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Enhancing lipid production of the marine diatom *Chaetoceros gracilis:* synergistic interactions of sodium chloride and silicon

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Abstract Silicon deficiency is a lipid-promoting stress for many oleaginous diatoms. Literature reports suggest that reduced salinity in seawater, a primary component of which is sodium chloride, may inhibit metabolism of silicon in marine diatoms. We hypothesized that lowering sodium chloride below ocean levels may thus be effective in creating silicon stress and enhancing lipid productivity. We examined the interacting effects of silicon supply (0.05, 0.1, 0.2, and 0.8 mM) and sodium chloride concentration (50, 100, and 400 mM) on growth and lipid production in *Chaetoceros gracilis*. This was done in batch culture to facilitate the application of severe stress. Low levels of either sodium chloride or silicon resulted in at least 50 % increases in lipid content. The synergy of simultaneous, moderate sodium chloride and silicon stress resulted in lipid content up to 73 % of dry mass and lipid productivity of 1.7 g m^{-2} day^{-1}; with a daily integrated photosynthetic photon flux of 17.3 mol photons m^{-2} day^{-1}, the efficiency of lipid synthesis was thus 0.1 g mol^{-1} of photons. Decreased silicon also resulted in a 5 % shift in lipid chain length from C18 to C16 fatty acids. We observed a strong sodium chloride/silicon interaction on total and ash-free dry mass densities that arose because low sodium chloride concentrations were inhibitory to growth, but the inhibition was overcome with excessive silicon supply. This observation suggests that low levels of sodium chloride may have affected metabolism of silicon. The findings of this study can be used to enhance lipid production in oleaginous marine diatoms.

Keywords Abiotic stress · Diatom · Biodiesel · Plant oil · Nutrient stress

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Introduction

Diatoms are a diverse and ecologically successful class of marine and freshwater algae. Diatoms are the primary constituent of the marine plankton community, typically representing more than 70 % of the total plankton, and are estimated to contribute up to 40 % of the total oceanic primary production (Sumper and Brunner 2008). A primary driver of the ecological success of diatoms is their use of silicon (Si) for formation of their exoskeleton, called a frustule. Some likely reasons for this are that it is energetically less costly to make a Si-based cell wall than a carbon-based cell wall and that, different from other limiting nutrients, diatoms have little competition from non-diatoms for Si (Martin-Jezequel et al. 2000). Huge diatom abundance has caused dissolved Si to be drawn down to low levels; however, there is an approximate average of 35 μM Si in surface waters (Hem 1985) and a global average of approximately 70 μM Si (Sumper and Brunner 2008). This has resulted in Si becoming a major limiting nutrient for diatoms in the oceans.

There are two primary Si pools in diatoms: cellular Si and frustule-associated or mineralized Si (Vrieling et al. 1999). At high concentrations, Si enters the cell by diffusion, but at low concentrations (≤30 μM), Si is actively taken up by sodium (Na)-dependent Si transport proteins (Bhattacharyya and Volcani 1980; Hildebrand et al. 1997; Thamatrakoln and Hildebrand 2008). Cellular Si is mineralized to form the frustule in specialized intracellular compartments called silicon deposition vesicles (SDVs). Cell division and Si metabolism are closely tied (Martin-Jezequel et al. 2000). In some species, including *Chaetoceros gracilis* (Lombardi and Wangersky 1991), the production of storage lipid (triacylglycerol or TAG) is stimulated when Si availability is limiting to cell division (Hildebrand et al. 2012; Araujo et al. 2011). Little is known, however, about the physiological mechanism by which deficient Si signals the accumulation of lipids (Merchant et al. 2012). Deficiencies of other

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nutrients, like N and P, have also been shown to promote lipid accumulation in diatoms, but several studies suggest that Si deficiency stimulates lipid formation more rapidly and can result in higher lipid content (Enright et al. 1986; Taguchi et al. 1987; Mortensen et al. 1988; Lombardi and Wangersky 1991; Parrish and Wangersky 1990; McGinnis et al. 1997; Shifrin and Chisholm 1981).

Some studies suggest that salinity may affect the metabolism of cellular Si in marine diatoms. At low salinity, Vrieling et al. (1999) and Tuchman et al. (1984) observed increases in total diatom Si concentration, with a larger cellular Si pool accounting for most of the increase. At some level, these observations suggest inhibition by low salinity of Si movement from the cellular pool to the mineralized pool. In two diatoms grown at low salinity, Vrieling et al. (2007) observed nanostructural changes in mineralized Si, including increased Si density. The density of mineralized Si is affected by the size of coalescing Si particles inside the SDVs, with smaller particles resulting in a denser frustule. The authors therefore suggested that salinity affects intracellular Si transport to the SDVs or the function of the SDVs themselves (Vrieling et al. 1999, 2007). Currently, little is known about the effect of salinity on these processes (Sumper and Kroger 2004; Vrieling et al. 1999, 2007). Observations of increased lipid content in marine diatoms grown at low salinity, including *C. gracilis*, may further suggest a connection between Si metabolism and salinity (Araujo et al. 2011; Chelf 1990).

The marine diatom *C. gracilis* is known to accumulate lipids when it is Si deficient and when cultivated at low salinity. These effects may have a connection, however, as studies suggest that low salinity may affect intracellular Si transport or mineralization. We thus hypothesized that reducing salinity (by reducing the concentration of NaCl) may be effective in enhancing lipid accumulation in *C. gracilis* at any given rate of Si supply. Testing combinations of wide ranges in Si and NaCl, we sought to characterize the respective effects of Si and NaCl and their interaction on lipid content, growth rate, and other parameters in batch culture. Our broad objectives were to provide useful information to the lipid producer and, secondarily, to provide data suggesting the nature of the relationship between NaCl and the metabolism of Si in a marine diatom.

Methods and materials

Experimental outline

Batch cultures of the marine diatom *Chaetoceros gracilis* (UTEX #LB 2658) were grown in glass, air-lift bioreactors to test combinations of three NaCl concentration treatments (50, 100, and 400 mM) and four Si supply treatments (0.05, 0.1, 0.2, and 0.8 mM). There were two replicate bioreactor tubes per combination of treatments, for a total of 24 tubes. The cultures were started by adding media and 100 mL of inoculum to the bioreactor tubes to a 1.2 L volume. The inoculum culture was pre-adapted to an intermediate NaCl concentration of 100 mM and a low Si supply of 0.1 mM. This was done by growing a culture at 100 mM NaCl and 0.1 mM Si for 7 days, then transferring 100 mL to a new 100 mM NaCl/0.1 mM Si medium and allowing it to grow for 4 days. An absorbance measurement was taken from each tube daily. Solution Si measurements were taken every other day. The cultures were harvested when the absorbance of all treatments had plateaued (2 days of consistent optical density). Terminal measurements of total and ash-free dry mass density, biomass lipid content, and lipid chain length were taken.

Culture apparatus and maintenance

The cultures were grown in glass, air-lift bioreactors that were autoclaved before use. The bioreactors had an outer diameter of 50 mm and inner diameter of 45 mm and were filled to an approximate height of 75 cm, giving a culture volume of 1.2 L. Under the same conditions and timeframe as the final experiment, we quantified Si leaching from the bioreactors and found that total leaching was 3.2±0.3 μM Si (n=3), which is only 6 % of the Si in the lowest treatment. As such, interference by Si leaching was expected to be minimal. The bioreactor tubes were placed in a plexiglass water tank that was maintained at a temperature of 25 °C. Filtered air (Whatman PolyVENT 0.2 μm PTFE filters, L#639) was bubbled into the bottom of each bioreactor through a 1-mm glass capillary tube at a rate of 0.5 L min^{-1}. Carbon supply and pH were managed by $NaHCO_3$ and CO_2 inputs, equally distributed to all bioreactors. pH was maintained between 7.2 and 7.5 (variation in pH among treatments did not exceed 0.15 pH units). pH was measured by a Mettler Toledo SG2 SevenGo pH electrode kit. During the lag phase (the first 2 days of growth) 0.05 mM $NaHCO_3$ was added once daily. For the remainder of the experiment, the air flow was enriched in CO_2; the enrichment rate increased over time in response to algal metabolism, up to ~1 % CO_2. Light was supplied by banks of fluorescent tubes that ran perpendicular to the bioreactor tubes, completely covering one side. The photosynthetic photon flux (PPF) was 300 μmol photons m^{-2} s^{-1} with a 16-h photoperiod. A 16-h photoperiod was chosen to approximate the natural photoperiod of summer days in the mid-latitudes. Growth of photosynthetic organisms is best determined by the daily integrated PPF (Bugbee and Monje 1992), which was 17.3 mol photons m^{-2} day^{-1} in this study. This is less than 50 % of the average daily PPF of 45 to 55 mol photons m^{-2} day^{-1} in the summer months in North America. For conversion of measurements made by volume (e.g., g L^{-1}) to a unit of area (e.g., g m^{-2}), the illuminated area of the bioreactor tubes containing algae was used as a conversion factor as follows: 1.2 L / πrh=1.2 L / (3.14×2.25 cm×75.5 cm)×(1 m^2 / 100^2 cm^2)=22.5 L m^{-2} and 22.5 L m^{-2}×g L^{-1}=g m^{-2}.

Media composition and preparation

Three media were prepared and autoclaved, differing in NaCl concentration: 50, 100, and 400 mM NaCl. Silicon was added separately to each bioreactor tube at four concentrations: 0.05, 0.1, 0.2, and 0.8 mM Si. Because of the effect of Si on pH, the pH of the bioreactor tubes was adjusted individually to pH 7.5 by addition of 1 M NaOH or 1 M HCl. The culture media had the following composition: 2.5 mM KNO_3; 0.9 mM $CaCl_2·2H_2O$; 7.0 mM $MgSO_4·7H_2O$; 6 mM KCl; 50, 100, or 400 mM NaCl; 1.5 mM KH_2PO_4; 0.05, 0.1, 0.2, or 0.8 mM $Na_2SiO_3·9H_2O$; 14.3 μM ferric ammonium citrate; 25 μM H_3BO_3; 3.0 μM $MnCl_2·4H_2O$; 0.25 μM $CuSO_4·5H_2O$; 0.75 μM $ZnSO_4·7H_2O$; 0.2 μM $Na_2MoO_4·2H_2O$; 0.2 μM $CoCl_2·6H_2O$; 1.5 nM vitamin B12; 4.1 nM biotin; and 150 nM thiamine. Differential addition of Na within each NaCl treatment—due to initial pH adjustment (NaOH) and the counter ions of Si ($Na_2SiO_3·9H_2O$)—were expected to have a minimal effect. NaOH was added only to the 0.05 and 0.1 mM Si treatments, resulting in up to a 1.6 mM increase in Na. The counter ion Na that came with Si most affected the high Si treatments, up to 1.6 mM.

Algal density measurement and harvest

Daily measurements of culture density were made spectrophotometrically at 750 nm with a Shimadzu UV-2401 PC, UV–VIS recording spectrophotometer. Ash-free dry weight was determined by filtering 10 mL suspensions of algae with Whatman GF/C filters. The filters were dried for 1 to 2 days at 105 °C, weighed to determine the total dry weight, then combusted at 550 °C for 15 min to determine ash. Cells suspended in media were concentrated for harvest by centrifugation at 7,500 rpm for 5 min. Following centrifugation, the biomass was loaded into 15-mL plastic sample vials, frozen at −80 °C, and lyophilized. For accurate determination of total dry mass (cellular mass, including ash), attempts were made to rinse the filters free of extracellular salts. This resulted in loss of algae from the filters. Therefore, ash content was determined by combusting 20 mg samples of lyophilized algae.

Solution Si measurements

Samples of algal suspension (3 mL) were collected from each bioreactor tube every other day and filtered with Whatman GF/C filters. Solution Si was measured on the filtered media with a Lachat QuikChem 8500 Automated Ion Analyzer using the QuikChem method 10-114-27-1-A available from the manufacturer (Lachat Instruments, USA).

Lipid extraction, conversion to FAME, and quantification

Simultaneous conversion and extraction of algal lipids to fatty acid methyl esters (FAME) was done by the method of Wahlen et al. (2011). This method effectively converts to FAME the fatty acids contained in membrane phospholipids and glycolipids, as well as free fatty acids and storage lipid triglyceride. The lipid or FAME content of 100-mg freeze-dried algal samples was determined with a gas chromatograph (Model 2010, Shimadzu Scientific, USA) equipped with programmable temperature vaporizer (PTV), split/splitless injector, flame ionization detector (FID) (GCMS-QP2010S, Shimadzu Scientific), and autosampler. Analytes were separated on an RTX-Biodiesel column (15 m, 0.32 mm ID, 0.10-lm film thickness, Restek, USA) using a temperature program of 60 °C for 1 min followed by a temperature ramp of 10 °C min^{-1} to 360 °C for 6 min. Constant velocity of carrier gas helium was set at 50 cm s^{-1} in velocity mode. Sample sizes of 1 μL were injected into the PTV injector in direct mode that followed an identical temperature program to that of the column. The FID detector was set at 380 °C. Each sample contained octacosane (10 μg mL^{-1}) as an internal standard. FID detector response to FAME was calibrated using methyl tetradecanoate (C14:0), methyl palmitoleate (C16:1), and methyl oleate (C18:1) at concentrations ranging from 0.1 to 1 mg mL^{-1} and tripalmitin at concentrations ranging from 0.05 to 0.5 mg mL^{-1}. Standards were obtained as pure compounds (Nu-Chek Prep, Inc., USA) and diluted with chloroform to obtain needed concentrations. Peaks were integrated using GC solution postrun v. 2.3 (Shimadzu) and concentrations were determined by linear regression analysis.

Statistical analysis

Analysis of variance was conducted using the general linear model (Proc GLM) in the SAS system (Statistical Analysis System, USA). Error bars in all plots represent the standard deviation.

Results and discussion

Silicon uptake, growth, and lipid content

Despite decreased growth rates with both decreasing Si supply and NaCl concentration, the removal of Si was nearly complete in all NaCl/Si treatments (Fig. 1). Averaged across all treatments, 12±2.8 μM Si was never recovered from solution. It is unclear whether this Si was

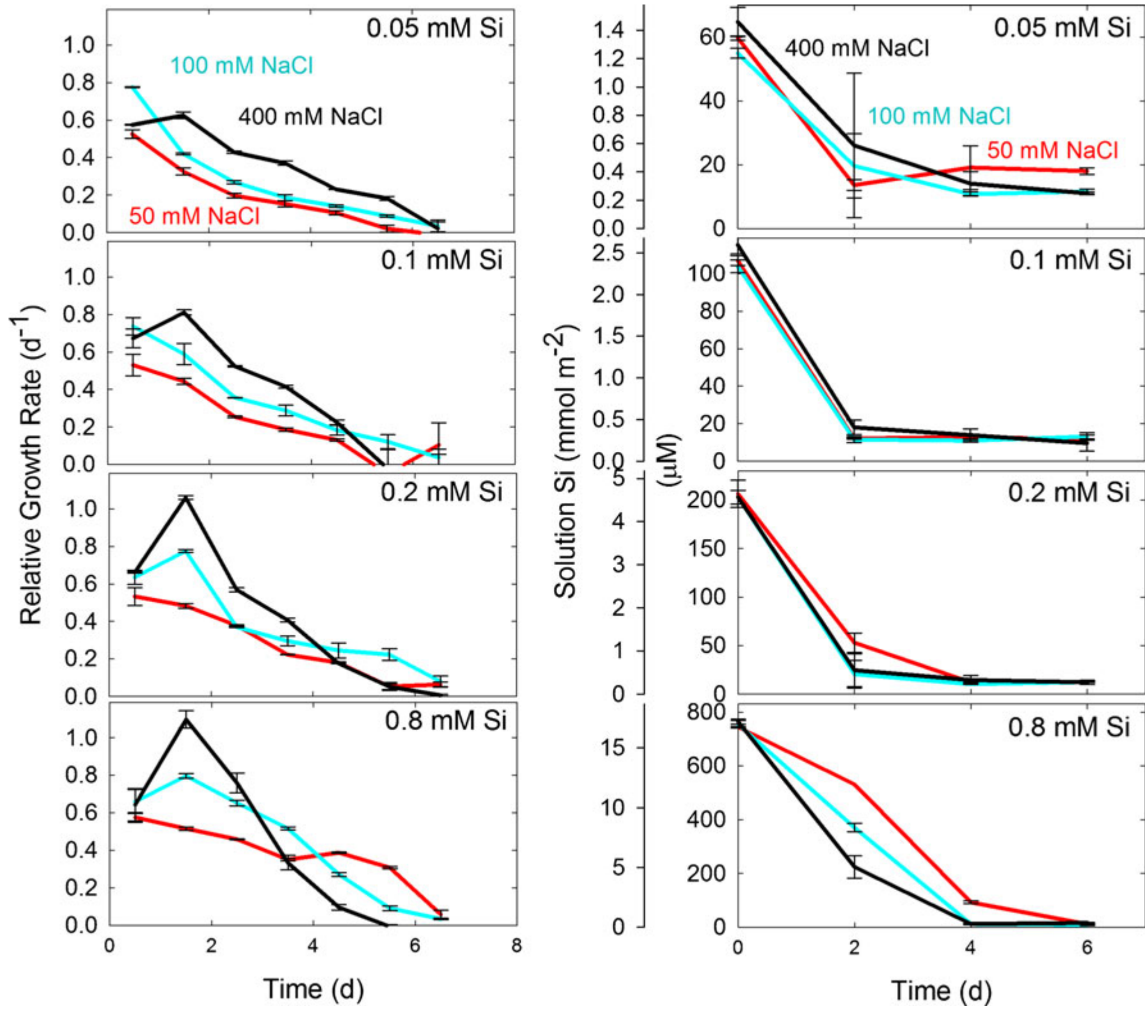

Fig. 1 Time series measurements of relative growth rate (*on left*) and Si in solution (*on right*) with combinations of four Si treatments (0.05, 0.1, 0.2, and 0.8 mM Si) and three NaCl treatments (50, 100, and 400 mM NaCl). The relative growth rate, determined by optical density at 750 nm, was negatively affected by decreasing NaCl concentration and Si supply rate. The diatom removal of Si was nearly complete in all treatments, with a fairly consistent, small amount of Si never recovered from solution: 12± 2.8 μM Si.

available for Si uptake, however, because soluble Si was measured by the molybdate assay in acidic conditions which could have solubilized polymeric forms of Si. Analysis of variance showed a strong interaction between NaCl and Si on total and ash-free dry mass densities, but that NaCl was the primary factor determining the outcome of these parameters (Table 1 and Fig. 2). The interaction arose because low NaCl concentrations were inhibitory to growth, but the inhibition was overcome with excessive Si supply rates. This suggests that low levels of NaCl may have affected the metabolism of Si.

In a synergistic effect, lipid content was positively affected by both decreasing Si and decreasing NaCl (Fig. 2). The relationships shown here are not perfectly linear, however, as a result of the lipid content in the 50 and 100 mM NaCl treatments peaking at 0.2 and 0.1 mM Si, respectively, and decreasing with lower Si supply. We speculate that the relationships are not linear because of lipid consumption or degradation before measurement. Traller and Hildebrand (2013) showed no reduction in lipid content over 4 days in a marine diatom with severe Si deficiency, but the diatoms in the present study had the added stress of low NaCl and thus the potential need to rely on their energy and carbon storage in lipids. Statistical analysis of the data showed that the relative effects of Si and NaCl on promoting storage lipids were similar—each increasing the lipid content by more than 50 %—with Si having a slightly larger effect (Table 1). With combinations of low Si (0.05, 0.1, and 0.2 mM) and low NaCl (50 and 100 mM), lipid content increased to more than 70 % of dry mass.

Lipid productivity

Lipid productivity is derived by multiplying total dry mass density by the lipid content and dividing by time (Fig. 2),

Table 1 Analysis of Variance in four response parameters (total dry mass density, ash-free dry mass density, lipid content, and lipid productivity) for two factors and their interaction (Si, NaCl, and Si*NaCl)

Source	df	*F* value	*Pr*>*F*
Total dry mass density			
Si	3	23.1	<.0001
NaCl	2	82.5	<.0001
Si*NaCl	6	8.9	0.0008
Error	12		
R^2	0.960		
Ash-free dry mass density			
Si	3	20.4	<.0001
NaCl	2	72.2	<.0001
Si*NaCl	6	11.0	0.0003
Error	12		
R^2	0.958		
Lipid content			
Si	3	19.4	<.0001
NaCl	2	17	0.0003
Si*NaCl	6	2.1	0.1313
Error	12		
R^2	0.897		
Lipid productivity			
Si	3	2.8	0.0834
NaCl	2	28.8	<.0001
Si*NaCl	6	11.9	0.0002
Error	12		
R^2	0.920		

The *F* values were used in determination of the relative impact of each factor on the response parameters and in assigning statistical significance

df degrees of freedom

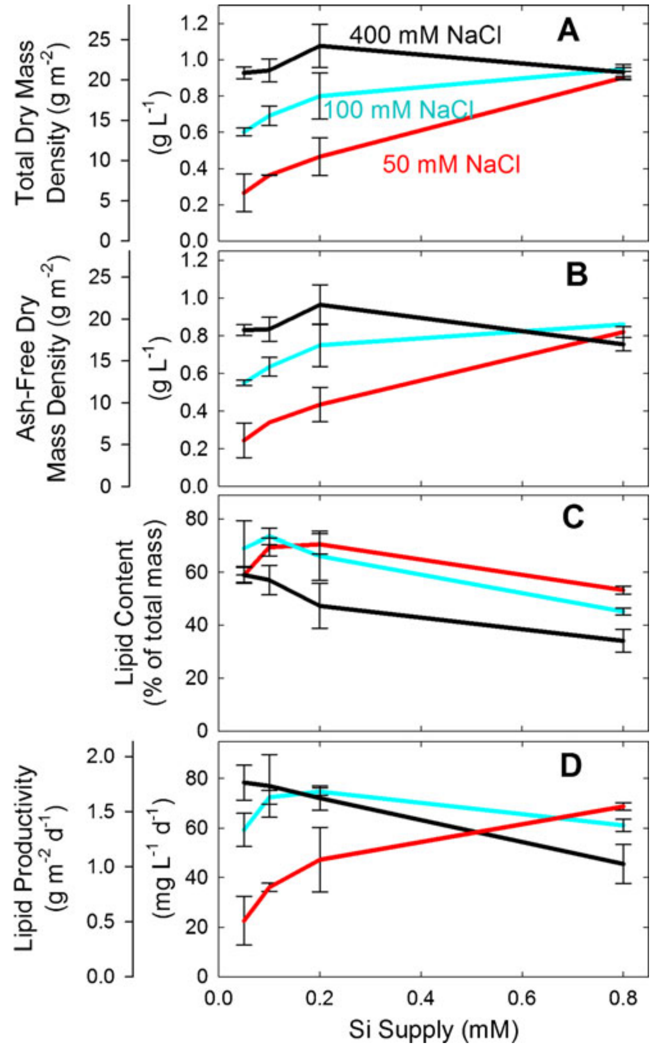

Fig. 2 Terminal measurements of total dry mass density, ash-free dry mass density, lipid content, and lipid productivity as a function of Si supply. Sodium chloride was the dominant factor determining total and ash-free dry mass yields (**a**, **b**), while NaCl and Si contributed approximately equally to the accumulation of storage lipid (**c**). Lipid productivity reflected the strong effect of NaCl on growth and a significant NaCl/Si interaction (**d**)

which integrates the combined effects of increases in lipid content and decreases in non-lipid biomass due to stress. This measure is important because stress can cause non-lipid biomass to decrease more than the increase in lipid fraction. Sodium chloride—because of its large impact on growth—and a significant NaCl/Si interaction were the dominant factors determining lipid productivity (Table 1). Lipid productivity peaked at a high NaCl concentration (400 mM) and a low rate of Si supply (0.05 mM), but was not significantly different than the productivity with intermediate levels of NaCl and Si (100 mM NaCl and 0.1 or 0.2 mM Si). These treatment combinations did differ in lipid content, however, with intermediate levels of NaCl and Si resulting in about 14 % higher lipid content. Thus, from a lipid production standpoint, intermediate levels of NaCl and Si stress resulted in a more favorable outcome. The contour plots of Fig. 3 illustrate the tradeoffs in total dry mass density and lipid content as a function of NaCl concentration and Si supply.

Fatty acid chain length distribution

Decreased Si resulted in a 5 % shift in lipid chain length from C18 to C16 fatty acids, but there was no effect of NaCl (Fig. 4). C14 fatty acids were unchanged. Together, C14, C16, and C18 fatty acids accounted for ~93 % of all lipids.

Efficiency of lipid production

Radiation is the ultimate limiting factor in all photosynthetic systems and thus it is appropriate to compare productivities

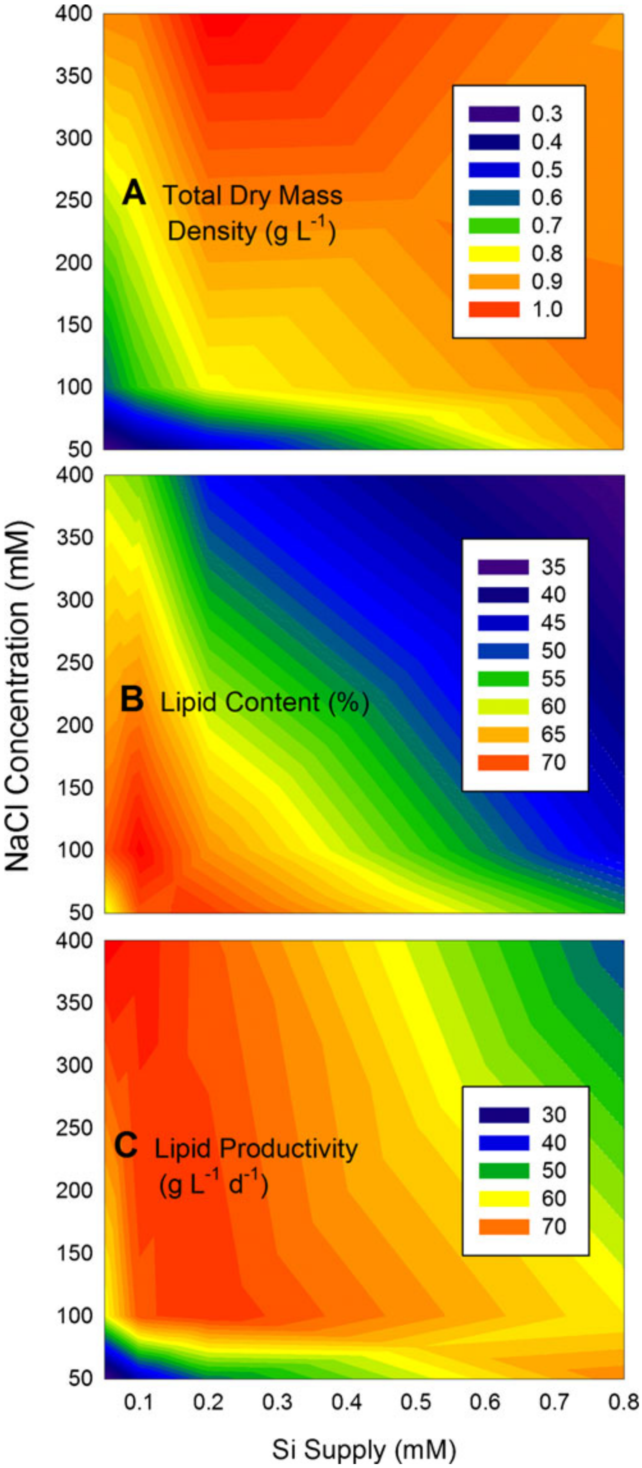

Fig. 3 Contour plots showing total dry mass density (**a**), lipid content (**b**), and lipid productivity (**c**) as a function of Si supply and NaCl concentration. The most favorable lipid production outcome—high lipid productivity and high lipid content—was observed with moderate levels of NaCl and Si stress.

based on the input of light (Bugbee and Monje 1992). In this study, peak lipid productivity was about 75 mg L^{-1} day^{-1} over a 7-day growing period and the illuminated surface-to-volume

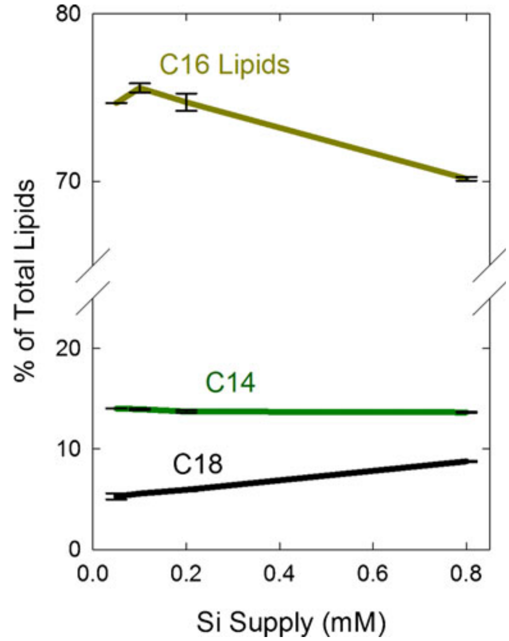

Fig. 4 The distribution in lipid chain length shifted with the rate of Si supply. An increase in C16 fatty acids with decreasing Si supply was offset by a similar reduction in C18 fatty acids. C14 fatty acids were unchanged. Sodium chloride had no significant effect on lipid distribution. The 50 mM NaCl treatment is shown here.

ratio of the bioreactor tubes was 0.044 m^2 L^{-1}. This gives a lipid productivity of 1.7 g m^{-2} day^{-1}. The daily PPF integral was 17.3 mol photons m^{-2} day^{-1}, giving an efficiency of lipid production of 0.10 g mol^{-1} of photons. For comparison, this was about 50 % of the best efficiency (0.19 g mol^{-1} of photons) found in green algae by using N deficiency to promote lipid accumulation in a recent publication using the same growth system (Adams et al. 2013).

Conclusions

Low levels of either NaCl or Si increased lipid content by about 50 % each. The synergy of simultaneous, moderate NaCl and Si stress resulted in lipid content up to 73 % with a lipid productivity of 1.7 g m^{-2} day^{-1}; with a daily integrated photosynthetic photon flux of 17.3 mol m^{-2} day^{-1}, the efficiency of lipid synthesis was thus 0.10 g $mole^{-1}$ of photons. The observation of a strong NaCl/Si interaction on total and ash-free dry mass densities suggests that low levels of NaCl may have affected the metabolism of Si. The findings of this study can be used to enhance lipid production in oleaginous marine diatoms.

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