Screening of growth phases of Antarctic algae and cyanobacteria cultivated on agar plates by chlorophyll fluorescence imaging

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Abstract

Recently, chlorophyll fluorescence imaging is frequently used non-invasive method to monitor the metabolic state and photosynthetic activities of vascular plants and other autotrophic organisms. In our study, we used the measurements of chlorophyll fluorescence kinetics to follow the development of culture of Antarctic algae (*Macrochloris rubrioleum*, *Zygnema* sp.) and cyanobacteria (*Hassalia antarctica*, *Nostoc commune*). On the cultures grown on agar plates, Bold´s Basal Medium (BBM), slow Kautsky kinetics supplemented with saturation pulses were measured repeatedly in a week interval. On the kinetics, typical points (OPSMT) were distinguished and species-specific and time of cultivation-dependent differences in shape of the OPSMT kinetics evaluated. We tested sensitivity of various chlorophyll fluorescence parameters to cultivation time on agar plates. In the algae, the most pronounced changes were the decrease in maximum quantum yield of photosystem II (F_V/F_M) and quenching of basal chlorophyll fluorescence qF_0 (*M. rubrioleum*, *Zygnema* sp.). In cyanobacteria, chlorophyll fluorescence parameters did not show clear trends with the time of cultivation. F_0 quenching (qF_0) reached positive values in *H. antarctica*, while it was negative in *N. commune*. In both cases, however, qF_0 showed an increase with cultivation time. The differences are discussed as well as the potential of the emerging area of the application of chlorophyll fluorescence imaging for evaluation of photosynthetic performance of algal/cyanobacterial cultures on agar plates.

Key words: microalgae, *in vitro* cultivation, slow Kautsky kinetics, OPSMT

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Abbreviations: F_V/F_M – maximum yield of photosynthetic processes in PS II, Φ_{PSII} – effective quantum yield of photosynthetic processes in PS II, OPSMT – slow Kautsky kinetics abbreviated according to the typical points reached on the curve: O – origin, P – peak, S – steady state, M – local maximum, T – terminal state, qF_0 – quenching of basal chlorophyll fluorescence, R_{Fd} – vitality index (relative fluorescence decrease)

Introduction

Since the discovery of the 'Kautsky effect' of chlorophyll fluorescence, the complex kinetics of the fluorescence have attracted the interest of many scientists, and interpretation of the polyphasic character of the kinetics reviewed by several authors (Govindjee 1995, Lazár 1999).

Within last decades, the use of chlorophyll fluorescence imaging by CCD cameras has been applied in many fields of plant physiology. Chlorophyll fluorescence imaging allows multiple plants to be monitored at the same time under identical conditions, providing an ideal screening platform. Therefore, slow Kautsky kinetics of chlorophyll fluorescence is advantageous for large-scale screening, and monitoring of large 2-D objects such as leaves or whole plants. Moreover, when chlorophyll fluorescence imaging of Kautsky kinetics is used repetitively in a course of time over the same photosynthesizing object, spatial and temporal heterogeneity of photosynthetic performance can be evaluated. In such studies, however, the shape of Kautsky kinetics is used for evaluation of physicological state (photosynthetic performance) of plants much less frequently than the changes in commonly used chlorophyll fluorescence parameters (maximum yield of photosynthetic processes in PS II- F_V/F_M, effective quantum yield of photosynthetic processes in PS II – Φ_{PSII}). However, the changes in Kautsky kinetics shape have been used to investigate direct effect of low temperature (Mishra et al. 2011 – vascular plant, Marečková et al. 2019 – lichen).

Differences in the shape of chlorophyll fluorescence kinetic have been used for screening of *Synechocystis* mutants and the regulation of photosystem stoichiometry (Fujimori et al. 2005, Ozaki et al. 2007, Ozaki and Sonoike 2009). In these studies, chlorophyll induction kinetics (Kautsky kinetics) were determined on agar plate-grown material by a fluorescence CCD camera (FluorCam 700MF, Photon Systems Instruments, Czech Republic) as described previously (Ozaki et al. 2007). To examine the effect of KCN, 10 μl of 100 mM KCN solution was dropped on a cyanobacterial patch (*Synechocystis* sp.) grown on an agar plate just before dark acclimation Ogawa (2017). The effect of osmotic stress was studied by Alonso (2018) in *H. antarctica* treated by NaCl. In the above-specified study, the shape of Kautsky kinetics responded to heavy stress (3 M NaCl) by a decrease in chlorophyll fluorescence signal and flattening of the curve. Gavel and Maršálek (2004) suggested to use slow Kautsky kinetics, the complementary area delimited by the kinetics and a horizontal line passing through the P point in normalized fluorescence, respecttively, for the assessment of phytotoxicity of tested compound applied to algae. Recent development of microscopy techniques allowed to use the measurements of slow Kautsky kinetics in algae and cyanobacteria *in vivo* by the combination of a high resolution optical microscopy and chlorophyll fluorescence technique (see *e.g.* Komárek et al. 2010 for *Scenedesmus quadricauda*, *Trichodesmium* sp.), Tolleter

et al. (2011) for *Chlamydomonas reinhardtii*.

The aim of our study was to distinguish different growth phases of Antarctic algae and cyanobacteria cultivated on agar plates by chlorophyll fluorescence technique. We hypothesized that shape of slow Kautsky kinetics of chlorophyll fluorescence would exhibit substantial changes in a course of time of cultivation from the start, *i.e.* the inoculation of algae on agar plates to the state of well developed culture. Therefore, we measured Kautsky kinetics in algal and cyanobacterial cultures in a week interval. We hypothesized that the changes in Kautsky kinetics shape would be rapid during the first several weeks, followed by much less pronounced changes in the month-lasting cultivation. Our aim was to identify sensitive indicators of the changes in Kautsky kinetics in several representatives of Antarctic algae and cyanobacteria.

Material and Methods

In this study, cyanobacteria (*Hassalia antarctica*, *Nostoc commune*) and algae (*Macrochloris rubrioleum*, *Zygnema* sp.) were used for the experiment. All the cultures were grown on Bold´s Basal Medium (BBM) on Petri dishes at temperature of 5°C. *H. antarctica* was provided from the Culture Collection of Autotrophic Organisms (CCALA, Třeboň, Czech Republic). The strain 955 was cultivated from the collections at the James Ross Island, Antarctica. *Zygnema* sp. (strain EEL-202), *N. commune* were collected in 2015 from the Long-term Research Plot (LTRP) situated near the Mendel station, James Ross Island and cultivated as axenic cultures in the Extreme Enviroment Life laboratory (EEL, Brno, Czech Republic). *M. rubrioleum* was collected from North-facing slope of the Berry Hill mesa, James Ross Island in 2014. The collection site was situated close to the seashore. After transfer to Brno, *M. rubrioleum* was cultivated on agars as axenic culture.

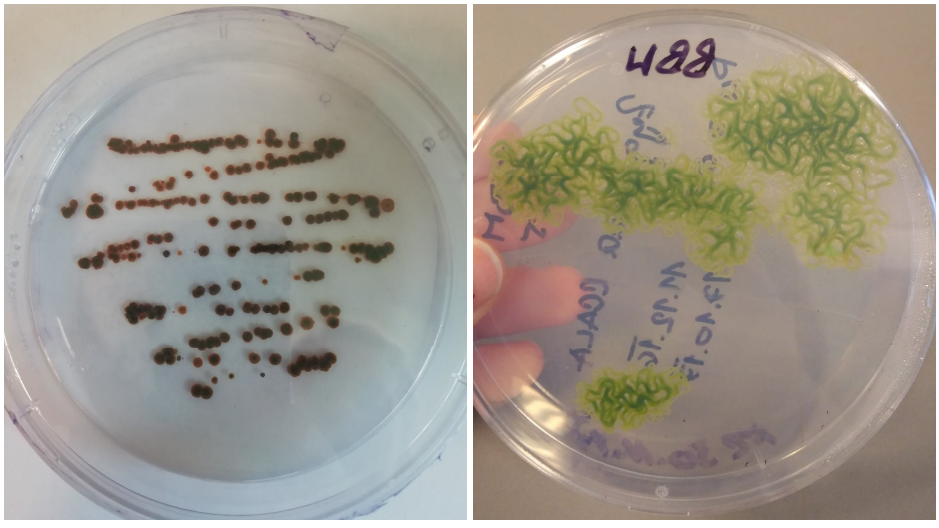

Fig. 1. The cultures of experimental species grown on agar plates: *Macrochloris rubrioleum* (left) and *Zygnema* sp. EEL strain 202 (right).

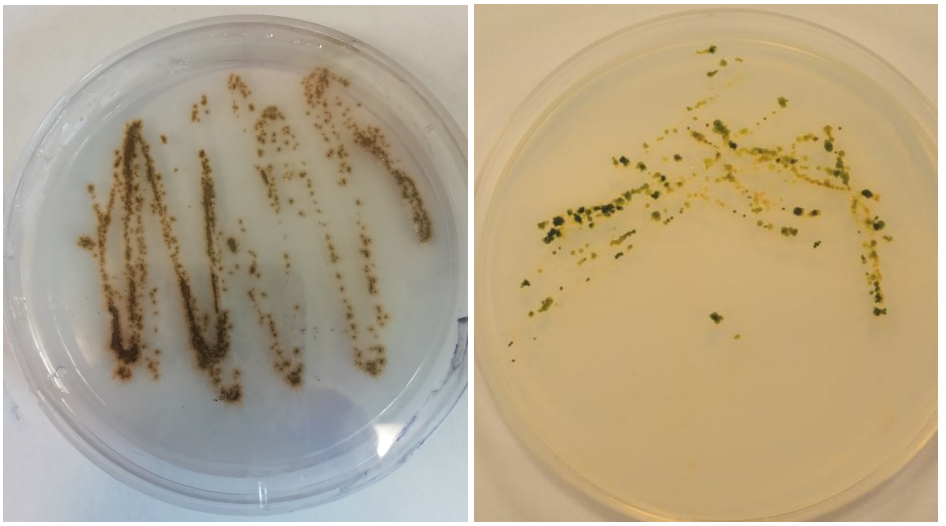

Fig. 2. The cultures of experimental species grown on agar plates: *Nostoc commune* (left) and *Hassalia antarctica* (right).

The species has been described recently by Kawasaki et al. (2015) based on strains isolated from both terrestrial and freshwater habitats in Continental Antarctica and Svalbard.

After 1.5 month cultivation of the above-specified four experimental species on agar plates the chlorophyll fluorescence parameters were monitored repeatedly in a period of 1 week (the first measurement is denoted as Day 0). Chlorophyll fluorescence (slow Kautsky kinetics) was measured directly from plate cultures and recorded by FluorCam (HFC-010, Photon Systems Instruments, Czech Republic). Each sample was dark-adaptated for 10 min. before measurements. The numerical data were recorded and slow chlorophyll fluorescence kinetic (OPSMT – *see* below for more details) plotted for particular species and time of cultivation. At the individual kinetics, fast OP phase was distinguished (O for Origin, P for Peak) and following slower transient SMT (S for Steady state, M for Maximum, T for Terminal state) – *see* Fig. 3. Further, the following chlorophyll fluorescence signals were evaluated: basic fluorescence (F_0), maximum fluorescence measured in dark-adapted sample (F_M), maximum fluorescence measured in light-adapted sample (F_M'), maximum chlorophyll fluorescence recorded after switching of the actinic light and 30 s of dark (F_M''). From the data, F_V/F_M - maximum yield of photosynthetic processes in PS II, Φ_{PSII} - effective quantum yield of photosynthetic processes in PS II and other chlorophyll fluorescence parameters were evaluated using standard equations (for equations see *e.g.* Roháček and Barták 1999). R_{Fd} was evaluated according by Lichtenthaler et al. 2005.

Shape of Kautsky kinetics was analyzed according to Riznichenko et al. (1996) by the following parameters: (1) presence of additional maximum M and minimum S, (2) location of maximum M and minimum S with respect to the steady-state level T(=F_S) (lower or higher), (3) the time at which steady-state level (F_S) was reached (*i.e.* before/after 200 s of actinic light time), (4) F_0 to F_S ratio (O/T).

Results and Discussion

The analysis of the Kautsky kinetics of fluorescence recorded in our experiments during growth on agar plates in Petri dishes showed that the shape of individual of kinetics varied considerably. The pattern of the PSMT part of the kinetics (*see* Fig. 3) exhibited cultivation time-dependent peculiarities.

Typically, the shape of kinetics changed much rapidly during the first three weeks after innoculation, while in the following period of cultivation time, the changes were much less pronounced. Similarly, some of chlorophyll fluorescence parameters showed cultivation time-dependent changes, while some of them did not changed. Here, we present only two of them: maximum yield of photochemical processes in PS II (F_V/F_M) and quenching of background chlorophyll fluorescence (qF_0 - according to Bilger and Schreiber 1986) - *see* Table 1. In *M. rubrioleum*, F_V/F_M remained constant during the cultivation period indicating high potential of photosynthetic processes in PS II. *Zygnema* sp., contrastingly, showed gradual decrease with the cultivation time and generally low values of F_V/F_M. Such behaviour indicated limitation in primary photosynthetic processes in PS II of the species. Cultivation time-dependent decrease in F_V/F_M was found also for *N. commune*, with absolute values similar to *Zygnema* sp. In contrast to that, *H. antarctica* showed an increase in F_V/F_M with cultivation time, however, the F_V/F_M values still remainned low. Functioning of light harvesting complexes remained more or less unchanged in both algal species (qF_0 about 0.15 in *M. rubrioleoum*, and 0.1 in *Zygnema* sp.). In the tested cyanobacteria, the most pronounced changes were decrease in qF_0 reached positive values in *H. antarctica*, while it was negative in *N. commune*. In both cases, however, qF_0 showed an increase with cultivation time.

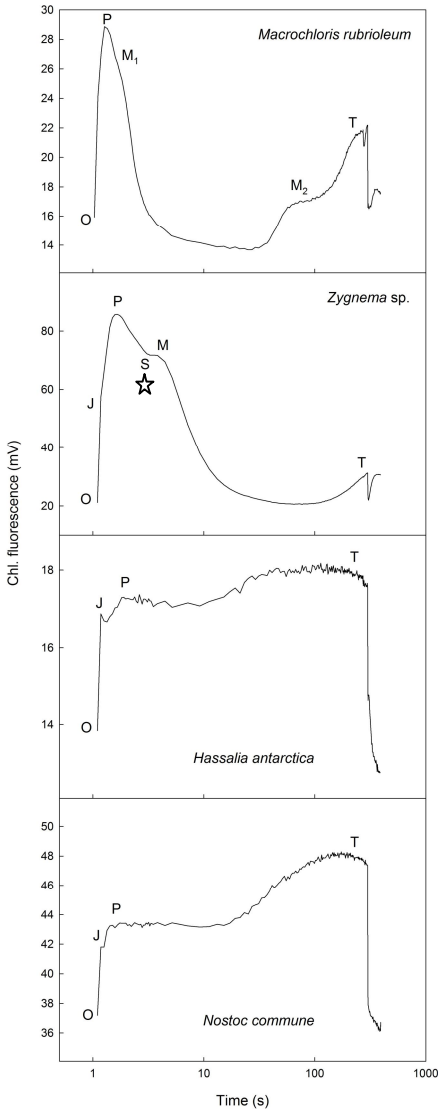

Fig. 3. Slow Kautsky kinetics recorded for experimental algae (*Macrochloris rubrioleum*, *Zygnema* sp.), and cyanobacteria (*Hassalia antarctica*, *Nostoc commune*) with indications of OPSMT points. Time axis is given in decadic logarithmic scale. Local minimum (S) is indicated by an asterisk. For more information *see* Discussion.

Species	Day of cultivation	F_V/F_M	std	qF_0	std
Macrochloris rubrioleum	14	**0.684**	±0.003	**0.135**	±0.013
	21	**0.690**	±0.010	**0.165**	±0.047
	28	**0.603**	±0.006	**0.424**	±0.026
	70	**0.687**	±0.003	**0.150**	±0.015
Zygnema sp.	14	**0.220**	±0.007	**0.106**	±0.006
	21	**0.233**	±0.013	**0.099**	±0.003
	28	**0.105**	±0.005	**0.042**	±0.007
	70	**0.118**	±0.019	**0.105**	±0.074
Hassalia antarctica	14	**0.018**	±0.028	**0.134**	±0.117
	21	**0.086**	±0.010	**0.112**	±0.024
	28	**0.291**	±0.007	**0.148**	±0.042
	70	**0.120**	±0.008	**0.221**	±0.032
Nostoc commune	14	**0.179**	±0.012	**0.053**	±0.010
	21	**0.148**	±0.014	**0.057**	±0.020
	28	**0.138**	±0.015	**0.187**	±0.057
	70	**0.138**	±0.015	**0.187**	±0.057

Table 1. Selected chlorophyll fluorescence parameters evaluated for experimental algae (*Macrochloris rubrioleum*, *Zygnema* sp.), and cyanobacteria (*Hassalia antarctica*, *Nostoc commune*) during cultivation on agar plates.

For *M. rubrioleum*, the most apparent change was the ratio of F_S signal (point T) to F_0 signal (point O). While it was over 1 (the value of 1.28) on 14 day of cultivation, it changed to the value below 1 (0.57) on 21 day and after of cultivation. In a course of time, F_S/F_0 ratio remained below 1. This indicates the presence of a strong quencher activated in light-adapted state of the sample. Such idea might be supported by F_0 exponential increase found in dark in the interval of 100 s after the actinic light switch of (*see* an arrow in the Fig. 3). Another remarkable characteristic is gradual decreease in F_M'peak found with time of cultivation. Such behaviour may be associated with gradual decrease of photosynthetic processes (Calvin-Benson cycle of CO_2 fixation) and increased mixotrophy of the culture. In such situation, demand for ATP and NADPH decreases and thus photochemical processes in photosystem II are strongly limited.

In *Zygnema*, the changes in OPSMT were much less pronounced than in *M. rubrioleum*. The F_S/F_0 ratio remained above 1. At the very beginning of cultivation, peak M2 was detectable (*see* an asterisk in Fig. 3) while it disappeared with further cultivation. Another change dependent on cultivation time, not seen in *M. rubrioleum*, was that local minimum S (indicated by an asterisk in Fig. 3) was found much sooner on 14 day than on the other days (S after P peak) while the time of appearance of the local minimum was higher with further cultivation. Similarly to *M. rubrioleum*, *Zygnema* sp. showed gradual loss of photosynthetic activity demonstrated by a decrease in F_M' (relative to F_S). In *Zygnema*, the changes in OPSMT were much less pronounced than in *M. rubrioleum*. The F_S/F_0 ratio remained above 1. At the very beginning of cultivation, peak M2 was detectable (*see* an asterisk in Fig. 3) while it disappeared with further cultivation.

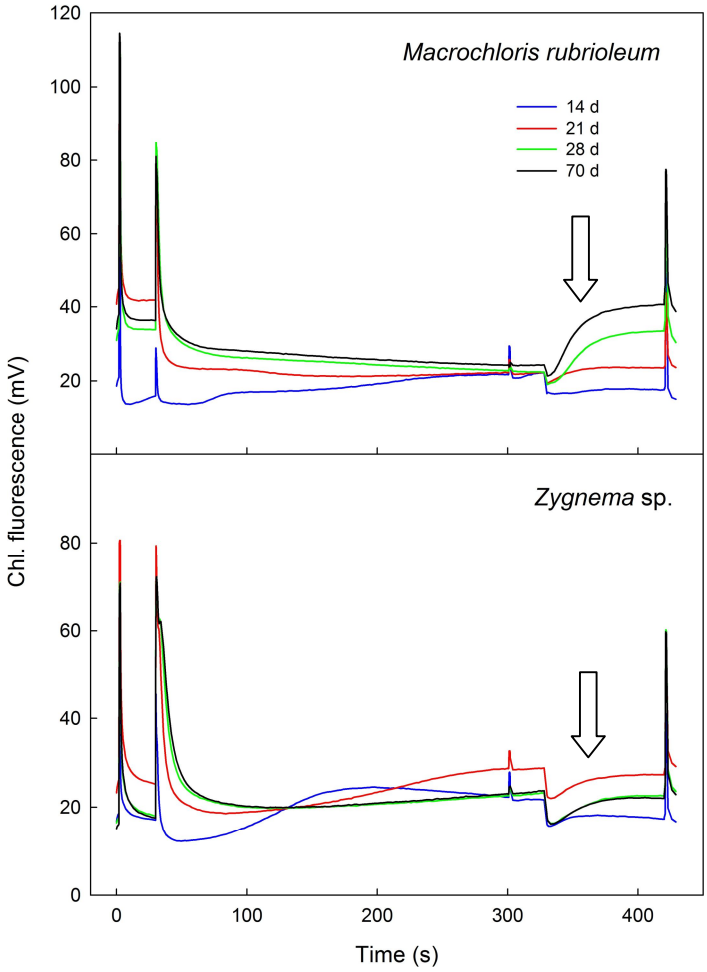

Fig. 4. Slow Kautsky kinetics recorded for *Macrochloris rubrioleum* (upper panel), and *Zygnema* sp. (lower panel) for 14 (blue), 21 (red), 28 (green), and 70 d-long (black) cultivation on agar plates. An arrow indicate exponential increase of the background chlorophyll fluorescence after the actinic light switch off.

For *Nostoc* and *Hassallia*, OPSMT curves typical for cyanobacteria were obtained. On days 14 and 21, T chlorophyll fluorescence level was found higher than P level in *H. antarctica* while T lower than P was apparent for the other days during further cultivation. In all cases, P chlorophyll fluorescence level was lower then maximum chlorophyll fluorescence (F_M - reached after saturation pulse in dark-adapted state).

Our data on slow Kautsky kinetics are in agreement with Papageorgiou et al. (2007) who reviewed the main differences between ´algal´ and ´cyanobacterial´ OPSMT. In algae and higher plants, the shape of PSM is dependent on actinic light intensity. Stirbet and Govindjee (2011) demonstrated that S and M chlorophyll fluorescence levels are distinguished when low but not high (over 320 μmol m^{-2} s^{-1} of photosynthetically active radiation) actinic

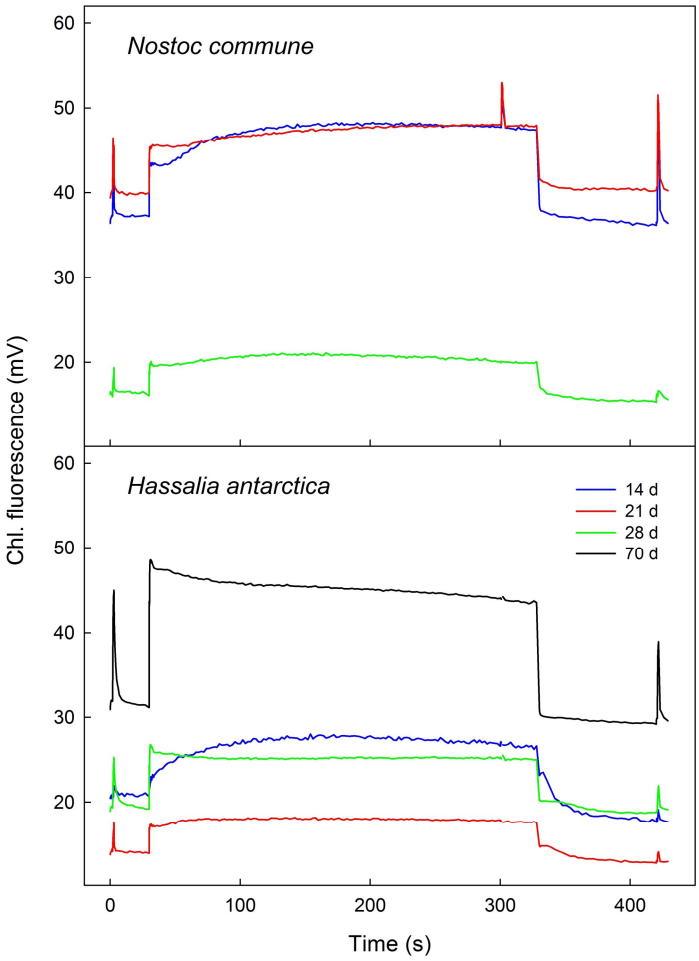

Fig. 5. Slow Kautsky kinetics recorded for *Nostoc commune* (upper panel), and *Hassalia antarctica* (lower panel) for 14 (blue), 21 (red), 28 (green), and 70 d-long (black) cultivation on agar plates.

light is used. Our OPSMT curves recorded for algae showed distinguishable S and M chlorophyll fluorescence levels.

Changes in chlorophyll fluorescence signal at the PSMT part of Kautsky kinetics result from combined effect of photochemical and nonphotochemical processes taking place in photosynthetic membranes at the same time. Photochemical quenching corresponds to the decrease of fluorescence emission due to oxidation of reduced Q_A by electron transport chain (ETC) carriers (PS II to PS I photosynthetic transport). It is considered that transitions of chlorophyll fluorescence on the SMT part of Kautsky kinetics as well as the appearance of additional maxima are mainly due to stimulation of dark reactions of Calvin cycle of CO_2 fixation in chloroplast stroma (Walker 1981, Walker et al. 1983, Ireland et al. 1985, Walker and Osmond 1986, Seaton and Walker 1990). Non-photochemical quenching of chlorophyll fluorescence at the SMT part of

Kautsky kinetics results from (1) formation of proton gradient across thylakoid membrane (Krause and Laasch 1987, Havaux 1990, Noctor and Horton 1990) and resulting energy quenching related to zeaxanthin formation. Other processes involved into non-photochemical quenching are (2) phosphorylation of the light-harvesting complex (Hodges and Barber 1984, Allen 1992), (3) oxidation of plastoquinone pool and photoinhibitory changes in structure and function of PS II complexes (Krause 1988).

Following the initial rise in fluorescence after the application of actinic light, the fluorescence signal then declines over a period of minutes, which is termed 'quenching' (Krause and Weis 1991). Quenching of the fluorescence signal can arise from a combination of processes: first there is the light activation of the process of photosynthesis itself. In particular, key enzymes in the Calvin cycle require activation in order to achieve full activity (Buchanan and Balmer 2005), and metabolite pool sizes in the stroma and cytosol need to increase. From darkness at ambient temperatures, this process typically can take several minutes or longer, with a large amount of variation caused by species and environment. Another factor is the opening of stomata, which increases the availability of CO_2 for Rubisco.

Complex appearance of SMT part of Kautsky kinetics is very common in cyanobacteria and in green algae (Kaňa et al. 2012, Kodru et al. 2015). It is, however, found in higher plants under specific conditions (Mishra et al. 2011). The underlying mechanisms of S-M rise and M-T decline are highly complex. It is believed that state 2 to 1 transition is the main phenomenon behind the S-M rise in green algae. The S-M rise is due to transition of low fluorescence state 2 to higher fluorescence state 1 (Kodru et al. 2015, Kaňa et al. 2012).

According to Riznichenko et al. (1996) relative share of particular factors affecting the slow fluorescence transients PSMT is still unclear. Fluorescence decay PS is assumed to result from the generation of pH gradient on the thylakoid membrane. Further increase of fluorescence yield SM is attributed to the activation of the enzyme Ferredoxin – NADPH reductase (FNR) or to the redox transformations of the NADPH pool (*e.g.* Hansen et al. 1993). The SMT part is dependent on combination of biochemical processes of photosynthesis. Among them, the light activation of the process of photosynthesis itself is of major importance. In particular, key enzymes in the Calvin cycle require activation in order to achieve their full activity (Buchanan and Balmer 2005). Consequently, metabolite pool sizes in the stroma and cytosol need to increase. From darkness at ambient temperatures, this process typically can take several minutes or longer, with a large amount of variation caused by species and environment (Murchie and Lawson 2013).

According to Riznichenko et al. (1996) who classified 4 classes and 4 subclasses of slow Kautsky kinetics, the recorded OPSMTs (*see* Fig. 2) belong to class 1B (*Hassalia antarctica*, *Nostoc commune*), 2A to 1A (*M. rubrioleum* from the beginning to the end of cultivation) and 3A to 4B (*Zygnema* sp. from the beginning to the end of cultivation). Our chlorophyll fluorescence imaging data showed that the shape OPSMT curves changes with cultivation time in all studied species in terms of particular signals (mainly P, T). Therefore, the slow Kautsky kinetics may be used for the evaluation of (1) growth on agar plates as well as (2) changes in quenching of chlorophyll fluorescence. Such approach might be potentially beneficial in screening of different microautotrophs and analysis of their vigor and primary processes of photosynthesis. If chlorophyll fluorescence imaging is applied in autotrophs cultivated on Petri dishes, spacial heterogeneity of inoculated species can be distinguished using different chlo-

rophyll fluorescence parameters. Such heterogeneity might be attributed either to patterened growth of sample species or an early indication of infection by another species.

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