Use of lanthanides to alleviate the effects of metal ion-deficiency in *Desmodesmus quadricauda* (Sphaeropleales, Chlorophyta)

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Lanthanides are biologically non-essential elements with wide applications in technology and industry. Their concentration as environmental contaminants is, therefore, increasing. Although non-essential, lanthanides have been proposed (and even used) to produce beneficial effects in plants, even though their mechanisms of action are unclear. Recently, it was suggested that they may replace essential elements. We tested the effect of low concentrations of lanthanides on the common freshwater microalga *Desmodesmus quadricauda*, grown under conditions of metal ion-deficiency (lower calcium or manganese concentrations). Our goal was to test if lanthanides can replace essential metals in their functions. Physiological stress was recorded by studying growth and photosynthetic activity using a pulse amplitude modulation (PAM) fluorometer. We found that nutrient stress reduced parameters of growth and photosynthesis, such as maximal quantum yield, relative electron transport rate, photon capturing efficiency and light saturation irradiance. After adding low concentrations of five lanthanides, we confirmed that they can produce a stimulatory effect on microalgae, depending on the nutrient (metal) deprivation. In the case of a calcium deficit, the addition of lanthanides partly alleviated the adverse effects, probably by a partial substitution of the element. In contrast, with manganese deprivation (and at even lower concentrations), lanthanides enhanced the deleterious effect on cellular growth and photosynthetic competence. These results show that lanthanides can replace essential elements, but their effects on microalgae depend on stress and the nutritional state of the microalgae, raising the possibility of environmental impacts at even low concentrations.

**Keywords**: algae, toxicity, calcium, manganese, metal requirements, rare earth elements

**INTRODUCTION**

Under normal conditions, the concentrations of essential metals inside any living cell are maintained within specific ranges. If the concentration of any biogenic metal is below a lower threshold level, organisms suffer from this metal ion-deficiency, but, on the other hand, an excessive amount of those metals usually turns toxic (Pakrasi et al., 2001). If the metals are not essential, the response is not so obvious. Numerous papers have reported that under particular conditions, elements that are not essential for (higher) plants can stimulate their growth and development (Kastori et al., 2010). Among non-essential heavy metals, lanthanides (Ln), also known as “rare earth elements” (REEs), have been demonstrated to exert low toxicity (Kastori et al., 2010) and therefore, in countries like China, for the last 30 years, they have been used as fertilizers (Hu et al., 2004). Despite their names, the content of REEs in the Earth’s crust is close to 0.015% (Kastori et al., 2010) and their total concentration matches that of copper, lead or zinc (Tyler, 2004; Brown et al., 1990; Hu et al., 2004 and in text references). Rare earth elements are, however, dispersed and not often found concentrated as minerals that are easily exploitable ore deposits. It was the very scarcity of these minerals ores (previously called “earths”) and the difficulties to isolate them that led to the term “rare earth.” Only some countries (India, South Africa) have sufficient deposits to produce rare earth concentrates, however, more than 95% of REE deposits are located in China. Paradoxically, due to their increasing agricultural and industrial uses, the concentration of these elements as environmental pollutants has risen (Loell et al., 2011). As other REEs, lanthanides were generally considered to exert low toxicity (Brown et al., 1990; Wang et al., 2003). Only recently, studies have focused on the ecological effects of Ln and their potential to affect life (Li et al., 2010, and references...
themselves. Those experiments have shown that effects of Ln on plant growth are diverse and dose-response relationships are complicated (Li et al., 2011). Algae, as primary producers and the basis of many food webs, are important and sensitive organisms with opportunities for exposure to Ln, although the effects of Ln on algae are poorly understood. Recently, stimulatory effects of low concentrations of Ln on microalgae were demonstrated at low concentrations, and toxic effects were seen at higher concentrations (Hu et al., 2001; Jin et al., 2009; Tai et al., 2010). However, the mechanisms of action of microalgal growth-promoting factors are still unknown and it is not clear whether the positive effect of Ln is due to their alleviation of symptoms of metal deficiency, as suggested previously for plants (see Wei and Zhou, 2000; Tyler, 2004; Gong et al., 2011), or if the elements participate in other physiological reactions.

There are a few experiments that present evidence that Ln addition, under metal-deficient conditions, could alleviate the symptoms of deficiency by partly substituting for essential elements (e.g., Ca$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$). Elements such as divalent cations like Ca$^{2+}$ and Mn$^{2+}$, among others, have essential biochemical and structural functions in plants and algae. They are involved in countless physiological processes such as signaling pathways, the activity of key enzymes and for the synthesis of essential controls) were similar to the standard medium (hereafter referred as “standard medium”). Cultures were aerated with air containing 2% carbon dioxide (v/v). The photobioreactors were illuminated from one side by fluorescent lamps (Osram DULUX L, 55W/840, Italy) at a surface incident irradiance of 500 μmol m$^{-2}$ s$^{-1}$. The starting pH of the suspension was 7.03 varying during 3 days between 6.9 and 7.2; final value of pH at the end of the third day was 6.9.

The compositions of metal-deficient nutrient solutions (deficient controls) were similar to the standard medium (Table 2) with the following equivalent modifications: (a) Mn$^{2+}$-deficient medium was prepared by replacement of MnSO$_4$ with 8.44 μmol L$^{-1}$ (1.20 mg L$^{-1}$) Na$_2$SO$_4$; (b) Ca$^{2+}$-deficient medium was prepared by replacement of CaCl$_2$ with 256.6 μmol L$^{-1}$ (15 mg L$^{-1}$) NaCl (Table 2).
To prepare cultures limited for Mn or Ca, the initial inoculum (from the agar plate) contained approximately 0.5 µg of algal biomass resuspended in 100 mL Mn- or Ca-free medium to a known final cell concentration (absorbance = 0.1). The element-limited cultures were cultivated for 2 weeks under the same conditions as control cultures. Finally, before the start of the experiment, the medium was again removed and replaced with Ca- or Mn-free medium. Changes in the concentrations of Ca and Mn during preparation of starved cultures were measured by ICP-MS, see Section Determination of Element Content (ICP-MS).

We tested different Ln (lanthanum, cerium, neodymium, gadolinium, and europium) that belong to the group of light rare earth elements (LREE). In the case of Ca²⁺-deprivation experiments, we used a reduced concentration of salt (2.5 mg L⁻¹), which was equivalent to 5.65 µmol L⁻¹ of Eu, 5.72 µmol L⁻¹ of La, 5.76 µmol L⁻¹ of Nd, 5.74 µmol L⁻¹ of Ce, and 5.61 µmol L⁻¹ of Gd. For the Mn²⁺-deprivation experiments, we used a manganese-equivalent concentration of salt (0.5 mg L⁻¹), which was equivalent to 1.11 µmol L⁻¹ of Eu, 1.13 µmol L⁻¹ of La, 1.09 µmol L⁻¹ of Nd, 1.14 µmol L⁻¹ of Ce, and 1.07 µmol L⁻¹ of Gd. In the second case, the concentration was equivalent to the amount of Mn added to the standard medium (Table 2). This was not the case for the Ca²⁺ concentration due to a potential risk of Ln toxicity (determined from a prior test, see below Concentration Range Finding Experiment). For that purpose we used analytical grade (99%, Sigma-Aldrich) chloride compounds (LaCl₃, CeCl₃, EuCl₃, NdCl₃, and GdCl₃).

### Concentration Range Finding Experiment

We performed several prior tests (standard medium + Ln) to observe the reaction of *D. quadricauda* to different amounts of Ln, in order to determine the range of concentrations to use in our photobioreactor experiments. Therefore, the experiments were carried out in polystyrene 96-well microplates (NuncMicroWell 96-Well Microplates, Thermo Fisher Scientific Inc., Germany) with flat bottom wells of 300 µL. Each well-received 200 µL of test solution, a nutrient spike (10 µL) and an algal inoculum (10 µL), with a final volume per well of 220 µL (Blaise and Vasseur, 2005). Peripheral wells were filled with distilled water to reduce evaporation. Eight different metal concentrations were tested, and five replicates per test solution were performed, from the highest to the lowest concentration. The negative control was the respective medium + algae + water (no metal), and we incorporated a blank well (medium + elements + water, no algae) for each metal concentration tested. The microplates were incubated under controlled experimental conditions of temperature (30°C) and continuous light (100 µmol m⁻² s⁻¹) in an incubation

| Plant species | Metal -def. | Ln | Physiological effects | References |
|---------------|-------------|----|-----------------------|------------|
| Sunflower (*Helianthus annuus*) | Ca²⁺ | La³⁺ | > Auxin transport | De la Fuente, 1984 |
| Rape (*Brassica napus*) | Ca²⁺ | Nd³⁺ | + Roots and seedlings < Membrane lipid peroxidation > Absorption of nutrients > Oxidizing capacity | Wei and Zhou, 2000 |
| Spinach (*Spinacia oleracea*) | Mg²⁺ | Ce³⁺ | > Chlorophyll > Synthesis proteins > Key enzymes CO₂ assimilation > Expression of rbcL, rbcS, rca > Oxidative stress resistance > Growth | Yin et al., 2009 |
| | Ca²⁺ | Ce³⁺ | < Electron transport > Membrane permeability > O₂ evolution rate > Reactive oxygen species > Phosphorylation > Mg²⁺ATPase, Ca²⁺ATPase > Rubisco | Huang et al., 2008a |
| Maize (*Zea mays*) | Mg²⁺ | Ce³⁺ | < Chlorophyll synthesis > N₂ and carbon assimilation > PSII activities > Growth | Zhou et al., 2011 |
| | Mn²⁺ | Ce³⁺ | > Chlorophyll biosynthesis > O₂ evolution rate > Key enzymes CO₂ assimilation < Photochemical reactions | Qu et al., 2012 |

Table 1 | Experiments on the effects of plants under metal deficiency and by exposure to lanthanides.
at 105°C and left overnight. The supernatant was discarded and pellets were dried in a centrifuge. The dried pellets were then transferred to pre-weighed tubes by centrifugation. The tubes were then weighed to determine gravimetrically. Aliquots (2 mL) of samples were harvested from the photobioreactors and passed through a 0.45-μm nylon filter. The filtrate was then diluted with distilled water before being analyzed.

Values were expressed in number cells mL$^{-1}$. Growth was observed by counting cells using a Bürker chamber. Growth rate was determined gravimetrically. Distilled and demineralised water (Millipore, Canada) equipped with a concentric PTFE nebuliser, a cyclonic spray chamber, a high-efficiency quartz torch and a dynamic reaction cell (DRC) for the elimination of spectral interference. The ICP-MS analytical method was used. ICP-MS measurements were performed using an Elan DRC-e (Perkin Elmer, Concord, Canada) equipped with a concentric PTFE nebuliser, a cyclonic spray chamber, a high-efficiency quartz torch and a dynamic reaction cell (DRC) for the elimination of spectral interference. The IS solution for the total metals concentration contained Ca and Mg. The DRC was used to obtain photosynthetic parameters i.e., photon-capturing efficiency of PSII in the light limited range ($\alpha$), maximum rETR ($\Delta F/\Delta F_m$), and the light saturation irradiance ($E_L$).

**Table 2 | Composition of the ŠS-medium (Zachleder and Šetlik, 1982).**

| Compound       | Weight (g L$^{-1}$) | Molar units (μmol L$^{-1}$) | Element | Molar units (μmol L$^{-1}$) |
|----------------|---------------------|-----------------------------|---------|-----------------------------|
| KNO$_3$        | 2.021               | 19990.11                    | N       | 19990.11                    |
| K$_2$HPO$_4$   | 0.14                | 803.67                      | P       | 3301.8                      |
| KH$_2$PO$_4$   | 0.34                | 2498.16                     | K       | 24995.6                     |
| MgSO$_4$·7H$_2$O | 0.99               | 4008.11                     | Mg      | 4008.1                      |
| CaCl$_2$·2H$_2$O | 0.011              | 78.83                       | Ca      | 78.83                       |
| FeNaEDTA       | 0.018               | 49.05                       | Fe      | 49.05                       |
| H$_2$BO$_3$    | 0.003               | 48.54                       | B       | 48.54                       |
| ZnSO$_4$·7H$_2$O | 0.00143           | 4.97                        | Zn      | 4.97                        |
| MnSO$_4$·4H$_2$O | 0.0012             | 5.38                        | Mn      | 5.38                        |
| CuSO$_4$·5H$_2$O | 0.00124            | 5.35                        | Cu      | 5.35                        |
| CoSO$_4$·7H$_2$O | 0.0014             | 6.17                        | Co      | 6.17                        |
| (NH$_4$)$_6$Mo$_7$O$_2$_4·4H$_2$O | 0.00184 | 1.49 | Mo | 10.4 |

All compounds are given in grams and molar units. The total content of each element in medium (with the exception of oxygen, hydrogen, and carbon) is given in molar units.

For the current experiment, samples were taken every 24 h for 3 days; although PAM samples were taken twice a day (every 24 and 30 h) (see Section Photosynthesis as Chlorophyll Fluorescence Measurements). Every treatment was independently replicated three times. To check that the microalgae were not seriously damaged by the deficit conditions, we carried out an experiment where we separately replaced the deficient medium with the original standard nutrient medium (hereafter as “recovery condition” or Rec).

Specific growth rates in the preliminary experiment (Table 3) were determined from the slopes of linear regressions of the natural log of cell concentration vs. time for the data plotted in Figure 1.

**PHOTOSYNTHESIS AS CHLOROPHYLL FLUORESCENCE MEASUREMENTS**

In vivo chlorophyll $a$ fluorescence was determined using a Junior-PAM fluorometer (Walz GmbH, Effeltrich, Germany), provided with blue light emitting diodes for measuring excitation and actinic light. Rapid light curves (RLCs) were carried out twice per day by sampling 5 mL of culture and transferring this to light-protected chambers for dark adaptation (15 min) in order to measure $F_o$ (basal fluorescence in dark adapted samples). After that, a saturating flash ($600$ ms $\sim 9000$ μmol m$^{-2}$ s$^{-1}$) was applied in order to obtain maximal fluorescence ($F_m$). Maximal quantum yield ($F_v/F_m$) was calculated according to Schreiber et al. (1986) and the effective quantum yield ($\Delta F/F_m$) was calculated as $\Delta F/F_m = (F_m - F_o)/F_m$ (Schreiber et al., 1995b), where $F_m$ represents the maximal fluorescence and $F_o$ the current steady-state fluorescence in light adapted algae. Samples were exposed for 20 s to twelve increasing $E_{PAR}$ levels between 0 and 1500 μmol photons m$^{-2}$ s$^{-1}$ to conduct RLCs according to Schreiber et al. (1995a). Relative electron transport rates (rETR, μmol electrons m$^{-2}$ s$^{-1}$) were computed by multiplication of $\Delta F/F_m$ and the incident irradiance ($E, \mu$mol photons m$^{-2}$ s$^{-1}$) as given by the Junior-PAM. rETR values were fitted according to Eilers and Peeters (1988) using a least squares error calculation and the Solver function of Excel (Microsoft, Redmond, U.S.A.) in order to obtain photosynthetic parameters i.e., photon-capturing efficiency of PSII in the light limited range ($\alpha$), maximum rETR ($\Delta F/\Delta F_m$), and the light saturation irradiance ($E_L$).

**STATISTICAL ANALYSIS**

All experiments were repeated at least twice. Two different Two-Way ANOVA analyses were performed: (1) to determine significant differences ($p < 0.05$) among Ca$^{2+}$ and Mn$^{2+}$ treatments and (2) to determine significant differences ($p < 0.05$) between treatments and controls. In the case of significant effects, the Student–Newman–Keuls post-hoc test was applied (Underwood, 1997). Three replicates ($n = 3$) of each nutrient treatment and controls were used for each sampling time. The software Statistica for Windows (version 7.0, Statsoft, Inc., 1984–2004) was used for analyses. Data were presented as means ± SD.

**RESULTS**

**CONCENTRATION RANGE-FINDING EXPERIMENT**

In prior rapid tests to establish the correct concentration range, we confirmed that non-essential Ln produce biological effects...
on algae and have demonstrated stimulatory and toxic effects on growth at lower (<25 μmol L\(^{-1}\)) and higher concentrations (>25 μmol L\(^{-1}\)), respectively (Figure 1).

In one graphic example of exposure to Ln, we showed that concentrations of cerium of 3, 6, and 12 μmol L\(^{-1}\) produced an increase of the specific growth rate of \(D. \text{quadricauda}\) of 13, 16 and 7%, respectively; which started to decrease after the exposure to 23 μmol L\(^{-1}\) (Figure 1, Table 3).

**CONCENTRATION OF ELEMENTS ON THE NUTRIENT MEDIA**

We measured the concentrations of Ca and Mn in each medium using ICP-MS. Replete medium contained 48.6 μmol L\(^{-1}\) Ca and 7.2 μmol L\(^{-1}\) Mn, respectively. Our depletion techniques greatly reduced their concentrations to 3.7 and 0.067 μmol L\(^{-1}\) for Ca and Mn, corresponding to 8 and 1% of the original medium concentration, respectively (see Table 4). In the case of treatments with lanthanides, we tested each medium by exposure to 9.9 or 1.9 μmol L\(^{-1}\) of neodymium, as an example of Ln, for Ca and Mn, respectively. There was a serious reduction in the concentration of Nd in each medium after 3 days of algal growth (Table 4).

**GROWTH KINETICS**

The growth of \(D. \text{quadricauda}\) under complete mineral medium as a control (Ctrl), or under calcium- or manganese-deficient mineral medium is shown in Figure 2. The control and deprived conditions are graphically represented with the red and blue lines, respectively. Our results showed that a deficiency in either metal (independently), but especially Mn\(^{2+}\), significantly decreased cellular growth of the microalgae (\(p < 0.05\)) (as “Def” in Figure 2).

Re-establishment of the standard medium resulted in recovery of growth in the metal-deprived strain (as “Rec” in Figure 2).

The effect of metal ion-deprivation (Ca\(^{2+}\) and Mn\(^{2+}\)) on the growth of \(D. \text{quadricauda}\) exposed to different lanthanides (at low concentrations) is also shown. In the case of the Ca\(^{2+}\)-deficient experiment, all Ln treatments increased cellular growth in comparison with the ion-deprived nutrient medium, to reach levels close to the standard conditions (Figure 2). By contrast, none of the Ln treatments alleviated the deleterious effects of Mn deficiency on cellular grow. Instead, the addition of Ce, Eu, and Gd led to a further decrease in cellular growth in the Mn deficient cultures (Figure 2). Significant differences in growth (expressed as dry weight) for the treatments, compared to controls, are shown in Supplementary information Figure S1, Table S1.

**PHOTOSYNTHETIC ACTIVITY**

The effects of treatment on \(in \text{ vivo}\) chlorophyll fluorescence of \(D. \text{quadricauda}\) are shown in Figures 3, 4, and Table 5. Under complete mineral medium, the maximum quantum yield of PSII (\(F_{v}/F_{m}\)) showed no significant differences (\(p < 0.05\)) between the first and second days. Under these standard (replete) conditions, the \(F_{v}/F_{m}\) mean value was 0.66 ± 0.00 (Table 5). However, omission of either Ca\(^{2+}\) or Mn\(^{2+}\) from the culture medium significantly decreased the maximum quantum yield. These nutrient stresses (metal-limited conditions) in microalgae were detected by a 21% ± 0.05 decrease (Ca\(^{2+}\)) or 88% ± 2.50 decrease (Mn\(^{2+}\)) compared with the controls (mean ± SD, \(n = 3\); Figures 3, 4,

### Table 3 | The effect of cerium concentration on specific growth rates of cultures of \(D. \text{quadricauda}\).

| Cerium concentration (μmol L\(^{-1}\)) | Specific growth rate (μL\((\text{cells} \times 10^{6}) \text{ L}^{-1} \text{ day}^{-1}\)) |
|--------------------------------------|-------------------------------------------------|
| 0                                   | 1.27                                            |
| 3                                   | 1.44                                            |
| 6                                   | 1.47                                            |
| 12                                  | 1.36                                            |
| 23                                  | 1.19                                            |
| 47                                  | 1.12                                            |
| 94                                  | 1.03                                            |
| 187                                 | 0.28                                            |

**Figure 1** | Changes in cell number in cultures of the alga \(D. \text{quadricauda}\) grown in the presence of different concentration of cerium. The suspensions of cultures were grown in a 96-well microplate for 3 days.

**Figure 2** | Changes in cell number in cultures of the alga \(D. \text{quadricauda}\) grown in the presence of different concentration of cerium.

**Table 4 | ICP-MS measurements of calcium and manganese in deficient media at 0 and 72 h in the absence or presence of neodymium.**

| Time (h) | Mn         | Ca         | Nd         | Mn         | Ca         | Nd         |
|----------|------------|------------|------------|------------|------------|------------|
|          | minus Nd   | plus Nd    | minus Nd   | plus Nd    | minus Nd   | plus Nd    |
|          |            |            |            |            |            |            |
| 0        | 7.2        | 3.7        | <0.00      | 7.2        | 3.7        | 9.90       |
| 72       | 0.7        | 6.2        | <0.00      | 1.8        | 2.4        | 0.09       |

**Table 5 | The effect of cerium concentration on specific growth rates of cultures of \(D. \text{quadricauda}\).**

| Cerium concentration (μmol L\(^{-1}\)) | Specific growth rate (μL\((\text{cells} \times 10^{6}) \text{ L}^{-1} \text{ day}^{-1}\)) |
|--------------------------------------|-------------------------------------------------|
| 0                                   | 1.27                                            |
| 3                                   | 1.44                                            |
| 6                                   | 1.47                                            |
| 12                                  | 1.36                                            |
| 23                                  | 1.19                                            |
| 47                                  | 1.12                                            |
| 94                                  | 1.03                                            |
| 187                                 | 0.28                                            |

All values in μmol L\(^{-1}\).
FIGURE 2 | Changes in dry weight in cultures of the alga *Desmodesmus quadricauda* grown either in complete mineral medium (Ctrl, red symbols, dashed curve) or in calcium- (upper raw of panels) or manganese-deficient mineral medium (bottom raw of panels) (Def., blue symbols, dashed curves). To calcium and manganese deficient cultures either the complete mineral medium was added (Rec, black symbols, solid line) or different lanthanides (Ce, Eu, Gd, La, Nd) as marked in individual panels. The curves (without symbols) from recovered (Rec) and deficient (Def) cultures are inserted in panels illustrating the growth in the presence of lanthanides. Supplementary information see Figure S1.

respectively. In the first 6 h, stresses produced by sampling, centrifugation and nutrient medium replacement was observable in all tests (control and treatments), with recovery within the first 24 h. Only in the case of Ca$^{2+}$-deficiency (Figure 3), but not Mn$^{2+}$-deficiency (Figure 4), did the addition of low concentrations of Ln$^{3+}$ produce a recovery in $F_v/F_m$ and apparently alleviated the symptoms of an ion-deficit.

$rETR_{\text{max}}$ values, determined from the RLCs, were significantly higher ($p < 0.05$) in the first hours under standard than under Ca$^{2+}$-deficient conditions (“Def” see Supplementary information Figure S3). After 30 h, the $rETR_{\text{max}}$ increased in all Ln treatments under Ca$^{2+}$-deficiency, reaching values significantly higher ($p < 0.05$) than in the controls (Figure 3). After that, values started to decrease, and although after 48 h, they were significantly higher than values under deficiency conditions (Def), no significant differences were found compared to the control.

On the other hand, treatments of the algae with Ln, under Mn$^{2+}$-deficiency, showed lower $rETR_{\text{max}}$ values compared to those of the deficiency conditions during the entire experiment (Figure 4, Table 5).

Photosynthetic efficiency ($\alpha$), obtained from the RLCs, was significantly higher in cultures grown in standard nutrient medium than under either ion-deficiency conditions, or in Mn$^{2+}$-deficient + Ln$^{3+}$ treatments. Almost no differences were observed between the Mn$^{2+}$-deficient control and those after exposures to Ln$^{3+}$ (Figure 4). However, under Ca$^{2+}$-deficient treatments exposed to Ln$^{3+}$, significant differences were observed. While in both controls, the photosynthetic efficiency ($\alpha$) tended to decrease, cultures in Ca$^{2+}$-deficient treatments exposed to Ce, Eu, and Gd achieved photosynthetic efficiency values significantly higher than those observed in the controls (Figure 3).

DISCUSSION
In preliminary experiments, we confirmed that at lower concentrations (<25 μmol L$^{-1}$), Ln can produce stimulatory effects on growth of *D. quadricauda* (Figure 1, Table 3). Within this range we were able to establish metal-deficiency experiments. SS-medium is considered as a rich mineral medium (Zachleder and Setlik, 1982), where cells may be able to accumulate metals. We initiated this experiment from an agar-plate culture, transferred it (±0.0005 g) into deprived-liquid medium and maintained it as a pre-culture for 2 weeks to effectively reduce the cellular content.
of the target metals (Table 4). Metal deficiency can, in this way, be induced at levels sufficient to partially inhibit physiological processes (e.g., to reduce photosynthesis), but not severe enough to reduce the survival of the population (Figure 2). The goal was not to grow algae in the complete absence of Ca\(^{2+}\) or Mn\(^{2+}\), which has previously been studied (see Dvořáková-Hladká, 1976; Adam and Issa, 2000), but to observe if Ln may compensate for levels of deprivation in microalgae. Under Ca\(^{2+}\)-deficient conditions, certain Ln were able to partly alleviate the symptoms of the deficiency: Increased growth and biomass production, to almost normal physiological levels for microalgae observed under standard conditions (Table 5). This was not true for any of those metals under Mn\(^{2+}\)-deficient treatments, where the deleterious effect on cellular growth and photosynthetic competence was increased even more (Figure 2).

Using ICP-MS, we have analyzed the content of Ca and Mn both in deficient mineral media, and in Ca- and Mn-deficient culture media after exposure to one lanthanide, Nd. The contents of
FIGURE 4 | Manganese treatment. The photosynthetic parameters in cultures of the alga *Desmodesmus quadricauda* grown either in complete mineral medium (Ctrl, red symbols, dashed curve) or in manganese-deficient mineral medium (Def blue symbols, dashed curves) are shown. To manganese deficient cultures either the complete mineral medium was added (Rec, black symbols, solid line) or different lanthanides (Ce, Eu, Gd, La, Nd) as marked in individual panels. The photosynthetic parameters were: light-limited photosynthetic efficiency ($\alpha$); maximum relative electron transport rates ($rETR_{\text{max}}, \mu$mol electrons m$^{-2}$ s$^{-1}$); maximal quantum yield ($F_v/F_m$); and light saturation irradiance ($E_k, \mu$mol electrons m$^{-2}$ s$^{-1}$). Supplementary information see Figure S3.

Both elements (Ca, Mn) in the deficient media were extremely low (Ca 3.7 and Mn 0.07 $\mu$mol L$^{-1}$), and would not be sufficient to support algal growth from 100 mg DW L$^{-1}$ to more than 5000 mg DW L$^{-1}$, which were the values achieved in control and recovery cultures (curves Ctrl and Rec, Figure 2). Consequently, growth was limited substantially by both deficiency-treatments. Cells grew slowly for about 2 days and then stopped growth completely (compare curves Ctrl and Def in Figure 2).

After adding complete mineral medium (for both cases of deprivation) or by exposure to REEs (only in the case of Ca-deficiency) growth recovered was substantial and in some cases near the level of the control culture (Figure 2). The amount of Nd measured in the medium after 3 days of algal growth decreased from 9.9 $\mu$mol L$^{-1}$ to 0.09 $\mu$mol L$^{-1}$ (Table 4). Although we don’t know the exact bioavailable concentration for each lanthanide, if we consider the growth response to lanthanides at low
Table 5 | Summary of the physiological parameters of *Desmodesmus quadricauda* measured under replete conditions (standard medium as control, “Ctrl”) and under selective nutrient stress (deficient condition, “Def”): Ca$^{2+}$-deficient media “a” and Mn$^{2+}$-deficient media “b”.

| Parameters | Standard conditions | Ctrl | Rec | Def | La$^{3+}$ | Ce$^{3+}$ | Nd$^{2+}$ | Eu$^{3+}$ | Gd$^{3+}$ |
|------------|---------------------|------|-----|-----|----------|----------|----------|----------|----------|
| **(a) CALCIUM-DEFICIENT MEDIA** | | | | | | | | | |
| $F_v/F_m$ | 0.66 | 0.64 | 0.52 | 0.66 | 0.64 | 0.64 | 0.67 | 0.66 |
| rETR$_{max}$ | 56.25 | 62.77 | 12.30 | 46.62 | 67.60 | 47.93 | 66.48 | 67.02 |
| $E_k$ (µmol m$^{-2}$ s$^{-1}$) | 89.29 | 91.09 | 35.30 | 59.71 | 104.82 | 80.18 | 84.41 | 91.92 |
| $a$ | 0.64 | 0.69 | 0.36 | 0.79 | 0.65 | 0.61 | 0.79 | 0.74 |
| **(b) MANGANESE-DEFICIENT MEDIA** | | | | | | | | | |
| $F_v/F_m$ | 0.66 | 0.65 | 0.08 | 0.09 | 0.18 | 0.09 | 0.19 | 0.17 |
| rETR$_{max}$ | 56.25 | 54.65 | 0.00 | 2.11 | 2.49 | 2.78 | 7.36 | 5.99 |
| $E_k$ (µmol m$^{-2}$ s$^{-1}$) | 89.29 | 76.57 | 0.00 | 32.76 | 36.57 | 35.19 | 60.96 | 71.33 |
| $a$ | 0.64 | 0.71 | 0.00 | 0.07 | 0.07 | 0.13 | 0.12 | 0.09 |

The effect of the re-establishment of standard medium (recovery, “Rec”) or by adding a low concentration of different Ln is described. Maximal quantum yield ($F_v/F_m$) and the ETR parameters obtained from the rapid light curves as maximal relative ETR (rETR$_{max}$), photosynthetic efficiency ($a$) and saturated irradiance ($E_k$) at 48 h are shown. Values are means ($n = 3$), with a complete listing, SD and statistics in Supplementary information Figure S2, Table S2.

levels of Ca, and if we use as a reference the values of the ICP-MS measurement of Nd, we strongly suggest that REEs can substitute for some functions of the missing (Ca) ions.

**METAL REPLACEMENT**

Ln are non-essential elements that have been shown to produce diverse physiological effects (Jin et al., 2009; Tai et al., 2010). In the last two decades, they have been suggested to play possible roles in terrestrial organisms as replacements for essential elements like Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ (Brown et al., 1990; Squier et al., 1990). This was also observed for microalgae in our Ca$^{2+}$ treatment experiments (Figure 3), but further studies are needed to identify specific mechanisms of action. In this sense, Wang et al. (2003) stated that the biological behavior of non-essential metal ions like Ln$^{3+}$ originates from the principle of analogy to an essential metal ion. Their properties are close to but not the same as the original. Therefore, displacement (by Ln) can have different consequences depending on the role of the native metal, possibly explaining why, in some cases, functionality can be retained (Yocum, 2008). This could explain our present (and divergent) results when exposing Ca$^{2+}$-deficient and Mn$^{2+}$-deficient algae to lanthanides (Figures 2–4).

**ESSENTIAL ELEMENTS**

Of all the metals, Ca$^{2+}$ may exert the widest range of effects on biological systems, including functions related to structure, regulation of enzymatic activity and intercellular and intracellular signaling (Brand and Becker, 1984; Yocum, 2008). In previous studies with microalgae under Ca$^{2+}$ deficient conditions, photosynthetic oxygen evolution and respiratory oxygen uptake were severely affected, in parallel with growth rate and chlorophyll content (Adam and Issa, 2000). These authors suggested that many enzymes (e.g., arginine kinase, adenosine triphosphatase, adenylyl kinase) that are regulated by this metal are directly involved in vital processes such as photosynthesis and respiration. Dvořáková-Hladká (1976) also associated Ca$^{2+}$ with energy, nitrogen and phosphorus metabolism of microalgae such as *Scenedesmus obliquus*. This could explain the observed decrease in physiological state of the deprived green algae (Figures 2–4).

Manganese (and Ca$^{2+}$) exist in the oxygen-evolving complex of plant and algal PSII, and participate in the water-splitting reaction; moreover, they could be involved in maintenance of the chloroplast structure. Photosynthetic water oxidation takes place at a catalytic Mn(4)-Ca site within the oxygen-evolving complex of PSII, which is embedded in the thylakoid membranes of green plants, cyanobacteria and algae (Yachandra and Yano, 2011; Hou and Hou, 2013). Mn$^{2+}$ can be also a redox cofactor or activator at metal-binding sites of many enzymes and coenzymes (e.g., manganese superoxide dismutase), so the redox balance and PSII are expected to be the prime targets of Mn$^{2+}$-deficiency in photosynthetic organisms (Cao et al., 2011). The important role of these two metals in algae, and the effects of deficiencies on algal physiology (e.g., slow growth, structural alterations, accumulation of less chlorophyll, loss of PSII and enzyme activity), have been previously reported for a few marine phytoplanktonic species and freshwater green microalgae (Constantopoulos, 1970; Dvořáková-Hladká, 1976; Adam and Issa, 2000; Allen et al., 2007; Cao et al., 2011; Hsieh et al., 2013, and references therein).

**PHYSIOLOGICAL RESPONSES**

Physiological stress by nutrient limitation was specifically measured not only by declines in cellular growth and reproduction, but also in terms of photosynthetic parameters. PAM fluorometry has been shown to be an effective method to study stress (see Komenda, 1998; Mallick and Mohn, 2003; White et al., 2011; Figueroa et al., 2013; Giovanardi et al., 2014), and in this work, demonstrated a significant decrease in $F_v/F_m$, rETR$_{max}$, $E_k$, and $a$ in comparison with standard conditions (Table 5, Figures 3, 4). For comparison, values of $F_v/F_m$ expressed for different species of the genus *Desmodesmus* under different standard nutrient media ranged from 0.60 to 0.74 (see Komenda, 1998; Koblížek et al., 2001; Karsten et al., 2007; Hu et al., 2013; Samori et al., 2013). Interestingly, by adding individual Ln to the Ca$^{2+}$-deficient treatment we observed different, although irregular, increases in
Fv/Fm, rETRmax, Ek, and α, toward the reestablishment of standard conditions in the control (Ctrl). This is surprising because previously, it was demonstrated that although Ln are successful competitors with Ca2+ for binding sites in PSII, none of them retained functionality and treatment did not result in reactivation of oxygen evolution activity (Ghanotakis et al., 1985; Bakou et al., 1992; Bakou and Ghanotakis, 1993; Ono, 2000; Yachandra and Yanou, 2011). There is some support for our observed Ln3+-stimulation of the growth of the control cultures (Figure 1) and Ca-limited treatments (Figure 2). Kruk et al. (2003) described a possible stimulation of oxygen evolution in PSII membranes by low concentrations of Eu3+ and Dy3+ ions. Under other nutrient-deficient conditions, Huang et al. (2008a,b) and Liu et al. (2008) demonstrated alleviation effects of Ce3+ on the photosynthetic rate (electron transport rate) of spinach chloroplasts under Ca2+-deficiency and the same metal improved ETR and yield values of Mg2+-deficient maize (Zhao et al., 2012). The stimulatory effect (or reduced stress) on D. quadricauda could be derived from secondary processes affecting algal physiology, but, because Ca2+ has a multifaceted role in photosynthesis (see Brand and Becker, 1984), it will be necessary to undertake a detailed molecular study. It has been suggested that Ln could modulate plant photosynthesis by interactions with K+, Na+, or Ca2+, ribulose-1,5-bisphosphate carboxylase/oxygenase, oxidative damage and redox systems, and indolyl-acetic acid (Chen et al., 2000; Wang et al., 2011, and references therein).

In the case of the Mn2+-deficiency treatments of D. quadricauda, there was no such stimulation by adding Ln to the deficient medium. Furthermore, in many cases the algae were more stressed than under deficient conditions, as was reflected by even lower values for the photosynthetic parameters (Figure T4). There is only one previous report of Mn2+-deficient maize treated with Ce3+ (Qu et al., 2012). The authors demonstrated that the Ln could significantly reduce losses in Fv/Fm, Y(I and II), ETR(I and II) and the photochemical quenching coefficient (qP), among other parameters, as compared to those of the control. They suggested that Ce3+ may improve the function of PSI and PSII under Mn2+-deprived stress, although the mechanisms are unknown. In our work, we did not observe any improvement. Furthermore, although certain chemical similarities with Ln, this metal serves a key redox role in water oxidation in the Mn4 clusters of PSII (Hou and Hou, 2013), a highly specific function that other metals are probably not able to duplicate.

ENVIRONMENTAL CONSEQUENCES

We want to make clear that these are simulation experiments where most of the conditions are controlled. We used a monoculture, a standard enriched medium, bubbling with a high CO2 level, and concentrations of Ln that were picked subjectively (related to algal behavior and the original concentration of the omitted metal). In this way, although it gave us valuable hints to study the effects of Ln on algae, it is by no means an ecological or environmental study, requiring more natural conditions.

Lanthanides represent a potential environmental threat, particularly in high metal-exposure areas such as sites for mining, refining and recycling of REEs (Tyler, 2004). As these elements have become indispensable for a number of critical technologies, the demand for REEs in the next few years is expected to increase (Loell et al., 2011). Similarly to trace elements, Ln exhibit both positive and negative effects on algal growth and development, at low concentrations and high concentrations, respectively (Chen et al., 2000). Nevertheless, as we have demonstrated, their behavior is not simple, and in certain cases, even at low concentrations, Ln can be toxic to microorganisms. These and further studies are essential to understand the physiological and ecological effects that Ln produce in nature.

CONCLUSIONS

At low concentrations, lanthanides Ln can produce a stimulatory effect on the growth of microalgae. These non-essential elements may replace certain metals in a few physiological roles, as was demonstrated by alleviation of Ca2+-deficiency in our experiments. It is not yet clear which pathways were affected by the metals, and at what stage they became either stimulatory or toxic. The same organism responds differently to the same non-essential element, depending on the cellular physiological state. This means that, depending on the stress that algae were suffering at any specific time, the same Ln concentration could have stimulatory effects, or may increase deleterious effects and finally suppress growth; this calls into question the safety of Ln at low concentrations.

AUTHOR’S STATEMENT

The work has not been published previously (except in the form of an abstract), and all authors have approved the final article.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Celia G. Jerez, Félix L. Figueroa, Franz Goecke, Kateřina Bišová, Tomáš Rezanka, Milada Vitová, Vilém Zachleder. Performed the experiments: Franz Goecke. Analyzed the data: Franz Goecke, Celia G. Jerez, Félix L. Figueroa, Milada Vitová, Tomáš Rezanka. Contributed reagents/materials/analysis tools: Félix L. Figueroa, Kateřina Bišová, Milada Vitová, Tomáš Rezanka, Vilém Zachleder. Wrote the article: Franz Goecke, Celia G. Jerez, Félix L. Figueroa, Kateřina Bišová, Milada Vitová, Vilém Zachleder. Graphics: Franz Goecke, Celia G. Jerez, Kateřina Bišová, Vilém Zachleder. Final approval of the version to be submitted: Celia G. Jerez, Félix L. Figueroa, Franz Goecke, Kateřina Bišová, Milada Vitová, Tomáš Rezanka, Vilém Zachleder.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmib.2015.00002/abstract

Table S1 | Raw data of growth kinetics and statistics.
Table S2 | Raw data of the photosynthetic parameters and graphics.

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