Microalgae respond differently to nitrogen availability during culturing

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Variations in the exogenous nitrogen level are known to significantly affect the physiological status and metabolism of microalgae. However, responses of red, green and yellow-green algae to nitrogen (N) availability have not been compared yet. *Porphyridium cruentum, Scenedesmus incrassatulus* and *Trachydiscus minutus* were cultured in the absence of N in the medium and subsequent resupply of N to the starved cells. Culture growth and in-gel changes in isoenzyme pattern and activity of glutamate synthase, glutamate dehydrogenase, malate dehydrogenase, aspartate aminotransferase, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase were studied. The results demonstrated that the algae responded to the fully N-depleted and N-replete culture conditions by species-specific metabolic enzyme changes, suggesting differential regulation of both enzyme activity and cellular metabolism. Substantial differences in the activities of the antioxidant enzymes between N-depleted and N-replete cells of each species as well as between the species were also found. In the present work, besides the more general responses, such as adjustment of growth and pigmentation, we report on the involvement of specific metabolic and antioxidant enzymes and their isoforms in the mechanisms operating during N starvation and recovery in *P. cruentum*, *T. minutus* and *S. incrassatulus.*

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1. Introduction

Microalgae are oxygenic photosynthetic organisms found in nearly all ecosystems. In many natural habitats, the availability of combined nitrogen is growth-limiting (Sterner and Elser 2002). Nitrogen (N) is an essential major element required for the synthesis of amino acids, proteins, nucleic acids, coenzymes, chlorophyll and other accessory photosynthetic pigments (phycobiliproteins in blue-green, rhodophytes and cryptophytes algae). Therefore, these ecologically successful organisms have evolved a number of mechanisms to overcome stress due to N limitation. The representatives of various phyla show much similarity in their responses to N starvation such as a reduced rate of cell division (Sciandra *et al.* 2000; Sinetova *et al.* 2006; Msanne *et al.* 2012), down-regulation of photosynthesis (Silva *et al.* 2009; Hockin *et al.* 2012; Zhang *et al.* 2013), transition of the electron transport chain to the cyclic regime (Berges *et al.* 1996; Zhang *et al.* 2013), maintenance only of essential protein synthesis in the cell (Hockin *et al.* 2012), accumulation of carbon- and energy-rich storage compounds such as polysaccharides, starch and triacylglycerols (Guschina and Harwood 2006; Hu *et al*. 2008; Silva *et al.* 2009; Msanne *et al.* 2012) and, as a result, cell survival. The reduction of the photosynthetic pigments content leads to yellow appearance of the cells, known as chlorosis or bleaching. Chlorosis

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under N starvation is observed in all photosynthetics, including higher plants, *Thalassiosira weissflogii* (Heterokontophyta) and *Dunaliella tertiolecta* (Chlorophyta) (Berges *et al.* 1996), *Nannochloropsis oceanica* (Heterokontophyta) (Dong *et al.* 2013), *Galdieria sulphuraria* (Rhodophyta) (Sinetova *et al.* 2006), various cyanobacteria (Sauer *et al.* 2001; Peter *et al.* 2010; Gigova and Ivanova 2014) and could serve as an indicator of the cellular N status. Besides the common responses, changes in some morphological features including cell size (Berges *et al.* 1996; Zhang *et al.* 2013; Razaghi *et al.* 2014) and ultrastructure (Sinetova *et al.* 2006; Dong *et al.* 2013) as well as in biochemical characteristics such as the level of some metabolites (Řezanka *et al.* 2011; Bromke *et al.* 2013; Razaghi *et al.* 2014), activity of nitrogen, carbon and energy metabolism (Hockin *et al.* 2012; Lee *et al.* 2012) have been also described in certain N-limited microalgae. Very little is known, however, about the role of antioxidant enzymes under N deficiency. No comparison of responses between red, green and yellow-green algae under N-depleted and N-replete conditions has been made yet. Therefore, the aim of the present work was to compare the effects of N starvation on *Porphyridium cruentum* (red alga), *Scenedesmus incrassatulus* (green alga) and *Trachydiscus minutus* (yellow-green alga) and the strategies applied by the algae to recover after N supply. For this purpose, the isoenzyme patterns and activities of some enzymes that link nitrogen and carbon metabolism as well as of the main antioxidant enzymes were analysed.

2. Experimental procedures

2.1 *Organisms and growth conditions*

Monoalgal, non-axenic cultures of *Scenedesmus incrassatulus* R-83 (Chlorophyta, Sphaeropleales, Scenedesmaceae, our collection), *Trachydiscus minutus* (P.Bourrelly) H. Ettl, Lukavský and Přibyl 2005/1 (Ochrophyta, Eustigmatales, Eustigmatophyceae) and *Porphyridium cruentum* (AG.) NAG Vischer 1935/107 (Rhodophyta, Porphyridiales, Porphyridiaceae), both from the culture collection of the Institute of Botany ASCR, Třeboň, Czech Republic) were used in this study. Algae were grown autotrophically in the appropriate liquid media: 1/2 medium of Setlik (1967) as modified by Georgiev *et al.* (1978), 3-fold Z (Staub 1961), and medium of Brody and Emerson (1959), for *S. incrassatulus*, *T. minutus* and *P. cruentum*, respectively) (controls, C). In the N limitation experiments, combined N was omitted from the media (designated as N-depleted culture, D). In both cases, exponential cultures grown in the respective full-strength medium, standardized at a density of about 0.6 mg mL^{-1} dry weight (DW; table 1) were used as initial experimental cultures. Algae were incubated at 28±1°C with continuous illumination (white fluorescent light, 132 mmol photons m^{-2} s^{-1}). A carbon source was provided by bubbling sterile 2% CO_2 (v/v) in air through the cultures. Nitrogen starvation was monitored by the changes in cellular coloration and growth retardation. When cell bleaching appeared, a half of the N-deprived cultures was spun for 15 min at 5000*g* at 4°C. The pellets were frozen and stored for analyses. To examine recovery, the N source was supplied to the remaining half of the cultures in the form and at a final concentration as in the respective original media (N-replete culture, R). During the next days of cultivation, each culture was monitored for coloration and growth recovery. In this case, cells were harvested by centrifugation (5000*g*, 15 min), frozen and subsequently used for analyses.

2.2 *Analyses*

Algal growth was evaluated gravimetrically by the increase in biomass DW. For biomass DW determination, algal suspensions (3×5 mL each) were filtered through Whatman GF/C glass filters (Whatman International Ltd, Maidstone, UK) and oven dried at 80°C to a constant weight. Specific growth rate (μ d^{-1}) was calculated as follows: $\mu=Ln(m_{t2}/m_{t1})/t_2–t_1$ (Levasseur *et al.* 1993), where m_t is the DW at different days (t_1 and t_2, $t_2–t_1\geq1$) (table 1).

The experiments were done in triplicate. Data are expressed as the means±standard deviation. The significance of differences between treatments was evaluated by one-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test using InStat (GraphPad Software, La Jolla, CA, USA). Values of $P<0.05$ were considered significant.

2.3 *Preparation of cell extracts, PAGE and enzyme activity staining*

Cells were mechanically homogenized in 60 mM TE (Tris-base with 0.1 mM EDTA) buffer (pH 6.8), at 4°C and centrifuged at 13000*g* for 15 min. The concentration of soluble proteins in the supernatant was determined by the method of Bradford (1976), with the BSA as a standard. Equal amounts (10 µg) of protein from cells grown at different N levels were subjected to discontinuous polyacrylamide gel electrophoresis (PAGE) under nondenaturing, nonreducing conditions as described by Laemmli (1970), but in the absence of SDS. Electrophoretic separation was performed on 10% polyacrylamide gels at a constant current of 35 mA per gel. Gels were stained for the activities of superoxide dismutase (SOD, EC 1.15.1.1) (Azevedo *et al.* 1998), catalase (CAT, EC 1.11.1.6) (Chandlee and Scandalios 1983), glutathione peroxidase (GPOD, EC 1.11.1.9) (Lin *et al.* 2002), glutathione reductase (GR, EC

Table 1. Changes in biomass dry weight (DW), specific growth rate (μ d^{-1}) and coloration during the cultivation of *Porphyridium cruentum*, *Trachydiscus minutus* and *Scenedesmus incrassatulus* in full-strength media (C), in the absence of nitrogen (D) or in nitrogen-replete (R) culture media

	t (day)	DW (g L^{-1})	Growth rate μ d^{-1}	m_{t2}/m_{t1}	t_2–t_1 (day)	Colour
P. cruentum	0	0.65±0.03	-	-	-	red
C	1	0.95±0.04	0.3796±0.0040[a]	m_1/m_0	1	red
D	1	0.84±0.04	0.2564±0.0014[b]	$m_{1(D)}/m_0$	1	yellow
R	3	1.56±0.15	0.3084±0.0244[c]	$m_3/m_{1(D)}$	2	red
T. minutus	0	0.58±0.03	-	-	-	yellow-green
C	3	0.85±0.04	0.1274±0.0015[a]	m_3/m_0	3	yellow-green
D	3	0.69±0.03	0.0580±0.0027[b]	$m_{3(D)}/m_0$	3	yellow
R	8	1.05±0.10	0.0835±0.0104[c]	$m_8/m_{3(D)}$	5	yellow-green
S. incrassatulus	0	0.52±0.05	-	-	-	green
C	4	1.53±0.09	0.2703±0.0094[a]	m_4/m_0	4	green
D	4	0.98±0.10	0.1583±0.0015[b]	$m_{4(D)}/m_0$	4	yellow
R	8	2.3±0.15	0.2138±0.0093[c]	$m_8/m_{4(D)}$	4	green

m_t is the dry weight at different days (t_1 and t_2).

Means for μ with different lowercase letters are significantly different (P<0.05) between treatments for each alga.

1.6.4.2) (Anderson *et al.* 1995), glutamate dehydrogenase (GDH, NAD^+ - EC 1.4.1.2; $NADP^+$ - EC 1.4.1.4) (Nash and Davies 1975), glutamate synthase (GS, EC 1.4.1.13) (Matoh *et al.* 1980), malate dehydrogenase (MDH, NAD^+ - EC 1.1.1.37; $NADP^+$ - EC 1.1.1.82) (Honold *et al.* 1966), and aspartate aminotransferase (AAT, EC 2.6.1.1) (Griffith and Vance 1989). All reagents used for enzyme activity staining were purchased from Sigma (Sigma Inc., St. Louis, MO, USA). Gel patterns were photographed immediately after staining using the UVItec gel documentation system (Cambridge, UK). Image analysis of the gels was performed on a PC using Gel-Pro32 Analyzer software (Media Cybernetics, Bethesda, MD USA). The activity of each isoenzyme (band) was measured as total integrated optical density (IOD), in arbitrary units. Most enzymes had multiple bands and the sum of their IOD values was considered as total enzyme activity.

3. Results

3.1 *Changes in biomass dry weight, cell growth rate and coloration during cultivation*

Our results indicated that signs of N deficiency (bleaching and growth retardation) occurred at the earliest in *P. cruentum* (after 24 h in culture), followed by *T. minutus* (72 h) and *S. incrassatulus* (96 h) (table 1). Upon addition of combined N to the N-starved cells, all cultures recovered their growth and coloration and this was accompanied by a significant increase (P<0.05) in the growth rate, although the duration of the recovery period was different (48 h, 96 h and 120 h for *P. cruentum, S. incrassatulus* and *T. minutus*, respectively) (table 1).

3.2 *Changes in isoenzyme patterns and activities of metabolic enzymes in response to N deficiency and resupply*

Three bands of GS activity (1–3) were detected in *P. cruentum* under all cultivation conditions examined (figure 1). Compared to control (C) and N-replete cells (R), the relative total GS activity was higher (by about 70%) in N-depleted cells (D) and a fourth activity band (4) appeared. GDH was presented by two isoforms (figure 1). Both isoforms were active in the presence of NADP as a cofactor, while only the faster-moving band (2) showed NAD-GDH activity. Under N deficiency, the activity of GDH was lower than in the control, especially that of NAD-GDH (about 80% decrease). The addition of N to the N-depleted culture led to an increase in both NADP- and NAD-GDH activities (by 110 and 400%, respectively) compared to the D levels. Two isoforms of NAD-dependent MDH were registered on the gel (figure 1). In the absence of N in the culture medium, the slower moving isoform (1) was invisible, but the intensity of the faster-moving isoform (2) was higher (by about 25%), leading to almost identical total MDH activity in both N-starved and C cells. In R culture the activity of MDH increased due to the activation of isoform 1. In the presence of NADP as a cofactor, the intensity of *P. cruentum* MDH bands was markedly lower and it remained unaffected by the

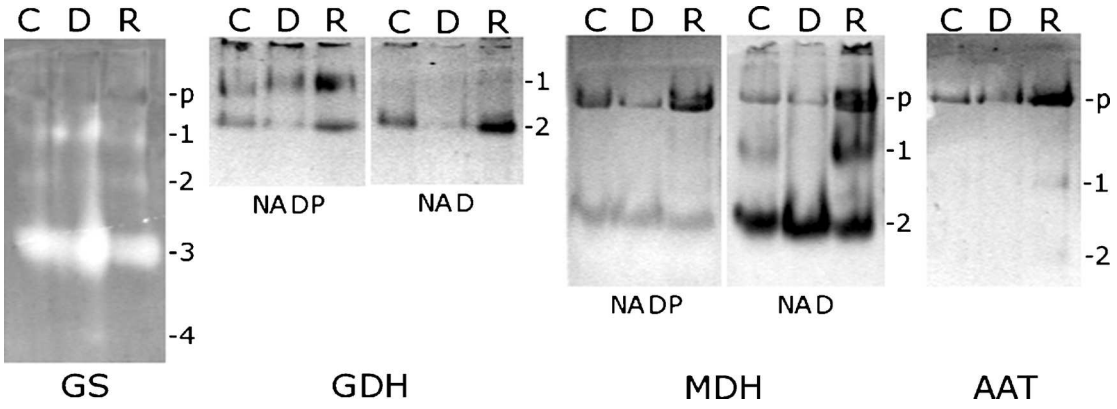

Figure 1. Changes in pattern and activity of glutamate synthase (GS), NADP- and NAD-dependent glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and aspartate aminotransferase (AAT) of *Porphyridium cruentum* in response to nitrogen depletion (D) and nitrogen resupply (R) in the medium. Control culture (C) was grown in standard full-strength medium. Numbers on the right indicate the bands of respective enzyme activity in order of increasing electorphoretic mobility on native polyacrylamide 10% gels. *P. cruentum* pigments are indicated with "p". Equal amounts (10 μg) of protein were loaded in each lane.

N levels in the medium. Two weak AAT activity bands were detected in R cells and they were almost invisible in C and D cells (figure 1).

Three bands of GS activity were distinguishable in *T. minutus* (figure 2). Although the relative total GS activity did not change markedly between the treatments (±10%), the intensity of each band varied considerably. GDH activity was presented by two bands with similar migration mobility. The only change in their intensity was observed in D cells, where total GDH activity increased by about 100 and 26% for NADP-GDH and NAD-GDH, respectively. Five weak bands of NAD-MDH activity (1, 2, 3, 4 and 6) were visible in control *T. minutus* cells (figure 2). In D and R cells, band 2 disappeared, but the intensity of bands 3 and 4 increased and a new band (5) appeared in R cells. Relative total enzyme activity in D cells was about 2.7 times higher than in C cells. In R cells, a slight increase (by about 13%) in MDH activity was observed compared to D cells. NADP-MDH and AAT activities were not detected in *T. minutus* cells.

In *S. incrassatulus*, four GS isoforms were seen on the gel (figure 3). The intensity of their bands varied between treatments, resulting in the lowest total enzyme activity in C cells, an increased activity (by 38%) in D cells which was additionally stimulated (by about 20%) in R cells. NAD-GDH activity of this green alga was presented by three bands (1-3) with different migration mobility and intensity. Relative total NAD-GDH activity in D culture was reduced twice compared with C and 2.5-times lower than in R culture. The fastest-moving band (3) showed also NADP-dependent activity which was most prominent under N-depleted conditions (about 10 times higher than in C cells). Under N-replete conditions, NADP-GDH activity was about 4.6-times lower than in D cells. Two bands of high NAD-MDH and weak NADP-MDH activities were registered in *S. incrassatulus.* The activity of NADP-MDH did not show N level-dependent changes (figure 3). Concerning NAD-MDH activity, isoform 1 was almost inactivated in the absence of N in the culture medium and reactivated after N addition, while the activity of isoform 2 varied very slightly. In contrast to NAD-MDH, the sole band of AAT activity in *S. incrassatulus* was most intense in D culture.

3.3 *Behaviour of the antioxidant enzymes*

Six bands of SOD activity, five bands of GPOD, five main bands of GR and one band of CAT activity were detected in *P. cruentum* cells (figure 4). The enzyme patterns did not change, but the intensity of some bands varied between the treatments leading to changes in the relative total activity of the respective enzyme. SOD activity increased (by 22%) in D cells as compared to the control and decreased (by about 31%) in R cells compared to D cells. A similar trend was observed in GPOD activity, but with higher increase (by 48%) and lower decrease (by 24%) in D and R cells, respectively. CAT activity was almost undetectable in D cells whereas under N-replete conditions it was highly stimulated. The activity of GR did not change considerably.

Three strong (3, 4 and 6) and two faint bands (2 and 5) of SOD activity were seen in *T. minutus* cells (figure 5). Relative total SOD activity was 2.5-fold higher in D cells compared with the control due to the activation of SOD4 and 6, and one new band (SOD1) appeared. N resupply to D culture led to a decrease in SOD activity by about 55% and disappearance of SOD1. Similarly, the intensity of CAT activity bands was highest in D cells (200% over the control) and approximately 2-fold lower in R than in D cells. In addition to the three common isoforms (1–3), one new fastest-moving

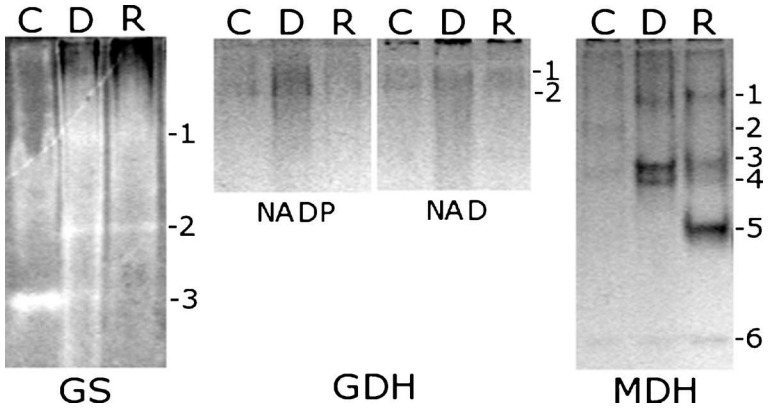

Figure 2. Changes in pattern and activity of glutamate synthase (GS), NADP- and NAD-dependent glutamate dehydrogenase (GDH) and malate dehydrogenase (NAD-MDH) of *Trachydiscus minutus* in response to nitrogen depletion (D) and nitrogen resupply (R) in the medium. Control culture (C) was grown in standard full-strength medium. Numbers on the right indicate the bands of respective enzyme activity in order of increasing electorphoretic mobility on native polyacrylamide 10% gels. Equal amounts (10 μg) of protein were loaded in each lane.

isoform (CAT4) appeared in D cells. Three bands of GR were detected in *T. minutus* under all cultivation conditions (figure 5). The intensity of the bands varied between treatments, leading to the lowest enzyme activity in D cells (35% from that of the control) and the highest activity in R culture (more than 3-fold over the D value).

In the green alga *S. incrassatulus*, GPOD and GR were presented by three activity bands each, whereas only a single band of CAT activity was detected (figure 6). Five SOD bands (1–5) were visible in C cells. An additional band of SOD activity (7) appeared in D cells and one more (6) in R cells. Relative total SOD activity was slightly higher in D cells (by 27%) than in the control and it was additionally stimulated in R cells (50% over D cells). Total GR and especially CAT activities decreased in the absence of N in the medium, being approximately 2- and 5-fold lower, respectively compared with C, but markedly increased after N addition (by 150 and 800%, respectively over the D levels). In contrast, the activity of GPOD was higher (by 15%) in D than in C cells. An increase in GPOD activity (by 20%) was found in R compared to D cells.

4. Discussion

4.1 *Metabolic responses to N starvation and resupply*

The signs of N starvation (growth retardation and bleaching) and subsequent recovery of *P. cruentum*, *T. minutus* and

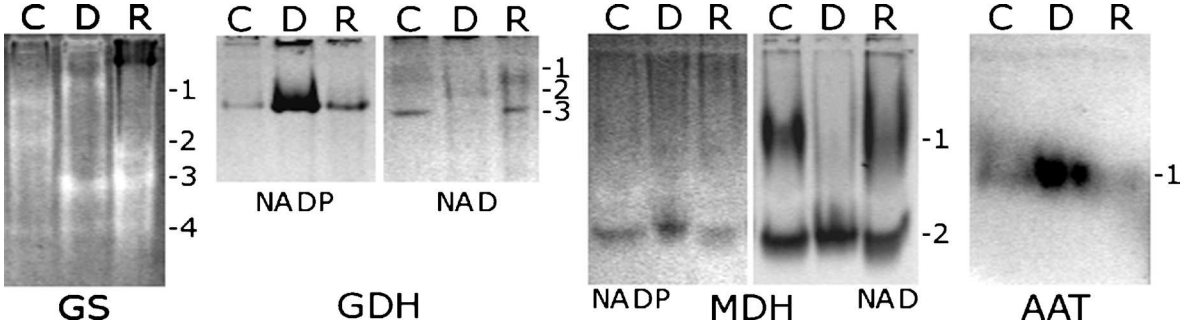

Figure 3. Changes in pattern and activity of glutamate synthase (GS), NADP- and NAD-dependent glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and aspartate aminotransferase (AAT) of *Scenedesmus incrassatulus* in response to nitrogen depletion (D) and nitrogen resupply (R) in the medium. Control culture (C) was grown in standard full-strength medium. Numbers on the right indicate the bands of respective enzyme activity, in order of increasing electorphoretic mobility on native polyacrylamide (10%) gels. Equal amounts (10 μg) of protein were loaded in each lane.

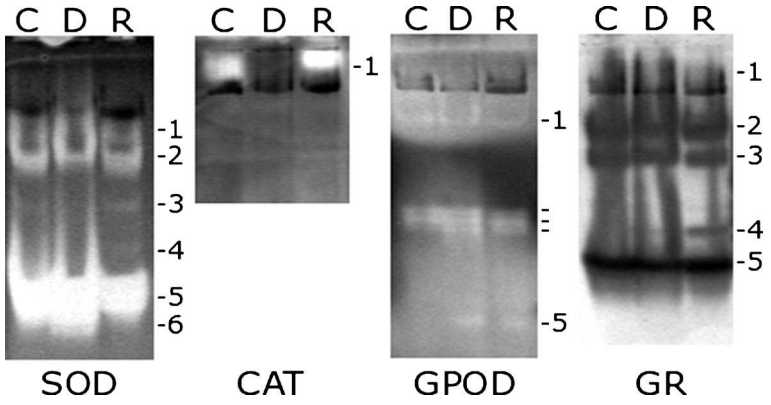

Figure 4. Changes in pattern and activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOD) and glutathione reductase (GR) of *Porphyridium cruentum* in response to nitrogen depletion (D) and nitrogen resupply (R) in the medium. Control culture (C) was grown in standard full-strength medium. Numbers on the right indicate the bands of respective enzyme activity in order of increasing electorphoretic mobility on native polyacrylamide 10% gels. Equal amounts (10 µg) of protein were loaded in each lane.

S. incrassatulus cells occurred after various cultivation times. The differential temporal behaviour of the studied species was related to the significant differences in their growth rate (table 1, μ d^{-1} of the controls, $P<0.05$) and was probably due to species-specific regulation of cellular metabolism. To study the metabolic responses of the algae to N deficiency and resupply, four enzymes that link nitrogen and carbon metabolism (GS, GDH, MDH and AAT) were analysed. GS, a partner in the glutamine synthetase/glutamate synthase cycle and GDH are key enzymes in the biosynthesis of glutamate, one of the central players in nitrogen metabolism (Hellebust and Ahmad 1989; Stitt *et al.* 2002). The activity of both pathways requires the input of carbon skeletons in the form of 2-oxoglutarate. MDHs catalyse the NAD/NADH-dependent interconversion of the substrates malate and oxaloacetate (Minárik *et al.* 2002). AAT participates in the use of oxaloacetate for producing 2-oxoglutarate in the citric acid cycle, the precursor for glutamate, or use of oxaloacetate directly for aspartate metabolism. The amino group transfer between aspartate or glutamate to the respective keto acids catalysed by this enzyme is crucial in both amino acid biosynthesis and degradation.

In *P. cruentum* cells, cultured in a nitrate-free medium, an increase in the total GS activity and appearance of new enzyme isoform, as well as more intense isoform 2 of NAD-MDH were detected. In addition, NAD-GDH activity was decreased whereas both NADP-GDH and AAT activities remained unchanged compared with the control. The response of *T. minutus* to N starvation was different. In this yellow-green alga, the activities of GDH (especially NADP-GDH), isoforms 1 and 2 of GS as well as MDH were stimulated. In *S. incrassatulus*, N starvation caused up-regulation of GS, NADP-GDH and AAT activities and down-regulation of both NAD-GDH and NAD-MDH (isoform 1) activities.

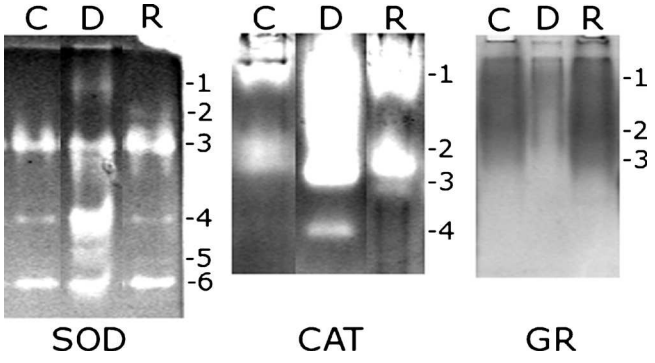

Figure 5. Changes in pattern and activity of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) of *Trachydiscus minutus* in response to nitrogen depletion (D) and nitrogen resupply (R) in the medium. Control culture (C) was grown in standard full-strength medium. Numbers on the right indicate the bands of respective enzyme activity in order of increasing electorphoretic mobility on native polyacrylamide 10% gels. Equal amounts (10 µg) of protein were loaded in each lane.

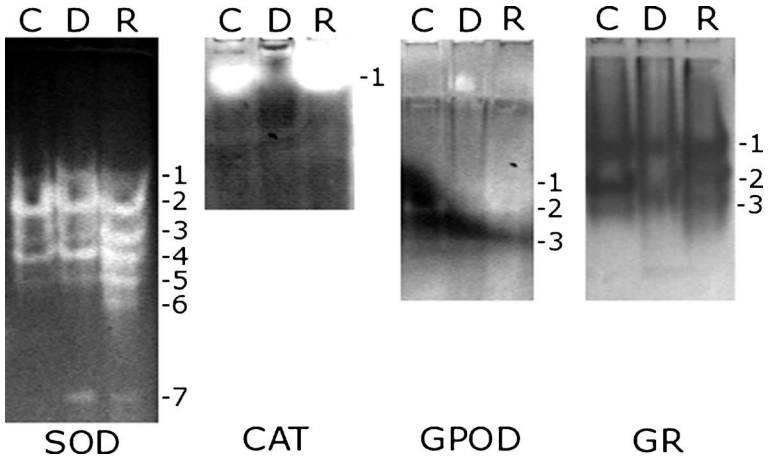

Figure 6. Changes in pattern and activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOD) and glutathione reductase (GR) of *Scenedesmus incrassatulus* in response to nitrogen depletion (D) and nitrogen resupply (R) in the medium. Control culture (C) was grown in standard full-strength medium. Numbers on the right indicate the bands of respective enzyme activity, in order of increasing electorphoretic mobility on native polyacrylamide 10% gels. Equal amounts (10 μg) of protein were loaded in each lane.

Compared to D cells, *P. cruentum* R cells exhibited higher metabolic activity as was indicated by the increased activities of all studied enzymes, except for GS. The response of N-starved *T. minutus* cells to N repletion included a decrease in GDH activity coupled with the appearance of a new highly active MDH isoform (isoform 5). N supply to *S. incrassatulus* D cells stimulated the activities of GS (especially isoform 2), NAD-GDH (isoforms 1 and 3) and MDH (isoform 1), while the activities of NADP-GDH and AAT were suppressed.

The identified different responses among the species may signify differences in N metabolism and/or metabolic strategies for handling N deficiency and resupply. In *S. incrassatulus* cells, NADP-GDH and GS might act together in the reassimilation of ammonium produced by cellular processes, such as protein and amino acid catabolism (AAT was up-regulated) under N-depleted conditions (figure 3), as was recently observed in the diatom *Thalassiosira pseudonana* (Hockin *et al.* 2012). In contrast, only GDH activities (operating in both ammonia assimilation and glutamate catabolism) were increased in *T. minutus* cells under these cultivation conditions. The up-regulation of the activities of all studied enzymes (except for GS, which maintained high activity in all experimental conditions) could account for the faster recovery of *P. cruentum* after N resupply. In addition, the impact of N uptake, rate of storage compounds accumulation and protein turnover on the observed differences between the studied species could not be excluded. The ability of a particular species to acquire, assimilate and store nutrients, when available, can ensure their use in conditions of nutrient starvation, thereby providing opportunities to faster overcome the stress period. For example, the phycobiliproteins of red algae and cryptophytes, reported as N-storage compounds in N-rich conditions, serve as N sources under N-limiting conditions (Sciandra *et al.* 2000; Mizuta *et al.* 2002). Many species of microalgae from different taxa accumulate neutral lipids as carbon and energy storage (Hu *et al.* 2008) and mobilize them after N resupply (Larson and Harrison 1997; Siaut *et al.* 2011; Přibyl *et al.* 2013). Lipid accumulation in *P. cruentum*, *S. incrassatulus* and *T. minutus* in response to N-starvation was also described (Cohen 1990; Arias-Penaranda *et al.* 2013; Řezanka *et al.* 2010, respectively). To understand how lipid responses are related to the observed metabolic differences between *P. cruentum*, *S. incrassatulus* and *T. minutus*, further research on the dynamics of deposition and mobilization of their lipids should be done.

4.2 *Role of antioxidant enzymes under N starvation and resupply*

Deficiency of combined N has been reported to create oxidative stress (Hockin *et al.* 2012; Zhang *et al.* 2013) due to increased generation of reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide (H_2O_2). Hence, activation of cellular antioxidant enzymes is important to eliminate or reduce the damaging effects of increased ROS levels. Superoxide radicals are converted to H_2O_2 by the action of SOD, and the accumulation of H_2O_2 is prevented by the activities of CAT, PODs and GR. In

agreement with increased oxidative stress under N starvation, the activity of SOD was elevated in all studied microalgae and new SOD isoforms appeared in *T. minutus* and *S. incrassatulus*. Accordingly, total GPOD activity was stimulated in *P. cruentum* and *S. incrassatulus*. The great increase in CAT activity found in *T. minutus* presumably serves to compensate for low (undetectable) GPOD activity in detoxification of excess H_2O_2. GR activity decreased (*T. minutus*, *S. incrassatulus*) or remained unchanged (*P. cruentum*), indicating that GR did not play an important role in these conditions. Substantial differences were found in the behaviour of the antioxidant enzymes between the species under N-replete conditions as well as between R and D cells of each species. N resupply probably led to a fast redox balance recovery in *P. cruentum* cells since on the following day the total activities of SOD and GPOD decreased compared to their levels in D cells. In *T. minutus*, such a decrease in the relative SOD and CAT activities was registered 5 days after N addition to the medium. On the contrary, all enzyme activities measured in *S. incrassatulus* were higher under N-replete when compared with N-depleted conditions. In both *T. minutus* and *S. incrassatulus* the activity of GR was highest after N resupply, suggesting the importance of its function in N-replete conditions.

Only a meagre amount of information is available on the effect of N starvation and resupply on antioxidant enzyme activities in microalgae. SOD, CAT and POD were reported to play important roles in removing ROS in the green algae *Dunaliella salina* and *Chlorella sorokiniana* C3 under N starvation (Abd El-Baky *et al.* 2004; Zhang *et al.* 2013). In the eustigmatacean alga *Nannochloropsis oceanica* IMET1, the protein level of 2-Cys peroxiredoxin increased more than that of the other peroxidases, and was proposed as a key antioxidant involved in the N stress response (Dong *et al.* 2013). The protein levels of SOD and a mitochondrial alternative oxidase were reported to increase approximately 2.2-fold in *T. pseudonana* at the onset of N starvation (Hockin *et al.* 2012). In the blue-green algae *Oscillatoria willei* BDU 130511 and *Chroococcidiopsis* sp., Fe-SOD was presumed to protect the cells against free oxygen radicals generated during oxidative stress due to N starvation (Billi and Caiola 1996; Saha *et al.* 2003). In addition to SOD, an increased number of peroxidase isoforms were observed in *Oscillatoria willei*, three of them being intensely stained (Saha *et al.* 2003). In *Symploca* sp., GR activity was suggested to play a major role during short-term N starvation, since its activity increased, while SOD and POD activities did not change significantly (Gigova and Ivanova 2014). In this study, we observed that *P. cruentum*, *T. minutus* and *S. incrassatulus* triggered different cascades of antioxidant enzymes, each of them being effective in helping cells to overcome N starvation and to successfully recover after N resupply.

In conclusion, similar to other microalgae, *P. cruentum*, *T. minutus* and *S. incrassatulus* possess the potential to use a range of strategies in order to tolerate changes in their chemical environment. In the present work, besides the more general responses, such as adjustment of growth and pigmentation, we report on the involvement of specific metabolic and antioxidant enzymes and their isoforms in the mechanisms operating during N starvation and recovery in the three studied species. To our knowledge, this is the first study to show such differing responses to N availability during culturing between red, green and yellow-green algae. The ability of these microalgae to overcome N starvation and successfully recover after N resupply may have ecophysiological and biotechnological importance.

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