universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

Effects of cell density on chlorophyll fluorescence in liquid algal cultures

verfasst von / submitted by

Andreas Ernst Neuwirth, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2019 / Vienna 2019

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 832
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Botanik UG2002
Betreut von / Supervisor:	ao.Univ.-Prof. Mag. Dr. Michael Schagerl

Überblick

Oxygene Photosynthese ist der Prozess, bei dem unter Verwendung von Licht und Wasser Biomasse aufgebaut wird, während als Nebenprodukt Sauerstoff freigesetzt wird. Dieser Stoffwechselvorgang existiert seit drei Millionen Jahren auf unserem Planeten und wurde anfänglich ausschließlich von Bakterien genutzt [1]. Heute ist bekannt, dass oxygene Photosynthese in den Thylakoidmembranen von Cyanobakterien und Chloroplasten von Pflanzen stattfindet [2]. Die Moleküle, die diese Reaktion ermöglichen, heißen Chlorophylle. Erst durch sie wird das emittierte Licht der Sonne nutzbar gemacht. Doch nicht die gesamte absorbierte Energie wird für diesen Prozess verwendet: Ein geringer Teil des absorbierten Lichts wird von den Chlorophyll-Molekülen als messbare Strahlung remittiert. Dieser Prozess ist bekannt als Chlorophyll-a Fluoreszenz [3].

Seit der ersten Hälfte des 20. Jahrhunderts wird Chlorophyll-a Fluoreszenz erforscht [4]. Chlorophyll-a bezeichnet ein Pigment, das in allen Organismen zu finden ist, welche oxygene Photosynthese betreiben [5]. Kohlenstofffixierung und Chlorophyll-a Fluoreszenz sind jedoch nicht die einzigen Pfade, die die absorbierte Energie einschlagen kann: Eine weitere Alternative ist die Abstrahlung in Form von Wärme [6–8]. Die drei Pfade Photosynthese, Wärmedissipation und Fluoreszenz stehen in Wechselwirkung zueinander. Nimmt also die Verwendung eines Pfades zu, muss zumindest einer der anderen abnehmen [9]. Wird beispielsweise ein Anstieg der Chlorophyll-a Fluoreszenz beobachtet, kann das nur auf Kosten der Photosynthese und/oder der Wärmeabgabe erfolgen. Dieser Zusammenhang erlaubt, die zur Photosynthese beitragenden Anteile der absorbierten Photonen zu erfassen [10].

Heute werden spezielle Fluoreszenzmethoden genutzt, um Einblicke in die Photophysiologie von Algen und Gefäßpflanzen zu gewinnen. Einer der großen Vorteile dieser Messung liegt darin, dass sie nicht-invasiv ist, also die Organismen durch sie nicht beeinträchtigt werden. Das ist einer der Gründe, warum Chlorophyll-a Fluoreszenzmessungen ihre Verwendung sowohl in unterschiedlichen Feldern der Photosynthese-Forschung als auch im angewandten Bereich finden.

In dieser Arbeit wurde das sehr verbreitete Fluoreszenzmesssystem PAM-2500 der Firma Walz (Effeltrich) – ausgestattet mit einer Suspensionsküvette für flüssige Proben – eingesetzt, um Fluoreszenzmessungen an Algenkulturen durchzuführen. Das Gerät ist mit verschiedenen Lichtquellen und Fluoreszenzdetektoren ausgerüstet, die eine Messung ermöglichen, mit deren Hilfe Rückschlüsse auf den Status der Zellen gezogen werden kann. Ein Standardparameter ist der sogenannte „*maximum quantum yield of Photosystem II*“ oder „*Fv/Fm*“, der Aufschluss über die Photosynthese-Effizienz gibt, und auch als Stressindikator eingesetzt wird. Weitere Anwendungsmöglichkeiten sind „*rapid light curves“* (RLCs). Hier wird eine vorverdunkelte Probe über kurze Intervalle im Sekundenbereich ansteigenden Lichtintensitäten ausgesetzt, während die Photosynthese-Effizienz regelmäßig gemessen wird. So bekommt man Einblicke in den Akklimatisierungsstatus der Probe. Daraus lässt sich schließen, ob die Untersuchungsobjekte an hohe oder niedrige Beleuchtungen angepasst sind. Diese Informationen geben essentielle Einblicke über Leistung und Stresslevel der Zellen. Interessant sind diese Ergebnisse auch für die kommerzielle Produktion von Algenbiomasse, da man so potentielle Probleme besser erkennen und verhindern kann.

Eine Besonderheit der Messungen von Algenkulturen im Vergleich zu Algen in natürlich vorkommenden Konzentrationen liegt in der Natur der Proben: Es handelt sich häufig um Suspensionen mit sehr hoher Dichte. Ein wichtiges Thema stellt dabei die Beleuchtung dar, wie bereits des Öfteren in der Literatur diskutiert wurde [11,12]. Der Zusammenhang zwischen

Lichtabsorption, -streuung und Zelldichte wird zum Beispiel genutzt, um Rückschlüsse auf die Zunahme der Algenbiomasse zu ziehen (Lambert-Beer'sches Gesetz; [13]). Ähnliche Effekte, die letztendlich zu Messartefakten führen, sind auch bei Fluoreszenzmessungen zu erwarten. Trotzdem werden die Zusammenhänge zwischen Zellzahl und Lichtabsorption oftmals negiert [14]. Bei Fluoreszenzmessungen können jene Interferenzen zwischen Strahlengang und Objekt sogar zweimal auftreten, nämlich beim Anregungslicht und bei der Fluoreszenzantwort der Zellen.

PAM-Fluoreszenzsysteme werden in erster Linie für Messungen an Gefäßpflanzen benutzt, weshalb in den publizierten Protokollen nur unzureichend auf flüssige Algenkulturen Rücksicht genommen wird [3,10,15–19]. Um in Zukunft eine spezifische Anwendung von Chlorophyll-a Fluoreszenzmessungen zu ermöglichen, und Fehlerquellen von vornherein zu minimieren, wurde im Zuge dieser Arbeit der Einfluss der Probendichte auf die Ergebnisse der Fluoreszenzmessungen untersucht. Ziel war es, Richtlinien zu erstellen, die ermöglichen, Proben unterschiedlicher Dichte vergleichbar zu machen. Hierzu wurden diverse Experimente an Kulturen von *Chlorella* durchgeführt.

Wir stießen auf einige Herausforderungen beim Vergleich flüssiger Algenkulturen unterschiedlicher Dichte, da die Messung von Chlorophyll-Fluoreszenz-Werten einer optischen Messung entspricht und hier Effekte der Selbstbeschattung Auswirkungen haben. Darum haben wir eine Reihe von Experimenten durchgeführt, die helfen sollen, den Effekten unterschiedlicher Kulturdichten entgegenzuwirken.

Im ersten Schritt wurde untersucht, welcher Biomasseparameter sich am besten eignet, um die Dichte der zu testenden Proben zu bestimmen. Hier haben sich optische Dichtemessungen als passend erwiesen. Diese sind schnell und unkompliziert durchzuführen und zeigen eine hohe Übereinstimmung mit Trockenmasse-Bestimmungen. Für Messungen von Fluoreszenzwerten zur Feststellung des physiologischen Status, ist es nötig, die Probe dunkel zu adaptieren, bevor man Werte erheben kann. Hier gab es Unklarheiten darüber, welche Dauer am optimalsten für die verwendeten Kulturen sei. Wir können anhand unserer Ergebnisse eine Verdunklungsperiode von zehn Minuten empfehlen. Aus unserer Arbeit erschlossen sich außerdem neue Möglichkeiten für Vorgehensweisen, um die Dichte von Algenkulturen anzupassen, und die Effekte von Selbstbeschattung zu minimieren. Durch Verdünnungen mit unterschiedlichen Medien können wir zeigen, dass vollwertiges Medium als Verdünnungsmedium keine Abweichungen der Messergebnisse im Vergleich zu Verdünnungen mit einem Kontrollmedium verursacht. Das erarbeitete Wissen wurde angewandt, um die Auswirkungen der Probendichte auf die Ergebnisse von Fluoreszenzmessungen zu überprüfen. Bei dem Vergleich von „*rapid light curve*“-Daten einer Kultur, die einmal auf eine optische Dichte von 1 und einmal von 0,1 verdünnt wurde, konnten wir sehen, dass die Werte einiger Parameter um fast 50 % auseinanderwichen. Diese enormen Unterschiede können zu drastischen Fehlinterpretationen führen.

Das entstandene Protokoll wurde während eines Wachstumsverlaufs einer Kultur erprobt, um zu zeigen, auf welch unterschiedliche Ergebnisse man bei Missachtung der Kulturdichte stößt. Als letzten Versuch konnten wir erfolgreich zeigen, dass die Filtration als Methode zur Aufkonzentrierung von optisch dünnen Suspensionen hilft, durch unterschiedliche Kulturdichten bedingten Effekten entgegenzuwirken. Die dadurch erhaltene photosynthetisch aktive Schicht auf dem Filter ähnelt einem Blatt und erlaubt eine Messung, die keine Rücksichtnahme auf die Dichte der Probe verlangt.

Das Ergebnis dieser Arbeit ist ein Protokoll, dessen Anwendung einen vereinfachten Vergleich von Algenkulturen unterschiedlicher Dichte mittels PAM-Fluoreszenzmessungen ermöglicht.

Effects of cell density on chlorophyll fluorescence in liquid algal cultures

Abstract

Chlorophyll fluorescence techniques found their way into algal mass production. Guidelines on how to perform and interpret measurements are on hand for higher plants, but it is hard to find protocols focusing on algal suspensions. We faced challenges comparing algal cultures of different concentrations. That is why we performed a series of experiments to counteract the effects of sample density during the measurements. We chose to work with *Chlorella* cultures and a PAM-2500 (Walz) to show how the concentration of cells changes the outcome of the measurements. First, we tested which biomass parameter fits best for evaluating algal cell concentration. Optical density turned out to be the most feasible method to determine and adjust density. Another point not focused enough on is the pre-darkening duration before measurements. We could show that depending on how long samples are pre-darkened mean *Fv/Fm* values can differ more than 60 %. We consider ten minutes as a proper pre-darkening duration. With our work we can also present a way of adjusting the liquid algal sample density to overcome issues of mutual shading. We diluted our samples with different media and could show that dilution with adequate medium shows no significant differences compared to the control medium. We also applied the gained knowledge to see how a properly performed adjustment of density affects the measurements' outcome. We saw differences of nearly 50 % in some parameters of rapid light curves when comparing the same culture once diluted to an *OD* of 1 versus an *OD* of 0.1. This protocol was eventually tested in an application example. Finally, we successfully tested filtration as a method that allows to increase sample density and to overcome effects of mutual shading by filtering samples to create a flat surface of photosynthetically active cells resembling a leaf.

Keywords

PAM-2500, Chlorophyll Fluorescence, *Chlorella vulgaris*, density, concentration, liquid algal sample

Abbreviations

Chlorophyll fluorescence (CF), rapid light curve (RLC), optical density (OD), dry mass (DM), Chlorophyll-a (Chl-a), minimal chlorophyll-a fluorescence in the dark-adapted state (*Fo*), maximal chlorophyll-a fluorescence in the dark-adapted state (*Fm*), maximal photochemical yield in the dark-adapted state (*Fv/Fm*), actual fluorescence (*F*), maximal chlorophyll-a fluorescence in the light adapted state (*Fm'*), Photosystem (PS), photosynthetically active radiation (PAR), effective photochemical quantum yield of PS II (Y(II)), maximum relative electron transport rate (rETRmax), relative electron transport rate (rETR), initial slope (α), minimum saturating irradiance (I_K),

1 Introduction

Chlorophyll fluorescence (CF) has been applied for nearly 90 years to get insight into the photophysiology of photosynthetic organisms [20]. CF is used to gain information about photoinhibition [21], photosynthetic productivity [22], and overall stress on photosystem II of vascular plants and algae [3,23], as well as to study their acclimation to changing light supply [15]. Furthermore, it can be applied to make assumptions about biomass content of a sample [24]. By

treating samples with specific irradiation that causes detectable fluorescence of chlorophyll molecules, conclusions on their photosynthetic activity can be drawn [25]. Especially PAM fluorescence, which, in contrast to previously applied fluorescence techniques, allows to perform measurements in ambient light [16], is increasingly used in algae cultivation and agricultural facilities [26]. Although there exist excellent reviews and guidelines of fluorescence techniques to introduce this topic to a broader group of applied users [3,10,15–19], the needed level of expertise for conducting the measurements in a proper way is not always guaranteed in practice [9,16,18]. Moreover, the published literature mostly focuses on vascular plants, often neglecting specific demands of suspension measurements. Fast growing microalgae cultures have doubling times of less than one day [27], which result in big variations of cell density. Increased cell numbers cause strong mutual shading [28], which interferes with the optical path of the measuring beam emitted by the fluorometer. Although dense suspensions obviously affect both excitation and emission of fluorescence, details of the measurements (i.e., sample preparation, dilution) are often lacking, hindering reproducibility [29,30].

There exist two different approaches to monitor the physiological state of liquid algal cultures via CF, which are *online* and *offline*. During *online* measurements, a fibre-optic light guide is mounted on a reactor wall [21,31] or submerged in the culture [32]. In contrast, *offline* measurements are performed outside the culture vessel with an aliquot of the harvested culture [33]. In this study, we focused on the latter type, which facilitates the treatments such as sample dilution and the so-called pre-darkening needed for the evaluation of fluorescence key parameters [10].

Besides highly sophisticated fluorescence devices specifically developed for diluted cell suspensions down to less than 1µg L^{-1} Chl-a (e.g., WATER-PAM, Walz company; FL 6000-S, PSI company) and mainly used in basic research, instruments such as the PAM-2500 (Walz), FMS2 (Hansatech) or even handheld instruments (Aquapen, PSI) are on the market. As these devices are easy to handle, robust and cheaper than their laboratory counterparts, they are commonly used for routine measurements. These PAM fluorescence devices have been developed for leaf measurements, which require high fluorescence signals for accurate readings. Although high signals also can be obtained in suspensions, interferences between the sample and the measuring beam increasingly occur with cell density, which must not be neglected. Extreme care must be taken to minimize this problem, otherwise measuring artefacts will impede interpretation of the results. We used the PAM-2500 (Walz), which is often applied on suspensions [33–35], but also a common device for measuring vascular plants [36–38].

In the current study, we focused on the potential problem of cell density for routine PAM fluorescence measurements. We specifically investigated effects on the key parameter, namely the maximal photochemical yield in the dark-adapted state (*Fv/Fm*), which is measured on dark-adapted material and expected to be highest in unstressed organisms. For vascular plants a value around 0.8 is expected [9] while in different algal groups *Fv/Fm* is lowered due to the variations in light harvesting complexes. [39]. Besides, we addressed rapid light curve (RLC) measurements, a technique that allows to gain insight into the actual acclimation status of cultures [25]. We first tested different methods for algal density estimations. There exist direct parameters for estimating algal biomass such as dry mass (*DM*) [40] and carbon content [41,42], but these are not appropriate as decision making tools for fast growing cultures, as they take too long to determine [43]. For commercial algae cultivation, indirect, rapid and easy to handle methods such as optical density (*OD*), and in-vivo Chl-a fluorescence measurements should be the techniques of choice. Especially *OD* measurements can easily be performed in most algal production facilities within seconds, thus suggesting it as the preferred indicator for biomass [44]. Another prerequisite for correct RLCs and

specifically for *Fv/Fm* measurements is pre-darkening of the sample to relax the photosystems (PS). There are no standards regarding the pre-darkening period's length, however an appropriate duration of pre-darkening is essential. Dark periods from less than 10 min [45,46] to more than 10 min [25,30,47–49], up to 1 h [50,51] have been published. Too short periods might be insufficient to oxidize all the photosystems, while elongated pre-darkening already results in acclimation to low light conditions.

Industrial algae cultivation usually shows high cell densities exceeding 4 g L^{-1} [52,53] to increase efficacy of harvesting and downstream processes. From the theoretical point of view, *Fv/Fm* and related parameters are relative parameters and therefore independent of biomass, but the interferences between the optical path and the measuring beam might occur especially at higher densities thus creating measuring artefacts. Attention was therefore paid on suspension density effects on fluorescence signals. To eliminate effects of mutual shading, algal suspensions should be set to densities below a point where mutual shading has major impacts, preferably by dilution. We first tested different dilution media, as CF is very sensitive to all kinds of stress [9,10,54]. It was shown that adding nutrients to nutrient-limited cultures significantly affected CF values [50,55]. Specific care was taken to avoid any kind of osmotic stress [56] when diluting the samples. In a further step, we searched for an optimal range of algal density, where reliable and reproducible results could be obtained. A final application example compared results of a batch culture with and without considering potential effects of suspension density.

Challenges of optical measurements in algal cultures are scattering and absorption through cells, which finally disturb the light path in the measuring device. For PAM fluorescence, this is true for excitation and actinic light, and for the fluorescence response of the cells [57]. Fluorescence parameters relativized on overall fluorescence may partly compensate this effect, but actinic light and saturation pulses might still strongly influence the results. To minimize density effects, we simulated a leaf by filtrating the suspension followed by measurements, as it has been done to test effects of dehydration on algal cells [58]. This approach might be advantageous in two respects. Low density samples can be concentrated to increase their fluorescence signal, thus facilitating comparisons between cultures of high and low cell concentration. Additionally, the optical path is minimized from the 3-dimensional measuring cuvette to nearly two dimensions, which may reduce artefacts originating from optical path-sample interferences. Moreover, the method simplifies the comparison between cultures of high and very low density.

2 Methods

2.1 Cultivation

Unialgal batch cultures of *Chlorella vulgaris* SAG 211-11b were grown in 2 L cultivation flasks in autoclaved cultivation medium adjusted after Allen [59] known as BG11 [60] (see 7 Attachment for a detailed recipe). For the pre-darkening experiment, *Chlorella minutissima* UTEX 2219 was used. Cultures were kept in a temperature-controlled room at 22 °C and exposed to permanent light (fluorescent tubes, Lumilux Cool White, 36W/840, Osram) of approximately 150 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) reaching the bottle's light-facing side. Sterile filtered air enriched with 5 % CO_2 was delivered to the bottom of the flask via a silicone hose. A second silicone hose was used as an exhaust, covered with cotton and aluminium foil. We placed the bottles on magnet stirrers to prevent sedimentation. Cultures were always handled aseptically.

2.2 Monitoring and algal density measurements

For monitoring parameters, three replicates were taken whenever PAM measurements were performed. A defined sample volume depending on density was filtered via a suction unit through pre-combusted, pre-weighed glass microfibre filters GF/C (Whatman). Filters with material were dried again at 100 °C for 24 h, reweighed and DM calculated by subtraction of the initial filter mass from the filter + material. OD at 750 nm was measured by means of a spectrophotometer (U-2001, Hitachi). Pure BG11 medium was used as a blank and for dilution, if values of undiluted samples exceeded 0.7. For Chl-a content, a defined volume of the culture was filtered through glass microfibre filters (GF/C, Whatman) and stored at -20 °C until analysis to facilitate cell burst. Filters were then homogenized by ultrasonic treatment (S-250A, Branson Ultrasonics Sonifier) in 90 % acetone and stored in the dark at 4 °C for 12 h. The extract was then centrifuged, and the supernatant was measured photometrically (U- 2000, Hitachi) to determine Chl-a:

$$Chl\ a\ [\mu g \times L] = (11{,}85\ E_{664} - 1{,}54\ E_{647} - 0{,}08\ E_{630}) \times v \times V^{-1} \times d^{-1}\ [49]$$

$E_{664}, E_{647}, E_{630}$...Extinction at specific wavelength in nm; v... volume of extract in ml; V... volume of filtered sample in l; d... light path in cuvette in cm

2.3 Chlorophyll fluorescence

For all fluorescence measurements seven replicates of one culture were taken. We used a PAM-2500 device (Walz), equipped with a suspension chamber KS-2500 (Walz) with a micro magnetic stirrer. The temperature of the sample was kept at 22 °C by using a thermostat (RC 6, Lauda). The system was operated with the PamWin_3 software (Walz). After 10 min of pre-darkening (see details below), minimal chlorophyll-a fluorescence in the dark-adapted state (*Fo*) was determined with measuring light turned on (< 0.15 µmol m^{-2} s^{-1} PAR), followed by a saturation pulse closing all photosystems (> 8500 µmol m^{-2} s^{-1} PAR), and measurement of maximal chlorophyll-a fluorescence in the dark-adapted state (*Fm*). These values were used to calculate maximum efficiency of PS II (*Fv/Fm = (Fm-Fo)/Fm*) by comparing the dark-adapted fluorescence state *Fo* with the maximum fluorescence *Fm*. For construction of RLCs, ten incrementally increasing actinic light intensities were provided (10, 20, 75, 145, 225, 310, 435, 595, 790, 1035 µmol m^{-2} s^{-1} PAR according to PamWin_3) for 10 s each and followed by a saturation pulse. At every light step, actual fluorescence (*F*) (just before the saturation pulse) and maximal chlorophyll-a fluorescence in the light adapted state (*Fm'*) (when reaction centers are closed by the saturation pulse) were determined. These values were used to calculate the effective photochemical quantum yield of PS II: *Y(II) = (Fm'-F)/Fm'*. [61] For RLCs, relative electron transport rates (rETR) were calculated: rETR=PAR*ETR-Factor*P_{PS2}/P_{PPS}*Y(II). The ETR-Factor was set to 0.84 and P_{PS2}/P_{PPS} gives the number of photons absorbed by PS II in relation to photons absorbed by photosynthetic pigments [61]. The cardinal points of RLCs are maximum relative electron transport rate (rETRmax), quantum efficiency of photosynthesis (α) and minimum saturating irradiance (I_K) values. They were calculated by the software PamWin_3 (Walz) [61] according to the model of Eilers and Peeters (1988) [62].

2.4 Experiments conducted

Our experimental design consisted of five steps:

(1) We first compared direct and indirect algal biomass parameters for practical applicability. We prepared a dilution series of a dense culture with an *OD* of around 5.00. Dilution steps were approximately 0.43 down to an *OD* of 0.05; dilutions were done with BG11 and for all dilutions

Chl-a, *OD* and *DM* were obtained. *Chl-a* measurements were performed in duplicates, while for *OD* and *DM* three replicates were considered.

(2) A 2nd series addressed the pre-darkening period, followed by measurements of *Fv/Fm* and RLCs performed on a culture with an *OD* of approximately 4. Pre-darkening times ranged from 0 min to 60 min in following intervals: 0, 1, 2, 5, 10, 15, 20, 25, 30, 60 min. Seven technical replicates were tested for each pre-darkening duration.

(3) For interferences of algal density with CF-signals, dilutions of highly concentrated samples need to be performed. Here, special care must be taken to avoid changes of the physiological state due to dilution media. We therefore tested various approaches to minimize such dilution effects. A dense culture with an *OD* of 4.57 was diluted to lower densities of *OD* = 1 and *OD* = 0.5 right before a ten-minute pre-darkening period. We used BG11, BG11 enriched with 10 % NaCl to induce stress, reverse osmose filtered water, and the actual culture medium which is left after removal of cells via centrifugation followed by filtration through glass-fibre filters (GF92, Whatman). The filtrate was used as a control, as this medium has no impact on the environment of the tested cells (no changes in osmotic pressure nor alterations of the composition). For each test medium seven measurements were considered.

(4) Linked to the previous experiment, dilution and filtration series were performed with a special focus on the fluorescence signal. For the dilution series, a culture of *C. vulgaris* with an *OD* of 1.08 was diluted to different *OD*s = 0.5, 0.1, 0.05 using the current medium as dilution medium at the beginning of the ten-minute pre-darkening period. *Fv/Fm* values and RLCs were measured to see the influence of different *ODs* on the outcome.

We additionally applied gentle vacuum filtration followed by measurements of algal cells on wet filters to mimic measurements of leaves. Two cultures were compared, one with an *OD* = 0.469, the other one with an *OD* = 4.01. Both cultures were grown as described above. The dense culture can be addressed as low light culture, the more diluted one as high light culture. Samples were filtered through GF/C filters (Whatman) with gentle vacuum filtration until the filters were almost clogged. They were then immediately pre-darkened for 10 min in a chamber with water-saturated air to prevent drying. Fluorescence measurements (*Fv/Fm*, RLCs) were done with *dark leaf clips* (Diving LC, Walz). For a comparison, original liquid cultures were also measured with adjusting the low light culture to the same *OD* ~ 0.469 as the highlight culture right before darkening.

(5) In an application example, we monitored a culture over 6 days with and without considering potential effects of suspension density. RLC measurements were performed three times during the growth period, always with the original densities of the culture and with samples diluted to an *OD* of 0.2.

2.5 Statistical analysis

Statgraphics Centurion 18 (Statgraphics Technologies, Version 18.1.06) was used for one-way ANOVAs, multiple range tests and the calculation of Fisher's least significant difference test as well as for the construction of box-and-whisker plots.

3 Results

The regression of *OD* to *DM* resulted in a function that is described by the formula $y = 5.839x - 0.0761$ (Figure 1). The coefficient of determination was found to be $R^2 = 0.9973$. A similarly high $R^2 = 0.973$ was found between Chl-a content and *DM*, the relation is described by the formula $y = 23.549x - 0.3133$.

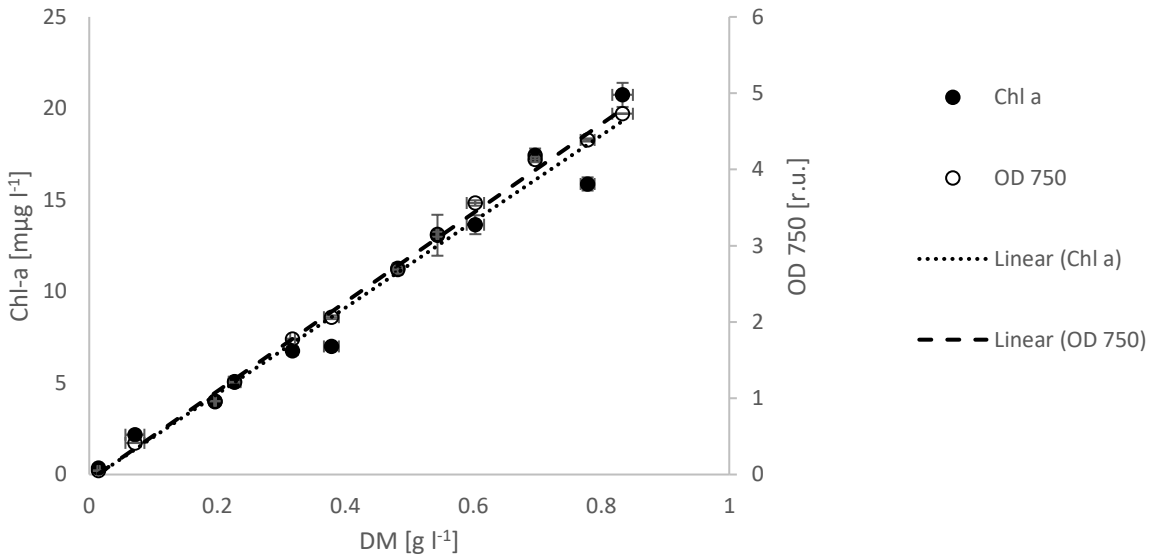

Figure 1 Regression of mean values of *OD* (*n* = 3) and *Chl-a* (*n* = 2) to *DM* (*n* = 3) of *Chlorella vulgaris* is relatively strong. Error bars show standard deviation.

We performed *Fv/Fm* measurements for a series of different pre-darkening times (Figure 2). Samples pre-darkened for less than ten minutes had high variations, in contrast to samples with ten or even more minutes of pre-darkening phases, they all grouped in one homologous group (Fisher's least significant difference test at the 99.99% level). RLCs of different pre-darkening phases are plotted in Figure 3. In Figure 4 rETRmax, I_K and alpha values of the various pre-darkening durations are shown. In Figure 4A the decrease in rETRmax during longer pre-darkening times can be observed showing signs of acclimation to low light conditions. However, only the 60 minutes period turned out be significantly (95% Fisher's least significant difference) different to all the shorter pre-darkening durations. With I_K and alpha values, the big variation in shortly dark-adapted samples (below five minutes) becomes visible (Figure 4B, C). These tested periods also showed unstable RLCs with high variations. When pre-darkening time exceeds one minute there can be no significant (95% Fisher's least significant difference) difference found in I_K and alpha values. The setup with 10 minutes of pre-darkening had some of the highest relative ETRs with low standard deviations. Runs with pre-darkening time exceeding 15 minutes already resulted in lower RLCs.

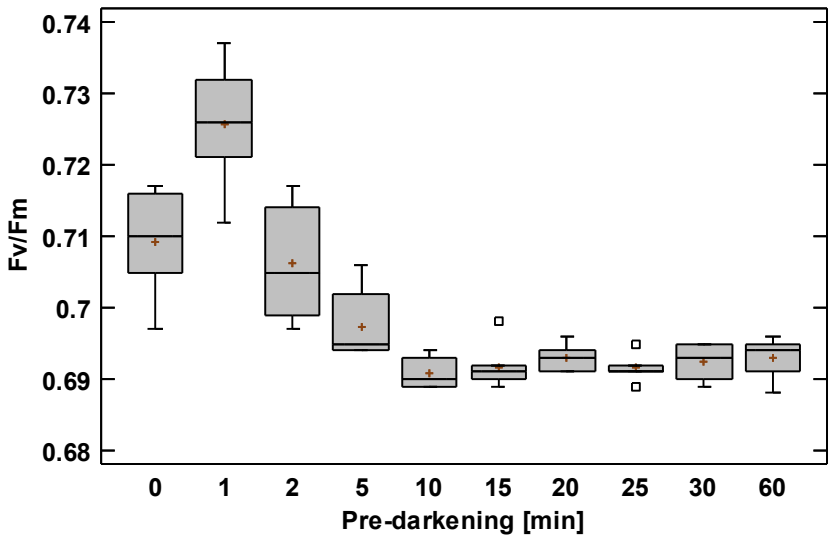

Figure 2 Box-and-whisker plot showing *Fv/Fm* of a *Chlorella* culture with increasing pre-darkening periods. (F (9, 60) = 37.56, $p < 0.001$, $n = 7$).

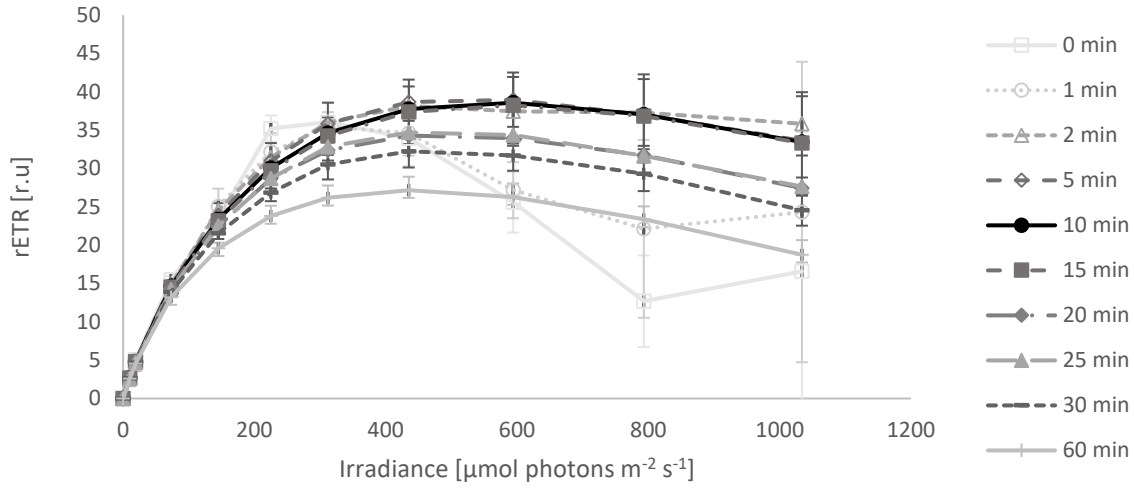

Figure 3 Mean RLCs of a *Chlorella* culture after increasing durations of pre-darkening. rETR in relative units was plotted against irradiance in µmol photons m^{-2} s^{-1}. Error bars show standard deviation ($n = 7$).

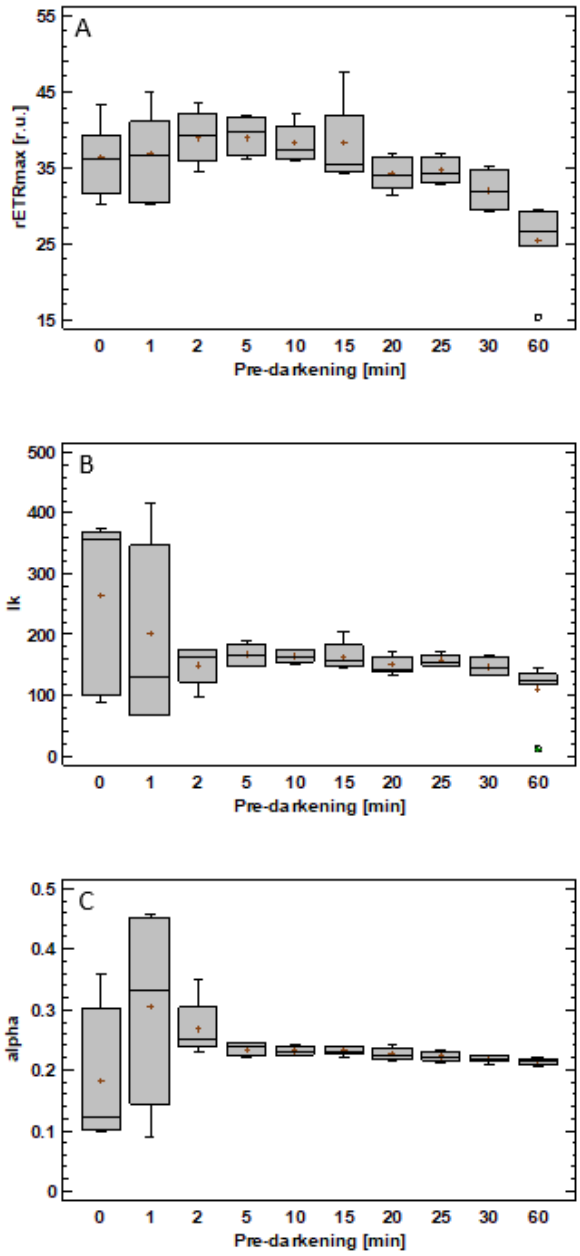

Figure 4 A: Box-and-whisker plot showing rETRmax values, grouped by pre-darkening time. The tested groups differ significantly in their rETRmax (F (3, 60) = 8.99, $p < 0.001$, $n = 7$). B: Box-and-whisker plot showing I_K values, grouped by pre-darkening time. The tested groups differ significantly in their I_K values (F (3, 60) = 2.63, $p < 0.015$, $n = 7$). C: Box-and-whisker plot showing alpha values, grouped by pre-darkening time. The tested groups differ significantly in their alpha values (F (3, 60) = 2.15, $p < 0.04$, $n = 7$).

Dilution of a culture to different *OD*s using various dilution media clearly revealed that dilution itself has a much bigger effect on readings than the media used for dilution. Four groups of RLCs can be differentiated: The undiluted sample had highest values for rETRmax and I_K (Figure 5A). Diluted samples are separated into two groups according to their dilution factor (Figure 5B): Cultures with $OD = 1$ have higher rETRs than those diluted to $OD = 0.5$. The negative control's samples treated with NaCl enriched medium showed hardly any electron transport. In figure 6A, rETRmax of the different dilutions are plotted in a box-and-whisker diagram. The samples grouped for *OD* were significantly different in rETRmax values employing the Fisher's least significant difference test at the 99.99 % level. The same is true for I_K ($F(2,46) = 302.10$, $p < 0.001$) and α

($F(2,46) = 101.85$, $p < 0.001$). Figure 6B shows a box-and-whisker plot with groups sorted according to the respective dilution medium to an *OD* = 1. The test with the undiluted original samples and the negative control samples diluted with NaCl enriched medium turned out to be significantly different from the rest employing the Fisher's least significant difference test at the 99.99 % level. In contrast, the three other trials (BG11, actual cultivation medium, reverse osmosis water) are all grouped together in one homologous group. Dilutions to an *OD* of 0.5 resulted in a similar outcome. Figure 6C shows that *Fv/Fm* of the same cultures significantly ($F(2,46) = 101.34$, $p < 0.001$) rises with higher *OD*. For *OD* = 0.5 *Fv/Fm* did not differ between tested dilution media (BG11, actual cultivation media, and reverse osmosis water; Figure 6D). Regarding *OD* = 1, reverse osmosis water showed increased *Fv/Fm* values compared to actual cultivation media and BG11 ($F(2,18) = 4.69$, $p < 0.03$).

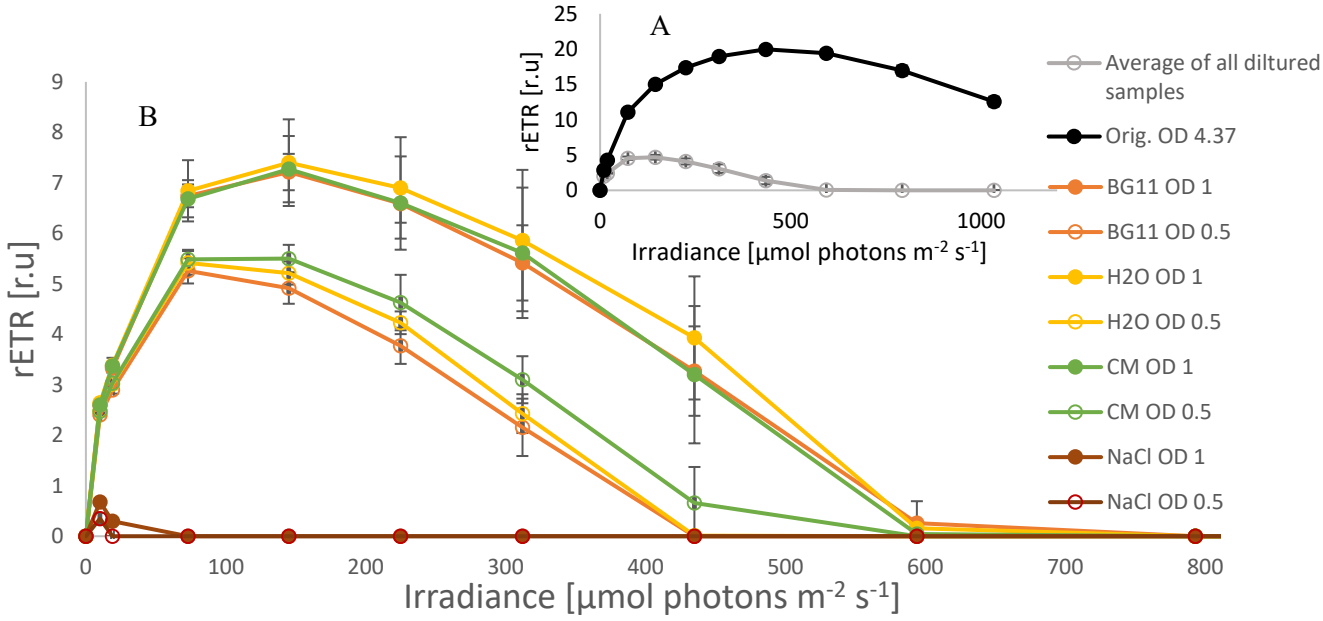

Figure 5 A: Overview showing RLC of original undiluted *Chlorella vulgaris* culture (Orig. *OD* = 4.37, black full circles, *n* = 7) versus average RLC of all diluted samples (grey empty circles, *n* = 56). rETR in relative units plotted against irradiance in µmol photons m^{-2} s^{-1}. Error bars show standard deviation. B: Detailed graph of mean RLCs of a *Chlorella vulgaris* culture diluted to *OD* = 1 (full symbols), *OD* = 0.5 (empty symbols). Various dilution media were applied BG11 (BG11, orange), reverse osmosis water (H_2O, yellow), actual cultivation medium (CM, green), and BG11 enriched with NaCl (NaCl, red). rETR in relative units plotted against irradiance in µmol photons m^{-2} s^{-1}. Error bars show standard deviation (*n* = 7).

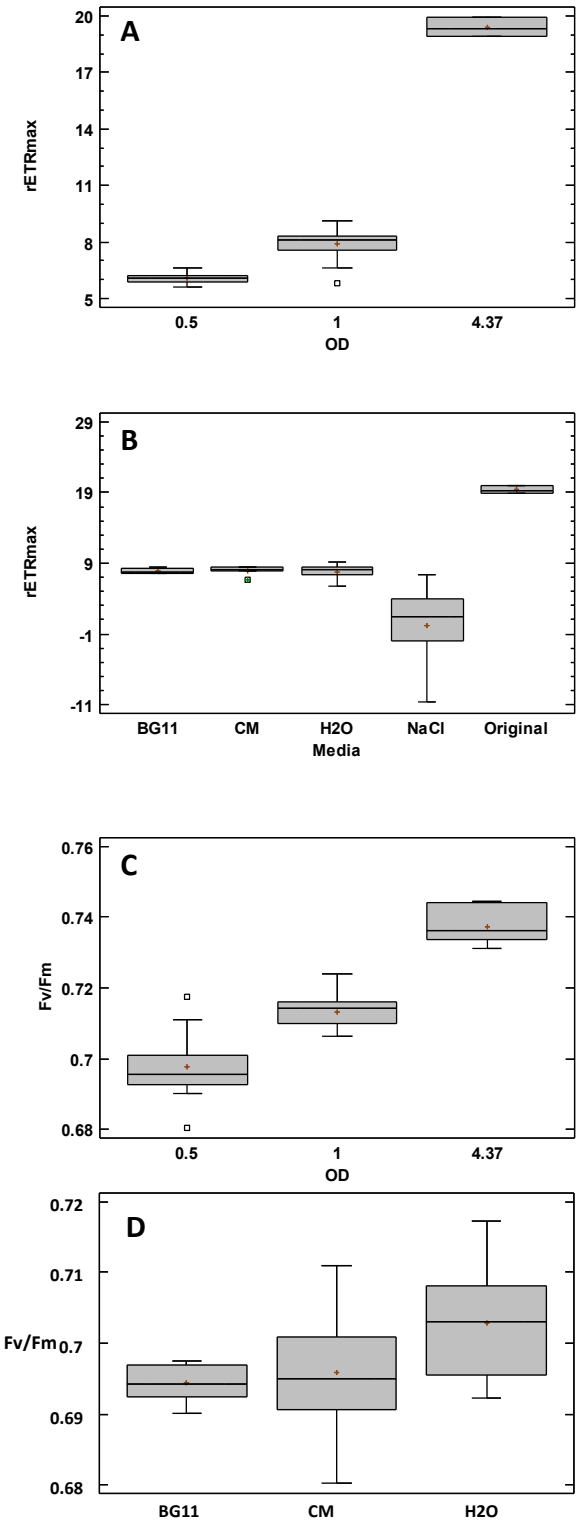

Figure 6 A: Box-and-whisker plot showing rETRmax values, grouped by the samples' *OD*. Negative control with NaCl enriched medium is excluded in this plot. The three tested groups of different *OD*s differ significantly in their rETRmax (F (2, 46) = 1717.08, $p < 0.001$, $n = 7$). B: Box-and-whisker plot showing rETRmax values grouped according to the media used for dilution. BG11, actual culture medium (CM), reverse osmosis water (H_2O) were used to dilute the samples to an *OD* of 1 and the undiluted culture (Original) had an *OD* of 4.37. Only the undiluted samples and the ones diluted with NaCl enriched medium differ from the other samples in rETRmax (F (4, 29) = 2295.99, $p < 0.001$, $n = 7$). C: Box-and-whisker plot of *Fv/Fm* values grouped by *OD*. Results of all tested media are combined except for NaCl enriched medium. The three densities showed to have significantly different values for *Fv/Fm* according to the Fisher's least significant difference test on the 99.99 % level (F (2,46) = 101.34, $p < 0.001$, $n = 7$). D: Box-and-whisker plot showing *Fv/Fm* grouped according to used dilution media for an *OD* = 0.5. *Fv/Fm* did not differ significantly between BG11, actual cultivation medium, and H_2O (F (2, 18) = 2.49, $p = 0.11$, $n = 7$).

To test, if an optimal range of suspension density for fluorescence measurements does exist, one culture was diluted to different *OD*s. Lower *OD*s resulted in lower rETRs (Figure 7). The dilution down to *OD* = 0.05 did not show any photoinhibition at highest irradiances but had a notably high standard deviation, especially at higher irradiances. This effect results from very similar *F* and *Fm'* values at higher actinic light levels leading to unstable Y(II) values. It was therefore excluded from further analyses. The resulting rETRmax values of the remaining dilutions are significantly different from each other at the 99.99 % level of Fisher's least significant difference (Figure 8A); the same pattern was found for I_K values (Fisher's least significant difference test at the 95 % level). Concerning α, only *OD* = 1 differed significantly from the others (Fisher's least significant difference 99.9 %). *Fv*/*Fm* values were smaller with lower density of the tested sample (Figure 8B). All three sample densities were significantly different concerning *Fv*/*Fm* according to Fisher's least significant difference of means with 99.9 %.

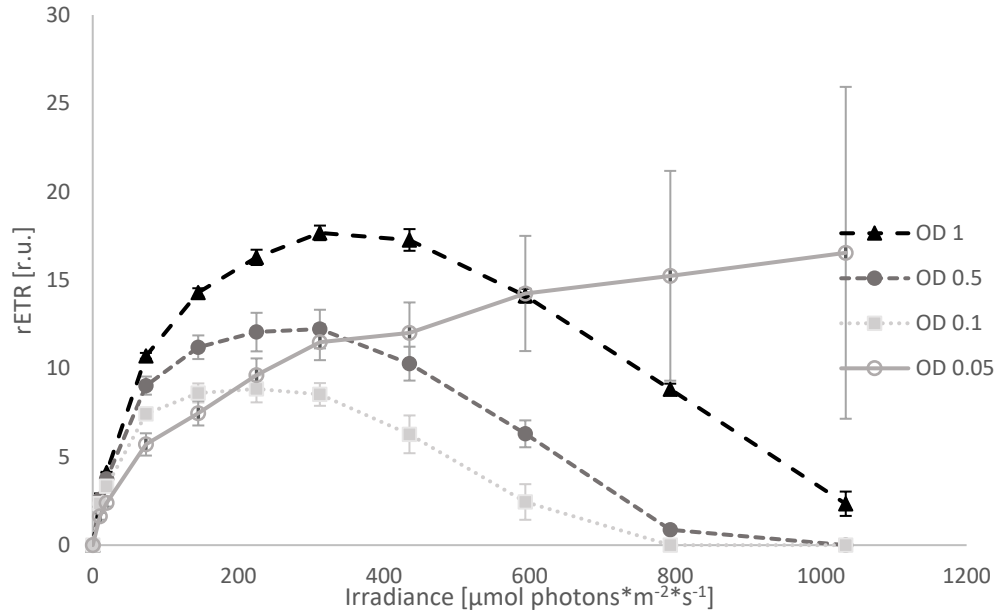

Figure 7 Mean RLCs of a *C. vulgaris* culture diluted to *OD*s of 1 (OD 1, triangles), 0.5 (OD 0.5, filled circles), 0.1 (OD 0.1, squares) and 0.05 (OD 0.05, empty circles). The sample with an *OD* of 0.05 shows that low density samples result curves with high standard deviation. Error bars represent standard deviation ($n = 7$).

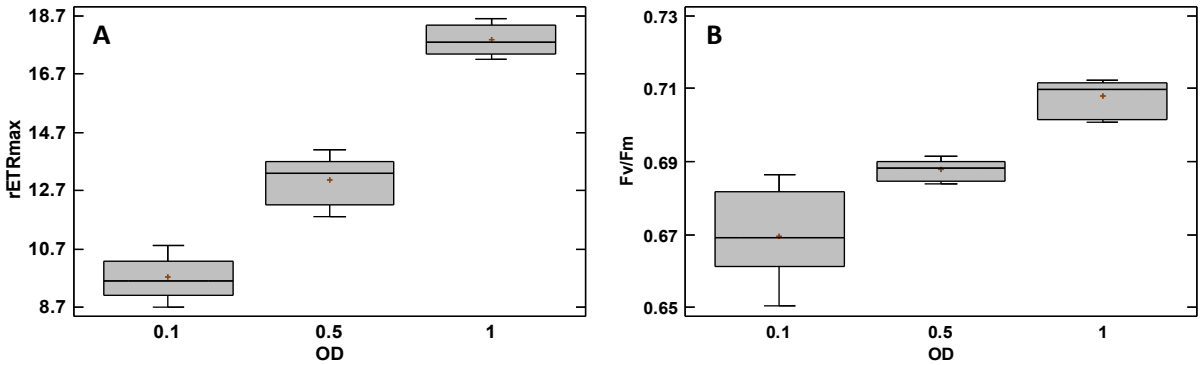

Figure 8 A*:* Box and whisker plot of rETRmax values of the same culture diluted to different *OD*s. All of them differed significantly ($F(2,18) = 239.28$, $p < 0.001$, $n = 7$). Samples with an *OD* of 0.05 are excluded from this plot. B*:* Box and whisker plot of *Fv/Fm* values, grouped by *OD*. All of them were significantly different ($F(2, 17) = 38.55$, $p < 0.001$). Data of *OD* = 0.05 are not considered (*OD* = 0.5 and *OD* = 1, $n = 7$; *OD* = 0.1, $n = 6$).

We compared suspensions and filtered samples and used two different cultures: a high light culture (*OD* = 0.469; *DM* = 0.124 g L^{-1}), and a dense low light culture (*OD* = 4.01; *DM* = 0.770 g L^{-1}). The plot of *Fv/Fm* values indicates that the high light adapted culture has very high variations in liquid state, while the values are more stable on filter (Figure 9). In the denser low light adapted culture the *Fv/Fm* values are quite similar on filter and in suspension. The adjustment of *OD* in the low light culture lead to a decrease in *Fv/Fm* becoming more similar to the high light adapted culture. Following groups are found to be significantly similar according to the Fisher's least significant difference test at a 95% level: High light on filter (HL Filter) and in solution (HL liquid); low light on filter (LL Filter) and low light in solution with adjusted *OD* (LL Liquid adj.). The plotted RLCs (Figure 10) resulted in substantially different shapes depending on both density and treatment. The diluted high light filtered sample represented a characteristic high light culture with high values of rETRmax and I_k. The opposite is true for the filtered low light culture. For the liquid culture, the pattern remains the same, but measurements resulted in more similar curves with less pronounced differences. If the dense low light culture was adjusted to an *OD* resembling that of the high light adapted culture, differences were again more pronounced.

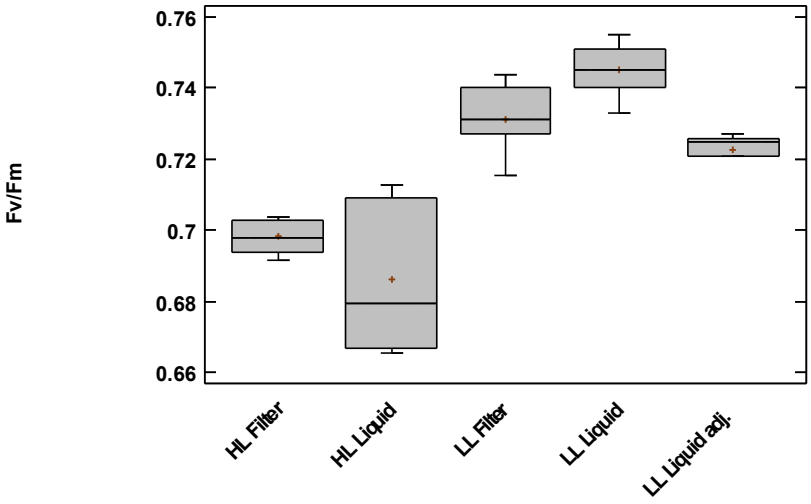

Figure 9 Box and whisker plot of *Fv/Fm* values of a high light (HL) and a low light culture (LL). Both were tested once on filter and once in suspension. Additionally, the LL culture was diluted to a similar *OD* as the HL culture (LL Liquid adj.) ($F(4,30) = 31{,}56$, $p < 0.0001$, $n = 7$).

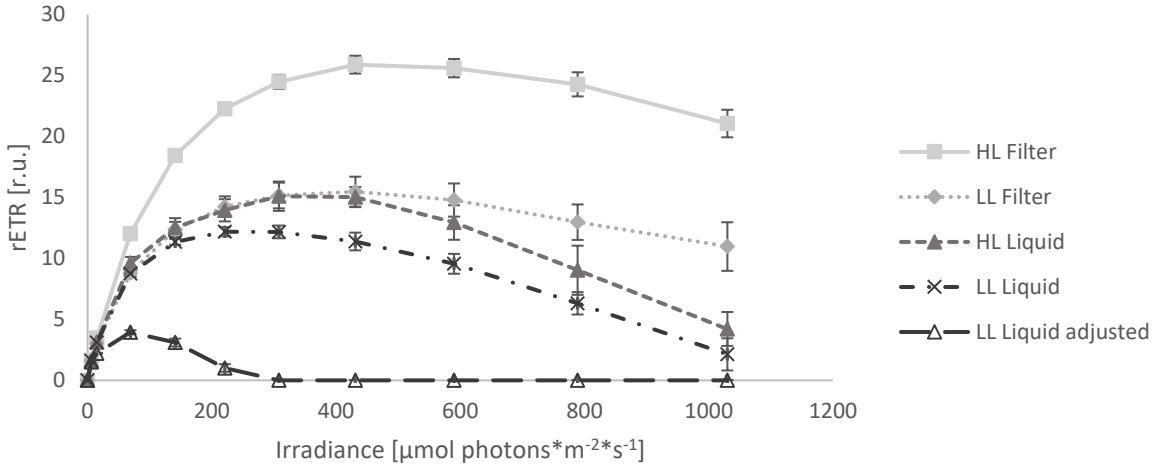

Figure 10 Mean RLCs of a dense low light (LL) and a diluted high light (HL) culture are plotted, showing that the samples measured on filters have more pronounced differences between LL and HL than the unadjusted liquid samples. Error bars show standard deviation (n = 7).

We performed a final application example to demonstrate effects of increasing density on fluorescence measurements. The growth curve of the batch culture shows the characteristic development with a short lag-phase followed by the exp-phase and the beginning of the stat-phase (Figure 11). The RLCs of undiluted samples taken on days 0, 2 and 6 clearly demonstrate large differences (Figure 12A). Day 0 has very low maximum rETR of 4.4 compared to day 2 with a maximum rETR of 20.2, and on day 6 a decrease to 15.1. If the samples are diluted to comparable densities as shown in figure 12B, the culture is hardly able to use any of the available light for electron transport after the actinic light reached intensities beyond 200 µmol photons m^{-2} s^{-1} on day 6. In both the diluted and undiluted test, maximum rETRs of day 2 exceeded the others, which points at optimum growth conditions. Big differences are found at day 6, with diluted samples clearly indicating low light acclimation and very strong photoinhibition at higher irradiances.

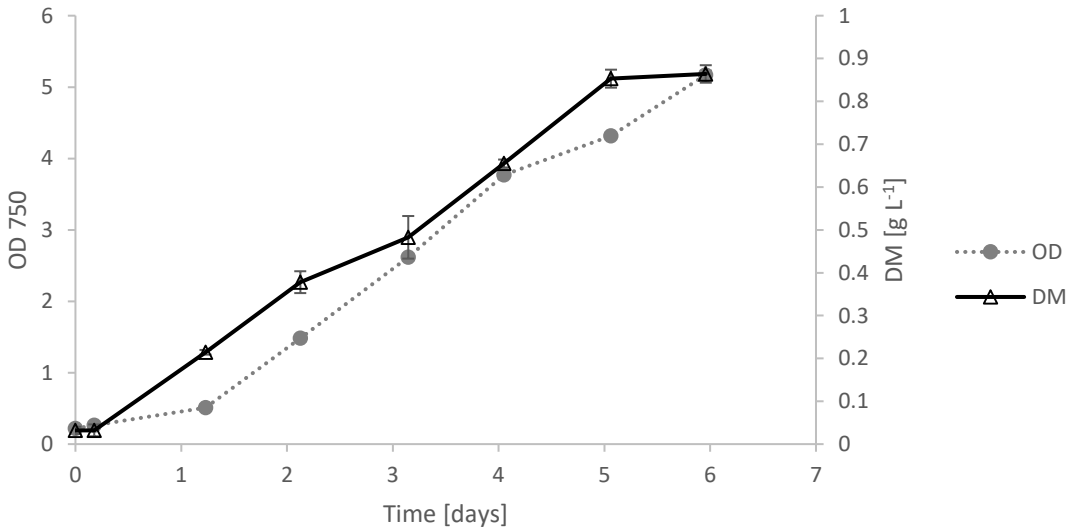

Figure 11 Growth of *C. vulgaris* culture represented by mean values of *OD* and *DM* are plotted against time. Samples were taken on day 0, 2 and 6. Error bars show standard deviation (n = 3).

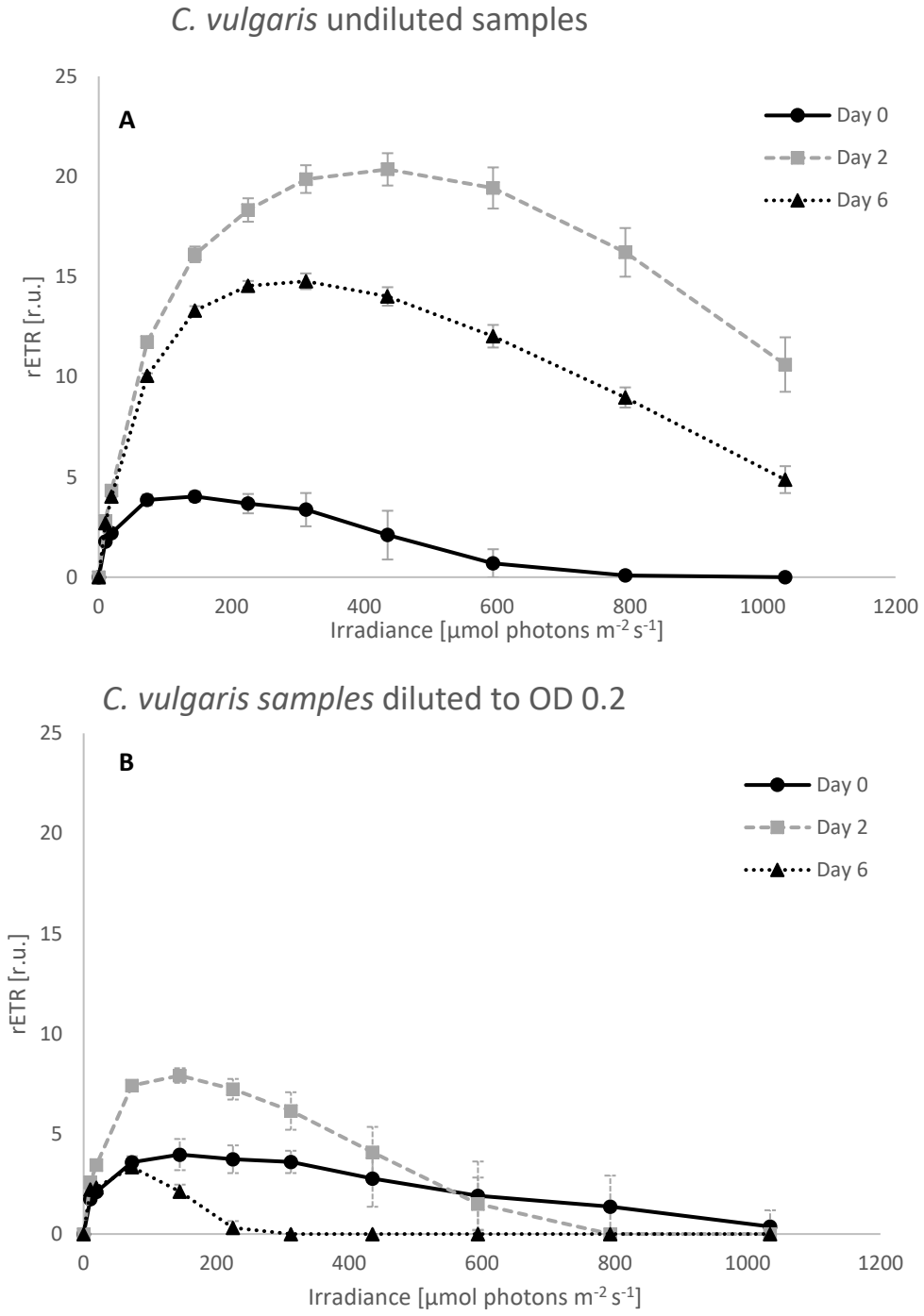

Figure 12 A: Mean RLCs of a *Chlorella vulgaris* culture on different days of cultivation. rETR values differ substantially depending on growth status. On starting day (Day 0, black circles), four hours after inoculation, an *OD* of 0.26 was measured. On the second day of cultivation (Day 2, grey squares) the culture had an *OD* of 1.48. Six days after inoculation (Day 6, black triangles) the culture reached an *OD* of 5.17. Error bars depict standard deviation ($n = 7$). B: Mean RLCs of a *Chlorella vulgaris* culture on different days of cultivation. Here, the samples were diluted to an *OD* of 0.2 before measurements were run. Fluorescence measurements were performed on inoculation day (Day 0, black circles, $n = 6$) and on the second (Day 2, grey squares, $n = 7$) and sixth day (Day 6, black triangles, $n = 7$) of cultivation. Error bars depict standard deviation.

4 Discussion

Both Chl-a and *OD* can were shown to be excellent predictors for *DM*. Nevertheless, this result should be treated with caution since ratios depend on culture age and status [44]. Specifically, *OD* measurements are easy and rapid to perform and recommended to use for determining biomass concentration [63]. This fact is relevant for fast growing cultures and applied fields because determination of *DM* and spectrophotometric Chl-a analysis take too long to make timely decisions. Furthermore, the necessary equipment and know-how is available in most applied algae culturing facilities. Nevertheless, *OD* is an indirect parameter for estimating algal biomass, providing only relative numbers and depending on size, shape and absorption properties of specimens. For each taxon, *OD* needs to be calibrated against direct biomass measurements. The same shortcomings are valid for Chl-a *in vivo* fluorescence measurements, although they are commonly used [33,34,64,65]. Chl-a monitoring systems are already available on the market and have the advantage of the possibility to perform *online* measurements. However, these readings should be interpreted with care, as interferences between the optical path and the sample are expected especially at higher cell densities. Moreover, only a small amount of Chl-a in intact cells shows fluorescence [66], and Chl-a per unit biomass may show big variations depending on culture conditions [67–69] and physiological state of the cells [69,70], which can lead to misinterpretation of biomass concentration [71].

The length of the pre-darkening period turned out to be crucial for *Fv/Fm* and RLC measurements. Suggested times start with five minutes of pre-darkening [45,46], which, however, were too short for generating reproducible values in our experiments. We suggest ten minutes of pre-darkening for stable, reproducible results with low variations, as it was also recommended by Malapascua and colleagues [33]. Also, RLCs with only brief pre-darkening periods as suggested in recent literature [46,72], turned out to be unreliable because of high variations of ETRs especially at increased light intensities. These effects could be caused by the high number of reduced photosystems. The high variation within the results of the short pre-darkening periods could be linked to the different proportions of oxidized to reduced photosystems between the replicates. On the other hand, if pre-darkening time was too long, cells already showed acclimation towards low light.

Concerning the dilution of samples, we could show that BG11 did not significantly influence overall performance of cultures concerning *Fv/Fm* when compared with actual culture medium as a control. However, we cannot draw any clear conclusions regarding the influence of dilution with reverse osmosis water on *Fv/Fm* values, since our results differed depending on the *OD*. The negative control, i.e. medium enriched with NaCl exceeding the tolerance limits of *Chlorella vulgaris* [73,74] led to substantial decrease of Y(II) and rETR. It needs to be highlighted, that dilution effects were rather caused by the process of sample dilution than by the medium.

Less diluted samples (higher *OD*) had increased values of rETRmax, I_k, and *Fv/Fm*, clearly demonstrating the effect of mutual shading: high cell density caused absorption of actinic light, which eventually leads to increased Y(II) and rETR.

Fv/Fm is used to get insight into the overall fitness of plants [61]. Our experiments however showed that measurements and interpretations need to be done carefully, considering suspension density. The higher the density of the tested sample the more mutual shading can be expected [12], thus influencing the amount of exciting photons. If cultures of highly differing densities are compared, fluorescence measurements might provide misleading information because of shortened light paths due to mutual shading [12,57,75]. It was also pointed out that such effects could influence the outcome of light response curves, which are comparable to RLCs [14,75]. Our experiments show that dilution of *OD*s of 0.1, 0.5, or 1 give reasonable CF values. Nevertheless, when comparing cultures of different cell number, density should be adjusted to one another to obtain reliable results. We are not able to give precise values

of *OD* for optimal outcome of the measurement. Rather we can talk about a range of sample density where mutual shading has no major impact and density is still high enough to give solid results with the given setup of the instrument. In our case we would recommend an *OD* that is higher than 0.05 but does not exceed 1.

It was pointed out that neglecting the relation between light absorption and sample density lead to a misapplication of light response curve measurements [75]. We would like to stress that similar trends can be seen in the application of RLCs.

Our experiments further indicated that PAM devices developed mainly for vascular plants [76], are not recommended for suspensions with low biomass. In our study, *OD* = 0.05 did not provide statistically reliable results [see also 51]. Here, concentrating cells without stressing them becomes obvious. We tested filtration followed by fluorescence measurements on filters, simulating a leaf surface. While we could not find a statistically relevant difference concerning *Fv/Fm* values between the diluted high light adapted culture in suspension or on filter, the dense low light culture shows that there is a significant decrease in *Fv/Fm* when the culture is filtered. We need to undertake further research to see if filtration of the sample might add some detectable stress to the culture. Contrarily to their liquid counterparts used as a control, obtained RLCs of filter material resulted in characteristic high light and low light curves as it was expected from the initial growth conditions. Respective suspension measurements without adjusting density resulted in comparable RLCs. The picture, however, changed drastically when the low light culture was diluted to the same *OD* as the high light sample. The density adjustment resulted in typical curves resembling those of the filter approach, but with one difference: rETR of the high-density sample adjusted to low-density is generally low and showed barely any electron transport at PAR > 300 µmol s^{-1} m^{-2} (Figure 10). More representative graphs were obtained when the samples of different densities were compared after filtration, and mutual shading effects were minimized. The expected differences of the cultures were still pronounced, and the range of actinic light steps is used sufficiently by the sample leading to curves with higher resolution.

Our findings enable us to show that filtration of the sample could help to overcome two challenges linked to fluorescence measurements of liquid algal cultures: First, the effects of different densities can be minimized, and secondly, the samples are not diluted to ranges where the signal becomes weak. This becomes notably helpful when very diluted cultures need to be compared to denser cultures. However, further tests are needed to clarify if the potential effects of filtration cause stress on the tested cells.

We combined the new findings in an application example to highlight the risks of misinterpretations if density was neglected for PAM measurements. Usually, measurements of high light adapted organisms result in RLCs with high maximum ETR and I_K [19,77–79]. In our experiment, we observed that after a lag phase, visible in both, biomass parameters and lowered RLCs, cells had optimal growth conditions already on the second day after inoculation with increased RLCs. The sixth day of cultivation can be seen as the onset of the stat-phase, visible in biomass parameters and lowered RLCs. The undiluted samples however showed still unexpectedly high RLCs because of strong interferences of the sample with the optical path (absorption, scattering of actinic light). The results from density-adjusted samples clearly reflect true acclimation towards low light.

When working with liquid algal cultures, RLCs are not only determined by the status of acclimation but also by sample density. These two factors are antagonists, as high *OD* causes mutual shading and thereby lower actinic light penetration. Therefore, the status of light acclimation cannot be directly inferred from RLCs when working with liquid algal cultures of different density.

5 Conclusion

Not only culture conditions, but also density of the measured sample have substantial effects on the results of fluorescence measurements. Highly concentrated suspensions result in increased rETRmax values and high *Fv/Fm* values indicating high light acclimation and high fitness of cultures, which, however, might not reflect reality. Our data highly suggest the dilution of the sample. We were able to show that *OD* is applicable to adjust sample densities, as this is a fast and easy method that can be used in most standard laboratories [44,63]. For drawing correct conclusions for culture developments, we suggest using samples with *ODs* between 0.05 and 1. Denser samples lead to effects of self-shading, so we recommend diluting dense cultures with media similar to the cultivation media. It needs to be stressed that for a proper comparison on two different samples their densities need to be set to similar values. We found pre-darkening essential not only for *Fv/Fm*, but also for RLCs. A pre-darkening period of ten minutes showed to be an ideal compromise between stable values and no interference with light acclimation towards darkness. Additionally, we found a novel approach of how to apply PAM fluorescence measurements on algal suspensions in a similar way as it is done on vascular plants, by filtering them to create a leaf-like surface of cells to overcome effects of culture density.

6 Acknowledgment

We are grateful to Mag. Dr. Silvia Fluch and all the colleagues from Eparella GmbH, who allowed me to use equipment and facilities for my studies. Special thanks to my family and especially Johanna Köllner who supported me as an assistant during the measurements, in proof reading and by motivating me.

7 Attachment

BG11

Table 1 Recipe for BG11

Components	Stock [g L^{-1} dH_2O]	Quantity Used per Liter Medium [ml]	Final Concentration [µM]
Citric Acid * H_2O	6	1	28.553
NH_4 Fe^{III} citrate	6	1	29.203
$NaNO_3$	150	10	17.648.097
K_2HPO_4	30	1	172.216
$MgSO_4$ * 7 H_2O	76	1	308.354
$CaCl_2$ * 2 H_2O	32	1	217.672
Na_2CO_3	20	1	188.697
NaFeEDTA	1	1	2.724
H_3BO_3	2.860	1	46.256
$MnCl_2$ * 4 H_2O	1.810	1	9.146
$ZnSO_2$ * 7 H_2O	0.222	1	0.772
Na_2MoO_4 * 2 H_2O	0.39	1	1.612
$CuSO_4$ * 5 H_2O	0.08	1	0.32
$Co(NO_3)_2$ * 6 H_2O	0.05	1	0.172
HEPES	200	1	839.274
Adjust pH to 7.5 with NaOH			

Summary

Chlorophyll fluorescence techniques have been applied since the middle of the 19th century. Guidelines on how to perform and interpret measurements are on hand for higher plants, but it is hard to find protocols with regards to algal suspensions. We encountered this lack of information as we focused on the growing industry of micro algal mass production, where dense suspensions of algal cells are the object of interest. We faced some challenges comparing fluorescence data of algal cultures of different concentrations. As fluorescence measurements are optical methods, we hypothesized that mutual shading could cause interference with the measuring lights. To test this, we performed a series of experiments to investigate how to counteract the effects of sample density during the measurements. We chose to work with *Chlorella* cultures and a PAM-2500 (Walz) equipped with a suspension chamber KS-2500 (Walz) to show how the concentration of cells in a sample changes the outcome of the measurements. First, we tested what biomass parameter fits best for evaluating algal cell concentration. Optical density turned out to be the most feasible method to determine and adjust density. It is faster and easier to assess optical density than dry mass or Chlorophyll-a content. We also addressed another issue that had not been in focus sufficiently enough: pre-darkening duration before measurements. This is the first step to perform certain fluorescence measurement techniques like rapid light curves and the determination of *Fv/Fm*. We could show that depending on how long samples are pre-darkened mean

Fv/Fm values can differ more than 60 %. We consider ten minutes as a proper pre-darkening duration because values gained with this setup or longer pre-darkening times had low standard deviation, and also rapid light curves show most satisfying results after a ten-minute long pre-darkening period. With our work, we could show that the density of a tested sample has indeed influences on the fluorescence-measurements' outcome. Due to our gained knowledge, we can also present a way of adjusting the liquid algal sample density to overcome issues of mutual shading by high cell concentration. We diluted our samples with different media and could show that dilution with adequate medium shows no significant differences compared to the control media. These results hint that a sample can be diluted without adding measurement artefacts to the outcome. We also applied the gained knowledge on a batch culture to see how a properly performed adjustment of density affects the outcome of the measurements. We saw differences of nearly 50 % in some parameters of rapid light curves when comparing the same culture once diluted to an *OD* of 1 versus an *OD* of 0.1. This protocol was eventually tested in an application example. This applied test gave insight into how drastic the effects of mutual shading and how misleading the outcome of such measurements could be. Finally, we successfully tested filtration as a method allowing to increase sample density and to overcome effects of mutual shading by filtering samples onto a paper filter to create a flat surface of photosynthetically active cells resembling a leaf. This method allowed us to gather solid data without any need for adjustment of sample density and risking the quality of our measurements because of the scattering we observed when very diluted samples were tested.

By this work, we managed to highlight the pitfalls that are involved in fluorescence measurements of high-density liquid algal culture. Based on our findings, we offer solutions of how to overcome the challenges involved, and thereby paved the way for further research concerning the optimisation of fluorescence measurements.

Zusammenfassung

Chlorophyll Fluoreszenz Techniken werden seit der Mitte des 19. Jahrhunderts angewandt. Richtlinien zur Verwendung der Methode an Gefäßpflanzen gibt es zur Genüge. Allerdings erwies es sich als schwierig, Protokolle zur Testung von flüssigen Algenproben zu finden. Wir stießen auf dieses Problem, da es unser Wunsch war, die Chlorophyll Fluoreszenz Messungen im wachsenden Sektor der Mikroalgenkultivierung anzuwenden. In diesem Bereich wird vor allem mit sehr dichten Algensuspensionen gearbeitet. Als die größte Herausforderung erwies sich der Vergleich zweier unterschiedlich dichter Kulturen. Da es sich bei den erwähnten Fluoreszenz Messungen um optische Messungen handelt, lautete unsere Hypothese, dass die Selbstbeschattung der Probe Einfluss auf die Ergebnisse der Messungen hat. Eine Reihe von Experimenten wurde aufgesetzt um dies zu überprüfen. Als Testobjekte wurde *Chlorella* Kulturen eingesetzt und Fluoreszenz Messungen wurden mittels eines PAM-2500 (Walz) und einer Suspensionsküvette KS-2500 (Walz) durchgeführt.
Als erster Schritt wurden verschiedene Wachstumsparameter verglichen, um zu testen welche Methode sich als geeignetste herausstellen würde. Besonderes Augenmerk wurde auf die Durchführung der Messung gelegt. Optische Dichte hat sich wegen der einfacheren und schnelleren Bestimmung im Vergleich zu Trockenmasse und Chlorophyllgehalt als vorteilhaft erwiesen. Ein anderes Thema, welches unserer Meinung nach nicht genug Aufmerksamkeit erhält, ist die Verdunklungszeit der Probe vor der Messung. Dieser erste Schritt ist notwendig bei „*rapid light curve*“ Messungen und der Bestimmung der *Fv/Fm* Werten. Wir konnten zeigen, dass *Fv/Fm* Werte je nach Verdunklungsdauer Unterschiede von bis zu 60% aufzeigen. Wir empfehlen 10-minütige Dunkelzeiten vor den Messungen. Diese Periode resultierte in geringen Schwankungen der *Fv/Fm* Werte und die erhalten rapid light curves hatten die höchsten Elektronentransportraten. Mit unserer Arbeit konnten wir außerdem zeigen, dass die Dichte der Proben tatschlich Auswirkungen auf die erhalten Werte hat. Mit dem erhaltenen Wissen konnten wir eine Vorgehensweise präsentieren, die Dichte von Proben anzupassen, bevor eine Messung durchgeführt wird. Die Rede ist von Verdünnungen. Um sicher zu gehen, dass durch diesen Schritt keine Stress-Artefakte entstehen, wurden unterschiedliche Verdünnungsmedien getestet. Die Daten zeigen,

dass vollwertiges Medium problemlos verwendet werden kann um die Dichte der Probe zu adaptieren. All unsere gesammelten Erfahrungen wurden an einer Batch Kultur getestet, um zu sehen, wie drastisch die Auswirkungen der Dichte sein können. Dieselbe Probe wurde sowohl mit einer optischen Dichte von 1 als auch 0,1 getestet. Manche Messwerte wiesen Unterschiede von bis zu 50% auf. In einem weiteren angewandten Versuch wurden bei einer Kultur während der Wachstumsphase mehrmals Fluoreszenz Messungen durchgeführt. Hier wurden die drastischen Auswirkungen der unterschiedlichen Dichten am besten sichtbar und wir konnten zeigen, wie sinnvoll eine Anpassung der Zelldichte sein kann. Schließlich wurde noch eine Filtration als alternative Methode zur Verdünnung getestet. Durch Filtration der Kulturen erhält man eine fast zweidimensionale Fläche von photosynthetisch aktiven Zellen, die einem Blatt ähnelt. Durch das Messen dieser Filter konnten wir ohne Verdünnung die Unterschiede der Dichte und der Selbstbeschattung ausgleichen. Die Technik verhindert außerdem, die Verdünnungen der Probe in störanfällige Bereiche, die zu schwer interpretierbaren Ergebnissen führen.

Mit dieser Arbeit konnten wir die Schwierigkeiten, die beim Messen von Fluoreszenzdaten hochkonzentrierter Algenkulturen auftreten, aufzeigen. Mit unseren Lösungsvorschlägen ist es möglich, den Effekten entgegenzuwirken. Dadurch ebneten wir den Weg für weitere Forschung und Optimierungen in diesem Bereich.

Outlook and future studies

We would like to point out that in this study we only targeted the methodological part of the topic, so no biological replicates were considered. In future works we would include also biological replicates for all the setups. We could not focus on all issues that might influence chlorophyll fluorescence measurements in liquid algal cultures. One important aspect that needs to be addressed is the setup of the PAM device. In previous tests we could see that the duration and intensity of the different lights can impact certain fluorescence values. Further tests could also put the spotlight on different algal strains with various morphological features. In future experiments semi-continuous cultivation should be preferred over batch cultivation.

8 Literature

[1] G. Torzillo, J. Masojídek, M. Koblížek, Photosynthesis in Microalgae, in: A. Richmond, Q. Hu (Eds.), Handb. Microalgal Cult. Appl. Phycol. Biotechnol., 2nd ed., John Wiley & Sons, 2013: pp. 21–36. doi:10.1007/978-1-4020-9237-4.

[2] G. Renger, Overview of Primary Processes of Photoynthesis, in: G. Renger (Ed.), Prim. Process. Photosynth. Princ. Appar., RSC Publishing, Cambridge, 2007: pp. 5–35. doi:10.1039/9781847558152-00001.

[3] G.H.. Krause, CHLOROPHYLL FLUORESCENCE AND PHOTOSYNTHESIS: The Basics, Annu. Rev. Plant Physiol. Plant Mol. Bioi. 42 (1991) 313–49. doi:10.1146/annurev.pp.42.060191.001525.

[4] H. Kautsky, A. Hirsch, Neue Versuche zur Kohlensäureassimilation, Naturwissenschaften. 19 (1931) 964–964. doi:10.1007/BF01516164.

[5] M.I. Queiroz, A.S. Fernandes, M.C. Deprá, E. Jacob-Lopes, L.Q. Zepka, Introductory Chapter: Chlorophyll Molecules and Their Technological Relevance, in: E. Jacob-Lopes (Ed.), Chlorophyll, IntechOpen, 2017: p. 64. doi:10.5772/32009.

[6] A. Jablonski, Efficiency of Anti-Stokes Fluorescence in Dyes, Nature. 131 (1933) 839. doi:10.1038/131839b0.

[7] D.O. Hall, K.K. Rao, Photosynthesis, 6th ed., Cambridge University Press, 1999. doi:10.1016/S0176-1617(00)80259-X.

[8] N.R. Baker, Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo, Annu. Rev. Plant Biol. 59 (2008) 89–113. doi:10.1146/annurev.arplant.59.032607.092759.

[9] K. Maxwell, G.N. Johnson, Chlorophyll fluorescence—a practical guide, J. Exp. Bot. 51 (2000) 659–668. doi:10.1093/jexbot/51.345.659.

[10] K. Roháček, J. Soukupová, M. Barták, Chlorophyll fluorescence: A wonderful tool to study plant physiology and plant stress, in: B. Schoefs (Ed.), Plant Cell Compart., Research Signpost, Kerala, India Plant, 2008: pp. 41–104.

[11] A. Richmond, Handbook of Microalgal Culture: Biotechnology and Applied Phycology, Blackwell Science, Oxford, 2004. doi:10.1002/9780470995280.

[12] M.R. Tredici, Photobiology of microalgae mass cultures: understanding the tools for the next green revolution, Biofuels. (2010). doi:10.4155/bfs.09.10.

[13] W. Mäntele, E. Deniz, UV–VIS absorption spectroscopy: Lambert-Beer reloaded, Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 173 (2017) 965–968. doi:10.1016/J.SAA.2016.09.037.

[14] A. Richmond, Biological Principles of Mass Cultivation of Photoautotrophic Microalgae, in: A. Richmond, Q. Hu (Eds.), Handb. Microalgal Cult. Appl. Phycol. Biotechnol. Appl. Phycol. Biotechnol., 2nd ed., Wiley-Blackwell, West Sussex, 2013: pp. 171–204. doi:10.1002/9780470995280.

[15] P.J. Ralph, R. Gademann, Rapid light curves: A powerful tool to assess photosynthetic activity, Aquat. Bot. 82 (2005) 222–237. doi:10.1016/J.AQUABOT.2005.02.006.

[16] M. Consalvey, R.G. Perkins, D.M. Paterson, G.J.C. Underwood, Pam Fluorescence: A Beginners Guide for Benthic Diatomists, Diatom Res. 20 (2005) 1–22. doi:10.1080/0269249X.2005.9705619 To.

[17] A.A. Fernandez-Jaramillo, C. Duarte-Galvan, L.M. Contreras-Medina, I. Torres-Pacheco, R. de J. Romero-Troncoso, R.G. Guevara-Gonzalez, J.R. Millan-Almaraz, Instrumentation in Developing Chlorophyll Fluorescence Biosensing: A Review, Sensors. 12 (2012) 11853–

11869. doi:10.3390/s120911853.

[18] E.H. Murchie, T. Lawson, Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications, J. Exp. Bot. 64 (2013) 3983–3998. doi:10.1093/jxb/ert208.

[19] H.R. Bolhar-Nordenkampf, G. Oquist, Chlorophyll fluorescence as a tool in photosynthesis research, in: D.O. (David O. Hall, J.M.O. Scurlock, H.R. Bolhar-Nordenkarnpf, R.C. Leegood, S.P. Long (Eds.), Photosynth. Prod. a Chang. Environ. a F. Lab. Man., R.C. Leego, C!taprnan & Hall, London, 1993: pp. 193–206. doi:10.1007/978-94-011-1566-7.

[20] Govindjee, Chlorophyll a Fluorescence: A Bit of Basic and History, in: G.C. Papageorgiou, Govindjee (Eds.), Chlorophyll a Fluoresc. A Signiture Photosynth., Springer, 2004: pp. 1–41. doi:10.1007/978-1-4020-3218-9.

[21] G. Torzillo, P. Bernardini, J. Masojídek, On-Line Monitoring of Chlorophyll Fluorescence to Assess the Extent of Photoinhibition of Photosynthesis Induced by High Oxygen Concentration and Low Temperature and Its Effect on the Productivity of Outdoor Cultures of Spirulina Platensis (Cyanobacteria), J. Phycol. 34 (1998) 504–510. doi:10.1046/j.1529-8817.1998.340504.x.

[22] N.R. Baker, K. Oxborough, Chlorophyll Fluorescence as a Probe of Photosynthetic Productivity, in: G.C. Papageorgiou, Govindjee (Eds.), Chlorophyll a Fluoresc. A Signiture Photosynth., Springer, 2004: pp. 65–82. doi:10.1007/978-1-4020-3218-9_3.

[23] N.R. Baker, E. Rosenqvist, Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities, J. Exp. Bot. 55 (2004) 1607–1621. doi:10.1093/jxb/erh196.

[24] C. Honeywill, D. Paterson, S. Hagerthey, Determination of microphytobenthic biomass using pulse-amplitude modulated minimum fluorescence, Eur. J. Phycol. 37 (2002) 485–492. doi:10.1017/S0967026202003888.

[25] A.J. White, C. Critchley, Rapid light curves: A new fluorescence method to assess the state of the photosynthetic apparatus, Photosynth. Res. 59 (1999) 63–72. doi:10.1023/A:1006188004189.

[26] J. Masojídek, A. Vonshak, G. Torzillo, Estimating Aquatic Productivity from Active Fluorescence Measurements, in: Chlorophyll a Fluoresc. Aquat. Sci. Methods Appl., Springer Netherlands, Dordrecht, 2010: pp. 227–293. doi:10.1007/978-90-481-9268-7_6.

[27] P. Roger, N2-fixing Cyanobacteria as Biofertilizers in Rice Fields, in: A. Richmond (Ed.), Handb. Microalgal Cult. Biotechnol. Appl. Phycol., Blackwell Science, Oxford, 2004: pp. 329–402. doi:10.1002/9780470995280.

[28] H. Tamiya, Mass Culture of Algae, Annu. Rev. Plant Physiol. 8 (1956) 309–334. doi:10.1146/annurev.pp.08.060157.001521.

[29] J. Serô, Á.S. Vieira, Á.S. Cruz, Á.H. Coelho, J.O. Serô, A. Só, V. Ae, S. Nia, C. Ae, H. Coelho, Rapid light-response curves of chlorophyll fluorescence in microalgae: relationship to steady-state light curves and non-photochemical quenching in benthic diatom-dominated assemblages, Photosynth Res. 90 (2006) 29–43. doi:10.1007/s11120-006-9105-5.

[30] E. Houliez, S. Lefebvre, F. Lizon, F.G. Schmitt, Rapid light curves (RLC) or non-sequential steady-state light curves (N-SSLC): which fluorescence-based light response curve methodology robustly characterizes phytoplankton photosynthetic activity and acclimation status?, Mar. Biol. 164 (2017) 175. doi:10.1007/s00227-017-3208-8.

[31] J.U. Grobbelaar, Physiological and technological considerations for optimising mass algal cultures, J. Appl. Phycol. 12 (2000) 201–206. doi:10.1023/A:1008155125844.

[32] C.G. Jerez, J.R. Malapascua, M. Sergejevová, J. Masojídek, F.L. Figueroa, Chlorella fusca (Chlorophyta) grown in thin-layer cascades: Estimation of biomass productivity by in-vivo chlorophyll a fluorescence monitoring, Algal Res. 17 (2016) 21–30. doi:10.1016/J.ALGAL.2016.04.010.

[33] J. Malapascua, C. Jerez, M. Sergejevová, F. Figueroa, J. Masojídek, Photosynthesis monitoring to optimize growth of microalgal mass cultures: application of chlorophyll fluorescence techniques, Aquat. Biol. 22 (2014) 123–140. doi:10.3354/ab00597.

[34] G. Torzillo, C. Faraloni, A.M. Silva, J. Kopecký, J. Pilný, J. Masojídek, Photoacclimation of Phaeodactylum tricornutum (Bacillariophyceae) cultures grown outdoors in photobioreactors and open ponds, Eur. J. Phycol. 47 (2012) 169–181. doi:10.1080/09670262.2012.683202.

[35] A. Babaei, K. Ranglová, J.R. Malapascua, J. Masojídek, The synergistic effect of Selenium (selenite,-SeO 3 2−) dose and irradiance intensity in Chlorella cultures, 7 (2017) 56. doi:10.1186/s13568-017-0348-7.

[36] L.J. Cooney, H.M. Schaefer, B.A. Logan, B. Cox, K.S. Gould, Functional significance of anthocyanins in peduncles of Sambucus nigra, Environ. Exp. Bot. 119 (2015) 18–26. doi:10.1016/J.ENVEXPBOT.2015.03.001.

[37] K. Chen, X. Sun, E. Amombo, Q. Zhu, Z. Zhao, L. Chen, Q. Xu, J. Fu, High correlation between thermotolerance and photosystem II activity in tall fescue, Photosynth. Res. 122 (2014) 305–314. doi:10.1007/s11120-014-0035-3.

[38] W. Hu, X. Lv, J. Yang, B. Chen, W. Zhao, Y. Meng, Y. Wang, Z. Zhou, D.M. Oosterhuis, Effects of potassium deficiency on antioxidant metabolism related to leaf senescence in cotton (Gossypium hirsutum L.), F. Crop. Res. 191 (2016) 139–149. doi:10.1016/J.FCR.2016.02.025.

[39] C. Büchel, C. Wilhelm, In Vivo Analysis of Slow Chlorophyll Fluorescence Induction Kinetics in Algae: Progress, Problems and Perspectives, Photochem. Photobiol. 58 (1993) 137–148. doi:10.1111/j.1751-1097.1993.tb04915.x.

[40] A.M. Wood, R.C. Everroad, L.M. Wingard, Measuring Growth rates in microalgal cultures, in: R.A. Anderson (Ed.), Algal Cult. Tech., Elsevier, 2005: pp. 269–285.

[41] L.H.S. Silva, V.L.M. Huszar, M.M. Mainho, L.M. Rangel, J. Brasil, C.D. Domingure, C.C. Branco, F. Roland, Drivers of phytoplankton, bacterioplankton, and zooplankton carbon biomass in tropical hydroelectric reservoirs, Limnologica. 48 (2014) 1–10. doi:10.1016/J.LIMNO.2014.04.004.

[42] V. Gosselain, P.B. Hamilton, J.-P. Descy, Estimating phytoplankton carbon from microscopic counts: an application for riverine systems, Hydrobiologia. 438 (2000) 75–90. doi:10.1023/A:1004161928957.

[43] A.D. Steinman, P.R. Leavitt, D.G. Uzarski, Biomass and Pigments of Benthic Algae, in: Methods Stream Ecol. Vol. 1, 3rd ed., Academic Press, 2017: pp. 223–241. doi:10.1016/B978-0-12-416558-8.00012-3.

[44] M.J. Griffiths, C. Garcin, R.P. van Hille, S.T.L. Harrison, Interference by pigment in the estimation of microalgal biomass concentration by optical density, J. Microbiol. Methods. 85 (2011) 119–123. doi:10.1016/j.mimet.2011.02.005.

[45] J. Seroio, A.S. Vieira, A.S. Cruz, A.F. Barroso, Short-term variability in the photosynthetic activity of microphytobenthos as detected by measuring rapid light curves using variable fluorescence, Mar. Biol. 164 (2004). doi:10.1007/s00227-004-1504-6.

[46] S. Lefebvre, J. Mouget, J. Lavaud, Duration of rapid light curves for determining the photosynthetic activity of microphytobenthos biofilm in situ, 95 (2011) 1–8. doi:10.1016/j.aquabot.2011.02.010.

[47] J. Kromkamp, J. Beardall, A. Sukenik, J. Kopecky, J. Masojidek, S. van Bergeijk, S. Gabai, E. Shaham, A. Yamshon, Short-term variations in photosynthetic parameters of Nannochloropsis cultures grown in two types of outdoor mass cultivation systems, Aquat. Microb. Ecol. 56 (2009) 309–322. doi:10.3354/ame01318.

[48] E. Pfündel, Junior-PAM Chlorophyll Fluorometer Operator's Guide, (2017). http://www.walz.com/downloads/manuals/junior-pam/jpm_071206.pdf (accessed April 18, 2018).

[49] D.J. Suggett, O. Prásil, M. Borowitzka, Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications, 1st ed., Springer Netherlands, Dordrecht, 2010. doi:10.1007/978-90-481-9268-7.

[50] S. Lippemeier, R. Hintze, K. Vanselow, P. Hartig, F. Colijn, In-line recording of pam fluorescence of phytoplankton cultures as a new tool for studying effects of fluctuating nutrient supply on photosynthesis, Eur. J. Phycol. 36 (2001) 89–100. doi:10.1080/09670260110001735238.

[51] B. Genty, J.-M. Briantais, N.R. Baker, The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence, Biochim. Biophys. Acta - Gen. Subj. 990 (1989) 87–92. doi:10.1016/S0304-4165(89)80016-9.

[52] M. Cuaresma, M. Janssen, C. Vilchez, R.H. Wijffels, Productivity of Chlorella sorokiniana in a Short Light-Path (SLP) Panel Photobioreactor Under High Irradiance, Wiley Intersci. (2009). doi:10.1002/bit.22394 ABSTRACT:

[53] A. Sánchez Mirón, M.-C. Cerón García, F.G. Camacho, E. Molina Grima, Y. Chisti, Growth and biochemical characterization of microalgal biomass produced in bubble column and airlift photobioreactors: studies in fed-batch culture, 2002.

[54] B. Demmig-Adams, W.W. Adams, Responses of plants to high light stress, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 599–626. doi:10.1146/annurev.pp.43.060192.003123.

[55] K. Shelly, D. Holland, B. John, Assessing Nutrient Status of Microalgae Using Chlorophyll a Fluorescence, in: D.J. Suggett, M.A. Borowitzka, O. Prásil (Eds.), Chlorophyll a Fluoresc. Aquat. Sci. Methods Appl., 1st ed., Springer, 2011: pp. 223–235. doi:10.1007/978-90-481-9268-7.

[56] J.G. Day, J.J. Brand, Cryopreservation Methods for Maintaining Microalgal Cultures, in: R.A. Anderson (Ed.), Algal Cult. Tech., 2005: pp. 165–187.

[57] J. Cosgrove, M. Borowitzka, Applying Pulse Amplitude Modulation (PAM) fluorometry to microalgae suspensions: Stirring potentially impacts fluorescence, Photosynth. Res. 88 (2006) 343–350. doi:10.1007/s11120-006-9063-y.

[58] A. Holzinger, K. Franziska, K. Blaas, B. Zechmann, K. Komsic-Buchmann, B. Becker, Reaction, Transcriptomics of Desiccation Tolerance in the Streptophyte Green Alga Klebsormidium Reveal a Land Plant-Like Defense, PLoS One. 9 (2017). doi:10.1371/journal.pone.0110630.

[59] M.M. Allen, Simple Conditions for Growth of Unicellular Blue-Green Algae on Plates, J. Phycol. 4 (1968) 1–4. doi:10.1111/j.1529-8817.1968.tb04667.x.

[60] R.Y. Stanier, R. Kunisawa, M. Mandel, G. Cohen-Bazire, Purification and Properties of Unicellular Blue-Green Algae (Order Chroococcales), 1971. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC378380/pdf/bactrev00074-0091.pdf (accessed June 3, 2019).

[61] H. Walz, Portable Chlorophyll Fluorometer PAM-2500 Handbook of Operation, (2008) 110. http://walz.com/downloads/manuals/pam-2500/PAM_2500_03pp.pdf (accessed November 2, 2017).

[62] P.H.C. Eilers, J.C.H. Peeters, A model for the relationship between light intensity and the rate of photosynthesis in phytoplankton, Ecol. Modell. 42 (1988) 199–215. doi:10.1016/0304-3800(88)90057-9.

[63] J.A. Myers, B.S. Curtis, W.R. Curtis, Improving accuracy of cell and chromophore concentration measurements using optical density, BMC Biophys. 6:4 (2013). doi:https://doi.org/10.1186/2046-1682-6-4.

[64] C.S. Ting, T.G. Owens, Limitations of the Pulse-Modulated Technique for Measuring the Fluorescence Characteristics of Algae, 1992. doi:10.1104/pp.100.1.367.

[65] S. White, A. Anandraj, F. Bux, PAM fluorometry as a tool to assess microalgal nutrient stress and monitor cellular neutral lipids, Bioresour. Technol. (2010) 1675–1682. doi:10.1016/j.biortech.2010.09.097.

[66] J. Cao, Govindjee, Chlorophyll a fluorescence transient as an indicator of active and inactive Photosystem II in thylakoid membranes, 1990. http://www.life.illinois.edu/govindjee/Cao_Govindjee_1990.pdf (accessed July 4, 2019).

[67] Q. Hu, Environmental Effects on Cell Composition, in: A. Richmond (Ed.), Handb. Microalgal Cult. Biotechnol. Appl. Phycol., Oxford, 2004: pp. 83–93.

[68] S. Boussiba, W. Bing, J.-P. Yuan, A. Zarka, F. Chen, Changes in pigments profile in the green alga Haeamtococcus pluvialis exposed to environmental stresses, 1999. doi:10.1023/A:1005507514694.

[69] E. Álvarez, E. Nogueira, Á. López-Urrutia, In Vivo Single-Cell Fluorescence and Size Scaling of Phytoplankton Chlorophyll Content, (2017). doi:10.1128/AEM.03317-16.

[70] Impact of seconds to minutes photoperiods on Chlorella vulgaris growth rate and chlorophyll a and b content, Algal Res. 36 (2018) 10–16. doi:10.1016/J.ALGAL.2018.10.007.

[71] M. Kruskopf, K.J. Flynn, Chlorophyll content and fluorescence responses cannot be used to gauge reliably phytoplankton biomass, nutrient status or growth rate, New Phytol. 169 (2006) 525–536. doi:10.1111/j.1469-8137.2005.01601.x.

[72] R.G. Perkins, J. Lavaud, J. Serôdio, J.L. Mouget, P. Cartaxana, P. Rosa, L. Barille, V. Brotas, B.M. Jesus, Vertical cell movement is a primary response of intertidal benthic biofilms to increasing light dose, Mar. Ecol. Prog. Ser. 416 (2010) 93–103. doi:10.3354/meps08787.

[73] L.E. Den Dooren de Jong, Tolerance of Chlorella vulgaris for metallic and non-metallic ions, Antonte van Leeuwenhoek 31. 31 (1965) 301–313. doi:https://doi.org/10.1007/BF02045910.

[74] R. Singh, A.K. Upadhyay, P. Chandra, D.P. Singh, Sodium chloride incites reactive oxygen species in green algae Chlorococcum humicola and Chlorella vulgaris: Implication on lipid synthesis, mineral nutrients and antioxidant system, Bioresour. Technol. 270 (2018) 489–497. doi:10.1016/J.BIORTECH.2018.09.065.

[75] A. Richmond, Microalgal biotechnology at the turn of the millennium: A personal view, J. Appl. Phycol. 12 (2000) 441–451. doi:10.1023/A:1008123131307.

[76] U. Schreiber, U. Schliwa, W. Bilger, Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer, Photosynth. Res. 10 (1986) 51–62. doi:10.1007/BF00024185.

[77] Y. Sharon, O. Levitan, D. Spungin, I. Berman-Frank, S. Beer, Photoacclimation of the seagrass Halophila stipulacea to the dim irradiance at its 48-meter depth limit, Limnol. Ocean. 56 (2011) 357–362. doi:10.4319/lo.2011.56.1.0357.

[78] E. Bañ Ares-Españ, J.C. Kromkamp, V. Ló Pez-Rodas, E. Costas, A. Flores-Moya, Photoacclimation of cultured strains of the cyanobacterium Microcystis aeruginosa to high-light and low-light conditions, (n.d.). doi:10.1111/1574-6941.12025.

[79] S. Cruz, J. Serôdio, Relationship of rapid light curves of variable fluorescence to photoacclimation and non-photochemical quenching in a benthic diatom, (2007). doi:10.1016/j.aquabot.2007.11.001.