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BIOENERGY/BIOFUELS/BIOCHEMICALS

Freshwater diatoms as a source of lipids for biofuels

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Abstract Until recently, biodiesel production has been derived from terrestrial plants such as soybean and canola, leading to competition between biodiesel production and agricultural production for source materials. Microalgae have the potential to synthesize 30 times more oil per hectare than terrestrial plants without competing for agricultural land. We examined four genera (*Cyclotella*, *Aulacoseira*, *Fragilaria*, *Synedra*) of common freshwater diatoms (Bacillariophyceae) for growth and lipid content in defined medium (sD11) that replicates hypereutrophic conditions in lakes and wastewater treatment plant effluents and optimized the medium for silicon content. *Cyclotella* and *Aulacoseira* produced the highest levels of total lipids, 60 and 43 μg total lipids/ml, respectively. Both diatoms are rich in fatty acids C14, C16, C16:1, C16:2,7,10, and C22:5n3. Of the diatoms examined, *Cyclotella* reached the highest population density (>2.5 × 10^6 cells/ml) in stationary phase when many of the cells appeared to be filled entirely with oil. Silicon enrichment studies indicated that for optimal utilization of phosphorus and nitrogen by diatoms growing in wastewater effluent, the amount of silicon present or added to the effluent should be 17.5 times the mass of phosphorus in the effluent. With high growth rates, high lipid contents, and rapid settling rates, *Cyclotella* and *Aulacoseira* are candidates for biodiesel production.

Keywords Biodiesel · Diatoms · *Cyclotella meneghiniana* · Lipids · Microalgae

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Introduction

Every day, the United States imports about 10 million barrels of oil from overseas sources. Two-thirds of this imported oil goes into transportation fuels, including gasoline, diesel fuel, and aviation fuel [32]. Imported fossil fuels have shown rising prices due to increased global demand and price instability due to political turmoil in producing nations. Rising and unstable prices of imported oil, coupled with concerns about global warming and rising levels of carbon dioxide in the atmosphere, have led many oil-importing countries to seek ways to reduce oil imports and to increase the development and production of biofuels.

Biodiesel is a type of biofuel derived from a variety of feedstocks through transesterification of triglycerides into fatty acid methyl esters and glycerol. Grease, waste cooking oil, and animal fats may be used as feedstocks, but they do not occur in sufficient amounts to meet even a small part of the United States' daily consumption of transport fuels [7]. Terrestrial crop plants such as soybean, corn, and canola (rapeseed) are the primary feedstocks for biodiesel production in the United States. Unfortunately, the use of food crops for biodiesel production leads to competition

between the use of agricultural land for food production and its use for fuel production with a resulting increase in food costs and potential habitat and biodiversity losses [22]. Food crops cannot meet the annual United States' demand for liquid transport fuels; if soybeans were planted to provide one-half the current annual consumption of transport fuels in the United States, a land area equal to 326% of total U.S. agricultural area would be required [7].

Microalgae are algal microorganisms that require a light microscope to see their characteristics and are generally smaller than about 20 μm in size. In contrast, macroalgae can be seen with the unaided eye. If microalgae were used to produce a feedstock for biodiesel production, the area of land in raceway ponds required to produce microalgal biomass to meet one-half the current annual United States' consumption of transport fuels would be only 1.1–2.5% of United States cropland area [7]. Microalgae, however, do not require the use of agricultural land and therefore would not compete with food for agricultural land.

Microalgae are currently grown commercially for various high-value products [33, 40]. The cyanobacterium *Spirulina* is grown in shallow raceway ponds as a nutritional supplement for its high protein content, B vitamins, and beta-carotene [35, 42]. The prasinophyte *Tetraselmis suecica* is used as a food organism in aquaculture [10]. The diatom *Phaeodactylum tricornutum* is cultivated in outdoor photobioreactors for production of long-chain polyunsaturated fatty acids, which are used as a human food supplement [1, 30], and the green alga *Haematococcus* is cultured in photobioreactors for extraction of the red pigment astaxanthin, a carotenoid used as a coloring agent in foods and cosmetics [16, 21].

Lipid production is a key characteristic in the investigation of algal species for biodiesel production, and a significant number of species have been studied for their growth and lipid content [15]. The majority of green algae studied to date are freshwater species. The green flagellate *Chlamydomonas* is being investigated because its genome is known and it can be genetically modified. The green alga *Botryococcus braunii* is known to be a rich source of lipids [29], but it is thought to grow too slowly for commercial purposes. A number of species in the green algal genus *Chlorella* have been studied, including *C. pyrenoidosa* [23] and *C. vulgaris* [36]. *Chlorella protothecoides* has been cultivated heterotrophicallly for the production of biodiesel fuel [17, 25, 45].

Heterotrophic production, however, requires an organic carbon source such as glucose, acetate, or corn powder hydrolysate as a feedstock that is ultimately derived from crop plants. In addition, *Chlorella protothecoides* is closely related to *Prototheca*, a serious pathogen in livestock and sometimes in humans. Other genera of green algae studied for lipids include *Ankistrodesmus*, *Monoraphidium*, *Scenedesmus*, and *Selenastrum* [15].

A number of genera of diatoms have been examined for lipid content, including *Amphora* [9], *Chaetoceros calcitrans* [36], *Cyclotella cryptica* [39], several species of *Nitzschia* [15], *Phaeodactylum tricornutum* [4, 23, 46], and *Thalassiosira pseudonana* [36]. The genomes have been sequenced for both *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* and triacylglycerol profiles obtained [47]. *Phaeodactylum tricornutum* is considered a model diatom for research purposes. Its lipid content is reported in the range of 21–26% of dry weight and its doubling time T_d has been reported as 25 h [15]. Cerón-García et al. [5] have investigated mixotrophic growth of *P. tricornutum* on various carbon sources. All diatoms produce and store lipids as food reserves, but the majority of diatom species studied for lipid content in connection with biofuels have been marine species [15]. The only exceptions before this paper are a few species of the freshwater genus *Navicula* [39].

Until quite recently it was generally believed that the cost of producing algal biomass was more than the cost of producing crops for biodiesel [7]. Recent engineering life cycle comparisons, however, indicate that linking algal production to wastewater cultivation has the potential to offset many of the environmental burdens of algae biomass production for biofuel feedstocks and outperform terrestrial crops [8]. Wastewater-linked algae cultivation also offers the prospect of effluent bioremediation [18], with the sale of algal feedstock potentially offsetting increased costs associated with new *N* and *P* reduction mandates. Even in cold temperate climates algal biomass production can outperform soybean-based biodiesel production if the algal production is carried out in a photobioreactor within a greenhouse warmed by waste heat from an adjacent power plant [2]. Consequently, in the present study, we investigated the concept of using natural freshwater periphyton communities, which include diatoms that are adapted to the high nutrient conditions of hypereutrophic lakes, for wastewater-linked biofuel feedstock production.

The results reported here focus on fatty acid production by four periphytic diatom species (*Cyclotella meneghiniana*, *Aulacoseira granulata*, *Synedra ulna*, and *Fragilaria brevistriata* (=*Pseudostaurosira brevistriata*) isolated from hypereutrophic Lake Mendota (Dane County, WI) that also occur in local wastewater effluent sampled from the nearby Madison Metropolitan Sewerage District. These four freshwater diatom species have not been examined previously for growth and lipid content in the microalgal biofuel literature.

Wastewater effluents in the United States are discharged into natural waters from wastewater treatment plants, which routinely monitor their effluents for phosphorus, nitrogen, or both. Dissolved silicon is not considered a pollutant or hazard, and it is rarely monitored at wastewater treatment

plants. The levels of silicon in effluents are therefore mostly unknown. Proposed new mandates for reduced levels of phosphorus and nitrogen in effluents could cost in the range of $54 billion to implement on a nation-wide basis in the United States. If diatoms or other algae are to be grown in wastewater effluents as a potential source of biomass feedstock for biofuels and as a cost-effective means of reducing nutrient pollution to natural waters, then the diatoms must be grown to the maximum biomass permitted by the levels of phosphorus and nitrogen in the wastewater effluent. In that event, the diatoms will reduce phosphorus and nitrogen in the effluent before discharge to levels that would not cause eutrophication. A discharge level of less than 50 μg P/l of effluent would be in the mesotrophic range for natural waters and one of <10–15 μg P/l would be in the oligotrophic range for natural freshwaters [43]. If wastewater effluents are limiting in silicon, however, silicon limitation will halt diatom growth before the levels of phosphorus and nitrogen are reduced sufficiently to achieve a non-polluting effluent and at the same time produce less than the maximum possible diatom biomass for use in biofuel. We therefore investigated the effects of Si enrichment of a "hypereutrophic" defined medium on growth of these four diatom species, with the aim of optimizing wastewater-linked algal productivity and phosphorus and nitrogen nutrient reduction.

Methods

Isolation, culture, growth, and SEM of diatoms

Diatoms were isolated from shoreline samples of the filamentous green alga *Cladophora glomerata* collected from Lake Mendota in Dane County, Wisconsin, from May through September, 2009. Individual cells were isolated by micropipette into well slides containing small volumes of sD11 medium [13, 14]. Unialgal isolates were used in growth experiments to determine growth rates, carrying capacities, and optimal silicon levels at the phosphorus (15 mg K_2HPO_4/l or 2.7 μg P/ml) and nitrogen (150 mg $Ca(NO_3)_2 \times 4H_2O$/l or 17.8 μg N/ml) concentrations in sD11 medium. The original sD11 medium contains 60 mg $Na_2SiO_3 \times 9H_2O$/l (5.9 μg Si/ml), an amount included to supply the requirements of *Cladophora glomerata* for silicon in its cell walls. To determine the optimal silicon level for diatom growth, growth experiments were conducted from the basic level up to 13 times the basic level or 780 mg $Na_2SiO_3 \times 9H_2O$/l (77.2 μg Si/ml). Growth experiments were conducted in 500-ml Erlenmeyer flasks with 250 ml sD11 medium at 21°C and 152 μmol quanta m^{-2} s^{-1} on a 16:8 light:dark cycle with continuous hydrated air bubbling through the flasks. Diatom cultures require mixing because the cells readily sink and settle out in stationary cultures. Cells were counted with a hemocytometer (*Cyclotella*, *Synedra*, and *Fragilaria*) or Sedgewick-Rafter sedimentation chamber (*Aulacoseira*) and sized with a calibrated optical micrometer.

Many genera of diatoms can be identified by light microscopy (LM), but some genera and all species of some genera can only be identified by observing microscopic structural features of the siliceous frustules by scanning electron microscopy (SEM) of cleared, metal-coated, dehydrated diatoms. Species of *Fragilaria* and *Synedra* can be identified with SEM images using keys in Patrick and Reimer [31]. SEM images in Lowe [27] permit identification of species of *Cyclotella*. The earlier name for *Aulacoseira granulata* was *Melosira granulata*; thus it may be found in older keys by this name. Round et al. [37] provides SEM images of *Aulacoseira* cells. Scanning electron micrographs (SEMs) were produced using a Hitachi S4800 at the University of Wisconsin-Milwaukee after metal-coating dehydrated diatoms and an environmental SEM at the University of Wisconsin-Madison.

Lipid extraction, methylation, and analysis

Lipid extractions were similar to the lipid extractions performed by [24]. Two and a half ml of cell culture was collected in a glass centrifuge tube, and 5 μl of 10 mg/ml heptadecanoic acid (internal standard) dissolved in ethanol was added. One hundred microliters of glacial acetic acid was then added and the tubes vortexed. Five ml of 1:1 $CHCl_3/CH_3OH$ was added by glass pipette and centrifuged at 1,000 × *g* for 10 min. The upper aqueous layer and all cell debris at the interface were removed by aspiration. The chloroform layer was evaporated under a nitrogen stream, and the residue was lyophilized for 30 min to remove residual water. Dried extract was added to 0.5 ml of anhydrous 1.25 M HCl in methanol, capped and incubated overnight at 50°C. Tubes were cooled before adding 0.5 ml hexane and 5 ml of 100 mg/ml $NaHCO_3$. The tubes were vortexed and then centrifuged at 1,000 × *g*, for 10 min. The hexane layer was collected for gas chromatography/mass spectrometry (GC/MS) analysis on a model 7890 Agilent GC (Agilent Technologies, Inc., Santa Clara, CA) with a 30 m × 0.25 mm DB-5 capillary column and a model 5975 mass spectrometer [24]. Fatty acid species were identified and titered using dilution series of Supelco 37 Component FAME mixture from Sigma-Aldrich (St. Louis, MO) run concurrently with the diatom lipid samples.

Results

SEMs of the four diatom isolates are shown in Fig. 1a–d. These and other SEMs allowed identification of the four

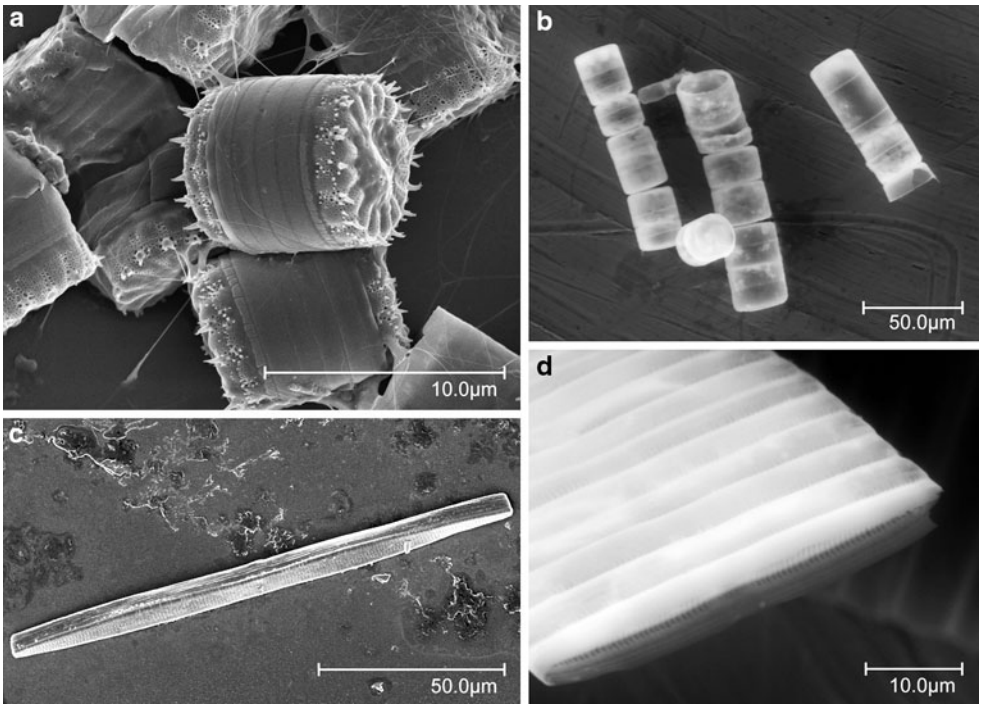

Fig. 1 SEMs of the four species of diatoms studied for growth and lipid content: **a** *Cyclotella meneghiniana*, **b** *Aulacoseira granulata*, **c** *Synedra ulna*, **d** *Fragilaria brevistriata*

species as *Cyclotella meneghiniana*, *Aulacoseira granulata*, *Fragilaria brevistriata*, and *Synedra ulna*.

The initial set of growth experiments focused on *Cyclotella meneghiniana*. The *Cyclotella* growth curves for sD11 with 60, 180, and 300 μg Na_2SiO_3/ml are shown in Fig. 2, and those for 120, 240, and 420 μg Na_2SiO_3/ml (where the nine water molecules of hydration are omitted for brevity) are given in Fig. 3. The values plotted are the natural logs of the means of 6–16 replicate counts. The graphs for 540, 660, and 780 are not shown because they are essentially indistinguishable from that for 420 μg Na_2SiO_3/ml. Table 1 gives the values for the maximum specific growth rate (μ_{max}), number of days to reach stationary phase, and the carrying capacity *K*, which is the mean population density in cells/ml in stationary phase. The average of the maximum specific growth rates from 60 to 540 μg Na_2SiO_3/ml is 0.84 ± 0.045 day^{-1} (mean ± standard error). Above 540 μg Na_2SiO_3/ml, the growth rate shows a noticeable decline. The natural logarithm of the carrying capacity *K* rose linearly up to 420 μg Na_2SiO_3/ml, followed by a slow decline at higher silicon levels. For *Cyclotella*, the addition of silicon above a concentration of 420 μg Na_2SiO_3/ml produced no further increase in population density. Silicon was no longer limiting growth and presumably phosphorus was now the limiting nutrient.

The growth curves for *Synedra ulna* at 300, 420, and 540 μg Na_2SiO_3/ml are given in Fig. 4. The average maximum specific growth rate for these three silicon levels is

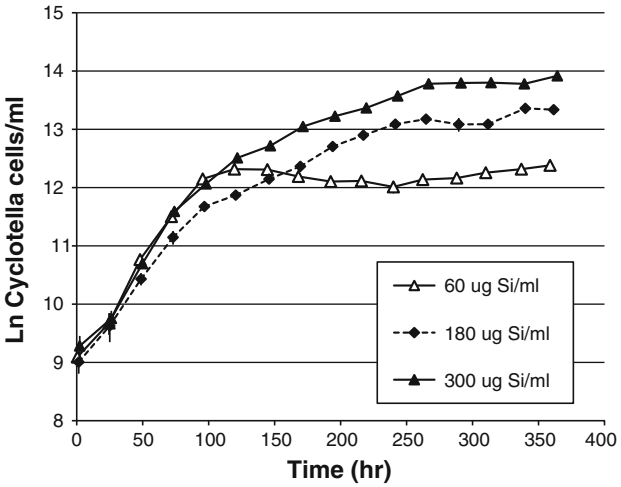

Fig. 2 Growth of *Cyclotella meneghiniana* at 60, 180, and 300 μg Na_2SiO_3/ml. *Error bars* denote the standard errors of the means. *Error bars* are frequently too small (about 0.03 to 0.06 log units) to be seen separately from the symbols

0.61 ± 0.015 day^{-1} (mean ± standard error). While the carrying capacity is highest at 540 μg Na_2SiO_3/ml, the number of cells at stationary phase is much less than for *Cyclotella* (Table 2).

Aulacoseira granulata growth curves for three silicon levels (180, 360, and 540 μg Na_2SiO_3/ml) are shown in Fig. 5. The average maximum specific growth rate is 0.35 ± 0.014 day^{-1} (mean ± standard error). For *A. granulata*, the number of

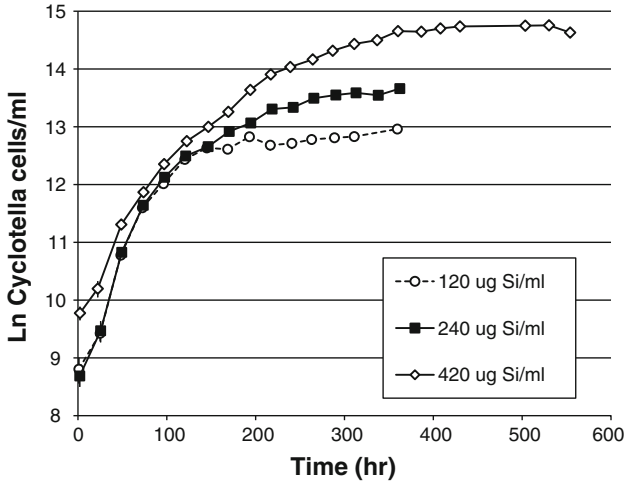

Fig. 3 Growth of *Cyclotella meneghiniana* at 120, 240, and 420 µg Na_2SiO_3/ml. *Error bars* indicate the standard errors of the means

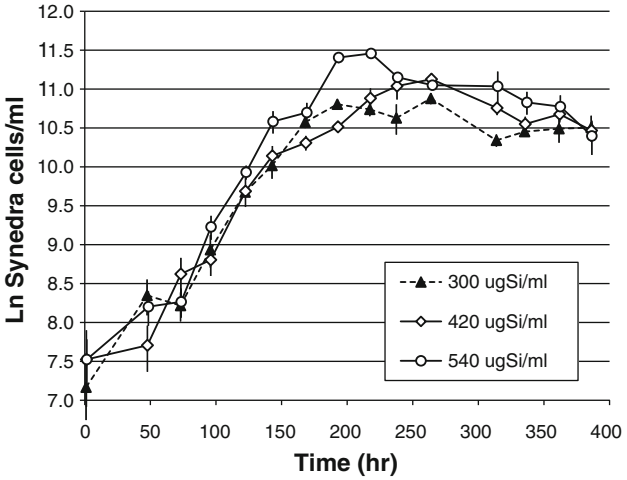

Fig. 4 Growth curves for *Synedra ulna* at 300, 420, and 540 µg Na_2SiO_3/ml. *Error bars* denote the standard errors of the means

Table 1 Maximum specific growth rate μ_{max} (day^{-1}), days to reach stationary phase, and stationary phase population density (carrying capacity *K* in cells/ml) of *Cyclotella meneghiniana* in sD11 medium for a range of silicon levels

µg Na_2SiO_3 $9H_2O$/ml	μ_{max} (day^{-1})	Days to reach stationary phase	Carrying capacity *K* (cells/ml)
60	0.80	5	201,000
120	0.98	8	362,000
180	0.73	10	532,000
240	1.03	11	780,000
300	0.78	11	999,000
420	0.73	17	2,510,000
540	0.85	19	2,318,000
660	0.69	16	2,192,000
780	0.60	16	2,229,000

The basic level of silicon in sD11 is 60 µg/ml as $Na_2SiO_3 9H_2O$ or 5.9 µg Si/ml

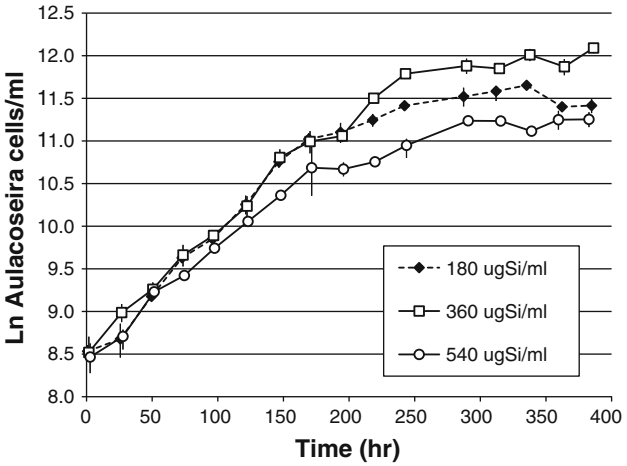

Fig. 5 Growth curves for *Aulacoseira granulata* at 180, 360, and 540 µg Na_2SiO_3/ml. *Error bars* indicate the standard errors of the means

cells at stationary phase is greatest at 360 µg Na_2SiO_3/ml, and markedly fewer cells are present at 540 µg Na_2SiO_3/ml (Table 2).

Figure 6 presents the growth curves for *Fragilaria* sp. at 180 and 360 µg Na_2SiO_3/ml. The average maximum specific growth rate is 0.32 ± 0.02 day^{-1} (mean ± standard error), the lowest growth rate of the four diatom genera. The population density at stationary phase was greater than 1 million cells/ml (Table 2) and the second highest carrying capacity after *Cyclotella*.

The total diatom lipids as µg lipid/ml are shown in Table 3 for the four genera examined. All four genera were grown into stationary phase in sD11 medium enhanced with 180 µg Na_2SiO_3/ml. Sixteen different fatty acids (FAs) were detected in the four genera. C16:1 was the most abundant single FA in the four diatoms, where it averaged 68% of total lipids. C14, C16, C16:1, C16:2,7,10, and C20:5n3 were the most common FAs except in *Fragilaria* where C20:4n6 was more abundant than C16:2,7,10. *Cyclotella* produced just over 60 µg lipid/ml, and *Aulacoseira* had 43 µg lipid/ml of culture. In comparison, *Fragilaria* and *Synedra* produced very low levels of total lipid.

Table 4 presents some of the cell characteristics of the four diatom genera, including population density, cell shape, mean cell size (MCV), biovolume/ml in the cultures, and surface to volume ratio (S/V). *Cyclotella* produced the densest population in the sD11 medium. *Cyclotella* and *Fragilaria* are both small cells, while *Synedra* and *Aulacoseira* are comparatively large, about ten times the MCV as the smaller genera. The two most productive genera for

Table 2 Maximum specific growth rate μ_{max} (day^{-1}), days to reach stationary phase, and stationary phase population density (carrying capacity K in cells/ml) for the diatoms *Aulacoseira*, *Fragilaria*, and *Synedra* grown in sD11 medium at a range of silicon levels

The basic level of silicon in sD11 medium is 60 µg/ml of $Na_2SiO_3 9H_2O$ or 5.9 µg Si/ml

Diatom	µg SiO_3/ml	μ_{max} (day^{-1})	Days to reach stationary phase	Carrying capacity K (cells/ml)
Aulacoseira	180	0.37	12	100,000
	360	0.35	12	153,000
	540	0.32	12	74,400
Fragilaria	180	0.30	14	1,378,000
	360	0.34	15	1,110,000
Synedra	300	0.59	8	47,000
	420	0.60	10	65,000
	540	0.64	8	92,000

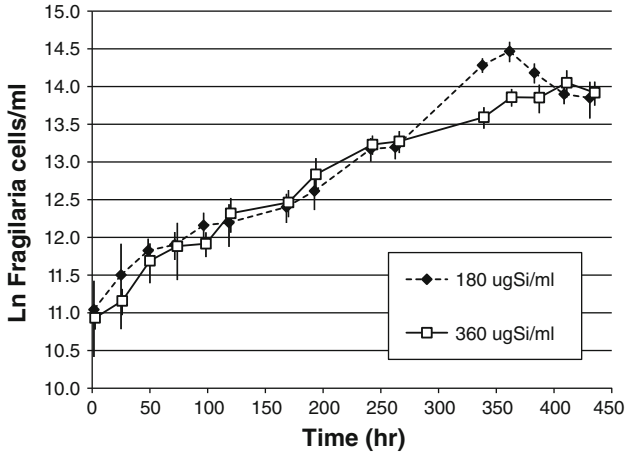

Fig. 6 Growth of *Fragilaria brevistriata* at 180 and 360 µg Na_2SiO_3/ml. *Error bars* denote the standard errors of the means

lipids were *Cyclotella* and *Aulacoseira*, which are both nearly square cylinders, where their diameters are about the same as their lengths. *Synedra* and *Fragilaria*, in contrast, are fusiform, or needle-shaped. As algal cells become larger, their S/V ratio normally declines, because volume increases as the cube of the cell radius while the surface area increases as only the square of the radius. A cylindrical shape is a very efficient way to contain a large volume inside a small surface area, while a fusiform shape has a large surface area around a small volume. Aside from differences in stationary cell density, *Cyclotella* and *Aulacoseira* had higher lipid yields per ml compared to the needle-shaped *Fragilaria* and *Synedra* because a cylinder has more volume to accumulate lipid than does a needle-shape.

The results of the culture phase studies on lipid production are shown in Table 5. The peak *Cyclotella* population densities were 440,800 cells/ml at 60 µg Na_2SiO_3/ml, 1,087,500 cells/ml at 180 µg Na_2SiO_3/ml, and 2,145,400 cells/ml at 420 µg SiO_3/ml. The population density in the 180 µg SiO_3/ml culture remained stable throughout the experimental period of 36 days. Those in the 60 and 420 µg SiO_3/ml cultures declined in the final samples by 57 and 34%, respectively.

Table 3 Fatty acid concentrations in four genera of freshwater diatoms

Fatty acid esters	*Cyclotella* (µg/ml)	*Aulacoseira* (µg/ml)	*Fragilaria* (µg/ml)	*Synedra* (µg/ml)
C12	0	0	0	0.06
C14	1.74	2.34	0.29	0.56
C16: 2	0	0	0.09	0
C16: 2,7,10	3.57	1.12	0.08	0.16
C16: 1	41.99	29.25	3.15	4.72
C16	6.47	4.33	0.47	0.62
C18: 3	0.44	0.32	0	0
C18: 2n6c	0.29	0.10	0.02	0
C18: 1n9c	0.17	0.10	0.02	0
C18: 1n9t	0	0	0.02	0
C18: 1	0.17	0.11	0	0
C18	0.07	0	0	0
C20: 4n6	0	0	0.20	0
C20: 5n3	5.11	5.23	0.72	0.28
C22: 6n3	0.53	0	0	0
Total	60.55	42.90	5.06	6.40

Cultures were grown to stationary phase in sD11 medium with 180 µg/ml $Na_2SiO_3 \times 9H_2O$ added to enhance growth. Values are the means of three replicates

Ten distinct fatty acids were detected in this time course study. Palmitoleic acid (C16:1) was the most abundant with an average of 18.4 µg lipid/ml at 60 µg SiO_3/ml, 43.6 µg lipid/ml at 180 µg SiO_3/ml, and 79.1 µg lipid/ml in the two samples from the 420 µg SiO_3/ml culture. The second most abundant fatty acid was palmitic acid (C16:0), which ranged from 4.98 to 13.25 µg lipid/ml. Arachidonic acid (C20:4n6) averaged 3.48 µg lipid/ml and myristic acid (C14:0) 2.49 µg lipid/ml over the eight samples. The remaining fatty acids averaged less than 2.0 µg lipid/ml. Heptadecanoic acid (C17:0) was only detected in samples from the lowest level of silicon examined. Except for stearic acid (C18:0), which averaged 1.77 µg lipid/ml, the other C18 fatty acids occurred at low levels and were often undetected in the samples taken at the beginning of stationary phase. The highest level of lipid observed was 98.6 µg lipid/ml

Table 4 Cell and population parameters of the four diatom genera grown in sD11 with 180 μg Na_2SiO_3 for lipid analysis (mean ± standard error)

	Cyclotella	*Aulacoseira*	*Fragilaria*	*Synedra*
Cells/ml	771,250 ± 43,340	15,650	72,410	16,480 ± 3,390
Mean cell volume (μm^3)	870 ± 90	13,184 ± 950	1274 ± 21	10,080 ± 615
Biovolume (μm^3/ml)	6.71×10^8	2.06×10^8	9.22×10^7	1.66×10^8
Shape	Cylinder	Cylinder	Fusiform	Fusiform
Surface/volume ratio	0.56	0.27	0.77	0.51

Table 5 Concentrations of fatty acids (μg/ml) in cultures of *Cyclotella meneghiniana* grown in sD11 medium at three different levels of Na_2SiO_3 (60, 180, and 420 μg/ml) and three different grown phases (entering stationary phase, 2 weeks into stationary phase, and 36 days from the start of growth)

Silicon level (μg/ml)	60	60	60	180	180	180	420	420
Growth (days)	8	24	36	16	30	36	24	36
Fatty acids (μg/ml)								
C14	2.56	2.17	2.20	2.86	2.41	2.46	2.51	2.78
C16: 1	10.25	24.83	20.02	53.37	39.25	38.25	41.46	69.16
C16	5.29	5.44	4.98	9.91	8.88	9.07	6.93	13.25
C17	0.63	1.40	1.28	0	0	0	0	0
C18: 3n6	0	1.35	1.43	1.35	1.44	1.39	1.32	1.59
C18: 2n6c	0	1.40	0.47	1.24	1.36	1.36	1.25	1.44
C18: 1n9c	0	1.62	1.69	0	2.17	2.23	0	2.16
C18: 1n9t	0	0.74	0.42	0	0	0	0	0
C18	0.85	2.08	2.14	1.72	1.88	2.02	1.60	1.86
C20: 4n6	1.46	2.24	2.35	3.87	3.87	3.22	4.43	6.38
Totals	21.04	43.27	36.98	74.32	61.28	60.00	59.50	98.62

All fatty acid concentrations are the means of three replicates

(range of three replicates 94.0–104.4 μg lipid/ml) which occurred at 420 μg SiO_3/ml after 2 weeks in stationary phase.

Discussion

Growth rate, carrying capacity, time to reach stationary phase, and lipid content are important parameters in the selection of a potentially useful algal strain for use in production of biofuels. If growth rates are expressed as day^{-1}, carrying capacity K in cells/l and time to reach stationary phase in days and lipid content in percent dry weight, there are sufficient published values for algae to obtain some idea of the range of parameters. In the present case *C. meneghiniana* had a growth rate of 0.864 day^{-1}, a *K* of 2.51×10^9 cells/l, and a time to stationary phase of 17 days. The maximum stationary population density of 2.51×10^9 cells/l represents a dry weight of 960 mg/l and an ash-free dry weight of 397 mg/l. With a methyl ester content of 100 mg/l, *Cyclotella* has a lipid content of 25% on the basis of ash-free dry weight. The light level used in the growth of the four diatoms in these studies (152 μmol quanta m^{-2} s^{-1}) is similar to numerous other algal growth and lipid content studies [11, 12, 28, 38]. Fuentes-Grünewald et al. [11] examined three genera of marine dinoflagellates and one raphidophyte species for their potential as a source of biodiesel. The most promising species was the dinoflagellate *Karlodinium veneficum* with a growth rate of 0.14 day^{-1}, *K* of 4.4×10^7 cells/l, a time to stationary phase of 30 days, and 39.7% saturated lipids. By comparison, *C. meneghiniana* grows faster, reaches stationary phase quicker, and sustains a much higher carrying capacity than these reported marine algae but has a lower percent of lipids.

The closest values to those we obtained for *C. meneghiniana* come from other diatoms. *Phaeodactylum tricornutum* has a growth rate of 0.665 day^{-1} and a lipid content between 21 and 26% [15], about the same as *Cyclotella meneghiniana* but a somewhat slower growth rate. The marine diatom *Thalassiosira pseudonana* had a growth rate of 1.0–1.9 day^{-1} (depending on the number of hours of light), a *K* of 1.0×10^{10} cells/l, and a time to stationary phase of 6 days [3]. McGinnis et al. [28] reported that *Chaetoceros muelleri* from a New Mexico playa had a

growth rate of 1.6 day^{-1}, K of 3×10^9 cells/l, and time to stationary phase of 3 days. *Nitzschia laevis*, grown heterotrophically on glucose, had a growth rate of 0.58 day^{-1} and a time to stationary phase of 5 days [6]. One advantage of *C. meneghiniana* as a potential source of biodiesel is that it occurs naturally in practically every type of freshwater environment from ponds and lakes to rivers and wastewater effluents without forming harmful blooms.

The silicon enrichment studies were undertaken to determine the optimal mix of phosphorus, nitrogen, and silicon to maximize the production of diatoms and therefore the production of lipids. In the basic sD11 medium there is 2.7 mg P/l, 17.8 mg N/l, and 5.9 mg Si/l. The mass ratio of these elements relative to phosphorus is therefore 1:6.6:2.2 P:N:Si. As silicon was added to the basic medium, the stationary population density of *Cyclotella meneghiniana* steadily increased up to a silicon level of 47.2 mg Si/l, after which further additions of silicon failed to produce any increases in population density. Phosphorus or nitrogen would now be limiting and depleted in stationary growth phase. This optimal modified sD11 medium has a nutrient ratio of 1:6.6:17.5 for P:N:Si. This nutrient ratio allows one to predict how much silicon would be needed in wastewater with a known amount of phosphorus and nitrogen to deplete that phosphorus and nitrogen and produce the maximum amount of diatom biomass, which is about 17.5 times as much silicon mass per liter as phosphorus.

In addition to these growth parameters, diatoms sink out of culture readily in the absence of stirring. All non-motile algal cells tend to sink in freshwater in the absence of mixing because their cytoplasm tends to be slightly denser than water. Diatoms sink more rapidly than many other groups of algae because their silicon impregnated cell walls are extremely dense (2,600 kg/m^3) [34]. The capacity of diatoms to sediment rapidly should be useful in harvesting their biomass for lipids.

Cyclotella and *Aulacoseira* were the most productive diatom genera in terms of μg lipid/ml, and both have cylindrical cells. *Cyclotella* may form short chains of up to four cells, but *Aulacoseira* forms long chains of many cells. *Fragilaria* and *Synedra*, in contrast, are fusiform (needle-shaped). *Synedra* grows as single cells, while *Fragilaria* grows as long ribbon-like chains in which the cells are attached side-by-side. In diatom lipid production, shape matters, and it may be possible to use diatom cell shape as an indication of the potential to accumulate significant amounts of lipids for commercial application.

In the present study, the most common fatty acids in the four genera studied were C14:0, C16:0, C16:1, and C20:5n3 species. These are the same four fatty acids that are most common across all the algal groups compiled by Hu et al. [20], including diatoms, greens, eustigmatophytes, cryptophytes, dinoflagellates, and cyanobacteria. A fifth common fatty acid in our study was C16:3, common in diatoms and greens but not in other algal groups tested so far. Hu et al. [20] reported the fatty acid composition for the diatoms *Biddulphia aurica* and *Chaetoceros* sp. in a table of algal groups. Chen et al. [6] studied only *Nitzschia laevis*, in which C14:0, C16:0, C16:1, and C20:5n3 accounted for 85.8% of total triacylglycerols, and Fuentes-Grunewald et al. [11] added *Pseudo-nitzschia delicatissima* and *Chaetoceros affinis*. Unlike our results, these latter two studies found a significant amount of C17 and C18 fatty acids in their diatom species. The present work adds information on the growth characteristics and fatty acid composition of four freshwater diatom isolates suitable for wastewater cultivation to the available biofuel feedstock options. The process of obtaining detailed lipid profiles for microalgae is slow and laborious, but as this paper was in preparation, a new technique was reported for the direct, quantitative determination of lipid profiles on living microalgal cells by single-cell laser-trapping Raman spectroscopy [44]. Lipid profiles were reported for single living cells of *Botryococcus braunii*, *Chlamydomonas reinhardtii*, and *Neochloris oleoabundans*, with the time to obtain spectra for fatty acids less than 10 s. Raman spectroscopy should make rapid monitoring of mass cultures of microalgae possible for determination of optimal harvest time.

In the present study, we determined that the time of growth in stationary phase and the level of silicon had a significant effect on the amount of lipid present in the cultures of *Cyclotella meneghiniana*. The cultures grown at the two lower levels of silicon examined, where the *Cyclotella* cultures were limited by silicon, had lower levels of lipids than the optimal level of silicon. At the optimal level of silicon, the *Cyclotella* culture was limited by depletion of phosphorus, nitrogen, and silicon, and the culture produced the highest level of lipid seen. The cultures grown at the lower silicon levels showed a decline in lipid content as the time in stationary phase increased. It would be useful to determine the optimal time in stationary phase to produce the maximum lipid.

If the measured lipid production of *Cyclotella meneghiniana* (100 μg/ml) were coupled to wastewater treatment on a national level in the United States, how much biodiesel might potentially be produced? At that rate, 1 kg of lipid, with the fatty acid composition indicated in Table 5, would result from algal growth (and nutrient reduction) in 10,000 l of wastewater. Lipid is less dense than water and has a density of 860 kg m^{-3} [34]. Therefore, 1 kg of lipid would have a volume of 1.163 l. If a wastewater treatment plant produced 1 million gallons per day (mgd) or 3.78 million liters per day of effluent, it could potentially produce 440 l or 116 gal of lipid per day. The algae would be grown in a continuous process and maintained at high density to maximize biomass production and nutrient reduction. Growth at

low nutrient levels will in turn promote lipid production. The average wastewater treatment plant (WWTP) in the United States processes 3.2 mgd, so the average WWTP could potentially produce about 371 gal of lipid per day or 135,500 gallons of biofuel per year. There are some 15,000 WWTPs in the United States [41], and their potential oil production could be two billion gallons of biofuel per year. That number could be increased substantially if the WWTP system were designed to maximize algal biomass production while meeting reduced target levels for nutrient discharge of nitrogen and phosphorus. Where supplementary silicon is needed to produce maximum yield of diatom biomass, that silicon could be recovered along with phosphorus and nitrogen after lipid extraction.

The lipid production of *Cyclotella meneghiniana* (100 μg lipid/ml of medium) is of the same order of magnitude as fatty acid production (197 ± 14 mg/l of culture) in genetically altered cyanobacteria [26]. The diatoms studied in the present report naturally co-occur in freshwaters and wastewaters with cellulose-rich, filamentous green algae. The cellulose in these periphytic algae can be more readily hydrolyzed than plant cellulose, and therefore could be a valuable and abundant feedstock for the support of microbes that have been genetically engineered to produce desirable lipids [19]. Grown together in wastewater effluent, diatoms and filamentous green algae have the potential to improve wastewater effluent by removing nitrogen and phosphorus and generate sustainable biofuel feedstocks.

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