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FORWARD ELECTRON TRANSPORT MEASURED *IN SITU* IN MICROBIAL MATS FROM A HOT SPRING IN N-W ROMANIA

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SUMMARY. Cyanobacteria, green algae and higher plants have highly conserved photosynthetic machinery. Cyanobacteria have a great plasticity and can be found in different environments, from Arctic zones to hot springs; they can be part of microbial mats. The hot spring near Ciocaia, in Bihor County has a range of different temperatures, from 65.5°C at the spring well, 60.5°C at 2 meters away from the spring well and between 32-38°C in the area where microbial mats are formed. In this study we showed changes in PSII function in mats grown at the location of the hot spring, comparative with microbial mats grown in laboratory conditions. We also showed a comparative study of some basic parameters of chlorophyll fluorescence. These results are very important to understand the functionality of photosystem II in the larger context of adaptation to environmental cues.

Keywords: chlorophyll, cyanobacteria, fluorescence, hot spring

Introduction

Photosynthesis is the fundamental process by which cyanobacteria, algae and higher plants use water as a source of electrons in reducing CO_2 to various organic compounds, this way being responsible for maintaining the oxygen level in atmosphere, and in a larger context, sustaining life on earth (Loll *et. al.*, 2005). In organisms performing oxygenic photosynthesis, the linear electron transport (light reactions) takes place in the thylakoid membrane-embedded proteins, which are Photosystem II (PSII), Cytochrome b_6f ($Cytb_6f$) and Photosystem I (PSI). Photosynthesis is initiated in PSII, a dimeric protein-cofactor membrane embedded complex. By capturing the light energy, PSII produces the energy required to oxidize water to atmospheric oxygen (Singhal *et. al.*, 1999). PSII is a complex that

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consists of more than 20 protein subunits and 77 co-factors (Loll *et. al*., 2007). The D1 and D2 proteins are involved in binding the most redox active components of PSII. The factors which damage the linear electron transport equilibrium can lead to the accumulation of toxic intermediates that will cause irreversible damage to the PSII reaction center, especially to protein D1. The D1 protein of Photosystem II, encoded by the *psbA* genes, is indispensable for oxygenic photosynthesis. The aminoacid structure of the *psbA* gene predicts a hydrophobic nature for D1 protein with five membrane spanning helices (Dwivedi and Bhardwaj, 1995). Due to strongly oxidative chemistry of PSII water splitting, the D1 protein is prone to constant photodamage requiring its replacement, whereas most of the other PSII subunits remain ordinarily undamaged. When the photoinhibition occurs, the D1 protein is accessed by the FtsH proteases and degraded. Subsequently, a new copy of D1 protein is inserted in the membrane, the dimers are formed, and the functional PSII is activated again (Mulo *et. al*., 2009). In normal growth conditions a D1 protein is replaced in the PSII at every 5h, and in different stress conditions the replacement occurs in 20 minutes.

During the light reactions of photosynthesis is described the pathway of electron transport from water to $NADP^+$, resulting in release of oxygen, the reduction of $NADP^+$ to NADPH and the accumulation of a high concentration of hydrogen ions in the thylakoid lumen, needed for ATP production. During the light reactions, fluorescence measurement reveal functional changes in the acceptor and donor side of PS II (Fig. 1). Mn is a Manganese center, has 4 manganese atoms and splits 2 water molecules into 4 protons ($4H^+$), 4 electrons ($4e^-$) and 2 oxygen atoms. Tyrosine (Tyr) shuttles electrons to the reaction center of PSII, P680. P680 absorbs a photon of light energy and reaches the excited state. Whereas P680 is the primary electron donor of PSII, Pheo is a pheophytin molecule, the primary electron acceptor of PSII. After absorbing the light, P680 sends one electron to Pheo; Pheo accepts the electron, it is reduced, and transfer than the electron to Q_A. The electron transfer until Q_A is very fast, and cannot be detected. The transfer between Q_A and Q_B takes a few milliseconds, and can be detected during chlorophyll fluorescence measurement. Q_A is a plastoquinone molecule fixed into the membrane which accepts and transfers one electron at a time. Q_B is a loosely bound plastoquinone, which at 2 cycles accepts 2 electrons from Q_A, than becomes mobile and goes to the plastoquinone pool from the thylakoid membrane. In cyanobacteria, the photosynthetic system is tightly connected to the other principal metabolic paths; therefore, chlorophyll fluorescence signals can provide rapid, real-time information on both photosynthesis and the overall acclimation status of cyanobacteria (Campbell e*t. al*., 1998).

At the hot spring drilling of Ciocaia, in Bihor County, Romania, a microbial community mat was formed, adapted to high water temperatures Visually, the microbial mats formed here have different colors: green, red and white, based on the temperatures they grow at. In this article we performed for the first time PSII functional studies *in situ* on a hot spring microbial mat, using chlorophyll fluorescence measurements with a submersible measuring head

attached to the double modulation fluorometer. We were able to get indications relative to the photosynthetic function of the microbial communities in their natural environment. Based upon the specific conditions of studied cyanobacterial mat we developed a fluorescence measurement protocol that can be further used for mat measurements. We also show a comparative analysis of the forward electron transport between the measurements *in situ* of microbial communities from Ciocaia, and laboratory grown Phormidium strains.

Materials and methods

The measurements were performed *in situ* at Ciocaia thermal drilling. The hot spring near Ciocaia (Bihor County, Nort-Western Romania) is geographically located at E 22°02.744' and N 47°20.471'. The chemical composition of water is 812 mg/l Cl^-, 7,283 mg/l $HCO3^-$, 27.9 mg/l $SO4^{2-}$, 7.9 mg/l $NH4^+$, 3,525 mg/l Na^+, 30 mg/l K^+, 790 mg/l Ca^{2+}, 2.6 mg/l Mg^{2+}, 0.2 mg/l Fe^{2+} and 3.7 wt.% total salinity (Ţenu *et al*., 1981). The fluorescence measurements were made with an FL3500 Fluorometer (Photon Systems Instruments, Brno, CZ), using a Q_{A-} reoxidation protocol (Reference). The field fluorescence measurements were performed with a submersible head, designed to measure fluorescence of underwater organisms, from Photon Systems Instruments. The submersible head was powered by 220 V car adapted convertor. We optimized the protocol for on field measurements considering the specific conditions of the mat-building communities: we had to modify the intensity of measuring flashes in order to decrease the actinic effect of the measuring flashes as well as the saturation of basic fluorescence caused by the huge cell density specific to the bacterial mat, (Table 1).

The double-modulation fluorometer from PSI (CZ) can measure the chlorophyll fluorescence with a resolution of 4 micro-seconds, it is adapted for liquid sample measurement, can measure many photosynthetic parameters and in combination with DCMU (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea), provides information about both the donor and the acceptor side of PSII (Fig. 1). .

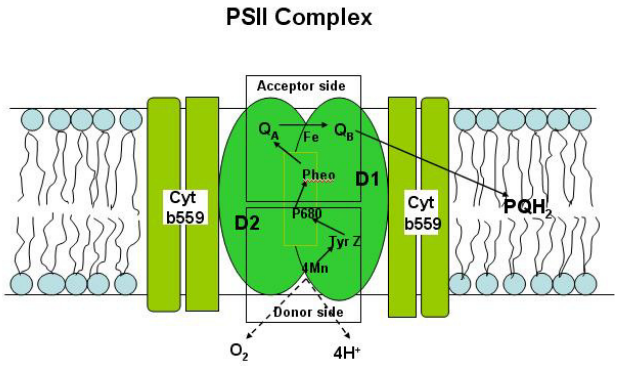


Figure 1. Functional structure of the Photosystem II.(original)

The transfer from Q_A to Q_B represents the acceptor side of PSII, so that the fluorescence measurement in absence of DCMU will give information about the functionality of the acceptor side of PSII (Fig. 1). DCMU is an herbicide that binds to the Q_B locus; this leads to the return of the electrons to the water oxidation complex (Metz *et.al*., 1986). In presence of DCMU, any changes in the functionality of the water oxidation complex will be observed at fluorescence measurement in presence of DCMU (Fig. 1).

Below we are showing the measurement protocol used in our experiments.

The Double-Modulation Fluorometer FL 3500 measures Chl-fluorescence signal with a time resolution of up to 4 µs in the Standard FL3500/S version. Measured fluorescence emission is excited by a set of light-emitting diodes that generate short measuring flashes. The photochemistry is driven by single-turnover flashes. Chlorophyll fluorescence is detected by a PIN photodiode and digitized by a 16-bit A/D converter. PSI fluorometers can measure various chlorophyll fluorescence parameters, ratios, and quenching coefficients that provide information on the functionality of the photosynthetic apparatus. In our experiments we followed the reoxidation of Q_A^- after a saturating actinic flash, probed with series of measuring flashes on a logarithmic time scale from microseconds up to 100seconds. In addition we recorded three basic fluorescence parameters: F0, Fm and Fv. A short description of these parameters is given below:

F0 represents the minimum chlorophyll fluorescence yield measured on a dark-adapted sample.

Fm represents the maximum chlorophyll fluorescence yield measured on a dark-adapted sample.

Fv represents the variable fluorescence obtained: Fv=Fm-F0.

All samples used for fluorescence measurements were dark-adapted for 6 minutes before measurement was done in absence and presence of DCMU.

The laboratory *Phormidium sp*. culture from Ciocaia was grown in the BG11 medium, at 38ºC and 50 µmol photons $m^{-2}s^{-1}$ light. The culture was mixed by bubbling air all the time.

Finally, data were processed in OriginPro 8 (OriginLab Corporation, Northhampton, MA 01060, USA) software.

Table 1.

Fluorescence measurement protocol parameters used in field and laboratory measurements.

Sample Measured	Ciocaia - Field sample	Ciocaia- laboratory sample
Measuring Flash (µs)	8	8
Measuring delay (µs)	6	6
Actinic Flash (µs)	20	20
Pre-Flash (µs)	0	0
M_Voltage	10	30
F_Voltage	100	100
A_Voltage	100	100

M_voltage= relative power of Measuring flashes, relative voltage of the Measuring LED's. The signal is proportional to the voltage in 20%-100%.
F_Voltage= relative voltage of the actinic Flash or relative power of actinic flashes.
A_voltage = relative voltage of the actinic light LED's.

Results and discussion

Recording the function of PSII, the key component of the photosynthetic electron transport chain, gives useful insight into cell metabolism and allows for understanding of molecular effect of stress factors. Use of the Flash induced Chlorophyll Fluorescence methods using modern fluorometers allow for functionally dissecting of PSII photochemistry. These measurements are usually performed under laboratory controlled conditions. We devised a measurement protocol allowing the measurement of the reoxidation of Q_A- under field conditions using a submersible measuring head.



Figure 2. Image of microbial mats formed around the Ciocaia hot spring. Depending on the temperature gradient the composition of the mat and the color change from white at highest to green at lower temperatures (bar is 20cm long).

. The hot waters from Ciocaia thermal drilling flow directly on the ground forming a small hot spring. Close to the point of emergence a series of microbial mats are formed depending on water temperature, between 35°C and 60°C (Fig. 2). Closest to the drill and at highest temperature white color mat is present, followed

by a red color mat at intermediate temperatures and a green mat at lower temperatures (Fig. 2).

A sample was collected from the green mat and photo-autothrophically grown in the laboratory at 38°C and BG-11 medium. Under light microscope the mat proved to be dominated by a *Phormidium sp*. strain (Fig. 3) (Coman, 2011). We used this strain as a control for our fluorescence measurements. Repetitions of fluorescence measurements were done in all of the above-mentioned mats.

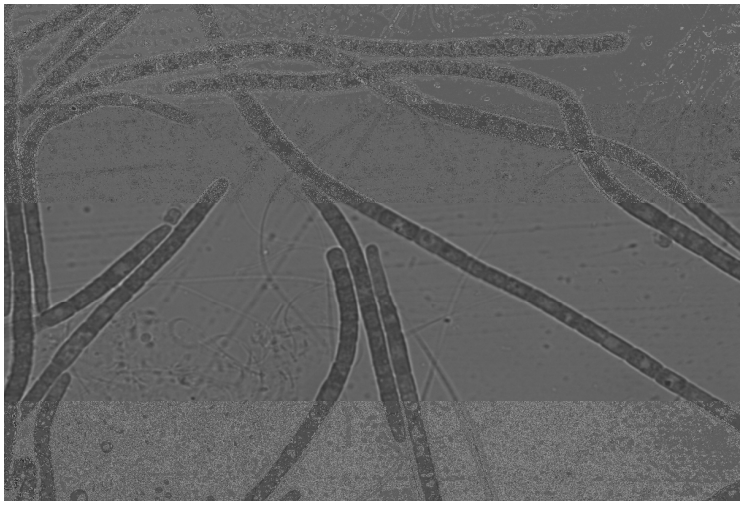


Figure 3. Typical shape of *Phormidium sp.* containing laboratory culture.
Laboratory culture isolated from the green mat from Ciocaia hot spring. Optical microscope image at 100x magnification.

Initially F0, Fm and Fv parameters were measured and calculated in all three microbial mats as well as the laboratory sample (Fig. 4). The highest F0 value was recorded in the laboratory grown sample followed by the green mat from the field. The red and white mats showed small F0 values (Fig.4, panel A). The highest Fm value was recorded at the laboratory grown sample, followed by the value from the green mat, while the values from the red and white mat are close to 0 (Fig.4, panel B). Also, the variable fluorescence parameter, Fv follows the same decrease, from the highest value at the laboratory sample, followed by the green mat from the field. The red and white microbial mats have small Fv values (Fig.4, panel C).

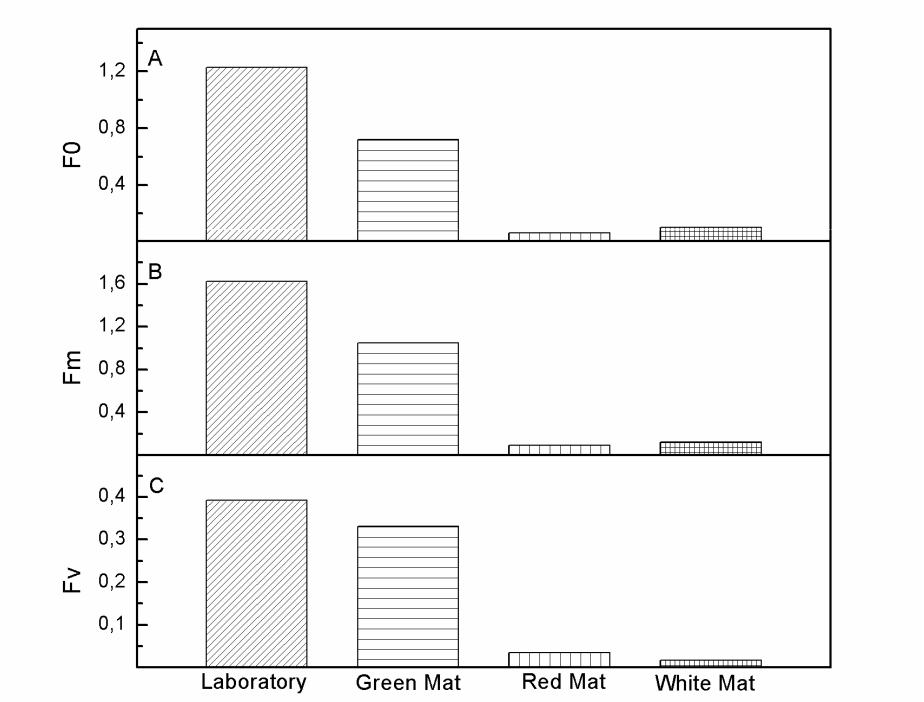


Figure 4. Basic fluorescence parameters F0 (panel A), Fm (panel B) and Fv (panel C) measured in all three microbial mats from Ciocaia as well as the laboratory grown sample.

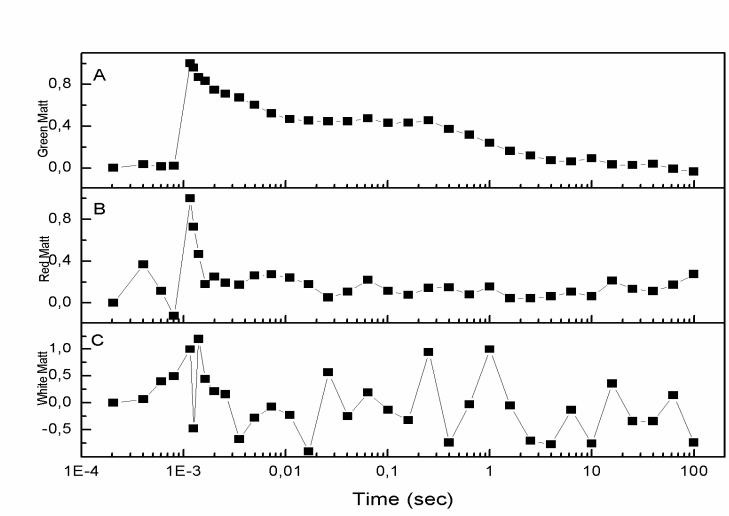


Figure 5. Changes of forward electron flow in the acceptor side of photosystem II at the green mat (A), red mat (B) and white mat (C) from Ciocaia hot spring.

Using the modified measuring protocol for the field measurements, we followed the forward electron transfer within PSII. In case of the green mat from the field (Fig. 5, panel A), the fluorescence decay curve looks close to the control one showing the typical features of a Q_A^- reoxidation decay curve. Following this experimental protocol we can evaluate the acceptor side of PSII. In our case there is a distinct slowdown of Q_A to Q_B transfer, and the Fm value is close to the 1.2 value.

The red mat (Fig. 5, panel B) displays a clear charge separation at the time of the saturating flash showing the formation of Q_A^-. However, the re-oxidation of Q_A^- is massively distorted, without the typical characteristics of a fluorescence decay curve. This will be consistent with an organism that does not exhibits typical photosynthesis, possibly bacteriochlorophyll-dependent photosynthesis still relying on energy from the light.

The white mat from Ciocaia did not show any signs of response to light excitation with the measurement being consistent with bacteria that has no light absorbance capacity but is probably adapted to high temperature (Fig. 5, panel C).

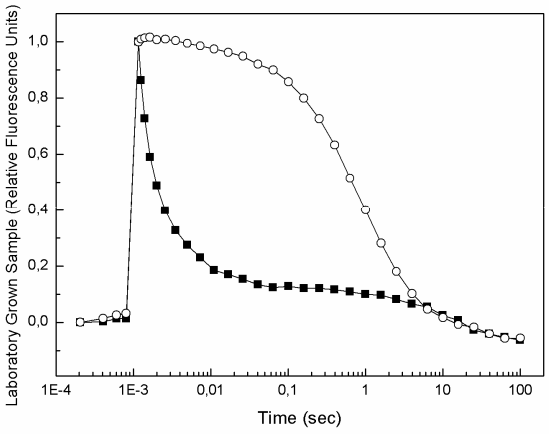


Figure 6. Characteristics of donor and acceptor side functionality of the photosystem II measured in the absence (solid squares) and presece of DCMU (open circle) in the laboratory sample isolated from the green mat from Ciocaia.

The laboratory-grown Phormidium sample started from the green mat from Ciocaia (Fig. 6), displayed a faster electron transfer between Q_A and Q_B corresponding to a "normally" functioning acceptor side of the photosystem II. In the presence of DCMU, that blocks the Q_A to Q_B transfer and prompts the back-recombination of Q_A^- with the donor-side components of PSII in particular the water-oxidation complex the fluorescence level is maintained at high values for longer time before the recombination. The laboratory-grown sample showed a typical, unmodified DCMU curve giving indication of a standard PSII donor side in this cyanobacterium. This type of measurement could not be performed in the

field samples, as we could not apply a DCMU treatment to the mat without fatally damaging the cells.

The fact that in the lab sample the fluorescence is decaying faster than the field measurement indicates the partial impairment of the electron transport that naturally occurs when the cells are exposed to sunlight. To our knowledge, this is the first measurement of forward electron transfer in a hot-spring cyanobacterial mat. This observation is very important, functionally, as most experiments are done under laboratory conditions and usually are claimed to mimic natural conditions.

Conclusions

Ciocaia hot spring is a good environmental model to study the physiology of microbial mat as it offers access to stable functional mats all year round. In our case, we performed field fluorescence measurements for the first time in three microbial mats of a hot-spring. From our experiments, we can conclude that:

- Due to the very high cell density, characteristic to a microbial mat, the optimization of the fluorescence measurement protocol was necessary in order to reduce noise and increase signal quality due to the field conditions;

- The fluorescence method we optimized, allows for the identification in the green microbial mat of members which have the capacity to use light as source of energy;

- A laboratory-grown sample isolated from the green mat was used to highlight the differences in forward electron transport between the laboratory condition and the natural conditions in the field. The first, showed a high amplitude of fluorescence characteristic to a high number of PSII active centers with a fast Q_A to Q_B electron transfer while in the field these traits were modified probably due to the effect of environmental conditions.

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