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Biomass Productivities in Wild Type and Pigment Mutant of *Cyclotella* sp. (Diatom)

Michael H. Huesemann · Tom S. Hausmann · Richard Bartha · M. Aksoy · Joseph C. Weissman · John R. Benemann

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Abstract Microalgae are expected to play a significant role in greenhouse gas mitigation because they can utilize CO_2 from power plant flue gases directly while producing a variety of renewable carbon-neutral biofuels. In order for such a microalgal climate change mitigation strategy to become economically feasible, it will be necessary to significantly improve biomass productivities. One approach to achieve this objective is to reduce, via mutagenesis, the number of light-harvesting pigments, which, according to theory, should significantly improve the light utilization efficiency, primarily by increasing the light intensity at which photosynthesis saturates (I_s). Employing chemical (ethylmethylsulfonate) and UV mutagenesis of a wild-type strain of the diatom *Cyclotella*, approximately 10,000 pigment mutants were generated, and two of the most promising ones (CM1 and CM1-1) were subjected to further testing in both laboratory cultures and outdoor ponds. Measurements of photosynthetic oxygen production rates as a function of light intensity (i.e., *P–I* curves) of samples taken from laboratory batch cultures during the exponential and linear growth phase indicated that the light intensity at which photosynthesis saturates (I_s) was two to three times greater in the pigment mutant CM1-1 than in the wild type, i.e., 355–443 versus 116–169 μmol/m^2 s, respectively. While theory, i.e., the Bush equation, predicts that such a significant gain in I_s should increase light utilization efficiencies and thus biomass productivities, particularly at high light intensities, no improvements in biomass productivities were observed in either semi-continuous laboratory cultures or outdoor ponds. In fact, the maximum biomass productivity in semi-continuous laboratory culture was always greater in the wild type than in the mutant, namely 883 versus 725 mg/L day, respectively, at low light intensity (200 μmol/m^2 s) and 1,229 versus 1,043 mg/L day,

M. H. Huesemann (✉) · T. S. Hausmann · R. Bartha
Marine Sciences Laboratory, Pacific Northwest National Laboratory, 1529 West Sequim Bay Road, Sequim, WA 98382, USA
e-mail: michael.huesemann@pnl.gov

M. Aksoy
Department of Biology, Brooklyn College, 2900 Bedford Ave, 200 NE, Brooklyn, NY 11210, USA

J. C. Weissman · J. R. Benemann
SeaAg, Inc., 705 27th Avenue S.W. Unit A, Vero Beach, FL 32968, USA

respectively, at high light intensity (1,000 μmol/m^2 s). Similarly, the biomass productivities measured in outdoor ponds were significantly lower for the mutant than for the wild type. Given that these mutants have not been completely characterized in these initial studies, the exact reasons for their poor performance are not known. Most likely, it is possible that the mutation procedure affected other photosynthetic or metabolic processes. This hypothesis was partially validated by the observation that the pigment mutant had a longer lag period following inoculation, a lower maximum specific growth rate, and poorer stability than the wild type.

Keywords Biomass productivity · Antenna mutants · Bush equation · Saturating light intensity · Light utilization efficiency · Semi-continuous culture · Outdoor ponds

Introduction

Currently, microalgae are being investigated in the USA and abroad for production of renewable fuels and for biofixation of CO_2 emitted by power plants to attain greenhouse gas abatement [1–3]. The advantages of microalgae are that they can produce high value liquid and gaseous fuels (biodiesel, hydrocarbons, ethanol, methane, hydrogen), that they have the potential of achieving very high biomass productivities approaching the theoretical limits of photosynthesis (about 10% solar conversion efficiency), and that they can function in greenhouse gas mitigation, because of their ability to utilize CO_2 from power plant and other flue gases directly [1, 4, 5]. However, these advantages must still be realized in practice, with many practical problems and issues remaining to be overcome, most importantly the selection and genetic improvement of algal strains that can be mass-cultured in open ponds, achieving high productivities [6, 7].

When microalgae are grown in open ponds in bright sun light, photosynthetic biomass productivities are often limited by a number of factors: First, the presence of light-harvesting pigments such as chlorophyll causes self-shading of cells, which, in turn, results in rapid light attenuation within the culture. Because of limited light penetration, just a small fraction of the culture, i.e., only those cells which are close to the pond surface, are photosynthetically active. Second, because of their exposure to the very high photon flux densities of direct sunlight, cells in the top layer of the culture are often unable to convert all absorbed photons into chemical energy, i.e., their rate of photon absorption by antenna chlorophylls exceeds their maximal rate of photosynthesis. Consequently, the excess photons absorbed are dissipated as fluorescence and heat, which translates into greatly reduced light utilization efficiencies. Finally, under high irradiance, normally pigmented cells are also particularly susceptible to photo-inhibition [8–10].

According to the Bush equation, enunciated in Burlew [11] and derived in Weissman [12], the light utilization efficiency (E_s) is a function of the incident light intensity (I_0) and the light intensity at which photosynthesis saturates (I_s), i.e.,

$$E_s = \frac{\text{Total Applied Light} - \text{Unutilizable Light}}{\text{Total Applied Light}} = \frac{I_s}{I_0}\left(\ln\left(\frac{I_0}{I_s}\right) + 1\right) \tag{1}$$

As shown in Fig. 1, a plot of photosynthetic oxygen evolution (P) as a function of light intensity (I) exhibits several distinct characteristics [8, 9, 13]. At low light, P increases

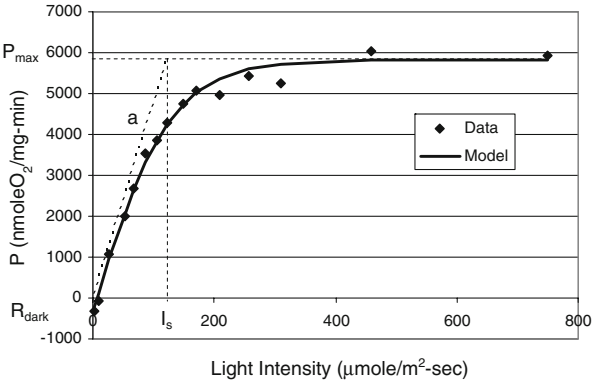

Fig. 1 Curve fitting of experimental *P–I* data (see text and Eq. 3 for more information)

linearly with light intensity (I). The magnitude of the initial slope of the *P–I* curve is an indicator of the solar energy conversion efficiency of photosynthesis which is related to the minimum number of photons needed to generate one molecule of oxygen. At intermediate light intensities, P increases non-linearly as a function of I until P reaches a maximum (P_{max}) at (approximately) the saturating light intensity (I_s). P does not increase beyond P_{max} at light intensities greater than I_s because dark reactions (i.e., either inter-photosystem electron transport or carbon fixation reaction) become rate-limiting under high irradiance, resulting in the wasting of excess photons absorbed as heat. Thus, the light utilization efficiency is directly related to the maximal quantum efficiency and the saturating light intensity: The greater I_s, the fewer photons are wasted as heat and the greater the E_s. For example, according to the Bush equation (Eq. 1), an algal culture illuminated by 2,000 μmol/m^2 s (I_0) would have a light utilization efficiency of only 20% if the saturating light intensity (I_s) were 100 μmol/m^2 s, but E_s would be 52% if I_s were 400 μmol/m^2 s.

Kok [14] postulated more than 50 years ago that a truncated chlorophyll antenna size of the photosystems should increase the light utilization efficiency of microalgae in mass culture since it would minimize the wasteful dissipation of absorbed sunlight. Recently, a number of investigators have tested this hypothesis by creating microalgae with reduced light-harvesting pigment content, and determined photosynthetic *P–I* curve parameters P_{max} and I_s, and, in some cases, compared biomass productivities in antenna mutants relative to highly pigmented wild-type cells. For example, Melis et al. [8] found that *Dunaliella salina* grown under high light intensity (2,000 μmol/m^2 s) was significantly less pigmented, had three times greater chlorophyll-specific P_{max} values, and more than 12 times greater saturating light intensities (I_s) than cells grown at low light (200 μmol/m^2 s). They postulated that these characteristics of the high light-adapted microalgae should translate into greater biomass productivities in outdoor mass culture, but no experiments were carried out to test this hypothesis. By contrast, a number of Japanese investigators compared the biomass productivities in mutants with reduced light-harvesting pigments to those in highly pigmented wild-type cells. For example, Nakajima and Ueda [15] reported that at high light intensity (2,000 μmol/m^2 s), the biomass productivity of a phycocyanin-deficient mutant of *Synechocystis* was about 50% greater than in the wild type. Similarly, Nakajima and Ueda [16] and Nakajima et al. [17] observed ca. 50% greater maximum biomass productivities in a mutant with small light-harvesting pigments compared to the normally pigmented wild type of *Chlamydomonas perigranulata*.

The objectives of this study were to (a) generate mutants of the diatom *Cyclotella* with reduced light-harvesting pigments, (b) characterize photosynthetic parameters (P_{max}, I_s, and the initial slope of the *P–I* curve) in both wild type and mutant, (c) measure biomass productivities in the wild type and the most promising mutant in low and high light semi-continuous cultures in the laboratory, and (d) compare biomass productivities of the wild type and mutant in outdoor ponds.

Materials and Methods

Cyclotella Wild Type

The original *Cyclotella* sp. wild type (CYCLOJ) was isolated using an enrichment process conducted over 10 years ago at the Sea-Ag, Inc. facility. It has been maintained since then in outdoor ponds under the original enrichment condition. Periodically, samples were plated and individual colonies (clones) were picked to grow test tube and flask cultures in the laboratory. Many of these have been maintained in the laboratory, offering readily available sources of unialgal inoculum for study. It is from one of these clones, CYCLOJ5, that culture samples were grown to provide wild-type strains for mutagenesis and subsequent biomass productivity measurements.

Generation of Antenna Mutants

Cell Culture Conditions

Wild-type and mutant strains of *Cyclotella* sp. were grown in artificial seawater medium. The medium consisted of 299.4 mL/L distilled water and 666.6 mL/L artificial seawater (28 ppt) made with Instant Ocean^{TM} seasalts from Aquarium Systems. In addition, each liter medium contained 2 mL Part A and 2 mL Part B of Kent Micronutrients (f/2), 25 mL of silica solution (40 mM, pH 7.5), and 5 mL of sodium bicarbonate (0.5 M, pH 7.5). After the medium was made, it was sterile-filtered before using it for culturing. To prepare agarose plates, the silica and sodium bicarbonate were added to the medium after autoclaving. Stock cultures were kept in 125 mL Erlenmeyer flasks in artificial seawater medium and illuminated continuously using daylight fluorescent lights at about 30 μmol/m^2 s.

Mutagenesis

For generating pigment mutants, 0.2 M ethylmethylsulfonate (EMS) or UV light was used according to Bennoun and Delepelaire [18]. After incubation with EMS for 2 h in the dark, cells were pelleted by centrifugation and washed three times with medium to remove excess EMS. The washed cells were then transferred onto agar plates and placed under daylight fluorescent lights (30 μmol/m^2 s) for ca. 10 days.

For UV mutagenesis, cells were grown in liquid culture to minimize clumping. If the cell density was too high, cells were diluted to prevent self-shading. Thirty milliliters of liquid culture was used for UV mutagenesis. The procedure was performed in a dark room to prevent light-mediated repair. Cells were kept in a Petri dish and were continuously mixed with a magnetic stirring bar during UV illumination. The distance between the Petri dish and the UV lamp was 10 cm. Cells were irradiated with two 15 W FC15T8 germicidal UV bulbs for 15–20 s. Following mutagenesis, cells were transferred into a flask, covered with

aluminum foil, and incubated in the dark on a shaker at 125 rpm for 16–18 h. After dark incubation, cells were diluted and transferred onto agarose plates. The plates were kept under continuous daylight fluorescent light (30 μmol/m^2 s) for ca. 10 days to allow the cells to grow.

Screening

Plates with mutant colonies were first inspected visually for those with aberrant coloration. Then colonies were transferred to index plates and grown for about 2 weeks. The index plates were screened for mutant colonies displaying low chlorophyll fluorescence emission using the FluorImager from Qubit Systems (Kingston, Ontario, Canada). Cells of colonies that demonstrated low chlorophyll fluorescence were transferred onto new index plates, and the process was repeated to also determine the F_v/F_m ratio which is an indicator for the photosynthetic efficiency of photosystem II [19]. Then, samples were transferred into liquid medium and grown in 125 mL Erlenmeyer flasks on a shaker under continuous light (80 μmol/m^2 s). Samples were taken from the liquid cultures, and the Chl-*a* to Chl-*c* ratios were determined by spectrophotometry using the protocol of Jeffrey and Humphrey [20]. Mutants that tested positive in all four screening steps were then grown outdoors in mass culture in small experimental raceway ponds or grown in simulated mass culture in the laboratory as described in the next section.

Performance of Laboratory Culture Experiments

Culture Conditions

All simulated mass culture experiments were carried out in the laboratory using sterile 1 L (flat) Roux bottles filled with 600 mL enriched seawater medium containing 28 g/L sea salts, 1,500 mg/L $Na_2O_3Si{\cdot}9H_2O$ (neutralized with HCl), 1,000 mg/L urea, 400 mg/L $NaH_2PO_4{\cdot}H_2O$, 100 mg/L Fe-sequestrate, 50 μg/L $CuSO_4{\cdot}5H_2O$, 125 μg/L $ZnSO_4{\cdot}7H_2O$, 50 μg/L $CoCl_2{\cdot}6H_2O$, 900 μg/L $MnCl_2{\cdot}4H_2O$, 32.5 μg/L $Na_2MoO_4{\cdot}2H_2O$, 500 μg/L thiamine HCl, 25 μg/L biotin, and 25 μg/L vitamin B_{12}. This medium is capable of supporting diatom growth to around 2.0 g biomass (AFDW)/L.

The Roux bottles were placed in a water bath with temperature controlled at 28 °C, sparged with CO_2 enriched air (0.5% *v*/*v* CO_2) at 3,015 mL/min (i.e., 3,000 mL/min air and 15 mL/min CO_2) to maintain a pH of 7.5. Each bottle was illuminated continuously (i.e., 24 h/day) from one side with a 500-W halogen (Regent PN PQS45) light at a light intensity of either 200 μmol/m^2 s (screened with neutral density filters, i.e., door screen) or 1,000 μmol/m^2 s (unscreened). The culture medium within the Roux bottles was mixed continuously with the help of magnetic stirrers.

Inoculum Preparation

Approximately 5 mL of cell suspension of either wild-type or CM1-1 mutant culture were aseptically transferred to a 125-mL Erlenmeyer flask containing 50 mL of sterile growth medium. The Erlenmeyer flask was closed with a cotton plug and placed under a fluorescent growth light (Vita-lite, light intensity=120 μmol/m^2 s) on a shaker table (150 rpm) for 3 to 5 days. As soon as the biomass concentration had reached an optical density (OD at 590 nm, OD_{590}) of approximately 1, the culture was used to inoculate sterile medium in the Roux bottles.

Measurement of Biomass Concentrations (AFDW and OD_{590})

The biomass concentration in algal cultures was measured both photospectrometrically as optical density (OD_{590}) and gravimetrically as ash-free dry weight (AFDW, mg/L). For the measurement of OD_{590}, the absorbance of the cell suspension at 590 nm was determined using a Unico 1100 photospectrometer. If the optical density reading was greater than 0.5, the sample was diluted with seawater medium to assure that the OD_{590} was measured in the linear range, i.e., when $0<OD_{590}<0.5$.

For the determination of AFDW, 10–25 mL (V) of cell culture was vacuum-filtered through a Whatman GF/F Glass microfiber filter. The filter was placed in an aluminum foil pouch and heated overnight in an oven at 100–105 °C to remove all water. The dried cells were weighed (M_1) and then heated in a 550 °C muffle furnace for 4 h to combust all biomass organic carbon. The weight of the inorganic residues remaining after combustion was determined and noted as M_2. The ash-free dry weight concentration of the cell culture sample was then calculated as $(M_1-M_2)/V$.

Determination of Maximum Specific Growth Rates (μ_{max}) in Batch Culture

The maximum specific growth rate for a given microalgal species was measured in unshaded batch cultures as follows: A temperature equilibrated Roux bottle filled with 600 mL enriched seawater medium was inoculated with the wild type or mutant and illuminated from one side with a screened 500 W halogen lamp at a light intensity of 200 μmol/m^2 s. The culture was then allowed to grow exponentially to a biomass concentration no greater than 50 mg AFDW/L which is required to ensure the absence cell self-shading. At the end of the growth period, a sample was taken to determine the biomass concentration (AFDW), and the batch culture was then diluted with new medium to repeat the growth experiment. The maximum specific growth rate ($\mu_{\max}$) during each exponential growth phase was calculated as:

$$\mu_{\max}\frac{1}{\Delta t}\cdot\ln\left(\frac{C_{\mathrm{f}}}{C_{\mathrm{i}}}\right) \tag{2}$$

where C_{i} and C_{f} are the initial and final biomass concentration, respectively, and Δt is the length of the incubation period. The mean maximum specific growth rate was determined as the average of at least five replicate specific growth rate measurements.

Measurement of Steady-state Biomass Productivities in Semi-continuous Culture

A set of three to four Roux bottles were inoculated with either wild type or mutant and grown under semi-continuous culture conditions by removing a fraction of the culture suspension and replacing it with fresh medium on a daily basis. The ratio of the rate of new medium addition F (L/day) to the total culture volume V (L) is defined as the dilution rate D (= F/V, day^{-1}). In general, these semi-continuous cultures were carried out at four different dilution rates, such as 0.3, 0.5, 0.7, and 0.85 day^{-1}. In some cases, additional dilution rates were tested to determine the dilution rate at which biomass washout occurs.

Prior to each daily dilution with fresh medium, a sample was taken from the Roux bottle and the cell density was measured photospectrometrically (OD_{590}) and gravimetrically (AFDW) as described above. The Roux bottles were cultured semi-continuously at the different dilution rates until steady state was reached, i.e., when daily OD_{590} and AFDW

readings stayed constant (i.e., fluctuated less than ±10% around the mean) for over a period of 3 to 5 days. When steady state was achieved, a sample was taken from each Roux bottle 22 h (t=22) after dilution for the measurement of P–I curves and chlorophyll content (see below). All semi-continuous culture experiments were carried out under continuous illumination at low and high light intensities, i.e., 200 and 1,000 μmol/m^2 s, respectively. The biomass productivity (BP, mg/L day) at each dilution rate was calculated as the product of the steady-state biomass concentration (C_B, mg/L) and dilution rate (D, day^{-1}), i.e., BP=$C_B \cdot D$.

Measurement of Photosynthetic O_2 Evolution as a Function of Light Intensity (P–I Curve)

The rate of oxygen evolution (P) was measured as a function of light intensity (I) using a computer-controlled Chlorolab system (Hansatech Instruments, Norfolk, UK) which consists of an illuminated temperature-controlled transparent incubation cell (ca. 2 mL) equipped with a dissolved oxygen (DO) meter and a magnetic stirrer. The DO probe was calibrated by measuring the dissolved oxygen concentration in artificial seawater that had been sparged for 10 min either with air (i.e., DO=250 nmol/mL) or nitrogen (i.e., DO=0 nmol/mL). To measure the oxygen evolution rate of a specific microalgal culture, a 5-mL sample was taken from the Roux bottle, homogenized, and diluted with artificial seawater down to an optical density (OD_{590}) of 0.5 to assure the absence of cell self-shading, and transferred (a 2-mL aliquot) to the Chlorolab incubation cell which was maintained at 28 °C. The culture sample was then sparged with a gas mixture consisting of 0.5% CO_2 and 99.5% N_2 until the dissolved oxygen concentration had been lowered to approximately 15 nmol O_2/mL.

At this point, the incubation cell was completely sealed off with a tight-fitting plunger which also removed any gas bubbles in the headspace, and the change of dissolved oxygen concentration was first measured as a function of time in the absence of light (dark respiration) and then at 18 different light intensities ranging from 10 to 1,000 μmol/m^2 s. Illumination at these different light intensities was generated by varying the distance between the incubation cell and the 100-W halogen lamp and by employing various combinations of neutral density filters in front of the light bulb. The light intensity for each specific combination of distance and filter arrangement was measured independently prior to the oxygen production rate measurements by placing a light meter (Hansatech Instruments, Norfolk, UK) inside the empty incubation cell. Oxygen evolution rates were determined in sequence from low to high light intensity settings and each measurement was taken for a period of ca. 90 s (i.e., 60 s to stabilize the dO_2/dt slope and 30 s to measure the slope). The resulting linear plots of oxygen concentrations versus time were converted into oxygen evolution rates by the Chlorolab computer software using previously obtained calibration data and AFDW and chlorophyll-a concentrations of the original microalgal culture.

The measured photosynthetic oxygen evolution rates (P) were plotted as a function of light intensity (I) as shown in Fig. 1. In order to determine the initial slope (a) and the maximum oxygen evolution rate (P_{max}), the experimental data were curve-fitted with a hyperbolic tangent function [21] using the Microsoft Excel least-squares solver algorithm:

$$P = P_{max} \cdot \tan h\left(\frac{a \cdot I}{P_{max}}\right) + R_{dark} \quad (3)$$

where R_{dark} is the rate of oxygen uptake in the absence of light (i.e., dark respiration). The light intensity at which photosynthetic oxygen production saturates, I_s, can then be obtained

as the intersection of the initial slope (a) and the maximum oxygen evolution rate (P_{max}), i.e., $I_s = P_{max}/a$.

Using an approximate stoichiometry of photosynthesis (i.e., 83 CO_2 + 58.5 H_2O + 6.5 CH_4N_2O (urea) + NaH_2PO_4 = $C_{83}H_{143}O_{29}N_{13}P$ + 97.75 O_2), 18.5 g of biomass is produced per mole of oxygen evolved. Thus, an oxygen evolution rate of 1 nmol O_2/mg AFDW min is equivalent to a specific biomass growth rate (μ) of 0.0268 day^{-1}.

Measurement of Chlorophyll Content

The chlorophyll-*a* content of *Cyclotella* cells was determined using a methanol extraction technique that was adopted from Tett et al. [22]. A 10-mL culture sample was taken from the Roux bottle, transferred to a 35-mL amber glass centrifuge tube, and centrifuged for 5 min at 500×*g* after adding 0.2 mL of 1% (*w*/*v*) $MgCO_3$. After decanting all supernatant, the cell pellet was resuspended in 10 mL of a 90% (*v*/*v*) methanol/water solution which then was boiled in a water bath at 90 °C for 2 min. After reconstituting the volume of the cooled extract with 90% (*v*/*v*) methanol/water back to 10 mL, the absorbance of both the extract and a 90% (*v*/*v*) methanol/water blank was measured with a Beckman DU-8 Spectrophotometer at 663 and 750 nm. If necessary, the extract was diluted with 90% (*v*/*v*) methanol–water to assure that the absorbance reading was in the linear range, i.e., less than 0.8. The chlorophyll-*a* (Chl-*a*) concentration (mg/L) was then calculated as:

$$\text{Chl}-a = 13 \cdot \text{DF} \cdot ((C_{663} - B_{663}) - (C_{750} - B_{750})) \tag{4}$$

where DF is the dilution factor, C_{663} and C_{750} are the absorbance of the extract, and B_{663} and B_{750} are the absorbance of the blank at 663 and 750 nm, respectively.

Performance of Outdoor Pond Experiments

Bioreactors

Figure 2 shows paddlewheel mixed, raceway ponds (RWP) that were employed in this study. The RWPs have a surface area of 2.8 m^2, are 77 cm deep, and have been used extensively in prior projects.

Media

Saline groundwater was used for all microalgal cultivation at the field site located at SeaAg, Inc., Fort Pierce, FL, USA. The salinity was relatively constant at 27–30 mg/L. Standard, commercial concentrates of Guillard's f/2 medium was used for stock cultures. In addition, the saline groundwater was supplemented with urea, iron sulfate (septahydrate), phosphoric acid, and sodium metasilicate pentahydrate (neutralized with sulfuric acid) in quantities sufficient to ensure that none of these nutrients were limiting biomass growth in the outdoor reactors.

Pond Operations

The ponds were diluted semi-continuously. Each morning, a prescribed proportion of the pond volume (determined as depth) was removed and replaced with fresh medium. The

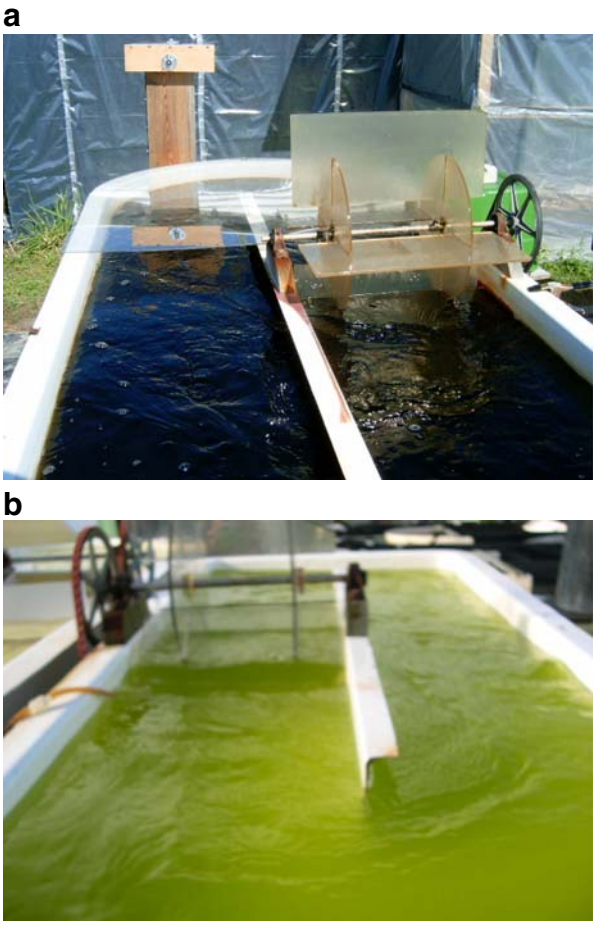

Fig. 2 Typical raceway pond reactor used in this study to cultivate the wild type (**a**) and the CM1-1 mutant (**b**)

daily operations were carried out as follows: (a) take temperature, pH, and depth measurements; (b) add fresh water to make up for any evaporation (e.g., bring the pond depth back to the prescribed level with fresh water); (c) take a 1-L sample of the culture; (d) remove a predetermined fraction of the culture to reduce the pond level to the new, desired depth; (f) refill with saline ground water to the standard depth; and (g) add nutrients as required.

Ash-Free Dry Weight Determination

Samples were taken for AFDW measurement every day. Enough volume of sample was filtered over a pre-ashed, pre-weighed Whatman GF/C filter to accumulate about 10 mg AFDW. Weights were determined using an analytical balance which measures to 0.01 mg. With this balance and this amount of biomass, replication was generally within 3%. Filtered samples were rinsed with a 20-g/L solution of ammonium formate prior to drying to remove salts. Samples were dried at 103 °C overnight and ashed for 15 min at 550 °C. Results were expressed as milligram per liter.

Biomass Productivity Measurements

Biomass productivity for the RWP reactors was calculated as grams of biomass per square meter of horizontal surface per day. For a given day, this is calculated as the standing biomass (grams per square meter) of the culture after dilution, subtracted from the standing biomass the next morning before dilution. The standing biomass is calculated from the biomass density (grams ash-free dry weight, AFDW, per liter) times the liters per square meter of pond surface.

Microscopic Examination

Cultures were examined under a microscope three times per week to determine culture characteristics including presence of grazers, clumps, and the species of algae present.

Results and Discussion

Generation and Screening of Mutants with Truncated Chlorophyll Antenna Size of the Photosystems

About 10,000 mutants of the diatom *Cyclotella* sp. were generated by treatment with EMS. An initial visual screening identified several mutants by aberrant coloration from brown wild-type *Cyclotella* colonies. From this first round of EMS mutagenesis, one mutant (CM1) originated that showed an olive-green coloration (Fig. 3). In comparison to the wild type, the olive-green CM1 mutant had similar chlorophyll fluorescence emission (Fig. 3). Concomitantly, the F_v/F_m ratio of mutant CM1 (0.73) was slightly reduced compared to the wild type (0.77), indicating somewhat reduced photosynthetic efficiency of PSII. In addition, mutant CM1 had a higher Chl-*a*/*c* ratio (19.1±1.1) as compared to wild type (12.7±1.7) suggesting a slightly truncated chlorophyll antenna size of the photosystems. As a result, the CM1 mutant was selected for a second round of UV mutagenesis which generated about 6,000 mutants. Screening of those UV mutants resulted in the identification of a green colony (CM1-1). The green coloration of CM1-1 (Fig. 3) indicated that cells of this mutant strain lost the ability to produce the pigment fucoxanthin. Loss of fucoxanthin in cells of the CM1-1 mutant was confirmed by HPLC analysis (data not shown). The CM1-1 mutant also had significant reduction of chlorophyll fluorescence emission (Fig. 3) and an increased Chl-*a*/*c* ratio (>30) suggesting drastic truncation of the chlorophyll antenna size of the photosystems.

Biomass Growth Kinetics in Batch Culture

When biomass growth was repeatedly measured during the exponential phase under light-sufficient, non-shading conditions, it was found that the wild type had a slightly greater maximum specific growth rate (μ_{max}) than the CM1-1 mutant, i.e., 3.45 versus 3.30 day^{-1}, respectively (see Table 1). While the exact reasons for this minor reduction of μ_{max} are not known, it is likely that it was caused by random mutations which negatively affect the growth and metabolism of the CM1-1 mutant.

In order to measure the rate of photosynthetic oxygen evolution as a function of light intensity (i.e., *P–I* curves) for samples taken during the exponential and linear growth phase (see next section), successive batch culture experiments were carried out for both the wild

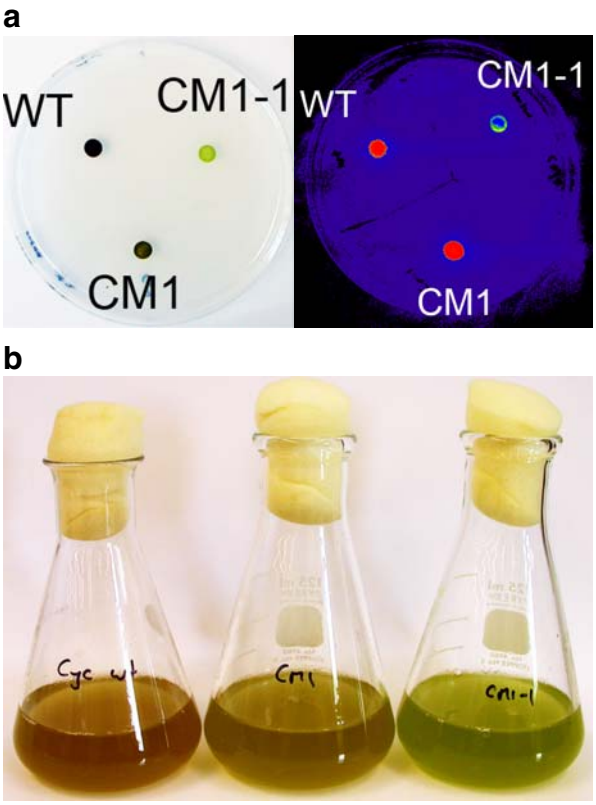

Fig. 3 Comparison of wild type, mutants CM1 and CM1-1 of *Cyclotella* sp. grown on agarose plate (**a**) or in liquid culture (**b**). Wild-type cells have the typical brown coloration while the CM1-1 mutant displays a green color indicating loss of the pigment fucoxanthin. The *upper right panel* shows a false color image of colonies grown on agarose plate

Table 1 Optical density (OD_{590}), AFDW and chlorophyll-*a* concentration, chlorophyll-*a* content, AFDW- and chlorophyll-*a*-normalized P_{max} values, saturating light intensity (I_s), and initial slope of the *P–I* curve (*a*) for exponential and linear phase samples taken from both wild-type and CM1-1 mutant batch cultures.

	Wild type		CM1-1 mutant	
	Exponential phase	Linear phase	Exponential phase	Linear phase
μ_{max} (1/day)	3.45 (0.11)	NA	3.30 (0.13)	NA
Optical density (OD_{590})	0.64	2.55	0.18	4.3
AFDW (mg/L)	239	922	136	1,337
Chl-*a* (mg/L)	3.6	27	1.2	17
Chl-*a*/AFDW (wt.%)	1.5	3.0	0.9	1.3
P_{max} (nmol/mg AFDW min)	92	274	90	202
P_{max} (nmol/mg Chl-*a* min)	6,200	9,250	9,900	15,800
I_s (μmol/m^2 s)	116	169	443	355
a (nmol/mg Chl-*a* min)/(μmol/m^2 s)	53	55	22	44

The maximum specific growth rate (μ_{max}) was measured in unshaded batch culture only during the exponential growth phase, with values in parenthesis denoting one standard deviation for at least five replicate measurements (see also "Materials and Methods" section).

NA Not applicable

type and CM1-1 mutant until reproducible biomass growth curves were obtained. As shown in Fig. 4a for the wild type, the lag phase decreased and the rate of biomass production (slope) during the linear, light-limited phase increased with successive batch culturing. Given that the growth kinetics in the third and fourth batch experiments were almost identical, it was decided to take exponential and linear phase cells from the fourth batch culture for *P–I* curve measurements (see arrows in Fig. 4a indicating sampling times). Similarly, three successive batch experiments were carried out for the CM1-1 mutant (see Fig. 4b), and exponential and linear phase cells were taken from the third batch culture. The biomass and chlorophyll-*a* concentrations for samples taken for *P–I* measurements are given in Table 1.

A comparison of biomass growth curves in the respective final successive batch cultures indicates that the wild type had a slightly greater growth rate during the linear, light-limited phase than the mutant, a finding that was also observed earlier in separate experiments during the exponential, light-sufficient phase (i.e., μ_{max} (wild type) > μ_{max}(mutant), see Table 1). In addition, the mutant culture reached a much higher final biomass concentration compared to the wild type, i.e., OD_{590}~6.5 versus OD_{590}~4.25, respectively. This was most likely due to the reduced chlorophyll content in the mutant relative to the wild

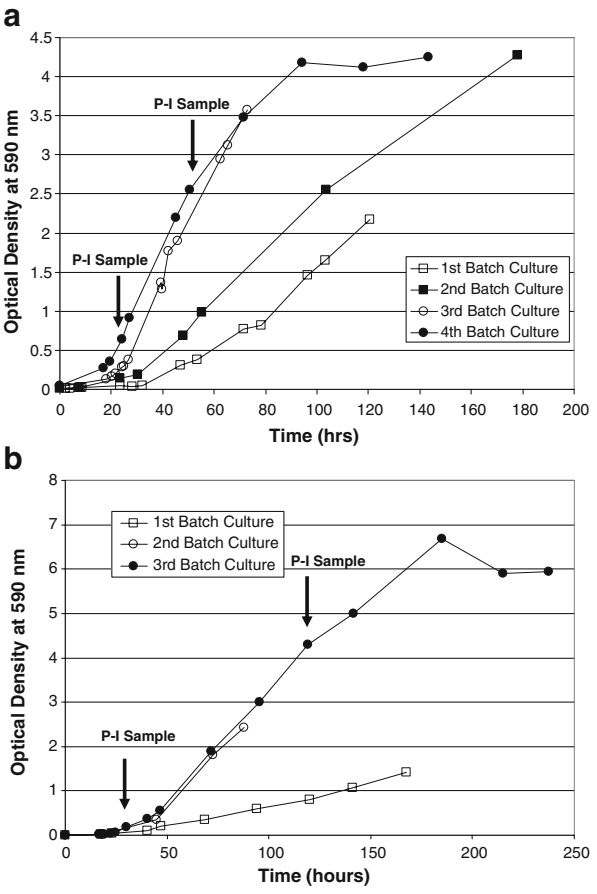

Fig. 4 **a** Optical density (OD_{590}) as a function of time in four successive wild-type batch cultures. Each successive batch culture was inoculated with 1 mL of linear phase cells obtained from the previous culture. The *two arrows* indicate when samples were taken for *P–I* curve measurements from the fourth batch culture during the exponential and linear growth phase, respectively. **b** Optical density (OD_{590}) as a function of time in three successive CM1-1 mutant batch cultures. Each successive batch culture was inoculated with 1 mL of linear phase cells obtained from the previous culture. The *two arrows* indicate when samples were taken for *P–I* curve measurements from the third batch culture during the exponential and linear growth phase, respectively

type (i.e., 1.3 versus 3.0 wt.% in linear phase cells, see Table 1), which results in less self-shading and deeper light penetration in the mutant culture compared to the wild type.

P–I Measurements in Batch Culture

Figure 5a shows the chlorophyll-*a*-normalized oxygen evolution rate as a function of light intensity for wild-type and mutant culture samples taken during the exponential growth phase. The chlorophyll-normalized maximum oxygen production rate (P_{max}) is at least 50% greater in the mutant than in the wild type while the AFDW-normalized P_{max} is about the same in both cultures (Table 1). The initial slope (a) is 200% steeper in the wild type than in the mutant. The light intensity at which photosynthesis saturates (I_s) is almost three times greater in the mutant than in the wild type, i.e., 443 versus 116 μmol/m^2 s (Table 1). Similar trends were observed in culture samples taken during the light-limited linear phase (Fig. 5b): The chlorophyll-normalized P_{max}, the initial slope (a), and the saturating light intensity (I_s) were all greater in the mutant than in the wild type (Table 1).

The AFDW-normalized P_{max} values during the exponential phase for the wild type and mutant were 92 and 90 nmol O_2/mg min (Table 1) which corresponds to a μ_{max} of 2.45 and

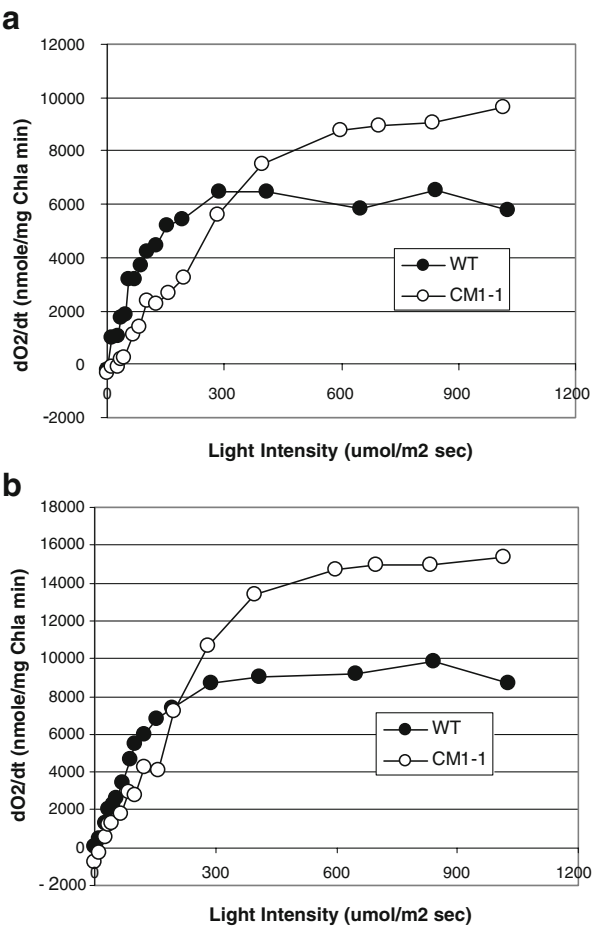

Fig. 5 **a** Chlorophyll-*a* normalized photosynthetic oxygen evolution rate as a function of light intensity for wild type and CM1-1 mutant during the exponential growth phase in batch culture. **b** Chlorophyll-*a* normalized photosynthetic oxygen evolution rate as a function of light intensity for wild type and CM1-1 mutant during the light-limited linear growth phase in batch culture

2.4 day^{-1}, respectively. Note that this is close to the μ_{max} values of 3.45 and 3.3 day^{-1} that were measured for the wild type and mutant, respectively, in non-shaded batch cultures during the exponential phase (Table 1). By contrast, the AFDW-normalized P_{max} values for the wild type and mutant during the linear phase were 274 and 202 nmol O_2/mg min (Table 1) which corresponds to a μ_{max} of 7.3 and 5.4 day^{-1}, respectively. These are much greater than the μ_{max} values measured in non-shaded batch culture.

The observation that a reduction in cellular chlorophyll content, which can be brought about either temporarily through adaptation of algal cultures to high light intensities or permanently through mutagenesis, is associated with significant increases in the saturating light intensity (I_s), the initial slope (a) of the *P–I* curve, and chlorophyll-specific P_{max} values, has been reported previously by a number of investigators. Melis et al. [8] showed that cells of *D. salina* grown under high light intensities were less deeply pigmented, had three times greater chlorophyll-specific P_{max} values, and about 12 times greater saturating light intensities (I_s) than cells cultured under low light intensities. Nakajima et al. [23] found half-saturating light intensities to be twice as high in a phycocyanin-deficient mutant of the *Synechocystic* compared to the wild type. In addition, mutant cells with small light-harvesting pigment content were shown to have significantly increased chlorophyll-specific P_{max} values compared to the wild type [16]. Finally, Nakajima and Itayama [24] also observed greater initial *P–I* curve slopes in wild types than in two mutants, which they attributed to the larger size of light-harvesting pigments in the wild type.

Biomass Productivities in Semi-continuous Culture

As was postulated earlier (see "Introduction"), a mutant with reduced light-harvesting pigment content should allow for greater light penetration into dense cultures, which, in turn, should result in greater biomass productivities compared to the wild type. In addition, according to the Bush equation (Eq. 1), the two to four times greater saturating light intensities (I_s) that were observed in the mutant should translate into significant improvements in light utilization efficiencies relative to the wild type, specifically at high light intensities. For example, per Eq. 1, at a light intensity of 1,000 μmol/m^2 s, a four-fold increase of I_s from ca. 100 to 400 μmol/m^2 s would more than double the light utilization efficiency from 33% to 76%.

In order to determine whether the reduced light-harvesting pigment content and the increased saturating light intensity in the CM1-1 mutant result in greater biomass productivities than in the wild type, both mutant and wild type were grown in semi-continuous culture at low (200 μmol/m^2 s) and high (1,000 μmol/m^2 s) light intensities (see Table 2). As shown in Fig. 6a where biomass productivity is plotted as a function of dilution rate for the low light intensity case, the wild-type cultures were always more productive in terms of CO_2 biofixation than the mutant. The maximum biomass productivity was observed in both mutant and wild type at a dilution rate of 0.7 day^{-1}. The mutant also appeared to be more prone to washout since a steady state could not be maintained, as in the wild type, at the high dilution rate of 0.9 day^{-1}, but only at D=0.7 day^{-1}. Similar results were also observed in the high light intensity case, where biomass productivities were always greater in the wild type than in the mutant (see Fig. 6b).

Samples from each steady-state semi-continuous culture listed in Table 2 were subjected to *P–I* measurements. As was the case in batch culture (Table 1), the chlorophyll-*a* content was two to three times greater in the wild type than in the mutant. Similarly, the saturating light intensities (I_s) were significantly higher in the CM1-1 mutant (i.e., 250 to 550 μmol/m^2 s) than in the wild type (i.e., 100–200 μmol/m^2 s). Under both low and high

Table 2 Steady-state AFDW concentration, biomass productivity (BP), chlorophyll-*a* concentration and content, AFDW- and chlorophyll-*a*-normalized P_{max} values, saturating light intensity (I_s), and initial slope of the *P–I* curve (*a*) for semi-continuous wild-type and CM1-1 mutant cultures operated at low and high light intensities at different dilution rates.

	Wild type							CM1-1 mutant						
	Low light (200 μmol/m^2 s)				High light (1,000 μmol/m^2 s)			Low light (200 μmol/m^2 s)				High light (1,000 μmol/m^2 s)		
Dilution rate (1/day)	0.4	0.5	0.7	0.9	0.5	0.7	0.85	0.3	0.5	0.7	0.85[a]	0.5	0.7	0.85
AFDW (mg/L)	1,448	1,442	1,261	760	1,522	1,755	1,351	1,180	1,340	1,036	323	1,498	1,491	607
BP (mg/L day)	579	721	883	684	761	1,229	1,148	354	670	725	275	749	1,043	516
Chl-*a* (mg/L)	39	36	29	12	24	27	13	8.4	9.4	1	NA	8.8	16	3.5
Chl-*a*/AFDW (wt.%)	2.4	2.5	2.5	1.4	1.3	1.9	1.5	0.9	0.8	0.5	NA	0.6	0.9	0.6
P_{max} (nmol/mg AFDW min)	304	272	319	229	72	121	154	164	152	55	NA	51	148	115
P_{max} (nmol/mg Chl-*a* min)	12,535	10,920	12,707	16,096	5,586	6,331	10,699	18,004	18,930	23,846	NA	8,658	15,580	17,643
I_s (μmol/m^2 s)	167	152	172	204	112	120	191	504	247	284	NA	353	331	548

NA Not applicable

[a] Slow washout (no steady state)

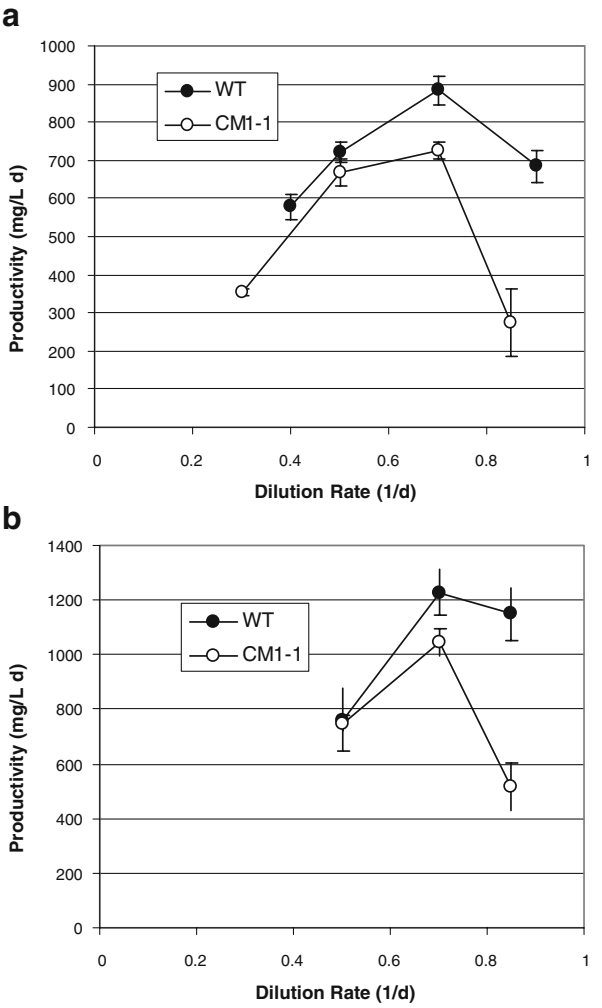

Fig. 6 **a** Biomass productivity of the wild type and CM1-1 mutant as a function of dilution rate in semi-continuous cultures operated a low light intensity (200 μmol/m^2 s). **b** Biomass productivity of the wild type and CM1-1 mutant as a function of dilution rate in semi-continuous cultures operated a high light intensity (1,000 μmol/m^2 s)

light conditions, chlorophyll-specific P_{max} values were considerably greater in the mutant than in the wild type (Table 2), a finding that was also observed in batch culture (Table 1) and by others [16, 24]. This is expected since the pigment concentrations are lower in the mutant. In addition, for both wild type and mutant, the chlorophyll-specific P_{max} values were always greater at low light than at high light intensity. This is contrary to the results reported by Melis et al. [8] who observed three times greater chlorophyll-specific P_{max} values in cells adapted to high light intensity compared to those cultivated under low light intensity.

If there were no light saturation effect, the rate of oxygen evolution would correlate linearly with light intensity, and a five-fold increase in light intensity from 200 to 1,000 μmol/m^2 s would translate into a five-fold gain in biomass productivity in semi-continuous cultures. Unfortunately, productivities did not increase in direct proportion to the incident light intensity (I_0) because oxygen evolution levels off at a saturating light intensity (I_s) which is generally much lower than I_0 (see also Fig. 1). Thus, photons in

excess of I_s are wasted as heat which results in low light utilization efficiencies. According to the Bush equation (Eq. 1), a five-fold increase in incident light intensity (I_0) should increase the high light to low light biomass productivity ratio much more in cells characterized with high than low saturating light intensities (I_s). However, despite the fact that the CM1-1 mutant had much greater I_s values than the wild type, the high light to low light productivity ratio was only slightly greater in CM1-1 than in wild type (see Fig. 7).

For both wild type and mutant, the high light to low light productivity ratio increases with dilution rate (Fig. 7), indicating that the effects of high light are most pronounced in the more dilute, less shaded cultures at high dilution rate. It is not clear why the mutant culture did not respond more significantly to the five-fold increase in I_0 in terms of biomass productivity, particularly in dilute semi-continuous culture. It is possible that the mutant is photo-inhibited at high light intensities, which may also explain its greater susceptibility to washout relative to the wild type (see Fig. 6a and b). However, no signs of photo-inhibition were observed during the *P–I* measurements. It is possible that the duration of the *P–I* measurement (i.e., 1–2 min) was too short to result in significant photo-inhibition.

Biomass Productivities in Outdoor Pond Cultures

Five mutant strains of *Cyclotella* were grown in the laboratory to produce the inoculum for the outdoor ponds. Pond cultures of the wild-type strains were operated in parallel to the cultures of mutants. Here we present only data for the selected mutant CM1-1 which was derived from CM1 by further mutagenesis (see above). As a result, CM1-1 lost most of the brown fucoxanthin pigment, so it appears green. In addition, this mutant also contained less chlorophyll.

As shown in Fig. 8, the wild type achieved an average biomass productivity of about 12 g/m^2 day, while the CM1-1 mutant could barely sustain a biomass productivity of 9 g/m^2 day. Thus, at best, the CM1-1 mutant was no better than the wild type at using light incident at high intensity despite lower pigmentation. The mutations may have caused not only a decrease in pigmentation in harvesting (antenna) pigments, but also may have reduced the number of photosynthetic reaction centers. We anticipated that CM1-1 would perform better since it exhibited a higher irradiance for the onset of light saturation of photosynthesis (I_s). But this was not the case. It is possible that the mutation process affected other sites which somehow limited net photosynthetic production.

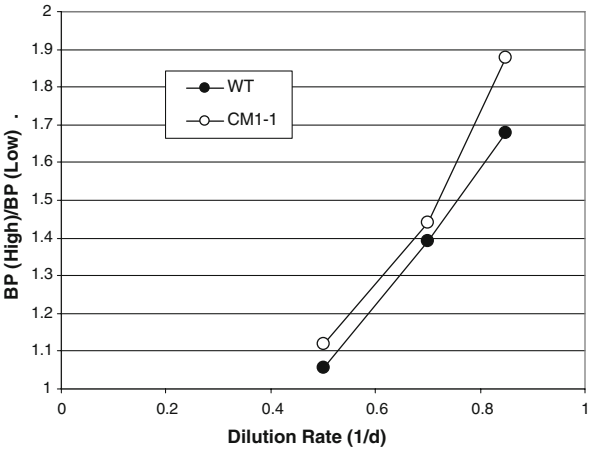

Fig. 7 Ratio of high light to low light biomass productivity for the wild type and CM1-1 mutant in semi-continuous culture as a function of dilution rate

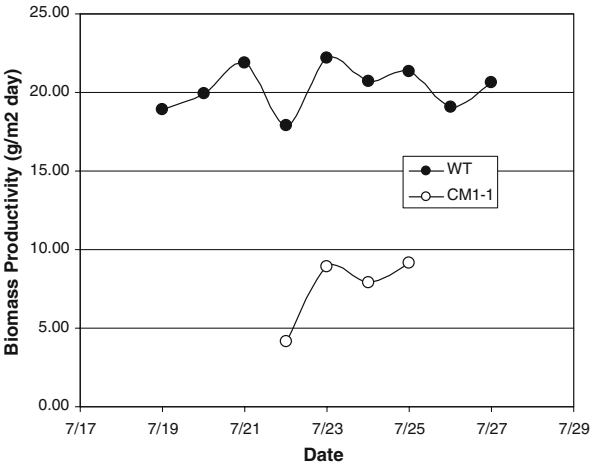

Fig. 8 Biomass productivities of the wild type and the CM1-1 mutant as a function of time in N-sufficient semi-continuous outdoor pond cultures operated at a daily dilution rate of 38% (D=0.38 day^{-1})

Whatever the reason, the consistent result was that none of the mutants was more productive than the wild type.

There are two other measurements which help to determine whether the mutants were generally competent or deficient in some unknown way. The first is the maximum specific growth rate attained (minimum doubling time) at low cell density, when light is totally saturating the photosynthetic systems. Table 3 shows the maximum specific growth rates for the wild type and mutants CM1 and CM1-1. While the minimum doubling time for the

Table 3 Maximum specific growth rates (i.e., doubling times) for the wild type and mutants CM1 and CM1-1.

Algal strain	Date	Daylight (h)	Sample interval (h)	AFDW start (mg/L)	AFDW finish (mg/L)	Doubling time (h)
WT	7/8	13.8	13.8	10.7	85.4	4.6
WT	7/8	13.8	13.8	11.4	88.1	4.6
WT	7/17	13.7	10.9	3.5	18.4	4.5
WT	8/8	12.5	12.5	7.9	41.0	5.3
Average						4.8
SD						0.3
CM1	3/3	11.7	11.7	22.4	71.4	7.0
CM1	4/10	11.9	11.9	13.5	60.7	5.4
CM1	4/24	12.2	12.8	8.0	30.6	6.6
CM1	5/7	12.6	12.1	36.0	101.4	8.1
CM1	5/13	12.8	12.8	11.3	46.4	6.2
CM1	5/27	13.7	13.7	12.2	48.1	6.9
CM1	7/18	13.7	14.3	13.4	68.0	6.1
Average						6.6
SD						0.8
CM1-1	7/22	12.7	12.7	19.0	42.7	10.3
CM1-1	7/29	12.7	11.0	4.3	10.9	8.2
Average						9.3
SD						1.5

WT Wild type

wild type was about 5 h, it was substantially longer for the mutants: about 30% longer for CM1 and 80% longer for CM1-1. In addition, most of the cultures of mutants exhibited a prolonged lag time after inoculation (data not shown). This can bias, or even obscure the measurement of growth rate, yielding artificially long doubling times. Although the mutations were not disabling cell growth, as evidenced by the biomass productivities achieved, long lag times and slow maximal growth rates indicate that the mutation process affected more than just light-harvesting pigment content.

The second measure of culture robustness is the length of time that cultures can be maintained. Wild-type cultures were maintained for weeks to months. They usually crashed only when blooms of predators or competitors, originating from the wall growth, occurred, or when addition of too much Si caused clumps to form. On the other hand, the mutant cultures could never be maintained in good condition for more than 7 to 10 days. While the wild type achieved a maximum biomass concentration of nearly 1,000 mg/L in batch culture, the CM1-1 mutant did not exceed 140 mg/L, which is very low. In addition, cultures involving the CM1-1 mutant either collapsed completely or became contaminated with other diatoms including the wild type. The cell surfaces became "dirty", the cells began to flocculate, and finally the culture crashed, i.e., it experienced a fast decline in biomass density within a few days. It may be possible that the decrease in pigment content along with a reduction in light energy processing centers rendered the mutant more susceptible to photo-inhibition.

In conclusion, antenna mutants of the diatom *Cyclotella* were generated that could be successfully cultured in laboratory bioreactors and outdoor ponds. However, despite reduced light-harvesting antenna content and increased saturating light intensity, the CM1-1 mutant exhibited lowed biomass productivities, both in laboratory and outdoor pond cultures, than the wild type. Given that the CM1-1 mutant was not completely characterized in these preliminary studies, the exact reasons for its poor performance are not known. Thus, future research is needed to create mutants, either by traditional chemical/UV mutagenesis or by genetic engineering techniques, which have specific mutations resulting only in truncated chlorophyll antennae without adversely affecting their functioning in terms of biomass growth and productivities.

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