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Physiological responses of freshwater oleaginous microalgae *Desmodesmus* sp. NMX451 under nitrogen deficiency and alkaline pH-induced lipid accumulation

Ling Xia · Haijian Yang · Qiaoning He · Chunxiang Hu

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Abstract Many microalgae are reported to accumulate TAG under high concentrations of bicarbonate and nitrogen deficiency, but their physiological responses remain unknown and the associated applied technology is rarely explored. We found that single high concentration of bicarbonate did not enhance lipid content, except when combined with nitrogen limitation in *Desmodesmus* sp. NMX451, and the lipid contents obtained were independent from nitrogen limitation and alkalinity stress. Further analysis showed that adding bicarbonate not only retarded the cell cycle, repressed starch accumulation but also promoted polar lipid content. Nitrogen limitation meantime caused protein and chlorophyll degradation with a corresponding increase in total lipid content. Following the aforementioned insights, combined cultivation was performed at an outdoor 5 L scale, and higher biomass and lipid productivities were obtained. These results suggest that bicarbonate can be used as a "trigger" for rapid lipid accumulation in microalgae during nitrogen depletion, and this is an effective new strategy for the cultivation of oleaginous microalgae.

Keywords *Desmodesmus* sp. NMX451 · Nitrogen deficiency · Bicarbonate addition · Oil accumulation · Microalgae cultivation

Introduction

Microalgae as an alternative for sustainable feedstock production of biodiesel is a hot topic within recent years. This is largely attributed to their widespread availability and higher oil content compared with conventional terrestrial plants (Chisti 2007). Moreover, lipid synthesis can be easily modulated in most of the species of microalgae by manipulating their cultivation conditions.

A number of factors are known to influence the lipid content of microalgae, and increased levels of lipid synthesis usually occur when cells are subjected to nutrient imbalances or culturing stresses. Among the unfavorable conditions, nitrogen starvation appears to be the most common and effective strategy to stimulate lipid accumulation. Lipid content can almost be doubled or even more in *Chlorella* spp., *Nannochloropsis* spp., *Neochloris oleoabundans*, and *Phaeodactylum tricornutum* (Hu et al. 2008; Rodolfi et al. 2009; Fan et al. 2012; Yang et al. 2013). Neutral lipids in the green algae, *Chlorella* and *Scenedesmus*, accumulate faster when cells are subjected to high pH (Guckert and Cooksey 1990; Gardner et al. 2010; Skrupski et al. 2012). However, neither nitrogen deficiency nor high pH induction contributes to lipid productivity of microalgae cells (Gardner et al. 2010; Han et al. 2013), because these cells grow slowly under the aforementioned stress conditions. Thus, developing a culture approach that regulates pH and controls nitrogen supplementation is necessary to promote both cell growth and lipid accumulation in microalgae, thereby enhancing overall lipid productivity.

Recently, in small, laboratory-scale tests, algae have been grown typically in nitrogen-replete conditions to enable rapid cell growth before transfer of the cells to a nitrogen-deprived medium to induce lipid stress response (Su et al. 2010; Mujtaba et al. 2012). However, this method is difficult to scale up because harvesting large amounts of algal cells requires large amounts of energy. Compared with nutrient stress, a pH-based stress strategy is easier to scale up because additional harvesting steps are unnecessary, and higher cell growth can be maintained

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until inducing conditions are applied, all within the same culture system (Skrupski et al. 2012). However, lipid accumulation is usually poor and the change is slow when a single pH-based stress method is used. In this study, combined nitrogen starvation and high alkaline pH stress condition is introduced to stimulate lipid accumulation.

Adding reagents (e.g., alkaline or biological buffer) is the main method to adjust pH during the culturing process to obtain an alkaline condition (Klein and Betz 1978; Mush 1980; Gardner et al. 2010). Among the reagent, $NaHCO_3$ has a profound effect on cell growth and oil accumulation in *Scenedesmus* sp., *Chlamydomonas reinhardtii*, *Tetraselmis suecica*, *Nannochloropsis salina*, and *P. tricornutum* (Gardner et al. 2012, 2013a, b; White et al. 2012; Mus et al. 2013). However, nothing is known of the physiological performance of *Desmodesmus* under alkaline conditions with high bicarbonate concentration.

The feasibility of microalgae production for energy purposes cannot be answered based on current available data from indoor cultures. Robust data from field experiments using free sunlight are necessary to investigate the potential in detail. Several datasets from outdoor experiments are available for biomass production using bicarbonate as carbon source or pH regulator (Ranga Rao et al. 2012; Moheimani 2013b), but few studies focus on the lipid production process under alkaline stress conditions by adding bicarbonate.

Numerous studies report on the use of bicarbonate addition and nitrogen starvation as trigger mechanisms for lipid accumulation in microalgae (Hu et al. 2008; Rodolfi et al. 2009; Fan et al. 2012; Gardner et al. 2012, 2013a, b; White et al. 2012; Mus et al. 2013; Yang et al. 2013), but thorough experimentations of the algal metabolic responses to the above conditions have not been well documented. This study aimed to investigate the effects of high concentrations of bicarbonate addition and nitrogen deficiency on the physiology and triacylglycerol (TAG) accumulation in *Desmodesmus* sp. NMX451. Detailed results on lipid accumulation from sodium bicarbonate addition and nitrogen starvation or a combination of both are presented. Fundamental physiological changes, including biomass, cellular cycle, photosynthetic pigment, protein content, and starch content, were collected and related to lipid synthesis. We also used our data to emphasize the importance of adding bicarbonate in the lipid production phase in outdoor cultures.

Materials and methods

Desmodesmus sp. NMX451, a gift of Prof. Xu Xudong of the Institute of Hydrobiology, was originally isolated from Erdos in Inner Mongolia, China. Stock culture was grown in modified BG-11 medium containing 300 mg $NaNO_3$, 30 mg K_2HPO_4, 36 mg $CaCl_2 \cdot 2H_2O$, 6 mg ammonium citrate monohydrate, 6 mg ammonium ferric citrate, 1 mg EDTA, 2.86 μg H_3BO_3, 1.81 μg $MnCl_2 \cdot 4H_2O$, 0.222 μg $ZnSO_4 \cdot 7H_2O$, 0.39 μg $NaMoO_4 \cdot 5H_2O$, 0.079 μg $CuSO_4 \cdot 5H_2O$, and 0.050 μg $CoCl_2 \cdot 6H_2O$ in 1 L sterile distilled water.

Batch cultures were started by inoculating 0.4 g L^{-1} log phase cells into 500 mL Erlenmeyer flasks filled with 300 mL of full BG-11 medium with (N) and without nitrate addition (N^-), combined nitrate deprivation and 0.3 M $NaHCO_3$ addition (N^-+), and full BG-11 medium and extra 0.3 M $NaHCO_3$ addition (N+). All tests were carried out in triplicate. The cultures were performed at room temperature (25±1 °C) with continuous photosynthetically active radiation (PAR, 400-700 nm) illumination of 100 μmol photons m^{-2} s^{-1}. Cultures were aerated continuously with sterile filtered air.

For outdoor experiments, the cultures were grown in 5 L bioreactors with 3 L medium. To ensure well mixed medium, we used a 5 cm magnetic stir bar (mixing at 150 rpm) placed at the middle of the bioreactor chamber for stirring. Air was bubbled in bioreactors at a constant rate (Xia et al. 2013, 2014). Prior to the tests, cells were cultivated in BG-11 medium with initial sodium nitrate concentration of 0.1 g L^{-1} for 3 days when the nitrate was depleted (below a concentration of 0.01 g L^{-1}). The cultures for N+ treatment were directly added with 0.3 M $NaHCO_3$. The control used the cultures for continuing incubation. The cultures for N^- treatment used fresh BG-11 medium deprived of nitrate. All tests were carried out in duplicate. The experiment was carried out during 6 days in the summer with an average radiation of 184.0±35.3 μmol photons m^{-2} s^{-1} on the surface of the bioreactor.

Analysis of biomass and biomolecule

The dry weight of the algal biomass was determined gravimetrically, and growth was expressed in terms of dry weight. A 20-mL sample was harvested by centrifugation. The pellets were then washed twice with distilled water, freeze-dried, and weighed.

To analyze the cellular constituents (chlorophyll, starch, and proteins), 1 mL of sample cells was harvested by centrifugation and grounded with mortar and pestle. Chlorophyll *a* was extracted in 95 % ethanol and measured spectrophotometrically as reported by Mush (1980). Protein was analyzed following the method of Bradford (1976). Starch was extracted by a modified version of the method of Klein and Betz (1978). In brief, the pellet was resuspended in 0.1 M acetate buffer (pH 4.4) and autoclaved at 110 °C for

15 min to solubilize starch. Next, 1.5 units of amyloglucosidase (Sigma–Aldrich, USA) was added, and the solution was maintained in a water bath at 55 °C for 1 h to hydrolyze starch to glucose. Glucose was determined by a glucose oxidase-peroxide enzyme system (containing 0.1 g L^{-1} *o*-dianisidine dihydrochlorid, 1,000 U L^{-1} glucose oxidase, and 0.1 g L^{-1} peroxidase) at a light absorption of 522 nm (Tang 1999).

Total lipid content of the cells was determined by the modified method of Bligh and Dyer (1959) using 1:2 chloroform:methanol. Polar and neutral lipid analysis followed the method described by Wang and Benning (2011). To determine the profile of fatty acids, lipid samples were trans-esterified (Soh and Zimmerman 2011), and the resulting fatty acid methyl esters (FAME) were analyzed by gas chromatograph mass spectrometry (GC-MS; Thermo Scientific ITQ 700, USA) equipped with a flame ionization detector (FID) and a fused silica capillary column (60 m×0.25 mm×0.25 μm; Agilent Technologies, USA). The injector and detector temperatures were maintained at 270 and 280 °C, respectively, with an oven temperature gradient of 50 to 170 °C at 40 °C min^{-1} after a 1-min hold time at 50 °C, then with an oven temperature gradient of 170 to 210 °C at 18 °C min^{-1} after a 1-min hold. All parameters of the FAME were derived from the calibration curves generated from the FAME standard mix (Supelco 37 component FAME mix, Sigma-Aldrich).

Analysis of media components

The pH of the medium was measured using a pH meter (Mettler-Toledo, Switzerland). For determination of nitrate uptake, the algal culture was centrifuged, and the supernatant used for measurement of nitrate concentration according to the Chinese state standard testing methods (Monitoring Methods for Water and Wastewater 2002).

Morphology observation

For microscopy analyses, oil bodies in the live cells of *Desmodesmus* sp. NMX451 were stained with Nile Red (9-diethylamino-5H-benzo(α)phenoxazine-5-one; Sigma-Aldrich) at a final concentration of 1 μg mL^{-1} in acetone and observed under a fluorescence microscope (Nikon Eclipse 80i, Japan).

For transmission electron microscopy (TEM), the cells incubated for 12 days were fixed at 4 °C with glutaraldehyde (2.5 % in 0.2 M phosphate buffer, pH 7.6) for 12 h, post-fixed in 4 % OsO_4 at 25 °C for 3 h. The materials were centrifuged, dehydrated in an acetone/water series, and embedded in resin of Spurr (1969). Ultrathin sections were stained with uranyl acetate and lead citrate. Micrographs were taken using transmission electron microscopy (H-7650, Hitachi, Japan).

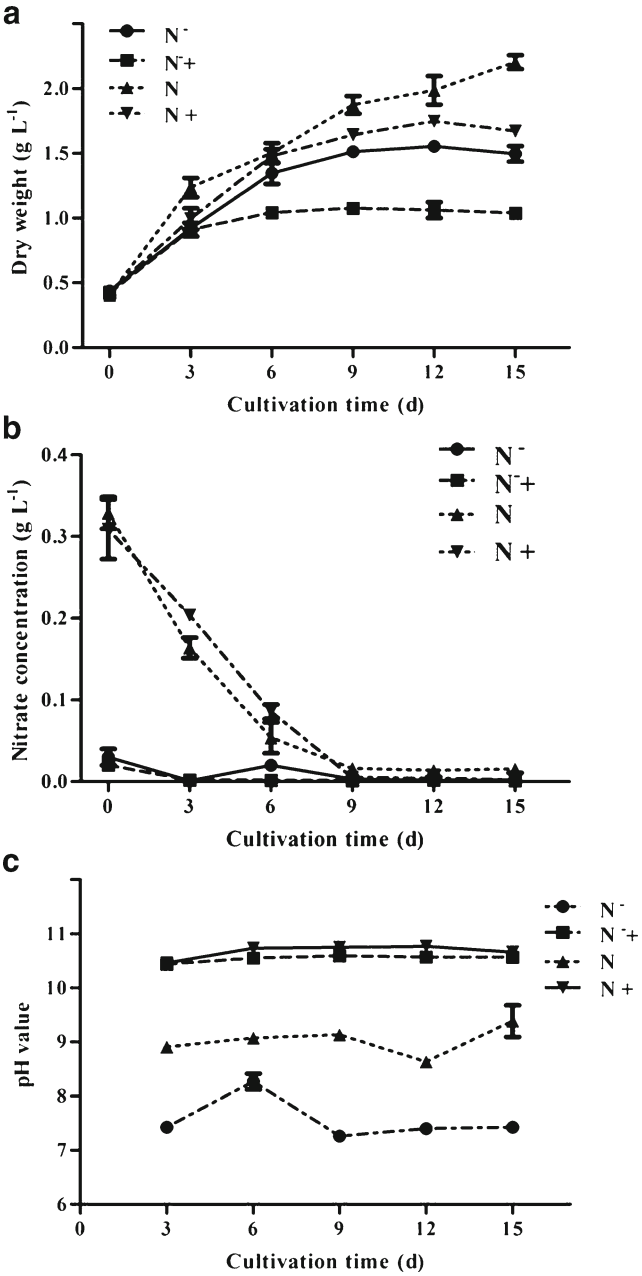

Fig. 1 Cell growth (**a**) of *Desmodesmus* sp. NMX451 and changes in medium nitrate concentration (**b**) and pH value (**c**) in response to nitrogen deficiency, bicarbaonate addition, or a combination of both. Data are means of triplicate samples±range

Statistical analysis

Results are averages of triplicates or duplicates, and the values in each graph and table are shown with 5 % error bars. Analysis of variance (ANOVA) was performed using SPSS 18.0 package (SPSS, USA), with values of 0.05 selected for significance.

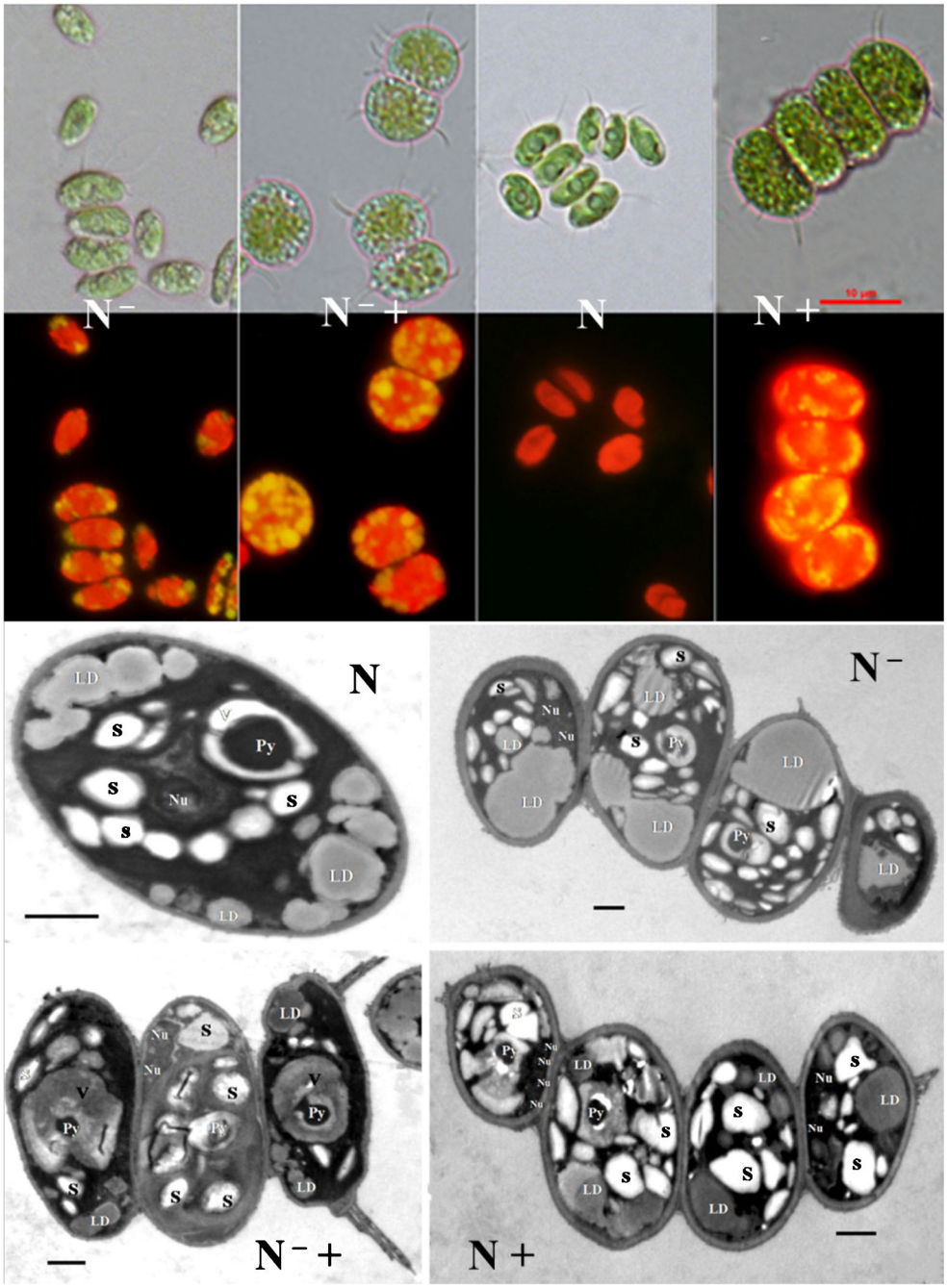

Fig. 2 Representative optical and epifluorescence (**a**) as well as electron micrographs (**b**) of *Desmodesmus* sp. NMX451 cells in response to nitrogen deficiency, bicarbaonate addition, or a combination of both. *LD* lipid droplets, *Nu* nucleus, *S* starch grains, *Py* pyrenoid, *V* vacuoles. Scale bar for micrographs, 10 μm; scale bar for electron micrographs, 1 μm

Result and discussion

Growth and lipid accumulation

To advance our knowledge of growth and lipid accumulation under high pH and nitrogen deficiency conditions, *Desmodesmus* sp. NMX451 was grown in batch cultures under full BG-11 medium with (N) or without sodium nitrate addition (N^-), full BG-11 medium with added 0.3 M $NaHCO_3$ at the same time with (N+) or without sodium nitrate (N^-+) addition. Time courses of cell growth rate, level concentrations for NO_3^-, and pH value of medium are shown in Fig. 1a, b, and c. The alga grew most rapidly in the N culture and achieved 2.2 g L^{-1} at day 15, after which the N+ culture

surpassed the N⁻ culture. Growth was most inhibited in the combined sodium bicarbonate addition and nitrogen deficiency (N⁻+) culture, with a final density of 1.0 g L^{-1}. Notably, the cell cycles in cultures of N⁻, N⁻+, and N+ were arrested, and the growth exhibited a turning point at day 6, followed by slow growth and even absence of growth. This time point corresponded to the low detection level concentrations for NO_3^- in the N+ culture, in which the nitrate was depleted after a 6-day cultivation (Fig. 1b). Medium pH maintained high values in cultures with sodium bicarbonate addition during the entire experiments; pH 10.5 and 10.7 were obtained in N⁻+ and N+ cultures, respectively (Fig. 1c). These results indicate that the growth was inhibited at varying degrees by nitrogen deficiency or alkaline pH stress or a combination of these (Gardner et al. 2010). It was also noticed in this study that the cell size largely increased during acclimation to high pH cultures of N⁻+ and N+ (Fig. 2a), presumably as a consequence of cell cycle (the process of a parent cell dividing into daughter cells) delay (Gardner et al. 2010, 2012, 2013a, b) and the greater accumulation of carbon storage compounds of starch granules and oil bodies (Fig. 2b; Msanne et al. 2012).

Lipid and TAG contents were analyzed at the growth turning point of day 6 and the end of the experiment on day 15 (Fig. 3). Total lipid content significantly increased with incubation time in all cultures, except in the N culture (Fig. 3a; $P< 0.05$). The N⁻+ culture had the highest lipid content of 43.8 % at day 6 and 49.5 % at day 15, followed by the N+ (32.8 % at day 6, 43.0 % at day 15), N⁻(29.0 % at day 6, 34.4 % at day 15), and N (26.4 % at day 6, 26.9 % at day 15) cultures. The same trend was also observed in the level of TAG content (Fig. 3b, Fig. 4a). Nile Red fluorescence images confirmed the results (Fig. 2a). It should be noted that the response of cells subjected to combined nitrogen deficiency and high pH stress condition was much stronger than either nitrogen starvation- or high pH stress-induced lipid accumulation independently. Thus, the combined stress condition caused an additive effect for lipid accumulation in *Desmodesmus* sp. NMX451. It is also noticeable that TAG accumulated in the N+ culture until near medium nitrate depletion on day 6 (Fig. 4a). Similar findings also have been observed in other species, such as *Scenedesmus* sp., *C. reinhardtii*, *T. suecica*, and *P. tricornutum* (Gardner et al. 2012, 2013a; White et al. 2012; Mus et al. 2013). Several reports also suggest that carbon dioxide addition had no effect on lipid content when nitrogen was replete (de Castro Araújo and Garcia 2005; Raghavan et al. 2008), and high TAG or lipid accumulation was observed when high CO_2 was used when nitrogen was limited (Gardner et al. 2013a; Toledo-Cervantes et al. 2013). However, the TAG or lipid obtained was unstable and followed by somewhat rapid diminishment in these conditions (Gardner et al. 2013a; Toledo-Cervantes et al. 2013). Although when bicarbonate and CO_2 were used equally as carbon source and pH regulator (Moheimani 2013a, b), bicarbonate exhibited more alkaline stress and resulted in stable TAG accumulation. These results provide further insight into the lipid production phase of microalgae for large-scale cultivation.

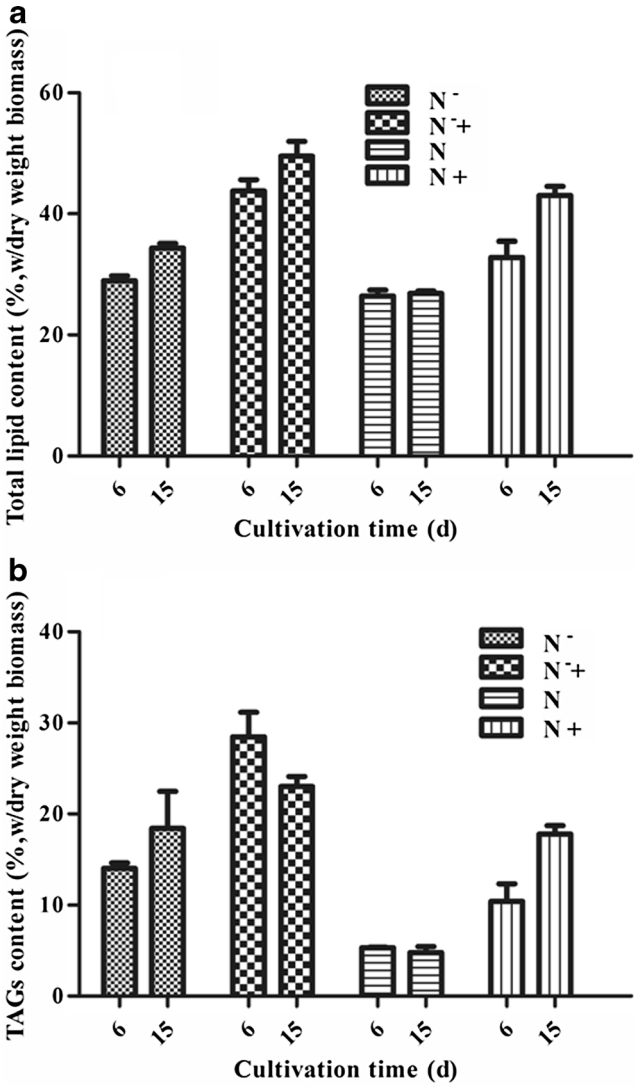

Fig. 3 Lipid (**a**) and TAG (**b**) content in response to nitrogen deficiency, bicarbaonate addition, or a combination of both. Data are means of triplicate samples±range

Lipid fraction and profile

In addition to TAG, the polar lipids phospholipids (PL) and glycolipids (GL) are the two main fractions of total lipids. These lipids are important components of the membrane system, including the external cell membrane and membranes associated with organelles, particularly the chloroplasts and endoplasmic reticulum. The conversion of existing membrane polar lipids into TAG may contribute to the overall increase in

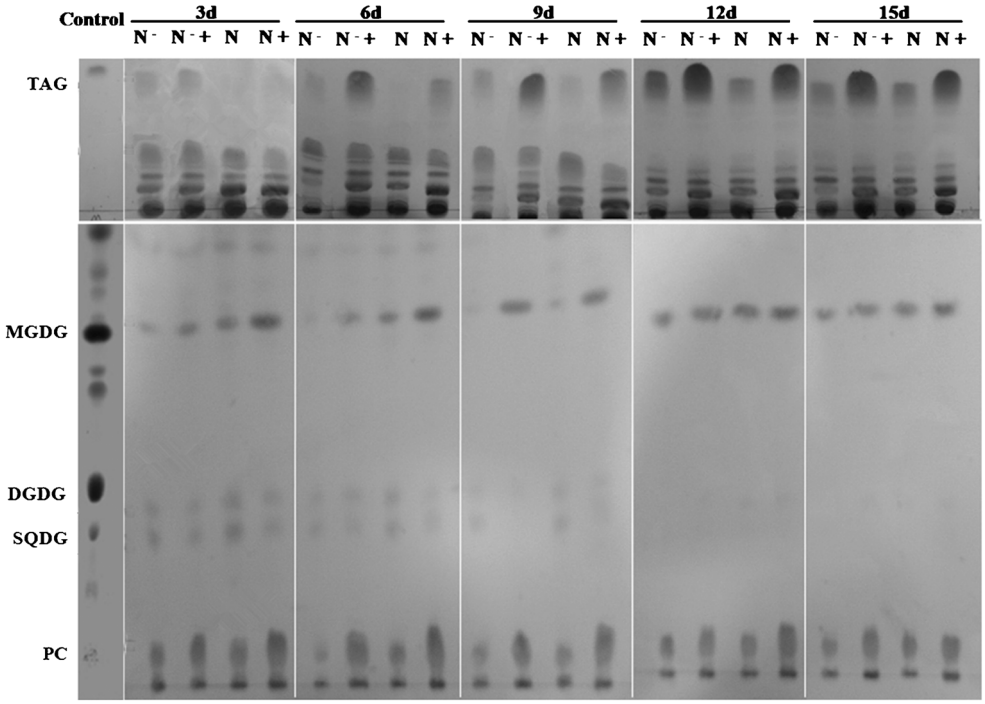

Fig. 4 Alterations in neutral and polar lipids of *Desmodesmus* sp. NMX451 in response to different treatments. The total lipids for chromatography of all samples were extracted from an equal amount of dry biomass. *TAG* triacylglycerol, *MGDG* monogalactosyldiacylglycerol, *DGDG* digalactosyldiacylglycerol, *SQDG* sulfoquinovosyldiacylglycerol, *PC* phosphatidylcholine

TAG under stress conditions in addition to the de novo biosynthesis of TAG (Hu et al. 2008; Mus et al. 2013). Thus, it is of importance to know the lipid turnover during nitrogen limitation or high pH stress. We followed a time course of changes in membrane polar lipids in *Desmodesmus* sp. NMX451 during the cultivation. Fig. 4 shows that, glycolipids, which were mostly represented by monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), all reduced within the cultivation time, except for MGDG. In this study, the maintenance of the chloroplast-specific lipid MGDG may serve for fundamental basal photosynthesis in cells subjected to stress conditions (Guschina and Harwood 2006), in terms of nitrogen limitation. The phospholipid, phosphatidylcholine (PC), was also maintained to potentially serve for the activity of the cellular membrane system. In addition, the amount of the most abundant polar lipids, namely, MGDG and PC, was higher in cultures with bicarbonate addition (N^-+ and N+ cultures) than that in cultures without bicarbonate addition (N and N^- cultures). A similar trend was also observed in the amount of TAG, which was higher in cultures with bicarbonate addition (N^-+ and N+ cultures) than that in cultures without bicarbonate addition (N and N^- cultures). Given these results, lipid fractions were much more affected by bicarbonate availability than nitrogen supplementation. The rapid increase of neutral lipids in cultures with bicarbonate supplement (N+ and N^-+) could enhance membrane rigidity, potentially avoiding excessive flux of Na^+ and HCO_3^- into the cells and aiding organisms tolerating high alkaline conditions

Table 1 Fatty acids composition (relative mole percentage of total fatty acids) of total lipid in *Desmodesmus* sp. NMX451 from different treatments

	C16:0	C16:1	C18:0	C18:1	C18:(2–4)	Others	ΣSFA	ΣMUFA	ΣPUFA
N^-	34.5±0.0	1.7±0.0	3.8±0.2	30.6±0.2	29.0±0.3	0.5±0.1	38.6±0.4	32.3±0.2	29.1±0.3
N^-+	31.4±0.1	2.9±0.0	3.8±0.1	40.4±0.2	21.0±0.2	0.7±0.0	35.8±0.0	43.2±0.2	21.0±0.2
N	31.8±1.2	1.9±0.1	3.1±0.4	31.0±0.4	31.8±0.5	0.5±0.0	35.3±0.8	32.9±0.3	31.8±0.5
N+	33.9±4.9	2.1±0.3	0.2±0.0	42.5±0.4	20.9±1.8	0.5±0.3	34.5±5.2	44.7±3.5	20.9±1.8

Results are expressed as the mean±standard deviation (n=3)

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

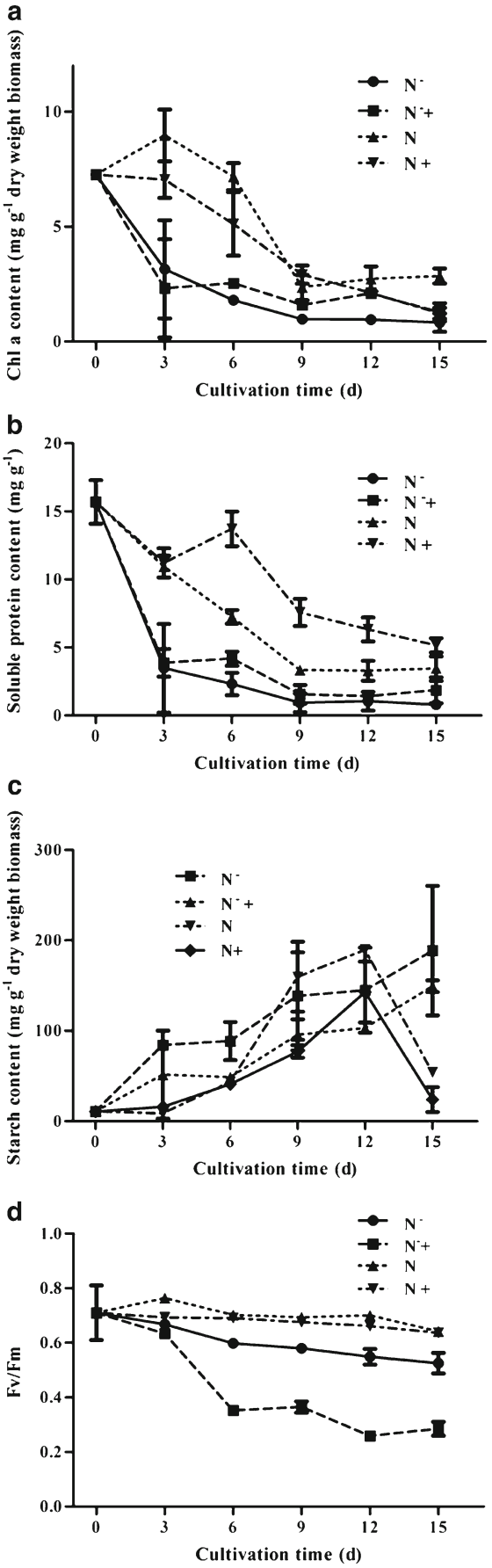

Fig. 5 Changes in chlorophyll *a* (**a**), protein (**b**), starch (**c**) content, and F_v/F_m (**d**) of *Desmodesmus* sp. NMX451 in response to nitrogen deficiency, bicarbaonate addition, or a combination of both. Data are means of triplicate samples±range

(Mansour and Salama 2004). The presence of more polar lipids in cultures with bicarbonate supplement (N+ and N⁻+) also aided salt stress tolerance in algae because a higher content of polar lipids could decrease membrane permeability and fluidity (Chen et al. 2008). Moreover, the accumulation of phospholipids in polar lipids in cultures with bicarbonate supplement (N+ and N⁻+) could stimulate membrane biosynthesis, which is vital for the export of Na^+ irons to maintain the ion concentration at the proper level inside the cells (Lu et al. 2012). This conclusion is also supported by the observation that the plasma membrane was strengthened in cultures with added bicarbonate (N+ and N⁻+; Fig. 2b). However, further work is necessary to demonstrate the relationship between membrane function and physiological changes caused by adding sodium and bicarbonate ions.

Bicarbonate availability affected not only the lipid fractions but also the fatty acid composition. Table 1 shows that monounsaturated fatty acids (MUFA; both C16:1 and C18:1) were significantly higher in cultures with added bicarbonate (N⁻+ and N+ cultures) than those in cultures without added bicarbonate (N and N⁻ cultures; $P< 0.05$). By contrast, polyunsaturated fatty acids (PUFA) showed opposite results, and saturated fatty acids showed no remarkable variations. Bicarbonate-induced conversion from PUFA to MUFA may favor the cellular oxygen-evolving activity/machinery tolerating environmental stress (Sakamoto and Murata 2002). The observed MUFA content also increased under high CO_2 concentration, although the other portions followed other trends (Rocarati et al. 2004; Yusof et al. 2011).

Photosynthetically fixed carbon

The decreased amount of polar lipids may be potentially for TAG incorporation, but the absolute increase in TAGs was much larger than the decrease in polar lipids (Fig. 4). Thus, the increase in TAG is largely depended on de novo photosynthetic carbon fixation (Pan et al. 2011; Fan et al. 2012). To determine how photosynthetically fixed carbon was directed into major metabolic pathways, we measured the contents of chlorophyll *a*, protein, and starch under N, N⁻, N+, and N⁻+ scenarios (Fig. 5, Table 2). Nitrogen deprivation (N⁻ and N⁻+ cultures) led to a reduction in nitrogen-containing chlorophyll *a* content (Fig. 5a). Chlorophyll *a* content also decreased with nitrate depletion after a 6-day cultivation in cultures initially supplemented with nitrate (N and N+ cultures; Figs. 1b, 5a). A decrease in protein content was also observed under nitrogen

Table 2 Cellular composition of major biomolecules of *Desmodesmus* sp. NMX451 under different conditions

	Chlorophyll *a* content (mg g^{-1})		Protein content (mg g^{-1})		Starch content (mg g^{-1})		Lipid content (mg g^{-1})	
	6 days	15 days	6 days	15 days	6 days	15 days	6 days	15 days
N⁻	1.8±0.2	0.8±0.4	2.3±0.8	0.8±0.3	88.7±21.0	188.7±71.7	289.6±8.2	343.8±7.6
N⁻+	2.5±0.2	1.3±0.4	4.2±0.5	1.8±0.9	48.6±1.5	149.4±6.4	437.5±18.6	495.4±24.2
N	7.2±0.6	2.8±0.3	7.2±0.5	3.4±0.9	43.3±0.7	53.9±2.0	264.3±10.0	268.8±4.1
N+	5.1±1.4	1.2±0.2	13.7±1.3	5.1±0.5	40.9±0.7	23.9±13.8	328.5±26.5	430.4±14.8

Results are expressed as the mean±standard deviation (n=3)

deprivation or depletion condition (Figs. 1b, 5b). Chlorophyll *a* and protein have been suggested to degrade under nitrogen limitation conditions. In contrast, starch content increased with cultivation time in nitrogen deprivation cultures of N⁻ and N⁻+ (Fig. 5c). The concomitant accumulation of starch and lipids under nitrogen limitation was consistent with that previously reported for *C. reinhardtii* and *N. oleoabundans* (Wattebled et al. 2003; Hamid Rismani-Yazdi et al. 2012). It should be noted that the starch content obtained in the N⁻+ culture was significantly lower than that in the N⁻ culture ($P<0.05$; Fig. 5c; Table 2). This restrained carbon flow into starch in the $NaHCO_3$-supplemented culture, possibly directed to biosynthesis and storage of lipids, since carbon availability is a key metabolic factor controlling carbon partitioning between starch and oil during nitrogen limitation (Fan et al. 2012; Valenzuela et al. 2012; Mus et al. 2013; Peng et al. 2014). Correspondingly, the starch content in the cultures initially supplemented with nitrate (N and N+) increased with cultivation time and then decreased at day 15. This decrease may be due to the sudden and sharp decrease in photosynthetic activity as represented by F_v/F_m (Fig. 5d). Fig. 5d shows that F_v/F_m in the stressed cultures of N⁻, N⁻+, and N+ decreased with time and was significantly lower than that in the N culture. This downregulation of photosynthesis under nitrogen deficiency or high pH stress condition is commonly observed in *C. emersonii*, *N. oleoabundans*, and *P. tricornutum* (Shelly et al. 2007; Hamid Rismani-Yazdi et al. 2012; Mus et al. 2013).

The observed changes suggested that nitrogen limitation drives the redirection of carbon flux from nitrogen-containing compounds (chlorophyll *a* and protein) toward the nitrogen-free storage TAG and starch accumulation. Bicarbonate amendment represses starch accumulation to save energy for TAG accumulation during nitrogen limitation. Furthermore, the delayed cell cycle observed in bicarbonate supplemented cultures may also prevent the oil from being assimilated for energy for cell division (Matusiak-Mikulin et al. 2006). Thus, bicarbonate was directly or indirectly responsible for induced TAG accumulation (Gardner et al. 2013b).

Application of results for outdoor cultivation

Our data indicate that nitrogen limitation was a prerequisite for lipid accumulation, and there is an additive effect for lipid accumulation in *Desmodesmus* sp. NMX451 under combined nitrogen limitation and high alkaline stress condition. This combined stress condition can be carried out in outdoor mass cultivation by adding $NaHCO_3$ to the culture when the nitrogen in the medium is about to be depleted. This technique enables rapid cell growth in the first stage before nitrogen depletion and accelerates lipid accumulation in the second stage with bicarbonate addition. Moreover, high bicarbonate concentrations can minimize contamination from invasive microorganisms (Richmond et al. 1982). This culture mode

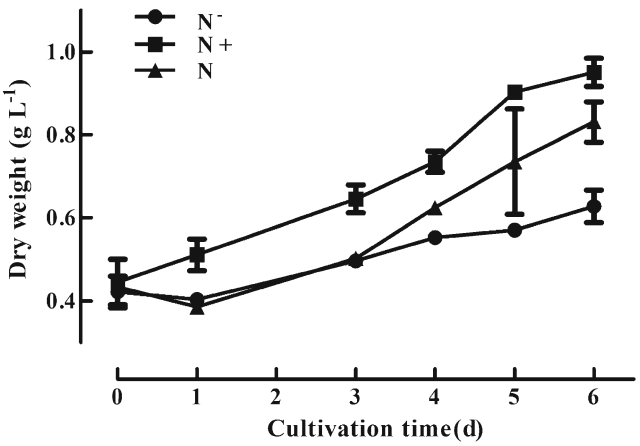

Fig. 6 Cell growth of *Desmodesmus* sp. NMX451 in outdoor cultures with full nutrition, nitrogen deprivation, and bicarbonate addition in lipid induction phase. Data are means of duplicate samples±range

Table 3 Biomass and lipid productivities of *Desmodesmus* sp. NMX451 under outdoor conditions

	Lipid content (%, w/w)	Biomass productivity (mg L^{-1} day^{-1})	Lipid productivity (mg L^{-1} day^{-1})
Control	31.8±1.7	66.3±0.1	21.3±2.3
N$^-$	32.5±3.1	34.4±0.0	10.9±0.5
N+	38.0±0.8	84.2±0.0	32.0±0.7

Results are expressed as the mean ±standard deviation (n=2)

was examined under outdoor conditions in the present study. During the experiments, the pH value was maintained at about pH 10. Fig. 6 and Table 3 show that the growth was inhibited in the N$^-$ culture and the lipid content was 32.5 %. Surprisingly, growth in N+ was much higher than that in control. This result may be due to the ample dissolved inorganic carbon in the medium where bicarbonate was bioavailable for carbon fixation, especially under high light intensity outdoors. In addition, the lipid content was also much higher in N+ than that in control and N$^-$, with a content of 38.0 %. The high growth rate and high total lipid content in N+ culture resulted in high lipid productivity of 32.0 mg L^{-1} day^{-1}, which is 1.5 times higher than that obtained in the control and 2.9 times higher than that in N$^-$ (Table 3). The biomass and lipid production ability of some *Desmodesms* isolates are summarized (Table 4). It shows that the total lipid content of 38.0 % and lipid productivity of 32.0 mg L^{-1} day^{-1} obtained in the present study in the lipid production process were comparable or even higher than those reported in literature. Therefore, adding bicarbonate into cultures can be used as a stable alkaline condition for enhancing oil production in microalgae, especially under outdoor conditions, since the pH changes drastically in outdoor cultures (Moheimani and Borowitzka 2006).

Conclusions

The highest biofuel potential was observed when bicarbonate amendment and nitrogen deficiency were combined. Arrested cell cycle, depressed starch accumulation upon sodium bicarbonate amendment, decreased cell pigment and protein content, and degraded polar lipid upon nitrogen limitation may all save the energy for TAG synthesis. Outdoor experiments further suggested that bicarbonate could be used as a stable stress factor during nutrient depletion to obtain lipids accumulatively. Our results also highlighted the adaptation of *Desmodesmus* sp. NMX451 in high alkaline systems with high lipid production. Cell cycle retardation under high pH stress causes the cells to become larger, further reducing the cost of harvesting in *Desmodesmus* sp. NMX451 and perhaps also in other industrially relevant microalgae. Therefore, this study, which used a combination of basic physiology and applied research, provides a novel and effective culture strategy for microalgal biodiesel production.

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Table 4 Biomass and lipid productivities of some *Desmodesmus* isolates

	Lipid content (%, w/w)	Biomass productivity (mg L^{-1} day^{-1})	Lipid productivity (mg L^{-1} day^{-1})	Culture volume (L)	Reference
Desmodesmus brasiliensis[a]	18.0	130.0	23.4	0.6	Nascimento et al. (2013)
Desmodesmus sp.[a]	12.8–13.5	–	–	–	Pan et al. (2011)
Desmodesmus sp. F2[a]	64.1	–	263.0	1	Ho et al. (2014)
Desmodesmus intermedius[b]	23.3	125.9	29.3	5	Xia et al. (2013)
D. intermedius XJ-1[b]	23.2	134.0	31.1	5	Xia et al. (2013)
Desmodesmus subspicatus[a]	18.0	–	–	30	Gressler et al. (2014)
Desmodesemus sp. NMX451[b]	38.0	84.2	32.0	5	Present study

[a] Culturing indoors

[b] Culturing outdoors

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