INTERREG CZECH REPUBLIC - AUSTRIA
Algenetics ATCZ15 - Společné česko-rakouské centrum řasových biotechnologií
Joint Czech-Austrial Centre for Algal Biotechnology

SUMMARY RESEARCH REPORT

Period	3
Activity	Implementation
Name	**Characterisation of microalgae in larger scale cultivation**

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1st experimental series (Třeboň)
Temperature optimum of Synechocystis sp. PCC 6803-NIX

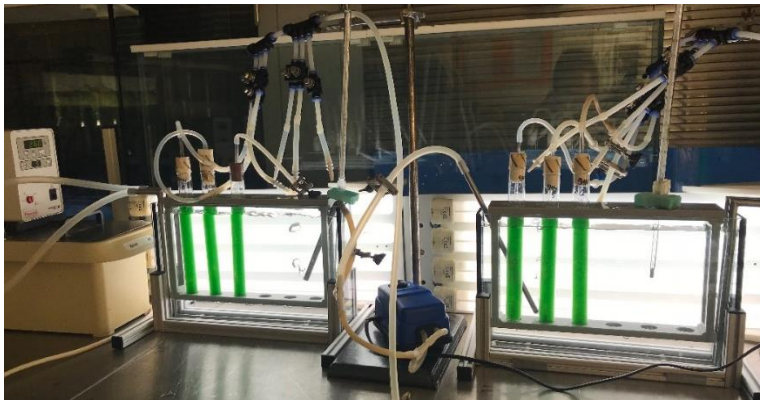
Strains:
- *Synechocystis sp.* PCC 6803-NIX

Aim:
- Growth test in 10-L and 25-L photobioreactors.

Experiments:
- Photosynthetic activity
- Growth

Culturing conditions/regimes:

Pre-growth in 300 mL cylinders



Temperature experimet in 50 mL cylinders

- Culture medium: BG-11
- Initial concentration ~0.7 g L^{-1} (diluted on the basis of Chl concentration $O.D._{680}$)
- Light intensity: 80 µmol photons m^{-2} s^{-1} inside exposed wall surface of temperated water bath (glass reservoir) using cosine-corrected light sensor
- Light/dark cycle: continuous light
- Temperature steps: 20, 25, 27.5, 30, 32.5, 35 °C
- Bubbling volume: ~ 0.2 L min^{-1} of air +1.5% CO_2

Methodology

Dry weight measurement:

Dry weight was measured every day. 2 ml samples were taken from each cylinders and stored in Eppendorf tubes at -20 °C every day until utilization. Wet biomass was obtained by centrifugation in pre-weighted Eppendorf tubes. The cell pellets were dried at 105 °C overnight. The mean of dry weight was calculated from triplicates.

Optical density

The growth rate has been determined by the optical density (OD) increment. The OD has been determined reading a sample, 2 mL, for each flask and measured at 750 and 730 nm in a spectrophotometer.

Cell counting

The growth rate has been also determined by cell counting under microscope.

Oxygen evolution

Oxygen evolution is measured by Oxylab plus device (Hansatech Instruments) using a close chamber (DW2/2). The electrode disc is a specialised form of electrochemical cell known as a Clark type polarographic sensor which comprises a resin bonded central platinum cathode and a concentric silver anode. Preparation of the electrode includes the addition of electrolyte (50% saturated potassium chloride) and the application of a thin, selectively permeable to oxygen, P.T.F.E. membrane to the electrode dome. Once prepared and positioned in the electrode chamber, the disc is connected to an electrode control unit which applies a small polarising voltage between the platinum and silver electrodes. In the presence of oxygen, a small current is generated proportional to oxygen activity in the sample.

Measurement: Samples were adapted for 5-10 minutes in dark at temperature 30°C. Thereafter we measured light-response steady state curve of oxygen evolution according to the protocol:

Time [min]	Light intensity [µmole photons m^{-2} s^{-1}]
2	0
2	200
2	400
2	600
2	1200
2	1800

First step (2 min in dark) provides the extent of respiration. During the measurements, light intensity is increased stepwise. Every light increase will affect oxygen production. By this method we get information about respiration, compensation point, photosynthesis [nm/ml/min] light saturation, maximum photosynthetic activity. Oxygen production is calculated per chlorophyll or cell.

OJIP kinetics

It is believed that the I step in OJIP kinetics reflects the heterogeneity of the PQ pool, fast-reducing and slow-reducing PQ pools, an idea originally developed by Lavorel and Etienne (1977).

Although slow reaction centres can be modified by light, their basic attributes remain unchanged, i.e., their inability to reduce the plastoquinone pool (Nedbal and Whitmarsh 1992). Thus slow reaction centres might convert to a form capable of protecting active ones from excess excitation energy. Hence, slow reaction centres may provide a mechanism for quenching excessive energy. The presence of slow reaction centres, and appearance of the I step is usually observed in shade adapted cultures or when dark adaptation is prolonged' for hours or overnight (Strasser et al. 1995). Thus, the OJIP transient can be used as a rapid monitor of the electron acceptor side reactions, the pool heterogeneity and pool sizes, the effects of inhibitors and mutations on these processes, as well as on the donor side (Govindjee 1995).

Glycogen

Samples were collected on the last day of the experiment and stored at -20 °C for later processing. For glycogen determination cultures were melted out and 1 mL volumes was transferred into 2 ml Eppendorf tubes. Samples were centrifuged and taken back three times in 50 mM pH 8 Tris-HCl buffer. After the addition of 0.5 mL sand to the washed samples, they were sonicated 2*15 minutes long in cold bath. The 100 µL from the supernatant of the centrifuged samples was added to 900 µL 100% ethanol. The mixture was heat treated at 90 °C for 10 minutes and cooled down on ice for 30 minutes. Finally, the samples were centrifuged at 20000 g for 30 minutes and the supernatant was removed.

The pellet was solubilized in 100 µL 50 mM pH 5 sodium acetate buffer. For glycogen digestion 50 µL of 8 U/mL amyloglucosidase and 50 µL of 2 U/mL α-amylase were added to the solution and incubated at 60 °C for 2 hours.

For glucose yield detection glucose assay kit (MAK263) from Sigma-Aldrich and plate reader (TECAN) were used. 50 µL from the digested samples were added to the reagent mix into the 96 well-plate. For colorimetric assay the plate was incubated at 37 °C for 30 minutes then measured at 570 nm. Unknown glucose contents were calculated from the calibration curve of the glucose standard.

Results

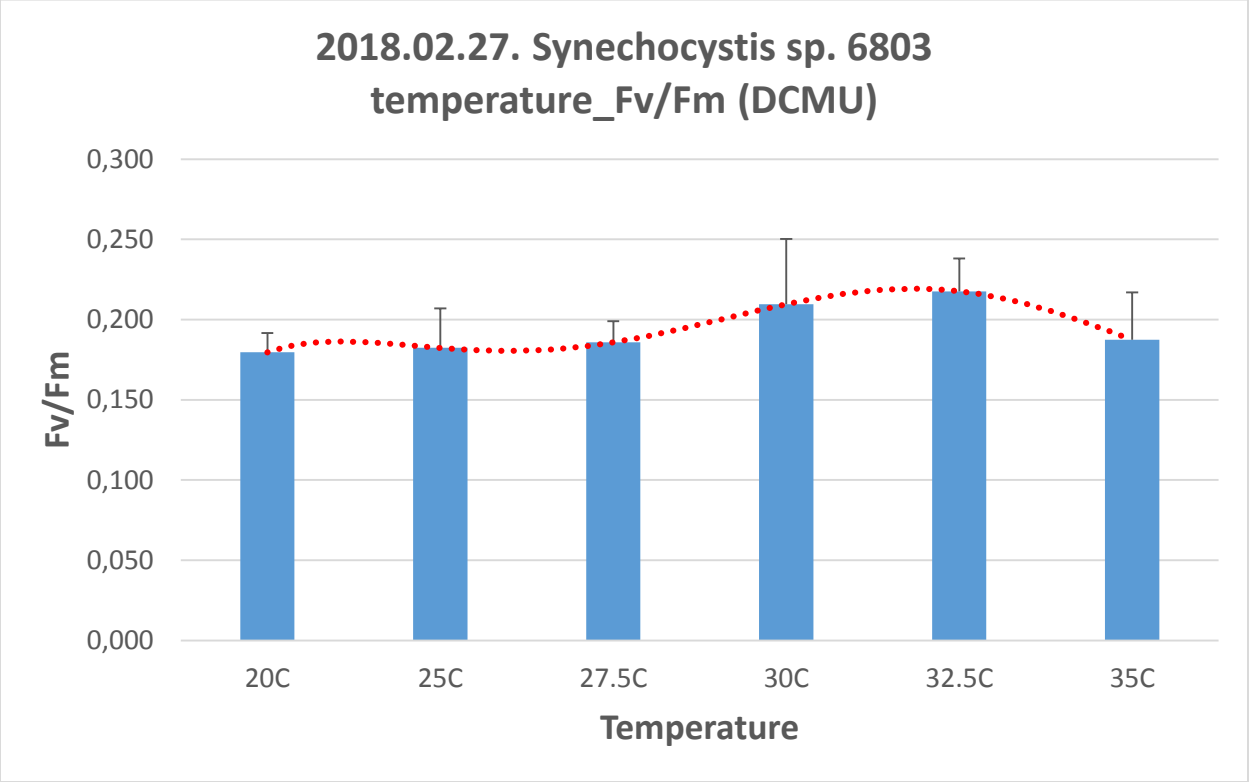


Fig. 1. Fv/Fm (DCMU) changes with different temperatures

Synechocystis sp. PCC 6803-NIX culture was grown in 350 mL cylinders then 50 mL of culture was transferred to 80 mL cylinders (i.d. 20 mm). Three replicates were incubated at specific temperatures for two hours (20, 25, 27.5, 30, 32.5, 35 °C) and then photosynthetic activities were measured (RLC curves, OJIP curves, and photosynthetic oxygen evolution). The most corresponding results for optimum temperature determination were Fv/Fm values measured in the presence of DCMU (herbicide blocking electron transport). Polynomial fitting was made over the columns which finally showed a 31-32 °C optimum temperature (Fig. 1.).

Conclusion

- Optimum temperature of cyanobacteria can be determined by measuring Fv/Fm values with the use of DCMU at different temperatures.
- Regularly, optimum Fv/Fm values with DCMU treatment are around 0.6. The values of the current experiment are far from this, cultures were presumably light stressed. Culture density for proper measurements must be considered especially when the diameter of the cultivation cylinder is also reduced.

2nd experimental series (Třeboň)

Testing Synechocystis growth in 10-L column and 25-L flat-panel photobioreactor

Strains:

- *Synechocystis sp.* PCC 6803-NIX

Aim:

- Growth test in 10L and 25-L photobioreactors

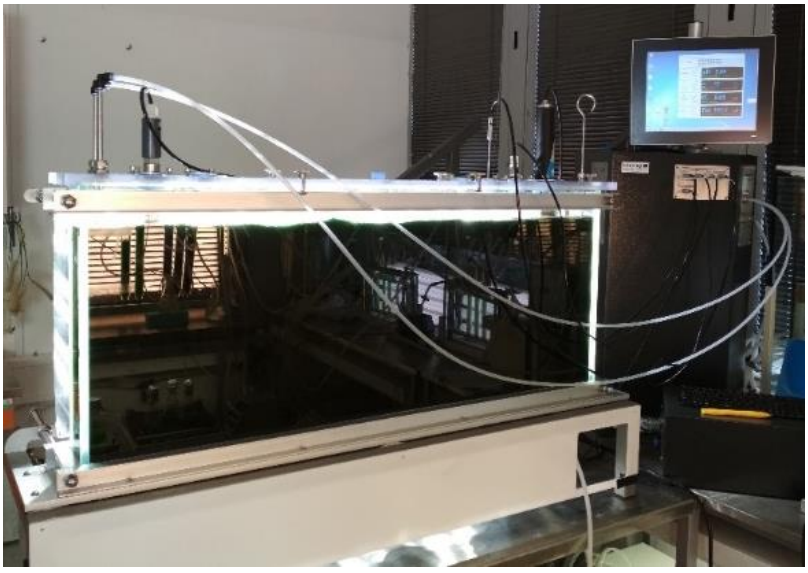
Experiments:

- Photosynthetic activity
- Growth

Culturing conditions/regimes:

- Cultivation volumes:

10-L cylindrical



25-L flat panel photobioreactor

- Culture medium: BG-11
- Initial concentration ~0.7 g L^{-1} in 25-L flat panel photobioreactor (diluted on the basis of Chl concentration $O.D._{680}$)
- Light intensity:
- Light source: LED panel for 25-L photobioreactor
- Light/dark cycle: continuous light
- Temperature: 30 °C
- Bubbling volume: 10%, pH 7 was set by CO_2 purging

Results

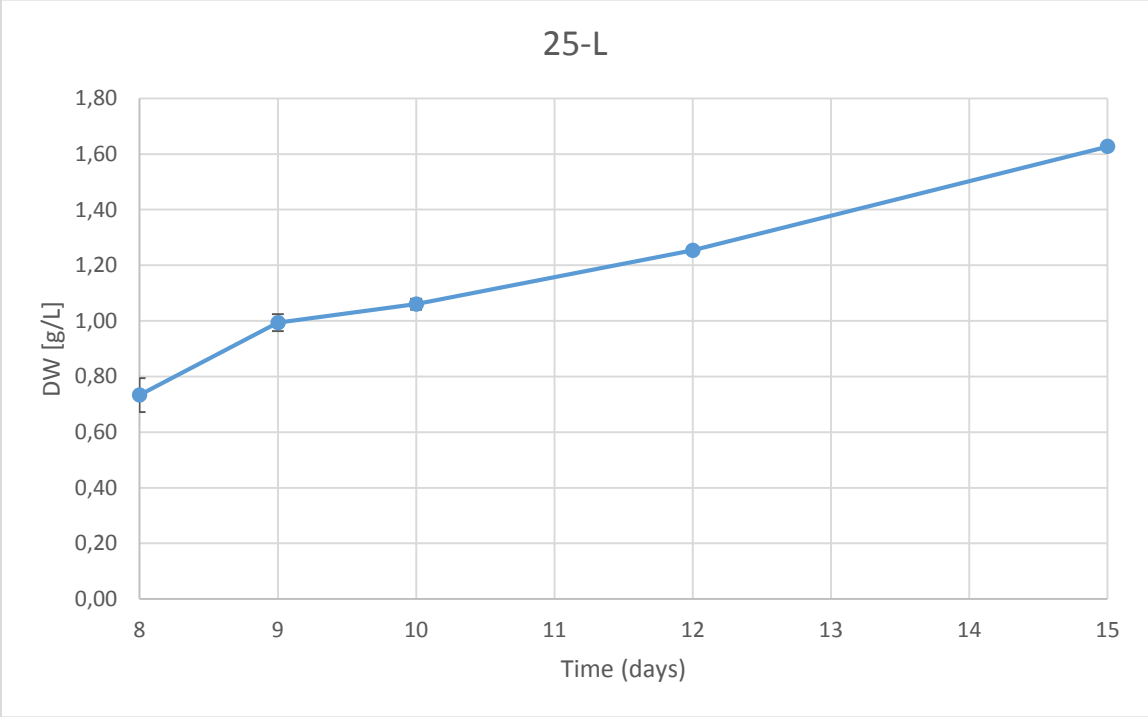


Fig. 1. Growth of Synechocystis in 25-L photobioreactor

Table 1. Growth rate between the measured time points

Growth rate changes during cultivation trial (on day #)	**g/L/day**
8-9	0.26
9-10	0.07
10-12	0.10
12-15	0.12
average	0.14

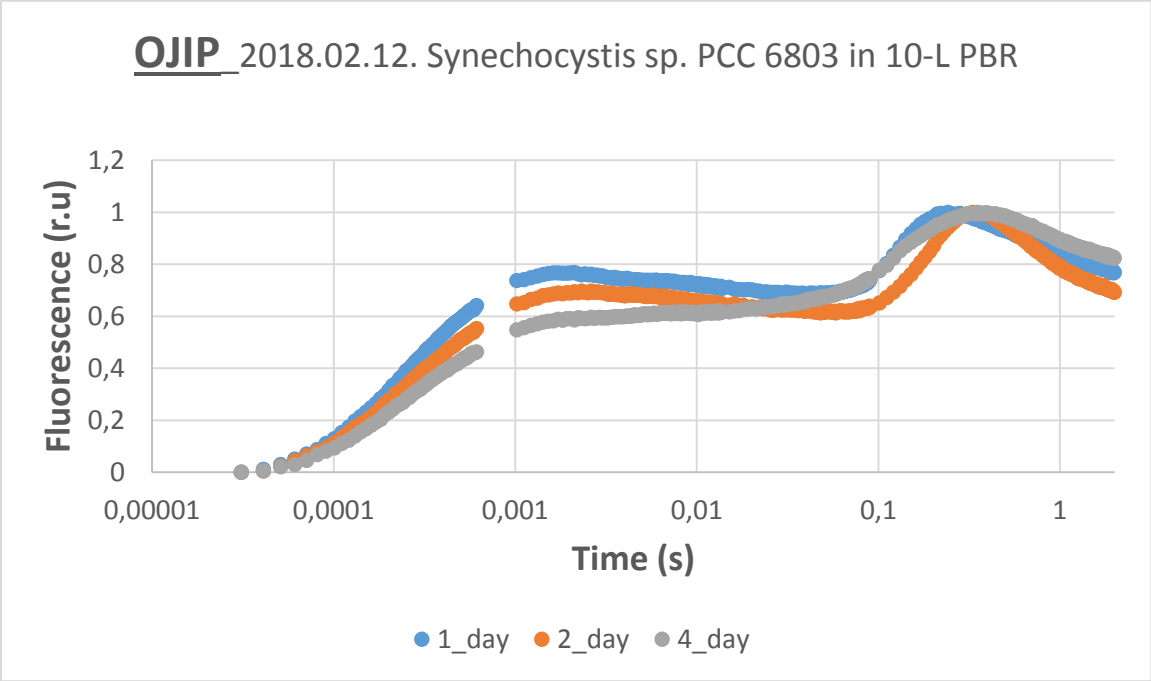


Fig. 2. Changes in OJIP curves during cultivation trial in 10-L photobioreactor

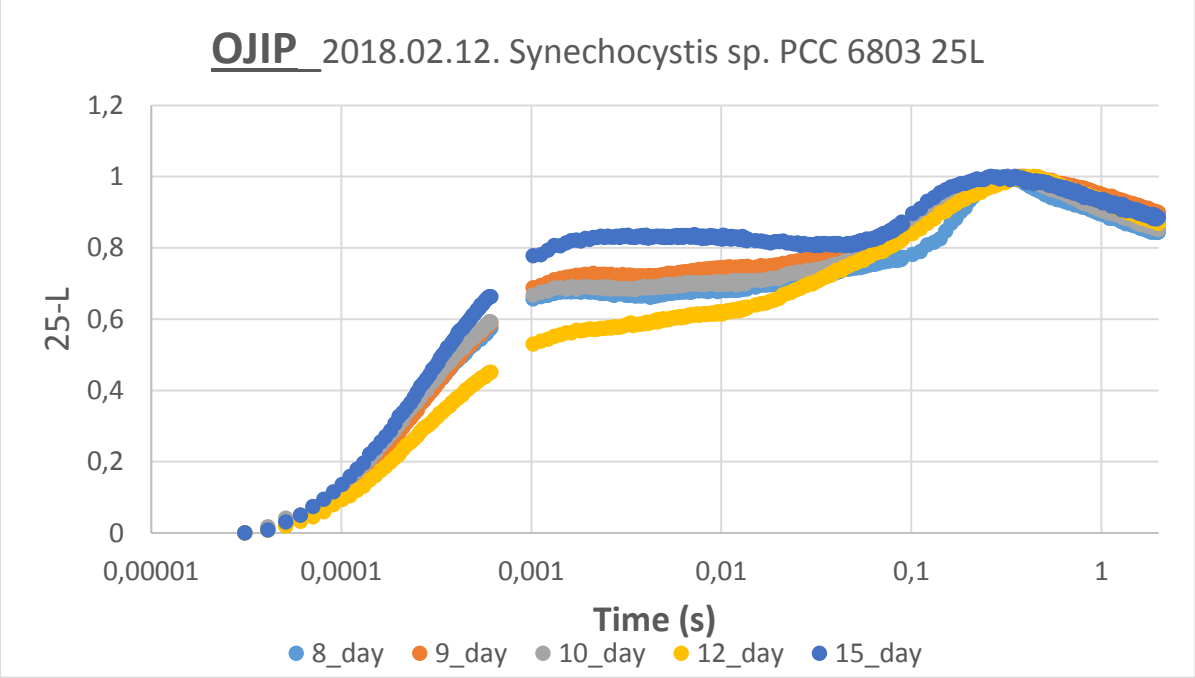


Fig. 3. Changes in OJIP curves during cultivation trial in 25-L photobioreactor

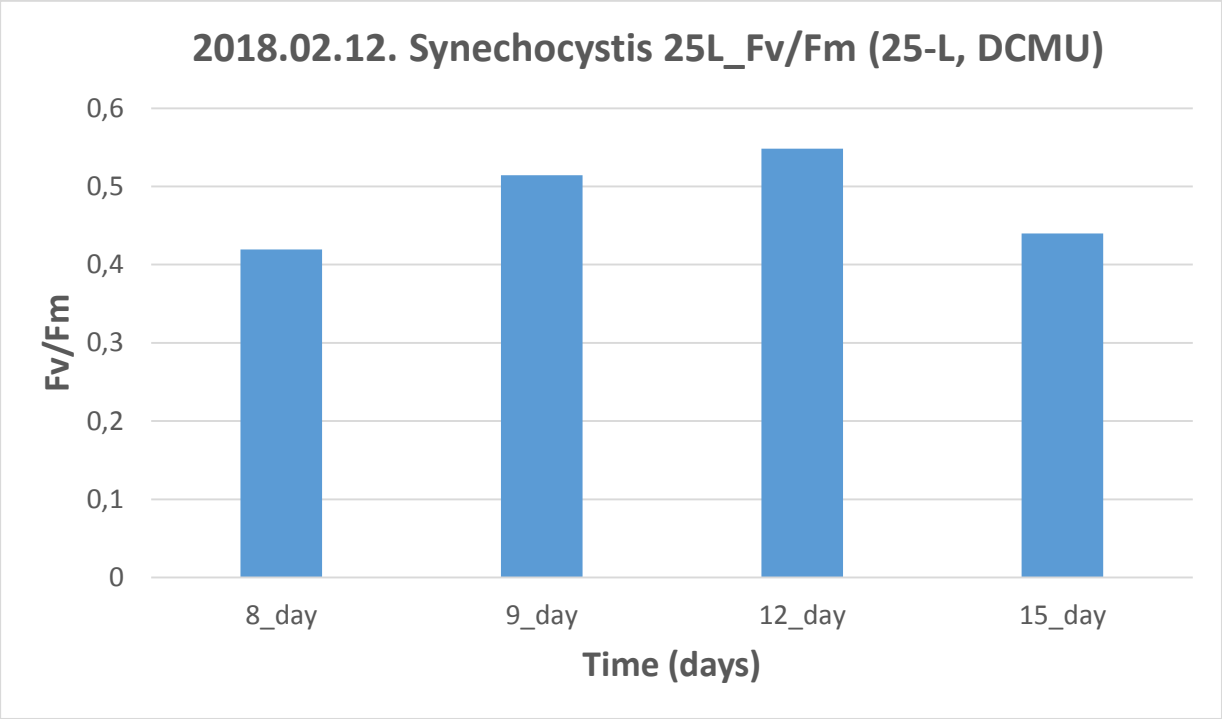


Fig. 4. Fv/Fm changes in 25-L photobioreactor with DCMU

Synechocystis sp. Culture was first pre-grown in 300 mL cylinders then transferred to 10-L photobioreactor to reach the sufficient biomass for the inoculation of the 25-L photobioreactor on 8th day. The culture conditions were continuously monitored by the sensors of the photobioreactor and by fluorescence measurements as well. The growth conditions provided by the photobioreactor were optimally adjusted. The highest growth rates were obtained when the culture was relatively diluted compared to the illumination intensity (Table 1, Fig. 1.). It occurred after the inoculation on the 8th day and after 12th-15th day when the light intensity was increased. On the other hand, the optimum conditions for the culture were obtained on the 12th day of the cultivation when the growth rate was moderate (Figs. 3 and 4).

Conclusions

- *Synechocystis sp.* PCC 6803-NIX grows well in higher volume photobioreactors
- The initial problems with temperature control and bubbling rate for culture mixing was solved and the maintenance of the culture was sufficient.
- With the regular fluorescence monitoring and DW/OD measurements of the culture the optimum set-up for the highest growth rate can be achieved. In this way, the too low or high irradiance which can decrease the growth rate can be avoided.

3rd experimental series (Třeboň)
Nutrient starvation experiment

Period: 8-day experiment

Strains:

- *Synechocystis sp.* PCC 6803-NIX

Experimental data:

- Pigments
- Photosynthetic activity
- Growth

Culturing conditions/regimes:

- Cultivation volumes: in 350 mL cylinder (diameter 38 mm) bubbled with air+CO2 (duplicates)

- Culture medium: BG-11 or BG deficient
- Initial concentration ~0.5 g L^{-1} (diluted on the basis of Chl concentration OD680)
- Light intensity: ~80-100 µmol photons m^{-2} s^{-1} on inner wall surface of glass water bath exposed using cosine-corrected sensor – on Day 5 increased to 120 µmol photons m^{-2} s^{-1}
- Temperature: 30±1 °C
- Light source: warm-white high-frequency horizontal fluorescence tubes
- Light/dark cycle: continuous light

Treatments

- Control - BG-11
- BG0-N – BG without nitrate
- BG0-P – BG without phosphate
- BG00-NP – BG without nitrate and phosphate

Results

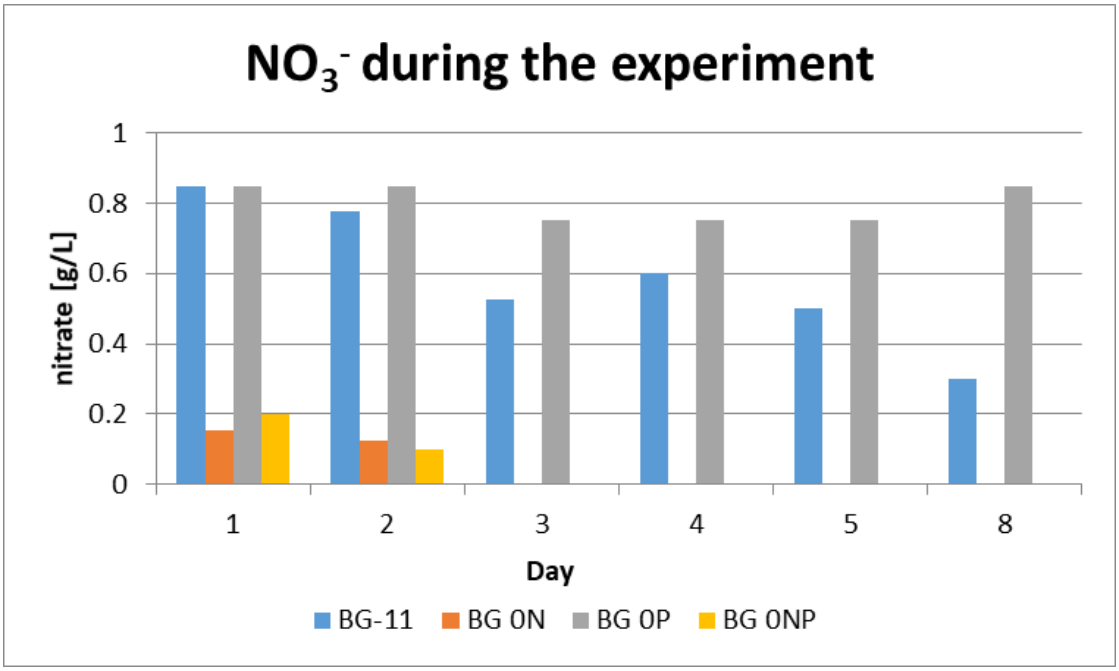


Fig. 1. Nitrate consumption in the different cultures

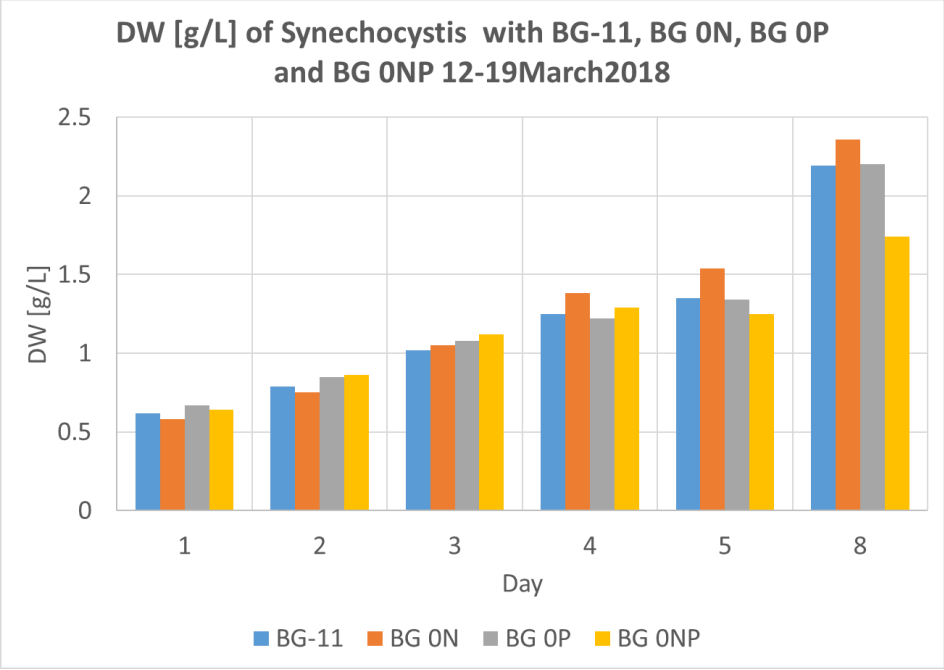


Fig. 2. Dry weight changes in different cultures

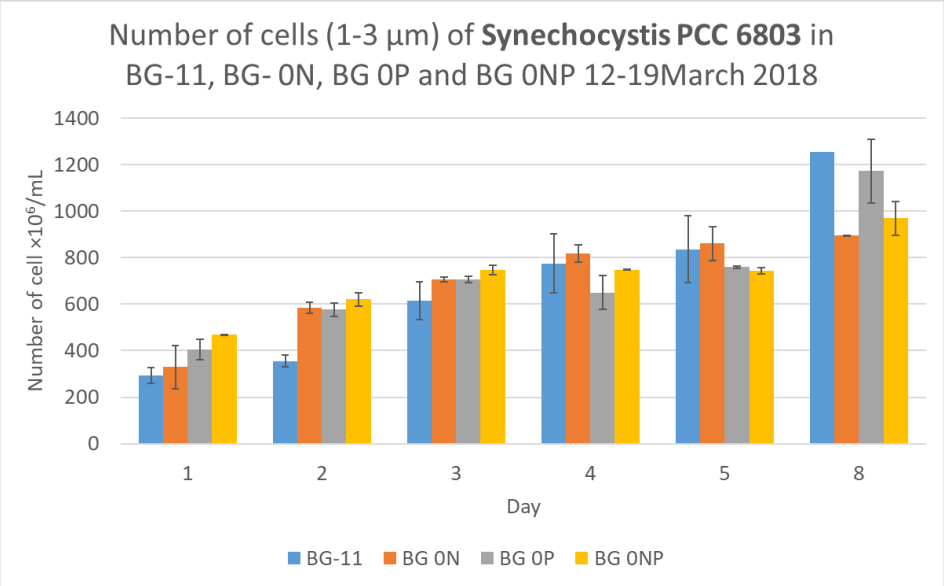


Fig. 3. Cell number changes in different cultures

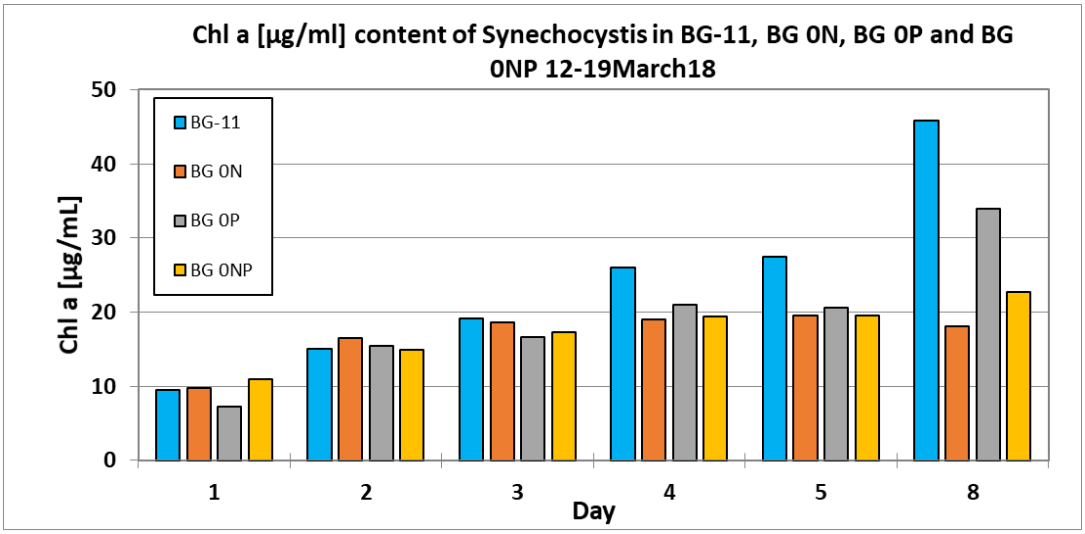


Fig. 4. Chlorophyll a changes in different cultures

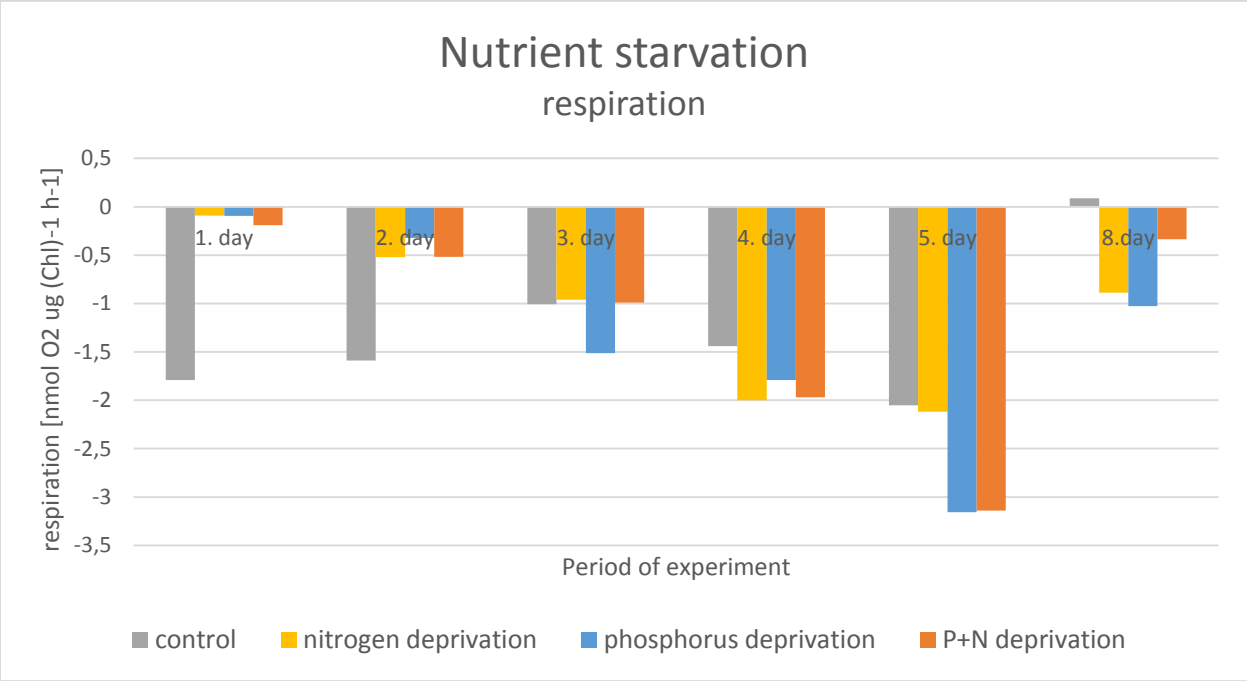


Fig. 5. Maximum respiration rate in the different cultures

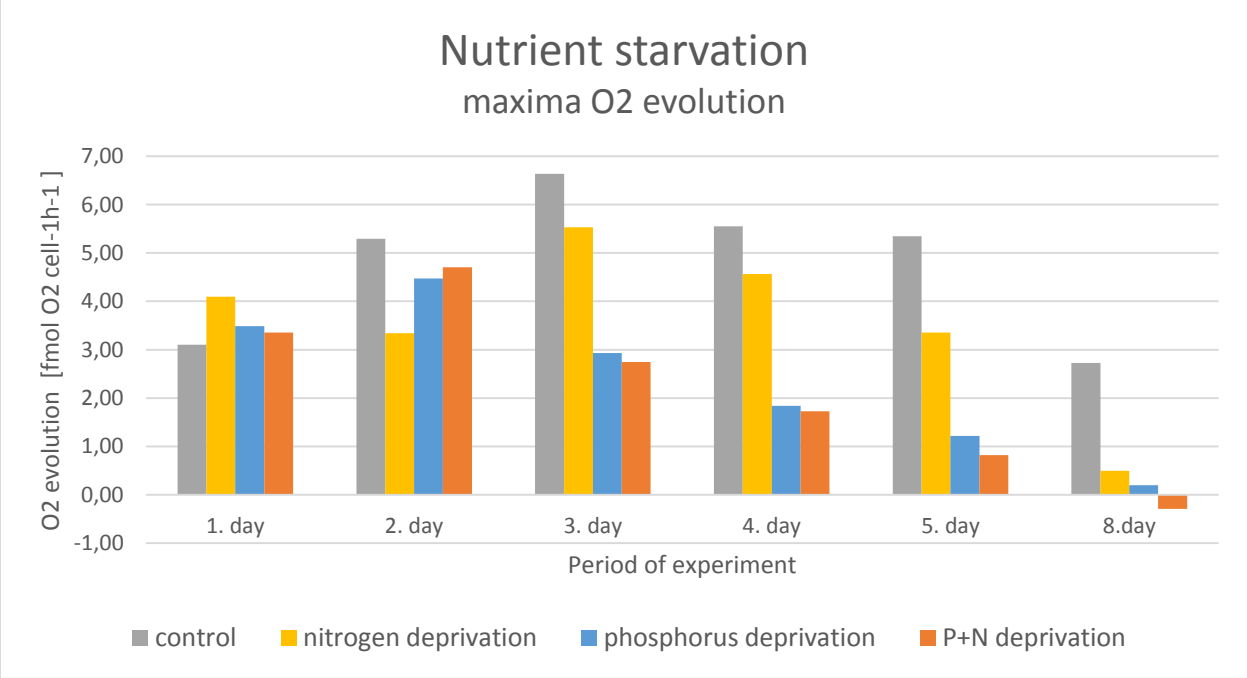


Fig. 6. Maximum O_2 evolution in different culture

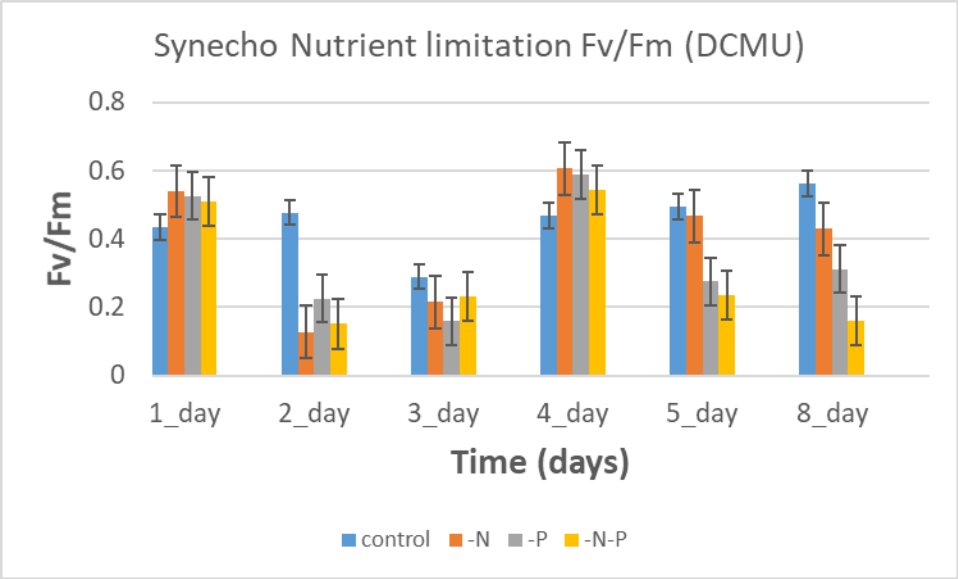


Fig. 7. Fv/Fm values in different cultures after DCMU addition

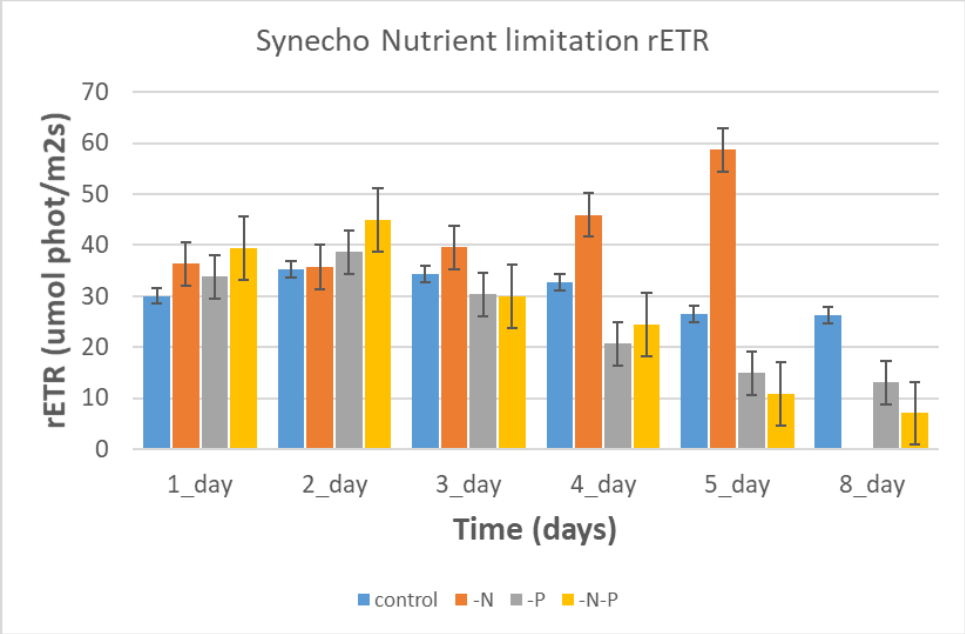


Fig. 8. Relative electron transport rETR values in different cultures after DCMU addition

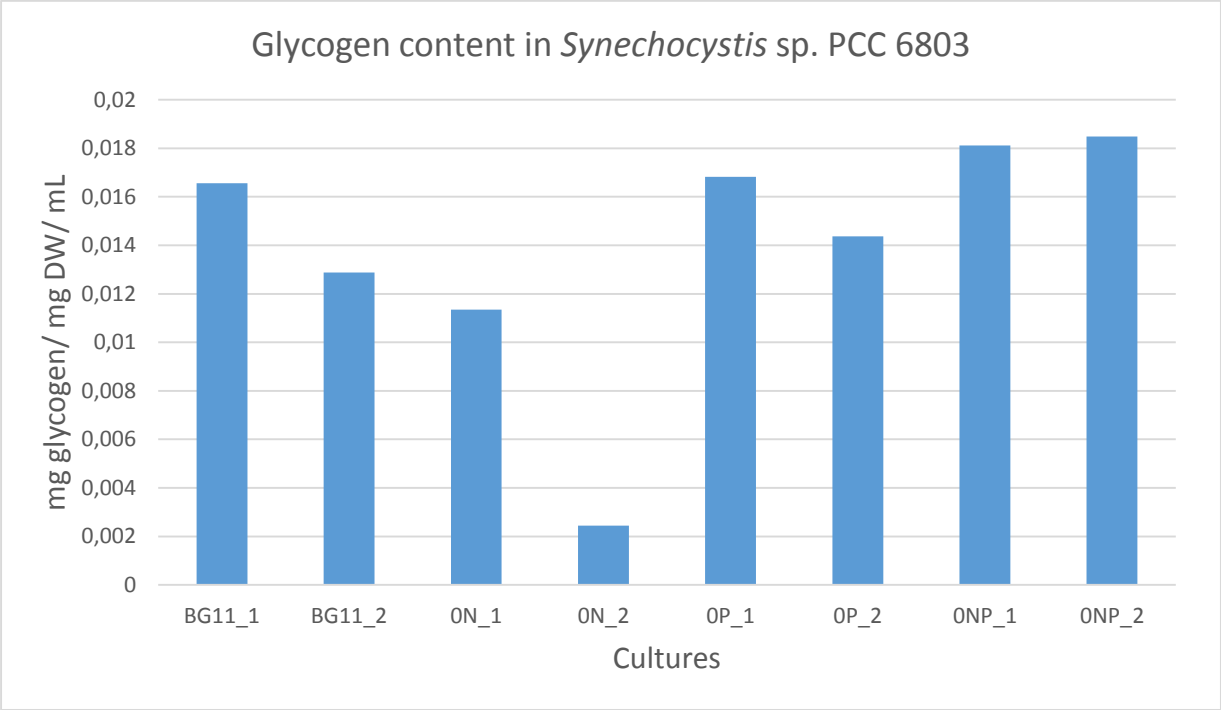


Fig. 9. Glycogen content in different cultures

After double washes of the pre-grown cultures tiny amount of nitrate was still presented (Fig. 1.). It was totally consumed in two days from the start of incubation in 0N and 0NP cultures. In the control culture the consumption rate was in correlation with the growth rate, while in the 0P cultures remarkable nitrate concentration decline was not measured during the whole period of cultivation. All in all, the different cultivation media could indicate efficient nutrient starvation stresses.

The changes of the cell numbers, chlorophyll a content and dry weight shows noticeable differences (Figs. 3, 4 and 5). Distinguishably, it can be seen that nitrogen starvation resulted the inhibition of cell division because the cell numbers are the lowest when nitrate is not presented in the media. On the other hand, highest dry weights were measured when only nitrate was deprived, which can be caused by the increase of the cell sizes instead of cell division. Phosphorous deprivation didn't inhibit cell division, but together with nitrogen starvation it inhibited the growth of dry weight.

Respiration was balanced in the control cultures but increasing trends can be observed in the three other cases. Highest respiration was obtained in the phosphorous deprived cultures (0P and 0NP) (5.). Oxygen evolution rate was supremely decreased in the phosphorous deprived cultures as well (Fig. 6.). In the nitrogen deprived cultures oxygen evolution rate was slightly decreased compared to the control samples.

Fv/Fm values shows an initial stress during the cultivation period on the send and third days. For the fourth day it increased back to the normal level (approximately 0.6). From the fifth day starts the real effect of the nutrient starvation on the cultures which can be seen from the decrease of the Fv/Fm values (Fig. 7.). Relative electron transport rate showed similar decrease.

The most inhibited cultures were the phosphorous deprived cultures. Interestingly, intensive growth of relative electron transport rate was observed in the case on nitrogen starvation (Fig. 8.).

Glycogen content was also measured and normalized on dry weight content, but for reliable data further improvement of method is necessary (Fig. 9).

Conclusion

- Cultures were grown at biomass density of 0.5-0.6 g DW/L in tubes with 38 mm light-path. In this set-up the onset of light saturation measured by oxygen production and fluorescence was between 100-200 µmol photons m^{-2} s^{-1}, it means that the cultures can be grown at irradiance between 100-200 µmol photons m^{-2} s^{-1}, depending on biomass density.
- Growth of Synechocystis cultures at various treatments showed that it is affected not as much under phosphate limitation, but under nitrate and phosphate-nitrate limitation at the end of 8-day treatment (graph cell count, probably due to cell size).
- Per biomass cultures are growing (g/mL) even at nutrient deficiency when after two days there is low content of N and P under deficiency??
- Changes of pigment content correlate with nutrient changes as highest Chl value was found in control and then under P deficiency (25% decrease), in Phosphate and Nitrate-phosphate deficient was about 40-50% of control after 8 days.
- Photosynthetic oxygen production evolution (represent steady-state measurements) measured per cell – the data show that nitrogen limitation decrease O_2 production starting from Day 3-5 by about 30-40%, but much more significantly under phosphate and phosphate-nitrate limitation, finally to about 5-10%.
- The onset of light saturation (saturating irradiance) at about 100-200 µmol photons m^{-2} s^{-1} in this set-up
- Fluo RLC measurements showed inhibition of photosynthetic activity - Fv/Fm (maximum photochemical yield) and rETR (electron transport rate) starting from Day 4.
- The nutrient analyses showed some content (0.1 g/L) of nitrogen even in deficient samples which start to limit growth on Day 5, in Photosynthetic activity it is manifested on Day 4.
- Samples of cultures were taken for glycogen content analysis at Day 8 which is in progress.

4th experimental series (Wels)
Photosynthesis based pre-screening to determine suitable growth irradiation of *Synechocystis* sp. PCC6803

Strains:
Synechocystis sp. PCC 6803-NIX
Aim:
Screening procedure for genetically modified cyanobacteria. Photosynthesis based pre-screening to determine suitable growth irradiance of *Synechocystis* sp. PCC6803

Experiments:
- Photosynthetic activity: RLC, LRC, OJIP
- Oxygen production
- Growing rate: OD and cell number
-

Culturing conditions/regimes:
- Culture flasks of 225 ml placed in vertical position, working volume 140 ml

- Light intensity: 30, 60, 90 µmol photons m^{-2} s^{-1}
- Culture densities: OD_{750}: 0,1; 0,2; 0,4; 0,8; 1,6
- Continuous light
- Temperature 31 °C
- CO_2% : 1%
- Media: BG 11

Experimental scheme:
- Day 1:
  - Equipment setup, culture into the incubator,
  - 1st trial inoculation in the afternoon, measuring OD 750/730, cell counting
  - Exposure overnight: cultures OD750: 0.1, 0.2, 0.4, 0.8, 1.6
    - Triplicates: 15 culture flask, 140 ml
    - Light intensity, 30 µmol photons m^{-2} s^{-1}

- Day 2
  - Measuring in the morning: LRC, OJIP, oxygen production, OD 750/730, cell counting
  - 2nd trial inoculation in the afternoon, measuring OD 750/730, cell counting
  - exposure overnight:
  - Cultures: OD750: 0.1, 0.2, 0.4, 0.8, 1.6
    - Triplicates: 15 culture flask, 140 ml
    - Light intensity: 60 µmol photons m^{-2} s^{-1}

- Day 3
  - Measuring in the morning: LRC, OJIP, oxygen production, measuring OD 750/730, cell counting
  - 3rd inoculation in the afternoon, measuring OD 750/730, cell counting, exposure overnight:
  - Cultures: OD750: 0.1, 0.2, 0.4, 0.8, 1.6
    - Triplicates: 15 culture flask, 140 ml
    - Light intensity: 90 µmol photons m^{-2} s^{-1}

- Day 4
  - Measuring in the morning: RLC, LRC oxygen production, OJIP, measuring OD 750/730, cell counting

Results

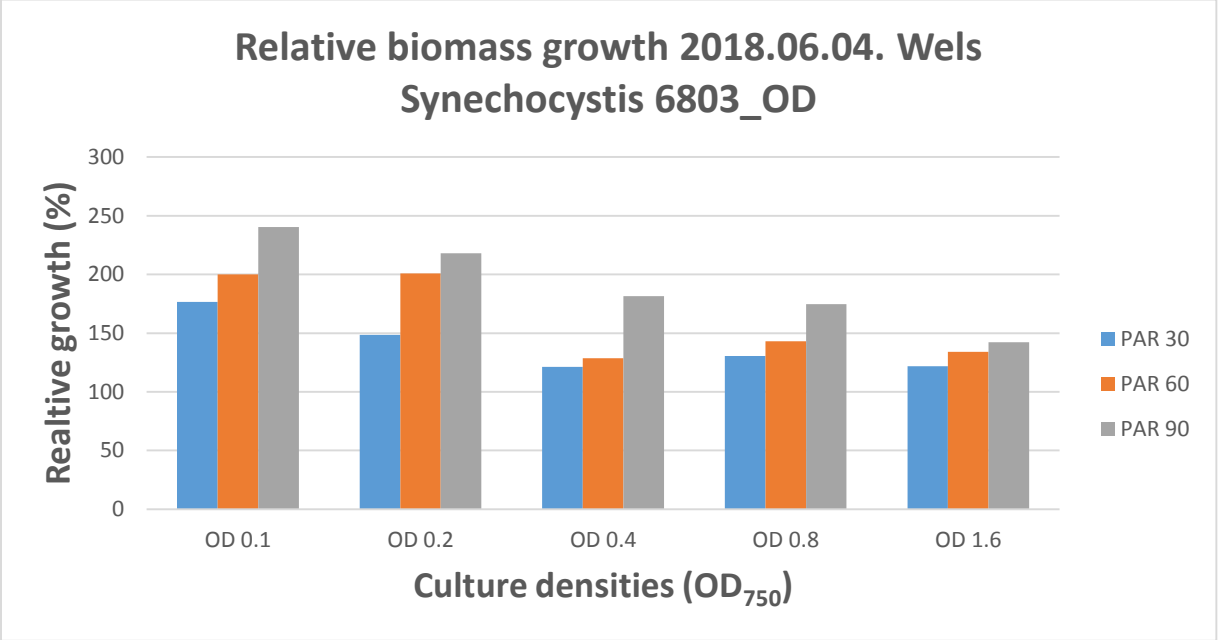


Fig. 1. Relative biomass growth in different cultures under different illumination

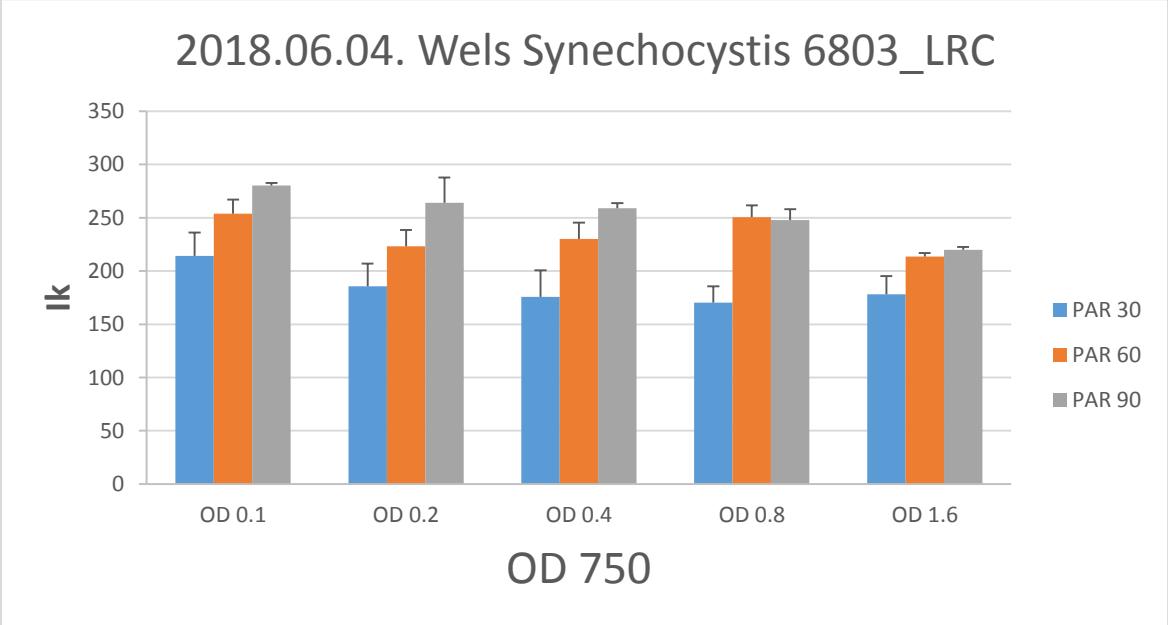


Fig. 2. Light level saturating photosynthesis Ik values of different cultures under different illumination

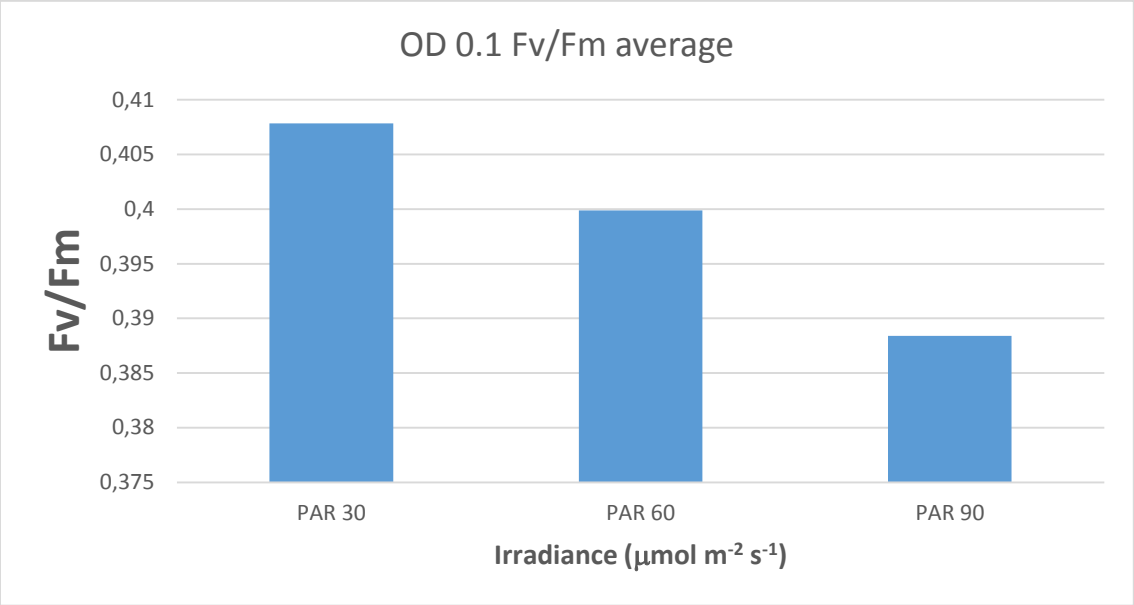


Fig. 3. Fv/Fm (DCMU) values of cultures with OD 0.1 dilution under different illumination

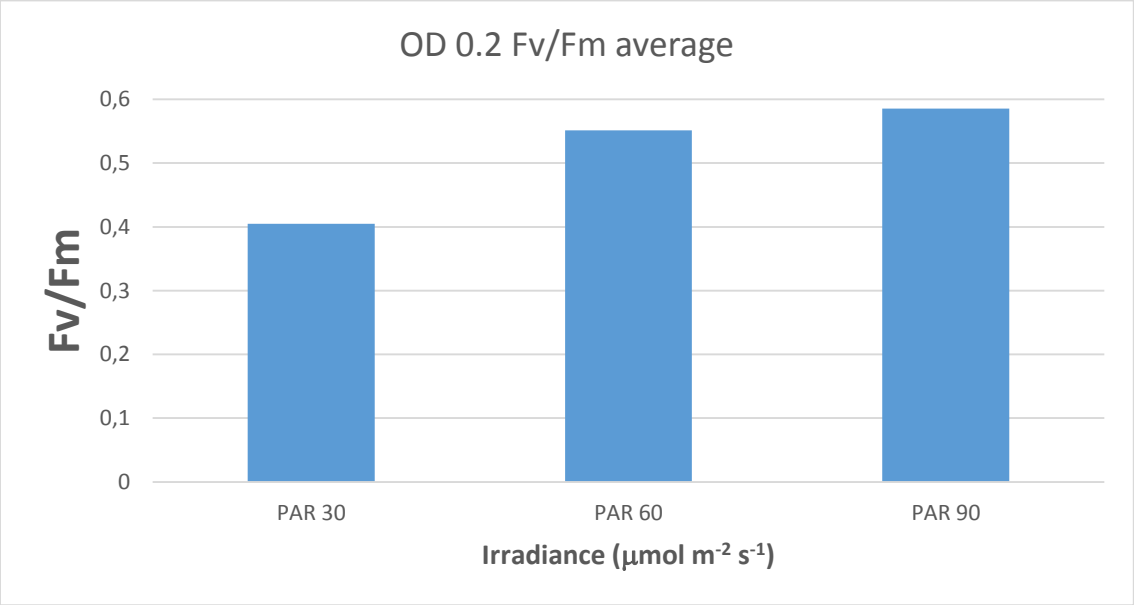


Fig. 4. Fv/Fm (DCMU) values of cultures with OD 0.2 dilution under different illumination

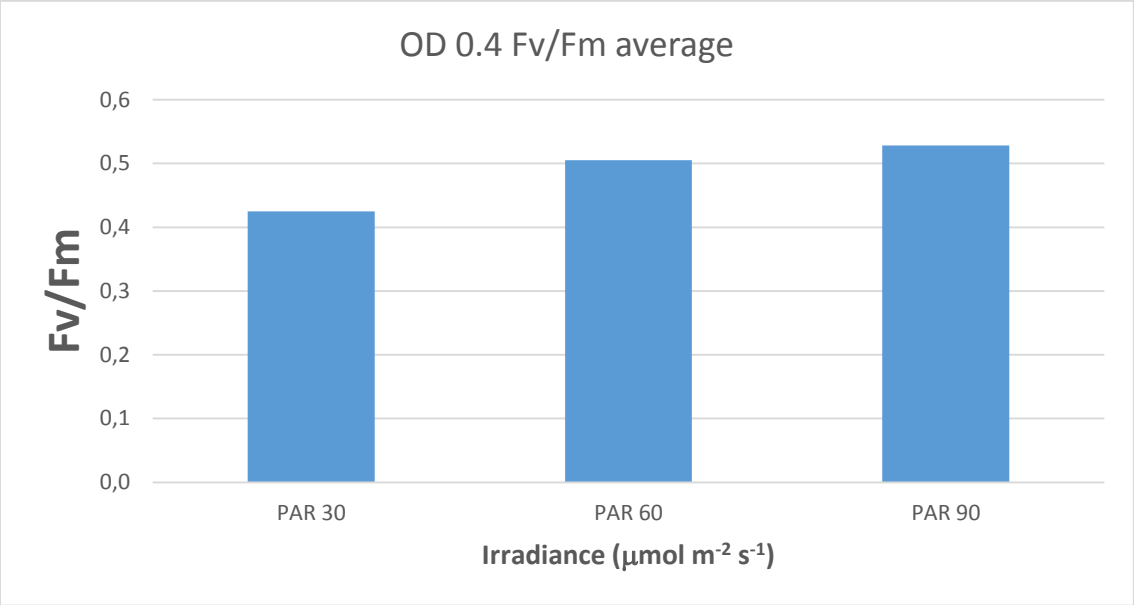


Fig. 5. Fv/Fm (DCMU) values of cultures with OD 0.4 dilution under different illumination

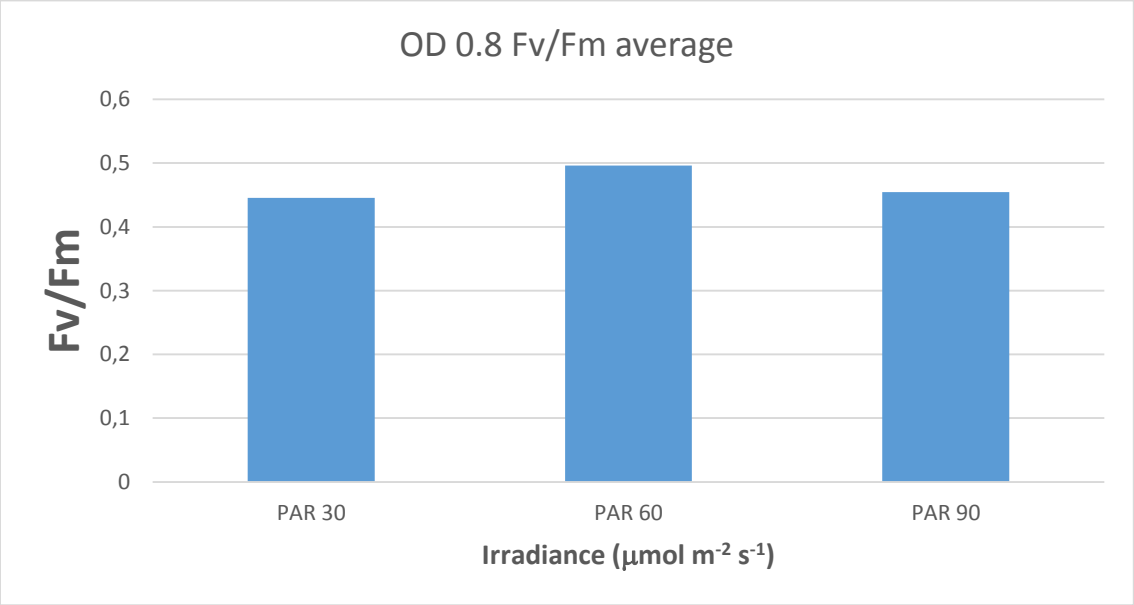


Fig. 6. Fv/Fm (DCMU) values of cultures with OD 0.8 dilution under different illumination

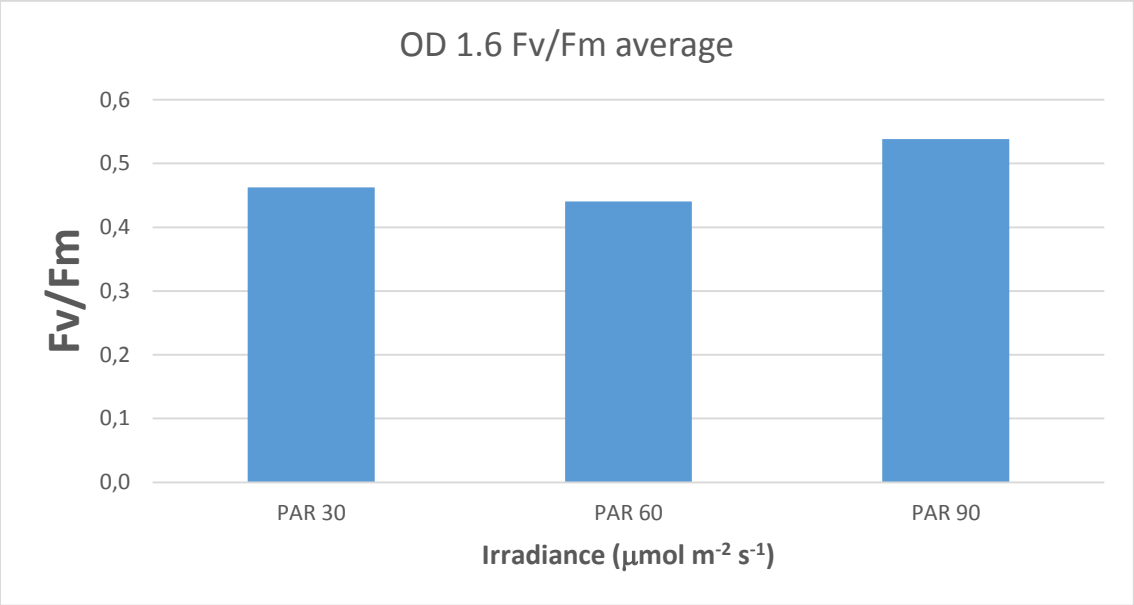


Fig. 7. Fv/Fm (DCMU) values of cultures with OD 1.6 dilution under different illumination

Synechocystis sp. PCC 6803 NIX cultures were incubated overnight under three illumination intensities at five different densities. The study focused on the determination of the optimum irradiation strength for efficient growth without any factors causing stress symptoms on cyanobacterial culture. Highest relative growth rate was obtained by the most diluted cultures under the highest irradiation (Fig. 1). However, under 90 µmol photons m^{-2} s^{-1} cell aggregation appeared at the bottom of the culture flasks at OD 0.1, 0.2 and 0.4. This indicates the influence of the light stress in these cultures which is supported by the results of Fv/Fm (DCMU) measurements (Fig. 3-7). The highest rate of aggregation was observed in the cultures with OD 0.1 dilution. On the other hand, the most intensive growth of biomass occurred in these cultures which show that the low level of light stress could be overcome via the formation of aggregates in the culture. Handling of relatively strong light was promoted by the 6-cm culture depth as well. Earlier experiments showed more intensive aggregate formation in the liquid and sedimentation on the bottom of Erlenmeyer bottles in *Synechocystis* cultures using similar light regimes. The symptoms of light stress can be reduced by using standing culture flask with high culture depth even in very low starting culture densities.

The other major point of this experiment was to find correlation between the growth rates (and productivity!) of the various cultures and some fluorescence variables (indicators) and oxygen production rates. A good indicator to determine the optimum irradiance related to a given culture density would remarkably reduce the time period which is necessary to define the optimum light intensity. Utilization of time consuming growth curves could be reduced. Fluorescence measurements resulted in curves and trends. Fv/Fm values after DCMU addition showed strong decline as the irradiance became stronger, when the culture density was set to OD 0.1. In the other cases the highest Fv/Fm values were obtained when the irradiance was the highest. The most suitable graph which follows the growth curves is the value of Ik. It is a promising candidate for this purpose.

Conclusions

- Cultures were grown in sufficient light path and density to avoid induction of light stress. Increase of irradiance and decrease of culture depth would cause the stress.
- Ik values directly correlate with growth curves. In the future it can be the major factor for the identification of optimum light intensity for growth.

5th experiment (Třeboň)
Determination of ethanol loss from the culture media in 350 mL bubble columns

Aim:

- Measuring the loss of ethanol from water in 350 mL bubble column PBR during 7-day period.

Experiment:

- Dissolved ethanol measurement – instrument LabQuest Mini (company Edufor) with ethanol sensor

Culturing conditions/regimes:

- Media: distilled water + 100 % ethanol, 3% final concentration
- Cultivation volumes: in 350 mL column PBR (i.d. 38 mm) bubbled with air+CO_2 (duplicates)
- Light intensity: ~80 µmol photons m^{-2} s^{-1} on the inner wall surface of the glass temp-controlled bath exposed to irradiance using cosine-corrected sensor
- Temperature: 30±1 °C
- Light source: warm-white high-frequency horizontal fluorescence tubes

Methods

Vernier ethanol sensor was placed 5 cm above the surface of the water. The sensor was connected to the transmitter and computer which registered the measurements. Evaporated ethanol was measured by the sensor and the concentration in the water was calculated by the software. The sampling time was 15 min during 7 days. The initial ethanol concentration was 3% (v/v)

Results

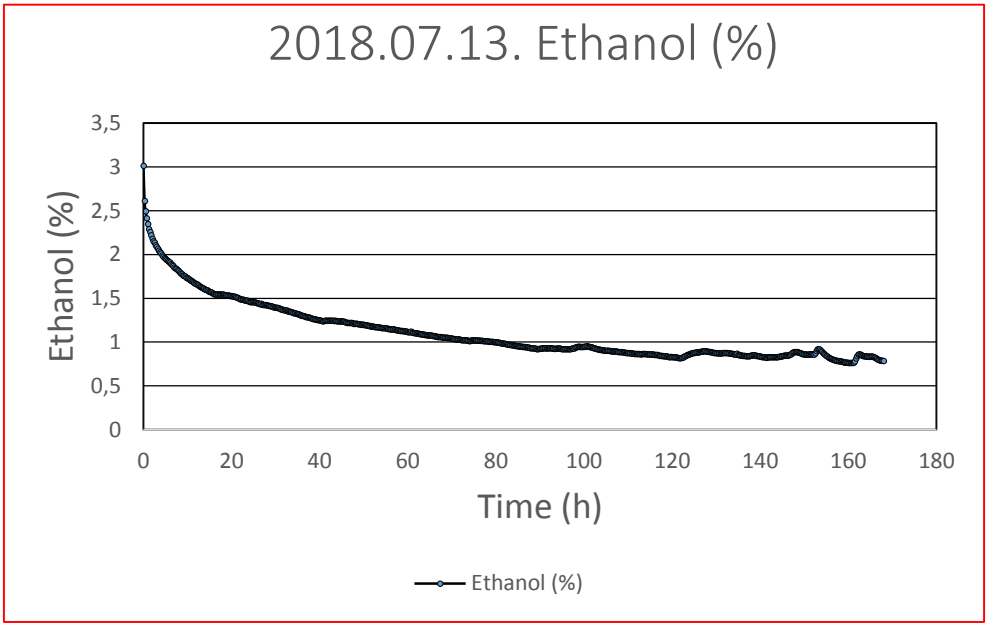


Fig. 1. Ethanol concentration in distilled water in 350 mL cylinder

Ethanol evaporation was very high above 1% (per hour?). Logically, the higher ethanol concentration, the higher ethanol evaporation was observed. Around 0.8-0.9 the evaporation rate was remarkable reduced.

Conclusions

- This equipment Ethanol sensor can be efficiently used for ethanol production experiments in non-invasive way. The risk of contamination can be reduced.
- In cylinders the ethanol evaporation won't cause serious loss at low concentration, thus it can be used for screening tests as Ethanol production can be followed continuously.