

Biodiversity and characterization of high lipid content microalgae in Porong River Estuary East Java, Indonesia

SRI NURHATIKA, DINI ERMAVITALINI*, TRIONO BAGUS SAPUTRO, YUDI APRIYATMOKO

Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Teknologi Sepuluh Nopember. Jl. Arif Rahman Hakim, Keputih, Sukolilo, Surabaya, East Java 60111, Indonesia. Tel./fax.: +62-31-5963857, *email: dinierma@bio.its.ac.id

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Abstract. Nurhatika S, Ermavitalini D, Saputro TB, Apriyatmoko Y. 2018. Biodiversity and characterization of high lipid content microalgae in Porong River Estuary East Java, Indonesia. *Biodiversitas* 19: 627-632. Microalgae are aquatic unicellular or multicellular microscopic photoautotroph organisms. High lipid content in microalgae biomass can be used as raw material for biodiesel. Porong river estuary is one of the sites of industrial waste disposal. The polluted waters considered will affect the lipid microalgae metabolism. The main research aims are to isolate and characterize of high lipid content microalgae found in Porong river estuary through the selection of qualitative lipid content. Sampling was conducted at three different stations. Isolation was conducted in solid agar media enriched with Porong River Estuary sterile water and using streak plate method. The isolated microalgae were characterized using a light microscope and to be matched with identification books. The lipid content was determined qualitatively by coloring the isolated-microalgae cells with Nile Red, an intracellular lipid dye then observed its luminescence color under a fluorescence microscope. Several genera of microalgae that can be isolated in this study are *Oscillatoria*, *Nitzschia*, *Merismopedia*, *Navicula*, *Nannochloropsis*, and *Melosira*. The results of qualitative lipid analysis show that the genus *Nannochloropsis* and *Nitzschia* have high intracellular lipid accumulation.

Keywords: Biodiesel, *Nannochloropsis*, *Nitzschia*, Porong River Estuary, qualitative lipid content

INTRODUCTION

The fossil-based fuel energy sources that are continually becoming diminish will provide a significant problem for every country in the world. So it needs an alternative for sustainable fuel that is renewable. Alternative energy that is potentially replacing the fossil-based energy sources comes from the biomass of organisms that are processed into biofuels (Patil et al. 2008). The use of biofuel as an alternative source of energy has several advantages compared to the fossil-based energy that is more environmentally friendly and contains high octane value (Hidayat and Syamsul 2008). One of promising biofuel is biodiesel. Biodiesel fuel derived from plant or animal oil through the transesterification process of triacylglycerol with alcohol to fatty acid methyl ester (Teresa et al. 2009). Biodiesel accounts for less pollution than fossil fuels but has a higher cost of production (Fakuda et al. 2001). Therefore, some efforts are needed to find out alternative raw materials to obtain cheaper biodiesel. Microalgae are organisms that have potential as raw materials of biodiesel. These unicellular organisms have greater photosynthetic capability than higher plants (Huesemann et al. 1987). Based on several studies, microalgae can produce lipids of more than 60% of their dry weight (NREL Report 2003). □

The growth and production of lipid in microalgae are completely influenced by the environment i.e nutrients, temperature, salinity, pH, DO, and water brightness (Bajpai and Bajpai 1993). A non optimal growth environment

condition will trigger the microalgae to produce higher intracellular lipids (Guschina and Harwood 2006). Microorganisms that accumulate high lipids have potential as a raw material for biodiesel (Zuhdi 2003). The production of lipids in microalgae varies with environmental parameters. High phosphorus and low nitrogen content in the waters will increase the production of intracellular lipids in microalgae (Borowitzka 2005). Waters with a pH of 5.9-7.5 with temperatures higher than normal will increase lipid content in microalgae (Bajpai and Bajpai 1993). One of water environment that predicted does not optimal for the microalgae growth is the estuary waters of the Porong river. Porong river estuaries are included in coastal ecosystems that are widely used for human activities and a mud disposal site by PT. Lapindo Brantas (Abida 2009). These conditions affect the physical and chemical changes in water river includes nutrients, pH, brightness, temperature, and salinity. These physical and chemical changes are thought to affect the abundance and productivity of lipids in microalgae. The research that has been done by Environmental Agency of East Java (2010) states in the waters of the Porong River has obtained the genus of microalgae of diatoms and dinoflagellates. The genus of microalgae found in the waters of the Porong River has never been tested for its intracellular lipid levels. So this research was conducted with the aim to characterize the morphology of microalgae that was successfully isolated from the waters of the Porong River and tested the qualitative content of intracellular lipid microalgae.

MATERIALS AND METHODS

Time and location of the research

This research was conducted in February-June 2015, and located in estuary waters of Porong River, Sidoarjo East Java Indonesia. There are three sampling points of microalgae communities in this study covering the river, estuary and sea area. Microalgae isolation, identification, culturing and Nile-red staining for qualitative analysis of intracellular lipids were done in Biosains and Plant Technology laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Teknologi Sepuluh Nopember (ITS), Surabaya Indonesia.

Collection of environmental parameter data □

The environmental parameter data taken at the sampling location were the water chemistry and physics data. Chemical and physical parameter data were taken at three stations include on water brightness, temperature, salinity, pH, DO (Dissolved Oxygen), Ammonium (NH₄⁺) content, Nitrate (NO₃⁻) content, Nitrite (NO₂⁻) content and Phosphate (PO₄²⁻) content.

Microalgae sampling

The sampling of microalgae is done on waters surface area with a depth of ± 50 cm with the *horizontal towing method*. Microalgae samples were taken from each station using a plankton net with mesh size of 35 µm. The filtered water sample at the cod end was then poured into the sample bottle (Arinardi et al. 1997). The calculation of the microalgae abundance was done with the following formula:

$$N = n_i \times \frac{1}{V_d} \times \frac{V_t}{V_s}$$

N = Total number of individuals or microalga cells per m³ (cell/m³)

n_i = Number of individual or microalgae cell species

V_d = The volume of filtered water (L)

V_t = The volume of water that filtered (ml)

V_s = The sample volume is below the cover glass (ml)

Microalgae identification, isolation, and culture □

Microalgae samples were identified using a light microscope based on Bellinger and Sigeo (2010), Vuuren et al. (2006) and Jomas and Carmelo (1997). Microalgae isolation was carried out using a solid agar medium enriched with water from the sampling site. The microalgae growth medium was prepared by mixing the filtered sample water with 1.5 % bacto agar. Isolation is done by using dilution method and streak method. Medium containing microalgae isolates were incubated at 27°C under 30 Watts fluorescent light for 7-14 days until the microalgae grow. The microalgae that grow were separated by a scratch method on the solid agar medium for 16 scratches by using the ose needle to obtain homogeneous microalgae. □

Qualitative analysis of microalgae intracellular lipid content

Qualitative analysis of lipid content was done by Nile red staining. Microalgae isolates were stained by

suspending microalgae cells on an agar solid medium to 0.5 ml of Nile red solution (0.1 mg Nile red/ml acetone) and incubated for 10 minutes at room temperature. Next rinsing with aquadest and taken one drop of microalgae cells were stained and placed on top of the object glass to be observed under a fluorescence microscope (Olympus fsx 100 bioimaging navigator) at a wavelength of 450-490 nm (Vuuren et al. 2006). □

RESULTS AND DISCUSSION

Parameters of aquatic environment

Waters physics parameters such as brightness and temperature affect the growth of microalgae in water. The amount of brightness value at each station indicates the ability of sunlight in penetrating the water column. Considering that, the brightness value tell us to some extent the process of photosynthesis of phytoplankton especially microalgae can running well (Odum 1971). Because of the depth of the water column and the intensity of the incoming sunlight (Nybakken and Bertness 2005), the brighter water will result in increasing the temperature relative to the feculent water. Water temperature is one environmental factor that can affect the rate of photosynthesis and growth of phytoplankton in the waters. Temperature values in each station show the appropriate value for the growth of phytoplankton, according to Nybakken and Bertness (2005) who states that the optimum water temperature for phytoplankton growth ranges from 20-30oC.

The salinity value consecutively increases from station 1 to station 3. The lowest salinity value is at station 1 which is 0‰, while the highest salinity value is at station 3 which is 20‰. The volume and flow of freshwater from the upstream of river stream considered influence the low salinity value at station 1. Conversely, the sea tides and reduced of freshwater exposure from the river dedicated to the high salinity value at station 2 and station 3. The presence of salinity in the estuary produces salinity gradients, ranging from sea water dominance to freshwater dominance in upstream estuaries. The salinity gradient changes dynamically following the changes in river water discharge, tides and coastal waters (Nybakken and Bertness 2005). The salinity value in estuarine waters of the Porong river suggests within the minimum range according to definition by Isnansetyo and Kurniastuty (1995), which states that the optimal salinity for plankton is between 20-35‰.

DO (dissolved oxygen) has an important role in the presence of organisms in the waters, especially in the process of cellular respiration and photosynthesis. DO is also a factor regulate the composition and abundance of aquatic species. The average DO value varied among stations ranging from 3.6 to-10 mg/L. The lowest value is in station 1 while the highest is in station 3. According to the decree of Indonesian State Ministry of Environment (MENLH) number 51, 2004, the quality standard of sea waters DO is > 5. Based on this quality standard, the levels of DO at station 2 and station 3 are the optimum condition for aquatic organism growth., whereas in station 1, because of the DO value is smaller than the quality standard, it is a

suboptimum condition for growth. The smaller value of DO at station 1 is an impact of still exposure of the waters to mud and organic matter from the land. According to Hutagalung and Rozak (1997), the main factor that lowers the oxygen content of the waters is the entry of organic waste that can easily decompose. The pH of the Porong river is classified as alkaline. The pH value at all three stations showed in the appropriate range of the quality standard, i.e. 7 to 8.5. Thus it suggested that pH value in Porong waters supports the growth of marine life including microalgae. Measurements of nitrate, nitrite and phosphate elements in the waters obtained values in the optimal category for the growth of phytoplankton based on MENLH decree number 51 the year of 2004. The level of ammonium in these waters is lower than the specified quality standard. According to (Raymont, 1980), there are two types of phytoplankton in their preference in consuming both nitrate and ammonium for nitrogen sources. The phytoplankton in these waters is allegedly the type that consumes ammonium in the beginning and then switches to uptake nitrate after the progress of nitrate levels higher than the ammonium levels.

Composition and abundance of microalgae

There were five classes of microalgae obtained from this research namely Bacillariophyceae, Chlorophyceae, Cyanophyceae, Dinophyceae, and Chrysophyceae. Bacillariophyceae is the most common group of microalgae found at all sampling stations. According to Nybakken and Bertness (2005), the Bacillariophyceae is more able to adapt to existing environmental conditions, this class is cosmopolitan and has high tolerance and adaptability. Here is a diagram of the composition and abundance of microalgae found at each observation station (Figure 2).

Figure 2 displayed the abundance of each genus of microalgae observed in each station. The genus *Nitzschia* and *Oscillatoria* are the genera found in all stations while the other genera are found only in particular stations. The genus *Nitzschia* is abundant at station 3 with 120,895 cells/m³, while the genus *Oscillatoria* was easily found at station 2 with an abundance of 394,836 cells/m³. Overall, the total abundance of microalgae in estuary waters of Porong river was 923.126 cell/m³.

Microalgae Isolation

As much as 11 isolates identified in 6 genera of microalgae was individually grown as a single culture (table 2), they are *Oscillatoria*, *Nitzschia*, *Merismopedia*, *Navicula*, *Nannochloropsis*, and *Melosira*. While all of the six genera presented and successfully isolated from station 1, only three genera (*Oscillatoria*, *Nitzschia*, and *Navicula*) and two genera (*Oscillatoria* and *Nitzschia*) which found and isolated from station 2 and 3 respectively. Genus *Skeletonema* that dominates the waters of station 2 and station 3 can not grow at the time of isolation. The composition of the agar the medium used during isolation might be unlikely to be suitable for the growth of the genus *Skeletonema*. The previous report by Renaud et al. 1998, suggested that the use of the medium with the addition of f/2 fertilizer (not accommodated in our experiment) can accelerate the growth of *Skeletonema* compared with other diatoms. Microalgae which have wide tolerance range to environmental conditions is one of the characters of microalgae which can be cultivated with large scale especially for the purpose as a source of biodiesel (Borowitzka and Borowitzka 1988).

Table 1. Environmental parameters of estuarine water Porong River, Sidoarjo East Java Indonesia

| Environmental parameters | Station 1 | Station 2 | Station 3 | Quality standards |
|----------------------------|-----------|-----------|-----------|-------------------|
| Physical parameters | | | | |
| Temperature (°C) | 28 | 27 | 28 | 20-30 |
| Brightness (m) | 0,1 | 0,14 | 1,06 | - |
| Chemical parameters | | | | |
| pH | 8,2 | 8,0 | 8,4 | 7-8,0 |
| Salinity (‰) | 0 | 10 | 20 | 20-35 |
| DO (mg/L) | 3,6 | 7,85 | 10,0 | >5 |
| Nitrate (mg/L) | 5,16 | 4,619 | 1,348 | 0,008 |
| Nitrite (mg/L) | 0,058 | 0,143 | 0,029 | <0,5 |
| Ammonium (mg//L) | 0,058 | 0,036 | 0,082 | 0,3 |
| Phosphate (mg/L) | <0,22 | <0,22 | <0,22 | 0,015 |

Note: Station 1 = River; Station 2 = Estuary; Station 3 = Sea. The analysis was conducted by Balai Penelitian dan Standardisasi Industri Surabaya. Valid from 8 May 2015 to 8 August 2015. Quality standards are in accordance with MENLH decision number 52 year of 2004

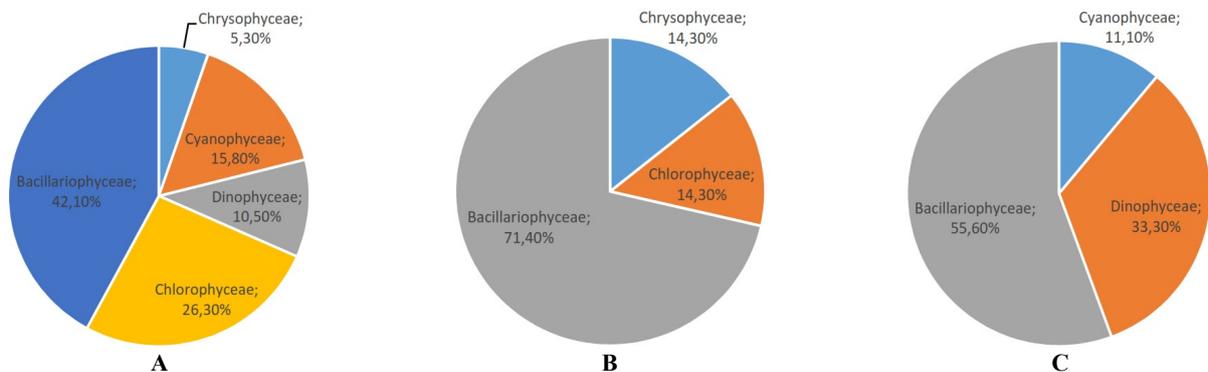


Figure 1. Microalgae class composition obtained from Porong River Estuaries, Sidoarjo East Java Indonesia. A. Station 1 (River), B. Station 2 (Estuary), C. Station 3 (Sea)

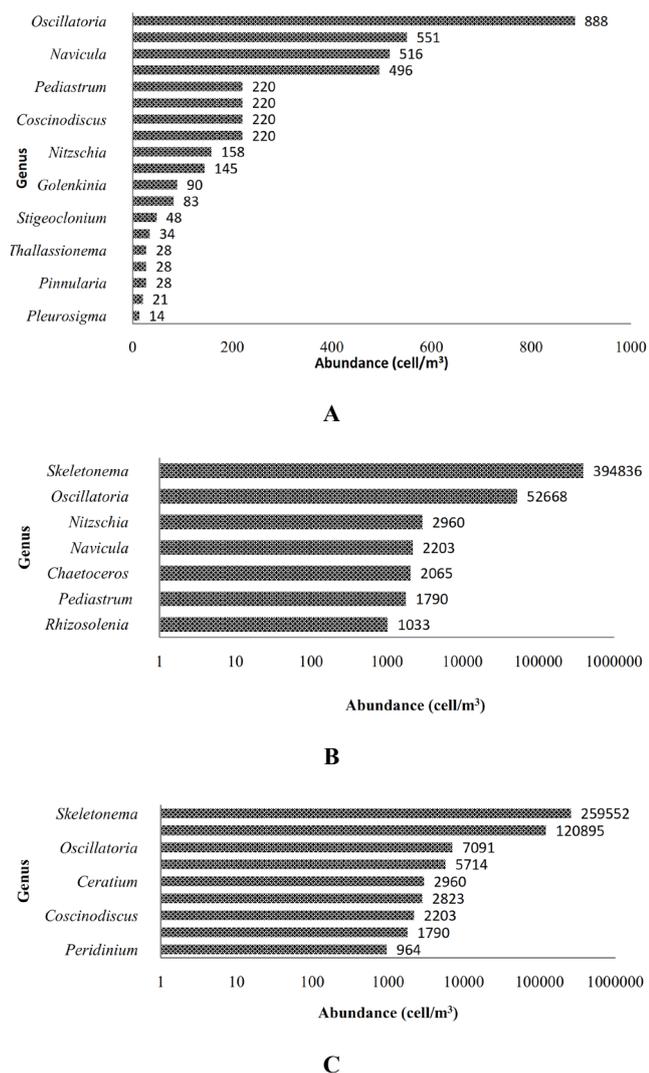


Figure 2. The abundance (cell/m³) of each microalgae genus obtained from each observation stations. A. Station 1 (River); B. Station 2 (Estuary); C. Station 3 (Sea)

Qualitative analysis of lipid microalgae

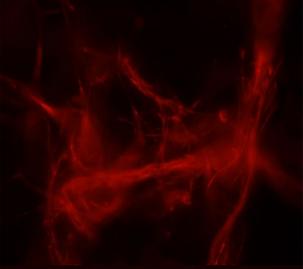
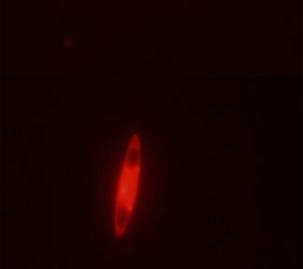
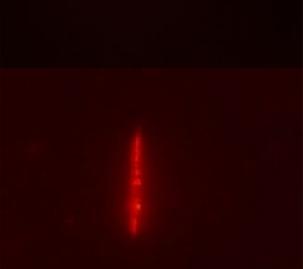
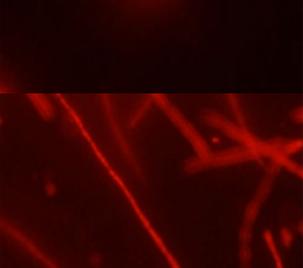
Table 3 displayed the color of luminescence in microalgae cell after Nile Red Staining under a fluorescence microscope. Those eleventh isolate showed different color after treated with Nile red. The content of lipid in microalgae cell that has been colored with Nile red is indicated by the luminescence of color. Yellow and red are two main colors formed after observation under a fluorescence microscope (Table 3). The yellow luminescence of the microalgae cells under the fluorescence microscope indicates the presence of lipids. While the red luminescence showed no lipid on the colored cells. The differentiation of that results is mainly caused by the reaction between the Nile red and the microalgae cells. The addition of acetone solvent facilitates the permeability of the cell membrane so that the Nile Red can enter the cells and tissues. The mechanism begins with water induction across the lipid bilayer membrane by altering membrane fluidity (Huang et al. 2009; Gunawan 2010).

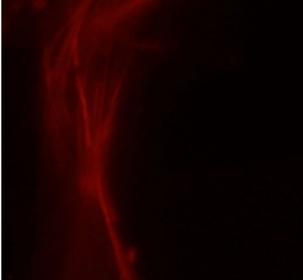
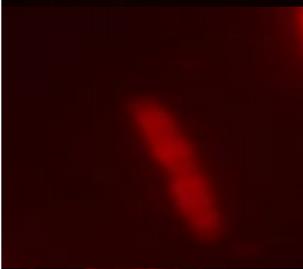
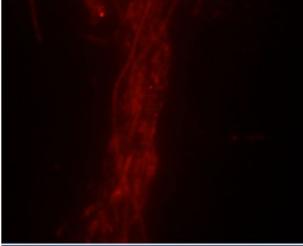
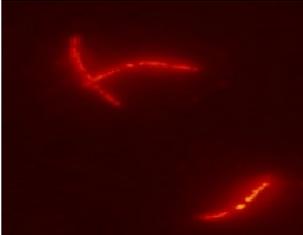
Table 2. Microalgae successfully isolated as single culture

| Genus | Single culture | Under microscope, M = 100x |
|-----------------------|----------------|----------------------------|
| <i>Oscillatoria</i> | | |
| <i>Nitzschia</i> | | |
| <i>Merismopedia</i> | | |
| <i>Navicula</i> | | |
| <i>Nannochlorosis</i> | | |
| <i>Melosira</i> | | |

Lipid synthesis especially TAG in microalgae cells occurs during photosynthesis, especially during light reaction. Those lipids then stored in the cytosol, producing non-polar lipids and then will be synthesized into polar lipids in a dark reaction (Thompson 1996). Sato et al. (2008) reported that microalgae have relatively low lipid level in an optimal condition which is about 5-20% by dry weight. In contrast, when the environmental conditions are less than optimal; the microalgae will be stressed and alter the lipid biosynthesis pathway to the formation and accumulation of neutral lipids (20-50%) of the dry weight, especially in the form of TAG. The TAG is used for defense in less than optimal environmental conditions. The red colored microalgae in Nile red may live in sub-optimal condition. Microalgae that live in optimal or sub-optimal environmental conditions still producing lipid in their cell, but the ability of lipid accumulation of each genus are varied. It is also mentioned by Hu et al. (2008) that lipid synthesis in TAG form can occur in non optimal conditions and lipid in the form of TAG is a precursor of biodiesel feedstock. Furthermore, The difference in salinity level assumed to play the main roles in creating the sub-optimal condition. The adaptation of microalgae to environmental condition causes the various lipids content (Sato et al. 2008).

Table 3. Color luminescence on microalgae cell after Nile red Staining and observation under fluorescence microscope

| Station | Genus | Color luminescence on microalgal cell | Intensity |
|---------|------------------------|---|-----------|
| 1 | <i>Oscillatoria</i> |  | - |
| | <i>Merismopedia</i> |  | - |
| | <i>Navicula</i> |  | ++ |
| | <i>Nitzschia</i> |  | +++ |
| | <i>Nannochloropsis</i> |  | +++ |
| | <i>Melosira</i> |  | - |

| | | | |
|---|---------------------|---|-----|
| 2 | <i>Oscillatoria</i> |  | - |
| | <i>Navicula</i> |  | ++ |
| | <i>Nitzschia</i> |  | + |
| 3 | <i>Oscillatoria</i> |  | - |
| | <i>Nitzschia</i> |  | +++ |

Note:-: red luminescence it means no lipid accumulation; +: low yellow luminescence it means low lipid accumulation; ++: medium yellow luminescence it means medium lipid accumulation; +++: strong yellow luminescence it means high lipid accumulation

Environmental parameters become a critical factor affecting the ability of microalgae in accumulating lipids in their body. Table 3 shows microalgae isolates that produce a strong yellow luminescence at station 1 and station 3. Microalgae isolates are *Nitzschia* and *Nannochloropsis*. Environmental parameters in observation stations that are not optimal for microalgae growth are salinity at station 1 and station 2, pH at station 1 and station 3, and ammonium at all observation stations. Based on (Table 1) the pH value

of the waters shows a pH value above normal that is 8.2 and 8.4. In general, alkaline pH will inhibit the growth of microalgae so that microalgae will adapt to form non-polar lipids in the form of the TAG (Guckert and Cooksey 1990). According to (Patil et al. 2008) alkaline pH in the culture environment can increase the content of the genus *Nitzschia* lipids. The pH of the environment affects the formation of polar lipids on the cell membrane of the microalgae so that when the pH of the environment changes then the growth of the microalgae will be disrupted so that the lipid synthesis path is converted into non-polar lipids through regulation in cells (Smith and Raven 1979). Nitrogen in water is found in the form of ammonia, nitrite, and nitrate. In general, nitrogen is absorbed by microalgae in the form of nitrate and ammonia. Nitrogen compounds are strongly influenced by the free oxygen content in water. When the oxygen content is low, nitrogen changes to ammonia (NH₃-), whereas the oxygen content is high, nitrogen changes to nitrate (NO₃-) (Effendi 2003). According to Hu et al. (2008) nitrogen deficiency of some diatom microalgae and dinoflagellates showed a significant increase in lipid production in cells. □

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