

Coupled effect of silicate and nickel on the growth and lipid production in the diatom *Nitzschia perspicua*

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Abstract The object of this study was to determine the growth rate and biochemical composition of the diatom *Nitzschia perspicua* cultivated with five Si and two Ni concentrations (which we will refer to as treatments) during a “growth” and “lipid” phase (GP and LP). The studies multi-factorial experimental design will provide information on the biochemistry of lipid accumulation under Si-replete and Si-deficient conditions in media and with high and low Ni concentrations. Growth rates ranged from 0.14 to 0.24 day⁻¹ during GP; they were higher in the low rather than high Ni treatments but did not vary in response to Si between 0.2 and 10.6 mmol m⁻³. Dry weights were higher when *N. perspicua* was grown in the LP than those in the GP (irrespective of Ni or Si treatment; with two exceptions). When comparing the same Si treatment, high Ni cultures had higher biomass (C% of dry weight (DW)) than low Ni cultures. High Si treatments generally had significantly lower N% content in both GP and LP. Fourier transform infrared spectroscopy revealed that high Ni in the medium induced higher lipid, protein, and polysaccharide contents regardless of the Si concentration during the GP. After being transferred to LP, cultures grown in lower Si concentrations generally accumulated more lipids than those grown at higher Si, consistent with findings from the oil index. The work described herein provides a better understanding of

the biochemistry of lipid accumulation of a representative diatom, *N. perspicua*, with a range of Si and Ni concentrations.

Keywords Lipid · Biofuels · Silicate · Nickel · *Nitzschia* · Diatoms

Introduction

Interest in the production of microalgae as a biofuel is increasing due to their high oil content; rapid biomass production and small carbon foot print (Chisti 2007; Gonzalez-Fernandez et al. 2012; Schenk et al. 2008). While the crisis of conventional fuel (petroleum for vehicles) during 1970s spurred the first real focus on using microalgae as alternative fuel source, the present interest in reducing CO₂ emissions has motivated a renewed attention in microalgae (Benemann et al. 1977; Brennan and Owende 2010; Chisti 2008; Sawayama et al. 1995). Over the decades, there has been a vast amount of research done on numerous microalgal species; studies have found that the quantity and quality of lipids varies in a species-specific manner as the result of changes in growth conditions (nutrient concentrations, temperature, and light intensity) (Hu et al. 2008; Illman et al. 2000; Sheehan et al. 1998). Microalgae have a higher photosynthetic efficiency than plants (Usui and Ikenouchi 1997) and have been suggested to be able to produce up to 100,000 L ha⁻¹ year⁻¹ (depending on their oil content, which can range from 30 to 70 % of dry weight), which is appreciably higher than other biofuel crops such as rapeseed, sugar cane, sugar beet, soybean, maize, and palm oil (Chisti 2007, 2008; Anemaet et al. 2010).

In terms of microalgae, many in the biofuel industry believe that diatoms have a great potential to be one of the top producers but more research is needed. Diatoms are strong candidate because of their high productivity, high diversity,

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trophic flexibility, and their efficient nutrient utilization (Hildebrand et al. 2012). They are found in a wide range of habitats and variable environments (Falkowski and Raven 2007). Nutrient availability is of considerable importance to the growth and primary production of microalgae. Unlike other microalgal groups, diatoms have an absolute requirement for silicon (Si) for growth and reproduction, that is, without sufficient Si in the medium, diatoms are not able to generate their highly ornamented external cell wall, the frustule, composed of biogenic amorphous silica. Si deprivation has been clearly documented to lead to cellular physiological stress (Jiang et al. 2014a) and cease cell cycle progression (Brzezinski et al. 1990; Martin-Jézéquel et al. 2000). Other taxa which also use Si to build cell structures (e.g., Chrysophytes) are more flexible and can continue to proliferate even in its absence (Falkowski and Raven 2007). Reducing Si concentration has been shown to be used as an effective strategy by the biofuel industry to limit diatom growth and/or induce lipid accumulation in actively growing strains (e.g., Chagoya et al. 2014; Jiang et al. 2014b).

Further, diatoms can use a variety of nitrogen (N) forms in the aquatic systems, including inorganic (e.g., nitrate and ammonium) and organic forms (e.g., urea and amino acids) (e.g., Dortch et al. 1991; Peers et al. 2000). Diatoms can grow fast on ammonium and urea, partly due to the low energetic costs associated with assimilation of these reduced N forms. In both field and laboratory studies, urea has been shown as an important N source for the growth of phytoplankton communities (Dupont et al. 2010; Joubert et al. 2011; Tamminen and Irmisch 1996; Becker 1994; Hsieh and Wu 2009; Matsudo et al. 2009; Oliveira and Antia 1986a, b). Diatoms use the enzyme urease for urea hydrolysis (Rees and Bekheet 1982), through which urea is metabolized to ammonium and carbon dioxide. Urease requires nickel (Ni) at its catalytic site (Mobley and Hausinger 1989; Oliveira and Antia 1986a, b). Urea-based medium therefore has a parallel requirement for Ni in sufficient quantities (Oliveira and Antia 1984, 1986a).

Nitzschia spp. are simple to cultivate and have fast growth rates and high lipid production (Chagoya et al. 2014; Jiang et al. 2014b), important characteristics for large-scale outdoor cultivation. The objective of this study was to determine the growth rate and biochemical composition of the diatom *Nitzschia perspicua* grown with five Si and two Ni concentrations during a “growth” and “lipid” phase (GP and LP, respectively). Growth rate, dry weight, ash-free dry weight, cellular carbon and nitrogen contents, oil index (measured using Nile Red staining), and composition (analyzed using Fourier transform infrared spectroscopy) were measured. The study’s multi-factorial experimental design will provide information on the biochemistry of lipid accumulation under Si-replete and Si-deficient conditions in media with high and low Ni concentrations.

Materials and methods

Cultivation conditions

All experiments were conducted in a 6020 series Caron diurnal incubator with a light/dark cycle (12:12 h) and light intensity of 130–150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Li-Cor, USA; LI-1888 integrating quantum radiometer photometer). Photoperiod and temperature ramping from 10 to 25 °C (see Table 1) were chosen to simulate spring/fall conditions in the Southern USA. These were based on average values recorded for each season in Midland, TX (2007–2010), by the data of the Texas High Plains Evapotranspiration Network meteorological station (Pinowska et al. 2011). All cultures were grown in triplicate 125 mL polycarbonate bottles and stirred by hand three times each day to ensure mixing. Samples were always taken at the same time of day, between 9:00 A.M. and 10:00 A.M. and then again between 4:00 P.M. and 5:00 P.M. Samples for analysis of parameters aside from cell density (e.g., ash-free dry weight (AFDW) and Fourier transform infrared (FTIR)) were always performed in the morning. This was important to avoid any issues which may have arisen due to cell cycle effects.

The artificial brackish growth medium (see Pinowska et al. 2011 for more details) used to grow *N. perspicua* included monoammonium phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$, 0.26 mol m^{-3}) as the phosphate (P) source and urea ($\text{CH}_4\text{N}_2\text{O}$, 4.26 mol m^{-3}) as the N source. The medium was ameliorated with Gaffron’s trace metal solution (1 mL L^{-1} ; Hughes et al. 1958; Zehnder and Gorham 1960) and f/2 vitamins (1 mL L^{-1} ; Guillard and Ryther 1962). This did not include added iron. The medium was adjusted to 15 practical salinity units with NaCl (Tru-soft, solar crystals, United Salt Corp.) and a saturating solution of sodium bicarbonate added as the carbon source (see Pinowska et al. 2011; Jiang et al. 2014b).

Table 1 Light and dark schedule and temperature ramping to simulate spring/fall conditions in Midland, TX

Time	Temperature (°C)	Light
8:00 A.M.	13	On
10:00 A.M.	17	On
12:00 P.M.	21	On
2:00 P.M.	25	On
4:00 P.M.	23	On
6:00 P.M.	21	On
8:00 P.M.	19	Off
10:00 P.M.	17	Off
12:00 A.M.	15	Off
2:00 A.M.	13	Off
4:00 A.M.	11	Off
6:00 A.M.	10	Off

For Si treatments, final concentrations of Si added, in the form $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$, were 0.2, 1.1, 2.1, 4.2, and 10.6 mol m^{-3} . The corresponding pH in the media was 8.85 ± 0.01 , 8.91 ± 0.01 , 8.91 ± 0.06 , 9.03 ± 0.06 , and 9.27 ± 0.05 , respectively. These high concentrations of Si required extra time in medium preparation, that is, the medium was allowed to stir overnight in order to ensure that there were no precipitates before experiments started. Further, to avoid potential dissolution issues, the medium was double-filtered rather than autoclaved for sterilization. Zeng et al. (2014) provide details on the constraints for dissolving high Si concentrations (e.g., NaCl, pH, and temperature). The two Ni concentrations used in these experiments, 0.025 and 0.278 mmol m^{-3} , will be referred to as low and high, respectively. These Ni concentrations were chosen to represent the base Ni concentration in the media plus a concentration tenfold higher. Urease requires Ni concentrations in the medium above background levels. Both concentrations used would have been more than sufficient to supply the enzyme even at the high cell densities present in our cultures.

Acclimation, growth, and lipid phase experiments

In all cases, *N. perspicua* was acclimated to each of the treatment conditions for approximately three generations before starting experiments, which were performed in semi-continuous batch mode. For the growth phase experiments, on day 0, each culture was inoculated at a cell density equivalent to an optical density at 750 nm (OD_{750}) of 0.3 to 0.5. Thereafter, OD_{750} was measured with a spectrophotometer (UV-2501PC UV-VIS, Shimadzu) two times daily at the same time each day (early morning and late afternoon). Fresh medium was used for blank measurements. The specific growth rate (day^{-1}) in the exponential phase was calculated from OD_{750} measurements: $\mu = 1/T(\ln N_{t+1} - \ln N_t) \text{days}^{-1}$, where N_t is the OD_{750} reading at time point t , N_{t+1} is the OD_{750} reading at time point $t+1$, and T (days) is the interval between observations. After the cultures reached exponential growth phase, half of the volume was harvested and replaced with fresh medium of the same composition. This process was repeated two times as part of the growth experiment. Growth rates from the last growth cycle were used in the statistical analyses. At the end of the growth phase experiment, fresh medium was prepared minus the Si but with a full complement of all the other nutrients. This was mixed with an equal volume of growth phase culture which had reached stationary phase. This designated the start of the lipid phase experiment. For simplicity, we will refer to the lipid phase treatments by the corresponding Si concentrations.

NR fluorescence of microalgae

Changes in the intracellular neutral lipid content at the end of GP and during the LP were followed by measuring

fluorescence intensity of Nile Red (NR) stained cultures (Cooksey et al. 1987; Elsey et al. 2007). Briefly, cells were stained with 9-diethylamino-5H-benzophenoxazine-5-one (final concentration in acetone, $0.75 \mu\text{g mL}^{-1}$), shaken gently for 10 s, incubated at room temperature for 3 min, and measured using a spectrofluorophotometer (RF-5301, Shimadzu) at 490-nm excitation and 500–750-nm emission wavelengths. The relative fluorescence intensity of NR was obtained after subtraction of the self-fluorescence of NR. The relative NR fluorescence intensity was then normalized to cell density using $\text{OD}_{750 \text{ nm}}$ to determine the lipid index, as lipid index = total area calculated under 580 nm peak/absorbance measured at $\text{OD}_{750 \text{ nm}}$.

DW, ash content, AFDW, and CHN

Algal cultures were filtered onto precombusted (500°C , 4 h) glass fiber filters (Whatman GF/F, 47 mm, nominal pore size $0.7 \mu\text{m}$) and rinsed with a solution of 0.34 N NaCl in 0.1 N HCl, followed by a rinse with 0.43 M ammonium formate to remove the NaCl (Britton and Greeson 1987; Zhu and Lee 1997). Samples were then dried at $103\text{--}105^\circ\text{C}$ for 2 h. After the dry weights (DWs) were measured, the samples were ashed at 500°C for 4 h, and the ash content was measured. AFDW was calculated by subtracting the ash weight from the total DW. Ash content was expressed as the percentage of ash weight in the total DW. Cellular carbon (C) and N were measured using a CHN analyzer (Perkin Elmer 2400 CHNS/O Analyzer) as described previously (Jiang et al. 2012).

FTIR spectroscopy

Integrated infrared FTIR band areas were used to identify the major macromolecules present in the samples (Dean et al. 2010; Giordano et al. 2001; Jiang et al. 2012). Briefly, freeze-dried whole cell pellets (1–3 mg) were allowed to come to room temperature before analysis was performed using a Variant 3100 FTIR with Varian Resolution Pro 4.0 software and connected to a single-reflection horizontal attenuated total reflection (ATR accessory) PIKE Technologies, Inc. (Madison, WI) according to Jiang et al. (2012). Twenty-five scans (absorbance) were collected with a spectral resolution of 8 cm^{-1} between 2,000 and 600 cm^{-1} ; these were co-added and averaged. The integrated FTIR band areas were used to calculate the relative ratios of cellular macromolecules (i.e., proteins, lipids, and nucleic acids).

Statistical analysis

Data represent means \pm standard deviations (SD). A three-way analysis of variance (ANOVA) was performed on the growth

rate, DW, lipid index, and C/N ratios to assess the effect of different Si concentrations and Ni concentrations under GP and LP. Data were log-transformed when non-normal distribution occurred. The homogeneity and normality of the data sets were analyzed with Levene's test and the Shapiro-Wilk test at a significance level of 95 %. The differences among Si were also investigated by conducting a one-way ANOVA followed by post hoc pairwise comparisons among treatments using Tukey's HSD test for each treatment. Independent *t* test with Bonferroni correction was used for Ni treatments and phase comparisons. Non-linear regression analysis was performed to assess the relationship of cellular C and logarithmic lipid index among treatments. The relationship was fitted using a quadratic regression equation ($y=y_0+a*x+b*x^2$). All statistics were performed using SPSS 20.0.

Results

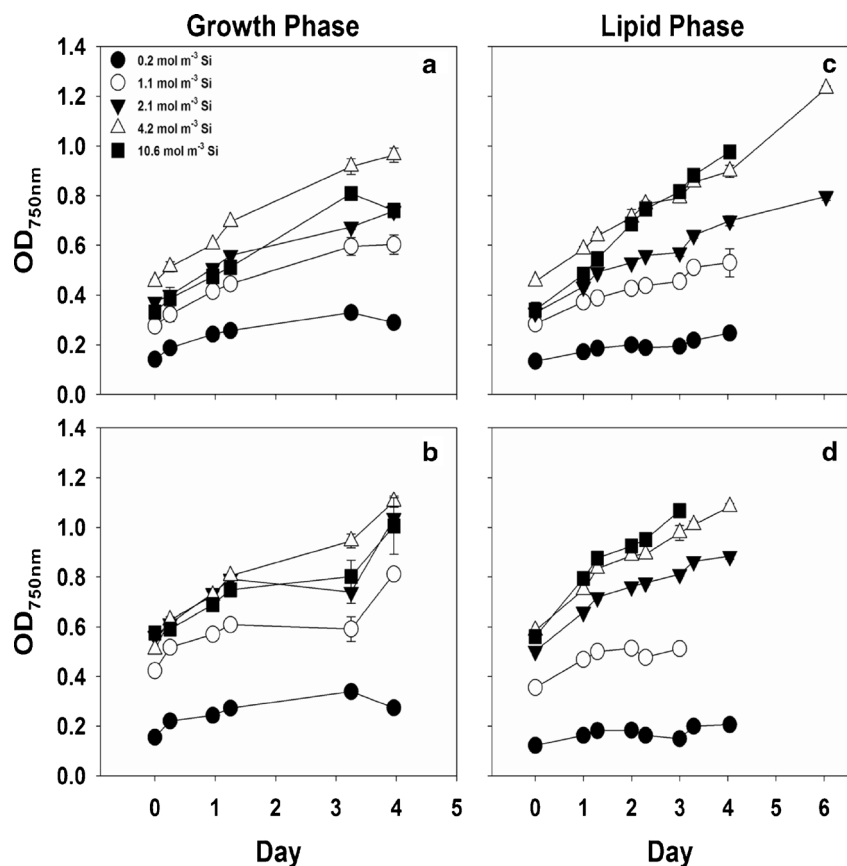
Growth rates during growth and lipid phases

The changes in OD_{750 nm} are shown for both low and high Ni concentrations with different Si treatments during the growth and lipid phases (Fig. 1). Growth rates in the different Si treatments ranged from 0.14 to 0.24 day⁻¹ during GP

(Fig. 2a). High Si concentrations in the media did not correspond to higher *N. perspicua* growth rates. In comparing the growth rates under the high and low Ni concentrations during GP, they were consistently between 11 and 45 % higher in the low Ni treatment (mean low Ni=0.21±0.02 day⁻¹; mean high Ni=0.16±0.02 day⁻¹; independent *t* test, $p<0.0001$, $n=15$) (Fig. 2a). Despite having ten times more Ni in the media, growth rates were not enhanced by elevating the Ni in the media.

After being transferred to LP medium, *N. perspicua* continued to grow for several days at same pace as in the GP medium despite the absence of Si in the new medium (Fig. 1). During LP, the highest Si (10.6 mol m⁻³) treatments had significantly higher growth rates than in other Si treatments, and in treatments with both low (mean 0.29±0.03 day⁻¹, HSD, $p<0.05$) and high Ni (mean 0.21±0.005 day⁻¹, HSD, $p<0.01$; Fig. 2b). The growth rates in the high Ni treatments were mostly lower compared with the low Ni cultures (Fig. 2b), ranging from 0.13 to 0.29 day⁻¹ (mean low Ni=0.2±0.05 day⁻¹, mean high Ni=0.17±0.03 day⁻¹, independent *t* test, $p<0.05$). The results of the three-way ANOVA found that there was a significant three-way interaction ($p<0.001$) between the concentrations of Si and Ni and phase (i.e., GP and LP) for growth rates (Table 2).

Fig. 1 Changes in OD_{750 nm} of *N. perspicua* for the different Si concentrations with low Ni (a, c) and high Ni (b, d) during the growth phase and lipid formation phase, respectively. Error bars represent standard deviation ($n=3$). If the error bars are not visible, they are smaller than the symbol



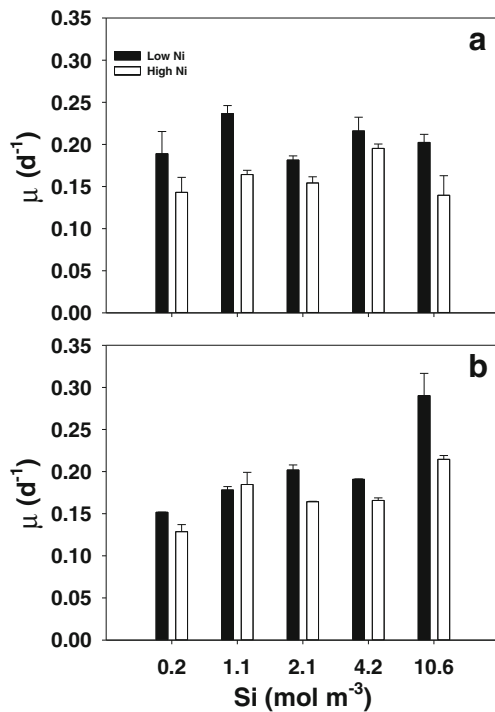


Fig. 2 Growth rates of *N. perspicua* cultivated in different Si and Ni concentrations media during the growth phase (a) and lipid formation phase (b). Errors bars represent standard deviation ($n=3$). If the error bars are not visible, they are smaller than the symbol

Changes in biochemical composition during growth and lipid phases

A summary of measured biochemical characteristics in both GP and LP is given in Table 3 for DW (g L^{-1}), ash content (%), g g^{-1} DW), C/N ratios, and C% and N% of DW. In GP, the highest DWs were measured when *N. perspicua* was grown with 2.1 mol m^{-3} Si/low Ni ($0.38 \pm 0.05 \text{ g L}^{-1}$) and

1.1 mol m^{-3} Si/high Ni ($0.44 \pm 0.04 \text{ g L}^{-1}$; Table 3). Dry weight was significantly higher in the 2.1 mol m^{-3} Si treatment compared to all other Si treatments with low Ni medium in both GP (HSD, $p < 0.05$) and LP (HSD, $p < 0.0001$). DW showed no significant differences among the five Si treatments with high Ni concentration in GP (one-way ANOVA, $F_{4,10} = 1.938$, $p = 0.198$). The DW in the 4.2 mol m^{-3} Si treatment was significantly higher than other Si treatment with high Ni medium in LP only (HSD, $p < 0.0001$). Dry weights were higher when *N. perspicua* was grown in the LP than those in the GP regardless of the Ni or Si concentrations with only a few exceptions (independent t test, $p < 0.0005$; Table 3).

The ash% of DW increased with increasing Si concentration regardless of the concentration of high or low Ni and in the GP (Table 3). The highest ash% of DW, from 37 to 47 % of DW, was measured when *N. perspicua* was grown in 4.2 or 10.6 mol m^{-3} Si irrespective of the Ni concentration in GP treatments (Table 3). During LP, ash% of DW only significantly increased in the 10.6 mol m^{-3} Si treatment (HSD, $p < 0.0001$; Tables 2 and 3).

In most cases, when comparing the same Si treatment, high Ni cultures had higher biomass than those grown under low Ni in terms of C% of DW (Table 3). Lower Si concentration (0.2 mol m^{-3} Si) resulted in the highest values of C% of DW and C/N ratios under both growth and lipid formation phases regardless of the concentration of Ni (HSD, $p < 0.01$). The higher C/N ratios in the higher Ni treatments may be the result of faster increases in the intracellular C content. The N% of DW ranged from 4.37 ± 0.29 to 6.59 ± 0.33 (Table 3). In both GP and LP, the high Ni treatments had higher or similar cellular N contents of DW than the low Ni (independent t test, GP, $p = 0.18$; LP, $p = 0.41$) When comparing the five Si treatments with the low Ni medium, high Si concentrations

Table 2 The results of a three-way ANOVA examining the significance of the effect of phase, different Si and Ni concentrations on the growth rates, dry weight (DW), lipid index, and C/N ratios of *Nitzschia perspicua*

Source of variation	df	Growth Rate (μ, d^{-1})		DW (mg L^{-1})		Lipid index		C/N	
		F	p	F	p	F	p	F	p
Phase	1	2.24	NS	148.70	***	19.26	***	882.37	***
Si	4	35.26	***	18.96	***	134.81	***	596.69	***
Ni	1	137.63	***	14.50	***	3.08	NS	158.40	***
Phase*Si	4	39.66	***	27.46	***	15.86	***	31.96	***
Phase*Ni	1	5.24	*	0.10	NS	10.57	**	1.16	NS
Si*Ni	4	5.97	***	25.66	***	6.13	***	39.15	***
Phase*Si*Ni	4	6.85	***	30.90	***	3.94	*	11.69	***

Phase: growth phase (GP) and lipid phase (LP); Si—0.2, 1.1, 2.1, 4.2, and 10.6 mol m^{-3} Si; Ni—0.025 and $0.278 \text{ mmol m}^{-3}$ Ni. Phase, Si, and Ni were treated as fixed variables

NS not statistically significant at least at $p < 0.05$ level

* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$

Table 3 Changes in biochemical characters of *Nitzschia perspicua* in the growth and lipid phase media with different Si and Ni treatments

	Treatment									
	0.025 mmol m ⁻³ Ni					0.278 mmol m ⁻³ Ni				
Si (mol m ⁻³)	0.2	1.1	2.1	4.2	10.6	0.2	1.1	2.1	4.2	10.6
Dry weight (g L ⁻¹)										
GP	0.10±0.03	0.20±0.05	0.38±0.05	0.20±0.01	0.28±0.02	0.37±0.1	0.44±0.04	0.34±0.06	0.30±0.01	0.31±0.03
LP	0.42±0.06	0.42±0.02	0.89±0.03	0.41±0.02	0.48±0.02	0.20±0.02	0.29±0.03	0.48±0.01	1.58±0.16	0.53±0.04
Ash% DW										
GP	10.1±1.33	18.3±2.69	45.8±0.2	36.9±5.57	47.3±0.82	13.8±4.48	23.5±7.43	22.6±6.24	41.0±3.2	40.7±2.8
LP	–	23.3±0.59	19.5±1.99	27.4±1.32	37.3±2.29	26.8±2.71	20.1±3.22	21.7±1.2	15.1±1.15	41.4±2.64
C/N										
GP	7.76±0.35	6.82±0.12	6.35±0.06	6.08±0.14	5.91±0.14	10.4±0.12	6.96±0.08	6.56±0.13	6.28±0.09	6.09±0.03
LP	11.1±0.47	8.44±0.33	7.9±0.17	6.86±0.2	7.04±0.38	12.6±0.24	9.75±0.24	8.11±0.33	7.88±0.14	6.91±0.09
C% DW										
GP	42.6±0.42	36.2±0.67	32.5±0.43	26.8±0.21	25.6±0.36	44.9±0.58	39.3±1.55	36.0±0.21	30.0±0.06	29.3±0.11
LP	49.8±0.32	46.8±4.75	40.1±0.43	34.4±0.49	26.4±3.1	50.8±0.09	45.7±0.48	43.0±0.28	39.4±0.91	27.0±0.55
N% DW										
GP	6.41±0.34	6.19±0.22	5.97±0.13	5.13±0.15	5.06±0.19	5.03±0.01	6.59±0.33	6.41±0.16	5.57±0.08	5.6±0.04
LP	5.26±0.21	6.47±0.65	5.92±0.17	5.85±0.25	4.37±0.29	4.69±0.08	5.47±0.09	6.19±0.25	5.84±0.19	4.57±0.15

Values are means±SD. (GP growth phase, LP lipid phase, – no data)

GP growth phase, LP lipid phase, – no data

(10.6 mol m⁻³ Si) had significantly lower N% content (HSD, $p<0.05$) in both GP and LP. The results of three-way ANOVA found significant three-way interactions ($p<0.001$) between the concentrations of Si and Ni and phase for C/N ratios and algal biomass (dry weight) (Table 2) with one exception. DW did not vary significantly between Ni concentrations but varied considerably depending on the concentrations of Si in the medium (Table 2).

Lipid index measured during the growth and lipid phases

The lipid index, ranging from 0.05 to 2.2×10^5 , declined from lowest to highest Si concentrations and for both Ni concentrations, in *N. perspicua* grown during the GP and LP (Fig. 3a, b, respectively). Highest lipid indices were therefore measured when *N. perspicua* was grown at the lower Si concentration; these were significantly higher than those measured at other Si concentrations for GP (0.2 mol m⁻³ Si, HSD, $p<0.0001$ for both low and high Ni) and LP (0.2 and 1.1 mol m⁻³ Si, HSD, $p<0.05$ for high Ni). Further, in the 0.2 mol m⁻³ Si treatment, the lipid index was not significantly different between low and high Ni concentration for GP (independent t test, $p=0.28$) or LP (independent t test, $p=0.83$). The results of three-way ANOVA showed that lipid index also varied significantly with Si treatments. Ni concentrations and phase ($p<0.05$) (Table 2).

To examine the potential to use *N. perspicua* carbon fixation to predict the oil content at high and low Ni

concentrations, C% of DW and oil index response curves were compared (Fig. 4a, b, Table 4). After log transformation, the

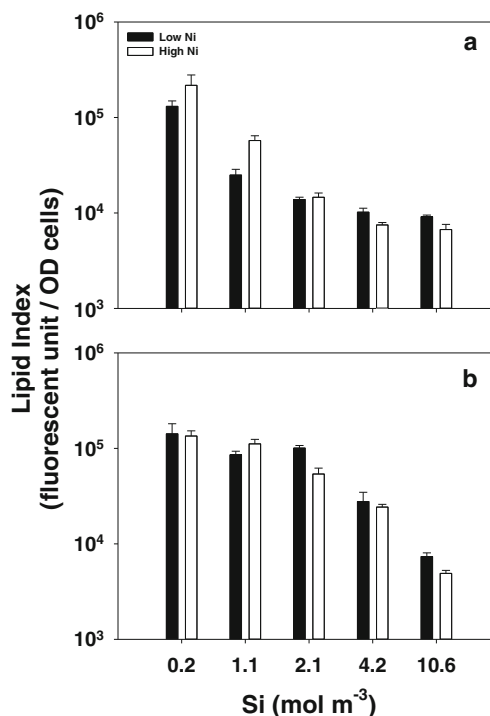


Fig. 3 Neutral lipid content of *N. perspicua* stained with Nile Red per OD₇₅₀ unit cultivated in different Si and Ni concentrations during the growth phase (a) and lipid formation phase (b). Errors bars represent standard deviation ($n=3$). If the error bars are not visible, they are smaller than the symbol

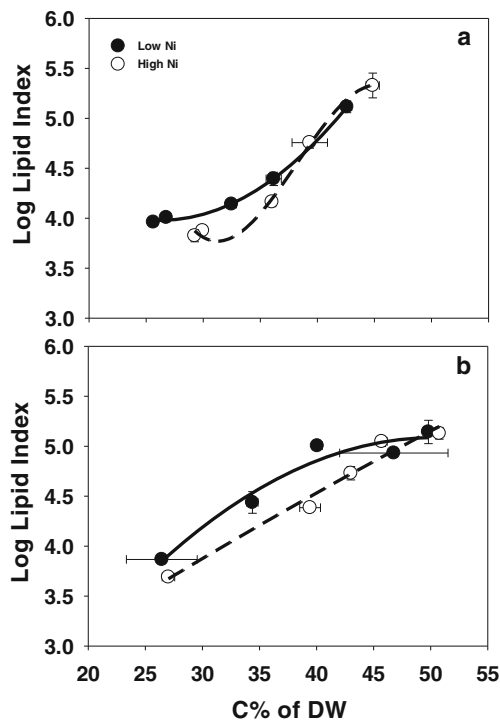


Fig. 4 The logarithmic changes in lipid content (measured by the oil index) of *N. perspicua* as a function of cellular carbon content under low (solid circles) and high Ni (open circles) and in the five Si treatments during the growth phase (a) and lipid formation phase (b). Errors bars represent standard deviations ($n=3$). If the error bars are not visible, they are smaller than the symbol. Quadratic regressions are shown for both low (solid line) and high (dash line) Ni treatments (see Table 4 for the regression models and parameters)

Shapiro-Wilk test on the oil index from the five Si treatments in both GP and LP indicated the data were normally distributed ($p>0.05$; Table 4). Further, fitting to quadratic regression models yielded generally highly significant regression coefficient (Table 4). Algal cells showed significantly carbon-dependent increase of the lipid in the five Si treatments with high and low Ni in the

growth (high Ni, $r^2=0.980$, $p<0.05$; low Ni, $r^2=0.999$, $p<0.005$; Fig. 4a and Table 4) and lipid formation phases (high Ni, $r^2=0.969$, $p<0.05$; low Ni, $r^2=0.954$, $p<0.05$; Fig. 4b and Table 4). Quadratic fits for all treatments combined had a significant regression between the lipid and carbon content ($p<0.0001$; Fig. 5).

FTIR spectra during the growth and lipid phases

By using FTIR, we examined the macromolecular composition of *N. perspicua* during the lipid and growth phases. The spectral range from 2,200 to 800 cm^{-1} is shown in Fig. 5 for a variety of treatments. The integrated peak areas of the FTIR spectra which represent polysaccharides (1,180–870 cm^{-1}), lipids (1,762–1,720 cm^{-1}), proteins (1,578–1,490 cm^{-1} ; amide II), and nucleic acids (1,256–1,186 cm^{-1}) were measured from the whole cell spectra. *N. perspicua* produced characteristically different FTIR spectra when grown under different Si and/or Ni concentrations. As there was a clear transition in the macromolecular composition of cells between the Si treatments, only spectra obtained from cultures grown with 0.2, 2.1, and 10.6 mol m^{-3} Si are shown (Fig. 5). High Ni in the medium induced higher lipid, protein, and polysaccharide contents regardless of the Si concentration during the GP (Fig. 5). After being transferred to the lipid formation medium, cultures grown in lower Si concentrations ($<1.1 \text{ mol m}^{-3}$) generally accumulated more lipid than cultures grown at higher Si concentrations (Fig. 5) consistent with findings from the oil index (Fig. 4).

We calculated the integrated peak area to examine the changing spectral ratios of macromolecules in *N. perspicua* grown under in different Si and Ni treatments (Fig. 6). The lipid/protein ratio responded to Si concentrations, showing a decrease in this ratio with increasing Si concentration due to the decrease in the lipid band. High Si treatments (4.2 and 10.6 mol m^{-3}) induced significantly lower lipid/protein ratios

Table 4 Results of quadratic regression parameters in cellular carbon content with logarithmic lipid index in both growth (GP) and lipid formation phases (LP) under low and high Ni concentrations

		y_0	a^*	b^*	df	r^2	p value	Normality test (p) (Shapiro-Wilk)
GP	Low Ni	6.908	-0.223	0.004	4	0.999	0.001	0.556
	High Ni	5.351	-0.151	0.003	4	0.980	0.020	0.915
	All (GP)	7.253	-0.248	0.005	9	0.966	<0.0001	0.089
LP	Low Ni	-0.400	0.218	-0.002	4	0.954	0.046	0.732
	High Ni	1.689	0.078	0.000	4	0.969	0.031	0.672
	All (LP)	0.771	0.143	-0.001	9	0.908	0.0002	0.895
All (GP + LP)		1.561	0.098	-0.001	19	0.867	<0.0001	0.240

Lipid index= $y_0+a^*(\text{carbon}\%)+b^*(\text{carbon}\%)^2$ where y_0 is the intercept while a and b are correlation coefficients. Significant results ($p<0.001$) are shown in bold

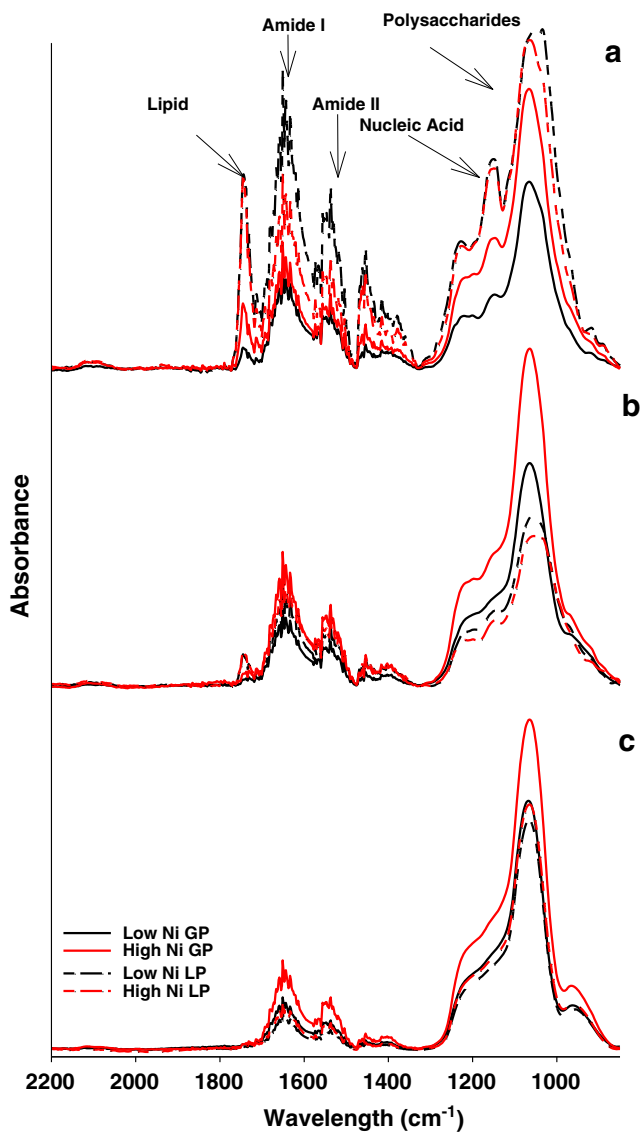


Fig. 5 FTIR spectra (2,200–800 cm^{-1}) collected for whole cells of *N. perspicua* in growth (solid line) and lipid formation phase media (dash line) with low Ni (black) and high (red) concentrations under 0.2 (a), 2.1 (b), and 10.6 mol m^{-3} (c) Si conditions (color figure online)

irrespective to the Ni concentration (HSD, $p < 0.05$; Fig. 6a, d). By contrast, high Ni combined with low Si (0.2 mol m^{-3}) induced a significantly higher lipid/protein ratio than in the corresponding low Ni treatments in GP (independent t test, $p < 0.0005$) and LP (independent t test, $p < 0.01$; Fig. 6a, d). Hence, the effect of Ni concentration was only observed in the lowest Si treatment.

Increasing Si concentrations in the medium resulted in a decrease the lipid/nucleic acid ratio up to a factor of 11 (low Ni in GP, Fig. 6b) to 41 (low Ni in LP, Fig. 6e). At the same time, increasing Si concentration resulted in a rise of the carbohydrate/protein ratios regardless of the Ni concentration and phases (GP and LP, Fig. 6c, e). The treatment with high Si led to a significantly greater carbohydrate/

protein ratio than other Si treatment in both GP (4.2 and 10.6 mol m^{-3} Si, HSD, low Ni, $p < 0.05$; high Ni, $p < 0.01$) and LP (10.6 mol m^{-3} Si, HSD, low Ni, $p < 0.001$; high Ni, $p < 0.0001$) (Fig. 6c, f).

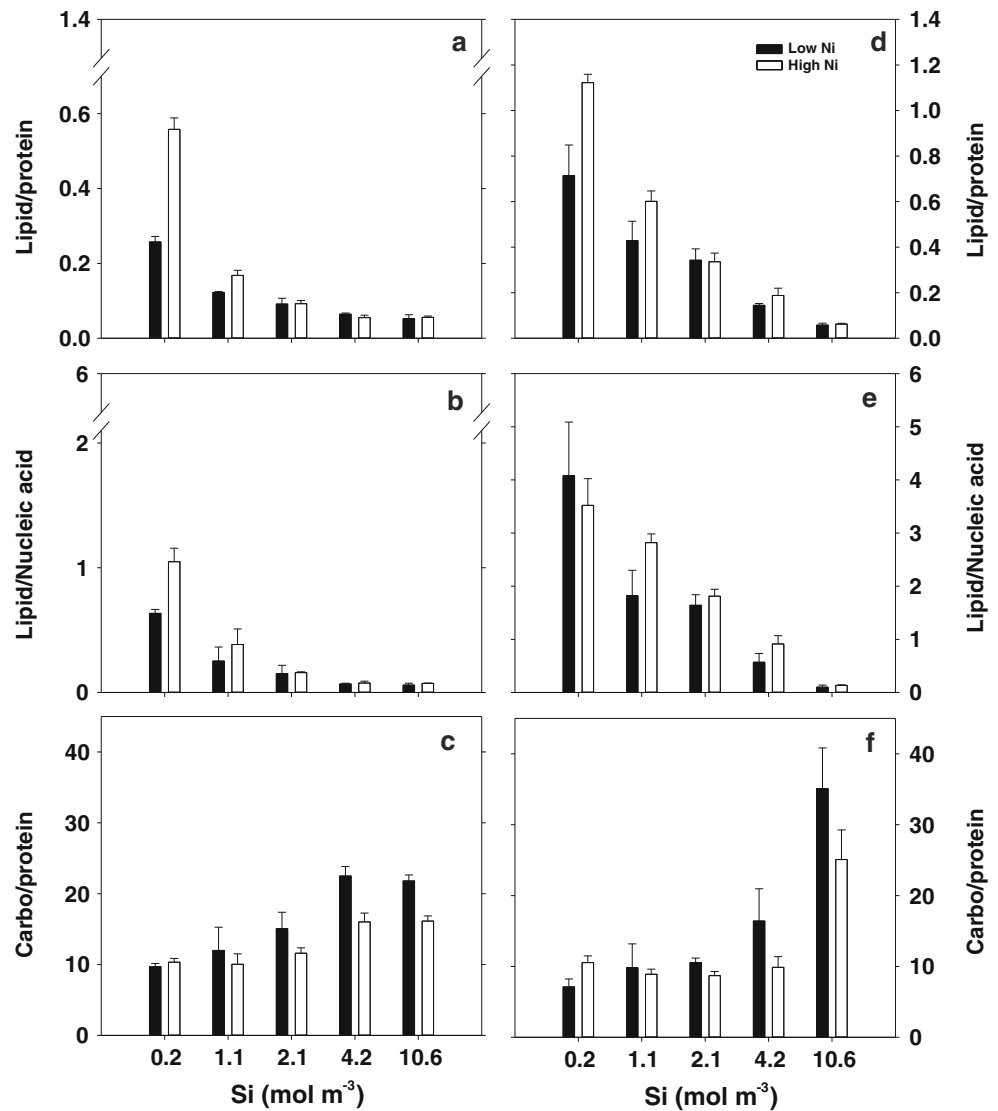
Discussion

Microalgal biomass accumulation depends on their ability to acquire major (C, N, and P) and minor (e.g., Fe, Mn, Zn, Cu, Co, and Ni) nutrients. There are species-specific preferences for some but not all nutrients (e.g., Quigg et al. 2003, 2011) including diatoms which have a unique absolute requirement for the major nutrient silicate (Brzezinski et al. 1990; Martin-Jézéquel et al. 2000; Jiang et al. 2014a). Diatoms are known to also have differing requirements for Ni, depending on the primary source of N in the medium (Oliveira and Antia 1984, 1986a, b; Peers et al. 2000). In this paper, we explored influence of simultaneously differing Si and Ni concentrations on growth and lipid formation in the diatom *N. perspicua*. We focused on a diatom since this group is considered a good feedstock for biodiesel based on their known oil and carbohydrate production (Dempster and Sommerfeld 1998; Hildebrand et al. 2012).

Si requirements for diatoms From our results, Si concentrations had to be significantly less than 0.2 mol m^{-3} before there was a marked change in the growth rate of *N. perspicua* during the lipid phase (Figs. 1 and 2). This is not surprising given that most artificial media and natural waters have significantly lower (mol m^{-3}) Si concentrations. The high concentrations used in this study greatly enhanced the carrying capacity (cells m^{-3} or g DW L^{-1}) of the medium (data not shown) but apparently not the growth rate of the diatom. This is an important trade-off when growing diatoms for biofuels versus other kinds of activities. Our findings contribute to the body of work addressing the need to balance medium optimization for high biomass versus that for high lipid production.

Diatom cells undergoing nutrient limitation introduce metabolic acclimatization, which often results in marked changes in their biochemical composition. For example, silicate (Si) limitation leads to lipid accumulation in *Chaetoceros gracilis*, *Hantzchia* sp., *Nitzschia* spp., and *Cyclotella* sp. (Roessler 1988; Taguchi et al. 1987). In terms of lipid production induced by Si, we found that the oil index did vary with the Si concentration in the media (Figs. 3, 4, 5, and 6). *N. perspicua* growing on the lowest Si (<1.1 mol m^{-3}) had the highest oil index and cell C content during both GP and LP. This suggests that the changing Si in the media did stress the diatom cells so that they did accumulate additional carbohydrate and lipids, but without significantly impacting the growth rate. Cultures of the diatom with high Si concentration were likely not experiencing the same level of Si depletion as

Fig. 6 Changes of the lipid/amide II (a in GP; d in LP), lipid/nucleic acid (b in GP; e in LP) and polysaccharide/amide II (c in GP; f in LP) ratios determined from FTIR spectral bands for low Ni (black bars) and high Ni (white bars). Errors bars represent standard deviation ($n=3$). If the error bars are not visible, they are smaller than the symbol ($n=3$)



those with low Si. Since lipid formation was allowed for only a few days before the lipid index was determined, there is a correlation between Si depletion to lipid formation/ index. Protein levels also decline as lipids are formed which also explains the increasing lipid-to-protein ratio with decreasing Si. The significant enhancement of lipids with low Si concentrations (increase of the lipid/protein and lipid/nucleic acid ratios) is also consistent with the findings of lipid index (Figs. 3 and 6).

Ni requirements for diatoms In biofuel research, several N forms have been considered for use in the cultivation of microalgae (Becker 1994). Urea-based media are more frequently used than ammonia because they are cheaper, and this form of N is more readily available than all other possibilities combined (Becker 1994; Hsieh and Wu 2009; Matsudo et al. 2009). However, in order for microalgae to use urea effectively, there must be concurrently sufficient concentrations of Ni

present in the medium as this metal ion is required as the catalytic enzyme component for urease synthesis (Muysen et al. 2004; Oliveira and Antia 1984; Rees and Bekheet 1982). Many algal species have been found to grow well on urea as sole N source, including chlorophytes, diatoms, dinoflagellates, and haptophytes (Berman and Chava 1999; Levasseur et al. 1993; Strom and Bright 2009). In some cases, microalgae will grow faster on a urea-based medium relative to a nitrate-based medium (Oliveira and Antia 1986a), but this observation is species-specific.

We found that high Ni stimulated higher lipid, protein, and polysaccharide production in *N. perspicua* but was inhibitory on the growth rate, compared with the tenfold lower Ni concentration we used, especially at lower Si concentrations in the medium. Collectively, the findings indicate that low Ni concentration used in this study ($0.025 \text{ mmol m}^{-3}$) is optimal for lipid production using urea as N source for *N. perspicua*. This is consistent with previous findings which showed that

the optimal Ni concentration to support the urea growth of the diatom *Cyclotella cryptica* was 0.1–1 mmol m⁻³ (and a minimal concentration of 5–10 μmol m⁻³) (Oliveira and Antia 1984). Peers et al. (2000) demonstrated the development of in vitro urease enzyme by adding 63 μmol m⁻³ Ni to the culture medium for *Thalassiosira weissflogii* and *Thalassiosira pseudonana*. Oliveira and Antia (1986a) investigated the optimal Ni concentration for 12 marine microalgae; this varied from 0.005 to 0.1 mmol m⁻³ for species investigated. The multi-species study demonstrated that 10 mmol m⁻³ Ni was severely deleterious to the growth all the microalgae tested (Oliveira and Antia 1986a) while our findings suggest that the high Ni concentration used (0.278 mmol m⁻³) may have been suboptimal to this diatom.

Ni and Si coupled effect When performing these multi-factorial designed experiments, it is important to rule out external factors which may contribute to the final outcome of the experiments. Our findings for *N. perspicua* growing on low Ni support the contention that the cultures were not nitrogen-limited during either the growth or lipid phase. That is, the growth rates on low Ni were significantly higher than those on high Ni in (independent *t* test, $p < 0.0001$) and LP (independent *t* test, $p < 0.05$) (Table 2). Hence, Si limitation was a factor influencing the experiments during the lipid phase but not during GP. Further evidence to support this conclusion comes from the biochemical (Table 3) and macromolecular composition of the diatom cells (Figs. 3, 4, 5, and 6). We found that *N. perspicua* had higher C/N ratios during the lipid phase compared to the growth phase in each of the corresponding five Si treatments. The change in N was driven by a correspondingly larger change in C, that is, a small change in the amount of protein relative to a greater production of energy storage products (lipids/carbohydrates). Similar such findings have been reported previously (Dean et al. 2010; Jiang et al. 2012) reflecting species-specific responses to changes in growth conditions.

The versatility of FTIR for biofuel studies has been documented with previous microalgal investigations (e.g., Giordano et al. 2001; Dean et al. 2010; Jiang et al. 2012). In the present study, the coupled effect of different Ni and Si concentrations on the composition of *N. perspicua* was compared (Figs. 5 and 6). The patterns are complex and reveal the plasticity of the cell physiology in responding to the treatments. For example, we found that high Ni induced higher lipid, protein, and polysaccharide peaks, especially with low Si concentrations (0.2–2.1 mol m⁻³ Si) in both the growth and lipid phases. Under high Si concentrations, low Ni showed a higher transfer from C to lipid than under high Ni, both in GP and LP. Multi-factorial experiments are required to understand the synergistic and potentially antagonistic relationships between different nutrients on algal growth and composition.

Cellular responses are certainly not linear nor are they ubiquitous. Previous studies have also found that inhibition of fatty acid synthesis can result in cell cycle arrest, suggesting that lipid synthesis is essential for the progression of the cell cycle (Jackowski 1996; Kwok and Wong 2005). Despite many studies, including those cited here and the present to investigate the response of cells to nutrient stress in terms of changes in proteins, lipids, and other properties, little is still known about how lipid synthesis is coordinated with the cell cycle. In order to manipulate the growth of different microalgae for biofuel production, a better understanding of how the cell coordinates the synthesis of different macromolecules during cell growth will be key and contribute toward a better understanding of lipid synthesis during cell growth and the cell cycle (Jackowski 1996; Kwok and Wong 2005; Martin-Jézéquel et al. 2000).

While diatoms have a great potential to be one of the top producers for the biofuel industry (Hildebrand et al. 2012), their absolute requirement for Si needs to be addressed. In the absence of sufficient Si, diatom cell cycle stops until Si is once again available (Brzezinski et al. 1990; Martin-Jézéquel et al. 2000; Jiang et al. 2014a). While this can be exploited to induce lipid production, prolonged cessation may lead to loss of cultures, which in a large-scale outdoor pond (tens of thousands of gallons) biofuel setting, would be devastating. Further consideration is the concentration of nutrients added. Traditional media used for cultivation of algae in the lab over the last five to six decades have significantly lower concentrations of nutrients than those used in this and other studies directed at cultivation of significant biomass for biofuels (e.g., Chagoya et al. 2014; Jiang et al. 2014b). In an effort to increase the carrying capacity of the medium (that is, number of cells milliliter), while avoiding nutrient limitation, the concentration of some elements used is close to or at saturating. This is the case for the Si concentrations used in the current study (see also Zeng et al. 2014). At the other end of the spectrum, toxicity must be avoided. In this study, we used two concentrations of Ni to induce urease activity and increase growth urea utilization as a nitrogen source. We did not find evidence of toxicity (e.g., a reduced growth rate). However, given that co-factors for enzymes are trace metals, the consequences of elevating their concentrations as part of the medium optimization process requires careful consideration.

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