A comparison of photosynthesis measurements by O$_2$ evolution, $^{14}$C assimilation, and variable chlorophyll fluorescence during light acclimatization of the diatom *Coscinodiscus granii*

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Photosynthetic rates of the large centric diatom *Coscinodiscus granii* were measured by means of multicolor variable chlorophyll fluorescence imaging, single cell $^{14}$C assimilation, and optical O$_2$ sensor measurements during light acclimatization of cultures grown at five different irradiances: 50, 150, 235, 332, and 450 µmol photons m$^{-2}$ s$^{-1}$. Photo-acclimatization was evident from changes of cellular chlorophyll a content, growth rates, and light response curves. Each of the applied methods evaluates different parts and reactions in the photosynthetic apparatus, which makes a direct quantitative comparison of rates difficult, although a different degree of correlation were found between all three methods. However, when used in combination, they provide information about the internal relationship of photosynthetic pathways as well as the variation in photosynthetic capacity between individual cells within a single algal culture.

**Key Words:** $^{14}$C incorporation; diatoms; O$_2$ evolution; photo-acclimatization; photosynthesis; variable Chl a fluorescence imaging

**INTRODUCTION**

Aquatic photosynthesis is typically monitored as oxygen production determined by titration or with electro-chemical or optical O$_2$ sensors, uptake of dissolved inorganic carbon (DIC) with infra-red gas analyzers (IRGA), inorganic carbon fixation using radioactive or stable isotopes, or via variable chlorophyll fluorescence methods. All these methods have limitations and drawbacks, including assumptions on parameters in their calculation on photosynthetic activity (Falkowski and Raven 2007). The three most commonly applied techniques today are 1) inorganic $^{14}$C radiotracer fixation (Steemann Nielsen 1952), 2) O$_2$ evolution (Qlark 1956, Pomeroy 1959, Delieu and Walker 1972), and 3) variable chlorophyll fluorescence (Papageorgiou and Govindjee 2004).

The $^{14}$C assimilation method for quantifying the photosynthetic inorganic carbon (DIC) fixation of phytoplankton was invented by Steemann Nielsen (1952). The method is based on the assumption that the uptake of added $^{14}$C-labeled DIC to the seawater is proportional to the uptake of unlabeled $^{12}$C DIC, when incubating a water sample in a closed container. Thus, knowing the initial DIC of a sample, the precise amount of $^{14}$C-labeled DIC added, as well as the amount of $^{14}$C incorporated in particulate organic matter during a known incubation-time,

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one can then calculate the rate of inorganic C fixation. As $^{14}$C is fixed by photosynthesis, some is also released again through respiration. Since the $^{14}$C assimilation method does not allow for simultaneous determination of respiration rates, it should thus be taken into account that only an intermediate value between gross and net photosynthesis can be obtained (Ryther 1955). Accordingly, the longer the incubation time, the higher the risk of underestimating gross $^{14}$C uptake, and potentially the same $^{14}$C molecule will pass through the cell several times. One should also be aware that heterotrophic respiration is also included in the measurements (Steemann Nielsen and Jensen 1957). This can be addressed by concentrating the phytoplankton biomass or increasing the amount of $^{14}$C added, as is done in the single cell version of the technique, and thus reducing the incubation time by increasing the phototrophic activity per volume of sample (Rivkin and Seliger 1981, Duarte and Ferreira 1997). One should also keep in mind the enclosed conditions in the flasks, “bottle effect,” which can lead to buildup of oxygen gradients in cases of low water movement or lack of stirring.

The O$_2$ evolution method quantifies the net consumption / production of O$_2$ in an incubated water sample over time. This can be done e.g., by simple Winkler titration (Strickland and Parsons 1972, Winkler 1888, Helm et al. 2009) of water samples taken at the beginning and end of incubation, or by following the change in O$_2$ in the sample with an electrochemical or optical O$_2$ sensor (Colman et al. 1998, Kühl 2005, Gloag et al. 2007, Woelfel et al. 2009). In recent years, especially optical O$_2$ measurements have been applied for quantifying photosynthetic oxygen production in closed incubation containers equipped with a patch of an O$_2$ sensitive fluorochrome on the inside, which can be monitored optically from outside; this alleviates the requirement for insertion of a probe into the incubation chamber (Warkentin et al. 2007, Woelfel et al. 2009, Cooper et al. 2011). There are several advantages of using optical O$_2$ sensors: no O$_2$ is consumed during measurements, and there is thus no stirring artifact in the measurements (Kühl 2005); they can also have a short response time, which makes them useful in experiments involving flow-through-chambers or other rapid changing conditions. However, one again needs to be aware of the potential “bottle effect”, as with the $^{14}$C assimilation method, in cases of low water flow or insufficient stirring. Another thing to keep in mind, especially during longer lasting experiments, is growth or sedimentation on the sensor surface. This can potentially lower their otherwise quick response time, or give false results. The O$_2$ evolution method measures net O$_2$ production or consumption in the incubation chamber and does not allow for measuring the respiration rate in the light, which is often assumed identical to the respiration in dark incubated samples. However, during measurements of a light response curve, light-dark shift measurements can be performed, thus measuring the net O$_2$ production rate in light, as well as the post-illumination respiration rate in darkness. From such measurements, it is possible to estimate the gross photosynthetic rate (Beardall et al. 1994, Raven and Beardall 2003, Cooper et al. 2011, Kliphuis et al. 2011). Another potential limitation occurs when working with non-axenic cultures / samples, where it is difficult to distinguish between bacterial and phototrophic respiration.

Variable chlorophyll fluorescence measurements of photosynthetic activity are based on monitoring the fate of absorbed light energy trapped by the photosynthetic apparatus. Absorbed light energy can be dissipated through three pathways: emission of fluorescence from the photopigments, heat dissipation (non-photochemical quenching), and photochemical quenching driving the photosynthetic electron transport chain between PSII and PSI, which is linked to photosynthetic activity. Any change in the latter two dissipation mechanisms will affect the fluorescence emission causing a variable fluorescence yield, especially from PSII. Several techniques have been developed to assess the state of the photosynthetic apparatus by means of variable fluorescence (Papageorgiou and Govindjee 2004, Kromkamp et al. 2008, Suggett et al. 2011), two of which are often used in aquatic systems: 1) The so-called saturation pulse method (Schreiber 2004), which is based on the use of pulse-amplitude modulated (PAM) fluorometry that employ multiple turnover flashes of weak non-actinic measuring light, to assess the status of PSII from the changes in chlorophyll fluorescence yield, $F$, between dark or ambient light conditions, and during a strong saturating light pulse; 2) fast repetition rate fluorometry (e.g., Kolber et al. 1998), uses single turnover flashes at increasing excitation energies to saturate PSII, while monitoring $F$. Both types of variable chlorophyll analysis quantify the quantum efficiency of PSII. By measuring PSII quantum yields at defined levels of photosynthetic active radiation (PAR), the relative electron transport rate (rETR) can be calculated and used to produce so-called light response curves (Schreiber 2004, Trampe et al. 2011). The variable chlorophyll fluorescence method is commonly used due to the versatility of their measuring formats. It is non-invasive and thus allows for repeated measures of the same sample, and it also allows for quick assessment of a range of photo-
synthetic parameters. The novel multicolor imaging PAM microscope system (see a detailed description in Trampe et al. 2011), allows for assessment of the photosynthetic capacities between single differently pigmented cells and even chloroplasts, and thus enables spatio-temporal analysis of photosynthetic capabilities and processes that are not averaged over a bulk water volume. However, despite the simplicity of measurement, variable chlorophyll fluorescence of PSII can be affected by many different processes in the photosynthetic apparatus (Shinkarev 2004), and it is not trivial to extrapolate from rETR rates to absolute measurements of photosynthesis in terms of unit O$_2$ produced or unit C fixed per time. It is thus often necessary to investigate correlations between rETR and O$_2$ or $^{14}$C-based measures of photosynthesis (e.g., Glud et al. 2002). In some systems, absolute values of photosynthetic ETR can be obtained by including measurements of the absorption cross section of PSII (e.g., Hancke et al. 2008), though this is difficult to measure with variable fluorescence measuring systems and often needs additional spectrometric methods to be employed. Fluorescence measurements can also be prone to optical artifacts in dense cultures and in surface-associated phototrophic communities and plant tissue, e.g., affecting the depth reached in a sample by the measuring and actinic light (Oxborough 2004, Nielsen and Nielsen 2008).

Each of the above-mentioned methods evaluates different parts of the photosynthetic pathway, thus evaluations of photosynthetic capacity are established from different physiological responses. Consequently, each method displays a distinctive reaction to environmental factors, such as light and temperature, (Dubinsky 1980, Vavilin et al. 1994, Geel et al. 1997, Flameling and Kromkamp 1998, Morris and Kromkamp 2003). Many other studies have compared methods for measuring photosynthetic activity. Longstaff et al. (2002) showed deviation at higher irradiances between measurements of O$_2$ evolution and ETR measured on macro algae. Demmig and Björkman (1987) found a strong correlation between O$_2$ evolution and variable chlorophyll fluorescence measurements in leaves of terrestrial plants, and Genty et al. (1989) found a direct proportional relationship between CO$_2$ assimilation and variable chlorophyll fluorescence measurements in terrestrial plants. However, only few studies to our knowledge have been done comparing all three methodologies commonly used in aquatic photosynthesis studies. Glud et al. (2002) found a reasonable linear correlation between O$_2$ exchange measurements and $^{14}$C incubations measuring on ice algal communities, but their measurements of variable chlorophyll fluorescence showed non-linear relations at higher algal density. Hancke et al. (2008) found good correlations between the methods; O$_2$ production, PAM, and $^{14}$C assimilation, according to relative responses to the effect of temperature on microalgae. Studies focusing on variation of photosynthetic performance between single cells in differently acclimated cultures are also very limited, however, see e.g., Gorbunov et al. (1999), Snel and Dassen (2000), and Ralph et al. (2005) for some examples.

The aim of this study was to investigate the correlation between the three most commonly applied techniques assessing photosynthetic performance, $^{14}$C incorporation, O$_2$ evolution, and variable chlorophyll fluorescence measurements. To evaluate how the different photosynthesis measures correlate, we set up a light acclimatization experiment, exposing subcultures of the centric marine diatom _Coscinodiscus granii_ to five different irradiances: 50, 150, 235, 332, and 450 $\mu$mol photons m$^{-2}$ s$^{-1}$ for a period of three months, resulting in five differently acclimatized cultures. The photosynthetic activity of _C. granii_ was assessed in bulk samples using $^{14}$C fixation and O$_2$ production methodology, and at the single cell level with microscopic variable chlorophyll fluorescence imaging measurements on the same cultures. Furthermore, the experiment provided detailed information on changes in photosynthetic capacity, as well as physiological changes during light acclimation of _C. granii_.

**MATERIALS AND METHODS**

The complete experiment was repeated twice, i.e., there are two sub experiments, Exp. 1 and 2, for comparing the different methods. Exp. 1 was performed after a short acclimatization period of one week, while Exp. 2 commenced after an acclimatization period of five weeks. Due to contamination of a $^{14}$C stock solution in Exp. 1, comparison of all three methods has been limited to Exp. 2.

**Algal culture and growth conditions**

Cultures of _Coscinodiscus granii_ (strain CCMP1817) were grown at 17°C as a semi-continuous culture in Si-enriched f/2 medium (Guillard and Ryther 1962) adjusted to a salinity of 29 and pH 8. The culture was growing for 4 months prior to the experiment under a photon irradiance of ~25 $\mu$mol photons m$^{-2}$ s$^{-1}$ provided by a white fluorescent light source (PHILIPS TLD 58W/827 tube; Philips, Guildford, Surrey, UK). Every other week, 2.5 mL...
of culture was transferred to a clean 60 mL filter cap culture flask (tissue culture flask, TPP 02AG; TPP Trasadingen, Switzerland), containing 35 mL medium filtered through a 0.2 µm sterile syringe filter (Minisart NML; Sartorius AG, Göttingen, Germany). At the beginning of each semi-continuous cycle, the culture was topped up by replacing harvested sample with new media, enhancing nutrient levels, and thus providing better growth conditions until growth leveled off under increasing nutrient limitation and / or cell density. The experimental growth setup consisted of a custom built light table divided into five compartments coated with light reflecting aluminum foil, each with room for triplicate culture flasks (Appendix 1). Three white fluorescent light sources (PHILIPS TLD 38W/830 tube; Philips) were placed above the compartments. Each compartment was covered with a combination of neutral density filter foil (Lee Filters; Panavision Inc., Woodland Hills, Los Angeles, USA), creating five different photon irradiances: 50, 150, 235, 332, and 450 µmol photons m⁻² s⁻¹, as measured inside a water filled culture flask with a calibrated miniature spherical quantum sensor (US-SQS/A; Walz GmbH, Effeltrich, Germany) connected to a quantum irradiance meter (LI-250A; Li-Cor Inc., Lincoln, NE, USA), creating five different photon irradiances: 50, 150, 235, 332, and 450 µmol photons m⁻² s⁻¹, as measured inside a water filled culture flask with a calibrated miniature spherical quantum sensor (US-SQS/A; Walz GmbH, Effeltrich, Germany) connected to a quantum irradiance meter (LI-250A; Li-Cor Inc., Lincoln, NE, USA). Both during the acclimation period and experiments, the cultures were kept under a light:dark cycle of 16 : 8 hours in a temperature controlled room set to 17°C. To avoid prolonged settling of cells, the culture flasks were carefully shaken (with a 2 day interval) until all cells were resuspended.

Measurements

Subsamples of the *C. granii* culture, from each triplicate within each light environment, were analyzed for Chl a content and had their photosynthetic capacity evaluated by means of photosynthesis light response curves measured with variable chlorophyll fluorescence, O₂ evolution, and ¹⁴C-assimilation.

**Cell abundance.** All cells were gently resuspended in the culture flask by shaking, thereafter 1 mL subsample was transferred with a pipette to a 1 mL Sedgewick Rafter Counting Cell Slide (Graticules Ltd., Tonbridge, UK), placed on a compound microscope where cells were counted at 100× magnification. Each count was based on at least 400 cells, and cell counting was done in triplicate at all time points. The average cell count at each time point was used in the calculation of chlorophyll-specific photosynthetic parameters as described below. To estimate cells mL⁻¹ at specific measuring days, we fitted a polynomial regression line to cell counts in cells mL⁻¹ d⁻¹. The growth rate (µ), was calculated from the change in cell numbers over time (d) as:

\[ \mu (d^{-1}) = \ln \left( \frac{N_t}{N_0} \right) r^{-1} \]  

where \( N_0 \) is the concentration of cells at time \( t_0 \) (cells mL⁻¹), \( N_1 \) is the concentration of cells at time \( t_1 \) (cells mL⁻¹), and \( t \) (d) is the time between \( t_0 \) and \( t_1 \).

**Chlorophyll a (Chl a) measurements**

Ten milliliter subsamples of each culture were filtered through a Whatmann GF/C filter by means of a vacuum pump, and the filters were transferred with a forceps to glass vials containing 10 mL ethanol (96%). The vials were then stored for 8 hours in darkness at 4°C for pigment extraction. After the extraction time, the vials were shaken and placed at room temperature for 1 h, and were then shaken again and spun down for 10 min at 4,000 rpm. The supernatant containing Chl a was transferred with a pipette to a cuvette. A spectrophotometer (UV-2101 PC; Shimadzu Corp., Tokyo, Japan) was used to measure the absorbance at 665 nm (in vitro Chl a absorbance maxima) and at 750 nm (background absorbance) for all samples. Reference measurements at the same wavelengths were done with clean ethanol. The Chl a concentrations (µg L⁻¹) were determined following the Lorenzen method (Strickland and Parsons 1972).

**Variable chlorophyll fluorescence measurements**

The photosynthetic capacity of individual cells in the culture samples was estimated as the reTR using variable chlorophyll fluorescence measurements with a new multicolor PAM microscope imaging system (RGB-Imaging-PAM; Walz GmbH) (Trampe et al. 2011) controlled via a PC and the ImagingWin software (V2.32; Walz GmbH). The system was mounted on an epifluorescence microscope (Axioskop Plus; Carl Zeiss MicroImaging GmbH, Jena, Germany), equipped with a 20× objective (Zeiss Plan-Apochromat NA 0.8; Carl Zeiss MicroImaging GmbH). We used blue light (470 nm) for both measuring and actinic light during the measurements. The RGB-Imaging-PAM system and its application for single cell / chloroplast photosynthesis studies is described in detail elsewhere (Trampe et al. 2011).

Prior to measurements, subsamples were transferred with a pipette from culture flasks onto a microscope slide with a -0.2 mm deep well, mounted with a cover slip, and placed on the microscope in a custom-built tem-
perature controlled slide holder, set to 17°C (Trampe et al. 2011). After getting 4-12 cells in focus, using the halogen light-source build into the microscope covered by a 715-nm long-pass glass filter (RG715; Schott GmbH, Mainz, Germany), to avoid actinic effects on the sample during focusing, the sample was allowed to dark-adapt for 15 min before further measurements. The pulse-modulated measuring light was sufficiently weak (<0.5 μmol photons m⁻² s⁻¹) and therefore considered non-actinic during assessment of the minimal fluorescence yield, $F_0$, of the dark-adapted sample. Using the saturation-pulse-meth-od (Schreiber 2004), images of the maximum quantum yield of dark-adapted cells, Φₘᵠ = ($F'_m - F_0$) / $F'_m$, and the effective quantum yield of PSII, $\Phi_{psii} = (F'_m - F) / F'_m$, under defined irradiance levels of actinic light (PAR). This information was obtained during rapid light curves (RLC’s), where a series of measurements are performed at steps of increasing irradiance with an incubation time of 10 s at each irradiance level. Relative rates of PSII-driven electron transport were then calculated as:

$$\text{rETR} = \Phi_{psii} \times \text{PAR} \times K$$  \hspace{1cm} (2)

where K is a constant representing the distribution of light energy between PSI and PSII; here we assume $K = 0.5$, i.e., an equal distribution of absorbed light between the two photosystems.

RLC data from individual cells could then be analyzed, by defining areas of interest encircling whole C. granii cells in the Imaging-Win software, thus the variable chlorophyll fluorescence parameters were averaged for single cells (Fig. 1).

In order to determine sufficient dark adaptation peri-
ods for the I-PAM measurements, PSII quantum yield vs. irradiance curves were for all samples followed by time curves measuring the recovery of PSII quantum yield after return to darkness (Appendix 2). These measurements were performed with a small portable variable chloro-
phyll fluorescence analyzer (Junior PAM; Walz GmbH) connected to a PC running WinControl-3 Software (Walz GmbH). For measurements, measuring / actinic blue light at 450 nm was used to estimate $\Phi_{psii}$ directly through the culture flasks via an optical plastic fiber light guide. Analyzing these data, a dark adaptation time of 15 min was chosen for the above-mentioned RGB-imaging-PAM measurements.

### O₂ evolution measurements

All O₂ measurements were carried out as light response curves, using a multi-channel fiber-optic O₂ meter (OXY-4 mini; Presens GmbH, Regensburg, Germany) con-
nected to measuring chambers with three polymer optical fibers and controlled by a PC running OXY-4v2_11 software (Presens GmbH). This setup facilitated parallel measurements of the dissolved O$_2$ concentration (DO) in 3 chambers. The measurement of DO is based on the dynamic collisional quenching of a luminescent optical indicator by O$_2$ as described in detail elsewhere (Lakowicz and Masters 2008).

The O$_2$ concentration in algal subsamples was monitored in small glass vials coated with an optical O$_2$ sensor spot on the inside. To ensure durable attachment of the indicator spot, the vials were washed in dry acetone, placed in a glass tray, and dried in an oven at 110°C for 12 h. Subsequently, the glass surface in the vials was covered with a 7.5% solution of dimethyldichlorosilane (synthesis grade; Merck, Darmstadt, Germany) in toluene for two hours, then washed twice in toluene and once in ethanol (96%). The O$_2$ indicator material consisted of a solution of 75 mg Pt(II) meso-tetra(pentafluorophenyl)porphine and 4 g of polystyrene (Goodfellow Cambridge Ltd., Huntingdon, UK) dissolved in 40 mL of 1,1,2-trichloroethane (96% stabilized with 2-propanol; Sigma-Aldrich, St. Louis, MO, USA), filtered and diluted to 60 mL. The polymer-dye solution was applied onto the inside of the vials with a glass pipette, carefully positioning a thin drop in the center of the bottom, followed by drying at room temperature and a relatively high humidity (>80%) in order to prevent crack formation. This procedure resulted in vials with an even thin circular layer of indicator dye at their bottom.

The vials were filled with culture sample to capacity and carefully closed with airtight lids avoiding formation of gas bubbles. Two glass beads, Ø1 mm, kept sufficient mixture in the sample during the shaking movements without damaging the delicate silica frustules enclosing the diatom cells. To prevent cell clumping and the development of O$_2$ gradients, three 2 mL glass vials containing subsamples of culture were attached to the 3 fiber-optic light guides in a custom build shaking apparatus based on a commercial whirley mixer. For all cells to experience identical light conditions, the shaker was wrapped in aluminum foil to reflect light back through the vials. A fiber-optic halogen lamp equipped with a collimating lens (KL 2500 LCD; Schott) was used as actinic light source. For all cells to experience identical light conditions, the shaker was wrapped in aluminum foil to reflect light back through the vials. A fiber-optic halogen lamp equipped with a collimating lens (KL 2500 LCD; Schott) was used as actinic light source. A fiber-optic halogen lamp equipped with a collimating lens (KL 2500 LCD; Schott) was used as actinic light source. A fiber-optic halogen lamp equipped with a collimating lens (KL 2500 LCD; Schott) was used as actinic light source. A fiber-optic halogen lamp equipped with a collimating lens (KL 2500 LCD; Schott) was used as actinic light source.

Photosynthetic carbon fixation of C. granii was measured using a slightly modified version of a previously described protocol (Skovgaard et al. 2000). For this, 20 μL of a NaH$^{14}$CO$_3$ stock solution (specific activity = 100 μCi mL$^{-1}$; DHI, Hørsholm, Denmark) was added to glass scintillation vials containing 2 mL subsample, with ~4500 cells mL$^{-1}$; this was done at two time points from each replicate, and for all experimental irradiances. For incubation, a custom built light table was used to determine photosynthesis vs. irradiance curves. The light table consisted of three white fluorescent light sources (PHILIPS TLD 38W/830 tube; Philips) positioned under 6 glass frames positioned at different distances above the light source (Appendix 4), to make 6 incubation points of increasing photon irradiance (50, 75, 96, 150, 195, and 321 μmol photons m$^{-2}$ s$^{-1}$), as measured inside a water filled scintillation vial with a miniature spherical quantum sensor (US-SQS/A; Walz GmbH) connected to a quantum irradiance meter (LI-250A; Li-Cor Inc.) was placed inside a water filled glass vial, and positioned as a sample in the setup (Appendix 3).

Prior to every measurement, a two-point calibration of the O$_2$ sensors was carried out. A zero O$_2$ measurement was obtained with a solution of 1 g sodium sulfite (Na$_2$SO$_4$) dissolved in 100 mL of water, and a measurement at 100% atmospheric saturation was obtained with air saturated water. The O$_2$ concentration in air saturated water at known salinity and temperature was calculated according to (Weiss 1970). Triplicate cultures from one light condition were assessed at a time. Samples were first allowed to dark-adapt for 15 min. Then light / dark response curves were carried out with 20 min cycles at increasing irradiances (53, 154, 245, 277, and 450 μmol photons m$^{-2}$ s$^{-1}$).

Photosynthesis / respiration rates were calculated from the linear change in O$_2$ concentration (% air saturation) over time. Further calculations deducting absolute values of DO as O$_2$ in mg L$^{-1}$ were performed according to Weiss 1970, by including cell counts and the molecular weight of O$_2$. The O$_2$ evolution rate was quantified in units of nmol O$_2$ cell$^{-1}$ h$^{-1}$. By adding the dark respiration measured immediately after each irradiance incubation to the net O$_2$ production, the gross photosynthetic rate ($P_{o2}$) could be expressed vs. irradiance (Beardall et al 1994, Raven and Beardall 2003, Cooper et al. 2011, Kliphuis et al. 2011).

$^{14}$C-assimilation

Photosynthetic carbon fixation of C. granii was measured using a slightly modified version of a previously described protocol (Skovgaard et al. 2000). For this, 20 μL of a NaH$^{14}$CO$_3$ stock solution (specific activity = 100 μCi mL$^{-1}$; DHI, Hørsholm, Denmark) was added to glass scintillation vials containing 2 mL subsample, with ~4500 cells mL$^{-1}$; this was done at two time points from each replicate, and for all experimental irradiances. For incubation, a custom built light table was used to determine photosynthesis vs. irradiance curves. The light table consisted of three white fluorescent light sources (PHILIPS TLD 38W/830 tube; Philips) positioned under 6 glass frames positioned at different distances above the light source (Appendix 4), to make 6 incubation points of increasing photon irradiance (50, 75, 96, 150, 195, and 321 μmol photons m$^{-2}$ s$^{-1}$), as measured inside a water filled scintillation vial with a miniature spherical quantum sensor (US-SQS/A; Walz GmbH) connected to a quantum irradiance meter (LI-250A; Li-Cor Inc.). Each glass frame fitted three vials, i.e., triplicate determination, and these were always accompanied by parallel dark incubated vi-
als wrapped in aluminum foil. The setup was placed in a temperature controlled laboratory at 17°C. After 4 h and 40 min of incubation, the specific activity was determined by transferring 100 µL from each vial to new vials containing 200 µL phenyl ethylamine. Then 2 mL of 10% glacial acetic acid in methanol was added to the remaining 1.9 mL sample to remove all dissolved inorganic C. The vials were dried in a heating block set to 60°C for 12 h. The residues within all vials, including those for specific activity determination, were re-suspended in 2 mL of distilled water and 10 mL of Insta-Gel Plus scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA). Activity was measured using a liquid scintillation counter (Tri-Carb; Packard Instrument Co., Inc., Downers Grove, IL, USA). Calculations of photosynthetic rates were done according to (Parsons et al. 1984):

\[
PA = \frac{DPM \times [DIC]_{added} \times h \times cells}{h_{added}}
\]

, where DPM is the measured disintegration rate (disintegrations min\(^{-1}\) mL\(^{-1}\)), DIC is the concentration of inorganic carbon (µg C mL\(^{-1}\)), \([^{14}\text{C}]_{added}\) is the specific activity in disintegrations min\(^{-1}\) mL\(^{-1}\), and \(h\) is the incubation time in hours, and cells is the number of cells in the vials. Prior to the irradiance experiments, a first preliminary experiment was performed using the techniques described above to proof the linearity of \([^{14}\text{C}]\) uptake of \(C.\) granii by incubating triplicate vials for 1, 2, 3, 4, 5, and 6 h. The \([^{14}\text{C}]\) uptake was linear for at least the first six hours of incubation \((r^2 = 0.98)\) (Appendix 5); an incubation time of ~5 h was selected for the light response experiments. This allowed measurements of low uptake rates at low irradiances.

DIC in all samples was measured in triplicates by adding 50 µL HgCl\(_2\) to 1.5 mL glass flasks filled with sample and sealed with a serum stopper in the lid. All samples were then analyzed for DIC concentration using an IRGA (225-Mk3; Analytical Development Corporation, Hoddesdon, UK). Using a bicarbonate standard for comparison, triplicate measurements were performed for each incubated sample. For each triplicate, three IRGA measurements were made with three measurements of a bicarbonate-standard before and three after.

Correlations

In order to evaluate the relationship between the three methods used in this study, rates of photosynthesis vs. irradiance for variable chlorophyll fluorescence-, \([^{14}\text{C}]\)-assimilation- and optical O\(_2\)-sensor-measurements were plotted as scatterplots against each other with simple linear regression to determine the strength and significance of correlation, as quantified by the correlation coefficient \((r^2)\). When the difference in irradiance were less than 5% between two methods they were used as equal values for correlation between their corresponding values of photosynthetic rates.

RESULTS

Growth rates

Semi-continuous cultures of \(C.\) granii were grown for a period of 100 days at five different irradiances. For both experiments the growth rate was highest early in a cycle, with slower growth late in the exponential growth phase (Fig. 2). The growth rate of the cultures increased during acclimatization between Exp. 1 and 2 (Fig. 2), as well as

![Fig. 2](image-url)
Optical O₂ sensor measurements

In Exp. 1 and 2, gross photosynthetic rates, \( P_{O2} \) (in units of nmol O₂ cell⁻¹ h⁻¹) (Fig. 4A & B), and normalized to Chl a, \( P^*_{O2} \) (in units of nmol O₂ [µg Chl a]⁻¹ h⁻¹) (Fig. 4C & D) were calculated from the O₂ evolution experiments under different irradiances. All cultures in Exp. 1 exhibited a relatively uniform response to increasing irradiance, though \( P^*_{O2} \) data indicated that culture #5 had a distinctive higher apparent \( P_{max} \). In Exp. 2, the O₂ production showed more variation in the response of the different cultures to increasing irradiance. The apparent maximum photosynthetic rate \( P_{max} \) for culture #1, growing at 50 µmol photons m⁻² s⁻¹ changed from ~0.27 to ~1.0 nmol O₂ cell⁻¹ h⁻¹ between the two experiments, going from the lowest apparent \( P_{max} \) to the highest between all cultures. Culture #5 showed the opposite trend going from the highest apparent \( P_{max} \) to the lowest, albeit with a less dramatic change. Culture #2 changed its apparent \( P_{max} \) from ~0.4 to ~0.6 nmol O₂ cell⁻¹ h⁻¹ from Exp. 1 to Exp. 2, while the other cultures stayed more or less at the same production level in both experiments.

Variable chlorophyll fluorescence measurements

Light response curves displaying relative values of photosystem II electron transport rates (rETR) as a function of irradiance, were measured, (Fig. 5A & B) and normalized to the amount of culture specific Chl a cell⁻¹ (Fig. 5C & D). The rETR vs. irradiance curves showed, like the O₂ measurements, a shift from relatively uniform rETR vs. irradiance curves in Exp. 1, towards more distinct rETR vs. irradiance curves between the individual cultures in Exp. 2. In contrast, the Chl a-normalized rETR vs. irradiance curves showed a higher variability between cultures in Exp. 1, with culture #3 and #4 having a higher maximum rETR (rETR_max). Culture #1 shifted from having the lowest rETR_max in Exp. 1 to the highest in Exp. 2, and exhibited quite a profound deviation from the rest of the cultures.

Table 1. Percentages of changes in Chl a content of Coscinodiscus granii (pg Chl a cell⁻¹) between Exp. 1 and 2

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. granii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Chl a change (%)</td>
<td>-9.1</td>
<td>-24.9</td>
<td>-46.0</td>
<td>-23.9</td>
<td>14.6</td>
</tr>
<tr>
<td>±1 S.E. (n = 3)</td>
<td>1.7</td>
<td>4.0</td>
<td>1.0</td>
<td>4.1</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Cultures #1-5 indicate cultures experiencing growth at an irradiance of 50, 150, 235, 332, and 450 µmol photons m⁻² s⁻¹, respectively.
**Fig. 4.** Photosynthesis vs. irradiance curves for cultures #1-5 as measured with oxygen optical O$_2$ sensors. (A & B) Gross photosynthetic rates vs. irradiance, in Exp. 1 and 2, respectively. (C & D) Chl $\alpha$-normalized rates of gross photosynthesis ($P^*$) vs. irradiance, in Exp. 1 and 2, respectively. Error bars indicate ±1 S.E. (n = 3).

**Fig. 5.** Photosynthesis vs. irradiance curves for cultures #1-5 as measured with a multicolor variable chlorophyll fluorescence microscope (RGB-imaging-PAM). (A & B) Relative electron transport rates (rETR) vs. irradiance, in Exp. 1 and 2, respectively. (C & D) Chl $\alpha$-normalized rates of rETR vs. irradiance, in Exp. 1 and 2, respectively. Error bars represent ±1 S.E. (n = 24).
There was an apparent negative relationship between growth irradiance of the cultures and the maximal photosynthetic rate. The $P_{\text{max}}^{14C}$ data showed e.g., that the lower the growth irradiance the higher the apparent $P_{\text{max}}$ except for culture #4 displaying a slightly lower apparent $P_{\text{max}}$ than culture #5.

**Correlations**

Rates of photosynthesis vs. irradiance as determined by variable chlorophyll fluorescence-, $^{14}$C-assimilation- and optical O$_2$-sensor-measurements, respectively, were plotted against each other to determine the strength and significance of linear correlation (Table 2, Fig. 7). We only used rate values obtained at quasi-similar irradiances; five pairs for PAM measurements vs. optical O$_2$-sensor in both Exp. 1 and 2, and $^{14}$C vs. PAM measurements in Exp. 2, four pairs for $^{14}$C-assimilation vs. optical O$_2$-sensor in Exp. 2. Generally, we found a good linear correlation ($r^2 \geq 0.9$) between data obtained with variable chlorophyll fluorescence and O$_2$ methodologies in Exp. 1 and 2, except for culture #1 in Exp. 1, and culture #5 in Exp. 2 (Table 2, Fig. 7), showing a less strong correlation of $r^2 = 0.86$ and $r^2 = 0.78$, respectively. $^{14}$C vs. O$_2$ measurements in Exp. 2 showed more variable correlations, with cultures #1 and #4 showing the strongest correlations of $r^2 = 0.85$ and $r^2 = 0.91$, respectively. In the comparison of $^{14}$C vs. PAM, cultures #1 and #3 showed a strong correlation with $r^2 = 0.87$, where culture #2 has a modest correlation of $r^2 = 0.64$, and culture #4 and 5 showed very strong correlation of $r^2 = 0.92$.

**DISCUSSION**

Cultures of *C. granii* from all experimental light conditions effectively modified their photosynthetic appara-

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**Table 2.** Correlation between three different methods for photosynthesis measurements in *Coscinodiscus granii*

<table>
<thead>
<tr>
<th><em>C. granii</em> culture No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
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<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAM vs. O$_2$</td>
<td>0.86</td>
<td>0.10</td>
<td>0.96</td>
<td>0.95</td>
<td>0.90</td>
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<tr>
<td>Exp. 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAM vs. O$_2$</td>
<td>0.95</td>
<td>0.98</td>
<td>0.90</td>
<td>0.96</td>
<td>0.78</td>
</tr>
<tr>
<td>$^{14}$C vs. O$_2$</td>
<td>0.85</td>
<td>0.53</td>
<td>0.76</td>
<td>0.91</td>
<td>0.61</td>
</tr>
<tr>
<td>$^{14}$C vs. PAM</td>
<td>0.88</td>
<td>0.64</td>
<td>0.87</td>
<td>0.92</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Variable chlorophyll fluorescence measurements (PAM), O$_2$ sensor measurements (O$_2$), and $^{14}$C-assimilation measurements ($^{14}$C), used to measure photosynthesis vs. irradiance, for cultures #1-5 of *C. granii* grown at different light conditions (50, 150, 235, 332, and 450 µmol photons m$^{-2}$ s$^{-1}$, respectively).
Trampe et al. Comparison of Photosynthesis Measurement

During acclimatization, the cells slowly began to up-regulate their photosynthetic apparatus, reaching a balanced light harvesting potential supplying sufficient energy required for optimum carbon fixation and growth rate at the given irradiance, without further photo inhibition. Among cultures #1-5, there was an increase in growth rate, corresponding to the increasing growth irradiance (Fig. 5). However, this change did not correlate with the amount of Chl a (Fig. 6) possibly attributed to internal cellular regulation, which has been shown to vary

tus by altering their Chl a content, as well as their growth rates (Table 1, Figs 2 & 3). The decrease in Chl a cell⁻¹ from the first to the second experiment (Fig. 3), was reflected by an increase in growth rates between the two experiments (Fig. 2). Similar changes were also found in earlier studies of photoacclimation in diatoms, and cyanobacteria (Kana and Glibert 1987, Anning et al. 2000) respectively. This suggests that cell division was prioritized above synthesis of photosynthetic pigments upon the shifting to higher irradiance levels.

Because the initial growth rate between Exp. 1 and 2 was constantly increasing, one could expect that a lower level of Chl a content of the algae was initiated to prevent photo inhibition by decreasing their light harvesting potential; e.g., by down regulating Chl a to avoid over excitation of PSII reaction centers (Post et al. 1985), or by spending energy on increasing their amount of photo protective xanthophylls, to cope with higher irradiances (Anning et al. 2000, Lavaud et al. 2002a). The observed increase in Chl a content in culture #5 may be attributed to a “shock” response, i.e., when going from ~25 to 450 µmol photons m⁻² s⁻¹, a protective response could have induced down regulation of Chl a and promoted synthesis of photo protective pigments.

Fig. 7. Linear regression comparing the three different methods for photosynthesis measurements in Coscinodiscus granii cultures #1-5 from growth conditions of 50, 150, 235, 332, and 450 µmol photons m⁻² s⁻¹, respectively. (A) Exp. 1. (B-D) Exp. 2. (A) Showing variable chlorophyll fluorescence (PAM) vs. O₂ evolution measurements (O₂). (B) PAM vs. O₂ measurements. (C) ¹⁴C-assimilation (¹⁴C) vs. PAM. (D) ¹³C vs. O₂ measurements.
according to irradiance and Chl a : C ratios (Geider et al. 1997), the measurement of which was outside the scope of this study.

Some general patterns emerged from the measurements of photosynthetic rates vs. irradiance, with all three applied methods (Figs 4-6). Photosynthetic rates measured with O₂ optodes, and variable chlorophyll fluorescence imaging, displayed an increasing variation in between the cultures from the first to the second experiment. Unfortunately, we cannot provide any data on this for ¹⁴C-assimilation measurements; however, we do see a significant variation between the cultures for the ¹⁴C-assimilation data from the second experiment, (Fig. 6).

All three methods for quantifying photosynthetic activity showed similar trends in the investigated cultures. However, we did not see a clear-cut trend in the variations between cultures from method to method. Variations between measurements can be attributed to several factors. Measurements were not performed on exactly the same days, and the duration of measurements varied between methods. We used three different methods, targeting three different parts of the photosynthetic apparatus. For example, when measuring photosynthetic capacity from variable chlorophyll fluorescence it correlates to the rate of charge separation in PSII reaction centers; however, this rate is not directly coupled to O₂ evolution or ¹⁴C-assimilation (Jakob et al. 2005), hence estimations will vary between methods. The findings of Lavaud et al. (2002b) showed that in diatoms a cyclic electron transfer path at PSII diminishes the production of O₂, as a protective mechanism against oxidative damage at high irradiances; consequently the O₂ measurements would show a lower photosynthetic capacity at high irradiances than the fluorescence measurements. However, this was not the case in our study comparing cultures exposed to high vs. low irradiances (Figs 3 & 4). The PSII reaction center activity monitored from variable fluorescence can deviate significantly from the measured O₂ production rate (Falkowski et al. 1986, Geel et al. 1997, Gilbert et al. 2000, Figueroa et al. 2003). Low correlation between fluorescence based measurements vs. O₂ measurements of photosynthetic capacity was observed both at low and high light intensities by Jakob et al. (2005). In parallel to the findings of Lavaud et al. (2002b), a CO₂ independent alternative electron transport pathway in the photosynthetic apparatus at high irradiance (Schreiber and Neubauer 1990, Geel et al. 1997) could explain the differences in measures between methods of assessing photosynthetic capacity. This could explain why culture #5 showed the lowest apparent Pₘₐₓ measured from O₂ evolution in the second experiment, while it had the second highest rETR based on variable chlorophyll fluorescence measurements in the same experiment.

Variations in the photosynthetic capacity between cells within each culture (shown as S.E. in Fig. 5) can to some degree be attributed to the internal rearrangement within the cells as described elsewhere (Trampe et al. 2011). Variation between cells according to age has also been shown for the dinoflagellates, Ceratium longipes and C. tripos, Boulding and Platt (1986), who found significantly higher photosynthetic capacity for older cells vs. newly divided ones.

Correlations between different methods

We found very strong linear correlations between O₂ evolution and variable chlorophyll fluorescence methods for all cultures, (Table 2, Fig. 7), except for culture #1 in Exp. 1, and culture #5 in Exp. 2, showing a slight reduction in correlation coefficients, but still with a strong linear relationship between the two methodologies. These results correspond well to the explanations and findings of others as described above.

The linear correlation of the ¹⁴C-assimilation measurements vs. both O₂ evolution and the variable chlorophyll fluorescence measurements was weaker. This could be attributed to the duration of incubation time, which was significantly longer than for the variable chlorophyll fluorescence measurements, and about 25% longer than for O₂ evolution measurements. The O₂ evolution method also involves an interval of darkness before increasing the light intensity during PI measurements, this time allows for partial reduction of the photosynthetic apparatus, whereas the ¹⁴C-assimilation measurements were based on continuous light during the experiment, which could cause oxidative stress at increasing irradiance. The variable chlorophyll fluorescence method has some advantages above both the O₂ evolution and ¹⁴C assimilation methods, due to rapid assessment of photosynthetic capacity. The effect of being incubated for longer periods, allowing for sinking / stacking of cells could be a pronounced source of error, as well as the longer duration at which the cultures were exposed to high irradiances could also alter the potential of photosynthetic capacity. Long incubation times furthermore allow for gradual adjustments of the photosynthetic apparatus during the measurement, i.e. cultures acclimated to low light had more time to acclimate to high light, and cultures adapted to high light had more time to down regulate.

The fact that cells are continuously suspended during
measurements by the O₂ evolution method, as compared to the stationary cells settling / stacking on the bottom of the ¹⁴C incubation vials, could somewhat lessen the significance of incubation effects on these cells. The O₂ evolution method also eliminated the cells’ exposure to high O₂ levels, since every change in irradiance was followed by a dark period. In contrast, the ¹⁴C method requires continuous light on the airtight incubation vials, potentially producing higher O₂ concentrations towards the end of the incubation period, and thus potential oxidative damage. However, such inhibition is not apparent when comparing O₂ production in Fig. 4B and D, where culture #1 is producing significantly more O₂ than the rest of the cultures, with Fig. 6 showing that culture #1 at the same time is having the highest apparent Pₘₐₓ measured by the ¹⁴C assimilation method.

It should be noted, that our correlation analysis only compares whether the shape of the light curve, i.e., the relative response to increasing irradiance, correlates or not between methods. Thus, we do not attempt to obtain correlations between the absolute values of photosynthetic capacity. In order to do so, it would be necessary to normalize to a common unit, e.g., O₂ evolution, C-incorporation, or absolute electron transport rates. However, this would rely on the strength of mathematical models as to predict one component of photosynthesis in relation to others. As described above, this includes many complex, often inaccurate, presumptions, and requires additional optical characterization of the samples, such as e.g., measurements of the absorption cross section of PSII, in order to convert rETR measures to absolute ETR values. Hancke et al. (2008) used the stoichiometric ratio of O₂ evolved per electron generated in PSII, together with a bio-optical measurements of the absorbance of PSII. This enabled calculation of O₂ production in absolute units directly from the measured PSII effective quantum yield. The calculation is based on assumptions of the amount of O₂ evolved per electron generated, and the accuracy in measuring the absorption cross section of PSII, which was assumed to stay constant during measurements. The PSII absorption cross section can, however, vary with irradiance, e.g., during so called state transitions. This involves the detachment and attachment of LHClII and CP29 during high and low irradiances respectively, as caused by enzyme driven phosphorylation and de-phosphorylation, which lowers the actual absorption cross section (Suggett et al. 2011).

Concluding remarks

We present a detailed study of photosynthesis of C. granii acclimated to different light levels. We found good linear correlations between variable chlorophyll fluorescence, photosynthetic ¹⁴C fixation, and O₂ production measurements done at increasing irradiance. Correlations were strongest between O₂ evolution and variable chlorophyll fluorescence measurements, but we also observed modest to very strong correlations to the ¹⁴C assimilation method. However, it is very difficult to quantitatively compare results since each method measures the activity of different reactions in the photosynthetic pathway. Furthermore, typical duration of measurements and treatment of cells differ between methods. The strong correlations do nevertheless indicate that each method performs as a good alternative to each other in evaluating the photosynthetic capacity of planktonic phototrophs. Such insight to the function and state of the photosynthetic apparatus is a good indicator of the general fitness of the phototrophs under investigation, and provides valuable information for most ecophysiology studies. Thus, chlorophyll fluorometry is a valuable tool of assessing photosynthetic performance of marine phototrophs in combination with O₂ evolution and ¹⁴C assimilation measurements. rETR provides a good quantitative measure for assessment of the photosynthetic performance of single cells in a culture, as well as between different cultures. Further testing and alteration of the single cell variable chlorophyll fluorescence method, e.g., by incorporating a way of obtaining the absorption cross section of PSII, could provide valuable estimates close to absolute ETR enabling a more direct comparison of absolute photosynthesis rates between variable chlorophyll fluorescence, C¹⁴ and O₂-based measures of photosynthesis.

ACKNOWLEDGEMENTS

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Appendix 1. Experimental growth setup. Light box, containing triplicate culture flasks, A, B, and C, within each of the five light environments: 1 (50), 2 (150), 3 (235), 4 (332), and 5 (450 µmol photons m⁻² s⁻¹).

Appendix 2. PSII quantum yield measurements as a function of irradiance and as a function of time after return to darkness, showing the recovery of the photosynthetic apparatus to the dark-adapted state. PAR, photosynthetic active radiation.

Appendix 3. Experimental setup for optical O₂ measurements, consisting of a halogen lamp, a multichannel O₂ meter controlled by a PC. The setup allowed triplicate monitoring of sample vials attached to a custom build shaking device and connected to the O₂ meter via 3 optical fibers.

Appendix 4. Schematic drawing of ¹⁴C incubation light table with different irradiance levels 50, 75, 96, 150, 195, and 321 µmol photons m⁻² s⁻¹.

Appendix 5. ¹⁴C uptake of Coscinodiscus granii cells, as a function of incubation time under an irradiance of ~100 µmol photons m⁻² s⁻¹. The line represents a linear regression, r² = 0.98, error bars indicate ± 1 S.E. (n = 3).