and run at a flow rate of 1 mL/min. Under these conditions, standard fucoxanthin (Sigma-Aldrich) was given a maximum absorbance at 475 nm. For calibration standard (0.1–8 mg L^{-1}) was prepared from the stock solution and then standard curve was determined concentration of fucoxanthin (Erdoğan et al., 2016).

2.7. Nile red staining

The lyophilized diatom cells were re-suspended into 500 µL phosphate buffer (pH-7.0). Then 10 µL of Nile red solution (stock 1 mg 100 mL^{-1} isopropanol) was added to the cell suspensions and incubated for 5 min in the dark. Stained diatom cells were observed by Leica fluorescent microscopy.

2.8. Scanning Electron Microscopy (SEM)

Lyophilized diatom biomass powders were mounted on an aluminum stub with double-sided tape (diatom frustule was not treated with acid before mounting) and then coated with a thin gold layer using Emitech K550X sputter coater, with Edwards RVS vacuum pump. SEM imaging was observed using Philips XL 30 S FEG by Izmir Institute of Technology, Turkey.

2.9. Statistical analysis

Experiments were conducted in triplicate and the data were expressed as mean ± standard deviation. Statistical significance ($p < 0.05$) for the data was analyzed using one-way analysis of variance (ANOVA) and multiple comparisons among means were made using the Tukey's post hoc test with IBM SPSS Statistics version 23.0 software.

3. Results

3.1. Morphological changes

The scanning electron micrographs showed that varied nitrogen and iron conditions triggered the morphological changes in both *Nitzschia* sp. and *Nanofrustulum shiloi* (Figs. 1 and 2) compare to standard conditions. The size of *Nitzschia* sp. cells grown in the standard f/2 medium was approximately 21 ± 2 µm long (Fig. 1a), whereas, the cell size of *Nitzschia* sp. cultured in N-rich and N-deficient f/2 medium decreased slightly and the length of the cells was approximately 20 ± 1 µm (Fig. 1b and c). The length of the *Nitzschia* sp. cells was 45 ± 2 µm in Iron-rich f/2 medium (Fig. 1d). While the cell size was decreased in Iron-deficient cultures relative to Iron-rich cultures and the length of the cells varied from 35 ± 2–40 ± 2 µm (Fig. 1e). In case of *N. shiloi*, the cells are rectangular, forming chains linked by interlocking marginal spines, valves are circular, 3.1–3.7 ± 0.3 µm in diameter and there is no significant changes observed in the diameter of the valve cultured under nutrient rich and deficient conditions. However, changes in cells linking and interlocking marginal spines were observed. The cells cultured in N-rich and Iron-rich media showed poor linking (Fig. 2b and d), whereas, cells cultured in N- and Iron-deficient media shown strong linking and tight association of marginal spines (Fig. 2c and e).

3.2. Effect of nutrient-rich and deficient conditions on growth and biomass

The two marine diatom strains presented different responses in terms of maximum specific growth rates and biomass concentrations under nutrient-deficient and nutrient-rich conditions (Table 2). In this study, two different media have been used for the cultivation of marine diatoms. The *Nitzschia* sp. has been cultivated in the f/2 medium. Whereas, *N. shiloi* grown well in BG11 medium than f/2 medium. Therefore, BG11 medium has been selected for the cultivation of *N. shiloi*. The time-course profiles of cell growth obtained with different nutrient-stress conditions are illustrated in Fig. 3. The *Nitzschia* sp. cultured in N-rich f/2 medium grew faster and reached highest cell density of 55.9 ± 1.4 × 10^4 cells mL^{-1} with the specific growth rate of 0.313 ± 0.02 day^{-1} followed by cell density of 51.67 ± 1.4 × 10^4 cells mL^{-1} with the specific growth rate of 0.294 ± 0.00 day^{-1} in the Iron-rich f/2 medium. The specific growth rates of *Nitzschia* sp. cultured in N- and Iron-deficient f/2 medium were not significantly different

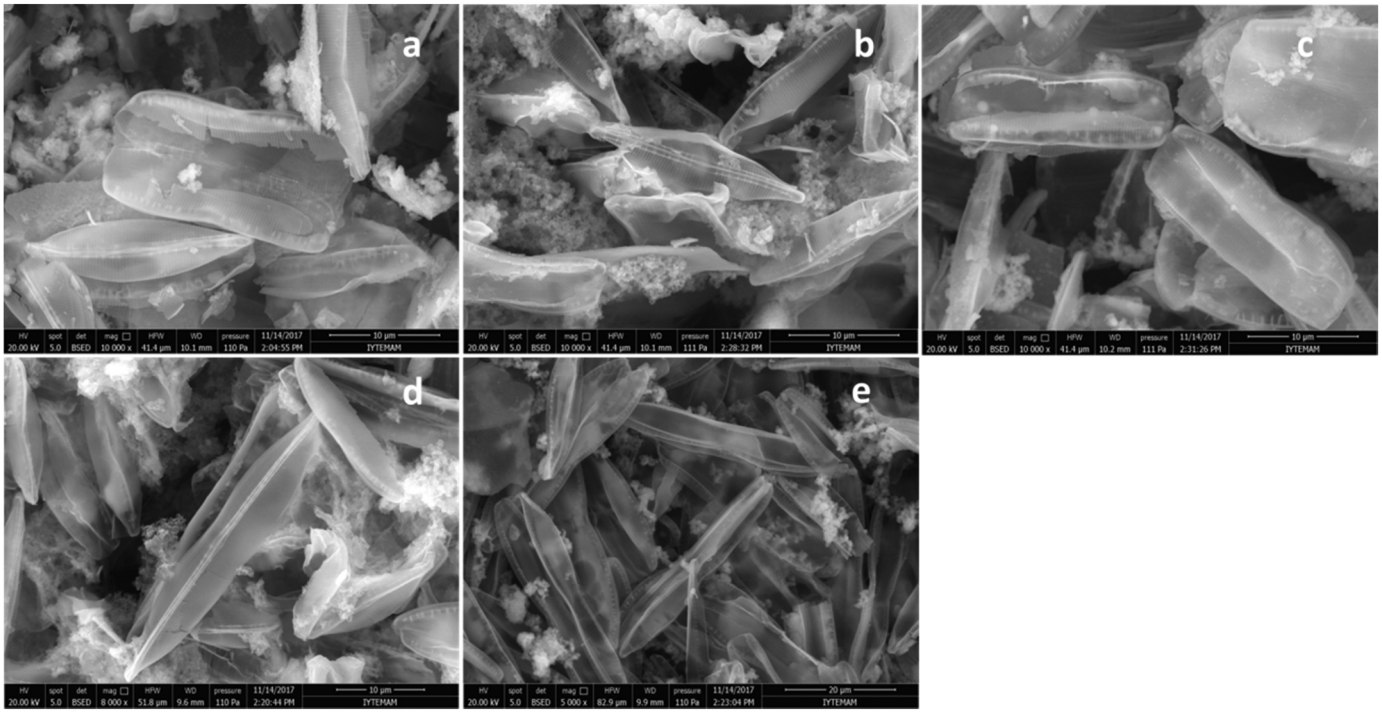

Fig. 1. Scanning electron microscope (SEM) images of *Nitzschia* sp. grown under (a) standard f/2 (b) N-rich (c) N-deficient (d) Iron-rich (e) Iron-deficient conditions.

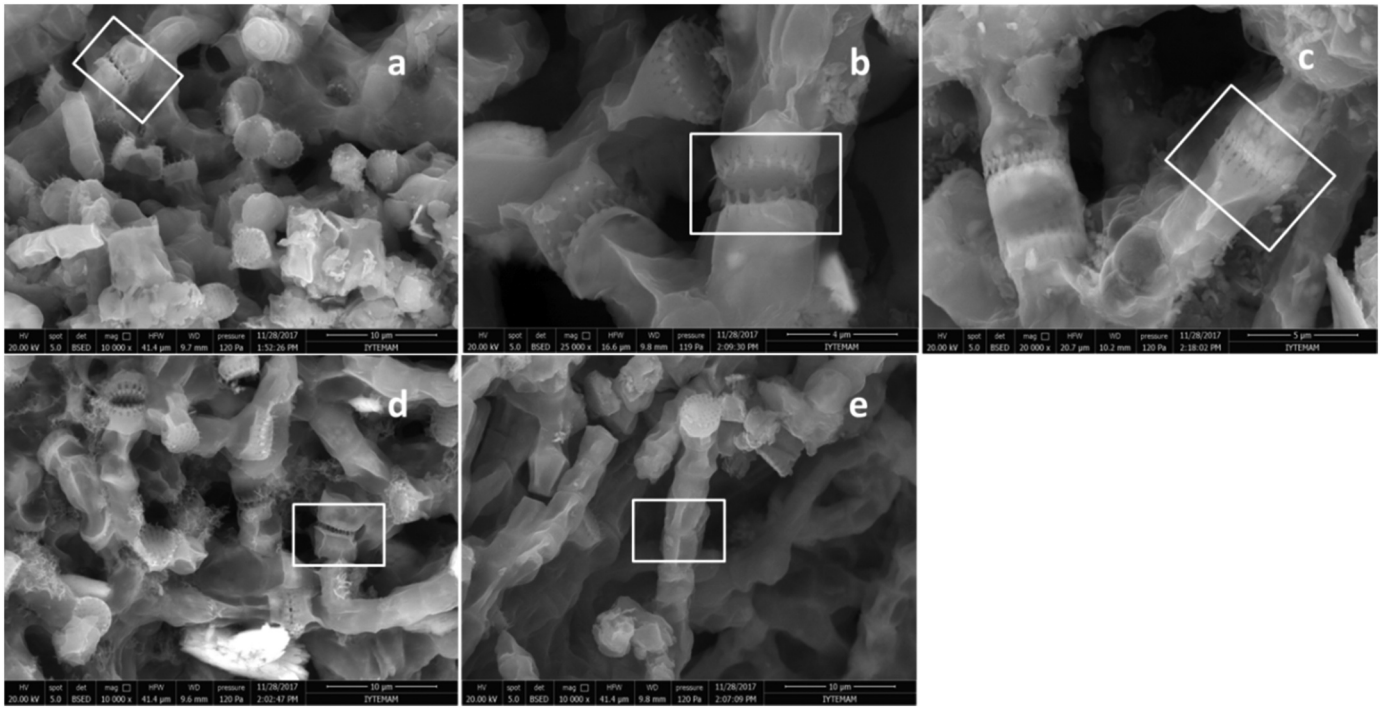

Fig. 2. Scanning electron microscope images of *Nanofrustulum shiloi* grown under (a) standard BG11 (b) N-rich (c) N-deficient (d) Iron-rich (e) Iron-deficient conditions.

($p > 0.05$) and the average maximum specific growth rates were 0.218 ± 0.01 (30.58 ± 1.4 ×10^4 cells mL^{-1}) and 0.206 ± 0.00 day^{-1} (26.33 ± 1.3 ×10^4 cells mL^{-1}), respectively. The diatom *N. shiloi* cultured in N-rich BG11 medium exhibited fastest specific growth rate of 0.368 ± 0.01 day^{-1} and reached the highest cell density of 313.10 ± 6.3 × 10^4 cells mL^{-1}, followed by specific growth rate of 0.323 ± 0.01 day^{-1} and the cell density of 310.25 ± 6.3 × 10^4 cells mL^{-1} in the Iron-rich BG11 medium. While in standard BG11 medium the cell density and specific growth rate were 296.00 ± 2.9 × 10^4 cells mL^{-1} and 0.305 ± 0.01 day^{-1}. There is no significant difference between the growth rate of N- and Iron-deficient cultures ($p > 0.05$).

The diatoms *Nitzschia* sp. and *N. shiloi* cultured in N-rich medium produced the highest biomass concentration of 2.66 ± 0.07 g L^{-1} and 3.034 ± 0.00 g L^{-1} followed by the values of 1.868 ± 0.02 g L^{-1} and 2.42 ± 0.03 g L^{-1} cultured in the Iron-rich medium. In standard f/2 medium, the obtained biomass concentration of *Nitzschia* sp. was 0.638 ± 0.02 g L^{-1}. Whereas, in standard BG11 medium, the obtained average biomass concentration of *N. shiloi* was 1.540 ± 0.03 g L^{-1}. While, cultures grown in Iron-deficient f/2 and BG11 media had the lowest biomass concentrations.

3.3. Variation in lipid and fucoxanthin content

The two diatoms stained by Nile red were observed under fluorescence microscope for the presence of intracellular lipid droplets. As shown in Fig. 4, cells emitted red fluorescence indicating the presence of neutral lipids. The intensity of the Nile red fluorescence confirmed the presence of a large number of lipids accumulated in the cells. The lipid yield (% dry cell weight) of the two diatoms grown under standard, nutrient-rich and deficient conditions are shown in Fig. 5. Among the two strains investigated, *Nitzschia* sp. cultured in N-deficient f/2 medium shown significantly higher lipid content of 37.7 ± 0.77% ($p < 0.05$) compares to N-rich f/2 medium, while the corresponding lipid content in the N-rich f/2 medium was 21.62 ± 0.64%. Furthermore, the lipid content of *Nitzschia* sp. cultured in Iron-rich and deficient f/2 medium were 37.03 ± 0.61% and 17.82 ± 0.72%, respectively. Statistically there is no significant difference between the lipid content of Iron-rich and N-deficient cultures ($p > 0.05$). Whereas, in the case of *N. shiloi*, the highest lipid content of 37.44 ± 0.54% was observed in the cultures of the Iron-rich BG11 medium. However, the corresponding lipid content in the N-deficient BG11 medium was 28.06 ± 0.54%.

Table 2
Specific growth rate, biomass, total lipid and fucoxanthin content of marine diatoms grown under different nutrient conditions.

Strain	Culture medium	Specific Growth rate μ (day^{-1})	Doubling time (days)	Fucoxanthin mg g^{-1}
Nitzschia sp.	Standard f/2	0.227 ± 0.01	3.05 ± 0.18	2.23 ± 0.67
	N- rich f/2	0.313 ± 0.02	2.21 ± 0.15	7.83 ± 0.31
	N-deficient f/2	0.218 ± 0.01	3.17 ± 0.17	0.72 ± 0.04
	Iron-rich f/2	0.294 ± 0.00	2.35 ± 0.01	6.16 ± 0.55
	Iron-deficient f/2	0.206 ± 0.00	3.36 ± 0.11	4.89 ± 0.10
N. shiloi	Standard BG11	0.305 ± 0.01	2.26 ± 0.10	3.52 ± 0.00
	N-rich BG11	0.368 ± 0.01	1.88 ± 0.09	5.71 ± 0.50
	N-deficient BG11	0.294 ± 0.01	2.35 ± 0.08	3.12 ± 0.06
	Iron-rich BG11	0.323 ± 0.01	2.14 ± 0.06	7.83 ± 0.14
	Iron-deficient BG11	0.292 ± 0.01	2.37 ± 0.11	5.23 ± 0.07

*values mean ± SD (n = 3).

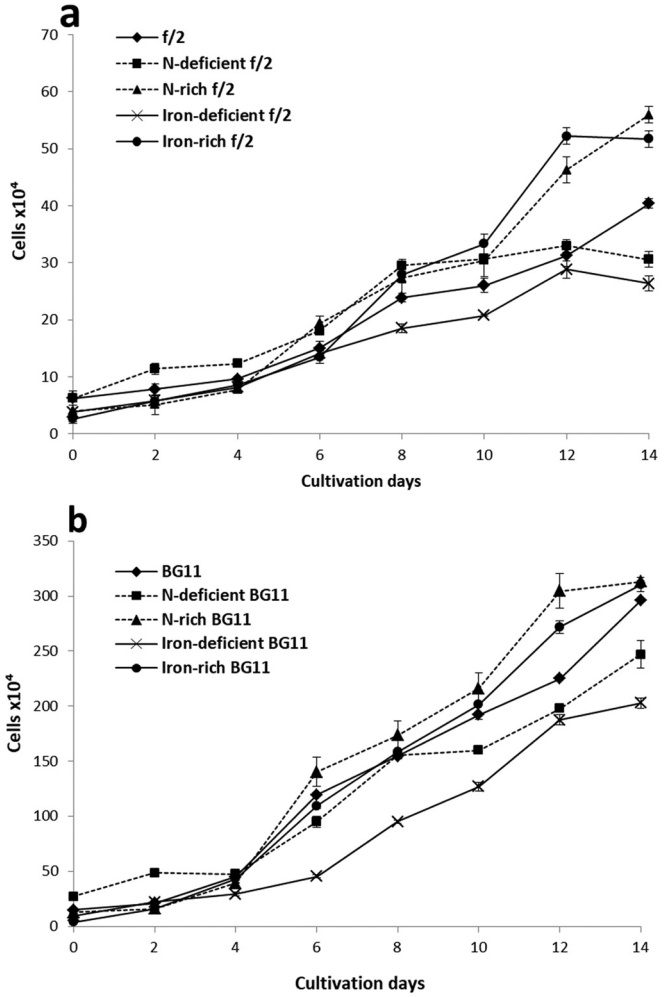

Fig. 3. Growth profile of (a) *Nitzschia* sp. (b) *N. shiloi* cultivated under different nutrient conditions.

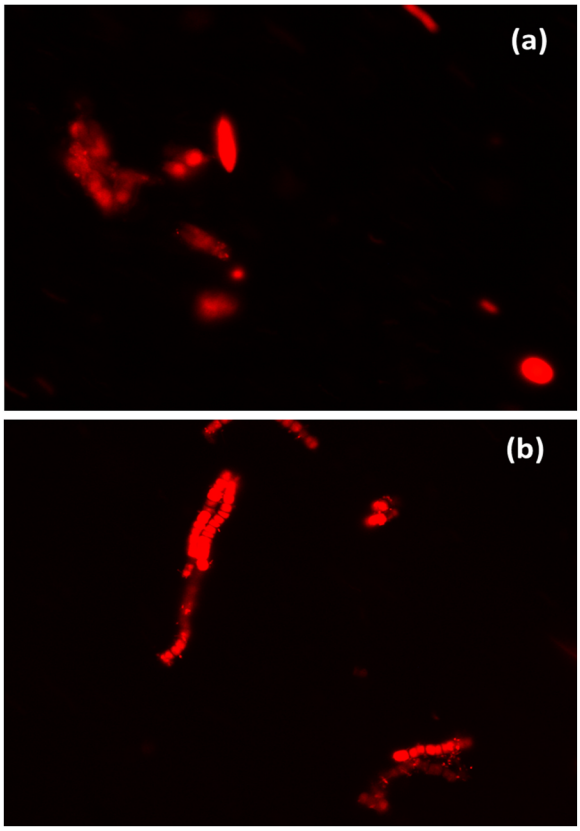

Fig. 4. Nile red fluorescence of representative (a) *Nitzschia* sp. (b) *N. shiloi* cells.

The influence of varied nutrient conditions on fucoxanthin production of both *Nitzschia* sp. and *N. shiloi* is summarized in Table 2. The fucoxanthin content of *Nitzschia* sp. was significantly different in nutrients-stress induced cultures compared to standard f/2 cultures ($p < 0.05$). The highest fucoxanthin content of 7.83 ± 0.31 mg g^{-1} dry weight was found in the *Nitzschia* sp. grown in N-rich f/2 medium which was 3.5 times higher than that of standard f/2 culture (2.23 ± 0.67 mg g^{-1}); whereas the lowest content of 0.72 ± 0.04 mg g^{-1} dry weight was obtained in the cultures of the N-deficient f/2 medium. The fucoxanthin content of *Nitzschia* sp. cultured in Iron-rich and deficient f/2 medium were 6.16 ± 0.55 and 4.89 ± 0.10 mg g^{-1} dry weight, respectively. On the other hand, *N. shiloi* grown in the Iron-rich BG11 medium produced the highest fucoxanthin content of 7.83 ± 0.31 mg g^{-1} dry weight which was 2.2 fold higher than that of standard BG11 culture (3.52 ± 0.00 mg g^{-1}). The concentration of fucoxanthin was lower in the cultures of N-rich (5.71 ± 0.50 mg g^{-1}) and N-deficient BG11 medium (3.12 ± 0.06 mg g^{-1}) in comparison with the cultures of Iron-rich BG11 medium and statistically, there is no significant difference between the fucoxanthin content of N-deficient and standard BG11 cultures ($p > 0.05$).

3.4. Variation in fatty acids

The fatty acid profile of *Nitzschia* and *N. shiloi* grown in the standard, nutrient-rich and deficient medium are summarized in Tables 3, 4. Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), palmitoleic acid (C16:1), linoleic acid (C18:2) and linolenic acid (C18:3) were found to be major contributing fatty acids in both diatoms lipid profile. However, there was a difference in the percentage composition of saturated and unsaturated fatty acids (Fig. 6). The proportions of saturated (SFAs), mono-unsaturated (MUFAs) and poly-unsaturated fatty acids (PUFAs) of *Nitzschia* sp. cultivated in the standard f/2 medium were 63.43%, 27.58% and 9.03% of the total fatty acids, respectively. Fatty acid profile of *Nitzschia* sp. showed a decrease in SFAs, an increase of MUFAs under nutrient-rich and deficient when compared to the control condition (standard f/2 medium). The percent composition of SFAs of *Nitzschia* sp. decreased from 63.43% to 37.16%, 42.46%, 41.36% and 32.48%, while, the sum of MUFAs increased from 27.58% to 55.59%, 49.85%, 52.79% and 57.80% in the cultures of N-rich, N-deficient, Iron-rich, and Iron-deficient f/2 medium, respectively. Whereas, the total PUFAs of 7.23%, 7.68%, 5.84%, and 9.71% was observed in the cultures of N-rich, N-deficient, Iron-rich, and Iron-deficient f/2 medium, respectively.

In case of *N. shiloi*, the sum of SFAs 64.79% was observed in the cultures of standard BG11 medium, whereas, sum SFAs reduced to 57.38%, 55.60% and 53.68% in the cultures of N-deficient, Iron-rich, and Iron-deficient BG11 medium, respectively. However, the slightly higher value of 65.81% SFAs was observed in the cultures of N-rich BG11 medium compare to standard BG 11 medium (64.79%) (Fig. 6). The proportion of MUFAs and PUFAs of the total fatty acids fraction observed in the standard BG11 medium were 29.85% and 5.35%. Compare to standard BG11 cultures, the higher values of 37.39% and 34.71% MUFAs was observed in the cultures of N-deficient and Iron-rich BG11 medium, while, the lower value of 24.30% was observed in the cultures of N-rich BG11 medium. The maximum sum of PUFAs 17.13% was observed in the cultures of the Iron-deficient BG11 medium which was 3.2 fold higher than the control cultures (5.35%). Whereas, the total PUFAs 9.88%, 4.44%, and 9.68% were observed in the cultures of N-rich, N-deficient, and Iron-rich BG11 medium, respectively.

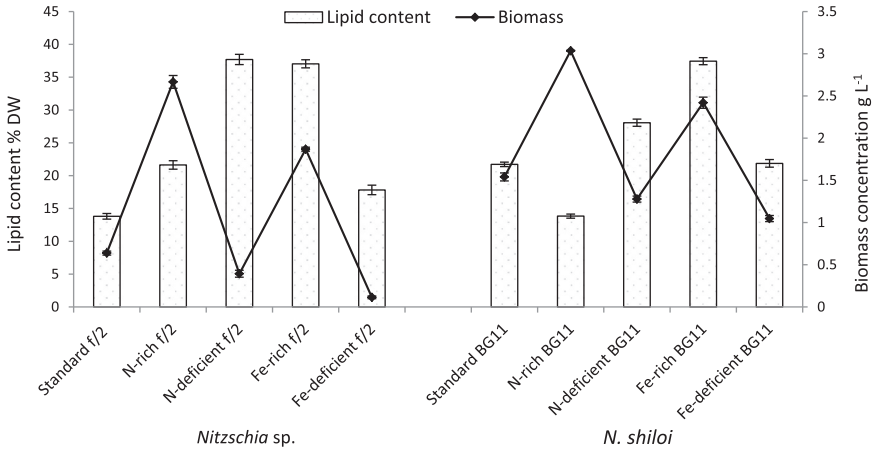

Fig. 5. Biomass and lipid content of *Nitzschia* sp. and *N. shiloi* grown under different nutrient conditions.

4. Discussion

Diatoms are microscopic, single-celled or colonial algae having complex two-sided cell walls composed of silicon and exhibit a large variety of shapes. Nutrient-stress generally induces changes in both morphology and biochemical composition of diatom and microalgae. Many studies reported the changes in diatoms cells in response to Iron-limitation or deficiency (Allen et al., 2008; Leynaert et al., 2004; Marchetti and Cassar, 2009). The iron concentration may affect the cell size of algal species differently. For example, an increase in *Nitzschia* sp. cell size under iron stress was reported earlier (Takeda, 1998). Similarly, a decrease in *C. fusiformis*'s cells size with decreasing iron concentrations was also reported (Leynaert et al., 2004). Moreover, the nutrient-stress induced change in cell size was also viewed in diatom *Attheya longicornis*. Under the Iron-depleted conditions, the surface area to cell volume ratio, silicon cell quota and siliceous horn length of the *Attheya longicornis* increased 2.3-, 2.3- and 1.4-fold, respectively. Whereas, N-depleted conditions led to decrease the cells size (Sugie and Kuma, 2017). The iron induced change in cell size and an increase in surface area to cell volume were also observed in *Phaeocystis antarctica* and in other diatom species (Zhu et al., 2016). Stress conditions, especially, N-deficiency triggers the degradation of nitrogenous compounds such as DNA, protein and chlorophyll results in cellular chlorosis and leads to a reduction in photosynthetic capacity, which in turn negatively affects cell growth (Pancha et al., 2014). Hence, lower growth rates and biomass concentrations have been observed under N and Iron-deficient conditions. The decrease in growth rate under Iron-deficient conditions has also been reported in the literature (van Oijen et al., 2004; Wan et al., 2014).

Nitrogen (N) is an essential major element required for the synthesis of nucleic acids, amino acids, proteins, coenzymes, chlorophyll, and other photosynthetic pigments, while Iron require for photosynthesis, nitrogen assimilation and other metabolic pathways. Based on the results obtained, the strategy of N-deficient and Iron-rich conditions can dramatically promote the lipid content of both diatoms. However, the N-deficient strategy had less impact on lipid accumulation in *N. shiloi*, which has the highest lipid content under Iron-rich conditions. For *N. shiloi*, the increase in lipid content triggered by N-deficient conditions was only 1.29-fold relative to standard BG11. Under N-deficiency majority of the genes associated with photosynthesis and chlorophyll biosynthesis were repressed and intracellular proteins may be degraded to supply nitrogen to maintain the metabolic functions which ultimately leads to a reduction in photosynthetic rates and metabolic flux towards lipid biosynthesis (Alipanah et al., 2015). It has been reported that the effects of N-deficiency on growth and lipid accumulation are species-specific, for example, cyanobacteria (*Arthrospira platensis*) and microalgae (*Dunaliella primolecta*, *Tetraselmis suecica,* and *Isochrysis* spp.) cultured under N-depletion conditions showed increases in carbohydrate contents instead of lipid (Panyakampol et al., 2016; Thomas et al., 1984). However, most microalgae (including diatoms) increase

Table 3
The fatty acids composition as the percentage of total fatty acids in *Nitzschia* sp. Values are the results obtained from single sample (n = 1).

Fatty acids (%)	Standard f/2	N-rich f/2	N-deficient f/2	Iron-rich f/2	Iron-deficient f/2
Myristic acid (C14:0)	6	9.62	8.62	3.21	5.46
Pentadecanoic acid (C15:0)	1.32	0.61	0.88	0.60	0.65
Palmitic acid (C16:0)	32.11	21.36	27.42	26.97	24.32
Heptadecanoic acid (C17:0)	N.D.	N.D.	N.D.	N.D.	0.11
Stearic acid (C18:0)	19.85	4.58	3.24	7.42	N.D.
Tricosanoic acid (C23:0)	1.89	0.59	1.69	1.29	1.36
Lignoceric acid (C24:0)	2.24	0.37	0.58	1.84	0.57
Sum of saturated	**63.43**	**37.16**	**42.46**	**41.36**	**32.48**
Palmitoleic acid (C16:1)	24.03	53.77	48.20	48.15	56.18
Oleic acid (C18:1n9c)	3.54	1.81	1.648	4.64	1.617
Sum of mono-unsaturated	**27.58**	**55.59**	**49.85**	**52.79**	**57.80**
Linoleic acid (C18:2n6c)	1.60	0.87	0.80	1.26	1.041
Trans Linolenic acid (C18:3n6)	1.39	1.49	1.12	1.26	1.62
cis-8,11,14- Eicosatrienoic acid (C20:3n6)	0.83	N.D.	0.21	N.D.	0.31
cis-13,16 - Docosadienoic acid (C22:2)	N.D.	N.D.	N.D.	N.D.	0.36
cis-5,8,11,14,17- Eicosapentaenoic acid (C20:5n3)	3.04	4.18	3.92	1.32	5.42
cis-4,7,10,13,16,19-Docosahexaenoic acid (C22:6 ns)	2.15	0.68	1.62	1.98	0.95
Sum of poly-unsaturated	**9.03**	**7.23**	**7.68**	**5.84**	**9.71**

*N.D. Not detected.

Table 4
The fatty acids composition as the percentage of total fatty acids in *Nanofrustulum shiloi*. Values are the results obtained from single sample (n = 1).

Fatty acids (%)	Standard BG11	N-rich BG11	N-deficient BG11	Iron-rich BG11	Iron-deficient BG11
Myristic acid (C14:0)	1.83	1.98	1.87	2.43	1.91
Pentadecanoic acid (C15:0)	0.67	0.91	1.07	1.04	1.07
Palmitic acid (C16:0)	42.77	30.95	45.47	32.78	33.34
Heptadecanoic acid (C17:0)	N.D.	N.D.	0.67	0.73	0.62
Stearic acid (C18:0)	16.71	23.35	4.71	11.72	8.76
Tricosanoic acid (C23:0)	2.79	7.99	3.58	6.88	7.96
Lignoceric acid (C24:0)	N.D.	0.61	N.D.	N.D.	N.D.
Sum of saturated	**64.79**	**65.81**	**57.38**	**55.60**	**53.68**
Palmitoleic acid (C16:1)	27.32	18.03	35.75	29.51	23.14
Oleic acid (C18:1n9c)	2.52	6.26	1.64	5.19	6.03
Sum of mono-unsaturated	**29.85**	**24.30**	**37.39**	**34.71**	**29.18**
Linoleic acid (C18:2n6c)	2.02	2.25	1.31	2.04	5.99
Trans Linolenic acid (C18:3n6)	0.61	0.91	0.60	1.19	1.84
cis-11,14- Eicosadienoic acid (C20:2)	N.D.	N.D.	N.D.	N.D.	1.32
cis-8,11,14- Eicosatrienoic acid (C20:3n6)	0.50	1.13	N.D.	N.D.	1.18
cis-5,8,11,14,17- Eicosapentaenoic acid (C20:5n3)	2.20	5.59	2.52	6.44	6.78
Sum of poly-unsaturated	**5.35**	**9.88**	**4.44**	**9.68**	**17.13**

*N.D. Not detected.

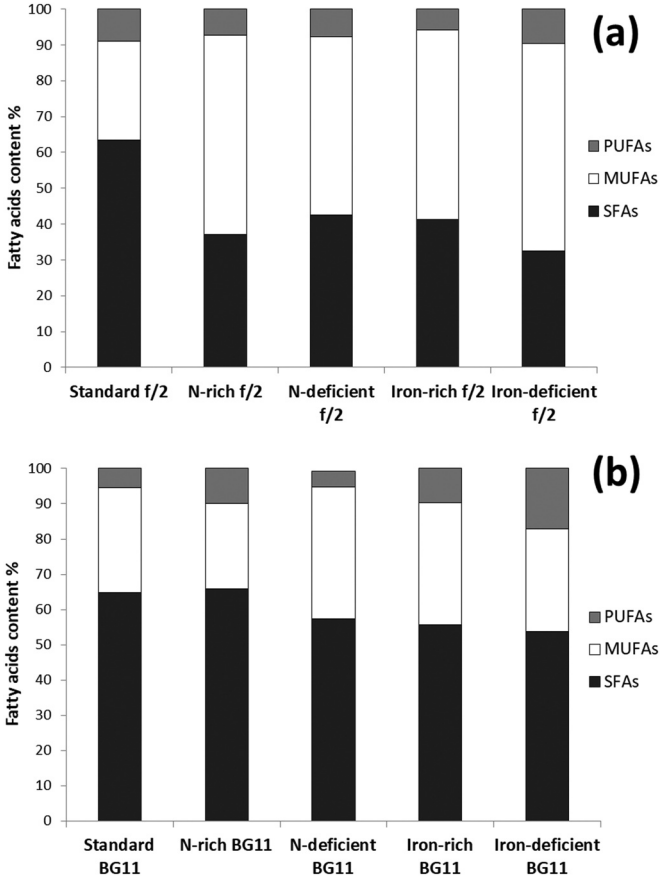

Fig. 6. The distribution shows the percentage of total fatty acids as saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) of (a) *Nitzschia* sp. (b) *N. shiloi* grown under different nutrient conditions.

the lipid content under N-limitation (d'Ippolito et al., 2015). Among the trace metals, iron is known to influence the lipid accumulation. Metal stress can lead to the formation of reactive oxygen species and other oxidant compounds. Therefore, under stress conditions microalgae induce more lipid synthesis to counteract the damaging effect of reactive oxygen species (Sivaramakrishnan and Incharoensakdi, 2017; Miazek et al., 2015). In the present study, *Nitzschia* sp. and *N. shiloi* accumulated higher lipid content (37.03 ± 0.61% and 37.44 ± 0.54) under Iron-rich conditions and a balance between cell growth, biomass concentration, and lipid accumulation have been noticed in Fe-rich conditions. This phenomenon had also been observed in other microalgae. In a previous study, *Chlorella vulgaris* was cultivated in the medium containing a higher level of iron and 3–7 folds increase in lipid accumulation as compared to the cultures of lower iron concentration was observed (Liu et al., 2008). Similarly, the maximum lipid yield of 28.12% was reported for *Scenedesmus obliquus* grown in a Fe-rich medium. Studies have shown that the Fe-rich conditions increased the lipid content in *Ankistrodesmus falcatus* (Singh et al., 2015), *Scenedesmus dimorphus* (Ruangsomboon et al., 2013), and *Botryococcus* spp. (Yeesang and Cheirsilp, 2011).

Earlier reports have shown that the diatom *Phaeocystis* sp. cells grown with high iron concentration contained fucoxanthin as the major carotenoid, while; Iron-depletion resulted in a decrease of all light-harvesting pigments. The iron and nitrogen limited cells show a high susceptibility to photooxidation, therefore, under such high light and low iron/nitrogen conditions cells activate the photoprotective cycle of diato/diadinoxanthin, which results in a relative increase of photoprotective pigments compared to light-harvesting pigments (van Leeuwe and Stefels, 1998). In the current study, the maximum fucoxanthin concentration was found in the cultures of N- and Iron- rich conditions, whereas, the lower concentration was found in the N- and Iron-deficient cultures. The decrease in fucoxanthin content in response to N-depletion has also been reported in *P. tricornutum* (Gao et al., 2017).

Among the environmental factors, light has a marked effect on the lipid production and fatty acid profiles of diatom. It has been reported that high light promotes the accumulation of SFAs and MUFAs, whereas, low light promotes PUFAs (He et al., 2015; Minhas et al., 2016). Based on results obtained, the light intensity (300 μmol photon $m^{-2}s^{-1}$) promoted the accumulation of saturated (C16:0, C18:0) and MUFAs (C16:1), and showed a decrease in the percentage PUFAs in both diatoms. The results of *Neochloris oleoabundans* also showed that high light intensity strongly increased the percentages of C16:0, C18:1 (Sun et al., 2014). It was interesting to find that, the combination of the SFAs and MUFAs content of the two species accounted for more than 90% of the total FAs when cultured in control and nutrient-stress conditions, Similar results were also reported in *Chaetoceros muelleri* and *Dunaliella salina*, which had more than 80% of the saturated and MUFAs content when cultured in f/2-N, f/2-P medium and seawater. The variation of fatty acid in diatoms and microalgae under high light and nutrient-stress conditions has also been reported by others (Qiao et al., 2016; Yodsuwan et al., 2017). Similarly, N-deficiency/limitation also showed variation in the fatty acid profile. An increase in the percentage of SFAs and MUFA, and a decrease in PUFA under N-limitation

have been observed in *P. tricornutum* as well (Qiao et al., 2016). Both diatoms in the present study followed this trend. The percentage of SFA and MUFA were increased while PUFAs was decreased under N-deficient conditions. However, it has been reported that higher nitrogen concentration increases the percentage of PUFAs (Kim et al., 2016). Similarly, in the present study, a two-fold increment has been observed in *N. shiloi* grown under N- and Iron-rich conditions relative to N-deficient cultures. Here, the diatom cultures gave a higher level of SFAs and MUFAs which considered as good choices for biodiesel production. SFAs can resist degradation and autoxidation which are suitable for long storage. While high levels of unsaturated FAs are not resistant to oxidation during prolonged storage. Furthermore, an appropriate combination of SFAs and MUFAs enhances the cetane number, cold flow and oxidative properties of biodiesel (Stansell et al., 2012). According to the requirement of European standard (EN 14214), the permissible level of linolenic acid (C18:3) is less than 12% for biodiesel production (Arias-Peñaranda et al., 2013; Chisti, 2007; Knothe, 2012). Interestingly, here, the C18:3 content of *Nitzschia* and *N. shiloi* was less than 2% in the culture of control and nutrient-stress conditions which meets the requirement of the European standard for biodiesel production.

In conclusion, Growing the diatoms under higher iron concentrations dramatically increased the lipid content (37.7 ± 0.77% of total lipid in *Nitzschia* sp. and 37.44 ± 0.54% in *N. shiloi*) which were 2.7 and 1.7 times higher compared to those obtained with standard f/2 (13.8 ± 0.42%) and BG11 (21.71 ± 0.34%) medium, respectively. In addition, higher iron and nitrogen concentrations also favored the production of fucoxanthin. The fatty acids in the lipid of both diatoms produced under nutrient-stress conditions comprised over 90% SFAs and MUFAs suggesting that these diatoms are promising candidates for biodiesel production. The main fatty acids produced by both diatoms were palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1) and oleic acid (C18:1n9c). The fraction of Eicosapentaenoic acid (C20:5n3) increased under N- and Iron-rich conditions in *N. shiloi*.

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