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METHOD

Rapid assessment of different oxygenic phototrophs and single-cell photosynthesis with multicolour variable chlorophyll fluorescence imaging

Erik Trampe · Jörg Kolbowski · Ulrich Schreiber · Michael Kühl

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Abstract We present a new system for microscopic multicolour variable chlorophyll fluorescence imaging of aquatic phototrophs. The system is compact and portable and enables microscopic imaging of photosynthetic performance of individual cells and chloroplasts using different combinations of blue, green, red or white light. Automated sequential exposure of microscopic samples to the three excitation colours enables subsequent deconvolution of the resulting fluorescence signals and colour marking of cells with different photopigmentation, i.e., cyanobacteria, green algae, red algae and diatoms. The photosynthetic activity in complex mixtures of phototrophs and natural samples can thus be assigned to different types of phototrophs, which can be quantified simultaneously. Here, we describe the composition and performance of the new imaging system and present applications with both natural phytoplankton and microalgal culture samples.

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E. Trampe · M. Kühl (✉)
Marine Biological Section, Department of Biology,
University of Copenhagen, Strandpromenaden 5,
3000 Helsingør, Denmark
e-mail: mkuhl@bio.ku.dk

J. Kolbowski · U. Schreiber
Julius-von-Sachs Institut für Biowissenschaften,
Lehrstuhl Botanik I, University of Würzburg,
97082 Würzburg, Germany

M. Kühl
Plant Functional Biology and Climate Change Cluster,
University of Technology Sydney, PO Box 123,
Ultimo Sydney, NSW 2007, Australia

Introduction

The state and function of the photosynthetic apparatus in oxygenic phototrophs has been extensively studied by means of variable chlorophyll fluorescence measurements (Papageorgiou and Govindjee 2004; Baker 2008) and this methodology has become widespread in aquatic photosynthesis studies. Mainly two measuring techniques are applied in aquatic systems: (A) The so-called saturation pulse method (Schreiber 2004) is based on the use of pulse-amplitude modulated (PAM) fluorometers employing repetitive pulses of 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. (B) Fast repetition rate (FRR) fluorometry (e.g. Kolber et al. 1998) uses a train of short non-saturating flashes with a constant intensity, where the energy in the sum of the flashes is more than enough to close all PSII, and the duration of the flashlet train is short enough to approximate a single turnover event saturating PSII, while monitoring *F*. Both types of measurement can be done with commercially available fluorometers (e.g. Walz GmbH, Germany; Satlantic Inc., USA, Photon Systems Instruments, Czech Republic) modified for specific applications in the laboratory and in situ. A more detailed assessment of the pro and cons of these two methodologies is outside the scope of this paper (see e.g., Kromkamp and Forster 2003; Suggett et al. 2003), but both types of variable chlorophyll fluorescence analysis quantify the quantum yield of PSII and a range of other important parameters characterising photochemical and non-photochemical quenching of light energy.

Investigations of photosynthetic performances in single phytoplankton cells have previously been performed with fluorescence microscopy (e.g. Olson et al. 1999; Snel and

Dassen 2000) and non-imaging PAM fluorometry e.g., on single planktonic diatoms (Villareal 2004) and individual expelled zooxanthellae of corals (Ralph et al. 2005). Such applications are often limited by the need to define and isolate each individual target cell within the field of view prior to performing measurements. A more advanced FRR fluorometer setup allowed for detailed measurements on single cells at higher throughput (Gorbunov et al. 1999) but variable chlorophyll fluorescence imaging (reviewed in Oxborough 2004; Kühl and Polerecky 2008) has many advantages over such sequential single cell approaches and allows investigations on intact heterogeneous samples. The ecophysiology of individual diatoms in natural biofilms has, e.g., been investigated by means of microscopic variable chlorophyll fluorescence imaging (Oxborough et al. 2000), and advanced kinetic microscopy allows very detailed variable chlorophyll fluorescence imaging of eukaryotic microalgae (e.g. Šetlíková et al. 2005) and cyanobacteria (e.g. Berman-Frank et al. 2001). However, most systems for microscopic chlorophyll fluorescence imaging are still rather complex laboratory instruments that are not easily portable and require intensive user training and adjustment.

As an alternative to traditional microscopy and/or pigment extraction analysis of samples, it is also possible to analyse pigment fluorescence "fingerprints" to differentiate the abundance of different functional groups of microalgaé and cyanobacteria in complex systems (Yentsch and Phinney 1985; Thar et al. 2001; Xupeng et al. 2010), e.g., using commercially available instruments such as the *Phyto-PAM* (Kolbowski and Schreiber 1995; Jakob et al. 2005; Walz GmbH, Germany) and the *FluoroProbe* (Beutler et al. 2002; BBE. Moldaenke GmbH) systems. Such measurements take advantage of the unique excitation and emission spectral characteristics of different chlorophylls and antenna pigments to monitor fluorescence "fingerprints" specific for different groups of phototrophs. Whereas the *FluoroProbe* system measures the algal type and chlorophyll concentration, the *Phyto-PAM* systems quantifies both relative abundance and photosynthetic activity of different phototrophs; both systems only analyse bulk samples.

In this study, we present a new system for the rapid assessment of different oxygenic phototrophs and single-cell photosynthesis via variable chlorophyll fluorescence imaging using a multicolour imaging PAM microscope capable of using red, green and blue (or combined white) excitation light from a special RGB LED excitation unit. We demonstrate the functionality of this new experimental tool in aquatic photosynthesis and explore its use for mapping and discriminating the abundance and photosynthetic performance of individual cells and chloroplasts in complex mixtures and communities of different algae and cyanobacteria.

Materials and methods

Experimental setup

The experimental setup (Fig. 1A) was composed of a high-speed CCD camera, connected via a 0.5x video adaptor (IMAG-AX, Heinz Walz GmbH, Effeltrich Germany) on an epifluorescence microscope (Axiostar Plus FL, Carl Zeiss GmbH, Germany), fitted with high-numerical-aperture objectives (10x and 20x, Plan-Apochromate, Carl Zeiss GmbH, Germany). Actinic, saturation and measuring lights were provided by a Red–Green–Blue LED excitation lamp (IMAG-RGB; Heinz Walz GmbH, Effeltrich Germany) mounted to the epifluorescence port of the microscope via a liquid-filled light guide joined by an adaptor holding collector optics. The RGB LED lamp and the CCD camera were connected to a control unit (IMAG-CM; Heinz Walz GmbH, Effeltrich Germany) interfaced to a portable PC running the *ImagingWin* software (Heinz Walz GmbH, Effeltrich, Germany). The whole system is compact and portable.

A custom-made aluminium slide holder was fixed on the microscope cross-table (Fig. 1B) and connected to a thermostated heater/cooler unit (Julabo GmbH, Germany). Dijkman and Kromkamp (2006) showed that by using such a thermostated holder the sample slide can be used a lot longer without affecting the general condition of the algae. The actual temperature of the slide holder was monitored by a small mounted thermistor connected to a temperature controller (CL100, Warner Instruments Inc., USA) and the slide temperature could be kept constant at ±0.5°C.

In contrast to previous microscopy PAM systems (see e.g. Schreiber 2004), this apparatus employs three different measuring lights, i.e. red (λ_{max}: 622 nm, HBW: ~65 nm), green (λ_{max}: 520 nm, HBW: ~90 nm) and blue (λ_{max}: 460 nm, HBW: ~80 nm) that can be used separately or together as white light. We measured the spectral composition of the 3 types of measuring lights using a fibre-optic spectrometer (USB2000, Ocean Optics, USA). The actual levels of measuring and actinic light reaching a sample (PAR, 400–700 nm, in units of µmol photons m^{-2} s^{-1}) had to be known to produce a correct list of light levels for the *ImagingWin* program, and we thus did a detailed calibration of the RGB microscopy PAM system. Light emitted from the RGB LED Lamp was measured with a calibrated micro quantum sensor (MC-MQS, Heinz Walz GmbH, Effeltrich Germany) connected to a control unit (PAM-Control; Heinz Walz GmbH, Effeltrich Germany) and placed on the cross-table of the microscope. The actinic light in the focal plane was then measured by turning on the RGB LED lamp for each of the 21 PAR-steps corresponding to all intensity settings as controlled through the *ImagingWin* software. Such irradiance measurements

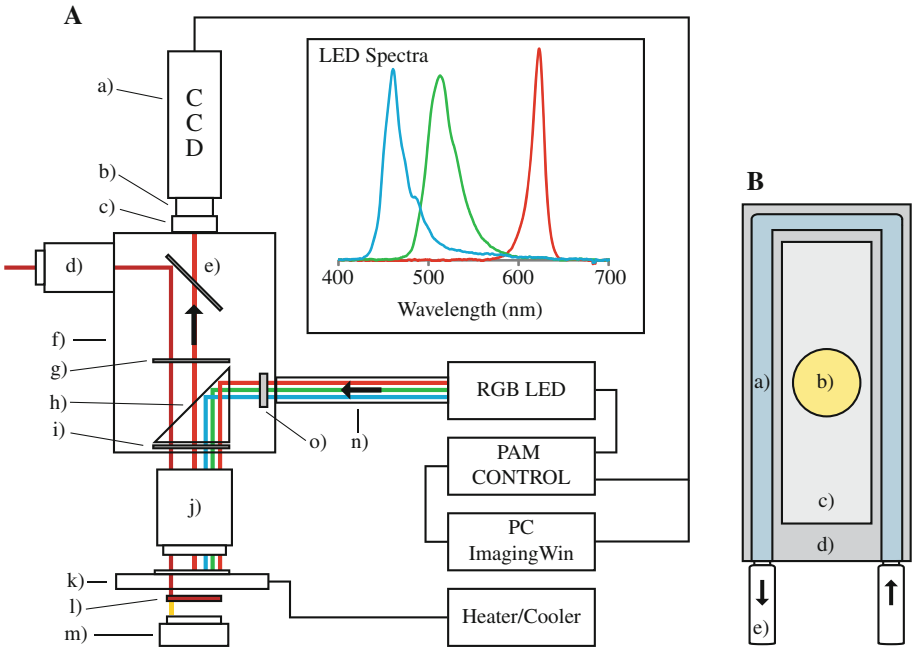

Fig. 1 **A** Schematic drawing of the measuring system and experimental setup, (*a*) CCD camera, (*b*) short pass and long pass filter, (*c*) C-mount 0.5x video adaptor, (*d*) ocular, (*e*) mirror, (*f*) epifluorescence microscope (Zeiss Axiostar Plus FL), (*g*) Dichroic filter, (*h*) dichroic beam splitter cube, (*i*) emission filter, (*j*) microscope objective, (*k*) heating/cooling block with sample, (*l*) Schott RG715 glass filter, (*m*) transmitting light source, (*n*) liquid light guide, (*o*) collector optics. Insert displays the emission spectra of the 3 different excitation ranges of the RGB LED unit. **B** Schematic drawing of the thermostated slide holder, (*a*) enclosed cooling liquid channel, (*b*) window for transmitted light, (*c*) slide cavity, (*d*) aluminium block, (*e*) tubes connected to the heater/cooler. *Arrows* indicate flow direction

were carried out for red, green, blue and white light emitted through the different Plan-Apochromat objectives (Carl Zeiss MicroImaging GmbH, Germany). Measured values were used to produce new objective and colour specific PAR-files for *ImagingWin*. The calibration procedure described above was accompanied by control measurements with a LI-190 Quantum sensor (LI-COR Biosciences GmbH, Germany) in the focal plane.

Sample preparation

Phytoplankton samples were collected from various surface waters in Øresund (Denmark) and around Heron Island, Australia, with a plankton net (mesh size 20–100 µm). For imaging, plankton cells were transferred with a pipette to a thermostated well slide; which was then sealed with a cover glass using petroleum jelly (Vaseline™) to avoid evaporation. Location of target organisms and final adjustments for field of view and sharpness were assessed through the "Live NIR Video" function in *ImagingWin*, using the bright field halogen light source of the microscope covered by a 715-nm long-pass glass filter (RG715, Schott GmbH, Germany), in order to avoid actinic light effects during focusing and observation.

Measurements

Samples were allowed to dark adapt for ~15 min prior to measurements. In the dark adapted state, all reactions centres of PSII are open, and we imaged the minimal fluorescence yield (F_0), with non-actinic modulated measuring light. During a subsequent high-intensity saturation pulse, all reaction centers are closed enabling imaging of the maximal fluorescence yield (F_m) over the sample. From these data, images of maximum PSII quantum yield could be calculated as (Schreiber 2004): $F_V/F_m = (F_m - F_0)/F_m$. Similarly, based on imaging the fluorescence yield, F, measured under illumination of the sample with a preset level of actinic light (PAR, in µmol photons m^{-2} s^{-1}), and the maximum fluorescence yield, F_m', measured under a following saturation pulse, images of the effective PSII quantum yield could be calculated as $\Phi_{PSII} = (F_m' - F)/F_m'$. In the presence of significant non-photochemical quenching of fluorescence, e.g., due to heat dissipation F_m' is $< F_m$. Images of non-photochemical quenching were calculated as $NPQ = (F_m - F_m')/F_m'$. The *Imaging-Win* software allows a large number of other derived parameters describing photochemical and non-photochemical quenching of excitation energy to be calculated from the measured

images of fluorescence yield before and during saturating light pulses, but in the following, we focus on quantum yields of photosynthesis and derived measures of relative photosynthetic activities.

From these measurements, images of relative rate of photosynthetic transport (rETR) could be derived, which in first approximation may be assumed to correspond to the relative charge separation rate at PSII reaction centers: $rETR = \Phi_{PSII}$. PAR, with PAR denoting the incident quantum irradiance. For estimating absolute electron transport rates, the absorbed quantum irradiance would have to be known. Relative ETR measurements can be done with blue, green, red or white (all three colours in concert) measuring and actinic light and can be automated, e.g., for generating a series of images of quantum yield and rETR as a function of irradiance. We used the latter to obtain a series of measurements at increasing irradiance with 10 s incubation at each irradiance step. While these so-called rapid light curves (RLC) of photosynthetic activity formally are analogous to classical light response curves (photosynthesis vs. irradiance, P-E curves), they provide distinctly different information: RLC measurements will not disturb the acclimation status of the plant as they measure the rETR at rapidly increasing irradiance levels, with the time interval at each level being too short (in the range of 10–20 s) to achieve steady state, which is a prerequisite for P-E curves (White and Critchley 1999. Thus, RLC represent snapshots of the photosynthetic capacity of a sample (or specimens within a sample), its current light acclimation and capacity to handle increasing irradiance over time scales, where various energy dissipating mechanism such as e.g. the xanthophyll cycle are not activated (Ralph and Gademann 2005, Kühl et al. 2001). It is also possible to obtain steady state P-E curves (rETR vs. irradiance) curves with the new imaging system by using longer periods of incubation at each irradiance level, which is feasible with the use of a thermostated slide holder (Dijkman and Kromkamp 2006).

The *ImagingWin* software aquires fluorescence images and controls the timing and levels of actinic light. Image data can subsequently be viewed in the program, where images of calculated key parameters can be observed for each time frame during the RLC. With the system software, it is possible to freely select areas of interest (AOI) of arbitrary shape in such images, from which the software calculates mean values of fluorescence parameters and rETR, which can be exported for further analysis such as curve fitting in other software programs.

Multicolour fluorescence imaging and deconvolution

Besides high-resolution imaging of photosynthetic performance at the cellular and subcellular level, the new RGB Imaging PAM allows automated discrimination between different microalgae and cyanobacteria by using sequential multicolour fluorescence imaging and subsequent deconvolution of the obtained fluorescence images into four different pigmentation types. The principle is based on differences in fluorescence excitation spectra of different microbial oxyphototrophs (Yentsch and Phinney 1985; Xupeng et al. 2010) and is in this respect similar to the deconvolution applied in the PHYTO-PAM Chlorophyll Fluorometer (Jakob et al. 2005) albeit with some important differences described below. For example, most cyanobacteria, with the exception of prochlorophytes and the Chl *d*-containing *Acaryochloris marina*, display maximal fluorescence yield with red–orange excitation (around 620 nm, phycocyanin absorption) and almost no fluorescence yield with blue excitation, as they are lacking (divinyl) Chl *b* and have most of their Chl *a* associated with the weakly fluorescing PS I. In contrast, chlorophytes and diatoms are characterized by strong fluorescence excitation by blue light (460 nm) overlapping with Chl *b* and Chl *c* absorption bands. Chlorophytes and diatoms can be further distinguished as diatoms are effectively excited by green (525 nm) light due to presence of antenna such as fucoxanthin, while green excitation is distinctly less effective with green algae. Red algae show exceptionally high fluorescence upon green excitation due to the presence of phycoerythrin.

The *Imaging-Win* software employs a fixed set of "RGB-Fit Conditions", which are based on well established differences of fluorescence yield with red (R), green (G) and blue (B) excitation for the four main pigmentation types mentioned above. The validity of these conditions relies on defined relative R, G and B excitation intensities. The software allows to determine the relative RGB intensities with the help of a plastic fluorescence standard ("RGB Gain" measurement) and to correct for any deviations from the original intensities, with which the "RGB-Fit Conditions" were established. The "RGB-Fit" results in a discrete "all-or-nothing" assignment of one of the four main pigmentation types or of "no fluorescence" (black) to each pixel. In this respect, the deconvolution method differs fundamentally from that employed by the *Phyto PAM*, which deals with mixed signals. In the case of microscopic images, it may be assumed that a particular pixel represents one of the four pigmentation types or none. To produce deconvoluted images showing different algal groups by discrete false colours, fluorescence yield images of a sample are averaged for 15 s for each of the three LED excitation colours. Detection and analysis of the differences in the fluorescence excitation spectra between the previous three measurements and the reference spectral matrix then produces a colour-coded deconvoluted image identifying cyanobacteria, red algae, diatoms and chlorophytes.

Additional documentation of sample composition was obtained through digital photography of samples done with a EOS 50D digital SLR camera (CANON Europe Ltd., Middlesex, UK) mounted onto the microscope via an DSLRCC, C-Mount adapter (LM-Scope, Micro Tech Lab, Graz, Austria).

Results

We tested the ability of the new imaging system to differentiate between distinct algal groups in phytoplankton samples from Danish coastal waters (Fig. 2). The micrograph (Fig. 2A) displays the sample in true colour. Three fluorescence images were each recorded after 15 min of dark adaptation. In the fluorescence image obtained with the red LED, exciting chlorophyll and phycocyanin, all phytoplankton cells were distinctly visible in the sample. In the fluorescence images obtained with green and blue LED excitation, the fluorescence pattern changed with some cells showing almost no fluorescence, if any. The result of the deconvolution of the three images shows the different functional groups in false colours; A, B and C indicating the presence of cyanobacteria, chlorophytes (*Pediastrum* sp.) and diatoms (*Coscinodiscus* sp), respectively.

Further analysis was done in a natural sample obtained in November 2009 from a bloom of the planktonic cyanobacterium *Trichodesmium* sp. nearby Heron Island, Great Barrier Reef, Australia, where we imaged the presence and activity of different phototrophs on dense tufts of *Trichodesmium* (Fig. 3). The tufts were mainly composed of densely packed and highly fluorescent *Trichodesmium* filaments, but imaging the PSII quantum yield as a function of irradiance using white light revealed the presence of numerous microalgae, mostly pennate diatoms adhering to the filament tufts (Fig. 3A–C). A RGB deconvolution enabled identification of AOI with predominantly cyanobacteria and diatoms, respectively (Fig. 3D). Using such areas of interest, it was possible to construct rETR versus irradiance curves for the two types of phototrophs indicating that the cyanobacteria generally were more efficient in using low irradiance and saturated at a lower irradiance than the diatoms, which exhibited a much slower saturation of photosynthesis at increasing irradiance. However, one should be aware that these findings are based on incident light, not on absorbed light. A more accurate evaluation of such apparent differences would involve a spectral correction based on the absorption properties of the algae and the light colour of the PAM.

We also used the new variable chlorophyll imaging system to map the variability in photosynthetic activity of single individual cells and the linkage thereof to the internal arrangement of single chloroplasts in a marine diatom culture of *Coscinodiscus granii* (Fig. 4). The RGB-fit (Fig. 4B–C) produced by fluorescence yield measurements acquired with the three different wavelengths of light produced by the RGB lamp, classified the organism by a false colour (yellow) as a diatom containing

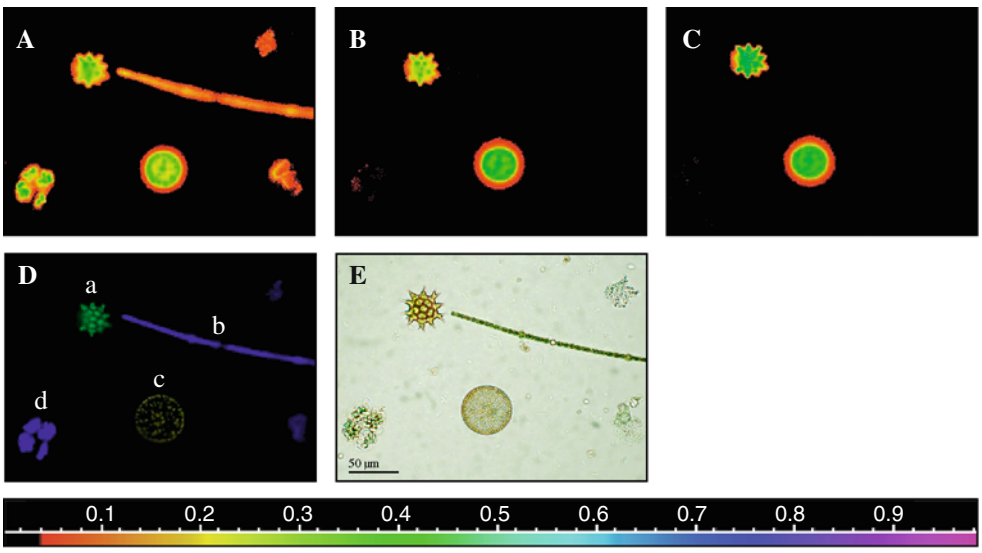

Fig. 2 Multicolour variable chlorophyll fluorescence imaging combined with spectral deconvolution of a mixed sample of planktonic phototrophs; a *green* algae (*a*, *Pediastrum* sp.), a heterocystous filamentous cyanobacterium (*b*), a diatom (*c*, *Coscinodiscus* sp.) and an aggregate of unicellular cyanobacteria (*d*). Panels **A**–**C** shows maximal fluorescence (F_m) images obtained with *red*, *green* and *blue* excitation, respectively. Panel **D** shows the corresponding RGB-Fit image with colour-coded marking of the three differently pigmented types of phototrophs. Panel **E** shows a micrograph displaying the sample in true colours

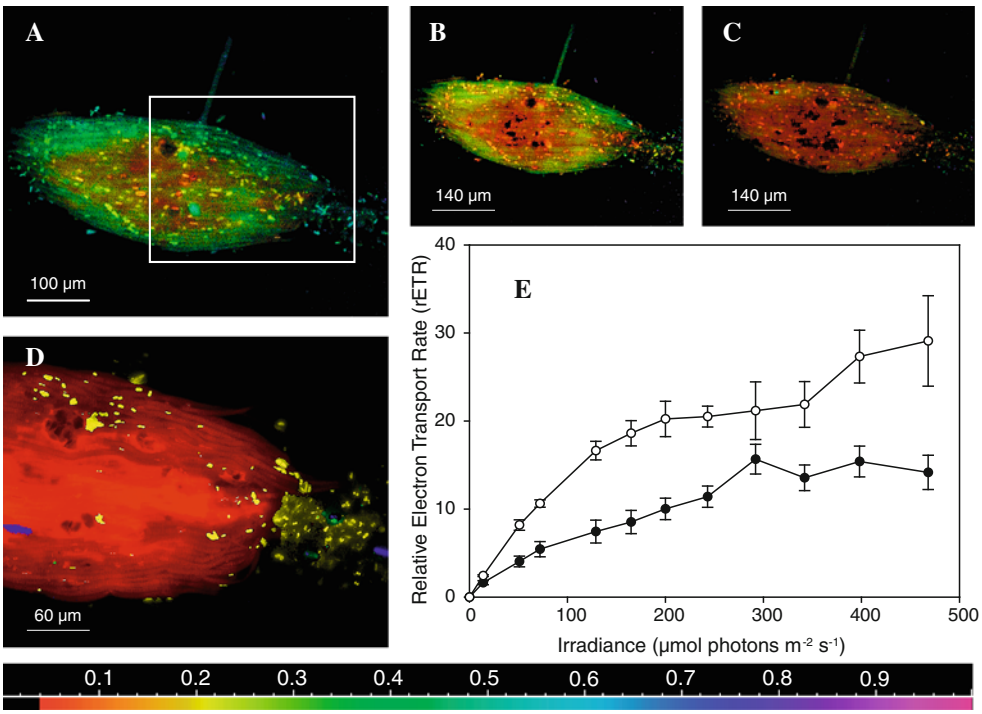

Fig. 3 Imaging of photosynthetic activity on a dense tuft of the cyanobacterium *Trichodesmium* sp. with patches of microalgae, mainly pennate diatoms. Panel **A–C**, display PSII quantum yield images in false colours, values correspond to the colour *bar* at irradiances of 0, 129, 468 µmol photons m^{-2} s^{-1}, respectively. Panel **D** is a RGB-Fit image showing deconvoluted fluorescence images, displaying the diversity of phototrophs on the tuft as red cyanobacteria and yellow diatoms. Panel **E** displays rETR versus irradiance for the two main phototrophs, (*open circle*) *Trichodesmium* sp. and pennate diatoms (*closed circle*)

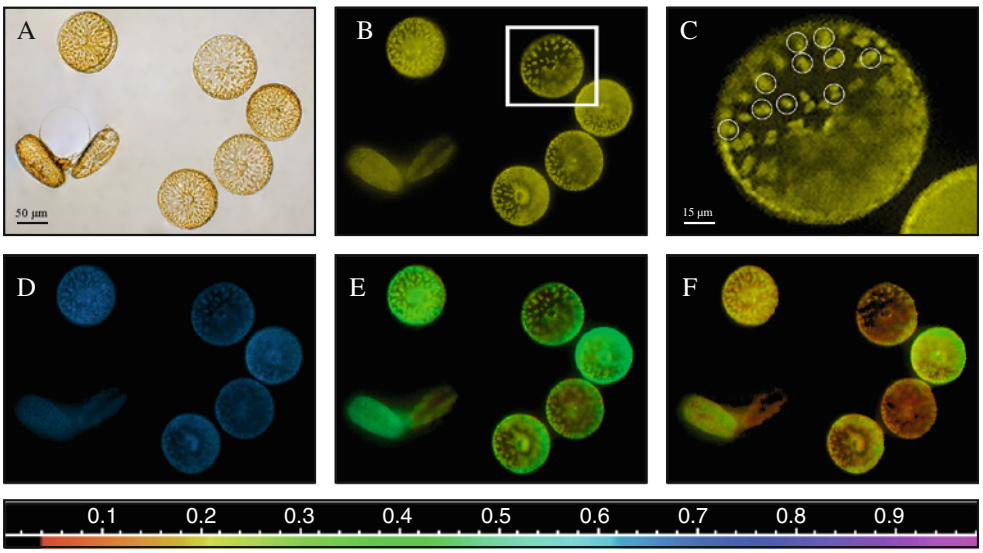

Fig. 4 Variable chlorophyll fluorescence imaging of *Coscinodiscus. granii* cells (**A**), displaying the heterogeneity in photosynthetic activity at the level of single cells and at the level of single chloroplasts. Panels **B–C** display RGB-Fit images at two magnification serving to identify AOI's used for further measurements. Panels **D–F** display PSII quantum yield images, values correspond to the false colour bar at irradiances of 0, 59, 159 µmol photons m^{-2} s^{-1}, respectively

chlorophyll and fucoxanthin, which can be effectively excited by blue and green light. Figure 3d–f displays high-resolution images of PSII quantum yield (Φ_{PSII}) obtained during RLC measurement with green light. It was clearly possible not only to map the photosynthetic activity of individual diatoms but also the photosynthetic activity of individual chloroplasts within a single diatom cell.

Based on these measurements, we could compare the variability in photosynthetic performance between individual cells of *C. granii* and individual chloroplasts within a single cell (Fig. 5). The quantum yield versus irradiance and the derived rETR versus irradiance curves generally showed more variability between cells (Fig. 5A, C) as compared to variations between chloroplasts within a cell (Fig. 5B, D).

Discussion

The new variable chlorophyll fluorescence imaging system presents a major advancement for microscopic assessment of phototrophs at high spatial resolution, i.e. at the level of individual cells and even chloroplasts, and enables exciting new directions for studies of photosynthetic performance in natural samples and cultures of specific phototrophs. While we have presented examples of application using 10x or 20x objectives, similar analysis can be done at higher magnification and we have done single cell analyses on small algal cell using a 100x objective. However, for each type of objective, careful calibration of the system is necessary. With the help of automated multi colour variable chlorophyll fluorescence imaging, in combination with a novel integrated fitting routine, deconvoluted RGB images are obtained, which show different types of phototrophs in false colours according to their pigment composition. These new means of assessing photosynthetic performance within a mixed phytoplankton sample, provides easy determination of the dominant phototrophs, and in principle also allows monitoring of the performances between different types of microalgae and cyanobacteria. For detailed analysis of the photosynthetic capacity of the various organisms in white light, however, further development work will be required to obtain estimates of absorbed PAR in differently pigmented phototrophs.

Variability of photosynthetic parameters in microalgae and cyanobacteria has mostly been studied on larger scales, e.g. the vertical (mixed surface layers versus deeper layers), horizontal (near shore versus offshore), seasonal (Mitchell and Kiefer 1988) and short-term variability (Morán and Estrada 2001), and photosynthesis measurements have either been performed as bulk measurements or integral measurements of larger individual cells (e.g. Villareal 2004). Although several studies of e.g., microphytobenthos (Oxborough et al. 2000) or algal cultures (e.g. Boulding and Platt 1986) have shown cell to cell variability in photosynthetic parameters, such phenomena are still underexplored in aquatic biology.

Our study showed differences in the variability of photosynthetic performance among individual cells and among individual chloroplasts within a cell (Figs. 4, 5). The S.E. shown in Fig. 5 can possibly be accredited to the internal rearrangement of chloroplasts seen in *Coscinodiscus* sp., which is capable of optimizing its light-harvesting capacity

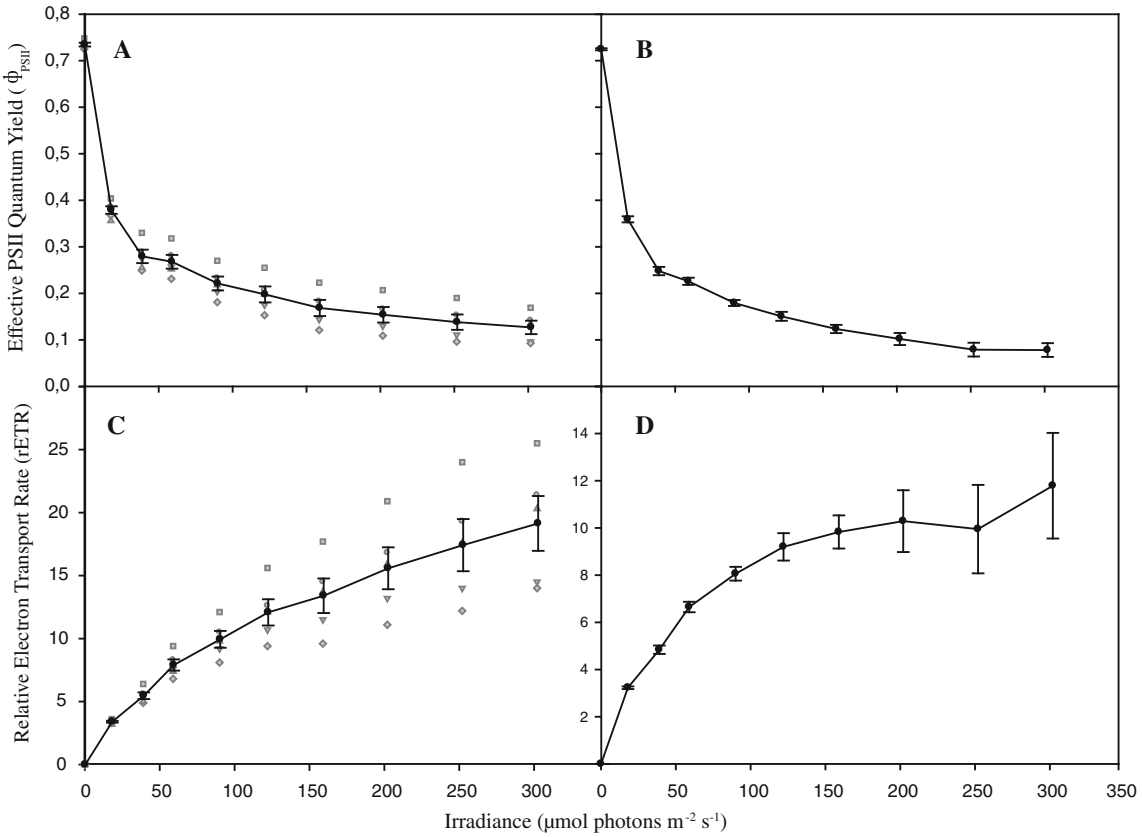

Fig. 5 Effective PSII quantum yield (**A**, **B**) and rETR (**C**, **D**) as a function of irradiance, measured on single cells of the diatom *Coscinodiscus. granii* (**A**, **C**) (±1 SE, n = 5) and on single chloroplasts (**B**, **D**), (±1 SE, n = 10). *Solid black* symbols represent average values, while *grey symbols* represent measurements on individual specimen of *C. granii*

by distributing chloroplasts evenly during low light situations and protect it from photo inhibition during high light intensities by condensing the chloroplasts near the centre. We speculate that the variation seen here might be a result of intracellular specialization, where shade acclimated chloroplasts can be protected by those acclimated to higher intensities. The new imaging system facilitates investigation of such hypothesis, and it would be interesting to further investigate the physiology and photosynthetic capabilities of *C. granii* by comparing the intracellular photosynthetic competence and arrangement of chloroplasts in *Coscinodiscus* sp. adapted to low versus high light, or when exposed to various microenvironmental changes; as well as comparing "natural" samples, adapted to varying light intensities with those of a controlled culture sample always experiencing the same light conditions.

While we have presented applications with phytoplankton, the new imaging system is also well suited for investigating biofilms and other surface-associated phototrophic communities. Recently, we have, e.g., used the system for studies of the in situ photosynthetic performance of the chlorophyll *d*-containing cyanobacterium *Acaryochloris marina* living in an endolithic habitat below encrusting coralline algae on coral reefs (Behrendt et al. 2011), and we have also investigated the performance of expelled zooxanthellae during coral bleaching (E. Trampe, unpub data). The new imaging system will thus enable many new experimental applications of microscopic variable chlorophyll fluorescence analyses in aquatic photosynthesis.

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