Journal of Fluorescence, Vol. 10, No. 3, 2000

Measurement of Light and pH Dependence of Single-Cell Photosynthesis by Fluorescence Microscopy

Jan F. H. Snel[1,2] and Hans H. A. Dassen[1]

Received October 1, 1999; revised February 1, 2000; accepted February 2, 2000

Measurement of algal photosynthetic performance with conventional methods requires thousands of cells obtained by isolation and subsequent cultivation. This is a time-consuming process for many species. We describe a new method to study photosynthetic performance of single algal cells under various environmental conditions by a combination of modulated chlorophyll fluorescence, light microscopy, and sample manipulation techniques. Single cell fluorescence was measured with a modulated microfluorometer integrated in an inverted microscope. The algal cell was sucked onto the tip of a glass microcapillary and positioned in the center of the field of view of the microscope by a micromanipulator. A superfusion device was used to generate a flow of experimental solution of variable composition along the alga. The light dependence of *Scenedesmus obtusiusculus* single-cell photosystem II (PSII) electron flow was measured at various pH. At a high light intensity PSII electron flow was inhibited at pH 6.5 and higher, while at a low light inhibition occurred at pH 9.5. This is in agreement with inhibition of photosynthesis by substrate (CO_2) limitation at alkaline pH. This approach can easily be extended to study the *in vivo* effects of other abiotic parameters (temperature, nutrients, toxicants, oxygen) on the photosynthetic performance of algae.

KEY WORDS: Algae; ecophysiology; electron flow; micromanipulation; photosystem II; *Scenedesmus*.

INTRODUCTION

Management of marine and freshwater ecosystems requires information about the structure and function of the system. One of the key parameters in the food web of an ecosystem is primary biomass production. Photosynthesis is the first step in biomass production and photosynthesis begins in the photosystems with the absorption of light by chlorophyll, the major light harvesting pigments in plants and eukaryotic algae. A photosystem (PS) consists of a light harvesting system and a reaction center where photochemistry takes place. The excitation energy is transferred from the light harvesting chlorophyll to a primary donor in the reaction center where a charge separation occurs. The photosynthetic apparatus of plants and algae contain two types of photosystems, called PSI and PSII, which operate in series to oxidize water and reduce $NADP^+$. The charge separations in PSII lead to electron flow and result in the oxidation of water and the reduction of plastoquinone. PSI activity results in the oxidation of plastoquinol and the reduction of $NADP^+$. During this electron flow from water to $NADP^+$, vectorial proton transport leads to a proton motive force which is used by the ATP synthase/hydrolase to phosphorylate ADP. The NADPH and ATP are required for the reduction of CO_2 to sugars.

The efficiency of photosynthesis can be estimated from the efficiency of PSII electron flow, which can be determined from chlorophyll fluorescence yield measurements with the saturating pulse method [1; see Refs. 2 and 3 for reviews]. Measurements of photosynthesis by

[1] Laboratory for Plant Physiology, Wageningen University, Arboretumlaan 4, NL-6703 BD Wageningen, The Netherlands.
[2] To whom correspondence should be addressed. Fax: +31 317 484740. e-mail: jan.snel@algem.pf.wau.nl.

1053-0509/00/0900-0269$18.00/0

means of chlorophyll fluorescence was used as a rapid and sensitive method to determine biomass production by macrophytes [4]. In many aquatic ecosystems, however, the planktonic algae are the main primary producers. Nevertheless, a detailed characterization of the relationship between algal photosynthesis and the abiotic environment has been achieved for only relatively few species. Modeling and prediction of photosynthetic performance in a given abiotic environment are therefore possible for these species only. One of the causes for this is that conventional algal photosynthesis measurements by means of, e.g., oxygen evolution, carbon uptake, or chlorophyll fluorescence, require thousands of cells. Obtaining enough material for conventional photosynthesis measurements by isolation and subsequent cultivation is often a tedious and time-consuming process, if possible at all.

In the last decade the use of chlorophyll fluorescence-based methods has attracted interest in the assessment of phytoplankton primary production [5]. Application to a water sample containing a mixture of algal species, however, is problematic, as the light absorption cross section of PSII is species dependent. Although the PSII efficiency can be measured with a high accuracy, the relationship between photosynthetic oxygen evolution and PSII electron flow, as estimated from the product of PSII efficiency and photon flux density (PFD), differs considerably among algal species [6] belonging to different pigment classes. Unless the PSII absorption cross section is measured, estimation of algal photosynthesis and primary production by means of chlorophyll fluorescence measurements from samples of unknown composition is of limited use due to the errors resulting from the differences in PSII absorption cross section.

In studies involving the short-term effects of the abiotic environment on algal photosynthesis, problems of this kind might be circumvented by studying the photosynthetic performance of a single algal cell. Very recently imaging [7] and nonimaging [8] chlorophyll fluorimeters for single-cell measurements have been described. In this work we make use of these new techniques and describe a novel method to study the photosynthetic performance of single algal cells under a variety of environmental conditions by an integrated approach using modulated chlorophyll fluorescence, light microscopy, sample micromanipulation, and sample superfusion. The algal cell can be visually identified under the light microscope, attached to a micropipette, and subsequently characterized by exposing the algal cell to various environmental conditions and measuring photosystem II efficiency. As an example we have measured the light dependence of photosynthetic electron flow of a single *Scenedesmus obtusiusculus* cell at various pH levels and show that the method can be used to assess effects of (changes in) the abiotic environment on algal photosynthesis.

EXPERIMENTAL

Scenedesmus obtusiusculus was grown in batch culture in an enhanced CO_2 atmosphere as described by Van Rensen [9]. Cells were directly transferred from the culture into the sample cuvette of the microscope. Media at pH 5.5, 6.5, 7.5, 8.5, and 9.5 were prepared from fresh culture media to which the buffers MES, HEPES, MOPS, and Tricine, respectively, were added at a concentration of 10 m*M*. The pH of the medium was adjusted with KOH, except in the case of Tricine, where HCl was used. Before filling the syringes of the DAD-12 superfusion system, the media were degassed as described in Ref. 10. All experiments were carried out at room temperature (21–23°C).

Microscopy-PAM

Single-cell chlorophyll fluorescence was measured with an epifluorescence microscope consisting of the Microscopy-PAM (WALZ, Effeltrich, Germany) and an Axiovert 25CFL inverted microscope (Carl Zeiss, Germany). This setup is similar to the one described in Ref. 9. The microscopy-PAM employs a single 470-nm LED to generate modulated light for the generation of chlorophyll fluorescence, intense light to saturate photosynthesis and actinic light to drive photosynthesis. In contrast to earlier models, the Microscopy-PAM uses a pulse repetition modulation protocol to adjust the integrated photon flux density during the different stages of the measurement. The LED emitter was fitted into the collector HBO 103/XBO 75 W/2 using an adapter piece and placed into the fluorescence excitation port of the Axiovert microscope. The excitation light was guided from the LED to the sample via a color glass filter (BG 39; Schott) and a dichroic beam splitter (RG 65; Balzers) and the objective (fluar 40 × /1.3; Carl Zeiss). Fluorescence was detected by the photomultiplier (Hamamatsu) attached to the photo port of the Axiovert microscope. The photomultiplier was shielded from stray light by a dichroic (DT Yellow; Balzers) and a color glass (RG 645; Schott) filter. The LED and PM detector were controlled by the PAM-Control unit of the Microscopy-PAM, which was connected to a PC running the WinControl (WALZ, Effeltrich, Germany) software. Incident light intensities were measured with the MC-MQS Micro Quantum Sensor (WALZ) in the focal plane at the site of the sample. This sensor is

equipped with a 200-μm pinhole in front of the sensitive area of the detector. As the diameter of the beam at the focal plane is larger than the diameter of the pinhole with the objectives used in this study, the measured light intensities are a fairly accurate reflection of the actual light incident on the algal cell. The photon flux density data include the excitation light required for the fluorescence measurement. For a further description of the Microscopy-PAM see Ref. 11. Saturating light pulses were given with a duration of 800 ms and maximal intensity.

The algal cell was sucked onto the tip of a glass microcapillary, which was positioned and kept in the center of the field of view by an MO-303 micromanipulator (Narishige, Tokyo, Japan), a procedure similar to the procedure to obtain a seal in patch-clamp measurements on isolated chloroplasts [12]. The field of view of the photomultiplier could be reduced to the area of a single alga and its direct environment by means of a diaphragm in front of the photomultiplier.

Superfusion System

A 12-channel superfusion drug application device (Adams & List Associates, New York) was used to generate a smooth flow of experimental solution along the alga. The DAD-12 consists of 12 pressurized reservoirs with experimental solution connected to a micromanifold via a valve and PE tubing. The micromanifold, with its single 100-μm output nozzle, was attached to a second Narishige micromanipulator and positioned near the center of the field of view. In this way rapid (<50-ms) changes in the composition of the experimental solution can be achieved by selecting another channel.

Figure 1 shows a schematic overview of the sample manipulation part of the experimental setup featuring the alga, the tip of the glass capillary, and the exhaust nozzle of the DAD-12 superfusion device. The figure also indicates the approximate field of view seen by the detector of the Microscopy-PAM as set by the diameter of the iris diaphragm in front of the photomultiplier.

Analysis of Chlorophyll Fluorescence Measurements

The nomenclature and calculation of the various fluorescence parameters were as in Ref. 13; the rate of PSII electron flow was calculated as the product of PSII efficiency and the local PFD as measured with the MC-MQS Micro Quantum Sensor. The experiment shown in Fig. 3 is from a series of three experiments, each carried out on a different algal cell. The results shown are from a single experiment using a single algal cell.

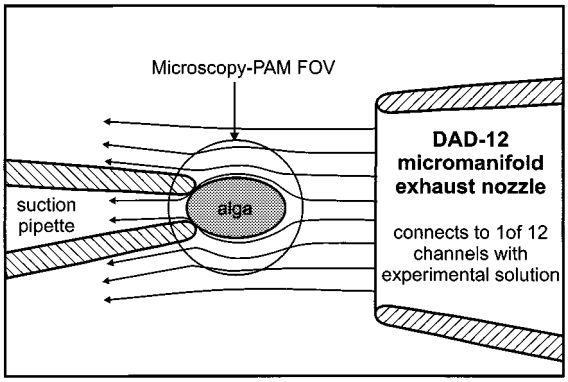

Fig. 1. A schematic overview of the measuring configuration shows the alga attached to the tip of a micropipette by maintaining underpressure in the interior of the micropipette. The exhaust nozzle of the DAD-12 superfusion device is positioned close to the alga to maintain a smooth laminar flow of experimental solution along the alga. The micoscopy-PAM field of view is indicated by a circle.

RESULTS AND DISCUSSION

Time Course of Fluorescence Induction

A change in light intensity results in induction of photosynthetic enzymes and metabolic intermediates in the photosynthetic apparatus. This adaptation requires a certain amount of time. A sudden increase in the intensity of nonmodulated light results in an sudden increase in the fluorescence yield. The adaptation of the algae to the new light intensity is reflected in a relaxation of the fluorescence yield to a new steady state. The light dependence of PSII electron flow was determined by applying stepwise increases in light intensity. Figure 2 shows the fluorescence yield of a single *Scenedesmus* cell during a typical light-dependence measurement. Upon an increase in the light intensity the characteristic transient increase in fluorescence yield in observed. At the end of each 30 s illumination step the fluorescence yield was relaxed to a new steady state in which the efficiency of PSII electron flow was determined from the fluorescence yield just before and the fluorescence yield during a saturating light pulse. From the PSII efficiency and the local PFD, the rate of PSII electron flow was calculated. The results for a series of data sets similar to the one in Fig. 2 are shown in Fig. 3. At low PFDs, PSII electron flow is proportional to the PFD, but above 50 μmol m^{-2} s^{-1} the rate of PSII electron flow shows saturation until a maximum is reached at PFDs above 100 μmol m^{-2} s^{-1}. This light

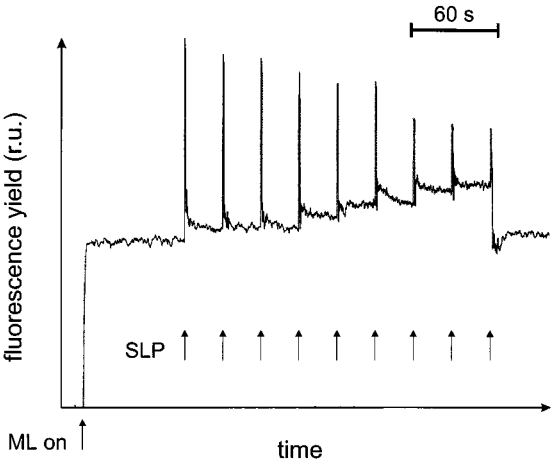

Fig. 2. Registration of the fluorescence yield of a single *Scenedesmus* alga during a typical light response measurement. The measurement starts by switching on the measuring light (ML). At the end an 0.8 s saturating light pulse (SLP) was given to measure the PSII efficiency. Immediately after the SLP the actinic light intensity was increased, and after 30 s the next SLP was given. This procedure was repeated until the maximum light intensity was reached. After the last SLP the actinic light was switched off.

dependence of PSII electron flow provides information on the photosynthetic performance of an alga in a (slowly) fluctuating light environment, which is the case in many natural aquatic ecosystems.

Light and pH Dependence of PSII Electron Flow

The potential of the method in the study of algal ecophysiology was further exploited by studying the pH dependence of PSII electron flow. The light dependence of photosynthesis was determined as a function of pH (Fig. 3). At a high PFD the rate of PSII electron flow shows an optimum near pH 6, and at pH 9.5 PSII electron flow is almost completely inhibited. It is noteworthy that the maximal rate of PSII electron flow is not observed at the highest light intensity. This may have been caused by the relatively short (5-min) adaptation period after the change in pH and by the fact that the light dependence was always measured starting at the lowest light intensity. At the time of the measurement at a high PFD the alga had been exposed for a longer time to a high pH and the decrease in the rate of PSII electron flow at a high PFD thus may have been caused by a progressive inhibition by the alkaline pH. At a low PFD the rate of PSII electron flow is hardly inhibited by an alkaline pH. At pH 9.5 a small inhibition was observed. The absence of inhibition is not caused by a too short incubation time at alkaline pH. This can be concluded from the fact that in the experiment at pH 9.5 the alga had already been exposed pH 7.5 and 8.5, but at pH 9.5 the rate of PSII electron flow is only slightly lower than at pH 6.5.

The data in Fig. 3 show that the effect of the pH on PSII electron flow is dependent on the light intensity. At a high PFD the substrate CO_2 is limiting photosynthesis. At a pH higher than 6.4, the pK_a for the dissociation of carbonic acid, the CO_2 concentration is lowered due to conversion of CO_2 into bicarbonate, causing inhibition of CO_2 reduction by substrate limitation. At a high PFD this results in a lower rate of PSII electron flow. This is clearly shown in Fig. 4. In contrast, the rate of PSII electron flow at a low PFD is not limited by CO_2 reduction but is, instead, determined by the PFD and the maximal (intrinsic) efficiency of PSII electron flow. Apparently the maximal PSH efficiency is not very sensitive to a more alkaline pH; only at a pH higher than 8.5 is a reduction of PSH efficiency observed at a low PFD (Fig. 4, data points at 17 μmol m^{-2} s^{-1}).

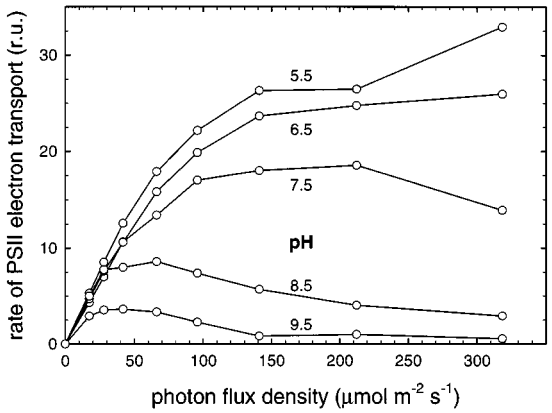

Fig. 3. Light dependence of PSII electron transport measured at various values of medium pH. Numbers indicate the pH of the medium.

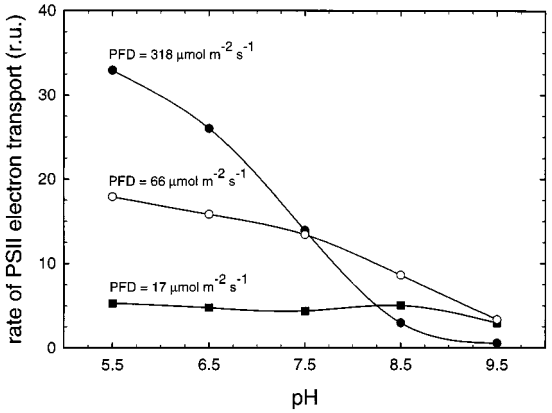

Fig. 4. Effect of the pH of the medium on the rate of PSII electron flow at three light intensities. The data were taken from Fig. 3.

The observed inhibition of the maximal rate of PSII electron flow at an alkaline pH is more severe than expected. The effect is caused by the low oxygen concentration in the medium. The DAD-12 superfusion system works properly only with degassed experimental solutions, resulting in a low oxygen concentration. The lack of oxygen prevents CO_2 independent photosynthetic electron transport linked to photorespiration and electron transport linked to the reduction of oxygen (see, e.g., Ref. 14 for a review). Under our low-oxygen conditions the rate of PSII electron transport is thus closely linked to the reduction of CO_2 in the Calvin cycle and therefore closely linked to CO_2 availability in the external medium.

Prospects of the Method

The results of this study show that the photosynthetic performance of algae can be studied at the single-cell level under a variety of experimental conditions. The combination of photosynthesis measurements in a controlled chemical and light environment and visual identification allows assessment of the biodiversity of phytoplankton responses to changes in the light and chemical composition of the water environment. The differences in PSII efficiency depend on the nature of the changes in the environment; both the magnitude and the duration of the perturbation influence the effect on PSII efficiency. A recent study on the variation of PSII efficiency between *Arabidopsis thaliana* ecotypes showed that chlorophyll fluorescence can be used to determine very small (< 0.06; i.e., about 10% change) differences in PSII efficiency between ecotypes (Snel, Vreugdenhil, and Koornneef, unpublished data).

In this study a single-cell technique was used to establish a relationship between external pH and photosynthetic performance. This relationship, however, is dependent on the set of properties of the individual used; other individuals with their own set of properties (genotype, developmental stage, environmental history) will give different results. In both ecological and ecotoxicological applications a significant number of single cells of a given species will have to be measured to be able to extrapolate the single-cell data to the population level. In ecotoxicological applications such a method would be useful to study the effects of toxicants on phytoplankton in aquatic ecosystems. In eco(physio)logy the method could be used to provide information on the role of environmental factors in the photosynthesis and growth of a given species. The single-cell approach described here yields information on single-cell photosynthetic performance under a wide range of environmental conditions which could be used for individual-based ecological simulation models to identify causal relationships at the ecosystem level.

ACKNOWLEDGMENTS

We thank Ria van den Noort for *Scenedesmus obtusiusculus* samples, Prof. Dr. Wim Vredenberg for making the Microscopy-PAM and the Axiovert microscope (NWO Project 047.01.008.96) available for this pilot study, and Dr. Ulrich Schreiber for help in setting up the Microscopy-PAM and for calibration of the PFDs.

REFERENCES

1. B. Genty, J. M. Briantais, and N. R. Baker (1989) *Biochim. Biophys. Acta* **990**, 87–92.
2. H. Dau (1994) *J. Photochem. Photobiol. B* **26**, 3–27.
3. H. Dau (1994) *Photochem. Photobiol.* **60**, 1–23.
4. J. F. H. Snel, J. H. Vos, R. Gylstra, and T. C. M. Brock (1998) *Aquat. Ecol.* **32**, 113–123.
5. P. G. Falkowski and Z. Kolber (1995) *Aust. J. Plant Physiol.* **22**, 341–355.
6. C. Geel, W. Versluis, and J. F. H. Snel (1997) *Photosynth. Res.* **51**, 61–70.
7. K. Oxborough and N. R. Baker (1997) *Plant Cell Environ.* **20**, 1473–1483.
8. U. Schreiber (1998) in *Photosynthesis: Mechanisms and Effects, Vol. V,* pp. 4253–4258, Kluwer Academic Publishers, Dordrecht.
9. J. J. S. Van Rensen (1975) *Physiol. Plant.* **33**, 42–46.
10. T. Van Voorthuysen, A. A. Bulychev, J. H. A. Dassen, J. F. H. Snel, and W. J. Vredenberg (1996) *Physiol. Plant* **98**, 156–164.
11. C. H. Goh, U. Schreiber, and R. Hedrich (1999) *Plant Cell Environ.* **22**, 1057–1070.
12. A. A. Bulychev, V. F. Antonoy, and E. V. Sevchenko (1992) *Biochim. Biophys. Acta* **1099**, 16–24.
13. O. Van Kooten and J. F. H. Snel (1990) *Photosynth. Res.* **25**, 147–150.
14. K. Asada (1999) *Annu. Rev. Plant Physiol.* **50**, 601–639.