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Metabolic profiles of *Nannochloropsis oceanica* IMET1 under nitrogen-deficiency stress

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Abstract

To understand the mechanism of lipid accumulation and the corresponding metabolic changes of the microalga *Nannochloropsis oceanica* IMET1, the lipid content, fatty acid composition and metabolic profile were investigated via batch culture under nutrient deficiency and chemostatic culture under nitrate limitation. The results indicated that the triacylglycerol-neutral lipids were significantly accumulated through an acyl-CoA dependent pathway, while the polar lipids were partially converted to triacylglycerol through an acyl-CoA independent pathway. The fatty acid compositions of the polar lipids changed concurrently with the length of time of the nutrient deficiency, while the fatty acid compositions of the neutral lipids remained nearly consistent. The concentrations of several major osmolytes were significantly changed under chemostatic conditions with different nitrogen concentrations, which reflect the membrane property changes caused by the alteration of the polar lipid

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composition.

Keywords *Nannochloropsis oceanica*; lipid; metabolism; biodiesel; nitrogen limitation

Abbreviations

TAG, triacylglycerol; EPA, eicosapentaenoic acid; FA, fatty acid; NL, neutral lipid; PL, polar lipid; GL, glycolipid; PUFA, poly unsaturated fatty acid; DHA, docosahexaenoic acid; GC-MS, gas chromatography-mass spectrometry; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; LN, low nitrogen; MN, medium nitrogen; HN, high nitrogen; FAME, fatty acid methyl ester; PB, phosphate buffer; DCW, dry cell weight; DAGAT, diacylglycerol acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; TMA, trimethylamine; DMA, dimethylamine; GABA, -aminobutyrate; SFA, saturated fatty acid; USFA, unsaturated fatty acid

1. Introduction

Biodiesel from microalgae as an alternative fuel has attracted increasing attention worldwide in the past several years, but the metabolic engineering of microalgae, which is vital for reducing the production cost, is hampered by the lack of understanding of the metabolism and an effective regulation mechanism for cellular lipid accumulation, as well as how the metabolism is affected by environmental factors in microalgae (Lü et al., 2011; Hu et al., 2008). The eukaryotic marine microalga *Nannochloropsis* has been studied as a potentially powerful candidate for biodiesel production. Rodolfi et al. (2009) reported that three *Nannochloropsis* strains have a lipid content of 30% or higher and lipid productivity ranging from 55 to 61 $mg \cdot L^{-1} \cdot day^{-1}$, making them the best lipid producers among 30 marine and freshwater microalgae in terms of both lipid content and lipid productivity. *Nannochloropsis oceanica* IMET1 is commonly cultivated in fish hatcheries as feed for rotifers and to create a ‘‘green-water effect’’ in fish larvae tanks (Lubzens et al., 1995). Due to its high content of eicosapentaenoic acid (EPA, C20:5), a high-value omega-3 polyunsaturated fatty acid (PUFA), *Nannochloropsis* has been proposed as an alternative source for commercial EPA production (Sukenik, A., 1991). At present, it has been successfully grown in indoor and outdoor systems for biodiesel or biomass production (Moazami et al., 2012; Quinn et al., 2012; Rodolfi et al., 2009). However, oil accumulation in the algal cells, commonly obtained via nitrogen-deficiency, was accompanied by low biomass productivity, which is contradictory for obtaining both high biomass and high lipid production in one step (Rodolfi et al., 2009).

The oil production of microalgae can be modulated by varying the growth conditions, which have been extensively studied to overcome many of the bottlenecks in biodiesel production. The strategies for utilizing environmental stress have been successfully developed by controlling the concentration of CO_2 aeration, light intensity, salinity, temperature and nitrogen concentration to manipulate *Nannochloropsis* metabolism in favor of high lipid (primarily triacylglycerols (TAGs)) accumulation for biodiesel production (Chiu et al., 2009; Simionato et al., 2011; Pal et al., 2011; Rodolfi et al., 2009; Van Vooren et al., 2012). The majority of these studies used batch or semi-continuous cultures; however, in batch cultures, the culture conditions continuously change, which is not optimal for investigating the long-term effects of only one environmental factor on lipid accumulation in *Nannochloropsis,* because other nutrient deficiencies may cause particularly misleading results. In continuous cultures, fresh medium is continually added as the culture medium is withdrawn, which allows the maintenance of a steady-state concentration of cells by the concentration of a single limited nutrient, i.e., chemostat (Wood, 2005). The chemostat system is more suitable for investigating a single environmental factor on lipid biosynthesis.

Algae synthesize fatty acids (FAs) as building blocks for the formation of various types of lipids. In general, saturated and monounsaturated FAs are predominant in most algae. Specifically in *Nannochloropsis* sp., the major FAs are C14:0, C16:0, C16:1, C20:4 and C20:5 (Sukenik et al., 1993). FAs exist in different classes of lipids, including neutral lipids (NLs), polar lipids (PLs) and glycolipids (GLs), and the

distribution of the various FAs in the different types of lipids often vary in relation to the species (Vanitha et al., 2007; Yu et al., 1999; Meireles et al., 2003). The composition and distribution of fatty acids in neutral and polar lipids have great impact on the strategy for lipid separation and purification and also contribute to the protocols for algae culture and metabolic engineering for specific purposes (Meireles et al., 2003; Guedes et al., 2010; Huang et al., 2010). For example, when algae are cultured as food for fish and animals to supply PUFAs, such as EPA and docosahexaenoic acid (DHA), polar lipids are more effective for nutrition than those esterified into neutral lipids (Guedes et al., 2010). When algae are cultured for biodiesel production, NLs, particularly TAGs, are the most suitable product (Huang et al., 2010).

Recently, the first draft genome sequence and genetic transformation methods of *Nannochloropsis gaditana* and the high-efficiency homologous recombination transformation method of *Nannochloropsis* sp. have become available (Kilian et al., 2011; Radakovits et al., 2012). Based upon the genome sequence, the pathway and regulation of lipid biogenesis have been detailed, and the metabolic pathway map has been more clearly identified. New transformation and homology recombination methods have permitted the genetic and metabolic engineering of *Nannochloropsis*. With these advances in the investigation of *Nannochloropsis*, it is quite urgent to obtain further insights into the lipid and metabolite profiles of the algae, which could provide clues, targets and strategies for improving productivity and overcoming the current barriers in oil production from *Nannochloropsis*.

Although the FA composition and lipid classes in many algae have been reported, few investigations have been performed in regards to their accumulation and regulation due to environment effects. Guedes *et al.* (2010) investigated the effects of light intensity on the lipid class and FA composition of the microalgae *Pavlova lutheri* and found that a misleading conclusion will be obtained for EPA and DHA production if their distributions in various lipids were not considered. Nitrogen deficiency is a common method for accumulating oil in algae, but no information is available in terms of changes to the lipid classes and their FA composition under nitrogen-limited conditions. In the present study, both batch and chemostat cultures were used to evaluate the effect of nutritional deficiency and nitrate limitation on the lipid content, fatty acid distribution and metabolic profiles of *Nannochloropsis oceanica* IMET1, which will further the understanding of lipid metabolism and provide further basis for genetic and metabolic engineering.

2. Materials and methods

2.1 Chemicals

Trichlormethane, methanol, sodium chloride, $K_2HPO_4 \cdot 3H_2O$, $NaH_2PO_4 \cdot 2H_2O$ (all analytical grade) and other inorganic chemicals were purchased from Guoyao Chemical Co. Ltd. (Shanghai, China) and used without further treatment. Nile red was purchased from Sigma-Aldrich Co. LLC (St Louis, MO, USA) for the lipid staining. N-hexane was purchased from Thermo Fisher Scientific Inc. (Fair Lawn, NJ, USA) and used for the gas chromatography-mass spectrometry (GC-MS) analysis. Vitamin

B12, biotin, thiamine·HCl and 9-diethylamino-5H-benzo(α) phenoxazine-5-one (Nile red) were purchased from Sigma-Aldrich Co. LLC (St Louis, MO, USA). D_2O (99.9%) and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) were purchased from Cambridge Isotope Laboratories (Miami, FL, USA).

2.2 Microalgae and growth conditions

The microalgae cells of *Nannochloropsis oceanica* IMET1 were maintained in annular glass columns, each containing 700 mL of autoclaved sea water and modified F/2 medium, which had the following composition (per liter): 200 g KNO_3, 25 g $NaH_2PO_4{\cdot}2H_2O$, 5 g $FeCl_3{\cdot}6H_2O$, 4.5 g EDTA and 1 mL of trace elemental solution. The trace elemental solution was comprised of (per liter) 0.4 mg $MnCl_2{\cdot}4H_2O$, 0.02 mg $Na_2MoO_4{\cdot}2H_2O$, 0.02 mg $CoCl_2{\cdot}6H_2O$, 0.02 mg $CuSO_4{\cdot}5H_2O$, 0.04 mg $ZnSO_4{\cdot}7H_2O$, 1 μg vitamin B12, 1 μg biotin and 200 μg thiamine·HCl. The pH was adjusted to 7.6.

The cells were incubated at 20 ± 2 °C and illuminated continuously on one side with 59 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ using cool white fluorescent lamps. Pure CO_2, from cylinders, was supplied continuously to the air stream (1.5% ~2%, v/v) to provide a carbon source for the culture. The mixed stream was filtered through 0.22-μm filters and injected into the flasks. The cell density (OD_{750}) was determined as the transmission percent at 750 nm on a UV/Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) and was performed in triplicate.

The batch culture experiment was started with an OD_{750} of 0.11, and the cells

were harvested at certain time points (from 7 days to 23 days) for a dynamic study.

The chemostatic culture experiment was performed in as two-step cultivation. First, the cells were cultured for 6 days obtaining an OD_{750} of 5.14 in the batch culture. Next, the chemostat culture was continued with cells that were washed three times to remove the nutrients and incubated with an adjusted nitrate concentration. The light intensity in the chemostatic model was set at a fixed dilution rate (D) of 0.27 d^{-1} for the nitrate effect study. The nitrate concentrations chosen are given as follows: 60 $\mu mol \cdot L^{-1}$ (designated as low nitrogen, LN), 120 $\mu mol \cdot L^{-1}$ (designated as medium nitrogen, MN) and 2200 $\mu mol \cdot L^{-1}$ (designated as high nitrogen, HN). The nitrate concentrations were detected by Collos' method (Collos et al., 1999). The pictures of the cultivation, biomass and extracted lipids are shown in supplementary Fig. S1.

2.3 Cell harvest and dry biomass measurement

In the batch culture, 100 mL of wet biomass, growing for 7, 10, 14, 17 or 23 days, was harvested for the lipid, fatty acid, protein and total carbohydrate analyses. For the chemostatic culture, 700-mL samples were harvested after 29 days of cultivation. The wet biomass was centrifuged (21,700 x g) at 4 °C for 10 minutes and then washed three times with deionized water to remove the salt. The cell pellets were lyophilized with vacuum freeze-drying equipment (ALPHA1-2LD, Martin Christ, Osterode am Harz, Germany) for 24 h. After drying, the cell pellets were weighed and stored at 4 °C.

2.4 Analysis of the lipids, proteins and total carbohydrates

The protein content was assayed using the Bradford method. The total carbohydrate was analyzed with the phenol-sulfuric acid method (Dubois et al., 1956). Prior to the protein and carbohydrate tests, a 10-mg dry mass of *Nannochloropsis oceanica* IMET1 was dissolved in a 0.9% NaCl solution and lysed by ultrasonication for 15 minutes (2 s sonication and 1 s break) and then centrifuged (21,700 x g) at 4 °C for 10 minutes. The supernatant was used for the protein and carbohydrate analysis. The total lipids were extracted using Bligh and Dyer's method (Bligh & Dyer, 1959) and determined gravimetrically. The NLs and PLs were eluted using chloroform and methanol, respectively (Gamian et al., 1996). The different fractions were dried and concentrated with a rotary evaporator under vacuum.

2.5 Gas chromatography-mass spectrometry for fatty acid methyl ester analysis

The total lipids, NLs and PLs were converted into methyl esters using 2.5 mL of 2% H_2SO_4-methanol (v/v) (Yang et al., 2008) and spiked with an internal standard (nonadecanoic acid, C19:0). The fatty acid methyl esters (FAMEs) were analyzed on an Agilent 7890-5975 GC-MS system (Agilent Technologies Inc., Santa Clara, CA, USA). A capillary column, HP-INNOWAX (cross-linked polyethylene glycol), with a dimension of 30 m × 0.25 mm i.d., 0.25 μm film thickness was used for the separation of the FAMEs. The oven temperature was initially set at 100 °C and increased at 15 °C·min^{-1} up to 240 °C (held for 10 min). The split ratio was 1:20, and helium was used as the carrier gas at a flow rate of 1.0 mL·min^{-1} in the constant flow mode. The

ion source and quadrupole temperatures were 230 °C and 150 °C, respectively. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV with a scan range of 30–400 *m/z*. The injection sample volume was 1.0 μL. The assignment of FAMEs is shown in Fig. S2.

2.6 Soluble cellular metabolite extraction

Each sample was ground in liquid nitrogen with a mortar and pestle followed by lyophilization for approximately 24 hours. Approximately 25 mg of the freeze-dried material was added with 1 mL of pre-cooled (-40 °C) aqueous methanol (50%) and agitated in a 2-mL EP tube with a vortex at room temperature for 30 s, followed by 5 minutes of intermittent ultrasonication (30 s sonication and 30 s break) in an ice bath. After a 5 minute centrifugation (16,100 x g) at 4 °C, 600 μL of the supernatant from the extracts was transferred into a 2-mL EP tube. The insoluble residues were further extracted twice using the same procedure, after which the supernatants from the three extractions were combined. Following the removal of the methanol under vacuum, the supernatants were lyophilized in a freeze-drier for at least 24 h to obtain a powder for subsequent use. Two extraction blanks were always added in parallel during the extraction to ensure no contaminants were introduced during processing.

2.7 NMR measurements of the soluble cellular metabolites

The extracted metabolite powder (approximately 2 mg) was added to 500 L of D_2O together with 100 μL of phosphate buffer (PB). The PB (100 mM, pH7.4) was

prepared in H_2O containing 10% D_2O to provide an NMR field lock, and 0.02 mM of DSS was used as an internal reference. After 5 minutes of centrifugation (16,100 x g) at 4 °C, 500 µL of the supernatant was transferred into a 5-mm NMR tube for NMR analysis. No obvious insoluble matter was observed in the EP tubes.

All of the ^{1}H NMR spectra were recorded at 298 K on a Bruker AVIII 600 NMR spectrometer (600 MHz for ^{1}H) equipped with a 5-mm inverse cryogenic probe (Bruker Biospin GmbH, Karlsruhe, Germany). A standard one-dimensional pulse sequence (noesypr1d) was used to obtain the metabolic profiles of the extracts with a 90° pulse length of approximately 10 µs and a t_1 of 3 µs. Water suppression was achieved with a weak irradiation during the recycle delay (2 s) and mixing time (100 ms). Sixty-four transients were collected into 32,768 data points for each spectrum, with a spectral width of 12 kHz. An exponential window function with a line broadening factor of 0.5 Hz was applied to all of the free induction decays prior to Fourier transformation. For the resonance assignment, two-dimensional Jres, ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H COSY spectra were acquired from the chemostat culture samples. In the COSY and TOCSY experiments, 48 transients were collected into 2,048 data points for each of the 256 increments, and the spectral widths were 10 ppm for both dimensions. A phase-insensitive mode was used with a gradient selection for the COSY experiments, whereas MLEV-17 was employed as the spin-lock scheme in the phase-sensitive TOCSY experiment, with a mixing time of 100 ms.

2.8 Data reduction and multivariate NMR data analysis

The ^{1}H NMR spectra were manually corrected for phase and baseline distortions using TOPSPIN (Bruker Biospin GmbH, Karlsruhe, Germany), and the spectral region of 0.5-9.5 ppm was uniformly integrated into 3,166 buckets with a width of 0.003 ppm (1.8 Hz) using the AMIX package (Bruker Biospin GmbH, Karlsruhe, Germany). The region of 4.67-5.15 ppm was discarded to eliminate the effect of imperfect water suppression. The spectral areas of each bucket were normalized to the internal reference (DSS). The absolute levels of the metabolites were calculated, as milligram per gram of dry cell weight, from the least overlapping NMR signals of the metabolites and DSS with known concentrations, ignoring the inter-sample variations of spin-lattice relaxation time for the same protons. These semi-quantitative data were expressed in the form of mean ± standard deviation and were also subjected to a classical one-way ANOVA analysis using SPSS 13.0 software with a Turkey post-test ($p < 0.05$).

3. Results and Discussion

3.1 Dynamic study of the lipids, proteins, carbohydrates and fatty acids in a batch cultivation

3.1.1 Lipid accumulation in a time-dependent manner

The time course data of the lipid, protein and carbohydrate content accounting for the dry cell weight (DCW) in the batch culture are presented in Table 1. The results show that the lipid content significantly increased from 44.8% to 69.1% after the 7-day (in exponential phase, Fig. S1C) and 23-day (in deceleration phase, Fig. S1C) cultures,

respectively. The lipid-content value was slightly higher than the value (60.0% of the DCW) of several *Nannochloropsis* strains, as reported by Rodolfi et al. (2009). The intracellular protein and total carbohydrate contents were approximately 4.3~7.4% and 4.7~6.6%, respectively. Therefore, in the batch cultivation, the lipids were significantly accumulated in a time-dependent manner, but the proteins and carbohydrates only slightly fluctuated and had a tendency towards a decrease and increase, respectively.

3.1.2 Changes in the lipid class during the oil accumulation induced by nitrogen deficiency

To investigate the effects of nutrient deficiency on lipid class in IMET1, the NLs and PLs were determined and are shown in Table 2. Table 2 shows that the level of NLs (primarily TAGs, thin layer chromatography data not shown) in the 23-day culture (up to 69.7% of the total lipids or 48.38% of the DCW) was much higher than that of the 10-day culture (31.4% of the total lipids or 15.15% of the DCW). Meanwhile, the levels of the PLs decreased to 7.6% of the total lipids (5.25% of the DCW) in the 23-day culture when compared with a value of 26.4% of the total lipids (12.73% of the DCW) in the 10-day culture. Although the DCW of the 23-day culture was approximately two-fold that of the 10-day culture, the total amount of PLs still decreased in relation to the culture time. Therefore, the lipid classes changed during the lipid accumulation. The lipid accumulation was primarily due to an increase in NLs, while the PLs in the cell, or at least a portion them, were consumed. It is well

known that TAGs, the major components of NLs, are biosynthesized via two pathways. The direct glycerol pathway involves acyl-CoA-dependent diacylglycerol acyltransferase (DAGAT), which is the major contributor to TAG accumulation. In another acyl CoA-independent pathway, which has been reported in some plants and yeast (Hu et al., 2008; Merchant et al., 2012), PLs are converted into TAGs by a trans-esterification catalyzed by the enzyme phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000). The relative contribution of PDAT vs. DAGAT to TAG synthesis in yeast depends on the growth phase of the cell (Merchant et al., 2012). Based on the genomic information of *Nannochloropsis*, both PDAT and DAGAT were identified (Radakovits et al., 2012), which suggests that both the acyl-CoA-dependent and the acyl-CoA-independent pathways exist in *Nannochloropsis*. We observed that the level of PLs decreased upon nitrogen deficiency, which likely indicates the role of the PDAT pathway. Although this acyl-CoA-independent pathway does not significantly contribute to the TAG accumulation, this pathway may play an important role in the regulation of membrane lipid composition in response to environmental conditions in algae (Hu et al., 2008).

3.1.3 The fatty acid profile

The FA profile of the total fatty acids is presented in Table 1. The dominant FAs (> 5% of the total FA content) are C14:0, C16:0, C16:1, C18:0, C18:1 and C20:5. C16:0 and C16:1 were the most abundant FAs, and they maintained nearly constant levels within the total FAs during the cultivation. The level of C18:1 was significantly

increased from 8.6% to 14.7% between days 7 to 23, which is similar to the results reported previously (Rodolfi et al., 2009). The levels of C18:0, C18:2 and C20:5 in total the FAs were decreased, but when considering the accumulation of total fatty acids within the total biomass, there was no change in the levels of these fatty acids. Therefore, C18:0, C18:2 and C20:5 did not accumulate during the cultivation, and their levels decreased due to the accumulation of the other fatty acids. Our data agree with the previous report that the highest EPA content was obtained in a nutrient sufficient culture (Sukenik, 1991).

The FA compositions of the NLs and PLs were further studied (Table 2). The levels of the majority of the FAs in the NLs showed only slight changes in the 10- and 23-day cultures, which was in agreement with the FA composition of the total lipids, as the NLs accounted for the majority of the lipid accumulation. Conversely, the FA composition of the PLs changed significantly during the cultivation. The levels of the saturated FAs (SFAs, C14:0, C16:0 and C18:0) in the PLs were decreased, and all of the unsaturated FAs (USFAs), with the exception of C20:5, were increased in the 23-day culture when compared to the 10-day culture. Although the data for the total PLs indicated that the PLs did not accumulate but instead were partially consumed during the cultivation (section 3.1.2), their FA composition was changed, which suggests that the SFAs and C20:5 were consumed more than the other USFAs.

3.2 Metabolic profiles of IMET1 in an N-limited chemostatic cultivation

3.2.1 The lipid and fatty acid profiles of the chemostatic cultivation

To investigate the effect of nitrogen concentration on the lipid metabolic profiles of IMET1, a chemostat was designed and used. The nitrogen concentration effect on the biomass, lipids, NLs and PLs is shown in Table 3. The results show that under the LN cultivation, although the lipid (179.2 mg/L) and DCW (319.1 mg/L) yields were the lowest, the NL yields (123.6 mg/L), the lipid content of the DCW (56.2%) and the NL content of the lipid (68.95%) and DCW (38.7%) were the highest when compared with the other two nitrogen concentrations. In contrast to the LN condition, the highest yields of total lipid (305.4 mg/L), PLs (148.5 mg/L) and DCW (897.1 mg/L), as well as the highest PL content of the lipids (48.64%) and DCW (16.81%), were gained under the HN condition. Therefore, the oil accumulation induced by the LN condition was primarily due to the NL accumulation, which is the best overall condition in terms to biodiesel production, giving both the highest yield and levels of NLs. For the production of PLs, which is the preferred nutrient food for fish and animals (Guedes et al., 2010), the HN condition is the optimum choice due to both the highest yield and levels of PLs. The MN condition serves as an intermediate between the NL and PL yields.

The FA profiles for the different nitrogen concentrations under chemostatic cultivation are shown in Table 4. The results show that the content of C16:0, C16:1, C18:1 and C20:3 in the total FAs increased under the LN and MN conditions when compared with the HN condition. In contrast, the polyunsaturated FA C20:5 (i.e., EPA) accumulated preferentially under the HN condition (24.9% of the total FAs) when compared with the MN (18.0% of the total FAs) and LN (8.6% of the total FAs)

conditions. As a result, the total USFA content within the total FAs increased from 49.7% under the LN condition to 61.9% under the HN condition, despite the decrease of several USFAs (C16:1, C18:1 and C20:3). Therefore, the highest yield of EPA, in terms of the levels in either the total FA or DCW, was obtained under the HN condition.

From these results, it can be concluded that to obtain the highest yield of NLs for biodiesel production, the LN condition is the first choice, but for the highest yield of EPA (23.5 mg/L), the HN condition is the optimal condition. When EPA production is considered as a means to overcome the cost barrier in biodiesel production (Huang et al., 2010), it should be noted that the yield of EPA is low and partially accumulated in NLs, therefore the following separation procedures should be carefully designed in the coupling of the cultivation conditions.

3.2.2 The metabolites identified by NMR spectroscopy

The ^{1}H NMR spectra of the aqueous methanol extracts from IMET1 cultured with LN, MN and HN are shown in Fig. S3. The metabolite resonances were assigned with both the ^{1}H-NMR and 2D-NMR data from the JRES, COSY and TOCSY spectra and were further confirmed with the literature data (Fan 1996; Fan and Lane 2008) and the public database “Madison Metabolomics Consortium Database” (Cui et al., 2008). 35 putative metabolites could be detected, of which 29 were identified and quantified. It was apparent that the 35 metabolites of IMET1 were composed of 15 amino acids, 4 carbohydrates, 10 organic acids/amines, 5 nucleotide derivatives and 1 unknown

metabolite (among these metabolites, 10 amino acids, 4 carbohydrates, 6 organic acids/amines, 5 nucleotide derivatives and 4 other metabolites were quantified and are shown in Fig. S3 and Table 5). As shown in Fig. S3, considerably different metabolic profiles of IMET1 are observed when cultured under different nitrogen concentrations. In addition, the results also show that the USFA (5.28-5.3 ppm) content under the LN condition was higher than that of the MN and HN conditions, which was consistent with the GC-MS data shown in Table 4 (10.24%, 7.42% and 6.47% of the DCW under the LN, MN and HN conditions, respectively).

3.2.3 The metabolite changes under different chemostatic cultivations

The metabolites of IMET1 identified via NMR spectroscopy are distributed in wide metabolic pathways (Fig. 1), and the concentrations of the majority of these metabolites varied significantly under the three conditions (Table 5, Fig. S3). Upon comparing the metabolites from the different nitrogen conditions, it was notable that the majority of the metabolite levels assayed under the LN condition were lower than those in the HN or MN conditions (Fig. 1, S3 and Table 5), which likely reflects the growth inhibition due to the nitrogen deficiency. However, citrate, fumarate and trimethylamine (TMA) showed increased levels under the LN condition, while glucose, galactose, valine, isoleucine and dimethylamine (DMA) showed no significant changes between the LN and HN conditions. The accumulation of citrate under nitrogen deficiency significantly contributed to the TAG biosynthesis in algae, which has been extensively studied (Ratledge, 2004).

Aside from citrate, the metabolites with high concentrations included mannitol, proline and -aminobutyrate (GABA). Although different functions for these three metabolites have been reported, their most common function is to act as osmolytes and equilibrate the osmotic pressure in high salinity solutions, thus requiring high concentrations in cells (Klindukh et al., 2011; Iwamoto and Shiraiwa, 2005; Bouche and Fromm, 2004). Furthermore, it was notable that their concentrations changed under different cultivation conditions, i.e., more osmolytes accumulated in higher nitrogen conditions. Particularly, the concentration of proline in the HN condition was more than 7-fold higher than that in the LN condition. As far as we know, this is the first report that nitrogen deficiency may reduce the osmolyte concentration that accompanies oil accumulation. Because the salt concentrations of the three chemostat cultivations were nearly identical, the reducing osmolytes in the LN condition were likely compensated by another mechanism. One possibility is the change of membrane permeability. It has been reported that the change of lipid composition in the membrane is an adaption mechanism to salinity, and a decrease in the unsaturation of fatty acids is usually observed (López-Pérez et al., 2009). We observed that under the LN condition the yield of PLs and USFAs was reduced, which likely resulted in the changes to the membrane lipid composition and permeability. Further studies on the membrane structure under these cultivation conditions are required to verify this hypothesis.

Conclusion

The NL accumulation in *Nannochloropsis oceanica* IMET1 may be due to acyl-CoA dependent and independent pathways. The former pathway contributes primarily to the accumulation of TAGs, while the later pathway consumes PLs and results in the changes to the fatty acid composition of the PLs. These changes were accompanied by variations in the cellular osmolyte concentrations, which reflect alterations to the membrane properties caused by changes to its lipid composition due to nitrogen deficient conditions.

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Figure Legends

Fig. 1. Responsiveness changes in the metabolite levels of *Nannochloropis oceanica* IMET1 treated with different nitrates. The proposed metabolic pathways were based on the KEGG database (http://www.kegg.jp). The level of significance was set at $p < 0.05$. The metabolites with bold characters were detected while those with regular characters were not detected. In regards to the lipids and fatty acids, the level of significance was defined as above or below 30% of the value obtained under the high nitrate condition. The "b" and "c" significant differences are derived from a one-way ANOVA ($p < 0.05$) between the medium- vs. high-nitrogen level data and the low- vs. high-nitrogen level data, respectively. Metabolites: 3PG, 3-phospho-D-glycerate; alpha-KG, alpha-ketoglutarate; F6P, fructose-6-phosphate; FUM, fumarate; G6P, glucose-6-phosphate; PYR, pyruvate; Cit, citrate; DMA, dimethylamine; Frc, fructose; GABA, gamma-amino-n-butyrate; GB, glycinebetaine; Galac, galactose; Glc, glucose; Gln, glutamine; Glu, glutamate; Gly, glycine; Ile, isoleucine; MAL, malate; Phe, phenylalanine; Pro, proline; Shik, shikimate; Succ, succinate; TMA,

trimethylamine; Tyr, tyrosine; Uri, uridine; Val, valine; UMP, uridine monophosphate; IMP, inosine monophosphate; Aden, adenosine; His, histidine; PRPP, phosphoribosyl pyrophosphate; OAA, oxaloacetic acid; P5C, 1-Pyrroline-5-carboxylic acid; Ser, serine; FA, fatty acid; Acetyl-CoA, acetyl-coenzyme A; TCA, tricarboxylic acid cycle; PPP, pentose phosphate pathway.

Tables

Table 1 Dynamic study of the fatty acid content and the lipid, protein and carbohydrate yields from *Nannochloropsis oceanica* IMET1 in batch culture.

FAME	Culture days				
	7	**10**	**14**	**17**	**23**
C14:0	8.45	8.69	8.73	8.27	7.78
C16:0	33.40	35.64	35.05	35.11	34.86
C16:1$^{\Delta 9}$	26.44	26.37	26.89	26.66	27.55
C18:0	8.06	7.53	7.03	6.00	5.16
C18:1$^{\Delta 9}$	8.57	10.57	11.84	14.01	14.68
C18:2$^{\Delta 9,12}$	2.16	1.84	1.60	1.53	1.49
C18:3$^{\Delta 6,9,12}$	0.20	0.20	0.20	0.19	0.27
C20:4$^{\Delta 5,8,11,14}$	2.89	2.29	2.23	2.17	2.23
C20:5$^{\Delta 5,8,11,14,17}$	9.81	6.87	6.44	6.06	5.99
Total SFA	49.92	51.86	50.80	49.38	47.80
Total USFA	50.08	48.14	49.20	50.62	52.20
FA/DCW (%)	20.80	24.16	26.98	30.40	32.94
Lipid/DCW (%)	44.76±0.86	48.33±0.50	56.33±2.81	62.27±1.40	69.06±0.83
Protein/DCW (%)	7.35±0.22	5.38±0.20	4.26±0.24	4.81±0.18	4.95±0.03
Carbohydrate/DCW (%)	5.57±0.02	4.72±0.02	5.65±0.03	6.23±0.06	6.62±0.03

SFA, saturated fatty acid; USFA, unsaturated fatty acid; FA, fatty acid; DCW, dry cell weight.

Table 2 The fatty acid composition of the neutral lipids (NLs) and polar lipids (PLs) from the 10- and 23-day batch cultures of *Nannochloropsis oceanica* IMET1.

FAME	10 days		23 days	
	NL	PL	NL	PL
C14:0	8.67	6.73	6.81	4.61
C16:0	40.53	36.51	42.21	27.56
C16:1$^{\Delta 9}$	29.10	13.23	29.31	26.00
C18:0	4.38	17.03	1.32	13.02
C18:1$^{\Delta 9}$	11.23	3.67	13.74	9.51
C18:2$^{\Delta 9,12}$	1.50	1.20	1.25	1.56
C20:4$^{\Delta 5,8,11,14}$	1.39	5.43	1.42	5.80
C20:5$^{\Delta 5,8,11,14,17}$	3.20	16.21	3.95	11.95
Total SFA	53.58	60.27	50.34	45.19
Total USFA	46.42	39.74	49.67	54.82
Yield/Lipid (%)	31.35	26.35	69.72	7.57
Yield/DCW (%)	15.15	12.73	48.38	5.25

SFA, saturated fatty acid; USFA, unsaturated fatty acid; FA, fatty acid; DCW, dry cell weight; NL, neutral lipid; PL, polar lipid.

Table 3 The nitrogen effect on the total dry cell weight yield and the total, neutral and polar lipid yields of *Nannochloropsis oceanica* IMET1 in N-limited chemostatic cultures.

Conditions	Nitrate (μmol/L)	DCW (mg/L)	Lipid (mg/L)	NL (mg/L)	PL (mg/L)	Lipid/DCW (%)	NL/DCW (%)	PL/DCW (%)	NL/Lipid (%)	PL/Lipid (%)
LN	60	319.10	179.24 ±0.67	123.59±0.46	28.21±0.11	56.17±0.21	38.73	8.47	68.95	15.74
MN	120	727.00	256.41±3.86	92.59±1.39	87.85±1.32	35.27±0.53	12.74	12.21	36.11	34.26
HN	2200	897.10	305.37±4.58	60.49±0.91	148.53±2.23	34.04±0.51	6.74	16.81	19.81	48.64

LN, low nitrogen; MN, middle nitrogen; HN, high nitrogen; DCW, dry cell weight; NL, neutral lipid; PL, polar lipid.

Table 4 The fatty acid composition of *Nannochloropsis oceanica* IMET1in N-limited chemostatic cultures.

FAME	Culture conditions (FA/total FA%)			Culture conditions (FA/DCW%)		
	LN	MN	HN	LN	MN	HN
C14:0	5.05	4.62	5.07	1.03	0.60	0.53
C16:0	41.31	31.00	27.41	8.53	3.96	2.89
C16:1$^{\Delta 9}$	24.42	21.90	21.83	5.03	2.79	2.29
C18:0	3.96	6.21	5.57	0.83	0.81	0.60
C18:1$^{\Delta 9}$	9.50	5.93	3.60	1.97	0.77	0.37
C18:2$^{\Delta 9,12}$	2.29	3.64	4.14	0.47	0.47	0.43
C20:3$^{\Delta 8,11,14}$	1.35	0.62	0.42	0.27	0.07	0.03
C20:4$^{\Delta 5,8,11,14}$	3.50	8.09	7.07	0.73	1.04	0.73
C20:5$^{\Delta 5,8,11,14,17}$	8.60	17.99	24.88	1.77	2.28	2.62
total SFA	50.32	41.83	38.05	10.39	5.37	4.02
total USFA	49.66	58.17	61.94	10.24	7.42	6.47
SFA (mg/L)	33.15	39.04	36.06			
USFA (mg/L)	32.68	53.94	58.04			
Total FA				20.63	12.79	10.49

SFA, saturated fatty acid; USFA, unsaturated fatty acid; FA, fatty acid; DCW, dry cell weight; LN, low nitrogen; MN, middle nitrogen; HN, high nitrogen.

Table 5 Quantification of the soluble cellular metabolites from *Nannochloropsis oceanica* IMET1 cultured under low, medium and high nitrate chemostatic culture.

Metabolites	Metabolite quantity (Mean ± SD, mg/g freeze-dried algae)			Differences
	LN	**MN**	**HN**	
Amino acids				
Ile	0.241±0.00	0.229±0.01	0.261±0.01	
Val	0.386±0.01	0.522±0.01	0.683±0.02	
Ala	1.307±0.04	1.952±0.05	3.306±0.05	a, b, c
GABA	2.613±0.04	4.476±0.11	5.354±0.09	a, b, c
Pro	1.867±0.39	4.251±0.16	14.24±0.18	a, b, c
Gln	0.908±0.03	0.861±0.05	2.845±0.04	b, c
Tyr	0.129±0	0.185±0.01	0.173±0	a, c
His	0.038±0.01	0.091±0.01	0.101±0.01	a, c
Phe	0.092±0.01	0.15±0.01	0.159±0	a, c
Trp	0.061±0.01	0.105±0.01	0.104±0.01	a, c
Carbohydrates				
trehalose	0.106±0.10	0.398±0.16	0.427±0.03	a, c
mannitol	33.55±1.10	63.76±1.65	54.83±0.56	a, b, c
glucose	0.389±0.14	0.639±0.21	0.35±0.03	a, b
galactose	0.083±0	0.244±0.15	0.241±0.03	
organic acids/amine				
succinate	1.065±0.03	2.052±0.05	1.947±0.03	a, c

fumarate	0.016±0	0.01±0	0.008±0	a, c
citrate	9.66±0.28	6.267±0.11	3.683±0.04	a, b, c
pyruvate	0.142±0	0.204±0.01	0.559±0	a, b, c
formate	0.911±0.04	0.261±0.02	0.322±0.02	a, c
lactate	0.664±0.25	1.229±0.39	0.954±0.08	
Nucleotide derivatives				
Adenosine	0.043±0	0.066±0.01	0.061±0	a, c
ADP	0.217±0.01	0.284±0.01	0.27±0.01	a, c
uridine	0.043±0	0.057±0.01	0.085±0	a, b, c
NMND	0.022±0.01	0.046±0.02	0.071±0	c
NAD^+	0.087±0.01	0.123±0.01	0.144±0	a, b, c
Others				
choline	0.273±0.00	0.583±0.01	1.059±0.01	a, b, c
GB	0.117±0.00	0.272±0.00	0.439±0.00	a, b, c
TMA	0.2±0.01	0.076±0.02	0.105±0	a, b, c
DMA	0.09±0	0.082±0.01	0.093±0	

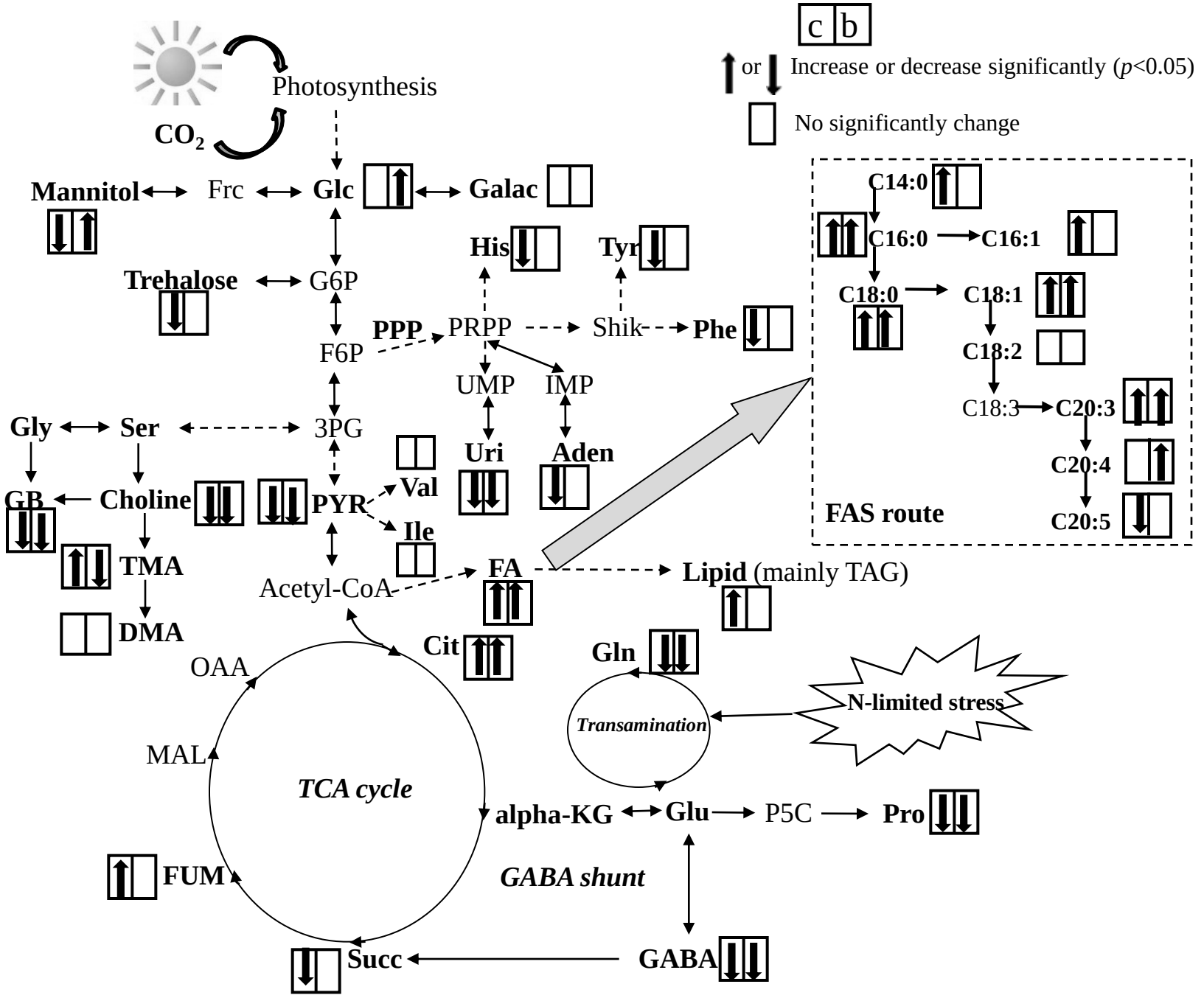
The “a”, “b” and “c” significant differences are derived from a one-way ANOVA analysis ($p < 0.05$) between the low- vs. medium-nitrogen level data, medium- vs. high-nitrogen level data and low- vs. high nitrogen level data, respectively. Metabolites: Ile, isoleucine; Val, valine; Ala, alanine; GABA, gamma-amino-n-butyrate; Pro, proline; Gln, glutamine; Tyr, tyrosine; His, histidine; Phe, phenylalanine; Trp, tryptophan; ADP, adenosine-5-diphosphate; NMND, N-methylnicotinamide; NAD^+, nicotinamide adenine dinucleotide; GB, glycinebetaine; TMA,

trimethylamine; DMA, di-methyl-amine.

Highlights

- The lipid, fatty acid and metabolite profiles of *Nannochloropsis* were studied.
- The accumulated neutral lipids had nearly consistent fatty acid compositions.
- The polar lipids were partially consumed, which changed the fatty acid composition.
- The concentration of cellular osmolytes varied with the nitrogen concentration.

Figure 1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Photosynthesis
CO_2
Mannitol
Frc
Glc
Galac
Trehalose
G6P
His
Tyr
F6P
PPP
PRPP
Shik
Phe
UMP
IMP
Gly
Ser
3PG
Uri
Aden
GB
Choline
PYR
Val
Ile
TMA
DMA
Acetyl-CoA
FA
Lipid (mainly TAG)
C14:0
C16:0
C16:1
C18:0
C18:1
C18:2
C18:3
C20:3
C20:4
C20:5
FAS route
Cit
Gln
OAA
Transamination
N-limited stress
MAL
TCA cycle
alpha-KG
Glu
P5C
Pro
FUM
GABA shunt
Succ
GABA