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ORIGINAL ARTICLE

The effect of lanthanides on photosynthesis, growth, and chlorophyll profile of the green alga *Desmodesmus quadricauda*

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Abstract Lanthanides (La, Gd, Nd, Ce) accumulated in the green alga *Desmodesmus quadricauda* but their intracellular localizations were distinctly different: lanthanum and gadolinium were localized in cytoplasm, while neodymium and cerium were in the chloroplast. The effect of lanthanum and neodymium, as representatives of these two groups, on growth, chlorophyll content and photosynthetic rate at different light intensities was studied. At the lowest light intensity used (50 μmol photons m^{-2} s^{-1}), in the presence of lanthanides (Nd), growth was enhanced by as much as 36 % over lanthanide free control, and the photosynthetic rate increased by up to 300 %. At high light intensities (238, 460, and 750 μmol photons m^{-2} s^{-1}), photosynthetic rate increased markedly, but there was no significant difference between rates in the presence or absence of lanthanides. However, growth, measured as a percentage of dry weight, if compared with lanthanide free control, increased at all light intensities (31, 39, and 20 %, respectively). The total amount of chlorophyll after lanthanide treatment increased by up to 21 % relative to the control culture, mainly due to an increase in the level of chlorophyll *b*. Addition of lanthanides caused a change in the chlorophyll *a/b* ratio from 4.583 in control cultivation, to 1.05. Possible mechanisms of lanthanide-induced photosynthetic change, alterations in photosynthetic structures, and increases in growth are discussed and compared with findings in higher plants. The hypothesis that the lanthanide effect could be due to formation of lanthanide-pheophytins was not confirmed as lanthanide pheophytins were not found in *D. quadricauda.* Furthermore, we have shown that the preferential incorporation of heavy isotopes of magnesium, namely ^{25}Mg and ^{26}Mg, into chlorophyll during photosynthesis that occurred in controls was diminished in the presence of lanthanides.

Keywords Chlorophyll · Green algae · Growth · High-resolution electrospray mass spectrometry · Lanthanides · Magnesium isotopes · Photosynthetic rate

Abbreviations

Chl	Chlorophyll
DW	Dry weight
ESI	Electrospray ionization
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-pressure liquid chromatography
HRMS	High-resolution-mass spectrometry
ICP MS	Inductively coupled plasma-mass spectrometry
LC-MS	Liquid chromatography–mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
NLS	Neutral loss scan
PS I	Photosystem I

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PS II	Photosystem II
REE	Rare earth element
Rubisco	Ribuloso-1,5-biphosphate carboxylase oxygenase
TIC	Total ion current

Introduction

Lanthanides plus scandium and yttrium make up a group of rare earth elements that are essential in many industries and technologies. They are also used, mainly in China, as fertilizers in agriculture, in aquaculture, or as animal growth enhancers. Lanthanides are biologically non-essential elements that have a high uptake and bioaccumulation in plant cells. At certain concentrations, they are known to stimulate plant growth, photosynthesis, and chlorophyll content (Goecke et al. 2015; Hu et al. 2004; Zeng et al. 2000). For example, Nd was found to enhance photosynthetic rate in spinach by forming a Rubisco–Rubisco activase supercomplex and causing an increase in carboxylation activity (Liu et al. 2006a, b).

Lanthanides are known to affect different aspects of photosynthesis. One described mechanism is their ability to coordinate the porphyrin ring in pheophytin to form a lanthanide-chlorophyll (Chl)-*a* complex (Hong et al. 2002). Complexes of lanthanides with Chl-*a* were described in the fern *Dicranopteris dichotoma*, where the content of La chlorophyll was 50.08 % and that of Ce chlorophyll 19.40 % (Hong et al. 1999; Wei et al. 2005). The presence of a Chl-*a* La-pheophytin-*a* sandwich complex was also demonstrated in spinach leaves (Wang et al. 2001). Unfortunately, the published data only allow speculation on the structure of pheophytins. Wang et al. used negative electrospray ionization (ESI) to partly identify Chl-*a* and its complex (Chl-*a*-La pheophytin) (Wang et al. 2003) but they failed to identify the presence of a lanthanide-Chl-*a* complex by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) (Wang et al. 2003). Similar results and conclusions were reached by others (Küpper et al. 2006).

Another phenomenon caused by the presence of lanthanides in plants is a change in the Chl *a/b* ratio. The ratio of Chl *a/b* in the fern *D. dichotoma* decreased with increasing content of lanthanides in the soil (Wang et al. 2005). This suggests stress caused by lanthanides since a similar effect in green algae was caused by heavy metals (Laszló et al. 2001; Omar 2002; Rocchetta et al. 2012).

We have studied the effect of lanthanides on growth, chlorophyll content, and photosynthetic rate of the green alga *Desmodesmus quadricauda* at different light intensities. Lanthanides were localized by fluorescence microscopy after staining with Fluo-4 dye. We describe a comprehensive analysis of pseudomolecular ions of chlorophylls and similar compounds related to the rate of photosynthesis, as influenced by lanthanides present in the culture medium.

Materials and methods

Strain and growth conditions

The chlorococcal alga *Desmodesmus quadricauda*, synonym *Scenedesmus quadricauda* (Turpin) Brébisson (strain Greifswald/15), was obtained from the Culture Collection of Autotrophic Organisms, Institute of Botany (CCALA, CAS, Třeboň). The microalga was grown under continuous illumination with incident light intensity 460 μmol photons m^{-2} s^{-1} at a constant temperature (30 °C) in liquid mineral medium (Goecke et al. 2015). In some experiments, different incident light intensities in the range of 50–750 μmol photons m^{-2} s^{-1} were used as well. Lanthanides were added to the liquid medium as analytical grade chloride salts ($LaCl_3$, $CeCl_3$, $NdCl_3$, and $GdCl_3$) (99 %, Sigma-Aldrich) at a concentration of 10 μM. The pH of the suspension was 6.9.

Laboratory photobioreactor

As a culture unit, cylindrical glass vessels of inner diameter 36 mm and 50 cm length were submersed in a temperature controlled water bath (30 °C). A panel of 10 fluorescent lamps (Osram DuLux L55W/840 daylight, Osram, Milano, Italy), dimmable in the range of the incident light intensity from 16 to 780 μmol photons m^{-2} s^{-1}, located at one side of water bath, was used for illumination.

The cylinders were ''aerated'' with a mixture of air and CO_2 (2 %, v/v). The volume of the algal suspension in each cylinder was 300 ml, each cylinder being aerated with gas at a flow rate of 15 l h^{-1}. The pH of cultures was maintained in the range of 6.5–7.5 by the addition of 1 M NaOH. To obtain a measure of light energy absorbed by a layer of cell suspension grown at different incident light intensities, the mean light intensity (I) was calculated according to the Lambert–Beer formula: $I = (I_i - I_t)/\ln(I_i/I_t)$, where I_i is the incident light intensity measured at the surface of the culture vessel, and I_t is the transmitted light intensity measured at the rear side of the culture vessel. Incident and transmitted light intensities were measured using a quantum radiometer–photometer (LI-COR, Inc.). All experiments for determination of photosynthetic rate (μmol mg^{-1} chl h^{-1}), dry weight (g l^{-1}), and chlorophyll (mg g^{-1}) were repeated at least 3 times.

Fluorescence staining

Algal suspensions (1 ml) from untreated cultures, and cultures treated with lanthanide chlorides were centrifuged in a 1.5-ml Eppendorf tube at 5000×*g* for 3 min. The cell pellets were washed with 1 ml of 0.1 % sodium dodecyl sulfate (SDS) followed by vortexing and centrifugation at 5000×*g* for 3 min. This washing step was repeated three times. The samples were then washed with 1 ml of 1×PBS (phosphate buffered saline) and centrifuged at 5000×*g* for 3 min, and the pellet was re-suspended in 1 ml of PBS. 100 µl of the algal suspension was transferred into a 2-ml Eppendorf tube and mixed with 150 µl of zirconium beads (diameter 0.7 mm), and 100 µl of 2.5 µM of Fluo-4 (Molecular Probes) freshly diluted in PBS and vortexed (Vortex Genie 2) for 1 min at full speed at 4 °C. After 2 h of incubation at room temperature, the suspensions were centrifuged at 5000 *g* for 3 min and re-suspended in 100 µl PBS. To identify the location of the lanthanides, 15 µl of each sample was put onto a glass slide, mixed with anti-fade mounting medium SlowFade Gold Antifade Reagent (Molecular Probes), and observed under the BX51 Olympus fluorescence microscope equipped with U-MWIBA2 filter block (excitation 460-490 nm/emission 510-550 nm). Photomicrographs were taken using a DP72 digital camera.

Measurement of photosynthetic rate

Photosynthetic activity was measured as the light-saturated steady-state rate of oxygen evolution in cell suspension using a Clark-type electrode every hour during algal cultivation. Each measurement was conducted with 6 ml of the algal sample supplemented with 200 µl ferricyanide and 150 µl benzoquinone as electron acceptors at 30 °C for 1 min in the dark and 1 min in light. The resulting data were expressed as µmol O_2 μg^{-1}Chl h^{-1}. Data in Figures are presented as mean ± SD of at least 3 experiments.

Dry weight determination

For dry weight determination, 2 ml of biomass was separated from the medium by centrifuging the cell suspension at 3000×*g* for 5 min in dried and pre-weighed 2-ml Eppendorf tubes. The sediment was dried at 105 °C for 12 h and weighed on a Sartorius 1601 MPB analytical balance. Data in Figures are presented as mean ± SD of at least 3 experiments.

Chlorophyll extraction and determination

For chlorophyll extraction, 10 ml (1.10^6 cells ml^{-1}) of *D. quadricauda* culture was centrifuged at 4000×*g* for 3 min. The cell pellet was covered by 1 ml of phosphate buffer (pH 7.7) containing a pinch of $MgCO_3$ to stabilize the chlorophylls and stored at −20 °C until use. 500 µl of zirconium beads (diameter 0.7 mm) was then added, and the sample was vortexed (Vortex Genie 2) at full speed for 10 min, with cooling on ice after every minute. The chlorophylls were extracted with 4 ml of 100 % acetone followed by centrifugation at 4000×*g* for 3 min. The supernatant was aspirated into calibrated testing tubes using an exhauster/air pump; the tubes were closed with a stopper and put in a dark block. 4 ml of 80 % acetone was added to the pellet and centrifuged at 4000×*g* for 3 min. The supernatant was combined with the previous one in the calibrated testing tubes. The tubes were filled with up to 10 ml of 80 % acetone. For analyzing chlorophyll content in the algal suspension, the samples were measured on a UV–Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) in 1-cm cuvettes at 750, 664, 647, 470, 450 nm with 80 % acetone as a blank. Calculations of chlorophyll content from absorbances measured at 645 and 664 nm were carried out using the method of MacKinney (1941). A modification of the procedure for *D. quadricauda* was described by Lukavský et al. (1979). The following equations were used for Chl-*a* ($12.25 \times A_{664\ nm} \times 2.79 \times A_{647nm}$) and Chl-*b* ($21.5 \times A_{647nm}$) $-5.1 \times A_{664nm}$ (Lichtenthaler 1987; Lukavský et al. 1979; MacKinney 1941). Data in Table 1 were presented as mean ± SD of at least 3 experiments.

Analysis of chlorophylls and their relative structures

Analysis of spinach extracts by nano-ESI (Hu et al. 2012) showed that they contain hundreds of compounds including many phospholipids and glycolipids, as well as plant pigments such as chlorophylls and carotenoids (Fig. 1). We therefore purified the samples using SepPack cartridges and separated them by HILIC (Fig. 2).

The combined acetone extracts were purified on a Sep-Pack cartridge (Sep-Pak Silica Cartridge Vac 3 cc, 500 mg of sorbent per Cartridge, 55–105 micron particle size). Pure hexane was used to elute non-polar compounds, and green pigments were eluted with isopropanol and, after concentration, stored at 4 °C in the dark until LC–MS analysis.

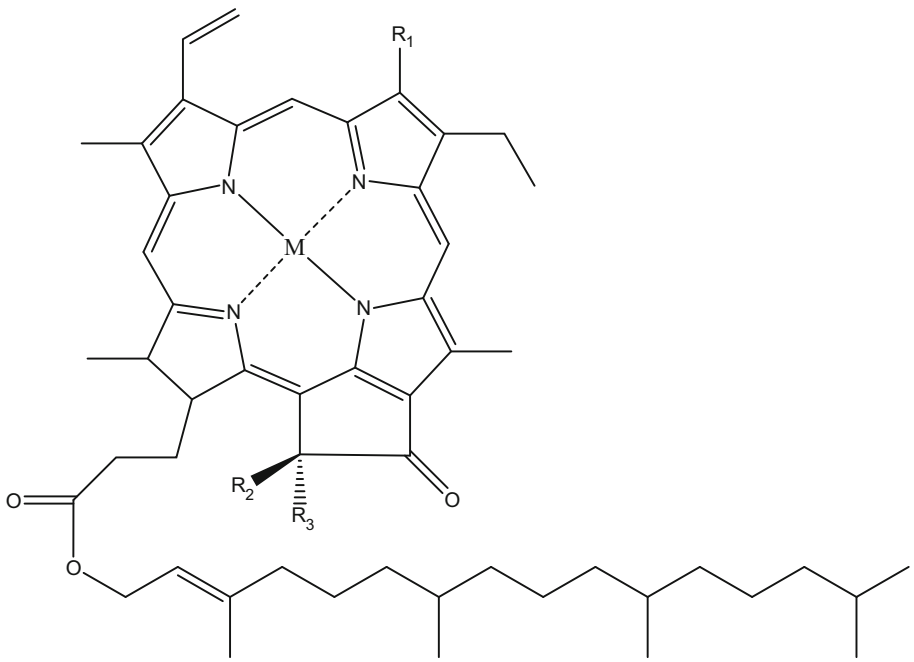
ICP-MS measurements were performed using NexION^{TM} 300D (Perkin Elmer). The organic material was extracted using a mixture of ethanol: acetone = 1:1 and the solution was evaporated. Extracts were digested with concentrated HNO_3 (Suprapur, Merck) and diluted with ultrapure water (18.2 MΩ.cm) to a final concentration of 0.1 mg l^{-1}. $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich) was used as standard.

LC–MS

High-pressure liquid chromatography (HPLC) equipment consisted of a 1090 Win system, PV5 ternary pump and

Table 1 Content of chlorophyll *a*, *b*, total chlorophylls and ratio of chlorophylls *a*/*b* in the alga *Desmodesmus quadricauda* in control culture or in the presence (10 μM) of different lanthanide chlorides (Ce, Nd, Gd, La) grown at incident light intensity 460 μmol photons m^{-2} s^{-1}

REE		Chl *a*	Chl *b*	Chl (*a* + *b*)	Chl *a*/*b*
Compound	mM	mg/g DW			
None	0	10.32 ± 0.12	6.52 ± 0.07	16.84 ± 0.23	1.58 ± 0.16
$CeCl_3$	10	10.88 ± 0.28	8.98 ± 0.11	19.86 ± 0.32	1.21 ± 0.12
$NdCl_3$	10	10.84 ± 0.39	10.62 ± 0. 9	21.47 ± 0.48	1.02 ± 0.05
$GdCl_3$	10	10.85 ± 0.32	10.40 ± 0.28	21.25 ± 0.28	1.04 ± 0.04
$LaCl_3$	10	10.88 ± 0.24	10.32 ± 0.21	21.20 ± 0.34	1.05 ± 0.06



Compound	Metal	R_1	R_2	R_3
OH-Chlorophyll **b**	Mg	CHO	OH	$COOCH_3$
Chlorophyll **b**	Mg	CHO	H	$COOCH_3$
Chlorophyll **b**´	Mg	CHO	$COOCH_3$	H
OH-Chlorophyll **a**	Mg	CH_3	OH	$COOCH_3$
Methoxychlorophyll **a**	Mg	CH_3	OCH_3	$COOCH_3$
Chlorophyll **a**	Mg	CH_3	H	$COOCH_3$
Chlorophyll **a**´	Mg	CH_3	$COOCH_3$	H
Hydroxypheophytin **a**	2H	CH_3	OH	$COOCH_3$
Pheophytin **b**	2H	CHO	H	$COOCH_3$
Pheophytin **a**	2H	CH_3	H	$COOCH_3$
Cu-pheophytin **a**	Cu	CH_3	H	$COOCH_3$
Zn-pheophytin **a**	Zn	CH_3	H	$COOCH_3$
Cu-pheophytin **b**	Cu	CHO	H	$COOCH_3$

Fig. 1 Structures of chlorophylls and their related compounds

automatic injector (HP 1090 series, Hewlett Packard, USA), and two Ascentis® Express HILIC HPLC columns (5 μm particle size, L × I.D. 25 cm × 2.1 mm (Supelco, Prague)), with an injection volume of 5 μl. Liquid chromatography (LC) of pigments was performed at a flow rate of 0.5 ml min^{-1}, with a linear gradient from the mobile phase containing methanol: acetone: aqueous 1 mM ammonium acetate (45:40:15, *v*/*v*/*v*) to methanol: acetone:

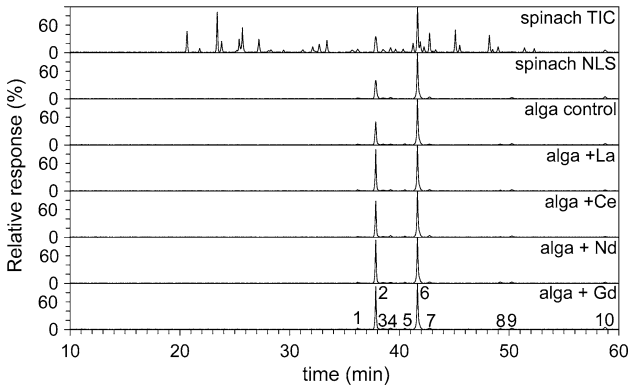


Fig. 2 LC–MS of chlorophylls and their related compounds in spinach and the green alga *Desmodesmus quadricauda.* Numbers of peaks correspond to the compounds in Table 2 (2 is for chlorophyll *b* and 6 for chlorophyll *a*); *TIC* total ion current method; *NLS* neutral loss scan method; Lanthanides added at 10 μmol l^{-1}

aqueous 1 mM ammonium acetate (15:70:15, *v*/*v*/*v*) for 60 min. The entire HPLC flow was introduced into the ESI source without splitting. The detector was an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany); a high-resolution hybrid mass spectrometer equipped with a heated electrospray interface was used. ESI–MS analysis was performed in the FT positive and negative ion modes. In positive mode, MS spectra were acquired with target mass resolution of $R = 105{,}000$ at *m/z* 800. The ion spray voltage was set at 3500 V and the scan range of the instruments was set at *m/z* 200–2000. Nitrogen was used as a nebulizer gas set at 18 arbitrary units (sheath gas) and 7 arbitrary units (auxiliary gas). In negative mode, the MS spectra were acquired with a target mass resolution of $R = 30{,}000$ at *m/z* 400 (lock mass 413.6662 Da). The ion spray voltage was set at 2500 V and the scan range of the instruments was set at *m/z* 200–2000. Helium was used as a collision gas for CID experiments. A CID normalization energy of 35 % was used for the fragmentation of parental ions. The MS/MS product ions were detected in the high-resolution FT mode. Further source conditions in the positive mode were as follows: vaporizer temperature 250 °C; capillary temperature 230 °C; capillary voltage 50 V; tube lens voltage 170 V.

Results

Intracellular localization of lanthanides, growth, and photosynthetic rate

A method for fluorescence staining was developed to identify the localization of accumulated lanthanides in *D. quadricauda* cells. The cells were stained with the fluorescence dye Fluo-4, a traditional calcium indicator, and observed under a fluorescence microscope with excitation/emission filters for both Fluo-4 and chlorophyll autofluorescence. The pictures were merged to see the position of the positive signal. Four lanthanides (La, Gd, Nd, Ce) were visualized, and their localizations in cell compartments were completely distinct. While lanthanum and gadolinium were localized in the cytoplasm (green dots in the merged pictures), neodymium and cerium accumulated exclusively in the chloroplast compartment (yellow dots in the merged pictures) of *D. quadricauda* (Fig. 3).

Lanthanum and neodymium, as representatives of lanthanides differently localized in cell ultrastructures, were observed for their impact on growth and photosynthetic rate. Algal cultures of the same initial biomass concentration (0.075 g DW l^{-1}) were grown at different incident light intensities 50, 238, 460, and 750 μmol photons m^{-2} s^{-1}, (the mean light irradiance of cells was found as 30, 190, 250, and 410 μmol photons m^{-2} s^{-1}, respectively). They grew for 4 days to attain stationary phase. As expected, the growth rate increased with increasing light intensities (Fig. 4), similarly as in control cultures grown in the absence of lanthanides. However, the presence of lanthanum and neodymium (10 μM) had a marked enhancing effect on the growth of *D. quadricauda* (Fig. 4a).

The dry weight, if compared with control (100 %) grown in the absence of lanthanides, increased. Neodymium supported growth more effectively than lanthanum and its presence caused a dry weight increase of about 36, 31, 39, and 20 %, respectively, at the different incident light intensities; lanthanum increased dry weight by about 14, 25, 28, and 8 %, respectively, at the same light intensities as above (Fig. 4b).

Photosynthetic rate measurements based on the Hill reaction (expressed in μmol O_2 mg^{-1} Chl h^{-1}) were carried out in the same cultures under the same conditions as those in which growth was monitored. With increasing light intensity (50–750 μmol photons m^{-2} s^{-1}), the photosynthetic rate increased in all experimental cultures (from 0.04 to 0.28 μmol O_2 mg^{-1} Chl h^{-1}). However, in contrast with the positive effect of lanthanides on growth at all light intensities, no effect on photosynthetic rate was observed at higher light intensities (238–750 μmol photons m^{-2} s^{-1}) compared with control cultures (Fig. 5a).

Surprisingly, an enormous increase in photosynthetic rate occurred at the lowest incident light intensity of 50 μmol photons m^{-2} s^{-1} (mean light intensity 30 μmol photons m^{-2} s^{-1}). The photosynthetic rate in the untreated culture was 0.04 μmol O_2 mg^{-1} Chl h^{-1}, while in the presence of lanthanum or neodymium it increased up to 0.11 or 0.12 μmol O_2 mg^{-1} Chl h^{-1}, respectively (Fig. 5a). The results pointed to a strong effect of lanthanides at low light intensities, with a 2.75-fold or 3-fold increase of the photosynthetic rate in the presence of lanthanum or neodymium, respectively (Fig. 5b).

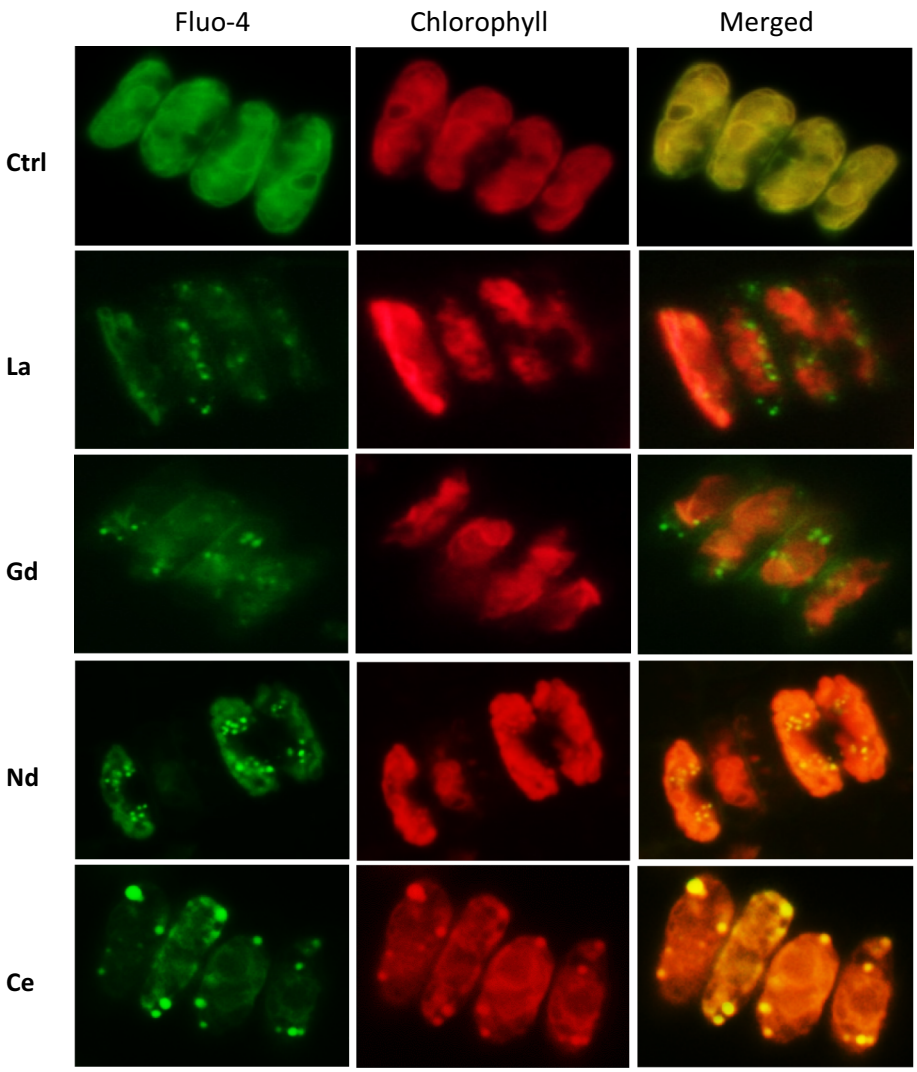


Fig. 3 Intracellular localization of different lanthanides in cells of *Desmodesmus quadricauda*. The absorbed lanthanides (*horizontal rows*) were visualized in cells stained with fluorescent dye Fluo-4 (*left column*). Chloroplasts are visualized by autofluorescence of chlorophyll (*middle column*). In merged photos (*right column*), the localization of lanthanides seen either inside chloroplasts (Nd, Ce) or in cytoplasm (La, Gd)

Total amount and ratio of chlorophyll *a/b*

The total amount and ratio of Chl *a*/*b* were determined on the basis of various absorbances of Chl-*a* and -*b* in the UV–Vis spectra. Values obtained by spectrophotometry were inserted into empirical equations (see note below Table 1). The total amount of chlorophyll in *D. quadricauda* biomass increased with treatment of all lanthanides. The untreated culture contained 14.52 mg g^{-1} of DW of total chlorophyll, while the cultures treated with cerium, neodymium, gadolinium, and lanthanum contained 19.86, 21.47, 21.25, and 21.20 mg g^{-1} of DW of total chlorophyll, respectively (Table 1). Enlargement of the chlorophyll pool was caused mainly by the increase in Chl-*b* content (from 4.20 in the control to 10.62 mg g^{-1} of DW in Nd^{3+}-treated cultures) (Table 1). From this table, it is clear that the results show three groups, i.e., a control cultivation, cultivation with Ce, and cultivation of the three remaining lanthanides (La, Nd, and Gd). The ratio varied from 2.458 (control culture) through 1.212 (Ce) to 1.021, 1.043, and 1.055 (Nd, Gd, and La, respectively).

Figure 2 shows that the Chl *a*/*b* ratio was similar in the higher plant spinach and the alga *D. quadricauda*. In the control culture of alga, the ratio was 2.458, decreasing to about 1 after adding three lanthanides (Nd, Gd, and La). In Ce, it was in the range between the control (1.12) and variants with the above three elements. This finding is fully consistent with the data (see above and Table 1) obtained by measuring the absorption coefficients and inserting these values into equations.

LC–MS of chlorophylls

Figure 2 shows the LC–MS trace of chlorophyll in spinach, as a representative of higher plants, and the alga *D. quadricauda*. The major peaks were identified as Chl-*a* and Chl-*b* (peaks no. 6 and no. 2, see Table 2). Other

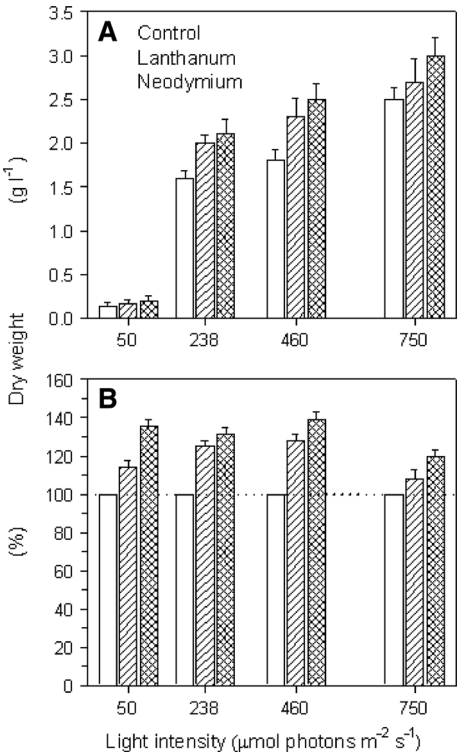


Fig. 4 Growth of *Desmodesmus quadricauda* in a control culture or in the presence of lanthanum or neodymium chlorides (10 µmol l^{-1}), at different incident light intensities. **a** Increase of DW after 4 days of growth at incident light intensities of 50, 238, 460, and 750 µmol photons m^{-2} s^{-1}, **b** increase in DW as a percentage of control values (taken as 100 %), at individual incident light intensities (*horizontal dotted line*)

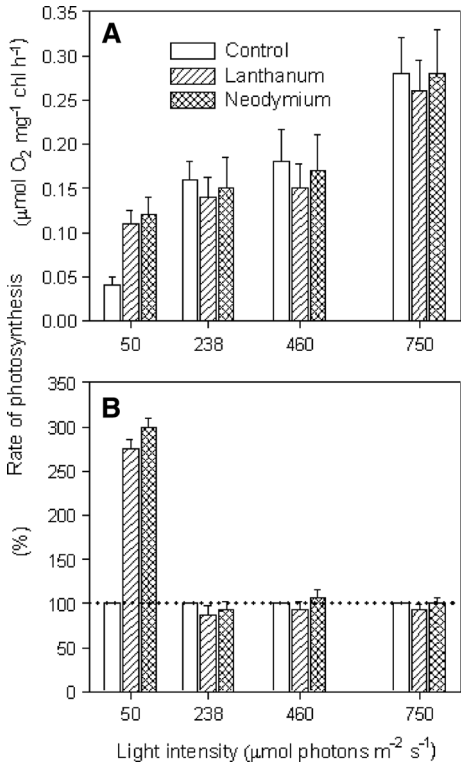


Fig. 5 Photosynthetic rate of *Desmodesmus quadricauda* in a control culture and in the presence of lanthanum or neodymium chlorides (10 µmol l^{-1}) at different incident light intensities. **a** Increase in photosynthetic rate after 4 days of growth at incident light intensities of 50, 238, 460, and 750 µmol photons m^{-2} s^{-1}, **b** increase in photosynthetic rate as a proportion of control values; taken as 100 %, at individual incident light intensities (*horizontal dotted line*)

peaks (for numbers see Table 2) are most likely degradation products.

Figure 6 shows a mass spectrum (positive ESI) in the interval from 400 to 2000 Da. Figure 6a shows 3 major peaks, i.e., $[M + H]^+$ ions at *m/z* 871.5738 (pheophytin-*a*), at *m/z* 893.5431 (Chl-*a*) and at *m/z* 907.5224 (Chl-*b*).

A mass spectrum in the interval from 920 to 2000 Da is shown in Fig. 6b, where the sensitivity or signal/noise ratio was raised to a maximum in comparison with Fig. 6a, and therefore, the response was linear within the range of 5 orders of magnitude (based on Chl-*a*). In other words, if there were other porphyrin complexes present, i.e., with metals other than Mg, they would be detected at a ratio of 100,000:1 (e.g., Chl-*a*: lanthanides-pheophytin). This fact is illustrated in Fig. 6b and far better in Fig. 6c (which is in greater detail from Fig. 6b in the interval from 920 to 950 Da). Three "chlorophylls" were identified that contained Cu and Zn, i.e., Cu-pheophytin-*a* (932.4877 Da), Zn-pheophytin-*a* (933.4872 Da), and Cu-pheophytin-*b* (946.4670 Da). All three were also found to generate primarily $[M + H\text{-}32]^+$, $[M + H\text{-}C_{20}H_{38}]^+$, and $[M + H\text{-}CH_3COOC_{20}H_{38}]^+$ ions. The remaining two peaks at ~932 Da were identified as hydroxy-Chl-*b* and methoxy-Chl-*a* (Fig. 1). The two arrows mark values that could correspond to ions constituted by complexes of lanthanides, rather than La, with a central atom, i.e., lanthanides-pheophytins. Although negative ESI was also used, no compounds having a porphyrin skeleton and at the same time having a molecular weight in the range of 950–2000 Da were identified.

All 10 peaks in the chromatograms were identified mainly based on HRMS and also using tandem MS (MS^2), see Fig. 7, which illustrates the MS^2 spectra of Chl-*a*, Chl-*b* and pheophytin-*a*. These figures clearly show cleavage of $[M + H]^+$ ions to form positively charged ions of lower values (Table 2). They are always the majority ions in tandem MS, at *m/z* 615 $[M\text{-}278]^+$ i.e., $[M\text{-}C_{20}H_{38}]^+$ and at *m/z* 555 $[M\text{-}338]^+$ i.e., $[M\text{-}C_{20}H_{39}CO_2CH_3]^+$. Other minor ions (e.g., $[M + H\text{-MeOH}]^+$ or $[M + H\text{-}HCOOCH_3]^+$, etc.) are listed in Table 2.

The ratio of three magnesium isotopes

Total chlorophyll extracted from the control cultivation (see "Materials and Methods" section) contained a higher proportion of heavier isotopes than the culture medium.

Table 2 The basic data from LC–MS of chlorophylls and their related compounds *Desmodesmus quadricauda* grown at 460 μmol m^{-2} s^{-1}

Peak no.	Compound	HRMS	Element composition	RT (min)	%[a]	$[M + H]^+$
1	OH-chlorophyll *b*	923.5173	$C_{55}H_{71}MgN_4O_7$	36.21	1.3	923.5 (30); 905.4 (100); **645.3** (15); 627.3 (15)
2	Chlorophyll *b*	907.5224	$C_{55}H_{71}MgN_4O_6$	37.85	21.7	907.5 (100); **629.1** (35)
3	Chlorophyll *b*′	907.5224	$C_{55}H_{71}MgN_4O_6$	38.48	0.9	907.5 (100); **629.1** (35)
4	OH-chlorophyll *a*	909.5381	$C_{55}H_{73}MgN_4O_6$	39.19	2.4	909.6 (20); 891.5 (100); **631.2** (10); 613.2 (15)
5	Methoxychlorophyll *a*	923.5537	$C_{55}H_{75}MgN_4O_6$	40.50	1.1	923.1 (15); 891.3 (100); **645.3** (10)
6	Chlorophyll *a*	893.5431	$C_{55}H_{73}MgN_4O_5$	41.61	63.2	893.5 (100); **615.5** (15)
7	Chlorophyll *a*′	893.5431	$C_{55}H_{73}MgN_4O_5$	42.74	2.1	893.5 (100); **615.5** (15)
8	Hydroxypheophytin *a*	887.5687	$C_{55}H_{75}MgN_4O_6$	49.20	1.3	887.4 (100) 869.5 (55). **609.2** (10). 591.1 (15)
9	Pheophytin *b*	885.5530	$C_{55}H_{73}MgN_4O_6$	50.23	2.3	885.4 (100); **607.2** (25)
10	Pheophytin *a*	871.5738	$C_{55}H_{75}MgN_4O_5$	58.75	3.7	871.5 (100); **593.2** (10)
	Cu-pheophytin *a*	932.4877	$C_{55}H_{75}CuN_4O_5$			932.5
	Zn-pheophytin *a*	933.4872	$C_{55}H_{73}N_4O_5$ Zn			933.5
	Cu-pheophytin *b*	946.4670	$C_{55}H_{71}CuN_4O_6$			946.5

Bold numbers—diagnostic ions, i.e., $[M + H]^+$—278 Da (phytol)

[a] Control cultivation of alga

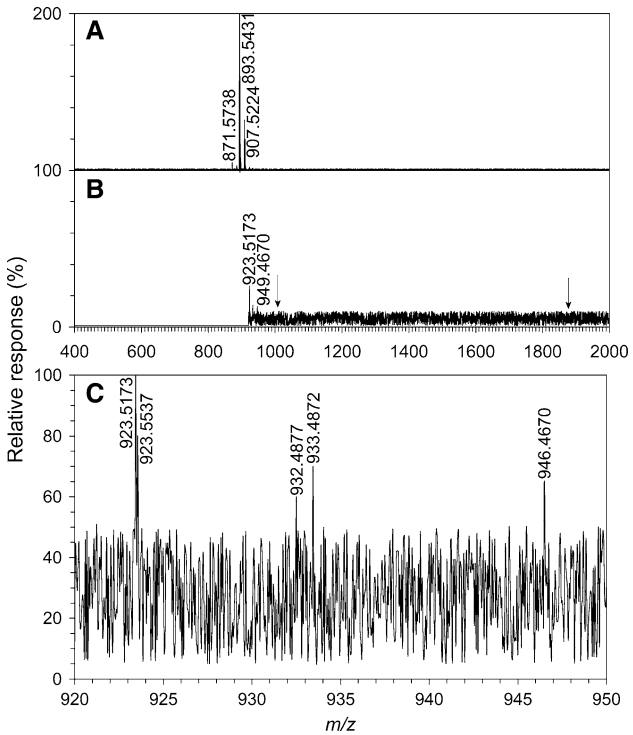


Fig. 6 HRMS of total extract in the interval of 400–2000 Da (**a**), for increased sensitivity in the interval of 920–2000 Da (**b**) and cut-out of total extract in the range of 920–950 Da (**c**)

The content of ^{25}Mg was about 0.61 ‰ higher and that of ^{26}Mg about 0.87 ‰ higher. The content of ^{24}Mg decreased proportionally. In the presence of La (10 μM), the ^{25}Mg content in total chlorophyll exceeded that in culture medium by some 0.22 ‰. When Nd was added, the content of ^{26}Mg in chlorophyll was about 0.09 ‰ higher than in the culture medium. The measured values were determined with an error of $\pm 2\delta = 0.11$ (Table 3).

Figure 8 includes values of $[M + H]^+$ ions as well as a molecular formula describing the isotopic Mg composition of chlorophylls. It shows two contradictory phenomena. The first is enrichment of Chl-*a* and -*b* relative to the culture medium in the control culture (Table 3), and the other is a clearly visible effect of lanthanides. In control culture, Chl-*a* was enriched with ^{25}Mg by 1.42 ‰ and with ^{26}Mg by 1.78 ‰; enrichment of Chl-*b* by ^{25}Mg was 1.70 ‰ and that by ^{26}Mg was 1.2 ‰. When lanthanides were added, enrichment decreased, the drop being practically the same for all four elements. For instance, in the case of cerium, Chl-*a* was 0.08 ‰ for ^{25}Mg and about 0.14 ‰ for ^{26}Mg.

Discussion

Intracellular localization of lanthanides

Information about the localization of lanthanides in *D. quadricauda* is valuable for predicting their possible functions in cells. In higher plants, hyper-accumulated metals are stored in compartments (at both tissue and subcellular levels) where they do the least harm to metabolism, becoming only weakly associated with oxygen ligands. In contrast, when metal(loid)s become toxic, they spread to various other sites depending on their toxicity targets and may disturb the correct compartmentalization of essential elements (Andresen and Küpper 2013; Andresen et al. 2013; Leitenmaier and Küpper 2013; Mishra et al. 2013; Thomas et al. 2013). Similarly, the observed localization of

different lanthanides in distinct compartments of algal cells could be related to different mechanisms affecting cellular functions, particularly photosynthesis and growth in the case of lanthanides (e.g., neodymium) located in chloroplasts. Stabilization of the cytoskeleton in the cytosol, as demonstrated in maize (Liu and Hasenstein 2005), could be assumed if lanthanum and gadolinium were localized in the cytoplasm of algal cells (Fig. 3).

Neodymium, which accumulated in the chloroplast, might enhance photosynthesis via the formation of a Rubisco–Rubisco activase super-complex similar to that observed in spinach (Liu et al. 2006a, b). Cerium was other

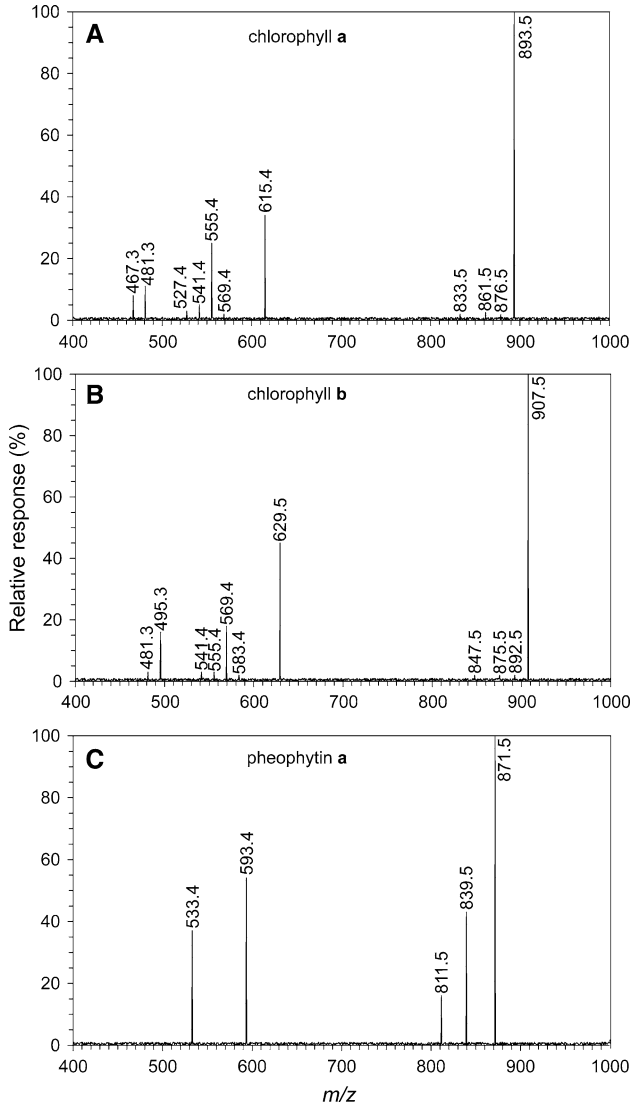


Fig. 7 Tandem mass spectrum of chlorophyll *a* (**a**) chlorophyll *b* (**b**) and pheophytin *a* (**c**)

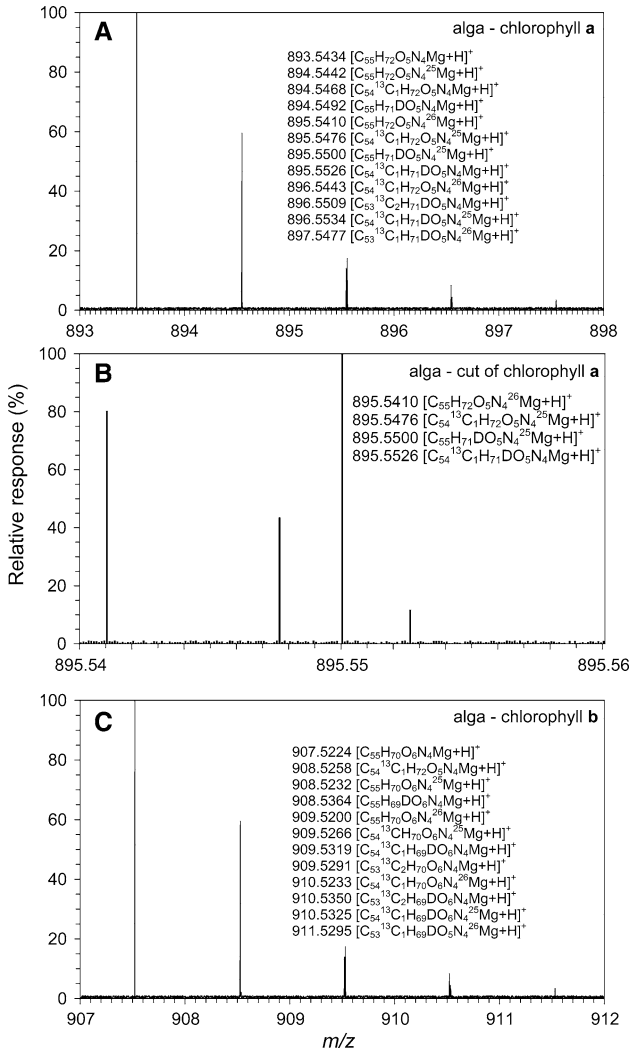


Fig. 8 HRMS of chlorophyll *a* (**a**), chlorophyll *a* (cut-out) (**b**) and chlorophyll *b* (**c**)

Table 3 Contents of chlorophylls with different Mg isotopes in the alga *Desmodesmus quadricauda* in control culture or in the presence (10 μM) of different lanthanide chlorides (Ce, Nd, Gd, La) grown at 460 μmol photons m^{-2} s^{-1}

Chlorophyll	Isotope	Natural abundance	Control (‰)	Ce (‰)	Nd (‰)	Gd (‰)	La (‰)
chl-*a*	^{25}Mg	1.00	1.42	1.34	1.34	1.33	1.36
chl-*a*	^{26}Mg	1.00	1.78	1.64	1.65	1.63	1.64
chl-*b*	^{25}Mg	1.00	1.70	1.61	1.60	1.59	1.61
chl-*b*	^{26}Mg	1.00	2.01	1.75	1.75	1.75	1.77

lanthanide localized in chloroplasts of *Euglena gracilis* (Ren et al. 2007) or in the fern *D. dichotoma* (Wei et al. 2005).

The present findings provide evidence that photosynthetic rate in *D. quadricauda* can be enhanced by neodymium, and also to a lesser extent by lanthanum, at a low mean light intensity (30 μmol photons m^{-2} s^{-1}), where the photosynthetic rate in the control is relatively low (Fig. 5). At this light intensity, the oxygen production rate in the presence of lanthanum or neodymium increased from 0.04 to about 0.11 or 0.12 μmol O_2 mg^{-1} Chl h^{-1}. This corresponds to a 2.75 and 3 fold increase, respectively, in culture grown in the absence of lanthanides (Fig. 5).

The increase in photosynthetic rate seems to be coupled with an increase in total chlorophyll content of *D. quadricauda* in the presence of lanthanides (Table 2). This is in agreement with results from tobacco seedlings (Chen et al. 2001) and maize plants (*Zea mays*) (Emmanuel et al. 2010) where chlorophyll content increased upon lanthanum treatment. The lanthanide-induced enhancement of plant photosynthesis is probably related to both increased chlorophyll content and enhanced enzyme activity (Chen et al. 2001); this could also be assumed in the algae. However, beneficial effects of lanthanides on photosynthetic rate (measured as μmol O_2 mg^{-1} Chl h^{-1}) occurred only under low light (a mean light intensity of 30 μmol photons m^{-2} s^{-1}), when the photosynthetic rate was limited (0.04 μmol O_2 mg^{-1} Chl h^{-1}); no significant difference was observed at higher light intensities (Fig. 5). Application of high incident light intensities alone (here 238, 460, and 750 μmol photons m^{-2} s^{-1}) (mean light intensities 190, 270, and 450 μmol photons m^{-2} s^{-1}, respectively) led to a significant increase in photosynthetic rate even in control cultures (0.16 to 0.28 μmol O_2 mg^{-1} Chl h^{-1}), which might be facilitated by activation of Rubisco, surpassing and overwhelming any possible additional effect of the lanthanides. Indeed, lanthanides were shown to cause a significant growth improvement of Ca^{2+}-limited algal cultures and less so promoting effects on growth of unlimited cultures (Goecke et al. 2015), suggesting that positive effects of lanthanides are more significant under limiting rather than optimal growth conditions.

Furthermore, Rubisco is known as a bifunctional enzyme, exhibiting both carboxylase and oxygenase activities (Jordan and Ogren 1981). In spinach, the oxygenase activity of Rubisco was not significantly affected but the carboxylase activity was increased more than two-fold and net photosynthetic rate (measured as μmol CO_2 mg^{-1} Chl h^{-1}) was also significantly increased in the presence of Nd (Liu et al. 2006a, b). Although we have not monitored carboxylase activity, we assume the same was also true for algae since the increase in carboxylase activity of Rubisco could explain the enhancement of dry weight of *D. quadricauda* at all light intensities in the presence of lanthanides, even though oxygenase activity was not affected. Interestingly, the increase in biomass yield in *D. quadricauda* treated with Nd was much higher (36, 31, 39, 20 %) at light intensities from 50 to 750 μmol photons m^{-2} s^{-1} (Fig. 4) than the highest increase in crops yields in China, where lanthanides used as fertilizers increased agricultural products in maize by 17 %, rice by 10.5 %, wheat by 24.2 %, and potato by 18.5 % (Liu et al. 2006b).

The chlorophyll *a/b* ratio

As shown in Fig. 2, total ion current (TIC) gave dozens of more or less separated peaks. All peaks other than peaks 2 and 6 were most likely degradation products, as has often been described (Canjura and Schwartz 1991; Kao et al. 2011; Lafeuille et al. 2014). To simplify the scan, we used a neutral loss scan (NLS) method, which greatly simplified the chromatograms of spinach and algae cultured under different conditions (Fig. 2).

The ratio of chlorophylls (*a/b*) in spinach and control (lanthanides free) cultures of *D. quadricauda* were about the same value, i.e., 100:35 (2.86) in spinach and 100:40 (2.5) for *D. quadricauda* (Fig. 2), which corresponded to a value of 2.458 if extracted chlorophylls were measured photometrically (Table 1). The values of the chlorophyll *a/b* ratio decreased to about 1 in algal cells treated by lanthanides, which was caused by an increase in chlorophyll *b*, while chlorophyll *a* was not affected (Table 1). This is not novel since the relatively constant chlorophyll a/b ratio can be substantially changed by environmental conditions (Gevaert and Rees 2015) such as light intensity, spectral composition (quality) of light (Melis 1991) or the presence of heavy metals (Laszló et al. 2001; Maina and Wang 2015; Rocchetta et al. 2012). Similarly to our results, lanthanides present in the soil improved photosynthesis and decreased the Chl *a/b* ratio of the fern *D. dichotoma*, although to a much lesser extent than in our experiments (Wang et al. 2005). Interestingly, the effect of lanthanides on photosynthesis differed depending on the type of REEs and improved either photosystem I or photosystem II (Wang et al. 2005). In contrast to our results, exposure to heavy metals (Cu and Zn) affected the chlorophyll *a/b* ratio by an interaction with chlorophyll-*a* and not *b* in lichen photobionts (Bačkor and Dzubaj 2004).

The other well-known effector of chlorophyll *a/b* ratio is variation in light intensity together with light spectral composition (Melis and Harvey 1981). Variations in light intensity are tightly coupled with antenna size and are accompanied by changes in chlorophyll levels in reaction centers (Guenther et al. 1988), since the number of chlorophyll molecules associated with each photosystem is not fixed but could vary substantially depending on environmental conditions (Anderson 1986; Bjőrkman et al. 1972; Melis 1991). Although most published papers deal

with higher plants (for detailed review, see Melis et al. 1996), there are also several reports on algae and cyanobacteria suggesting that the general mechanisms described here are the same. In the green alga *Chlamydomonas reinhardtii*, the PSI/PSII ratio varied in response to changes in light absorption and improved the quantum efficiency (Murakami et al. 1997; Melis et al. 1996). Thus, the response to light variation was similar to the effect of lanthanides in the fern *D. dichotoma* (Wang et al. 2005). More experiments would be required to prove if the changes in chlorophyll *a/b* ratio in our experiments were also connected to variations in PSI/PSII ratios.

Substitution of the central Mg^{2+} ion of chlorophyll by lanthanides

One possible mechanism underlying the increase in photosynthetic rate by lanthanides could be a substitution in vivo of the central Mg^{2+} ion of chlorophyll by lanthanides. In Chlorophyta, substitution of the central Mg^{2+} ion of chlorophyll with heavy metals varies with light intensity. Under low irradiance combined with a dark phase, the light harvesting complex II (LHC II) is the main target, while in high irradiance LHC II is inaccessible to substitution of Mg^{2+} (Küpper et al. 2006). Diverse lanthanide derivatives of chlorophyll (lanthanide Chls) occur in the fern *D. dichotoma* (Hong et al. 1999) and in *Spinacia oleracea* (Wang et al. 2001) after fertilization with lanthanide salts. Furthermore, it was claimed that these lanthanide Chl-*a* complexes could have an enhanced function in photosynthesis (Wei and Zhou 2000). The structure of chlorophyll in which the central Mg atom is replaced by a rare earth metal has been previously described using MS (Wang et al. 2001, 2003; Wei et al. 2005). In the case of *D. quadricauda,* the mass spectrum did not show any evidence of lanthanide chlorophylls (Fig. 6b) suggesting that this mechanism by which lanthanides might affect photosynthesis was not utilized by algae under conditions used in the experiments presented here.

The ratio of three magnesium isotopes

In our experiments, stable Mg isotopes accumulated in the algae against a concentration gradient; their concentration in the algae was significantly higher than in the nutrient medium. In the presence of nutrients, Mg stable isotopes were higher inside the algal cells but to a lesser extent than in the control culture (Fig. 8). We are not aware of any report dealing with the concentration of stable Mg isotopes in microalgae. Direct measurements of the proportion of Mg isotopes are quite difficult and are encumbered by many inaccuracies (Wiederhold 2015). It is generally claimed (Black et al. 2008; Bolou-Bi et al. 2010) that isotopic fractionation of Mg during uptake and transmission in plants, unlike other elements, results in enrichment with heavier isotopes. In the case of ryegrass and clover, it was in the interval from 0.26 to 1.05 ‰ (Bolou-Bi et al. 2010). The same effect was observed for wheat (Black et al. 2008), ivy (Black et al. 2007), and marine phytoplankton relative to sea water (Crotty et al. 2012; Ra and Kitagawa 2007).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. All the authors agree with the submission of the manuscript to Photosynthesis Research. The research does not involve any human participants and/or animals.

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