Applicability of the fluorescein diacetate assay for metabolic activity measurement of *Microcystis aeruginosa* (Chroococcales, Cyanobacteria)

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SUMMARY
The fluorescein diacetate (FDA) assay has been widely used to measure metabolic activity in phytoplankton. It was found that FDA fluorescence values did not decrease in some stressed cells, demonstrating that the applicability of the method needs to be assessed further in the context of growth-influencing conditions. In the present study, changes of FDA fluorescence values were studied in bloom-forming cyanobacterial *Microcystis aeruginosa* Kütz cells under stress conditions such as nitrogen (N) or phosphorus (P) deficiency, or darkness and low temperature (10°C), respectively. The results demonstrated that esterase activity decreased immediately in dark-stressed cells, which correlated with the decline of biomass and photosynthetic activity. Under the other three stress conditions, however, especially at low temperature, the cells lost photosynthetic activity but had the highest esterase activity, which was five times higher than the control group. These findings contrast with the assay criteria that the expression of a stain should reflect the change of photosynthetic activity and that stressed cells should have a lower staining intensity than the control cells. According to these results, the esterase activity response was dependent on environmental factors. Furthermore, higher fluorescence intensity did not mean higher metabolic activity, but a discrepant value indicated a severe stress.

Key words: esterase activity, fluorescein diacetate, low temperature, metabolic activity, metabolic vigor, photosynthetic activity.

INTRODUCTION
The determination of metabolic activity is essential for the ecological or physiological investigation of phytoplankton. Any influence on the metabolism of phytoplankton, such as nutrient availability, light, climate or the presence of environmental pollutants, is manifested in the metabolic activity of individual organisms and in the generation of new individuals (Brookes *et al.* 2000b), leading to changes in biomass levels. Studies on metabolic activity therefore enable the rapid assessment of phytoplankton growth compared to biomass investigations.

Metabolic activity in phytoplankton is often assessed by the use of parameters associated with photosynthesis, such as adenosine triphosphate (ATP) formation, 14C assimilation, oxygen production and, more recently, fluorometric analyses (Gilbert *et al.* 1992; Martinez 1992; Brookes *et al.* 2000b; Vinegla *et al.* 2006). These methods determine the metabolic activity at the population level. However, individual cells, even those in supposedly clonal populations, may differ widely from each other in terms of their genetic composition, physiology, biochemistry, or behavior (Brussaard *et al.* 2001; Brehm-Stecher & Johnson 2004; Nancharaiah *et al.* 2007). Methods that probe the physiological status at the single cell level are thus applied extensively, especially through a combination of fluorescent stains with flow cytometry technology (Jochem 2000; Adler *et al.* 2007).

The fluorescein diacetate (FDA) assay, which has proven useful in a variety of cell types, has also been applied to the study of phytoplankton physiology (Bentley-Mowat 1982; Brookes *et al.* 2000b; Jochem 2000; Brussaard et al. 2001; Latour *et al.* 2004). FDA is a non-polar, hydrophobic and non-fluorescent esterified compound, which enters the cells freely. Once inside the cells, FDA can be hydrolyzed by non-specific esterases to leave the brightly green fluorescent substrate, fluorescein. The esterases, mainly lipase and...
acetylcholinesterase, are essential for phospholipid metabolism in the cell membranes (Dorsey et al. 1989; Jochem 2000; Prado et al. 2009). Since membrane turnover is believed to be higher in healthy, rapidly growing cells and reduced in stressed cells, the esterase activity-based FDA assay enables changes in metabolic activity to be detected (Jochem 1999).

Brookes et al. (2000b) described five criteria to test whether a stain-based assay could be suitable for the assessment of metabolic activity:

1. The expression of the stain is a function of enzyme or photosynthetic activity.
2. The stain readily permeates the cell.
3. The stain is highly sensitive and easily measurable.
4. It stains both eukaryotic and prokaryotic cells.
5. The stain emits a fluorescence not masked by cell fluorescence.

Here we provide two additional criteria:

6. The stain intensity should be higher in routinely cultured log phase cells than in stressed cells.
7. It can be used to predict the development of biomass.

The FDA stain has been shown to fulfill the criteria 2 to 5 in some species, and 1, 6 and 7 under certain conditions. For example, the FDA mean fluorescence intensity per cell showed a reasonable correspondence with photosynthetic capacity under dark stress (Dorsey et al. 1989), and was decreased when cells suffered toxic exposure to heavy metals, high Cu\textsuperscript{2+} concentrations or acidic mine drainage (Regel et al. 2002; Wu et al. 2006). It was also able to distinguish cells grown under different light intensities and under phosphate replete or deplete conditions (Geary et al. 1998; Brookes et al. 2000b). However, the stimulation of esterase activity was also reported in a small subset of treated cells when exposed to stresses, such as herbicides, chlorine treatment, Cu\textsuperscript{2+}, cadmium and darkness (Franklin et al. 2001; Lage et al. 2001; Franklin & Berges 2004; Nancharaiah et al. 2007; Jamers et al. 2009; Prado et al. 2009), which did not comply with criterion 6. In addition, the correlation between the mean FDA fluorescence intensity and photosynthetic activity needs to be validated under further stress conditions.

Light, nutrients and temperature are key parameters that regulate phytoplankton growth, and are among the most important parameters that determine algal succession (Brookes et al. 2000b; Nicklisch et al. 2008). Therefore, the present paper studied the changes of esterase activity in Microcystis cells under stress induced by darkness, limited nutrients (nitrogen (N) or phosphorus (P)) and low temperatures, in order to establish whether the FDA assay could be used as a parameter to indicate metabolic activity under these conditions.

MATERIALS AND METHODS

Cell culture and treatments

The strain M. aeruginosa Kütz FACHB 905 strain was obtained from the Freshwater Algae Culture Collections of the Institute of Hydrobiology (FACHB-collection, Wuhan, China). It was grown under constant cool-fluorescent light at an intensity of 20–25 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), 12:12 LD (light : dark) cycle and a temperature of 25°C ± 1°C for approximately 5 days in BG11 medium. After 7 days, the cells were harvested by centrifugation at 8228 g for 3 min, washed three times with sterilized distilled water, and the pellets were inoculated into 250-mL flasks for treatment with an inoculation concentration about \( 7.0 \times 10^6 \) cells mL\(^{-1} \). Under dark conditions, the flasks were wrapped with aluminum foil to block light completely. Under N or P limited conditions, the BG11 medium was modified to contain no NaNO\textsubscript{3} or K\textsubscript{2}HPO\textsubscript{4}, respectively. With the exception of samples under stress from low temperatures at 10°C, all flasks were cultured at 25°C ± 1°C.

Determination of biomass

Biomass was estimated both by cell number and concentration of the pigment chlorophyll a. Cells were counted with a hemocytometer using light microscopy (Olympus BX-21, Japan). Chlorophyll a was extracted with 80% acetone and its concentration measured at 663 nm (Shen & Song 2007). The specific growth rate was calculated using the following equation (Pirt 1975):

\[
\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1)
\]

where \( X_2 \) and \( X_1 \) was the biomass at time points \( t_2 \) and \( t_1 \).

Chlorophyll fluorescence parameters determination

Chlorophyll fluorescence parameters were measured with a pulse-amplitude-modulated fluorescence monitoring system (Phyto-PAM, Walz, Effeltrich, Germany). Each measurement was performed after darkness adaptation of at least 15 min. Photochemical efficiency was calculated with the following formula:

\[
F_v / F_m = (F_m - F_o) / F_m \quad (\text{Maxwell & Johnson 2000})
\]

where \( F_v \) is the difference between the maximum fluorescence \( F_m \) and the minimum fluorescence \( F_o \) of the darkness-adapted stage of photosystem II (PSII). Photosynthetic electron transport rates (ETR) were measured with Phyto-PAM at 12 different irradiances in the range of 0 to 1265 \( \mu \text{mol photons PAR} \)}
(photosynthetically active radiation) $m^{-2} \cdot s^{-1}$. It was calculated by the following formula:

$$\text{Relative ETR} = \frac{\left( (F'_{m} - F_{r}) / F'_{m} \right) \times 0.84 \times 0.5 \times \text{PAR} \left( m^{-2} \cdot s^{-1} \right)}{}$$

where $F'_{m}$ and $F_{r}$ are the maximum and steady state fluorescence in light, respectively (Maxwell & Johnson 2000). Relative ETR is generally an increasing saturating function of irradiance, comparable with the photosynthesis-irradiance relationship. $ETR_{\text{max}}$ was defined as the maximum ETR.

**FDA staining protocol and esterase activity determination**

Fluorescein diacetate accumulates in cells as a product of the reaction between esterases and FDA, and the fluorescence intensity is dependent on the esterase activity when the substrate is sufficient (Brookes et al. 2000b), so the FDA assay has been used to quantify the esterase activity.

The FDA staining technique was performed according to the method described by Jochem (1999). A stock solution of 5 mg mL$^{-1}$ FDA (Sigma Chemicals F-7378) was prepared in acetone and stored at $-20^\circ\text{C}$. The stock solution was diluted 100-fold in distilled water to obtain the working solution, which was freshly prepared before the staining. Since FDA is only slightly soluble in aqueous solutions, the stock solution was injected rapidly into the distilled water and mixed quickly. Culture samples of 200 μL were withdrawn and FDA working solution was added to a final concentration of 30 μM. Reactions were incubated in the dark at 25°C for 5 min and stopped by ice-water incubation for 5 min. The dyes were then removed by centrifugation at 8000 g for 3 min and the cell concentration was diluted to $1 \times 10^6$ cells mL$^{-1}$ before detection using flow cytometry (Beckman Coulter Epic Altra; Brea, CA, USA). The FDA staining was measured instantly after removal of the dyes and the measuring period did not exceed 40 min post-removal.

The power output was 15 mW and a wavelength of 488 nm was used to excite the fluorescent probes. *Microcystis* cells were identified and distinguished from other particles by gating on two-parameter-plots of forward angle light scatter versus chlorophyll auto-fluorescence gathered through a 650 nm longpass filter. Green fluorescence was measured through a 535 ± 10 nm band-pass filter and at least $1 \times 10^5$ cells were collected. A value of two relative units was assigned based on the value of the heat-killed culture to separate live cells from dead ones. Cells with fluorescence readings above that value were considered FDA positive (Li & Song 2007), and the mean fluorescence intensity of the positive cells were adopted to calculate the esterase activity as the rate of fluorescein accumulation per cell per minute (Brookes et al. 2000b).

**Statistical analysis**

All experiments were performed in triplicate. Data in the study were presented as means ± standard deviations (SD). Significant differences between treated samples and control were determined by ANOVA. Relationship among growth characters such as cell number, chlorophyll a, specific growth rate, photosynthetic parameters $F_{r}/F_{m}$, $ETR_{\text{max}}$, and esterase activity were tested by Spearman’s nonparametric rank-correlation procedure (SPSS Version 14.0, SPSS Inc.). The values of $ETR_{\text{max}}$ and $F_{r}/F_{m}$ under low temperature were supposed to be zero after day 9 in the correlation process. Differences and correlations were considered to be significant at $P < 0.05$.

**RESULTS**

**Biomass**

Compared to the control under routine culture conditions, growth rates of the stressed cells were significantly inhibited ($P < 0.05$). This was shown by decreased cell numbers (Fig. 1a) and the chlorophyll content (Fig. 1b). Nevertheless, continual increases in biomass were observed in the first days under nutrient-limited conditions, especially when P was limited, under which both cell numbers and chlorophyll a concentration increased more than four times and did not stop rising in the first 20 days. Under dark and low temperature conditions, no significant increase was observed, but cell populations declined slowly in the first days. Loss rates increased only after prolonged periods of stress, and the time points at which the populations declined differed between stress conditions. These time points were 20 days for darkness, 9 for N limitation and 20 for P limitation, as shown by the change of chlorophyll a concentrations. The accelerated loss was less evident in low temperature treatments than under the other three stress conditions.

**Chlorophyll fluorescence parameters**

Photochemical efficiency $F_{r}/F_{m}$ and $ETR_{\text{max}}$ are both effective tools to investigate photosynthetic activity. However, under some conditions the two parameters did not respond in the same way (Fig. 2a,b). Generally, the decrease of $ETR_{\text{max}}$ was detected earlier than that of $F_{r}/F_{m}$. They changed synchronously only in cultures treated at low temperature, where $F_{r}/F_{m}$ decreased from 0.32 to 0.02 and $ETR_{\text{max}}$ from 75.0 to 3.7 μmol photons $m^{-2} \cdot s^{-2}$ both in 3 days, which correlated with the following decline in biomass. $ETR_{\text{max}}$ also correlated...
with the decline under N and light-limited conditions as measured by chlorophyll a concentrations. It decreased instantly when cultures were exposed to the stress conditions, whereas, $F_\text{v}/F_\text{m}$ did not decrease until 15 days later. The delayed decrease was useful to interpret the increased loss rates of chlorophyll a after the turning points under both conditions. Under P limited conditions neither of the two parameters showed significant changes, even in the period of steep decline.

Esterase activity

Cultures under stress maintained a high FDA positive ratio. With the exception of one sample taken on the last day from dark stress treatment, all samples presented a value above 90% (Fig. 3), which correlated with the slow declines of the stressed populations.

Routine cultures maintained an esterase activity of five to eight units. After they were transferred to the stress conditions, different responses were observed, depending on the treatment (Fig. 4). Under darkness, esterase activity decreased, which was consistent with the change of $\text{ETR}_{\text{max}}$ and decrease of biomass. Under P limited conditions, a significant increase was observed, while under N limited conditions, esterase activity increased significantly ($P < 0.05$) in the first 20 days. An increase was also observed in the control populations after 16 days. The most significant increase was observed in cells cultivated at low temperature, which showed a fivefold higher fluorescence intensity than the control group after 3 days, and remained at an increased value over the course of the studied period. This differed strongly with the changes in $F_\text{v}/F_\text{m}$, $\text{ETR}_{\text{max}}$, and biomass, which is contrary to the hypothesis that

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Spearman correlation

An ideal metabolic activity indicator should be able to predict or couple with changes of specific growth rates, but of all measured parameters only $F_v/F_m$ fulfilled this criterion under dark stress, referring to specific growth rate based on cell numbers ($r_s = 0.821$, $n = 21$, $P = 0.023$). Changes of the photosynthetic activity parameters, characterized by $F_v/F_m$ and $ETR_{max}$, correlated with changes of biomass compared to the control group. These were represented by chlorophyll a changes under conditions that were N limited, with dark stress and low temperatures, or P limited, with low temperature stressed populations are less metabolically active than conventionally cultured cells.

Fig. 3. Changes of fluorescein diacetate (FDA) fluorescence positive ratio. ■, N limitation; □, P limitation; ●, Darkness; ○, Low temperature; ◆, Control group.

Low temperature stressed populations are less metabolically active than conventionally cultured cells. However, they only correlated with esterase activity in cells under dark stress (Table 1). A correlation between esterase activity and photosynthetic activity parameters was also only observed under dark stress (Table 1). Moreover, negative relationships were found under low temperature stress. According to the conventional criteria the FDA assay was able to indicate metabolic activity under dark, but not under other conditions in our experiment.

DISCUSSION

The FDA assay has been widely used to assess metabolic activity, following the premise that esterase activity and photosynthesis correspond (Dorsey et al. 1989). Our experiments confirmed this relationship and were in accordance with the assumption that FDA cleavage rate was strongly affected by light intensity (Brookes et al. 2000b). However, to answer whether the FDA assay could indicate the metabolic activity under darkness, further investigations in different species are necessary. According to the data gathered already about the responses of esterase activity to dark conditions, microalgal cells can be classified into three types (Dorsey et al. 1989; Jochem 1999; Franklin & Berges 2004; Zhang et al. 2007). Type I cells, such as the diatoms *Brachimonas submarina*, the Chlorophyceae *Chlorella pyrenoidosa* and other algae, including *Chroomonas salina* and *Cryptomonas sp.*, decreased esterase activity to a constant low level to adapt to the prolonged darkness (Dorsey et al. 1989; Jochem 1999; Franklin & Berges 2004; Zhang et al. 2007). Type II cells (e.g. the diatoms *Prymnesium parvum*) did not alter their activity (Jochem 1999). Type III cells, such as the dinoflagellate *Amphidinium carterae*, showed an increase in esterase activity up to 10 days after transition to darkness (Franklin & Berges 2004). Cell numbers in Type I and II response species remained constant during the first few days of darkness (Jochem 1999), while in Type III, cells declined significantly and photosynthetic capacity decreased from a $F_v/F_m$ value of 0.6 to 0 within 3 days (Franklin & Berges 2004). The strain of *Microcystis* used in our experiment belongs to Type I, but inter-specific variability was also found, since only a slight decrease of esterase activity was reported when *Microcystis* suffered from dark stress (Zhang et al. 2007).

Light deprivation may block the duplication and expression of the esterase-related genes thanks to the lack of sufficient ATP supply, which could lead to the rapid decrease of esterase activity. However, similar effects were not observed under N/P limited conditions, though N and P are essential nutrients for algae. N is a constituent element of amino acids and thus of proteins and nucleic acids. P is also a component of nucleic acids, and also of ATP and phospholipids that form all
cell membranes (Brussaard et al. 1997). The regulation of free N/P levels by the storage products in cyanobacteria may explain this phenomenon. Excess N compounds can be stored as cyanophycin (Gorelova & Kleimenov 2003) or as phycobilin pigments (Kromkamp 1987), and P as polyphosphate (Powella et al. 2009). The storage products can be formed due to luxury consumption (Kromkamp 1987; Powella et al. 2009) and can be used again when the exogenous concentrations of N/P are in short supply (Kromkamp 1987), which would thus guarantee the synthesis of esterase at the onset of the limitations of nutrients.

Several explanations for the increase of FDA fluorescence under stress have been proposed. One of the explanations was that stress-induced membrane damage could lead to an increase in the uptake of FDA (Franklin et al. 2001; Nancharaih et al. 2007). However, Geary et al. (1998) found that FDA hydrolysis by M. aeruginosa saturated at 17 μM FDA. In our study, 30 μM was used to ensure saturation of FDA hydrolysis at all cell concentrations. In addition, 1 min exposure to a saturation concentration of FDA is sufficient to satisfy substrate demand of cells (Brookes et al. 2000a).

The increase of FDA fluorescence at a low temperature and N/P limitation can be explained by several other reasons. First, this enzyme activity stimulation might be a result of a general metabolic stimulation in order to adapt their physiology to adverse environmental conditions, which has been suggested in cells with increased esterase activity that were exposed to cadmium or paraquat (Jamers et al. 2009; Prado et al. 2009). Responses of esterase activity to dark stress and low temperature were similar to a previous study with regard to the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl tetrazolium bromide) reducing ability, which was quantified by a colorimetric method. MTT reduction was also applied extensively to study cell metabolic activity and viability. According to the MTT reduction results, cells cultured under darkness deposited much less of the MTT reduction product formazan and gave a lower value than healthy cells, while cells under 10°C deposited much more formazan and gave a higher value (Li & Song 2007). The MTT assay was dependent upon intracellular oxidoreductase. Cells increase their oxidoreductase quota to offset the weakened enzyme activity under low temperatures (Li & Song 2007). A similar mechanism could exist for esterase. In addition, the expression of specific FDA-cleavable enzymes is also possibly elevated, leading to the increase of esterase activity. It has been demonstrated that the desaturation of membrane lipids is essential to low temperature tolerance in cyanobacteria. The desaturation of fatty acids occurs without de novo synthesis of fatty acids during low temperature acclimation of cyanobacterial cells (Singh et al. 2002) through acylase, which has been implicated in the hydrolysis of FDA (Schupp & Erlandsen 1987). Second, substances that include nucleophilic and basic amino acids, including histidine and cysteine, have been reported to induce abiotic cleavage of FDA (Clarke et al. 2001; Wanandy et al. 2005). Some of these could be possibly induced under stress conditions, and the increase of cysteine biosynthesis has been proposed to be an initial stress response in plant (Tausz et al. 2004). Finally, the effect of the ion transporting system on the fluorescence intensity needs to be considered, as carboxyfluorescein (cF) and 2',7'-bis-(2-carboxethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester

### Table 1. Spearman correlation between fluorescein diacetate (FDA) cleavage activity, ETR$_{max}$ (maximum electron transport rates (ETR), $F_v/F_m$ and cells growth ($n = 24$)

<table>
<thead>
<tr>
<th></th>
<th>ETR$_{max}$</th>
<th>$F_v/F_m$</th>
<th>Cell number</th>
<th>Chlorophyll a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ps</td>
<td>ps</td>
<td>ps</td>
<td>P</td>
</tr>
<tr>
<td>FDA</td>
<td>N -0.833</td>
<td>0.01</td>
<td>0.476</td>
<td>0.233</td>
</tr>
<tr>
<td></td>
<td>P -0.357</td>
<td>0.385</td>
<td>0.31</td>
<td>0.456</td>
</tr>
<tr>
<td></td>
<td>D 0.81</td>
<td>0.015*</td>
<td>0.429</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
<td>T -0.627</td>
<td>0.096</td>
<td>-0.627</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>ck 0.143</td>
<td>0.736</td>
<td>0.587</td>
<td>0.126</td>
</tr>
<tr>
<td>ETR$_{max}$</td>
<td>N -0.095</td>
<td>0.823</td>
<td>-0.833</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>P 0.31</td>
<td>0.456</td>
<td>0.214</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>D 0.429</td>
<td>0.289</td>
<td>0.905</td>
<td>0.002*</td>
</tr>
<tr>
<td></td>
<td>T 1</td>
<td>0*</td>
<td>0.846</td>
<td>0.008*</td>
</tr>
<tr>
<td></td>
<td>ck 0.491</td>
<td>0.217</td>
<td>0.143</td>
<td>0.736</td>
</tr>
<tr>
<td>$F_v/F_m$</td>
<td>N -0.119</td>
<td>0.779</td>
<td>0.214</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>P 0.952</td>
<td>0*</td>
<td>0.714</td>
<td>0.047*</td>
</tr>
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<td></td>
<td>D 0.548</td>
<td>0.16</td>
<td>0.667</td>
<td>0.071</td>
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<tr>
<td></td>
<td>T 0.846</td>
<td>0.008*</td>
<td>0.873</td>
<td>0.005*</td>
</tr>
<tr>
<td></td>
<td>ck 0.85</td>
<td>0.007*</td>
<td>0.96</td>
<td>0*</td>
</tr>
</tbody>
</table>

*Significant difference at $P < 0.05$. ck, control; D, dark; N, N limitation; P, P limitation; T, low temperature.
(BCECF), which are derivatives of fluorescein, have been reported to be excreted from metabolically active cells (Molenaar et al. 1992; Bunthof et al. 1999; Lushchak et al. 2008). A weakened dye efflux system in stressed cells would also contribute to the increase of fluorescence, and vice versa, which has been used to explain the loss of labeling in Pseudokirchneriella subcapitata cells (De Mattia et al. 2007). Further studies are required to understand the changes on a cellular level induced under low temperature and N/P limited conditions.

The FDA assay could provide valuable insights into the relationship between the physiological and ecological functioning of phytoplankton cells, but esterase activity responses are dependent on the type of stress and on the species, and do not always correlate with changes in photosynthetic activity, which impairs its use as an indicator of metabolic activity. A possible solution would be to establish a new algorithm to decode the FDA information. According to our research, discrepant values that include increases as opposed to decreases of esterase activity indicate stress. As illustrated in our research, esterase activity in healthy Microcystis cells was maintained between 5 and 8 units. Although the N/P values were limited, these were increased by low temperature stress and a long duration of culture; however, this finding was not consistent in stressed cells. Therefore, it is crucial to establish levels of esterase activity in healthy growing cells to determine the correct metabolic activity after FDA treatment in further studies.

In conclusion, the responses of esterase activity were stress-dependent. They correlated with changes of photosynthetic activity under darkness, but not under N/P limited or low temperature stress conditions. Thus, any interpretation of fluorescence intensity should be revised, as deviations from normal fluorescence levels suggest stress conditions rather than higher metabolic activity.

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FDA for metabolic activity measurement


