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Data collection was performed by AMVV. Data analysis was done by AMVV and GP. TM and GP conceived and designed the experiments. All authors wrote the manuscript.

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Conflicts of interest
The authors declare they have no conflict of interest.

The robustness of NextGen-O2k for building PI curves in microalgae

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Abstract

The rate of oxygen evolution provides valuable information on the metabolic status and the photosynthetic performance of a cell, and it can be quantified by means of a photosynthesis-irradiance (PI) curve. Up to now, the construction of PI curves of unicellular organisms based on oxygen evolution has been difficult and time consuming due to the lack of sensitive instruments. Here we describe the set up of a reproducible method for constructing PI curves based on oxygen evolution using low amounts of sample in the microalga *Nannochloropsis gaditana*, easily translable to other algal species.

Keywords – photosynthesis, microalgae, oxygen evolution

1. Introduction

Photosynthetic eukaryotic organisms rely on two organelles, chloroplasts and mitochondria, for the synthesis of the molecules fueling their metabolism, NAD(P)H and ATP. The two organelles share common features such as membrane bound enzymatic complexes implicated in electron transfer coupled to proton translocation and the generation of a proton motive force driving the synthesis of ATP.

In chloroplasts, light energy fuels the electron transport from water to $NADP^+$ to generate NADPH via photosystem II (PSII), cytochrome b_6f (Cyt b_6f) complex and photosystem I (PSI), generating oxygen as byproduct. The electron transport is coupled to proton translocation from the chloroplast stroma into the thylakoid lumen and establishes a transmembrane electrochemical potential, exploited by the ATP synthase for the synthesis of ATP in a process called photophosphorylation.

Photosynthetic eukaryotes also present mitochondria, where electrons are transferred from the substrates NADH and succinate to molecular oxygen. In the dark, respiration is responsible of cells energy supply, but it is also active in the light when its activity is important for carbon fixation and optimal photosynthesis (Bailleul et al, 2015).

The same way as oxygen consumption is used as a proxy for quantifying the electron transport through the mithocondrial ETS and the respiratory activity, photosynthesis activity and the rate of electron transport through the plastidial electron transport system (pETS) can be quantified by the rate of oxygen evolution.

Oxygen evolution and, thus, the photosynthetic rate, depends on light intensity. At low intensities, photosynthesis is limited by the light availability and thus the energy to drive electron transport. Therefore, in this range, the relationship between light intensity and electron transport (and thus oxygen evolution) is linear. This linearity is lost at elevated light intensities where other factors (i.e. rate of carbon fixation) become limiting for photosynthesis and any further increase of photons does not result in a higher rate of electron transport. On the contrary, when photosynthesis is saturated at high intensities, light excess can drive to generation of Reactive Oxygen Species (ROS) and photoinhibition.

This relationship between light intensity and photosynthetic rate is depicted by the so-called PI curve. Historically, the proxy for photosynthesis quantification in microalgae has been the of rate of carbon fixation measured as ^{14}C-carbon assimilation (for examples see Perry, Talbot, and Alberte 1981; Jassby and Platt 1976). Another proxy for PI curves is the rate of evolution of molecular oxygen, as it is the direct acceptor of electrons that move through the pETS. Compared to CO_2, which is only indirectly linked to the transport of electrons as other metabolic pathways compete for the ATP and reducing power, O_2 is a better proxy for building PI curves. Quantification of O_2 was done first with the so-called clear and dark bottle method described in Strickland 1960, and then using low-resolution devices based on Clark's type electrodes. However, these techniques have limitations mainly due to the difficulty in controlling precisely the amount of light that reaches the sample. Also, they usually require elevated concentrations of cells, which can bring to inhomogenity due to internal shading.

The high resolution of the NextGen-O2k and the PhotoBiology module (PB-Module) allow to build PI curves with an unprecedented accuracy. The increased resolution comes from the two components of the system. First, the core FluoRespirometer is more sensitive than any of the common Clark's electrode-based oxygraphs popular in the labs of plant research. This makes it possible to do measurements of oxygraphy using a very low amount of sample, as we demonstrate here. Second, the PB-Module permits a very fine tuning of the light intensity that reaches the sample. The combination of these two factors makes it so that PI curves can be obtained in a fast and reproducible manner.

In this work we *i*) analyze the ability of the device to build PI curves with high resolution in short times and using low amounts of material and *ii*) titrate different cell concentrations and check for consistency and reproducibility of the measurements done in different days and from different samples.

We use as a model the oleaginous alga *Nannochloropsis gaditana*, an heterokont that is receiving a growing interest for its industrial applications.

2. Methods

2.1. Algal cultures

Nannochloropsis gaditana, strain CCAP 849/5, was purchased from the Culture Collection of Algae and Protozoa (CCAP) and maintained in F/2 solid media, with 32 g L^{-1} sea salts (Sigma Aldrich), 40 mM Tris-HCl (pH 8), Guillard's (F/2) marine water

enrichment solution (Sigma Aldrich), 1% agar (Duchefa Biochemie). Cells were pre-cultured in sterile F/2 liquid media in Erlenmeyer flasks irradiated with 100 µmol of photons m^{-2} s^{-1}, 100 rpm agitation, at 22 ± 1 °C in a growth chamber. Growth curves started from 5 $\times 10^6$ cells · mL^{-1} in F/2 supplemented with 10 mM $NaHCO_3$ to avoid carbon limitation and were kept in the same growth conditions of pre-cultures.

2.2. Cell counting

Cell concentration was monitored at the fourth day of the growth curve with an automatic cell counter (Cellometer Auto X4, Cell Counter, Nexcelom), to collect the different numbers of cells needed for high resolution respirometry. For high concentrations, cells were collected via mild centrifugation at 3,500 g for 10 minutes at room temperature.

2.3. High resolution respirometry

Oxygen consumption and production were measured at the 4th day of the growth curves set-up as described above. Measurements were performed at 22 °C using the NextGen-O2k and the PB-Module (Oroboros Instruments, Innsbruck), with the software DatLab 7.4.0.4. The measuring chambers were magnetically stirred at 750 rpm and the oxygen concentration of the chambers was measured with a frequency of 2 seconds. The light source was a blue LED (emitting wavelength range 439-457 nm with the peak at 451 nm) attached to the chamber of the instrument (provided by Oroboros Instruments, manufactured by Osram Oslon).

The 2-mL O2k-chambers were filled with growth medium containing 5 mM $NaHCO_3$ to avoid carbon limitation during the measurement and let equilibrate to experimental temperature (22 °C) for few minutes. Then, a small fraction of medium was replaced with an aliquote of cell suspension to reach the desired final concentration in chamber. The chambers were then closed and the oxygen consumption rate at dark was calculated.

2.4. Light curve protocol

After stabilization of the dark respiration signal, light was turned on at 10 µmol of photons m^{-2} s^{-1} until stabilization of the oxygen flow, indicatively 5-10 min (see Figure 1). This was done recursively for the following light intensities: 10, 25, 50, 75, 100, 150 and 200 µmol of photons m^{-2} s^{-1}. The reported values of oxygen evolution rates at each intensity correspond to the median of 40-50 points in the stable region of oxygen flow (pink regions in Figure 1A).

We automated the process by running the following script (read from left to right, one row at a time):

```
waitfor;600          pblue;10          pbrue;10
waitfor;300          pblue;25          pblue;25
waitfor;300          pblue;50          pbrue;50
waitfor;300          pblue;75          pbrue;75
waitfor;300          pblue;100         pbrue;100
waitfor;300          pblue;150         pbrue;150
waitfor;300          pblue;200         pbrue;200
waitfor;300          pblue;0           pbrue;0
```

2.5. Data analysis

After each experiment, the slope uncorrected data were exported from DatLab and analyzed with a spreadsheet template provided by Oroboros. For building PI curves, we used the oxygen flow normalized to cell concentration expressed in million cells: pmol of O_2 $s^{-1} \cdot 10^6$ $cells^{-1}$.

The data were fitted to the equation defined in Ye 2007 using a minimum mean square error-based approach with OriginPro Version 2020b (OriginLab Corporation, Northampton, MA, USA). After the fitting, we calculated the value of the light compensation point (I_{comp}), dark respiration rate (R_D) and maximal gross photosynthetic rate (P_{gmax}) parameters.

3. Results

3.1. Establishment of a protocol for PI curves

First tests were run with different cells concentrations, observing that 5-10 $\times 10^6$ cells $\cdot$ mL^{-1} were sufficient to obtain traces of good quality as shown in Figure 1.

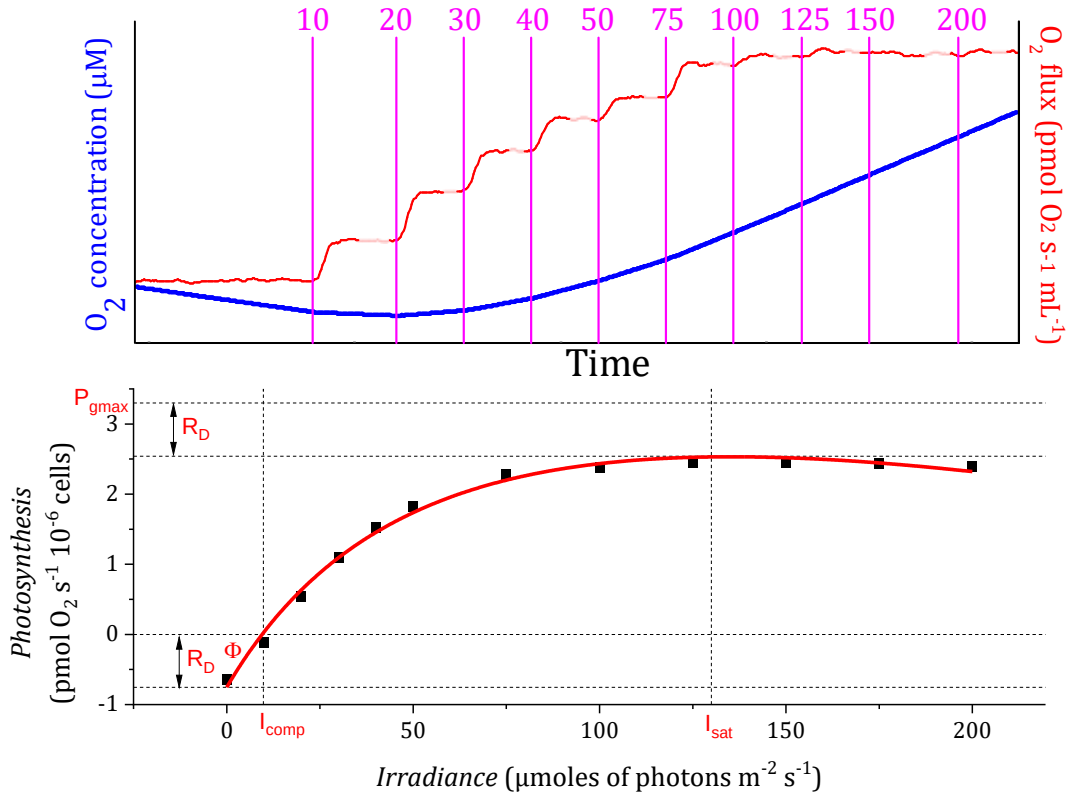

Figure 1. Construction of a PI curve. (A) Simplified diagram of a typical experiment as visualized by DatLab. After each increase in light intensity (vertical lines, values in µmol of photons m^{-2} s^{-1}), the O_2 flux (red plot, time-derivative of oxygen concentration in blue) first increases an then stabilizes. At high light intensities a plateau is reached. **(B)** Fitting of a PI curve to the data reported in A. Each point represents the median of 30-40 datapoints of the stable region of O_2 flux at each intensity (pink segments in A). The following parameters are shown: initial slope (Φ), compensation point (I_{comp}), rate of dark respiration (R_D), rate of maximal gross photosynthesis (P_{gmax}), saturation point (I_{sat}).

At a working concentration of 10 $\times 10^6$ cells · mL^{-1} (corresponding to 0.8 µg chlorophyll (Chl) mL^{-1}, see the corresponding plot in Figure 2), the rate of dark respiration was estimated as 0.95 ± 0.15 pmol O_2 s^{-1} · mL^{-1}. We increased light intensity progressively starting from 10 µmol of photons m^{-2} s^{-1}. After each light increase the O_2 flux stabilized within 2-4 minutes. The light compensation point (*i.e.* the point at which net photosynthesis is null) was estimated to be at 9.5 ± 1.2 µmol of photons m^{-2} s^{-1}, while saturation was reached at 148 ± 37 µmol of photons m^{-2} s^{-1}.

From first experiments as the one reported in Figure 1, we established and automated a protocol to increase the light every 5 minutes, followed for all the measurements reported hereinafter:

1) Add the sample into the chamber.
2) Wait 10 minutes to measure dark respiration.
3) Turn light on at 10 µmol of photons m^{-2} s^{-1}.
4) Measure for 5 minutes.
5) Increase the light to 25 µmol of photons m^{-2} s^{-1} followed by 50, 75, 100, 150, 200 µmol of photons m^{-2} s^{-1}, measuring for 5 minutes after each light change. These intervals were chosen as the most informative to describe the PI curve shape.
6) Wash the chambers and start next experiment.

A complete experiment lasted less than 1 h, allowing us to perform up to 10 experiments per day, each in two replicas, in the two chambers.

3.2. Testing the effect of cell concentration in PI curves

Once we defined our working pipeline, we performed different sets of measurements to check for the ideal range of cell concentration. In our samples, the chlorophyll content was approx. 0.8 µg of chlorophyll per million cells. It should be mentioned that *Nannochloropsis* cells are quite small (diameter of 2-3 µm). Working concentrations with other microalgae such as *Chlamydomonas*, that have a 10 µm diameter and thus approx. 50 times larger cellular volume, should be approx. 25-50 times smaller.

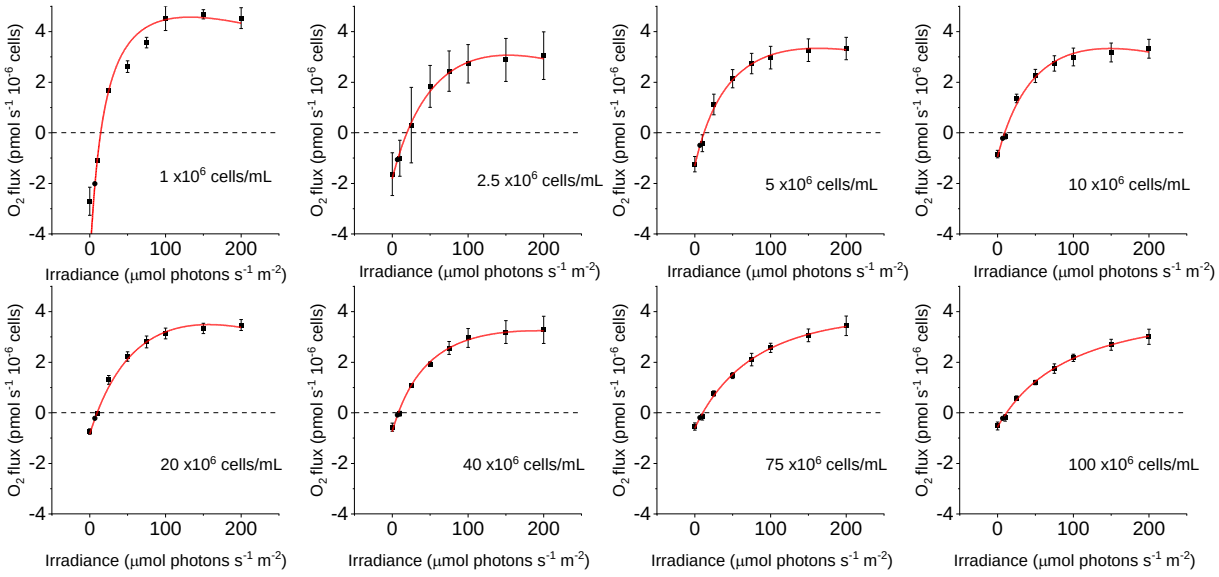

Figure 2. PI curves at cell concentrations ranging from 2.5 to 100 $\times 10^6$ cells · mL^{-1} and the photosynthetic parameters inferred from fitting.

We tested the following concentrations: 1, 2.5, 5, 10, 20, 40, 75 and 100 $\times 10^6$ cells · mL^{-1}. The respective PI curves are shown in Figure 2. Lower concentrations were also tested but were not retained as the signal was too noisy. In all the considered cases, the data points followed the expected shape represented in Figure 1B and could be fitted accurately to our PI model. We calculated the values of photosynthetic parameters reported in Table 1.

Table 1. Photosynthetic parameters at different cell concentrations.

Cell concentration (10^6 cells · mL^{-1})	Φ	I_{com}	I_{sat}	R_D	P_{gmax}
1	0.36 ± 0.10	14.6 ± 0.5	133 ± 59	5.28 ± 1.29	9.9 ± 1.2
2.5	0.09 ± 0.02	19.5 ± 2.3	153 ± 21	1.82 ± 0.21	4.9 ± 0.2
5	0.11 ± 0.01	12.2 ± 1.1	163 ± 27	1.31 ± 0.12	4.6 ± 0.2
10	0.1 ± 0.02	9.5 ± 1.2	149 ± 37	0.95 ± 0.16	4.3 ± 0.3
20	0.09 ± 0.01	9.9 ± 0.7	157 ± 26	0.85 ± 0.12	4.3 ± 0.2
40	0.09 ± 0.01	8.1 ± 1.0	194 ± 135	0.74 ± 0.17	4.0 ± 0.6
75	0.06 ± 0.01	10.7 ± 1.1	339 ± 449	0.62 ± 0.1	4.3 ± 1.2
100	0.05 ± 0.01	11.9 ± 1.1	597 ± 1889	0.57 ± 0.09	4.3 ± 2.5

Three groups of concentrations could be defined. First, for 1 and 2.5 $\times 10^6$ cells · mL^{-1}, data variability was larger, especially at low light intensities where signal was lower. The raw signal per volume (pmol O_2 s^{-1} · mL^{-1}) was low, affecting the signal-to-noise ratio. These cell concentrations are not optimal, even though these limitations could be addressed by increasing the number of experimental replicates and proloning sampling times.

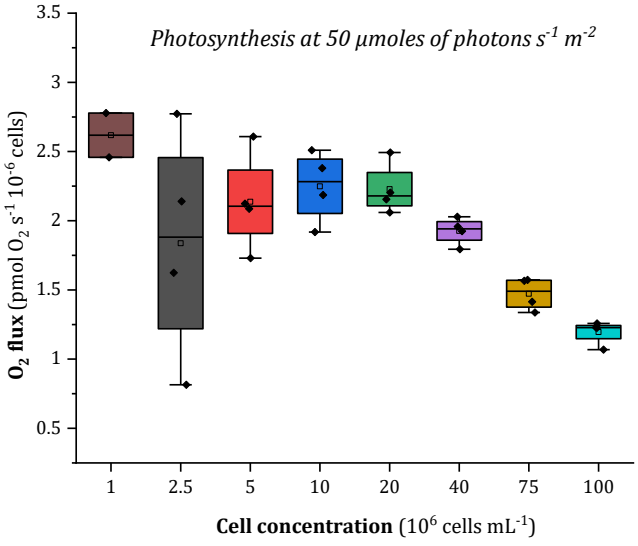

Figure 3. Comparison of O_2 flux values at a mid-light intensity (50 µmol of photons m^{-2} s^{-1}) at different cell concentrations.

Second, the range comprised between 5 and 20 $\times 10^6$ cells · mL^{-1} showed no major differences in terms of curve shape or internal variability among replicas. Since the signal was normalized to the number of cells this consistency suggest that the measurements are stable and not influenced by the cells concentration in this range (Figure 3). This also suggests that light shading is not affecting the measurements. Concentrations within this range seem optimal for future PI curves experiments.

Last, for 40, 75 and 100 $\times 10^6$ cells · mL^{-1}, there was instead an effect due to the cells concentration, especially during the linear phase (Figure 3). The light saturation point was higher with higher concentrations, likely explained because at higher concentrations there was a shading effect between cells due their high optical density that caused an inhomogeneous light distribution in the sample. This hypothesis is confirmed by the observation that P_{gmax} was the same for all concentrations. This suggests that even at high cell concentrations the value of maximal photosynthesis is correctly estimated because self shading is not a factor anymore when light is in excess.

4. Conclusions

Our results demonstrate that the protocol developed for building PI curves of *Nannochloropsis gaditana* cultures with the NextGen-O2k Fluororespirometer is robust and reproducible. The possibility of making programmed light changes enable to perform multiple measurements with limited time and efforts. The ideal working concentrations are those in the range of 5-10 $\times 10^6$ cells $\cdot$ mL^{-1}, corresponding to 0.4-0.8 µg Chl mL^{-1}. In our conditions, a 20 mL 4 days-old culture of *Nannochloropsis* was typically concentrated 25-45 $\times 10^6$ cells $\cdot$ mL^{-1}. Therefore, a single measurement needed a volume of culture of 200-800 µL, negligible in the total culture volume. Consequently, the same culture can be used for other experiments, optimizing the use of cultures. This can be of particular interest for strains with a reduced growth rate. The reproducibility of our approach makes it possible to compare the photosynthetic performance of different strains, mutants or conditions.

Another major advantage of working with low concentrations is that it is possible to work in a range where self shading effects are negligible. This is particularly important to study the effect of light on the photosynthesis parameters.

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