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Beneficial and toxic effects of REE in algae and plants

Ph.D. Thesis

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Annotation:

Lanthanides mainly represented by REE are the most frequently occurring elements as compared to arsenic and lead. REE consist of a group of elements associated with each other in terms of common physical and chemical properties, with studies concerning phytoremediation and physiological effects of such elements on living biota, is important to be addressed as these elements are frequently being considered as emerging pollutants because of excessive mining and release into the environment. Very important is to study the toxic effects of lanthanum in microalgae under environmental conditions. Experimental trials are evaluating especially potential risks on growth and photosynthesis under nanomolar-dose, with promising decrease and acute toxicity. To this end, the two most promising La-binding protein is currently investigated in green microalgae (*Desmodesmus quadricauda*) with high affinities. Subcellular localization patterns of La have been also shown to predict possible expression sites and to understand the metabolic response of La in microalgae.

We also identify accumulator plant species for LREE in contaminated mining areas for phytoremediation purposes, aim of this study was conducted in the Brazilian mining area for REE and as well as identifying the bioavailable content which can help in predicting the promising species. This field study was done for finding new accumulators which are involved in concentrating LREE in above-ground parts. Our study suggests toxic effects of La and identified preferentially good hyperaccumulator plant specie *Christella dentata* for phytomining of lanthanides. This could be used as a predictive bioaccumulator in phytoremediation and its further analysis can be a part of future studies for insight mechanisms using analytical techniques, involving the identification of La-binding proteins in *Desmodesmus quadricauda*.

DECLARATION

I hereby declare that I am the author of this thesis and that I have used only those sources and literature detailed in the list of references.

České Budějovice, 01.06.2022

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Nermeen Ashraf

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Survival of the fittest, interdependence of the economics, development of the countries, needs of the world and many other slogans and parameters have taken the quantum leap during the last 2 decade or so. The futuristic initiatives and approaches of the development keep oneself to be in an active mode all the time. The case leads us to some job oriented and task-oriented goals in our life. Every human being is a story in himself, some are deceived by the circumstances; some meet the total loss while some retrieve its dwindling power, scattered ideas in the reutilization by facing all sorts of the music. My dwindling power to fight against all the bitter realities were innovated once again by the **ALMIGHTY** and made me able to carry on this contribution for my personality and intellectual development. I have no suitable words to thank **ALMIGHTY** for His blessings.

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List of papers and author's contributions

The thesis is based on the following papers and manuscripts:

I. Küpper, H., Bokhari, S.N.H., Jaime-Pérez, N., Lyubenova, L., Ashraf, N., Andresen, E. 2019. Ultra-trace metal speciation analysis by coupling of sector-field ICP-MS to high-resolution size exclusion and reversed-phase liquid chromatography. Anal. Chem. 91, 1710961-10969. DOI: 10.1021/acs.analchem.9b00222. (IF: 6.78)

N. Ashraf participated in laboratory experiments (10%) and manuscript corrections; (5%) contribution.

II. Ashraf N, Vitova M, Cloetens P, Mijovilovich A, Bokhari SNH, Küpper H (2021) Effect of nanomolar concentrations of lanthanum on *Desmodesmus quadricauda* cultivated under environmentally relevant conditions. Aquat Toxicol 235: 105818. DOI: https://doi.org/10.1016/j.aquatox.2021.105818. (IF: 4.96)

N. Ashraf performed most of the laboratory experiments (80%), data evaluation, and manuscript writing and corrections (60%) contribution.

III. Ashraf N, Eduardo Rodrigues dos Santos, Eduardo de Almeida, Gabriel Sgarbiero Montanha, Cassio Hamilton Abreu-Junior, Vítová M, Rafael Henrique Lazzari Garcia, Küpper H, and Hudson Wallace Pereira de Carvalho (2022). Identification of potential plant species hyperaccumulating Light Rare Earth Elements (LREE) in a mining area in Minas Gerais, Brazil. (Manuscript)

N. Ashraf participated in laboratory experiments (50%), data evaluation, and manuscript writing and corrections (50%) contribution.

Co-author agreement

Hendrik Küpper, as corresponding author and main advisor of ALL papers, together with the supervisor of this thesis, fully acknowledges the contribution of Nermeen Ashraf as the first author of chapters IV and V and co-author of chapter III as stated above.

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Milada Vítová, the co-advisor of this thesis and co-author of chapters IV and V approves the contribution of Nermeen Ashraf as the first author and her contribution in chapter III as co-author as stated above.

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Centre Algatech, Institute of Microbiology (CAS).

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LIST OF ABBREVIATIONS

REE	Rare Earth Elements
USGS	United States Geological Survey
Ppm	parts per million
REO	Rare Earth Oxide
REM	Rare Earth Metal
RE	Rare Earths
NPs	Nano Particles
Ln	Lanthanide
E.coli	Escherichia coli
$LaCl_3$	Lanthanum chloride
$GdCl_3$	Gadolinium chloride
$CeCl_3$	Cerium chloride
µg/L	Microgram per litre
ng/L	Nanogram per litre
ln/L	Lanthanides per litre
LRRE	Light Rare Earth Elements
µM	Micromole
sp.	Specie
HRRE	Heavy Rare Earth Elements
var.	Variety
EDTA	Ethylene Dia-mmine Tetra-acetic acid
INAA	Instrumental Neutron Activation Analysis
HR-ICPMS	High Resolution-Inductively Coupled Plasma Mass Spectrometry
ROS	Reactive Oxygen Specie

TEM	Transmission Electron Microscopy
PAM	Pulse Amplitude Modulation
ATP	Adenosine Tri Phosphate
Nd-Fe-B	Neodymium-Iron-Boron
pH	$-\log[H^+]$
DNA	Deoxyribose Nucleic Acid
RNA	Ribose Nucleic Acid
SEP	Sequential Extraction Procedure
PSMs	Phosphates Solubilizing Microorganisms
FTIR	Fourier transform infrared
EDAX	Energy Dispersive X-ray Analysis
XRD	X-Ray Diffraction
ICP-MS	Inductively Coupled Plasma Mass Spectrometery
MRI	Magnetic Resonance Imaging
TETA	Triethylenetetramine
DOTA	Dodecane Tetraacetic Acid
PQQ	pyrroloquinoline quinone
MDH	Methanol Dehydrogenase
kDa	kilo Dalton
LanM	LanModulin
PSII	PhotosystemII
OD	Optical Density
nM	nanoMolar
PSIIRC	PhotosystemII Reaction Center
F_v/F_m	Variable Fluoresence
F_0	Minimum Fluoresence

NPQ	Non-Photochemical Quenching
mg/L	milligram per litre
OEC	Oxygen Evolving Complex
ESRF	European Synchrotron Radiation Facility
μXRF	Micro- Xray Fluorescence
Ppt	parts per trillion
WDXRF	Wavelength dispersive Xray Fluoresence
BCR	Community Bureau of Reference
HPLC	High Performance Liquid Chromatogram

CHAPTER 1

Introduction

CHAPTER 1: GENERAL INTRODUCTION

Lanthanides (commonly referred to as Rare Earth Elements) are the group of 17 elements of the REE series, which usually occur in the environment in the trivalent oxidation state. The term lanthanides originate from lanthanum, the first element of the series. The term "rare" seems to indicate the lack of large reserves of these elements worldwide, but this is not generally true. Some of them (e.g., cerium) are more abundant in the earth's crust than commonly known trace metals like copper, and they are commonly found in soils (Hu et al. 2006). They were isolated in the early 19th century in the form of oxides form rare minerals (Nicodemus, 2009) and they are a group of elements with common physical and chemical properties, ranging from lanthanum (La) to lutetium (Lu) along with scandium (Sc) and yttrium (Y) (Connelly et al. 2005). These elements occur widely in different forms, usually in granites, pegmatites, gneisses, and other commonly associated forms of rocks (Tyler, 2004). Among all elements in lanthanides, only promethium is radioactive and is very rare (Haque, 2014). The lanthanides are generally subdivided into three categories: light REE from lanthanum to europium, middle (samarium to holmium), and heavy ranging from gadolinium to lutetium. The wide use of these elements in the field of medicines, chemical engineering, and electronics (Massari & Ruberti, 2013) was, followed by an immense release of rare-earth into the ecosystems, and thus they are considered as emerging environmental contaminants (Yang et al. 2009). Application of REE in the field of agriculture, excessive mining, and refining of REE mineral ore, that leach potential toxic compounds from modern technologies are considered as the main source of REE pollution. Many studies revealed the "hormesis effect" of rare earth on physiological responses in plants for example growth and development that is mainly characterized by low dosage stimulation and high dose inhibition (Hu et al. 2016). Under high concentrations, the toxicity of these elements causes reduced net photosynthesis and chlorophyll content (Hu et al. 2016). More than 90% of the production of REE are located in China and therefore, it increases the interest of plant biologists, as it is widely being practiced in agriculture as fertilizer for increasing the quality and quantity of crop plants. According to Pang (2001), sufficient provision of lanthanides to agricultural soil has beneficial effects. The toxicity levels of lanthanides,

when compared with other metals, were also studied and it was found that REE are generally less toxic, as, for lanthanum, its toxicity is lower than Cu but higher when compared with Fe (Wheeler & Power, 1995). These elements exist in a diverse form of minerals usually as phosphates, fluorides, chlorides, and silicates (Tyler, 2004). The solubility of each rare earth element in the earth's crust depends on many factors that includes total soil REE content, type of soil, pH, soil organic matter composition, and composition of the parent material.

1.1 Abundance and distribution of REE

Distribution and occurrence of REE have raised a discussion in the last decade whether they are crucial for aquatic organisms, land vegetation, mammals, and for human beings. Among all 17 elements in the REE group, cerium and yttrium are the most abundant and occur on the earth's crust in higher concentrations when compared with other elements for example arsenic, lead, and molybdenum. Among all elements in the REE series, lanthanum and neodymium exist nearly in the same quantities as lead. On other hand, thulium is the rarest among them but occurs more than gold and platinum. Naturally, lanthanides usually occur as a group and are rarely alone in soil (Fu, 2001).

According to USGS statistics, 2019, four countries are well-known for REE reserves, including - China, Brazil, Vietnam, and Russia. The overall share of global rare earth deposits based on the data of 2018 is shown in figure 1.1. In 2019, the annual production of REE globally was 213,000 metric tons, with approximately 62% share of China (Statista USGS, 2021).

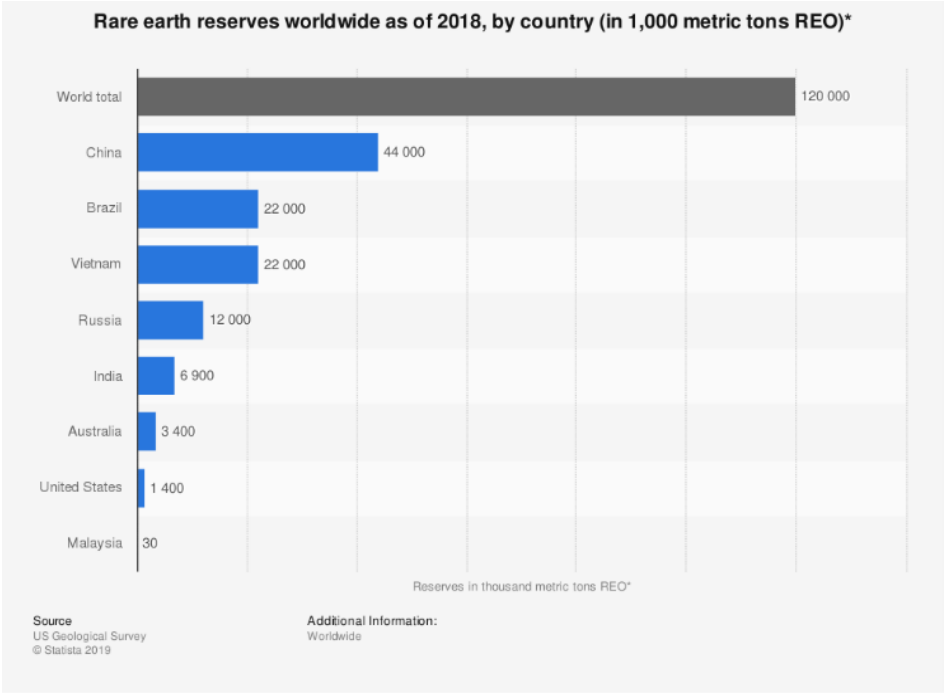

Figure 1.1: Rare earth elements distribution (USGS, 2019).

1.2 Opportunities, and landmarks in REE research

Rare Earth Metals (REM) can be frequently designated as those elements that are uncommon. Thus, these elements in soils allow scientists to understand their toxicity and harmful impacts on the ecosystem and associated pathways for their remediation. REE speciation is affected mainly by environmental conditions that vary with ionic and ligand content present and also the concentration that is accessible to living organisms (Borrego et al. 2012). Excess human activities damaged aquatic and terrestrial ecosystems because of large amounts of trace metal contaminants released into the environment (Khan, 2017). Predominant uses for REE are for automobile and petroleum refining catalysts, permanent magnets, batteries for electric and hybrid vehicles, and diverse medical tools. Lanthanides are fundamentally involved in making crucial tools involved in dentistry and other medical equipment (Eichler, 2015). Table 1.1 represents the common uses of lanthanides.

Element	Common uses
La	Optics, batteries and catalysis
Ce	Chemical applications
Pr	Magnets, lighting, and optics
Nd	Magnets, lighting, lasers, and optics
Pm	Radioactive and use in atomic batteries
Sm	Magnets and lasers
Eu	Lasers, color TV, lighting, and medical uses
Gd	Medical uses and X-rays generation
Tb	Lasers, lighting
Dy	Magnets and lasers
Ho	Lasers
Er	Lasers and steel making
Tm	X-ray generation
Yb	Lasers and chemical industry applications
Lu	Medical and chemical industry applications
Sc	Alloy‘s formations, lighting
Y	Lasers and microwave filters

Table 1.1: Summary of REE with their common uses (http:// metalpedia.asianmetal.com/metal/rare_earth/application.shtml).

Among plants and microorganisms, REE provide great opportunities to study biological effects that play either positive or negative physiological impacts depending on many factors including dosage (Zhang et al. 2013). The knowledge regarding the effectiveness of these elements on plant production is still hypothetical, ranging from their role on transportation mechanisms, fixing nitrogen by plants, impacts on calcium metabolism, and light-dependent photosynthetic reactions. In spite of this fact that the demand for rare earth for industries is high enough, alternative ways need to be explored to extract them, including agronomy in China as these elements are usually focused on experimentation in order to increase crop quality and productivity (Redling,

2006). Lanthanides can be rated as beneficial for crop plants and more than 100 crop species were claimed to show a positive response, with a yield increase of 5 to 15% (Hu et al. 2004). Above all positive effects, the consequences of high doses that lead to inhibitory effects cannot be excluded. There are further impacts of REE on plants and aquatic organisms that are still unknown. In terms of remediation, hyperaccumulators have the ability to accumulate larger amounts of REE with minimal negative consequences to the plant itself (Wu et al. 2013). Leaching of REE into the underwater environment and their further absorption by plants as mineral nutrition and thus enter in a human food chain is well-known from the past decade (Chua, 1998). Despite the research on REE is progressing in the last few decades, but certainly need more investigation in an aquatic and terrestrial ecosystem to answer the missing questions is required. Although, lanthanides are considered non-essential for life, however, they are biologically active and extensively being used in medicines (Thompson and Orvig, 2006; Major and Meade, 2009). Based on the toxicity of lanthanides (non radioactive compounds) are relatively less toxic for human beings (Eliseeva and Bünzli, 2010; Bünzli et al. 2008), however, this statement may not be true for other organisms (Eliseeva and Bünzli, 2010; Tai et al. 2010). Promethium occurs only as radioactive lanthanide with a short half-life, the longer-lived promethium isotope is ^{145}Pm with a half-life is 17.7 years (Emsley, 2020), however, very less health risk is associated with it as its abundance is so low on earth crust. Lanthanides interaction with biological systems have been explored in past decades. For example, higher content of phospholipids and hydroxylic groups of sugars like cellulose, modify cell surfaces of organisms in a way that enhances the adsorption capacity of rare earth (Moriwaki and Yamamoto, 2013; Merroun et al. 2003). Adequate levels of REE have extensively been utilized in China for increasing crop yield, however, an association of lanthanides for its utilization by plants is mainly associated with bacteria that affects plant growth and reflect plant-microbial interactions (Cotruvo, 2019). Crucial roles of lanthanides in a thermophilic bacterium (*Methylacidiphilum fumariolicum* SolV) were confirmed in 2014 with the discovery of roles of REEs related to growth, that is associated with XoxF (Pol et al. 2014), (PQQ; pyrroloquinoline quinone)-linked (MDH; methanol dehydrogenase), an enzyme that requires Ce^{3+} and La^{3+} ions, previously, this enzyme

was known to use calcium and PQQ-dependent MDH (Anthony and Williams, 2003). The discovery of Ln-dependent methylotrophic bacteria is crucial for maintaining biological functions, as they are found in water, soil, and plants, where they are involved in the carbon cycle thus opening the possibilities for the involvement of natural and efficient REE binding complex molecules, that might occur (Nakagawa et al. 2012; Martinez-Gomez et al. 2016; Cotruvo, 2019; Pol et al. 2014). In 2018, a small protein of approximately 12 kDa known as lanmodulin, LanM was first isolated from methylotrophic bacterium and thus categorized as Ln-binding polypeptide (Cotruvo et al. 2018). LanM is a macromolecule that acts as a chelator for REE ions and reversibly sequesters them (Deblonde et al. 2020). Studies revealed that lanthanides might be taken up by small molecules secreted in bacteria known as lanthanophores (Daumann, 2019; Semrau et al. 2018; Picone and Op den Camp, 2019; Cotruvo et al. 2018; Keltjens et al. 2014; Gu et al. 2016). Particular physiological behaviors of lanthanophores are already characterized in bacteria, however, the mechanism is unknown in plants and thus requires more thoughtful approaches to understand the benefits of REE, in plants and increasing growth (Tyler, 2004; Ramírez-Olvera et al. 2018). The direct targets can be cell walls and photosynthetic structures or effects the colonization of methylotrophic bacteria with plants because of their subsequent building up of methanol (Fall and Benson, 1996; Chistoserdova, 2016). It has been speculated that much of the uptake of REE occur along calcium absorption routes, like a wide range of metallophores and nicotianamine (White and Broadley, 2003; Guerinot and Morrissey, 2009). It is now known that plant takes advantage of lanthanophores secreted by bacteria (that utilize lanthanides) and reside in the phyllosphere, resulting in increased bioavailability of lanthanides (Cotruvo, 2019). In-vitro uptake of REE by cells can be demonstrated by the process known as endocytosis that resulted from the organization of insoluble forms of REE in aggregates (Wang et al. 2014; Yang et al. 2018 & Yang et al. 2019). Under conditions, such as in the plant, rare earth elements are present in soluble form and thus bioavailable to cells, for example, Ce^{3+} can be integrated into chlorophyll molecules under Mg-limited environment (Fashui et al. 2002; Zhou et al. 2011). It is also presumably estimated that REE can be taken up in place of calcium, such as Ca^{2+} ion present in oxygen releasing complex of PSII can be inserted with lanthanides, when

available (Yachandra and Yano, 2011). Ultimately, the growth-promoting effects of lanthanides in plants are generally associated with microbiome in soil biota, for example, Escherichia coli and Pseudomonas aeruginosa, both own PQQ-utilized dehydrogenase enzyme that might be activated by REE and promoted growth. Therefore, no obvious clue behind the mechanisms involved for the effects of lanthanides in higher organisms.

1.3 Ecotoxicology of REE – Impact on the environment

Majority sources of environmental contaminants including metals originating from industrial wastes represent a considerable role in natural environments in terms of pollution (Yusof et al. 2001). Besides industrial uses of rare earth, these elements are extremely important in the agricultural field as well. Examples include lanthanum and cerium as the main components of the fertilizers that have been used in Chinese plant production for the last two decades. Therefore, the amount of rare earth entering the environment increases and thus enters into the soil ecosystem (Zhang and Shan, 2001) and even disturb the aquatic ecosystem. Studies have shown that REE can have both beneficial and harmful effects that are dependent on bioavailability factors, as mainly the low concentration can enhance the plant biomass and improve fruit quality. However, if the dosage is high above the suitable range, they inhibit the growth and lead to mortality (Zhang et al. 2013). The biological role at the molecular level of REE is still a question that emphasizes researchers to study them in model organisms to better elucidate their impact on the environment because of its increasing applications (Ramos et al. 2016). One major factor that contributes to an increase of these elements in the ecosystem is the rapid release of minerals containing REE into the environment. Industrial activities present higher contents of REE in the soil and the zones where these metals are intensively used in fertilizers for agricultural purposes. Hence these areas if not properly managed or controlled, lead to the contamination of air, soil, and water (Khan et al. 2017). In addition to this, continuous application of rare earth metals into ecosystems via fertilizers to soils reversibly increases the amount of heavy transition metals, thus indicating its deposition in agricultural soil to toxic levels and thus causing a potential risk to the environment and health through accumulation along the human food chain. Extensive use of lanthanides in magnets, metal alloys, fertilizers,

ceramics, and as well as in freshwaters for eutrophication management (Zaragüeta and Acebes, 2017), have led to significant emission of REE into the environment, whereas limited knowledge of their fate and impact of the increasing release on the natural ecosystem is available (MacMillan et al. 2017). Most of the studies on ecotoxicity of lanthanides are laboratory-based, however, data regarding field measurements of natural background levels, are tremendously scarce (Weltje et al. 2002; Yang et al. 1999).

Excessive mining, leaching and processing of lanthanide ores is the main cause of environmental pollution with REE, including radioactive waste products. Lack of ecotoxicological data hinders the environmental risk assessment of REE for the determination of environmental thresholds (Gonzalez et al. 2014). It was commonly studied that REE promote chlorophyll biosynthesis and improve seed germination and availability of metals including calcium and manganese to soil biota (Liang et al. 2014), however, available information on the data collected from fields is mainly incompatible with laboratory experiments (Shtangeeva and Ayrault, 2007). For example, it was studied that both roots and shoot length were not improving with an application of lanthanum (La) and cerium (Ce). Disruption in the biogeochemical cycle of REE because of their agricultural applications shows abnormal enrichments of lanthanum, cerium, neodymium, samarium, and gadolinium, and are currently detectable in aquatic systems (Pereto et al. 2020). Excessive discharge of gadolinium (Gd) concentrations are observed all over the world in sewage water, consequently, in Germany elevated Gd is estimated to be more than 1000 kg/year (Kulaksiz and Bau, 2011). Thus, continuous application of rare earth indicates its potential risk to the ecosystem followed by health effects to humans by entering the food chain.

1.4 Behavior of REE in an aquatic environment

There are several routes through which rare earth metals could enter the aquatic ecosystems e.g., discharge from paint coverings and discharges released from sewage treatment industries (Keller et al. 2013). Ln are sometimes applied as nanoparticles in the form of oxides and the most commonly used Ln-based oxide NPs are cerium oxide nanoparticles that have main application in energy storage areas and about 3% of total

produced CeO_2 NPs enter into an aquatic ecosystem (Keller et al. 2013). Ce in the form of nanoparticles showed toxicity to algae *Chlamydomonas reinhardtii* and was taken up in the form of Ce-ions (Kosak née Röhder et al. 2018). Regardless of the hazardous effects, knowledge on toxicity of REM as an integral part of safety assessment is scarce. The level of knowledge on contamination associated with REE, based on concentrations in an environment, extent of toxicity, metal speciation along with bioavailability, uptake and bioaccumulation properties with reference to Ln ions is unusually far less in comparison with other harmful contaminants in an ecosystem, for example, heavy metals such as, Pb, Cd, Hg, As and so on (Gonzalez et al. 2014; Cobelo-García et al. 2015; Tchounwou et al. 2012). The data relevant to ecological toxicity of lanthanides and its fate are needed to be evaluated for potential risks of REE contamination to environment, in particularly, aquatic ecosystems. Soluble lanthanides based compounds leached from NPs and induce negative effects, in-case in *E. coli* dysprosium oxide nanoparticles was the main contributor in lanthanide toxicity (Anaya et al. 2016). Most of the studies in last decade were focused on both advantageous and lethal effects of lanthanides on various species including on humans, soil organisms and plants and microorganisms (bacteria), since these metals are widely being used in medicines and in fertilizers (Pang et al. 2001; Hanana et al. 2017; Rim, 2016). However, increasing levels of Ln in the ecosystem may cause potential accumulation in humans and cause detrimental effects, thus, this needs a long-term studies and observations for their potential roles in humans (Pagano et al. 2015; Wang et al. 2017; Poniedziałek et al. 2017; Marzec-Wróblewska et al. 2015). Comprehensive reviews regarding ecotoxicity of lanthanides-based NPs and salts on plants has been studies, however, limited data based on roles of REM on aquatic biota is available. Research has been done in past few years to evaluate the adequate levels of lanthanides in aquatic environment including fresh waters aquatic organisms in the past decade but still it is quite challenging because of lack of reliable studies. (Blinova et al. 2020). In addition to the information based on data from severe lethal response, long-lasting toxicity evaluation and bioconcentration were also considered to widely cover the broad range of ecologically important organisms in the aquatic environment. Toxicity studies based on Ln-based nano (particles), when compared with the corresponding soluble Ln-salts,

proved that NPs are less toxic than lanthanides salts, however, it is hard to distinguished between their toxicity levels, due to the limited knowledge in the test medium (Kurvet et al. 2017), as for example cerium based nanoparticles mostly in the form of oxides can show an alarming threat to environment, mainly to water-living organisms however, available information indicates that toxicity is more pronounced when the levels exceeds to 10 mg/L, depending on the exposure duration (Peng et al. 2017). Continuous exposure studies of lanthanides were done with $LaCl_3$ (Shen et al. 2018) and $CeCl_3$ (Wang et al. 2012) on cyanobacteria and research reveals that to the level of 0.1 to 0.5 mg/L of each (lanthanide) showed hormesis response. Studies on the long-term toxicity of lanthanides using microalgae are rare (about 4%) and 4% with other unicellular microorganisms (Blinova et al. 2020). Regarding the Ln bioaccumulation studies based on laboratory experiments mainly depends on model organisms, exposure conditions and duration and types of lanthanides compound in different studies varied considerably. It was shown that Gd accumulated 100 folds more when applied as $GdCl_3$ (Pang et al. 2001b). However, under laboratory based experiments, bioavailability of REE to microorganisms (e.g., microalgae) is mainly dependent on growth medium composition, as in the presence of hydroxides, phosphates and carbonates REE are hardly soluble, thus it is complex to assess toxicity as metals get precipitated in the test medium (Aharchaou et al. 2020) unless the precipitation is prevented by chelators (e.g. DOTA for REE). Most of the studies on lanthanide toxicity to aquatic organisms were done in artificial media that are enriched with nutrients but not taking care of REE chelation. Thus, REE precipitation restricted REE bioavailability, mainly by the presence of phosphate ions in medium that are necessary for growth of algae (Barry and Meehan, 2000; Joonas et al. 2017; Lürling and Tolman, 2010). Biological sequestration of metals either as free, dissolved ions or in the form of metal complexes are mainly dependent on uptake levels, consumption and storage capabilities of the model organisms. Studies on REE metabolism is similar to Ca in aquatic ecosystems, including algal cells that can concentrate REE from waters (lake or stream) and total REE content in cells may reach up to 1.3 mg/kg that is 10–20 times higher than total lanthanide concentrations determined in land plants (Xiao-jun et al. 1998). Although high lanthanides content in aquatic biota has been reported, Ln shows relatively higher

bioaccumulation capacity in aquatic organisms, being the highest for La and Ce and less for others. Detailed evaluation of the Ln discharge into the aquatic ecosystems, resulted in the accumulation of REM, for that, origins of emissions and pathways and fate of anthropogenic Ln should be considered. Thus, the only method for direct evaluation of REE in various environmental models for geoscientific studies include analytical methods e.g., ICP ion source coupled with various types of MS that allows researchers to develop ways for estimating the toxicity and contamination trends and their fate in an ecosystem. The figures obtained from the collected data indicates that generally lanthanides concentration is very low in surface waters ranging from less than 1 ng/L to 200 ng/L (Bau et al. 2006; Goldstein and Jacobsen, 1988; Han and Liu, 2007; Ingri et al. 2000; Neal, 2007; Spears et al. 2013; Weltje et al. 2002). However, Rzymski et al. (2019) reported that aggregate concentrations of Ln in river waters of Syr Darya reached up to the levels of 15.1-28.3 μg/L and in stream waters of Eastern Canada revealed excessive concentrations ranging from <5 - 11,540 ng/L of total lanthanides, with average of 253 ng ln/L from collected 498 samples (Leybourne and Johannesson, 2008). At highly contaminated sites, REE content may rise to 78 μg/L (Neal et al. 2005). Therefore, comparison of lanthanide concentrations determined in shallow waters with observed toxicity extent, gives solid data which concludes that despite having highly polluted waters, total lanthanides content is lower than the reported toxic values for water bodies (e.g., algae), but only exceptional cases (e.g., in the treated aquatic organisms) in which rare earth elements may serve as a significant pollutant that may disturbs the normal function of ecological cycle.

1.5 Concentration and distribution of REE in algae

For the past 50 years, algae have been extensively used both in pharmaceutical industries and in food preparations across the world, however knowledge on biological roles and their properties are not well understood, therefore applied research on algae in various fields need consequent development (Mabeau and Fleurence, 1993; Astorga-España et al. 2015). Algae are vital organisms for environmental purposes as they serve as producers in aquatic food chain and thus used as biological indicators in an ecosystem (Amer et al. 1999). Algal cells are capable of accumulating essential nutrient ions several degrees higher than other organisms found in surroundings (Serfor-Armah

et al. 2006), besides that metal disposition and distribution plays vital role in metabolism as well (Truus et al. 2001). Mostly present studies are based on the elemental content of toxic elements concentrated in algae originated through anthropogenic activities and their roles at levels of organizations (Castilla, 1996; Astorga-España et al. 2005). In past years, studies have been done that are usually based on toxicity of heavy metals like (As, Cd, Hg and Pb) on marine microorganisms (Astorga-España et al. 2005; Gaudry et al. 2007). However, few studies on biodistribution and compositional analysis of REE in ocean ecosystem are available and remain unresolved (Liang et al. 2014). As they are considerably neglected mainly because of non-toxic in particularly at low levels, lower abundance and no prerequisite roles of these elements (Goecke et al. 2015b). Various analytical techniques for example, ICP-MS are now widely being used for quantification of lanthanides using because of its high sensitivity, range of detection limits (less than ng/g) and its capability for multielement analysis simultaneously (Bulska et al. 2012). Three lanthanides, e.g. Pr, Nd, and Sm were firstly reported in France among Rhodophyceae *Lithotamnium calcareum* (Servigne and Tchakirian, 1939). Algae possess a diverse range of rare earth elements that diversity is independent of size (micro or macroalgae), structural basis, type of algal families or divisions e.g., Chlorophyta, or their biogeographic backgrounds (Hou and Yan, 1998; Fu et al. 2000; Kano et al. 2001a; Mashitah et al. 2012). According to Yan et al. (1998), total available REE in algal biomass reached up to 1.3 mg/kg and this concentration is far less when compared with other elements that are in macroelements range and serve as essential nutrients, for example Ca, K and Na ions (Hou and Yan, 1998; Amer et al. 1999). Most of the studies on lanthanides quantification and its composition on macroalgae have been performed on Asian species, among 37 published studies, two of them were studied in South American species until now, with total REE content determined were in the range from 0 to 226 mg/kg of dry weight. The potential of metal biosorption was functionally evaluated by pH, exposure time, metal content in the beginning and series of sorption and desorption cycles. The potential of cyanobacteria *Arthrospira* (commonly known as *Spirulina*) for biosorption of cerium ions has been explored already (Sadovsky et al. 2016). Algae either living or non-living were shown to concentrate rare earths

effectively because of their capability to develop metabolites that are chelated to allow efficient uptake, such as, with carbohydrates, nucleotides, and proteins etc. (Wang et al. 2014). In algal cells, REE can bind to pigments and sugars such as, polysaccharides, alginate, and fucoidan, etc. that are present in different concentrations (Okajima et al. 2010; Diniz and Volesky, 2005; Diniz and Volesky, 2005b; Gok and Aytas, 2009). Studies on the mechanisms for the uptake of REE in green microalgae *Desmodesmus quadricauda* are rare and some studies reveal the selective accumulation in chloroplasts or in cytoplasm (Řezanka et al. 2016). Lanthanide's entrance and its distribution within the cells of algae are still questionable. The cell wall is the first barrier that restrict the transfer of REE, however, its complex composition of algae and cyanobacteria makes the uptake mechanisms more complicated, thus its accumulation at certain specific sites or attached to cell walls or loosely bound to cell cytoplasm remain unclear. Therefore, research on hyperaccumulator species need to be done to answer these questions.

1.6 Role of REE and its potential effects on algal physiology

In past few years, lanthanides have been used excessively in China, because of its growth promoting effects in the field of agriculture, (Qinhai and Zhaojie, 1996). This causes bulk quantities of dissolved REE to be released into aquatic ecosystem. Though no acute lethal or deleterious effects have been reported, therefore, more attention has been put on their potential effects on aquatic environment (Qiang et al. 1994). Currently, research is mainly concentrated on maximizing the yield of important metabolites from algal cultures that are capable to produce various compounds in response to stress such as high temperature, nutrient deprivation, and metal stress (Miazek et al. 2015). In natural water bodies, organic ligands like organic acid and humic acids are vital in modifying the uptake of lanthanides by complexing them in an aquatic ecosystem (Sun et al. 1997). In marine algae it is well known that amount of rare earths is 102–106 times greater than in algae growing in seawater. The concentration of REE in algal material describe that the actions of lanthanides in a biological system are similar to most of the essential nutrient elements such as, Ca, Mg, and Zn because of similarity in their ionic radius and coordination number (Valcheva-Traykova et al. 2014). Lanthanides can interact with biological molecules like enzymes and proteins resulted in modifying their activities and polymerization of

macromolecules (Yoshimura, 1998). REE can chelate with the ion channels and thus, change the membranes organization and permeability that leads to the deficiency or surplus of ions in inner or outer sides of the membranes (Wang et al. 2003). Like described earlier, lanthanides are capable to interact with the receptors, uptake, bioaccumulate, and can block ion-channels. Lanthanide ions (Ln^{3+}) are extremely being studied to understand the mechanism of Ca^{2+} inflow in cells together with other subsequent reactions (Bauer et al. 1998). REE played an essential role in microalgal phototaxis, movement reaction and avoidance response (Yoshimura, 1998). Hill et al. (2000) blocked the movement reaction of flagella in freshwater microalgae *Spermatozopsis similis* treated with <50 μM gadolinium ions (Gd^{3+}). Pitta et al. (1997) observed that calcium is necessary for motility in marine non-flagellated cyanobacterium *Synechococcus sp*. but in the presence of Tb^{3+} that can act as a calcium blocker, movement can be prevented thus concluded that calcium is necessary for motility. In order to better understand algal physiological responses in association with cerium chloride ($CeCl_3$) that has wide range of applications to study biology of algal cells, as it can enter the membranes of living cells and thus produces cerium perhydroxide (an electron-dense deposits) that is insoluble when reacts in the presence of hydrogen peroxide, that could be featured by standard transmission electron microscope (TEM). In response to any stress either biotic or abiotic highly dangerous species known as Reactive Oxygen Species (ROS) are overly generated as a derivative of wide range of metabolic reactions occurring in cells, for example as studied earlier in single-celled Chlorophyceae (green alga) *Micrasterias denticulata* (Darehshouri & Lütz-Meindl, 2010) or among Rhodophyta *Gracilaria spp* that produces (ROS) for particular defense responses (Weinberger et al. 2005). Studies based on investigating role of REE in microalgae are particularly focused on its growth. For plants as suggested earlier, it is still unclear that beneficial effects of REE is because of alleviating nutrient deficiency (Qu et al. 2012; Goecke et al. 2015b) or if they are involved in other biological responses e.g. scavenging of free radicals (Ippolito et al. 2010; Valcheva-Traykova et al. 2014) or by compensating the toxic effects of other heavy metals (Volland et al. 2014). Elevated levels of lanthanides resulted in reduction in development, chlorophyll and K and Mg content essential for growth in general.

Lanthanides can serve as Ca channel blockers to understand the uptake mechanisms and transport pathways of other heavy transition metals, however in cyanobacteria calcium content raised with increasing exposure of lanthanum chloride in terms of concentration (Wang et al. 2012). Li et al. (2011), suggested that lanthanum can partially overcome calcium deficiency under low concentrations in green macroalga *Chara corallina*, thus allowing cytoplasmic movement. Lanthanides can have positive stimulatory effects on freshwater green microalga *Desmodesmus quadricauda* by adding low concentrations and also inhibits toxic effects of Ca^{2+} deficiency, but not in case for Mn^{2+} deficiency (Goecke et al. 2015b). Nutrient deficiency specifically effects physiology of any organism that can be determined by inhibition or declining in growth and division at cellular level in case of algae or by inhibiting photosynthesis by changing light dependent photosynthetic parameters, using a fluorimeter known as pulse amplitude modulation abbreviated by (PAM). Till now there is no proof for environmental effect of lanthanides, however their roles in agricultural field and for aquatic ecosystems need to be addressed. Reports on REE toxicity shows that they can intermingle with metabolic pathways of other transition metals that mainly act as nutrient elements (Pang et al. 2001b), therefore, usual biological activities of many important enzymes may vary like enzymes involved in catalyzing ATP synthesis typically termed as ATPases (Squier et al. 1990) or blocking of ion-channels. In algae, only a few species of Chlorophyta, Charophyta, Dinophyta and Haptophyta have been studied extensively to illustrate the potential biological toxicities of REE. However, almost all these studies on different microalgae lacked information about the bioavailability of lanthanides, concentrations, and stimulatory/inhibitory effects, however it was suggested toxic effects of lanthanides on algae involved either nutrient depletion or excess than adequate quantities may lead to toxicity and it was proposed that REE can sequester other nutrient elements like phosphates, that could induces growth.

1.7 REE content in soil minerals and factors regulating its adsorption and transport

Lanthanides exist together and mainly in a diverse form of associated minerals, for example in a variety of phosphates, carbonates, fluorides and silicates. In principle, REE usually occur in monazite, pegmatites, granites, and other associated volcanic rocks and form ores that are economically important (Forster, 1998). Fluorite-minerals also contain rare earths (Buhn et al. 2002; Gramaccioli et al. 1999) and silicates derived minerals, e.g., particularly allanites (Braun et al. 1993; Ercit, 2002) are rich in REE typically LREE. Lanthanide content in top-soil differs considerably with sub-soil that are mainly associated with the properties of the origin, such as organic matter content in parent material, biography, history of the region and weathering status of the rocks. Normally, total REE content in non-fertilized soil surfaces is lower when compared with the parent material. The available content of REE, found to be less than total in soil extracts or present in soil solution, some examples include the average concentration of lanthanides in unfertilized soil samples collected from Australian soils ranging from < 0.007 μM to 0.64 μM, with maximum content of lanthanum found was 0.13 μM and 0.51 μM for cerium (Diatloff et al. 1996). The average share in percentage for total REE concentration found in the soil REE extract of Cambisols soil samples (with low clay content) was found proportional to the ionic radius with an average of 0.019% La, 0.018% in Ce to 0.011% for Lu (Tyler & Olsson, 2002). Soil formation from rocks weathering and leaching processes are mainly but partly managed by soil biota (various microorganisms have a higher tendency for biosorption of lanthanides in ionic form) and partly done by decomposition of organic matter. REE phosphates in minerals are mainly surrounded by bacteria and fungi that are efficient producers of oxalates that act as good solubilizers for phosphate-containing minerals bounded with LRRE in slightly acidic soils (Schift & Byrne, 2001), and also for soil solutions. Besides soil biota, siderophores (natural Fe- transport chelator molecules) play a vital role in the fractionation of metals deposited at soil mineral surfaces by making a complex association and their subsequent release in soil solutions or further uptake by cells. In one of the studies on soil bacterium *Arthrobacter* sp. (Brantley et al. 2001), it was found that HREE were considerably released and consequently up taken by cells.

1.8 Rare earths in plants and their physiological effects

As discussed earlier, bioavailability of lanthanides from soil and their uptake by plants is affected by various factors and their concentration inside plants is much less in comparison with a total concentration in soils (Tyler, 2004). Since the ability of plants or crops to concentrate rare earth and its concentration may vary extremely depending on the plant species, growing conditions, and soil environment in context with rare earth element content in the parent soils or rocks (Fu et al. 2001), as an example studies have been done on investigating the rare earth concentrations in corn and rice plant species that presented different results, as higher content were found in rice when compared with corn that indicates the higher accumulation capacity for rice specie than corn for rare earth metals (Li et al. 1998). Studies on vegetal tissues were also observed in both plants that show higher concentrations in roots as compared to other parts such as in grains of both species Y, La, Ce, Pr, and Nd can be detected and measured quantitatively with concentrations ranging from < 0.78 ng/g to 5.58 ng/g of dry biomass, while other elements of lanthanide series were below the detection limits of mass spectrometry. In general, for ordinary plants, REE concentrations are typically in the ng/g range (Wyttenbach et al. 1994). In the above-ground biomass of vascular plants, concentrations of lanthanides are quite low. Studies have been done on plant REE concentration; however, it is really hard to distinguish between the content of rare earth present in various parts of plants, especially in vascular plants. The transfer factor calculated for lanthanides in grass *Agrostis capillaris* grown in soil, from soil A-horizon to plant was generally low and was between 0.04-0.09 (Tyler and Olsson, 2002). In commercially grown vegetables, such as cabbage (*Brassica oleracea* var. capitata), very low values for REE in dry weight were calculated (Bibak et al. 1999). Well-known pteridophyte (ferns) are considered good accumulators for REE. In one of the studies done by Japanese group on 96 different species of ferns to identify good accumulators for REE, they found good concentrations among 9 species of genera *Dryopteris*, *Asplenium*, *Adiantum*, and *Dicranopteris* (Ozaki et al. 2000). They found that in accumulator species, 10– 40 μg/g dry weight of La and 3–30 μg/g of Ce is present in leaf mesophyll tissues in compared to other studied species that contain 0.003–2.7 μg/g of La and 0.076–3.6 μg/g of Ce. Yttrium was also studied, and it was

found that in comparison with non-accumulators, accumulator species contained relatively much more Y than other determined lanthanides (Ozaki et al. 2002). Though, high contents of rare earth elements have been found generally for plants growing on fertilized grounds (Miekeley et al. 1994). Besides this, certain plants are highly capable of accumulating REE from soils and thus being termed hyper-accumulators. In aquatic biota, sediments are the main source of rare earth with almost 99% of REE being bioavailable. Most REE are available either in bound form to organic matter or suspended as sediments among different species in an ecosystem and thus become more available to biotic plants and microbes. Bioaccumulation of rare earth among organisms depends upon their availability to organisms feeding on them. Investigations have been done in the past decade to understand the association of rare-earth species and plant availability. Shan et al. (2003) investigated the accumulation and uptake mechanisms of light rare earth metals, in well-known hyperaccumulator fern *Dicranopteris dichotoma* and conclude that mostly LRRE were concentrated in vacuoles and cell walls of root endodermis using X-ray microanalysis. Generally, REE concentration in plants is lower than in soils (Cao et al. 2000). However, above-ground biomass such as plants can uptake REE through roots to other vegetal tissues and the uptake capacity of lanthanides depends on numerous factors that are already described previously however, it is noticed that transport of these metals in roots is mainly confined by Casparian strips in the endodermis. Thus, the use of organic ligands such as EDTA encourages the uptake of REEs in the soil (Kastori et al. 2010). The degree to which lanthanides can be transported from soil to plants is almost 20% and much of this is concentrated in roots and less in stems and other vegetal tissues including reproductive structures or parts. The uptake of REE affects the metabolism of other minerals, thus, enhancing the absorption of other mineral elements and eventually decreasing others. The impact of REE uptake is mainly focused on in association with calcium ions (Brown et al. 1990). China has been applied REE extensively in phosphate fertilizers for many years for agricultural crop plants such as maize, rice, wheat, and cabbage (Xiangsheng et al. 2006). Besides field studies, lab-based experiments have been done that mainly deals with the potential of REE to compete with proteins that have Ca binding sites on their surfaces, effects on membranes structural organizations

concerning its stability and permeability as they have very similar chemical properties as calcium, so it is mainly referred to lanthanum (Brown et al. 1990). It is assumed that under calcium deficiency, plant growth can be stimulated in the presence of lanthanides as it mainly reduces the effects of calcium deficiency, for example, lanthanum trivalent ions (La^{3+}) enhances the growth and development of tobacco seedlings and stimulates photosynthesis at appropriate amounts (Chen et al. 2001). Additionally, adequate quantities of lanthanides promote sprouting in seeds, vegetal tissues development among plants, improve resistance to biotic and abiotic stresses and nutrient uptake (Liu et al. 2013). REE usually enter into mesophyll cells via apoplastic and/or through cytoplasmic or plasmodesmatal connections and regulate the physiology of plants together with biochemical functions (Ye et al. 2008). REE concentration in any sample (biological or environmental) has been determined mostly by using instrumental neutron activation analysis (INAA) technique that cannot measure all elements in lanthanide series (Bangfa et al. 2008). In past decades, High Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS) is effectively being used to measure all the individual rare earth metals with higher sensitivity in desired samples, thus this highly distinct system will promote future studies on understanding the behavior of REE (Xu et al. 2003). It is suggested that lanthanides are vital for the physiology of plants to develop as they could encourage the absorption of other nutrients essential for growth and stimulate transport and assimilation as well (Pang et al. 2001). Ning and Xiao (1989) found that after using lanthanides in fertilizers, uptake of other nutrients, for example, N, P, and K increased by 16.4%, 12%, and 8.5% for each mentioned nutrient. REEs are commonly applied either in fertilizers or by directly spraying to plants is commonly practiced and considered as a much better method than mixing seedlings in lanthanides solution (Wang, 1995). Reports on physiological effects of REE suggest that lanthanides not only influence the growth of coleoptiles and membrane stability but also prevent loss of water by plants and enhance fixation of nitrogen from soil (Wen et al. 2001). Wen et al. (2001) reported in a field experiment in four provinces of China regarding distribution and bioconcentration of REE in agricultural plants and study the application of lanthanides in fertilizer at different levels to crops and determine the effects of these elements on crop growth and

production in hydroponic culture experiments and field experiments and their results suggests that these elements exert positive and negative effects similar to those of other trace metals at lower or higher level of treatments. Light mediated oxygen evolution, chlorophyll synthesis, photosystems activity, and other mechanisms of action related to photosynthesis including chlorophyllase enzyme synthesis was found to be affected by applications of REE at different concentrations (from low to high treatments) (Hu et al. 2002). However, it was suggested that higher concentrations of REE exert toxicity effects on soil and aquatic biota (Wang *et al*. 2005).

1.9 Biomining and phytoremediation strategies of REE

Rare earth elements are considerably being exploited in the past few decades around the world, causing lanthanides-based soil pollution, while using conventional mining techniques (Zhou et al. 2015). To protect the soil environment from excessive REE, release into an environment, bioremediation is a necessity and that needs establishing new technologies (Zhiqiang and Zhibiao, 2020). Phytoremediation is a low-cost and environment-friendly technique employed for the restoration of soils contaminated from heavy metals (Ji et al. 2015). Two main subdomains of phytoremediation include phytoextraction and phytostabilization. Extraction of heavy metals and their immobilization and uptake from highly contaminated soils through aboveground plants are commonly termed phytoextraction, while phytostabilization fixes metals in the rhizosphere of land vegetation. For bioremediation studies over the last few decades, plants among them bulk includes ferns and are considered as the most promising competitors for lanthanides biosorption and termed as REE hyperaccumulator (Lai et al. 2005). Hyperaccumulator species are mainly used to extract REE from soils through routine cultivation (Lokhande et al. 2013). These plants have exceptional mechanisms through which they can accumulate metals up to a maximum of 10% of their dry weight (Baker et al. 2020). Other strategies for REE bioremediation are often contradictory when plants remove these elements via leaching or by decaying of organic matter and leaf litters through microbes that cause plant clipping to be more complex and uncertain (Wu et al. 2014). This technique confirms the highest extraction of lanthanides that is more economically feasible (Ozkan et al. 2016). Besides this, many factors such as phenotypic characteristics of plant and organic matter content in the soil, have shown

major effects on bioremediation, therefore it is really hard to find out the prime clipping method. Phytoremediation (using green plants for extracting heavy metals from polluted soils) is among the most successful technology for the bioremediation of heavy metals. Plants have developed the mechanisms to withstand a higher concentration of metals and are typically termed as Metallophytes, thus considered as the best possible choice in phytotechnology (Whiting et al. 2001; Boularbah et al. 2006). Plants based on their extraction strategies for accumulating metals, called metallophytes are classified in three different groups, (a) normal accumulators that indicated the active metal transport and its transport mostly to vegetal tissues, (b) indicators, regulate the translocation of metals in a way to maintain internal concentrations that reflect surface metal and (c) excluders that prevent the absorption of metals from roots and its translocation to aerial parts (Baker, 1981; Barrutia et al. 2011). Few metallophytes are considered to have high accumulation capacities for phytoremediation, as for REE *Dicranopteris dichotoma* (broadly distributed ferns and well-known hyperaccumulator) found to be dominant among plant species at rare earth mines in (sub)tropical regions of southern China, that belongs to the perennial plant community of *Gleicheniaceae* family (Chen et al. 2016). According to (Wang et al. 2003), this plant species can accumulate up to 0.1% of lanthanides in dry biomass and has shown REE content in leaf mass ranging from 238.93 to 2364.51 mg/kg, whereas others vary from 6.81 to 92.17 mg/kg of total REE. Thus, *D. dichotoma* has been observed as the best suitable species for the phytoremediation of lanthanides polluted ecosystems because of its rapid growth, strong accumulation, translocation capacity, and excellent tolerance towards stress conditions (Chen et al. 2016). However, no knowledge on clipping techniques to aid phytotechnology using *D.dichotoma* at REE mining area for phytoremediation is available (Zhiqiang and Zhibiao, 2020).

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CHAPTER 2

Aims & Hypothesis

CHAPTER 2: AIMS & HYPOTHESIS

2.1 Experimental part of the thesis

In the presented Ph.D. thesis, experimental procedures and advanced technologies were applied to achieve desired aims. There are many studies showings the role of lanthanum (lanthanides) involvement in the physiology of plants with less focus on phytoremediation from polluted ecosystems under lab and field conditions, but to our best knowledge, almost all lab studies were done under artificial conditions providing metal treatments out of the normal ranges that exist naturally in the environment. Therefore, in the first part of the study, we combine physiological effects of lanthanum (the first element in lanthanide series) with natural conditions of aquatic ecosystem and involvement of uptake and bioaccumulation in microalgae using μXRF together with metalloproteomics and most results from this part belongs to original articles (Chapter 4). Chapter 3 shows the binding of lanthanum to proteins even when treated at a very low nanomolar range, this shows the ultra-sensitivity of ICP-MS in combination with HPLC for analysis of La-binding proteins in the green alga *Desmodesmus quadricauda*. In the second part of the study, we analyze soil samples with their total lanthanides content using WDXRF in comparison with extractable REEs using the BCR method and its further analysis using ICP-MS among Brazilian mining sites in combination with ICP-MS analysis of REE in plant species for phytoremediation grown under natural environment for determination of any suitable potential lanthanides accumulating plants and results from second part belongs to the original article (Chapter 5).

In the following section of the thesis, all desired goals were targeted to be achieved, together with the hypothesis associated with them are mentioned below.

2.2 Aims of the thesis

The main aim of the thesis was to evaluate REE phytoremediation using microalgae and plants that can predict the toxicity response of these elements to microalgae or in plants under environmental conditions.

The following aims were combined to achieve these principal goals.

1) To understand lanthanum (La) binding to proteins in extracts from microalgae as an example, describe a new system and methods for ultra-trace analysis using HPLC-ICPMS.
2) To comparatively analyze physiological effects (growth and photosynthesis) of green microalgae in response to La under environmentally relevant conditions.
3) To analyze the subcellular localization of La and detailed analysis of metalloproteomics (metal-binding proteins).
4) *In vivo* monitoring of soil total LREE by carrying out field screening in Poços de Caldas-MG, Brazil, together with acidic extractable contents for bioavailability, and searching for new potential hyperaccumulator species at LREE mining regions using analytical procedures

2.3 Hypothesis of the study goals

In scientific language, the above-mentioned goals can be questioned in a specific and verifiable combination of the working hypothesis and null hypothesis. Because of the high complexity of the goals and scope in our present thesis. Several different combinations of hypotheses ought to be formulated. Below we are describing several of these combinations based on a working hypothesis that represents a statement that is truly based on current knowledge on the topic or obtained information that supports our hypothesis in question. Besides, the working hypothesis, the null hypothesis indicates that there are no solid grounds for believing the working hypothesis and nullifying the hypothesis, as the name indicates. These hypotheses can be explained in our current thesis as followed:

Working hypothesis 1: Lanthanum in *D. quadricauda* has beneficial effects on algal growth and no acute effects have been reported.

Null hypothesis 1: Lanthanum in *D. quadricauda* has no beneficial effects on algal growth and acute effects have been reported.

Working hypothesis 2: Lanthanum can become toxic in *D. quadricauda* at environmentally relevant concentrations.

Null hypothesis 2: Lanthanum in *D. quadricauda* does not become toxic at environmentally relevant concentrations.

Working hypothesis 3: Lanthanum binds to proteins in *D. quadricauda* at environmentally relevant concentrations.

Null hypothesis 3: Lanthanum does not bind to any protein in *D. quadricauda* crude protein extracts.

Working hypothesis 4: High total REE content in mining area might have detrimental effects on naturally grown plants.

Null hypothesis 4: High total REE content in mining area showed no detrimental effects on naturally grown plant.

To achieve the previously listed goals, in the next chapters (3, 4, and 5) we examine the current knowledge on lanthanides and their physiological effects on growth and cellular photosynthesis in green microalgae in greater detail. Further, we analyzed REE binding proteins using a highly sensitive and dynamic system for ultra-trace metal detection and their binding targets together with its detailed analysis and localization of (La) at a cellular level. We also describe REE bioavailability to plants to counter the harmful impacts of lanthanides through phytoremediation or by the development of plants that have higher accumulating capacities. Furthermore, chapter 6 is the focal point of the summary of basic results and discussion with major conclusions and future possibilities to further develop the missing knowledge. There we take upon the task of investigating whether the working or the null hypothesis is in accordance with the experimental evidence.

CHAPTER 3

Ultratrace metal speciation analysis by coupling of sector-field ICP-MS to high-resolution size exclusion and reversed-phase liquid chromatography

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Ultratrace Metal Speciation Analysis by Coupling of Sector-Field ICP-MS to High-Resolution Size Exclusion and Reversed-Phase Liquid Chromatography

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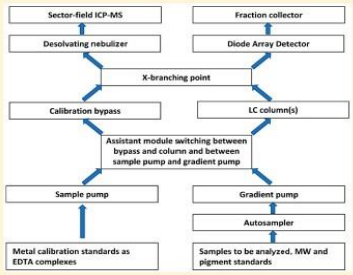
Supporting Information

ABSTRACT: Techniques for metal speciation analysis with subnanomolar (ppt) detection limits in complex matrices, with simultaneous quantification of matrix elements, have become a necessity for investigating targets of trace metal binding to macromolecules and pigments at environmentally relevant concentrations. In this work we optimized the analysis of such metal binding in a custom-built HPLC-ICP-MS system. Key elements of the optimization were the choice of components for the metal-free HPLC-DAD system and sector-field ICP-MS detection (ICF-sfMS) with desolvating injection and optimization of sample handling. Protein analysis was done using ammonium bicarbonate buffer and size exclusion chromatography (SEC-ICP-sfMS), with possible addition of anion exchange chromatography. Detection of metal exchange in pigments (chlorophylls and bacteriochlorophylls) was based on reversed-phase chromatography with a methanol-acetone gradient and coupling to the ICP-sfMS via a dedicated organic matrix interface (RPC-ICP-sfMS). The resulting HPLC-DAD-ICP-sfMS system has detection limits in the picomolar range in protein buffer, limited by the maximal achievable purity of buffers/solvents and not by system sensitivity. Tests for method optimization showed that sonication, meant to increase protein solubilization, leads to artifacts of metal loss from metalloproteins. Examples for Cd binding to soybean proteins and chlorophyll, Cr binding to *Arabidopsis thaliana* proteins, La binding to *Desmodesmus quadricauda* proteins, and Cu binding to *Rhodospirillum rubrum* proteins and pigments are shown. These application examples demonstrate that the system is sensitive enough to detect binding of metals to proteins and pigments at background concentration levels of typical nutrient solutions made from analytical grade chemicals, equivalent to ultratrace metal concentrations in nonpolluted environments.



Many metals are required for proper function of all organisms, because of their essential binding to proteins and metabolites. At deficient or toxic concentrations of essential elements and at toxic concentrations of nonessential elements, however, adverse effects on metabolism occur that can ultimately lead to death of an organism. Therefore, the analysis of the binding of metals to metabolites and proteins, as the core of the field called metallomics, has become a top priority of research.[1]

In order to analyze targets of trace metal binding at environmentally relevant, usually very low concentrations of trace metals,[2,3] analytical techniques with optimized sensitivity become increasingly important. Such environmental concentrations are usually in the nanomolar or even subnanomolar range, depending on the metal and habitat investigated.[2,3] The typical application for HPLC analysis of protein samples coupled to ICP-MS, besides diode array detection, is to analyze the problems in metal loading of proteins during metal deficiency or toxicity stress or to identify new metalloproteins. Similarly, HPLC-ICP-MS analyses of metal binding to low molecular weight substances are usually meant to investigate the involvement of such compounds in transport, sequestration, storage, or detoxification of metals. The often very low metal concentrations challenge the detection limits of conventional (quadrupole) ICP-MS. In addition, such samples have a matrix of buffer in high millimolar concentrations, causing further problems in analysis. First, such a concentrated matrix decreases the sensitivity of the ICP-MS. Second, the matrix leads to various interferences in quadrupole ICP-MS, which can only partially be eliminated by collision reaction cells (CRC) via use of reactive gases, because the introduction of the sample with even more matrix than the buffer would

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require adjustment of the collision chamber conditions, which is practically not possible. This applies also to the work with organic solvents in an HPLC-ICP-MS connection, which has rarely been done because such matrices lead to soot formation that blocks the sample introduction system unless a carefully tuned oxygen addition is used to burn that soot during sample introduction.[4]

Several enhancements of ICP-MS sensitivity and mass resolution have been made to overcome the problems of interferences, sensitivity, and dynamic range. The most significant single enhancement may be the development of a double focusing optics, leading to so-called sector-field ICP-MS. This has been shown to greatly enhance sensitivity, compared to conventional quadropole ICP-MS, while eliminating most matrix interferences.[5,6] Triple quadrupole ICP-MS improves the removal of matrix interferences compared to conventional single-quadrupole instruments as well, but a direct comparison to sector-field ICP-MS for biological samples was not yet published. Further sensitivity was gained in all ICP-MS types by replacing the standard spray chamber with a desolvating nebulizer (Apex Q, Elemental Scientific, Omaha, NE, USA[7]). A third enhancement, meant to be combined with a desolvating nebulizer in sector-field ICP-MS, is the so-called jet interface. This is essentially a strong dry interface vacuum pump behind the sample introduction system, which sucks more ions into the machine. The latter was not used for metallomics purposes so far, which might be because such applications include not only analytes at ultratrace levels but also elements at much higher concentrations, which would saturate the detector. Detector saturation can be overcome by using a Faraday cup as an additional detector,[8] but so far that was not combined with ultratrace metal analysis.

In the current HPLC-ICP-MS system, we combined all the different, previously described approaches for enhancement of sensitivity and dynamic range into one setup in order to be able to measure ultratraces and matrix elements simultaneously. Furthermore, to make the best use of this high level of sensitivity without artifacts, a completely metal-free HPLC was coupled to the ICP-sfMS, the choice of chromatography columns was optimized, and all available methods for reduction of background from chemicals and vessels were combined. This system fulfilled the aim of simultaneous measurement of ultratrace and the most abundant analytes in aqueous protein samples as well as pigments in organic solvents, of which a range of different examples is shown.

EXPERIMENTAL SECTION

General Precautions for Ultratrace Work. To achieve the lowest possible background levels of metals, all water used for protein work and cultivation of organisms under metal-limited conditions as well as standards was first purified by reverse osmosis and filtering (Demiwa 20 rosa, Watek, Ledeč N.S., Czech Republic), afterward double distilled (Destamat bi18e, QCS, Germany), and subsequently referred to as roddH_2O. All glass- and plasticware were acid-washed in 5% HNO_3 and afterwards with roddH_2O before use. PFA plasticware was used wherever possible, including the autosampler vials, to minimize surface adhesion of metals. Chemicals for metal-limitation treatments (inducing deficiency of essential trace elements) were of ultra- or suprapure grade whenever available.[9−11] Buffers for aqueous HPLC-ICP-MS were additionally batch-treated with Chelex-100 (Biorad, USA) that was regenerated with roddH_2O and suprapure NaOH.

Configuration of the HPLC-ICP-MS System. *HPLC.* A customized version of the Azura 6.1 L system (Knauer GmbH, Germany) was used, in which the complete flow path was free of metallic surfaces in order to minimize metal contaminations of the mobile phase. This was achieved by selecting ceramic pump heads, valves and tubes made of ceramics and/or PEEK, and a flow cell in the diode array detector made of PEEK +quartz. The flow path was as follows. For regular analysis, mobile phase was supplied with an Azura 6.1L binary gradient pump with solvent-switching valves. Samples were injected into the flow path via a custom metal-free version of the AS 6.1L autosampler. Afterward, the eluent was directed to the column, after which the eluent was split by a PEEK 4-way branch ("X-branch") between the DAD, from which the liquid flowed to the fraction collector, and the ICP-MS. The splitting ratio was adjusted by flow dynamics, i.e., the ratio of diameters and lengths of the tubes leading to the ICP-MS vs DAD/fraction collector. For calibration of the ICP-MS, an assistant module (ASM2.1) was used that allowed for switching from the column to a bypass (re-entering the regular flow pass at the 4-way splitter) and at the same time from the regular system pump to a sample pump (isocratic P6.1L pump). In this way, contamination of the column and system pump with the high concentrations needed for the upper limit of calibrations could be avoided.

ICP-sfMS. A customized version of the Element XR (Thermo Fisher Scientific, Waltham, MA USA) inductively coupled plasma sector-field mass spectrometer (ICP-sfMS) was used, in which a "jet interface" provided increased sensitivity, for aqueous samples together with a desolvating nebulizer (Apex Q). The Element XR as such already provides about a 1000× higher dynamic range compared to regular instruments by having a third detection mode via a Faraday cup, which is important for measuring the matrix elements together with the ultratraces at the maximized sensitivity level applied here. For minimizing background, also here the flow path was kept metal free, and a PFA nebulizer (Microflow PFA-20, Thermo Fisher Scientific, Waltham, MA USA) with a 125 $\mu L \cdot min^{-1}$ flow rate was used to minimize surface adhesion that would lead to peak tailing.

ICP-sfMS Tuning Conditions. *Tune in Aqueous Buffer.* A transfer capillary of 0.250 mm (i.d.) was used for aqueous buffers from the HPLC system to a desolvating nebulizer and ICP-MS. The sample gas was usually higher than 1 L/min. The nitrogen flow rate in the desolvating unit (ApexQ) was manually controlled; a sensitivity of >20 times higher was always achieved than in regular spray chamber mode. Ni sample and H-skimmer cones were used, which needed to be cleaned due to clogging with salts after approximately 2 days.

Tune in Organic Matrix. A transfer capillary of 0.18 mm (i.d.) was used for acetone and methanol as a mobile phase. Tuning was performed without a desolvating unit due to the risk of ignition of organic solvents. Thus, a Peltier cooled spray chamber was used at 2 °C instead of the desolvating unit. Pt skimmer and samples cones were applied, which are suitable for the higher heat load of burning the organic matrix. The plasma stability was highly influenced by sample gas and oxygen flow rates based on the different proportions of carbon to methanol and acetone. For methanol, a low flow rate of sample and oxygen gas (0−0.1 L/min oxygen and 0.6−0.8 L/min sample gas) was sufficient for plasma stability, which was

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also sufficient to remove the soot on cones. For acetone, a higher sample and oxygen gas flow rate was required for the plasma stability (0.25−0.3 L/min oxygen and 1.4 L/min sample gas). For gradient operation, the conditions for acetone were applied. The instrument could run easily for 4−5 days without any break for cleaning of cones.

Typical ICP-sfMS Operating Conditions. The instrument was optimally tuned to reduce the potential interferences by choosing medium resolution with an acceptably low oxide formation rate as monitored by CeO^+/Ce^+. The typical operating conditions of the ICP-sfMS Element XR-2 were as follows: RF power, 1250 W; oxide ratio CeO^+/Ce^+, 1.0−1.2%; doubly charged Ce^{2+}/Ce^+, 1.0−1.2%; auxiliary gas, 80 L/min; sample gas flow, 1.20 L/min (variable); cool gas, 16 L/min; extraction lenses, −2000 V; medium resolution, 4000 m/z. Examples of methods optimized for specific conditions are provided in Table S1.

Growth of Organisms. Soybean (*Glycine max*, variety Irin) plants were used for Cd exposure. Seeds were germinated in a mixture of roddH_2O-prewashed perlite:vermiculite (v:v, 1:3) before their transfer into 1/2 strength hyperaccumulator hydroponic nutrient solution (HHNS[12]) modified for soybean cultivation. The greenhouse experiment was performed with light supplementation and controlled temperature: 10 h night and 14 h day sinusoidal light cycle with maximal irradiation of 550 μmol photons·m^{-2}·s^{-1} achieved by regulated supplementation of daylight with white LEDs (Photon Systems Instruments, Brno, Czech Republic). Temperature was 16 °C minimum at night and 24 °C maximum during the day. Each pot contained 7 plants and 6.2 L of 1/2 strength HHNS nutrient solution without (control) or with addition of 20 nM Cd. The solution was persistently aerated with filtrated room air. It was exchanged constantly by peristaltic pumps at 2 L·d^{-1}, ensuring that the metal uptake into plants will not deplete the nutrient solution. The experiment ran for 5 weeks. At harvest, the leaves were frozen in liquid nitrogen and stored at −80 °C until analysis.

Arabidopsis thaliana (*A. thaliana*) plants (ecotype Columbia) were used for the test with chromium detection. They were grown on MS media (Sigma-Aldrich) instead of HHNS and grown with 8 h light:16 h dark cycle with max. 150 μmol·m^{-2}·s^{-1} but harvested after 5 weeks like the soybeans. Cr was present in the media only as the basal contamination of the chemicals, approximately 3 nM.

Rhodospirillum rubrum (*R. rubrum*) was batch-cultured in 4 L bottles with a defined medium,[13] gassed with 1% CO_2:99% N_2 (traces of oxygen entered through the walls of the tubing) at 30 °C. The bottles were illuminated with Osram Dulux L 55W/840 fluorescent tubes with a 12:12 h light:dark rhythm. During the light phase, a sinusoidal light curve with a maximum light intensity of 530 μmol·m^{-2}·s^{-1} (PAR) at noon was applied. The bacteria were inoculated at an initial $OD_{650\ nm}$ of 0.2 from a mother culture at the beginning of its exponential phase of growth ($OD_{650\ nm}$ of 4.0−5.0) and treated with 0.2 μM (control) or 2 μM Cu^{2+} (added as $CuSO_4$) in the medium. The cells were collected after 4 d (exponential phase in the control) by centrifuging at 6800 × g for 5 min. Harvested cells were kept at −80 °C. Further details are described in the full study of Cu-stress in this organism by Jaime-Pérez et al.[14]

Protein Isolation. *Protein Extracts for Metalloproteomics.* Soybean and *A. thaliana* proteins were extracted from 1 g of frozen leaves or roots as described in detail by Andresen et al.[9] In summary, they were ground under liquid nitrogen together with 1 mL of frozen isolation buffer (IB). After thawing and transferring the sample to an ultracentrifuge tube with the help of a second mL of IB, the plant-buffer-mixture was centrifuged at 4 °C for 90 min at 100,000 × g. The supernatant containing the soluble protein fraction was centrifuged immediately at 4 °C for 10 min at 16,000 × g in a microcentrifuge to remove resuspended parts of the pellet. The pellet from the ultracentrifugation was rinsed two times by resuspending it in solubilization buffer (SB) and ultracentrifuged again. The supernatant was discarded, and the pellet was resuspended in SB and stirred overnight at 4 °C. The supernatant after the final ultracentrifugation and an additional centrifugation with the microcentrifuge was used for the membrane proteins investigation on HPLC-ICP-MS. For *D. quadricauda*, the same protocol was followed with the exception that only approximately 100 mg of cells was extracted with 200 μL of buffer.

Sonicated Batch of Samples. The soybean samples planned to be sonicated were handled as the samples described in the previous section. One gram of frozen soybean shoot or root material was ground into a precooled mortar under liquid nitrogen conditions. IB was added to the fine powdered soybean material. The samples were sonicated, while being kept on ice, with a BioLogics Inc. ultrasonic homogenizer (Model 3000 MP) for 4 min (5 times of 20 s sonication with 40 s of pause in between pulses, at 50% amplitude = 150 W output, 20 kHz frequency). After sonication, the soybean extracts were processed with all ultracentrifugation and buffer exchange steps as described above.

Preparation of LH1 from R. rubrum. Light-harvesting antenna 1 (LH1) complexes were isolated from the harvested cells kept at −80 °C. The isolation of complexes was performed using the method described by Picorel et al.[15] with some modifications.[14]

Protein Measuring Conditions. Generally, for optimizing the molecular weight (MW) range and resolution in SEC, combinations of 2−3 Superdex Increase or Superose Increase 10 × 300 mm columns (all GE Healthcare, USA) were used in series. Which versions of Superdex/Superose Increase were combined depended on the MW range to be analyzed (see Results and Discussion). Proteins were eluted with 150 mM ammonium bicarbonate and 0.2 mM DDM, called metalloproteomics buffer (MP buffer). For anion exchange chromatography, a 10 × 300 mm column housing of one of the above SEC columns was filled with Source 15Q (GE Healthcare, USA). For protein elution from this column, a gradient of 0−1 M NH_4Cl was added to the buffer described for SEC.

The analysis of the membrane and the soluble proteins was done with a flow of 0.5 mL·min^{-1} using a splitting ratio of 0.12 mL·min^{-1} for the PDA and 0.38 mL·min^{-1} for the ICP-MS. For every run of membrane and soluble proteins, 100 μL of sample was injected. One sample run lasted for 270 min.

Pigment Isolation. *Tissues and Cells, Standards.* Pigment extracts from tissues/cells of plants, microalgae, and bacteria, as well as the [Mg]-Chl, [Cd]-Chl, and [Cu]-Chl standard, were prepared as described by Küpper et al.[16] Actually, the standards were those prepared for the previous publication[16] and stored at −80 °C ever since.

Proteins. Before extraction, the LH1 and RC protein preparations were dried in a vacuum concentrator (Savant SpeedVac SPD 111 V, Thermo Fisher Scientific, USA) in

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phosphate buffer (i.e., slightly alkaline and minimizing Cu solubility). Then, extraction was done with 100% acetone in which Cu has minimal solubility (in contrast to alcohols). Thus, best care was taken to prevent artifactual Mg-substitution by Cu during extraction. This method did not extract the most hydrophilic pigments efficiently. Therefore, afterwards a secondary extraction with 100% methanol was done, in which the hydrophilic pigments dominated, but compared to the acetone extracts no new pigments were found.

Pigment Analyses. All pigment extracts were run on a 4.6 × 250 mm C18 HPLC column (Nucleosil, Macherey-Nagel, Germany) with a 60 min gradient from 100% methanol to 100% acetone at a constant flow rate of 0.5 mL·min^{-1}. Of this flow, about 75% were sent to the fraction collector or waste, and the remaining 25% were sent to the ICP-MS. We had problems with elevated copper background from the steel housing of the HPLC column at the beginning of the gradient in methanol, drastically decreasing toward the 100% acetone, but the control samples still allowed us to see that the significant Cu-complexes did not originate from that background; this was verified with a recent test using a metal-free HPLC column (PRP-C18 5 μm 2.1 × 250 mm PEEK, Hamilton Bonaduz AG, Switzerland).

Metals in pigments were quantified using metallochlorophylls from the work of Küpper et al.[16] as standards. This was the easiest and most reliable method because the molar extinction coefficients of these pigments are known, and each molecule binds exactly one metal ion in its center. The molar extinction coefficients summarized by Küpper et al.[16] were used.

Data Analysis and Statistics. Data of the HPLC (DAD) were primarily processed by the software controlling the device, ClarityChrom (Data Apex, Czech Republic), from where 3D matrices of absorbance vs wavelength and time were exported in ASCII format. Data of the ICP-MS were primarily processed by the device controlling software (Element 3.1.2.242, Thermo Fisher Scientific, Waltham, MA USA) as well. Chromatograms consisting of count rate for each measure element vs time were exported into Excel worksheets. Both the DAD and the ICP-MS data were imported into Origin Pro 2015 (Originlab, USA) for visualization and further analysis including quantitative calibration.

■ RESULTS AND DISCUSSION

The Proof-of-Principle Measurements. *Sensitivity and Dynamic Range.* Sector-field ICP-MS overcomes both typical HPLC-ICPMS problems mentioned at the beginning of this article: interferences and limiting sensitivity. It is known to have, in low resolution mode, inherently up to 3 orders of magnitude higher sensitivity than quadrupole ICP-MS. At higher mass resolution, some of this sensitivity is lost, but interferences are efficiently removed as well. These features were already exploited for detection of phosphorus in small amounts of protein samples[17] and for detection of metal complexes in rat liver.[18] A direct comparison of sector-field ICP-MS and quadrupole ICP-MS with reaction cell showed that the interference removal with sector-field ICP-MS is more efficient for biological samples.[5,6] However, all of these previous studies suffered from the fact that increasing mass resolution causes loss of sensitivity - already the switch from low (m/z = 300) to medium (m/z = 4000) mass resolution causes an about 10× lowering of signal intensity. In all examples shown in the current study, medium resolution mode

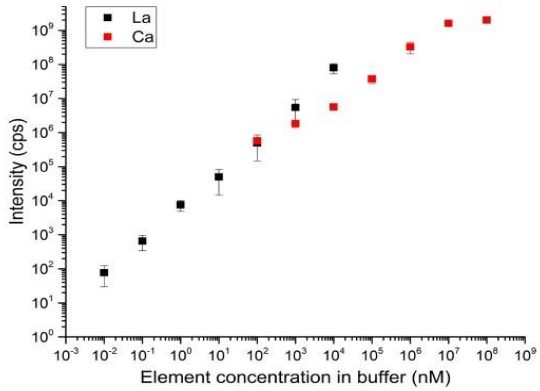

Figure 1. Calibration of Ca and La, showing the sensitivity and dynamic range of the system using the matrix and instrument parameters that were later used for the analysis of La binding in proteins.

was used, which resolved all interferences on all elements shown here. However, the loss of sensitivity compared to low-resolution mode was overcompensated by desolvating sample introduction via a desolvating nebulizer combined with a jet interface. As a result, even in medium resolution mode and at the low flow rate of the HPLC coupling (approximately 150 μL·min^{-1} entering the ICP-MS, the remaining 350 μL·min^{-1} going to the fraction collector), 1 nM La dissolved in 150 mM ammonium bicarbonate buffer (kept in solution by 1.2 nM DOTA) yielded about 7,500 cps for La[139]. The noise, as visible in all the HPLC traces shown in this work, was mostly caused by the injection system and is, therefore, over many orders of magnitude proportional to the signal itself; detector noise is low enough to have a theoretical detection limit (measured with closed sample valve) of 0.25 ppq (not shown). For this reason, even the approximately 78 cps in the 0.01 nM = 10 pM sample were still well measurable (Figure 1). Such a high sensitivity would be a problem for most ICP-MS systems because the more abundant elements that need to be measured besides the (ultra)traces, in our case elements like Mg, P, and S, would saturate the detector. To overcome this bottleneck, we chose the option of having the machine equipped with an additional Faraday cup as a detector for high count rate elements. This allowed, at the same high sensitivity as just mentioned, a linear calibration up to 10 mM; only the 100 mM (=10^8 nM) Ca sample was clearly out of the linear range because of matrix effects (Figure 1). This means, in a real-world application setting, a linear dynamic range of 10 orders of magnitude, which is enough for simultaneous measurement of ultratrace and matrix elements.

The optimization of column combinations allowed for the efficient separation of different sets of proteins (Figure 2). For maximal spread over a maximal MW range, for example, a combination of Superose Increase 12, Superose Increase 6, and one of the Superdex Increase columns for the most important MW ranges was used. In contrast, for a focus on a narrow MW range, two identical Superdex Increase columns plus one reaching from the middle to above or below the main MW range were optimal. For fully using the high resolution of such column combinations, it is decisive not to distort the peak

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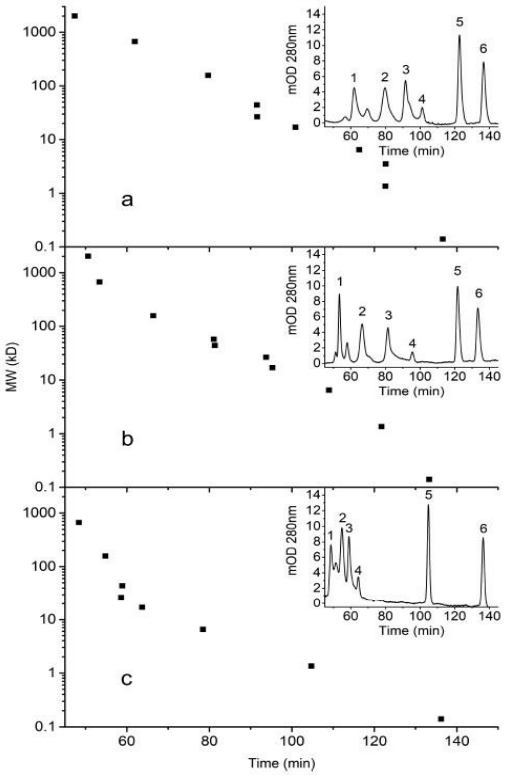

Figure 2. Examples of optimizing protein SEC resolution for different tasks by using different combinations of columns (10 × 300 mm each). For these tests, the Biorad (USA) SEC markers and the Sigma-Aldrich (USA) ultralow range MW marker were used in 1:10 dilution in the MP buffer, each supplemented with 5 $\mu g \cdot mL^{-1}$ 4-aminobenzoic acid (PABA, 0.14 kDa; Sigma-Aldrich, USA). Blue dextran (2 MDa, 0.5 $mg \cdot mL^{-1}$, Sigma-Aldrich, USA) was run separately. Examples of running the Biorad SEC marker (peaks: 1 = thyroglobulin, 670 kDa; 2 = bovine γ-globulin, 150 kDa; 3 = chicken ovalbumin, 44 kDa; 4 = equine myoglobin, 17 kDa; 5 = vitamin B12, 1.35 kDa) supplemented with PABA (peak 6) are shown in the inset graphs of each panel. (a) Widest MW range with increased MW resolution in the center: 1x Superose 6 increase, 1x Superose 12, 1x Superdex200 increase. (b) Focus on medium to high MW resolution: 1x Superdex75 increase, 2x Superdex200 increase. (c) Focus on ultralow MW range but medium MW range covered: 2x Superdex 30 increase, 1x Superose 12.

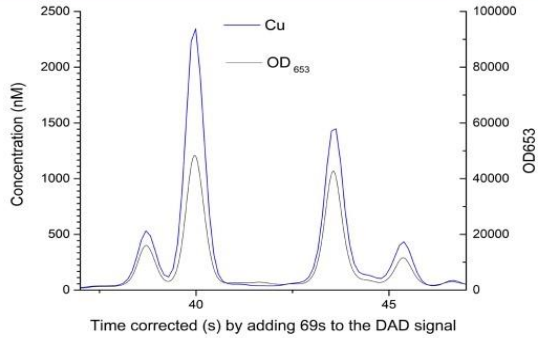

Figure 3. Comparison of peak shapes in the DAD and ICP-MS using a degraded [Cu]-Chl standard as an example. This sample was chosen because besides native [Cu]-Chl it contained various degradation products that allowed for better comparison of peak shapes.

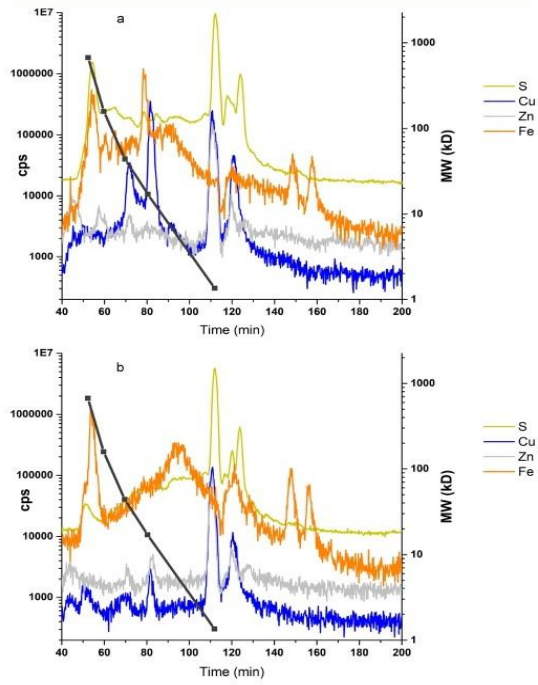

Figure 4. Effect of sonication on soluble metalloproteins from soybean leaves: (a) nonsonicated and (b) sonicated samples. The analysis was performed by a combination of Superdex 200 increase 10/300, Superdex 75 increase 10/300, and Superdex 30 increase 10/300 columns.

shape during transfer from the HPLC to the ICP-MS. Such distortions can result not only from the transfer capillary between the systems but also from the injection system of the ICP-MS. In the current case, they were negligible as shown by the direct comparison of the UV/vis absorption signal measured by the DAD of the HPLC and the Cu signal measured by the ICP-sfMS (Figure 3).

Aqueous Samples: Size Exclusion Chromatography of Proteins. *Technical Aspects.* For metalloprotein work, the use of a buffer is needed to maintain a physiological pH in which the protein conformation and metal binding remain stable. This results in a high concentration matrix, which can have very detrimental effects on ICP-MS in terms of signal quenching and clogging of the cones in the sample introduction system. This was overcome in a recent work by

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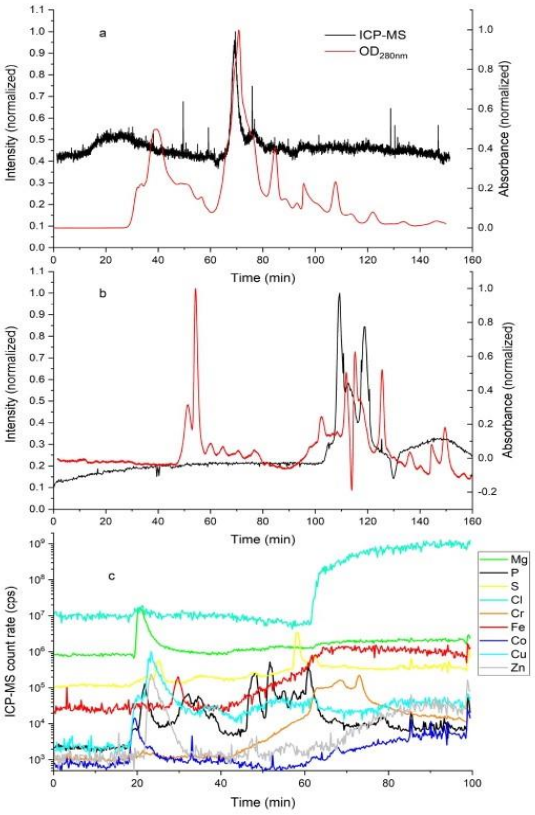

Figure 5. Analysis of chromium speciation in soluble protein fractions isolated from *A. thaliana* plants by a system based on a triple quadrupole ICP-MS and the sector-field ICP-MS presented here. a) Analysis with the old system as described by Andresen et al. (2016), using two Superose 12 10/300 columns in series. b) Analysis with the new system described here, with improved signal/noise ratio and chromatographic resolution. In the new system, the analysis was performed by a combination of Superdex 200 increase 10/300, Superdex 75 increase 10/300, and Superdex 30 increase 10/300 columns. Black line: ICP-MS signal; red line: UV/vis absorbance at 280 nm. c) Use of the system presented here for further separation of Cr binding from other proteins, using a gradient of 0−1.0 M NH_4Cl on a Source15Q (10 mm/300 mm) column.

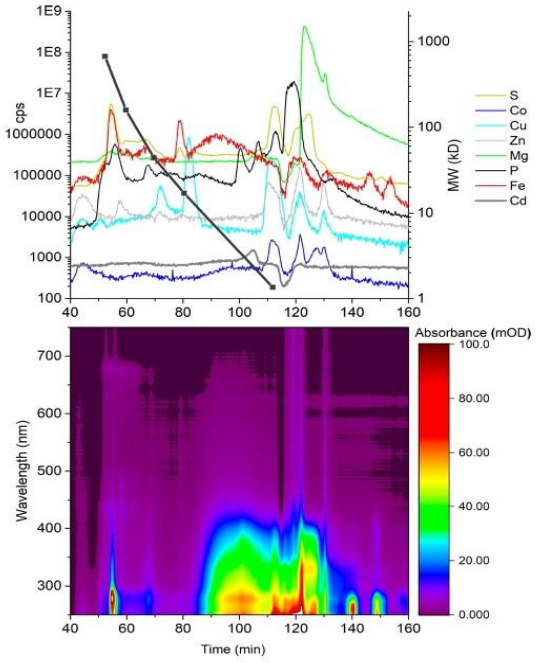

Figure 6. Analysis of protein binding of Cd in natural nontoxic abundance (as trace contamination in ACS-grade chemicals) to membrane proteins of soybean leaves. Top: ICP-sfMS signal; bottom: UV/vis absorbance measured by DAD. The analysis was performed by a combination of Superdex 200 increase 10/300, Superdex 75 increase 10/300, and Superdex 30 increase 10/300 columns.

the use of ammonium bicarbonate as a buffer,[9] which has a pH of about 7.4 without adjustment, and at the same time is optimally volatile in the ICP-MS. While at such a pH metal dissociation from the protein is not a problem if the protein is kept in its native state, it turned out that the latter condition is often violated by sample preparation procedures. In many laboratories, protein isolation and solubilization are aided by sonication of the samples, although it is not tested in how far this could cause problems of metal release. Therefore, this factor was tested now, leading to surprisingly drastic results (Figure 4). In extracts of soluble proteins from soybean leaves prepared without sonication, clear distinct peaks of proteins binding Cu, Fe, or Zn were found (Figure 4a). In the samples prepared from the same material with the same protocol but adding a step of mild sonication as usually used for aiding extraction, the peaks of medium MW Cu- and Fe-binding proteins were >100-fold quenched (Cu-peaks at about 70 and 80 min) or even disappeared (Fe-peaks at about 60 and 78 min) as shown in Figure 4b. As the most abundant soluble Cu-protein in plant leaves is well-known to be plastocyanin (the peak at approximately 10 kDa in Figure 4), which is a monomer, its decrease of intensity cannot be due to dissociation of oligomers. Further, the average metal levels in the extracts remained constant, showing that the extraction efficiency remained the same as the sonicated and non-sonicated samples were prepared from the same, and equal amount of, plant material. In the case of Zn, the peaks moved to lower MW, indicating a disintegration of protein oligomers. These artifacts can lead to entirely wrong conclusions in metalloproteomics studies for detection and quantification of physiological metal binding to proteins. Therefore, sonication should be avoided in such studies unless its safety for the particular type of samples is verified with appropriate standards.

Application Examples. As application examples for the analysis of metal binding to proteins under physiological conditions in nonstressed and metal-stressed organisms,

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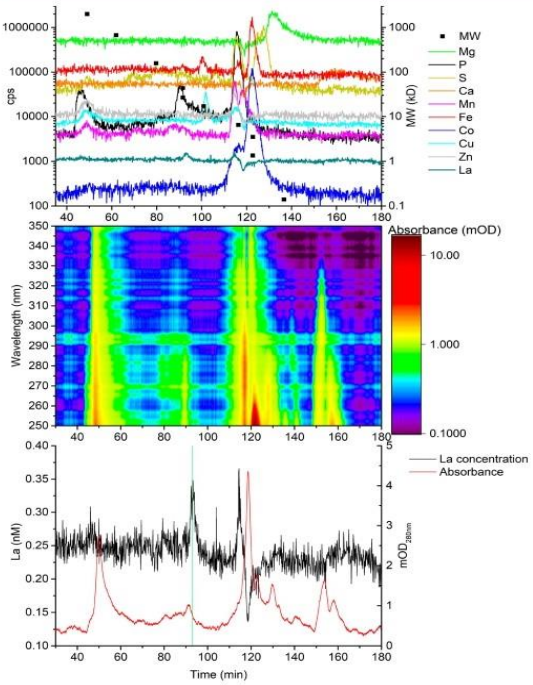

Figure 7. Analysis of binding of La in low environmental concentration (0.1 nM in nutrient solution) to soluble proteins from *Desmodesmus quadricauda*. The sample loaded to the column was extracted from approximately 5 mg FW of cells. Top: ICP-sfMS signal; middle: UV/vis absorbance measured by DAD; bottom: calibrated quantitative trace of the La signal. The analysis was performed by a combination of Superose 6 increase 10/300, Superdex 200 increase 10/300, and Superose 12 10/300 columns.

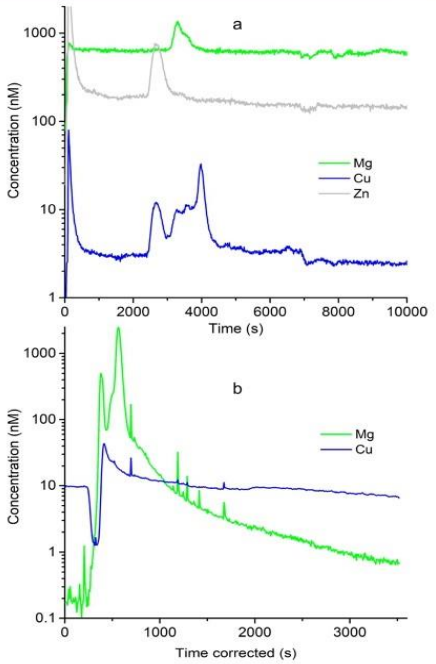

Figure 8. Analysis of copper abundance in an LH1 preparation from *Rhodospirillum rubrum* grown on 2 μM copper. (a) Preparation of LH1 from *R. rubrum*. The separation was performed with Superdex 200 increase 10/300, Superdex 75 increase 10/300, and Superdex 30 increase 10/300 columns. (b) Acetone extract from the protein preparation of a replicate experiment. The separation was performed on a C18 4.6 × 250 mm column.

several models were chosen. In all cases, P and S were measured in addition to the metals to support protein identification, and in all cases combinations of size-exclusion columns were chosen for separation.

Cr Binding to Proteins at Ultratrace Levels - Comparison to Quadrupole ICP-MS. As a first application example, a comparison was made with the system that was used for our previous publications on metalloproteomics.[9,10] The same samples were reanalyzed in the new system (Figure 5). The test investigated in which form chromium, for which no physiological function but toxicity has been reported in plants,[19] occurs in plant tissues. For this test, *A. thaliana* plants were grown on regular nutrient solution without addition of Cr; the Cr present originated only from the background levels in the chemicals. In the measurement with the old system, which was based on a Dionex HPLC system combined with a triple quadrupole ICP-MS (see Andresen et al.[9] for details), the Cr concentrations were close to the detection limit, although 200 μL of sample was injected, which is the maximum recommended amount of sample for these columns (Figure 5a). Further, despite the use and optimization of a collision chamber, in this ultratrace concentration range there were often artifactual peaks that were most likely caused by matrix interferences such as $^{40}Ar^{12}C$ (see the broad peak at 20−30 min and baseline oscillations afterwards). In the new system, although only half the amount of sample (100 μL) was injected, the signal/noise ratio was much improved, and the artifactual broad peak at 20−30 min disappeared (Figure 5b). Further, because of the optimized selection of columns, the resolution of the size exclusion chromatogram became much better. The resulting clear separation of peaks now allows for future studies that investigate Cr speciation in plants in detail. As a second step in purifying the Cr-binding proteins, an anion exchange column was used, where for the necessary salt gradient NaCl that clogged the cones very quickly was successfully replaced by NH_4Cl (Figure 5c).

Cd Binding to Proteins at Ultratrace Levels. One general concern of food safety is the uptake of Cd by crop plants, which always occurs to some extent (review by Küpper and Andresen[2]). In how far this constitutes a problem depends, among other factors, on the speciation and thus bioavailability of the Cd inside the cell. In the proof-of-principle study done here, an extract of membrane proteins from soybean leaves was analyzed (Figure 6). Although the plants had been grown without addition of Cd to their hydroponic nutrient solution, and all chemicals were at least p.a./ACS grade, so that the final

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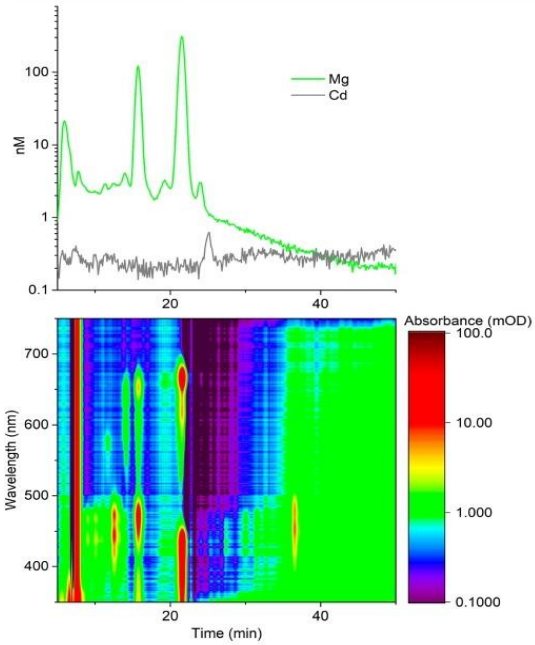

Figure 9. Proof-of-principle measurement for the detection of [Cd]-Chl in an extract from plant leaves. For this test, a 1000:1 mixture of the [Mg]-Chl a in the plant extract and a [Cd]-Chl standard[16] was prepared and injected into the HPLC-ICP-sfMS system. The identity of the Cd-peak in this chromatogram was verified by measuring the [Cd]-Chl standard alone at high concentration (1 μM peak) under identical conditions (Figure S2). The separation was performed on a C18 4.6 × 250 mm column.

Cd concentration in the nutrient solution was only about 6.5 nM, the sensitivity of the HPLC-ICPsfMS detection was sufficient to see a very clear peak of a Cd-complex at about 103 min (Figure 6). The peaks of the other measured elements show the separating capabilities of the applied column combination and help in identification of the Cd-binding protein. The intensity drop for all elements at about 115 min was caused by matrix effects of the rather concentrated aminocaproic acid buffer in which the protein isolation was done, which elutes from the column at this time. The exact identity of the protein of approximately 2 kDa that binds Cd with such high affinity remains to be resolved in future studies.

La Binding to Proteins at Ultratrace Levels. Rare earth elements (REEs) have become important for many branches of industry, but even their use as fertilizers in agriculture has become common in some countries (in particular China). The binding targets of REEs in plant cells at physiological concentrations, however, remain largely unknown. Therefore, the third trial in the current study focused on the possibility of detecting La in protein extracts from algae as a model system. To check practical detection limits of the system, the equivalent of only approximately 5 mg FW of cells, fed with only 0.1 nM La in the nutrient solution, was loaded as a protein extract to the column (Figure 7). Even at these low concentrations and sample amounts, a clear peak equivalent to approximately 0.09 nM La above background was detected at approximately 93 min. The identification of this protein, which obviously binds La with very high affinity and has a mass of about 20 kDa according to the mass calibration, is a task in ongoing research.

Cu Binding to Proteins during Sublethal Cu Toxicity Stress. While the previous three examples dealt with metal concentrations in the growth media that can be regarded as environmental background levels, the fate of metals under environmentally relevant sublethally toxic conditions is equally important. As an example, we investigated the binding of Cu to the light harvesting complex LH1 of the purple photosynthetic bacterium *R. rubrum*. When fed with 2 μM Cu, the cells were still able to grow and reached about 10% of the fresh weight of the control (not shown). At this sublethal stress level, Cu clearly bound to the LH1 protein (Figure 8a). An acetone extract from the protein-pigment complex showed Cu peaks as well (Figure 8b), leading to the next application area of the system: analysis of metals in pigments extracted with organic solvents.

Samples in Organic Solvents: Reversed-Phase Chromatography of Pigments. Analyzing organic matrices in HPLC-ICPMS is particularly difficult because such matrices normally lead to strong soot formation that extinguishes the plasma and/or blocks the sample introduction system by clogging of cones. The problems can be overcome by adding low concentrations of oxygen to the sample gas via an additional tunable mass flow controller. In the current system, for safety reasons a mixture of 20% oxygen and 80% argon proved to be successful in achieving a high sensitivity while eliminating the mentioned problems. The difficulty of such analyses is likely the reason why such analyses are scarce in the literature. For analysis of metal binding in chlorophylls only a single study was published so far,[20] and it only analyzed the usual and thus abundant central ion, Mg. Typical applications for analysis of extracts of biological material with organic solvents are, however, metabolomics and in particular pigment analyses where only metal traces are present. Therefore, two cases of pigment analyses were chosen as examples here. The first was the use of HPLC-ICPMS to identify more accurately the target of sublethally toxic Cu binding in the pigment–protein complexes of *R. rubrum*. The qualitative result that Cu-peaks can be seen in the acetone extract is shown in Figure 8b; the full analysis is the topic of a separate study.[14] A later repeat of the analysis on a metal-free column confirmed the Cu binding to the pigment but showed an improved signal/background ratio because no Cu leached from the column housing (Figure S1).

As a second example, the detection of the possible formation of [Cd]-Chl in pigment–protein complexes from soybean leaves was tested by a simulation, as Cd has been found to bind to LHC II under sublethally toxic conditions.[9] Traces of a [Cd]-Chl standard (Figure S2) were added to an acetone leaf extract from a plant grown with only background levels of Cd (see above) where no stress occurred and no Cd was previously detected in the light-harvesting complexes. Calculated from the added pigment, the OD of the [Cd]-Chl in this test would be 0.38 nM/1,000,000 nM/mM *90 1/mM*cm = 0.034 mOD, which is not measurable by any optical spectrometer. Nevertheless, it was well detectable with the ICP-sfMS (Figure 9), showing that this system should be able

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to resolve the question whether [Cd]-Chl is formed *in vivo*, which is the topic of an ongoing study.

CONCLUSIONS

In this work we could show that a task-dedicated optimization of hardware configuration, sample preparation, and measuring conditions allows for detection limits in protein SEC-DAD-ICP-sfMS and pigment RPC-DAD-ICP-sfMS. That makes analysis of metal binding possible under environmentally relevant metal ultratrace (<ppt, subnanomolar) concentrations for various analytes, with a dynamic range that enables the simultaneous analysis of matrix elements (high ppm, millimolar range). These features of the new system will allow for its use in many fields of basic and applied research, including the identification and characterization of metal binding to metabolites and proteins, environmental risk assessment, and assessments of food safety.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b00222.

Table S1, examples ICP-MS methods optimized for measurements shown in this work; Figure S1, analysis of copper abundance in a LH1 preparation from *Rhodospirillum rubrum* grown on 2 μM Cu^{2+}; and Figure S2, measurement of a [Cd]-Chl standard (PDF)

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Author Contributions

H.K. made the original plans for this work, designed the instrument configurations, and supervised the project; S.N.H.B. did most of the ICP-MS. H.K. designed the HPLC running parameters and coupling to ICP-MS. H.K. made the samples from *Arabidopsis thaliana*; N.J.P. made the samples from *Rhodospirillum rubrum*; N.A. and H.K. made the samples from *Desmodesmus quadricauda*. L.L., E.A., and H.K. made the samples from soybean. H.K. wrote the original draft of the manuscript. All authors contributed to further revisions of the manuscript.

Notes

The authors declare no competing financial interest.

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CHAPTER 4

Effect of nanomolar concentrations of lanthanum on *Desmodesmus quadricauda* cultivated under environmentally relevant conditions

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Effect of nanomolar concentrations of lanthanum on *Desmodesmus quadricauda* cultivated under environmentally relevant conditions

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ABSTRACT

Toxicity of lanthanides is generally regarded as low, and they even have been suggested to be beneficial at low concentrations. This research was conducted to investigate effects of Lanthanum (La) on *Desmodesmus quadricauda*, a freshwater green microalga. The algal cultures were treated with nanomolar La concentrations under controlled environmentally relevant conditions. Intracellular localization of La was analyzed with μXRF tomography in frozen-hydrated samples. At sublethal concentration (128 nM) La was in hotspots inside the cells, while at lethal 1387 nM that led to release of other ions (K, Zn) from the cells, La filled most of the cells. La had no clear positive effects on growth or photosynthetic parameters, but increasing concentrations led to a dramatic decrease in cell counts. Chlorophyll fluorescence kinetic measurements showed that La led to the inhibition of photosynthesis. Maximal photochemical quantum yield of the PSII reaction center in dark-adapted state (F_v/F_m) decreased at > 4.3 nM La during the 2nd week of treatment. Minimum dark-adapted fluorescence quantum yield (F_0) increased at > 13.5 nM La during the 2nd week of treatment except for control (0.2 nM La, baseline from chemicals) and 0.3 nM La. NPQ at the beginning of the actinic light phase showed significant increase for all the treatments. Metalloproteomics by HPLC-ICPMS showed that La binds to *a* >500 kDa soluble protein complex already in the sub-nM range of La treatments, in the low nM range to a small-sized (3 kDa) soluble peptide, and at >100 nM La additionally binds to a 1.5 kDa ligand.

1. Introduction

Lanthanides, also known as rare earth elements (REEs), have become indispensable for several critical technologies, and their input into the bio- and pedosphere has risen in parallel. Because of the wide usage of lanthanides in fertilizers, they are now considered as emerging pollutants (Yang et al., 2009). The toxicity of REEs is generally regarded as low. For example, the toxicity of lanthanum (La) in soil was found to be lower than copper (Cu) but higher than iron (Fe) (Wheeler and Power, 1995). REEs enter into the soil ecosystem in soluble form and even disturb the aquatic ecosystem (Wen et al., 2001). The knowledge on the effectiveness of REEs applications on plant production is still hypothetical. It has been found that net photosynthesis is reduced under high concentrations of REEs (Hu et al., 2016). In addition to the application in fertilizers (widely used in China), REEs beneficially increase the quantity and quality of crop yields (Pang et al., 2002; Tyler, 2004). REE accumulation in agricultural soil may cause potential environmental risks followed by health effects through bioaccumulation along the food chain. Excessive mining of REE mineral ore and the use of fertilizers are also considered as the main source of REE pollution, like phosphate fertilizers that are being excessively used in the field of agricultural biotechnology (Gwenzi et al., 2018; McLaughlin et al., 1999). To counter the harmful impacts of lanthanides, phytoremediation or the development of plants that have higher accumulating

Abbreviations: μXRF, Micro-X-ray fluorescence; FKM, Fluorescence kinetic microscopy; NPQ, Non-photochemical quenching; HPLC-ICPMS, High performance liquid chromatography-inductively coupled plasma mass spectrometry.

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capacities towards REEs in contaminated environments are the most promising strategies to clean up the environment.

Lanthanum, the first element of the REE series, is taken up by plants and microorganisms in ionic form such as La^{3+}. However, the capacity of plants or agricultural crops to accumulate REEs is extremely low and mainly depends on the plant species as well as on their growing conditions (Fu et al., 2001). Studies on REEs concentrations in corn and rice yielded different results for both plant species (Li et al., 1998). Higher values were found in rice, which indicated that rice has higher accumulation ability for REEs than corn. Some plants including ferns are considered as hyperaccumulators for REEs. However, intensity of uptake of REEs depends on numerous factors. Studies have shown that plants can uptake REEs through roots as well as the aboveground organs. The magnitude of mobility depends on numerous factors and in roots is usually limited by the endodermis. The use of organic ligands such as EDTA encourages the uptake by plants and increase desorption of REEs in the soil (Kastori et al., 2010). REEs usually enter into the cytoplasm of cells through channels and also by or symplastic channels (via plasmodesmata). Still it is unclear whether they are deposited at extracellular or intracellular locations and the mechanisms of regulation at biochemical level are not known (Ye et al., 2008).

Metal uptake by algae has many applications that range from sewage treatment to precious metal recovery (Mehta and Gaur, 2005). Further, many aspects of metal uptake and metabolism are common in algal, animal and even bacterial cells (Shanab et al., 2012). Macro- and microalgae can serve as a useful model because of their ability to accumulate high metal content (Wilde and Benemann, 1993). Though, the roles of REEs in microalgal cultures are not fully understood under environmentally relevant conditions (Rezanka et al., 2016). Reports have shown that bulk quantities of dissolved REEs are released into aquatic ecosystems (Qiang et al., 1994). Though, no acute effects have been reported so far. More attention has been put on their potential effects on the aquatic environment, mainly concentrated on maximizing the production of valuable compounds from microalgal cultures. REE complexation through organic ligands in aquatic ecosystem alters the bioavailability of these elements (Sun et al., 1997). Improving the knowledge about metal toxicity and bioavailability during exposure of aquatic organisms requires a study of the metal speciation. The low solubility of REEs in nutrient media is due to the precipitation of lanthanides as phosphates, but phosphates are essential for algal growth. In order to avoid this problem, the lanthanides need to be chelated by a specific ligand that is not too strong (otherwise the cells could not take the metal out of the complex) but strong enough to prevent the precipitation. Such a lanthanide ligand is DOTA (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid), which is well-known from various medical applications (Aime et al., 1997).

The concentration of REEs in algal material reached up to 1.3 $\mu g\ g^{-1}$, which is high compared to water bodies that contain low amounts of these elements (Liang et al., 2014). Lanthanide exposure at high concentrations, like other trace elements, induced negative impacts on aquatic ecosystems (Hu et al., 2016). It was reported that high concentrations of REEs exhibit inhibitory effects, which involve significant decrease in chlorophyll synthesis, and diminished photosystem (PS) I and PS II activity. Most of the previous studies on the potential effects of REEs on algal physiology focused on interactions of REEs with the Ca^{2+} influx mechanism and other downstream reactions. For example, the movement reaction of the flagella in the freshwater microalga *Spermatozopsis similis* can be blocked by using 50 µM Gd^{3+} (Hill et al., 2000). Most research to elucidate the effects of REEs has been done on agricultural plants. Experiments carried out with REEs and algae mainly focused on microalgal growth. However, it is still unclear whether these elements have some positive effects, as suggested previously for plants (Qu et al., 2012; Goecke et al., 2015). Research has shown that REEs were able to improve growth, apparently depending on stress and the nutritional state of the microalgae, raising the possibility of environmental impacts at even low concentration.

It is hypothesized that variation of REE concentrations in the nutrient solution should influence metabolic pathways, i.e. enzymes. Wen et al. (Wen et al. (2001)) reported effects of REEs on photosynthesis in terrestrial plants. However, the toxic effects of lanthanides and mechanisms behind the relationship among photosynthetic rate, electron transport efficiency and carbon assimilation are rather complex. Different mechanisms may be involved, and the photosynthetic apparatus may contain the most important sites of inhibition by REEs.

The aim of the present study was to analyze the effects of La in the green microalga *Desmodesmus quadricauda* under environmentally relevant conditions of light, temperature, nutrient supply and La concentrations. This study was performed in order to gain more insight into the mechanism of La toxicity and the microalgal response to toxic La concentrations in the natural environment.

2. Materials and methods

2.1. Microalgal strain and culture conditions

The freshwater chlorococcal alga *Desmodesmus quadricauda* (Turpin) Brébisson (strain Greifswald/15), was obtained from the Culture Collection of Autotrophic Organisms Institute of Botany (CCALA, CAS, Třeboň) (Czech Republic) (http://www.butbn.cas.cz/ccala/ccala.htm). The experimental microalgae were cultivated long-term (continuously since summer 2017) in a nutrient solution (submerged macrophyte nutrient solution, SMNS) that was optimized and designed previously for growing submerged macrophytic water plants (*Ceratophyllum demersum*) to reflect oligotrophic freshwater conditions of Central European habitats (Andresen et al., 2013). It was modified for this study by increasing the $CaCl_2$ concentration from 40 to 200 µM. This growth medium reproduces better the eutrophic conditions where *Desmodesmus quadricauda* naturally grows.

D. quadricuada was cultivated in batches in 300–500 mL glass tubes under computer-controlled simulations of a natural 12 h sinusoidal light cycle with maximum irradiance at noon of 50 µmol photons $m^{-2}s^{-1}$ and 12 h night. Temperature (30 °C) was controlled by a FP40/HL thermostat (JULABO GmbH, Germany). The light was provided by Dulux L 55 W/12–954 (OSRAM Munich, Germany) fluorescent tubes. The cultures in cylindrical vessels were aerated with normal air to prevent the sedimentation of cells. Stock cultures were prepared by inoculating 50 ml of green stock of *D. quadricauda* culture that was transferred to a cylinder containing 450 ml medium, to guarantee high cell density at the peak of the exponential phase ($72^{*}10^{4}$ cells/ml). Every experiment included ten 300 mL cultivation tubes (respective to ten lanthanum concentration treatments), which were inoculated from the stock cultures and subsequently run as chemostats. The nutrient solution was exchanged continuously by peristaltic pumps (ISMATEC Reglo Digital, Cole-Parmer GmbH, Wertheim, Germany) at a flow rate of 0.1 $lday^{-1}$ and aerated continuously. Stock bottles of the media pumped into the cultures contained different concentrations of La: 0.2 (control, La only from baseline contamination of chemicals), 0.3, 0.7, 2.3, 4.3, 13.5, 36.6, 128, 496, 1387 nM. Lanthanum was added in the form of $LaCl_3$ (99%, Sigma-Aldrich) and made fully soluble by adding DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetcic acid) (97%, Sigma Aldrich) as a ligand in a ratio of DOTA:La = 5:1. Toxic effects of DOTA were very unlikely because DOTA has already been tested and confirmed nontoxic for humans, and for this reason combined with its specificity for REEs it is extensively being used in medical applications as (REE) chelating agent (Wahsner et al., 2019; Herborn et al., 2007). Lanthanum treatments were started when the cultures attained a stable OD (Optical Density). Each of the experiments was performed independently and treatment was carried out for 2 weeks. Growth and photosynthetic rate were determined each week. After 2 weeks of La treatments, algae were harvested by centrifugation (BIOFUGE Stratos Thermo Scientific™) at 5000 *g* for 20 min at 30 °C to ensure the complete separation of algae from medium. The supernatant was discarded and the algal biomass was

resuspended in ddH_2O for washing. The algal suspension was centrifuged and the supernatant was removed in a final step. The pellets were immediately frozen and later stored in liquid nitrogen (−80 °C) until further analysis.

2.2. Determination of cell growth

Growth performance of *D. quadricauda* was analyzed by counting cells. Three counts were done from each culture and daily observations were made to estimate the growth rate. For counting, 2 ml of the individual algal samples were added in a test tube, centrifuged at 6000 *g* at 30 °C for 10 min. The supernatant was discarded. The pellet of algal cells was diluted with 10 µL of 1% glutaraldehyde and stored at 4 °C. These 10 µL samples were inserted into a Neubauer Improved cell counting chamber (Brand GmbH, Wertheim, Germany) and cells were counted in the squares of the chamber by observation under a microscope (Axioimager M2, Carl Zeiss AG, Oberkochen, Germany) with a 20x objective (W Plan-Apochromat 20x/1.0 DIC $D = 0.17$ M27 75 mm) and a CCD camera.

2.3. Photosynthesis biophysics

Two-dimensional microscopic imaging of in vivo chlorophyll fluorescence kinetic measurements (FKM) was performed to know the effects of La on photosynthetic light reactions in algal cells. For sample preparation, algal cells were centrifuged at 6000 *g* at 30 °C for 10 min, the pellet was resuspended in 100 µL of culture medium, then immediately mixed 1:1 with 2% Sea Kem agarose on the window of the measuring chamber, and covered with cellophane (Küpper et al., 2000; Šetlíková et al., 2005). There was a continuous flow of the culture medium in the chamber during the kinetics measurement. The detailed description of the microscope, the used protocols and analyzed parameters of the Kautsky induction can be found in Küpper et al. (2007a).

2.4. Harvesting

After 2 weeks of treatment, algal cultures were harvested by centrifugation, supernatant was removed and the pellet was washed in ddH_2O and centrifuged again to remove supernatant. The pellet was frozen in liquid nitrogen. Samples were stored at −80 °C until further analysis, e.g. by metalloproteomics.

2.5. Analysis of La binding to proteins (metalloproteomics)

For analysis of La binding to proteins with HPLC coupled to ICP-MS, proteins were isolated following the protocol of Andresen et al. (2016) with slight modifications. Instead of using a fixed ratio of biomass to buffer, due to the difficulty to determine accurate biomasses for the small algal sample a fixed volume of 100 µL of frozen buffer was added per sample of algae. The powdered mixture was placed on ice for thawing, and then it was mixed with another 100 µL of isolation buffer before the centrifugation. The analysis by size exclusion HPLC coupled to sector-field ICP-MS was carried out as described by Küpper et al. (2019) using the following set of three 10 × 300 mm columns in series: Superdex 200 Increase (2x) – Superdex 75 Increase (all GE Healthcare, USA). This choice was made because the first isolation published in the methods paper (Küpper et al., 2019) had indicated a main La-binding protein of a size around 20–30 kDa.

2.6. Localization of La by µXRF tomography

Algal cells exposed to 128 or 1387 nM La, representing respectively a sublethal and a lethal concentration, were used for synchrotron µXRF single-slice tomography mapping of La. Approximately 200 mL of algal culture from each treatment was centrifuged (5000 *g* at 30 °C). The supernatant was discarded and the pellet was thoroughly washed with 5 mL of nutrient solution with neither micronutrients nor La. The resuspension was incubated for 1 h at 30 °C with continuous rotation. Afterwards, the suspension was centrifuged at 5000 *g* at 30 °C for 15 min and the supernatant was discarded. The samples were prepared by sucking cell suspensions into 0.3 mm outer diameter polyimide capillaries (Professional Plastics, Fullerton, CA, USA), attaching them to holders made from magnet-holding (but not magnetic) stainless steel, and shock-freezing the whole assembly in supercooled isopentane (−140 °C). Multielement standards were prepared in the same way as the samples for calibration and full correction of absorption effects. The cells from two independent experiments exposed to sublethal and lethal concentration were frozen and the capillaries were stored in liquid nitrogen for analysis.

The synchrotron µXRF measurements were done at beamline ID-16A, i.e. the µXRF nanoprobe with cryostage, at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The measurements of X-ray fluorescence tomography and phase-contrast tomography were done on the shock-frozen hydrated samples to prevent element redistribution. The samples were measured at approximately −150 °C. ID16A-NI combines multi-element detection with both high sensitivity and spatial resolution producing high resolution elemental maps at organelle level with a beam down to 15 nm size (da Silva et al., 2017). For measuring µXRF at the La L edge, 17 keV excitation energy was used. Single-slice tomograms were measured by scanning lines across the sample at various rotation angles. X-ray fluorescence was detected with 2 multi-element silicon drift detectors (SDD's) coupled to xMap readout electronics (XIA LLC, Hayward, CA, USA). XRF spectra were fitted using the PyMca libraries (Sole et al., 2007) to yield elemental areal mass concentrations. Tomograms were reconstructed using a regularized Maximum-Likelihood Expectation-Maximization (MLEM) algorithm implemented with ESRF inhouse software using the ASTRA Toolbox and SPIRE wrapper (https://github.com/pierrepaleo/spire; Mokso and Cloetens, 2007). The tomograms were corrected for self-absorption on the basis of an equivalent tomogram of the multi-element standard. Therefore, the µXRF tomography provides virtual slices of the molar concentrations of multiple elements through-out the sample. Propagation based X-ray phase-contrast imaging was used to provide 3D tomograms of the density distribution in the sample (Mokso and Cloetens, 2007). Single distance phase retrieval was performed using the Contrast Transfer Function approach and carried out with ESRF in-house software using the GNU Octave programming environment (http://www.octave.org). From the 3D phase-contrast data sets, the slice corresponding to the µXRF analysis was selected. Further image processing (smoothing, contrast, color scales) of µXRF and phase contrast images was performed in ImageJ (http://rsbweb.nih.gov/ij).

2.7. Statistics

For statistical analysis of the results, non-parametric Mann-Whitney U tests of null-hypothesis were performed in ORIGIN Pro (software version 2019b; Originlab Corporation, Northampton, MA, USA). These tests were performed at a significance level of $p<0.05$ for weekly measured data of growth and all photosynthetic parameters as a comparison between control and La treatments. The results of all individual tests are shown in supplement 1. All these parameters were measured in three independent experiments as replicates and results are presented as means ± standard error (SE).

3. Results

3.1. Growth kinetics of algae

According to the cell counts, the best day for inoculations of new cultures was day 2 (middle of the exponential phase) or day 3 (end of the exponential phase) of the stock culture (Fig. 1A). The cell density of the stock culture reached a maximum value on day 5 and then started dying

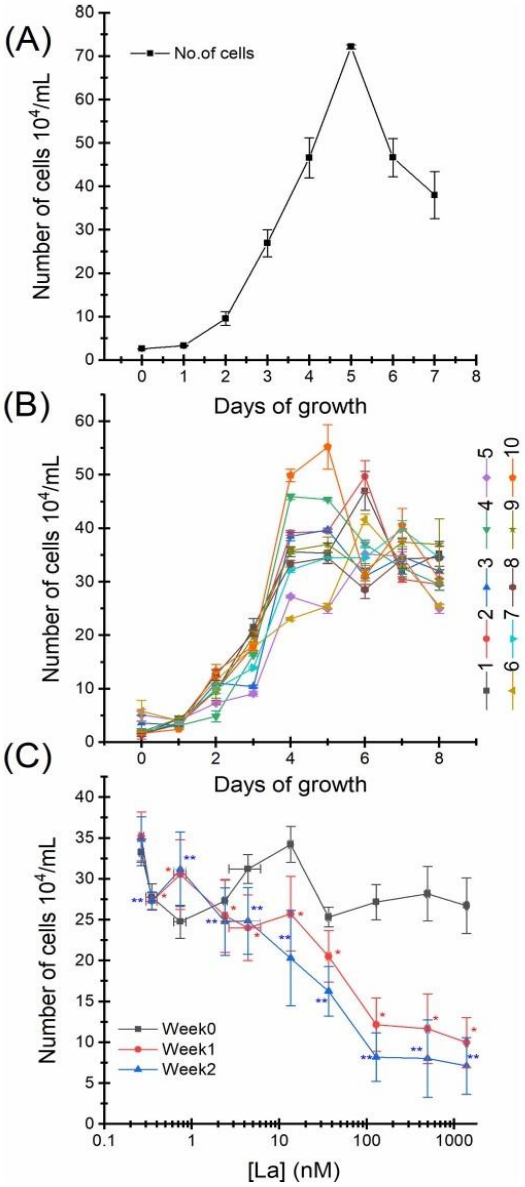

Fig. 1. Growth curve of *Desmodesmus quadricauda*, expressed as cell number; A) Stock culture; B) Chemostatic culture before start of La treatment, numbers refer to the 10 tubes that were later treated with different La concentrations; C) Chemostatic culture during La treatment. Single red asterisks represent significant differences during week1 for treatments vs. control. Double blue asterisks mark significant differences during week2 for different La treatments compared with control of respective week. Both was tested with the significance level of $p<0.05$ according to the Mann-Whitney U test. Each value is the mean of 2–3 different experiments. Error bars represent ±SE.

because of nutrient deficiency. In the chemostats, cultures reached stable growth on day 8 (Fig. 1B).

3.2. Effect of lanthanum on algal growth

Cell counts in algal cultures in the presence and absence of La under controlled conditions were determined during 2 weeks of exposure. The count rates of algae in the absence of La (also between chemostats before La addition i.e. in week 0) were constant and no significant differences ($p>0.05$) among the cultures were observed above the noise level. However, in the presence of La from 4.3 nM and upwards, already after one week of treatment algal counts decreased significantly ($p<0.05$) as compared to the controls (Fig. 1C). Cell counts declined to about 50% of the control at 13.5 nM La after the second week of exposure. In addition, the number of cells per coenobium started to decline during the course of treatment along with increasing concentrations (data not shown). Thus, a concentration of 4.3 nM La was determined for the onset of toxicity. The cell counts showed that lanthanum had no clear positive effects on algal growth, but was toxic already from 4.3 nM, during the first and second week of the culture.

3.3. Effect of lanthanum on photosynthesis

The effect of Lanthanum exposure on biophysics of the photosynthetic light reactions was determined by imaging analysis of chlorophyll fluorescence kinetic measurements (FKM). La exposure at 13.5 nM and upwards led to the inhibition of photosynthetic activity. The trend for F_0 (minimal dark-adapted fluorescence quantum yield) increased in response to toxic La treatments, which became statistically significant ($p<0.05$) for concentrations above the threshold at 13.5 nM during the first week of treatments (Fig. 2B). This trend became stronger during the second week for all the La treatments. The data for F_m (maximal dark-adapted fluorescence quantum yield) showed the same trend, but it was noisier (Fig. 2A). One of the most important parameters of fluorescence kinetics is the photosynthetic quantum efficiency of the PSII RC (Photosystem II Reaction centre) in the dark-adapted state measured as $F_v/F_m = (F_m-F_0/F_m)$ (Fig. 2C). This parameter decreased as compared to the control or week 0. However, in week 1 for all the La concentrations it was still quite stable, except at 1387 nM (the highest La concentration), which showed a significant difference ($p<0.05$) when compared with the control of week 1 and week 0. F_v/F_m showed a significant ($p<0.05$) decrease in week 2 for higher La concentrations, starting from the threshold level 4.3 nM.

The non-photochemical quenching (NPQ), i.e. the light-induced upregulation of exciton dissipation as heat, measured as $(F_m-F_m')/F_m$) at the end of the actinic light phase (i.e. NPQ_i6), was rather noisy with no statistically significant difference between the weeks of treatment (Fig. 3B). NPQ_i1 after 10 s of actinic light increased for most of the La concentrations, in particular the higher La treatments, during the first and second week as compared to control and to lower concentrations ($p> 0.05$) (Fig. 3A). NPQ at the end of the actinic light phase showed significant increase for all the treatments from the beginning, except for the control, at the end of La exposure (Fig. 5B). The data for the beginning and the end of the dark phase measured as NPQ_r1 and NPQ_r5 were rather noisy, respectively (Fig. 3C,D).

The light saturation parameter, which is a measure of the ratio of functional antennae coupled to functional PSII units (measured as $(F_p-F_0)/F_m-F_0$; Küpper et al., 2007a), decreased from the beginning of the treatment for almost all the La concentrations. However, an increase was observed during the 1st week of treatment compared to week 2 (Fig. 4). The Mann-Whitney test showed a significant ($p<0.05$) effect of La on the saturation parameter, which reached almost zero for the treatment with 1387 nM La. Light-acclimated photochemical quenching, which is a measure of functional electron transport from PSII to PSI (measured as $(F_m'- F_t')/F_m' = \Phi_{PSII}$, with Φ_{PSII} _i1 measured 10 s after the start of actinic light phase), was noisy (Fig. 5A) as compared to the light

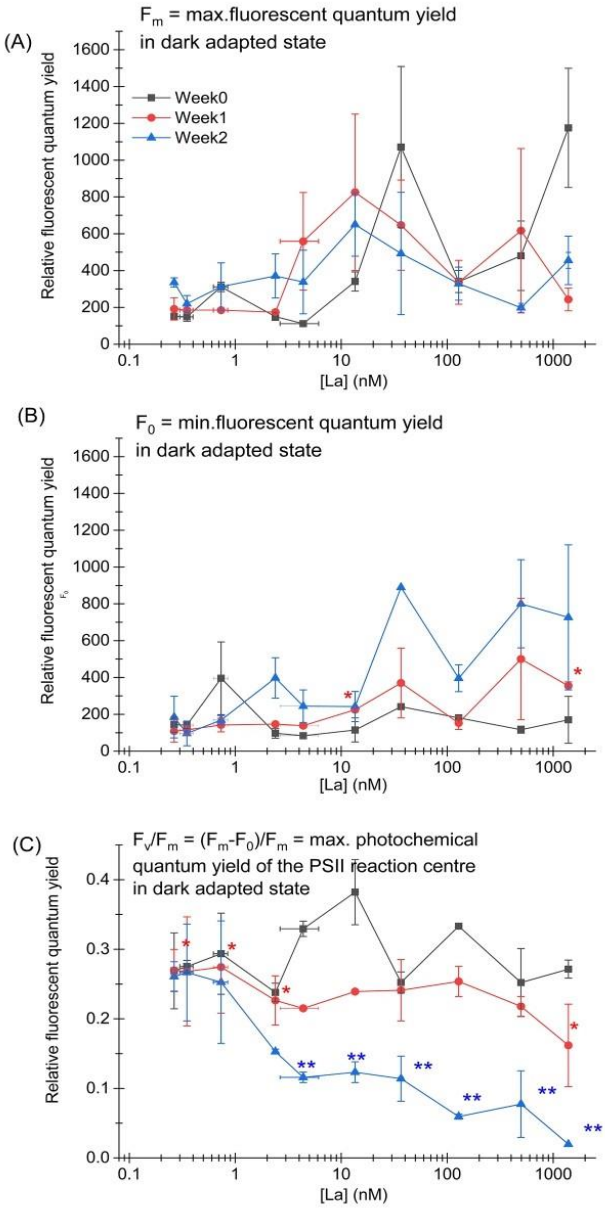

Fig. 2. Effect of La on photosynthetic parameters measured by FKM. A) F_m_d (Maximum Fluorescence in dark-adapted state); B) F_0_d (Minimum Fluorescence in dark-adapted state), single red asterisks mark significant differences for treatments at point 13.5 nM and 1387 nM during week1 vs control at the significance level of $p<0.05$; C) F_v/F_m (Maximum quantum yield of PSII in dark-adapted state). Single red asterisks indicate significant difference ($p<0.05$) during week1 for lower La treatments and highest concentration when compared to control. Double blue asterisks mark significant differences $p<0.05$ during week2 for F_v/F_m, for all higher treatments vs control, according to Mann-Whitney U test. Values are means from 3 different experiments. Error bars represent ± SE.

acclimated electron flow through PSII after 200 s of actinic light (Φ_{PSII}_i6). The latter parameter indicated a decrease in photochemistry in the light adapted sample during the second week of treatment for higher treatments from 36.6 nM and upwards as compared to the lower concentrations and control (Fig. 5B), but statistically there was not a significant difference at the level of $p> 0.05$.

3.4. Binding of lanthanum to proteins

To investigate the binding of La to soluble proteins, extracts from the La-stressed algae were prepared and measured by size exclusion chromatography coupled to sector-field ICP-MS ("metalloproteomics"). Because of the low signal/background ratio at the picomolar La concentrations in the chromatograms, several chromatograms had to be

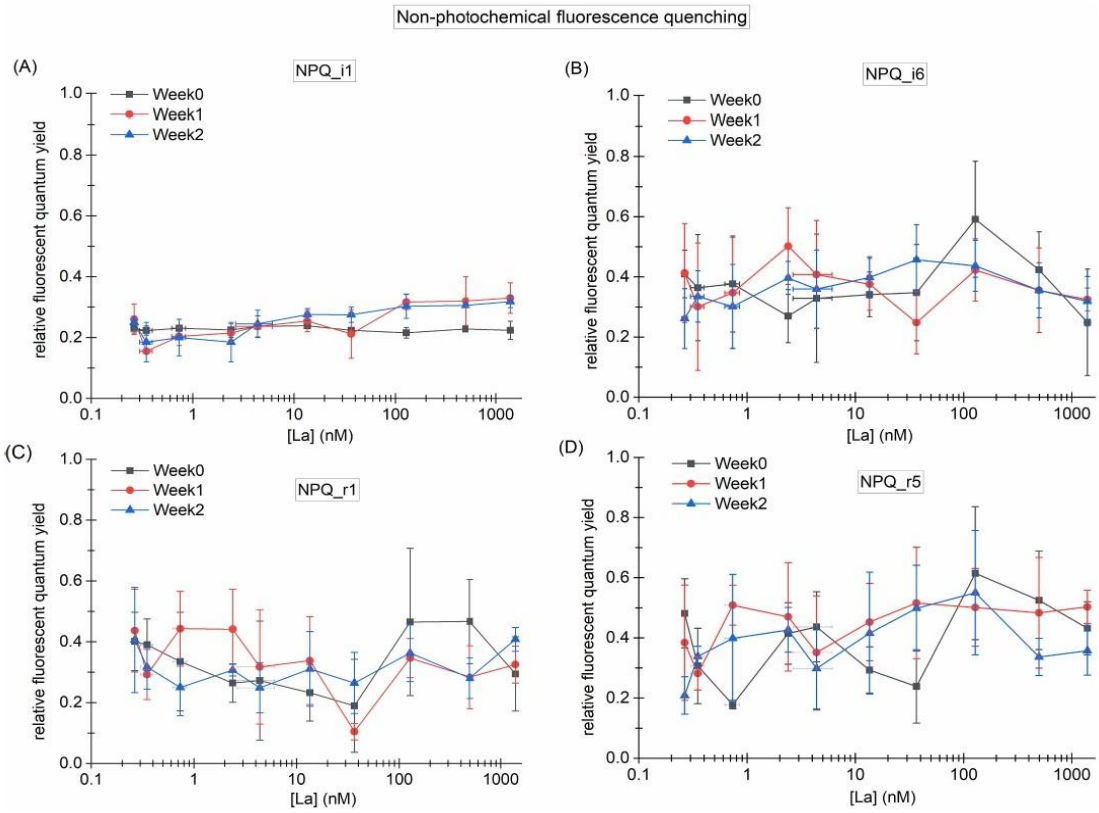

Fig. 3. Effect of different La treatments on non-photochemical exciton quenching measured as NPQ = $(F_m\text{-}F_m')/F_m$ in *Desmodesmus quadricauda*. A) NPQ_i1 = Non-photochemical exciton quenching after 10 s of actinic light phase. B) NPQ_i6 = Non-photochemical exciton quenching at the end of the actinic light phase. C) NPQ_r1= Non-photochemical exciton quenching after 10 s of dark phase i.e. during the beginning of the relaxation phase. D) NPQ_r5 = at the end of the relaxation phase. Values are means from 3 independent experiments; Error bars represent ± SE

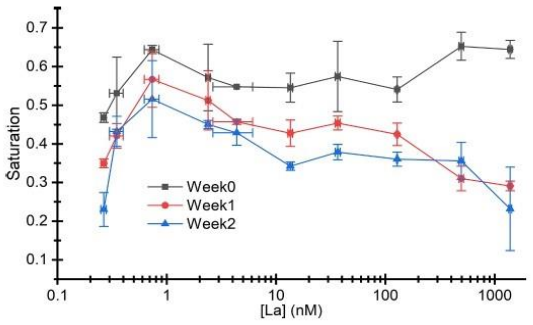

Fig. 4. Effect of the different La treatments on saturation measured by $(F_p\text{-}F_0)/F_m\text{-}F_0$: displaying antennae to Chl ratio in *Desmodesmus quadricauda*. Data represent the mean of three independent experiments with error bars representing ± SE.

averaged (Fig. 6) as described in detail in the following. With increasing applied metal concentration, La was detected not only with increasing peak size, but also the contributions of different La complexes changed. At low La treatment concentrations (chromatograms averaged for the 0.2 to 0.7 nM La treatments), the only La peak above the noise background was found in large protein aggregates (eluting at 46.5 min), above the size range where the columns could separate. This peak was in the range of 0.001 nM = 1 pM La peak height above a background of 9 pM La. At medium La treatment concentrations (chromatograms of 2.3–36.6 nM La treatments averaged), the peak in the large protein aggregates increased to an amplitude of about 2 pM La. In this concentration range, most of the La was detected in a protein peak with approx. 10 pM La amplitude that eluted at 108.7 min. This elution time indicates a small peptide of about 3 kDa. At the highest La treatments (chromatograms of 128–1387 nM La averaged), the high MW La peak increased to about 3 pM La, the 3 kD La peak to 18 nM La. Further, a new peak at about 1.5 kDa (115 min) appeared and became dominant with an amplitude of about 100 nM La. This likely represents either a very short oligopeptide or a non-protein La complex.

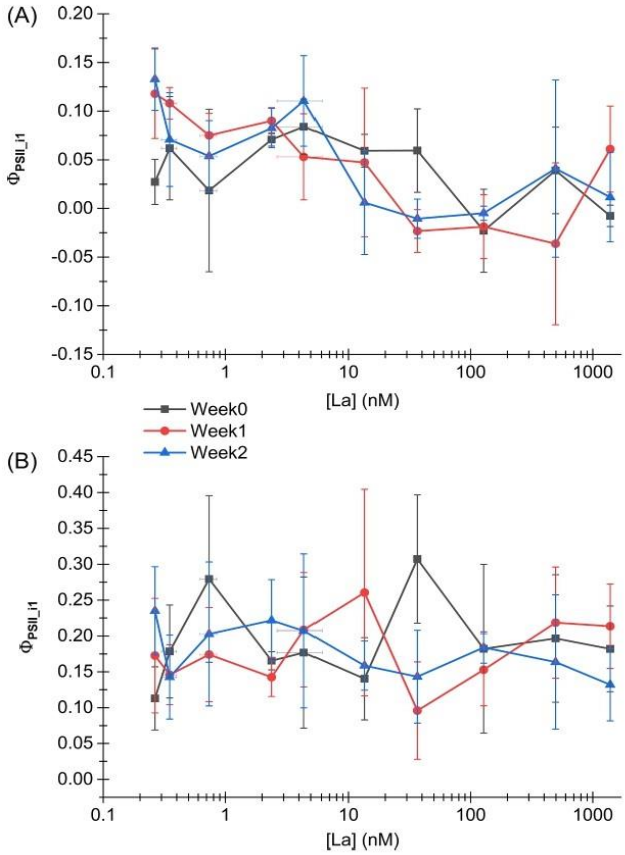

Fig. 5. Effect of La treatments on photochemical exciton quenching measured as $\Phi_{PSII=}$ ($F_m' - F_t'$)/F_m' indicating operating efficiency of PSII. A) during the beginning (1) after 10 s of actinic light and B) during end (6) after 200 s of actinic light in *Desmodesmus quadricauda*. Values are means from 3 independent experiments; error bars represent ± SE.

3.5. Intracellular lanthanum distribution at sublethal and lethal toxicity

Synchrotron μXRF tomography was used to analyze metal uptake on a single-cell level (Fig. 7) and in particular sub-cellular distribution of metals (Fig. 8). Other metals like K and Zn were analyzed simultaneously with La for comparison, because K is well-known to be highly mobile and therefore a good indicator of membrane leakiness, and Zn is well-known as an important micronutrient. In cells treated with lower La concentration, K and Zn predominantly showed a higher signal than in samples treated with a lethal La concentration. In these samples treated with high La, the concentrations of K and Zn inside the cells were low, likely caused by membrane leakage (Fig. 8). The tomographic reconstruction of the subcellular distribution of metals showed that at lower sublethal concentrations (128 nM La) most of the La was located inside the cells, often in small spots (biominerals?), and very little La was in the cell wall that should have appeared as a narrow ring around the cells (Fig. 8). When the concentration of La was increased to a lethal level (1387 nM), in the cells with highest total La most of the La appeared like a ring around the part of the cells that accumulated K if the tomographic section sliced the cell in a position where the K-accumulating interior was hit. In peripheral slices not hitting the K-filled interior, La looked rather homogeneously distributed. From the size and position of the K-filled portion of the cell compared to the phase contrast tomogram, we conclude that the K is in the chloroplast. While the ring-shaped La localization looks at first glance like La being mostly in the cell wall, the fact that La actually filled a large proportion of the whole cell volume leads to the conclusion that most of the La was in the cytoplasm. But even those most stressed cells seem to be able to keep La partially separated from the most sensitive photosynthetic apparatus in the chloroplast.

4. Discussion

In this study the levels of La (0.2 nM to 1387 nM) were chosen based on realistic La concentrations in unpolluted and La-polluted aquatic ecosystems. The first most obvious results of this study were the effects on the physiological parameters including growth and photosynthetic parameters, showing that even at very low concentrations (nanomolar range) La has inhibitory effects. Lanthanides are considered to be non-essential. So far, toxicity had generally been regarded as low, unlike other heavy metals, but the effects have been neglected especially in aquatic ecosystems (Liang et al., 2014). In the present study, it was

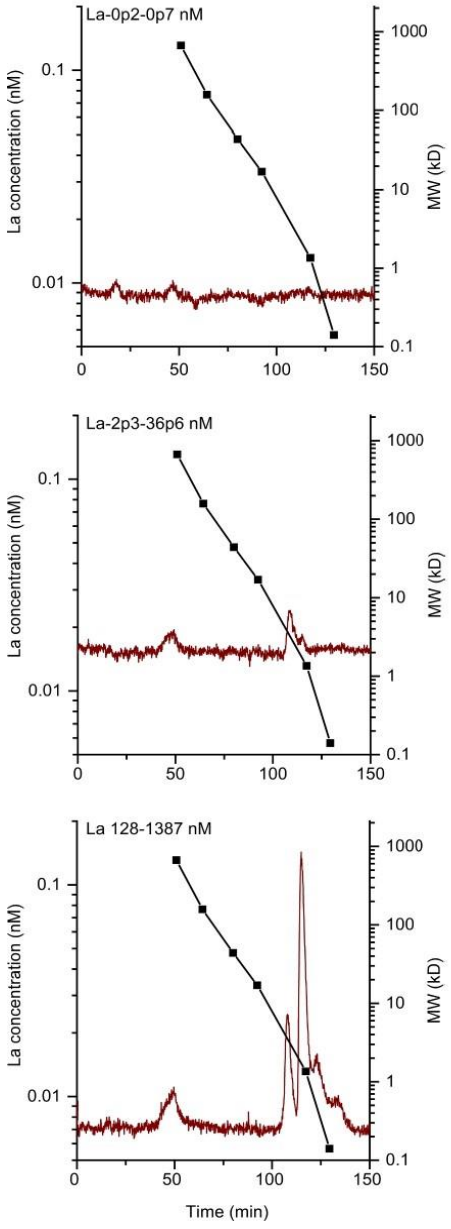

Fig. 6. Effect of La on soluble proteins of *Desmodesmus quadricauda*. ICP-sfMS signal. The analysis was performed by sector-field ICP-MS. The chromatograms are averages of 2–3 individual chromatograms in the specified La concentration range and from 2 to 3 independent experiments.

demonstrated that under environmentally relevant conditions, *D. quadricauda* possesses potential of La uptake in the nanomolar range, but it strongly suffers from this uptake. Under such conditions, the growth rate of *D. quadricauda* suffered already from La exposure in the nanomolar range, which could be a problem for phytoremediation and lanthanide recycling with such organisms, contrary to previous assumptions (Richards and Mullins, 2013). To make such technologies feasible with this organism, strategies to reduce toxicity to living cells such as increasing cell density e.g. by adding more nutrients, or use of dead biomass, would be required. The contrasting results of the current work compared to earlier studies about the La toxicity threshold could be explained by the precipitation of La in the former studies due to lack of a suitable ligand for La to remain in solution in the presence of phosphate. This was observed in preliminary experiments (data not shown), where precipitation makes La biologically unavailable. Lanthanum was previously reported to induce stimulatory effects on green microalgae by alleviating metal deficiency (Li et al., 2011; Rezanka et al., 2016; Goecke et al., 2017). This could not be confirmed in the current study under environmentally relevant conditions, although a slight stimulatory affect at 0.7 nM La cannot be ruled out with certainty, based on slight but statistically not significant changes of growth and NPQ_i1.

In order to analyze physiological stress under possible La limitation and toxicity stress, the decrease in cell growth and photosynthetic parameters were followed in the current study. These parameters were chosen as metals are extensively being studied related to performance of cellular functions and are affected by metal toxicity as mentioned already in the introduction. In general, growth measurements show the combined downstream effect of all inhibitions in metabolism of the affected organism. In contrast, photosynthetic light reactions are known to be a primary target point of metal toxicity. Correlating it with the growth measurements indicated that the inhibition of photosynthesis was ultimately causing most the inhibition of growth.

All photosynthesis-related parameters that were determined in current study decreased at 13.5 nM and higher La concentration. PSII photochemistry (F_v/F_m) was affected: La-treatment led to decrease in (F_v/F_m) compared to the start of the treatment and control. F_0 increased more than F_m for toxic La treatments, indicating that some reaction centers became inactive so that antennae coupled to them released their excitons as fluorescence. Interestingly, non-photochemical quenching (NPQ) at the beginning of actinic irradiance (NPQ_i1) increased for all the treatments compared to the control. This was contrasted by an almost unchanged NPQ in the light-acclimated state (NPQ_i6). This difference indicates that the La-induced inhibition of photosynthesis, leading to up-regulation of exciton dissipation by heat, affects a target close to the PSII RC so that feedback to NPQ is fast. At lethal La concentrations, NPQ_i6 decreased compared to the sublethal La levels, reaching values like the control. The difference between these two NPQ responses could indicate an additional site of inhibition at lethal La concentrations that is further away from the PS II RC, leading to a delayed response of NPQ. Altogether, photosynthetic parameters clearly revealed that La toxicity stress (La) directly inhibited photochemistry at nanomolar levels. As a consequence, this diminished energy capture by photosynthesis ultimately led to reduction in cell divisions that became obvious in terms of growth measured by cell density.

In recent years, bioaccumulation of metals in algae has been extensively studied. The uptake or removal of elements could be an active or passive process and is metabolically controlled (Davis et al., 2003). In our present study, X-Ray fluorescence tomography was used to study accumulation of La in algae. The accumulation of La inside the cells at sublethal La concentration (128 nM) can be explained by a possible binding to a protein or some enzyme. This means that under these conditions most of the La was in direct contact with the metabolism of the cells, which can explain the surprisingly high toxicity that was observed in our experiments. In our present studies metalloproteomics analysis showed that there are at least two soluble peptides (apparent

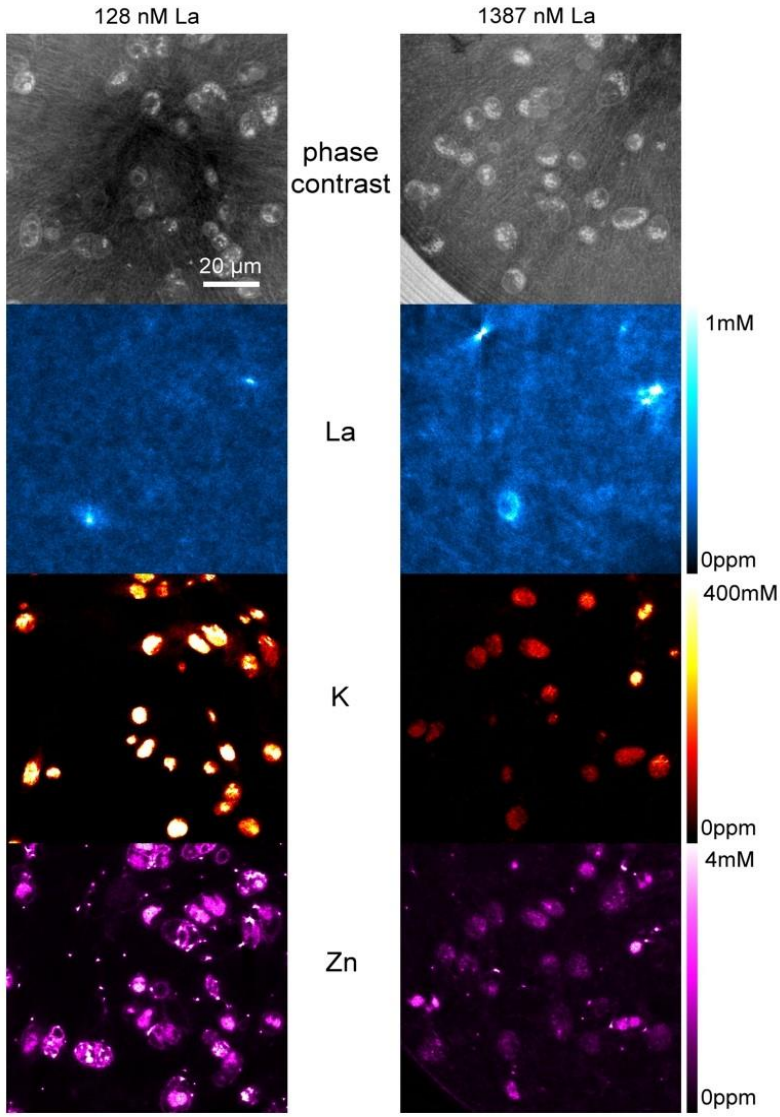

Fig. 7. In vivo phase contrast and micro-X-Ray Fluorescence (µ-XRF) tomograms showing cellular uptake of La, K and Zn by *Desmodesmus quadricauda* after 2 weeks of treatment at sublethal La concentration (128 nM) and lethal concentration (1387 nM). The size bar in the upper left image is valid for all images of the figure.

size 3 kDa, and >500 kDa) that have high affinity to La at nM concentration. Identification and characterization of the 3 kDa La-binding protein, as the most interesting of the observed complexes, is a task for further studies. As for the >500 kDa aggregates observed already at even lower La concentrations but with low abundance, the different response of the size of this peak compared to the 3 kDa peak to increasing La concentrations indicates that this is not the same La-binding protein. It may be aggregates of a 20 kDa protein that was found in a first test of the method (Küpper et al., 2019) and was not found again in the later systematic measurements published now. Revealing the identity of this large protein complex/aggregate will be a task for future work as well. Altogether, the different target proteins at different La concentrations indicate that mechanisms of La toxicity are different in the ranges of slight toxic effects vs. severe toxicity vs. lethal toxicity.

La at both 128 nM (sublethal) and 1387 nM (lethal concentration) was accumulated in the cytoplasm. But while at 128 nM La formed hotspots that may be biomineralization, at 1387 nM it filled most of the cell. At this La concentration, however, essential elements like K and Zn became released through leaky membranes. This indicated that at this

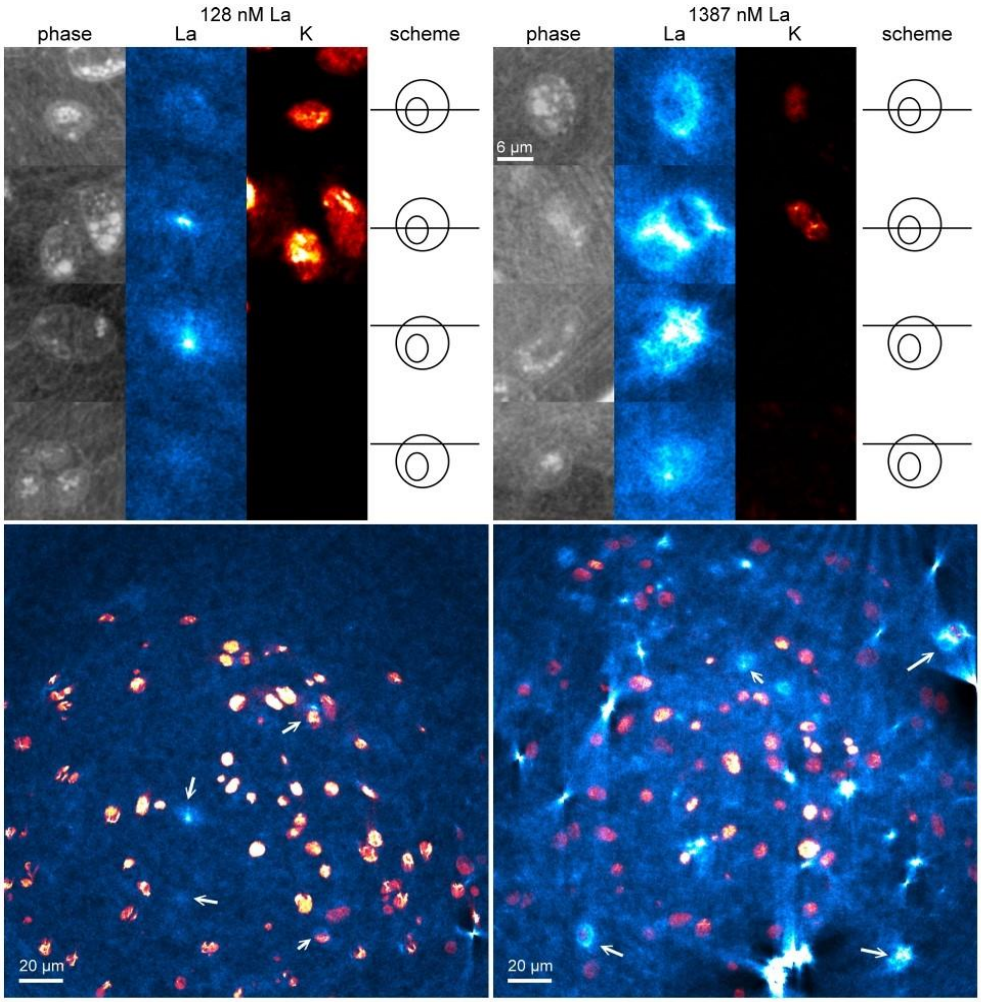

Fig. 8. In vivo phase contrast and micro-X-Ray Fluorescence (µ-XRF) tomograms of subcellular elemental distribution: La and K maps of *Desmodesmus quadricauda* after 2 weeks of treatment at sublethal La concentration (128 nM) and lethal concentration (1387 nM). The color scales for La and K are the same as in Fig. 7; the large bottom images are additions of the color channels of La and K. The scale bar in the upper-left one of the zoomed image is valid for all of the zoomed images. The schemes illustrate the position of the tomographic section relative to the chloroplast of the respective cell. The arrows in the La-K overlay images at the bottom indicate the positions of the cells that were studied in more detail in the magnified images above.

lethal La concentration, cells were extremely stressed, but not yet dead as shown by the K that is known to be highly mobile so that it would be lost from dead cells. In this scenario, effects of La were less specific, which is reflected by the additional La peaks in the metalloproteomics analysis at >100 nM La and the worsened inhibition of photosynthesis. Interestingly, it looks like even those very stressed cells were able to lower the La concentration in the chloroplast compared to the remainder of the cell. Many aspects regarding the uptake of lanthanides are still questionable, like for example, mechanisms of transport through the algae cell wall.

In conclusion, we showed that La is toxic already in the low nanomolar range, leading to inhibition of photosynthetic light reactions and, as an ultimate downstream consequence, growth reduction. The rather unspecific filling of most of the cell with La at 1387 nM compared to more restricted La localization inside the cell at 128 nM La, as well as changing target proteins of La binding from sub-nanomolar to low nanomolar to >100 nM, and more specific inhibition of photosynthesis at lower sublethal La, shows the necessity of working with sublethal concentrations under nature-like conditions for revealing physiologically relevant toxicity mechanisms.

CRediT authorship contribution statement

Nermeen Ashraf: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Milada Vítová:** Conceptualization, Writing – review & editing, Supervision. **Peter**

Cloetens: Methodology, Formal analysis, Investigation, Visualization, Writing – review & editing. **Ana Mijovilovich:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Syed Nadeem Hussain Bokhari:** Methodology, Formal analysis, Writing – review & editing. **Hendrik Küpper:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Visualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.105818.

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CHAPTER 5

Identification of potential plant species hyperaccumulating Light Rare Earth Elements (LREE) in a mining area in Minas Gerais, Brazil

(Under-review in Environmental Science and Pollution Research)

Identification of potential plant species hyperaccumulating Light Rare Earth Elements (LREE) in a mining area in Minas Gerais, Brazil

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Abstract

Phytoextraction of rare earth elements (REE) from contaminated soils has gained importance during the last few decades. The Poços de Caldas municipality in Brazil is known for its mineral richness, including large reserves of REE. In this study, we report light REE (La, Ce, Sm, Pr, and Nd) in soils and plants collected in an area. Composite soil samples and plant individuals were collected and total concentrations of LREE in soils were determined by wavelength dispersive X-ray fluorescence (WDXRF). The plant available LREE concentrations in soils were estimated upon the acetic acid method (F1 fractions) of the stepwise sequential extraction procedure, together with plant content that was analysed by inductively coupled plasma mass spectrometry (ICP-MS). The total sum concentrations of tested LREE in soils varied from 5.6 up to 37.9 g kg^{-1}, the bioavailable fraction was *ca*. 1%, and a linear relationship was found between them. The only exception was Sm, whose availability was lesser and did not show a linear relationship. The concentration of LREE in non-accumulator plants varied from 1.3-950 mg kg^{-1} for Ce, La 1.1-99 mg kg^{-1}, Sm 0.04-9.31 mg kg^{-1}, Pr 0.1-24.1 mg kg^{-1}, and Nd 0.55-81 mg kg^{-1}. The concentration of LREE among shoots did not show a linear relation either with the available fraction or total content. The screening also revealed *Christella dentate* (Forssk.) Brownsey & Jermy, *Thelypteridaceae* family, as a promising hyperaccumulator species. The concentrations of LREE among shoots of six individuals of this species were in the ranges from 115-1872 mg kg^{-1} for Ce, La 190-703 mg kg^{-1}, Sm 9-48 mg kg^{-1}, Pr 32-144 mg kg^{-1}, and Nd 105-478 mg kg^{-1}.

Keywords: Phytoextraction, Poços de Caldas, light REE, acetic acid method, *Christella dentata* (Forssk.) Brownsey & Jermy, hyperaccumulator.

CHAPTER 6

Summary

CHAPTER 6: SUMMARY

6.1 DISCUSSION

As discussed earlier in the introductory chapter, the ultimate physiological response of any living organism to any variation in the environment is growth, as a consequence of changes in various aspects of physiology. Most of the metal studies on algae or plants were started with their effects on physiology, either determined by cell growth (measured by cell number in case of microorganisms) or biomass in plants. An immediate consequence of this hypothesis is the proposition of mechanisms for the determination of cell division for growth under La with rather beneficial effects and no acute response. This beneficial concept led to the development of this hypothesis to a variety of model species to strengthen this putative connection between La and cell growth. However, the hypothesis of beneficial effects of La and increase in biomass was rather called out unexpectedly by our results in Chapter 4. The experiments on cellular growth were started with a previous study on lanthanides in microalgae and their effects on growth (Goecke et al., 2017). It is one of the fundamental ways to determine the growth response of algae in presence of certain metals. Cell counting is highly convenient and has been widely used, however the most critical step in counting *D. quadricauda* under a microscope was to count the individual cell in a coenobium and fix them properly during measurements on counting chamber under a microscope. The present method of growth analysis was contradictory to the absorbance OD (Optical Density) measurements and dry weight measurements used previously by (Goecke et al., 2017). However, OD measurements are not useful as they could not tell the real cell numbers growing in culture. In the present method, successful cell growth was achieved in algal stock and chemostatic cultures. Guruvaiah and Lee (2014) reported the cell counts using a hemocytometer in *Desmodesmus* sp. previously known as *Scenedesmus* sp. In the present study, *D. quadricauda* (green microalgae) was chosen as a model organism to study metal (La) effects on physiology as algae are ecologically important organisms and are frequently used in environmental studies for assessing the relative toxicity of various chemical wastes discharged from industries (Tai et al., 2010). Thus, in the present study, individual cell counts in algal culture were found to be a more effective way for estimating accurate cell growth and its phases and

influence of metal (nM La) treatments as reported by a number of research reports. Our experiments on cellular growth analysis had shown no clear positive effect of La even under nanomolar treatments but showed that it was toxic. Further, the present results are contradictory to previous studies on lanthanum, which indicated stimulatory effects on plants, but on aquatic organisms, very few studies are available that indicate positive effects by alleviating metal deficiency as reported by (Li et al., 2011; Řezanka et al., 2016; Goecke et al., 2017). To our surprise, the results falsified the first hypothesis. The contrasting results of our present study, when compared to earlier studies, were mainly caused by the fact that previous studies were based mostly on artificial conditions aiming at high biomass. Further, they often lacked suitable organic ligands that allow La to be up taken by algal cells, and thus most of La got precipitated by phosphates in nutrient media. It was also shown that REE may replace other metals among a few physiological roles, as was demonstrated by alleviation of Ca^{2+} deficit (Goecke et al., 2015b). As lanthanides are considered an emerging pollutant in recent years, few studies concluded toxicity associated with rare earth elements to aquatic and land biota. However, consistent and deep-scientific investigations need to be conducted regarding transportation and *in situ* studies associated with these scarce metals and phytoremediation. Previously, it was claimed that REEs were able to improve growth, apparently depending on stress and the nutritional state of microalgae, raising the possibility of studying environmental impacts at even low concentrations. Our studies on La and its physiological effects are based on environmentally relevant conditions with the choice of a suitable ligand, allowing La uptake and low biomass to nutrient ratio simulating natural conditions. In order to reveal the mechanisms that might be involved in altering growth, the physiological response in the present study was investigated. This involved light-mediated photosynthesis of algal cells under lanthanum treatments, and we conducted experiments for determining chlorophyll fluorescence by using fluorescence kinetic microscopy or macroscopic fluorescence imaging that measures *in vivo* photosynthetic activity of PSII and heterogeneity among cells in response to La treatments. Our results presented in chapter 4 provide evidence that inhibition of cellular growth directly affects all the downstream metabolic processes, especially the sensitive targets that regulate photosynthetic machinery of cells, inhibited in our study. This was best illustrated by the most common parameters

that were directly influenced includes, PSIIRC activity, determined by F_v/F_m, hence this parameter was significantly decreased during the course of nM (lanthanum) treatments. Our present results are in line with the fact that this diminished energy captured photosynthesis consequently led to a reduction in growth (see Chapter 4). The results presented in this thesis were consistent with or contrary to the previous research, which makes our present study novel. All the photosynthetic parameters that were determined in the present study showed inhibition at 13.5 nM La treatment and above as compared to the control. F_0 (Minimal fluorescence) increased in most of the treatments as compared to Fm, which indicates that reaction centers associated with antenna complexes become inactive and therefore release excess excitons as fluorescence. NPQ (Non-photochemical quenching) increased during the exposure duration, which indicates light-mediated upregulation of dissipation of excitons in the form of heat. These prominent parameters in photosynthesis were selected as we know already from previous studies that the primary targets of metal on any photosynthetic organism is their photosynthetic machinery. Nonetheless, the overall inhibition of photochemistry reveals, La toxicity that ultimately inhibits cell divisions. Our results are contrary to the previous hypothesis, however, conducted under non-environmentally relevant conditions that indicate the positive role of lanthanum or lanthanides in general on photosynthesis. Recent studies are mainly based on potential toxic effects as lanthanides are now considered as an emerging pollutant, however, our conclusive physiological study based on environmentally realistic concentrations in aquatic biota indicates toxicity of La on microalgal cells in a nutrient-depleted media to understand physiology under environmental conditions similar to water bodies and thus our results when compared to the recent studies conducted by Xiao et al. (2021) showed net inhibition of leaf photosynthesis in response to lanthanum oxide nanoparticles in radish plant at >100 mg/L, but the study was conducted under more artificial conditions and treatments were out of the physiological range. To our surprise, the results confirmed the hypothesis that La can become toxic at environmentally relevant concentrations and conditions and has no beneficial effects on *D. quadricauda.* In order to build upon the results indicated the physiological response of microalgae to La further, experiments on intracellular lanthanum compartmentation and distribution were conducted in algal cells to understand the uptake mechanisms

(see Chapter 4) because metal localization at the cellular level decides all possible cellular processes that La might come into contact. Further, many aspects of metal uptake and metabolism are common in algal, animal, and even bacterial cells, therefore our study on microalgae can serve as a useful model for other organisms, as well. *In situ* experiments at ESRF (European Synchrotron Radiation Facility) were done with the aim to reveal the true localization of lanthanum inside the cells, in order to understand its role in cellular metabolism. To elucidate the mechanistic approaches of the effects of REE is hardly known, therefore, little attention has been paid to their impacts on aquatic species, thus in our present experiments, localization was investigated by carrying out X-ray fluorescence (XRF) at microscopic level on frozen-hydrated algal cells. Previously, studies were conducted mainly on the impacts of REE on pigment profile, lipids, and fatty acids content in algal cells for biotechnological purposes (Goecke et al., 2017). For localization of La, fluorescent dyes, for example, Calcium Green I (which has a higher affinity for lanthanides) showed Ce, La, and Gd in the cytoplasm, while Nd is in the chloroplast (Řezanka et al., 2016). However, this method is not validated for true localization because of uneven loading of dye in different cellular compartments and these dyes only show weakly bound ions, so strongly bound REE would remain invisible. Therefore, for correct localization μXRF is quite feasible and is insensitive to the aforementioned problems of dyes. Our results were mainly based on the synchrotron μXRF, for intracellular localization of La together with other metals for comparison, most of the algal cells cultivated under lethal and sub-lethal (nM) La, were located inside the cell, mainly in the cytoplasm as at high La the entire cell was filled homogeneously with lanthanum. This could explain the possible binding of lanthanum to enzymes or proteins, which could explain the direct role of lanthanum in metabolism that surprisingly led to the cell death and toxicity response, as what was concluded for physiological studies in algae. However, at increasing La concentration (lethal concentration), cells were extremely stressed and most of the K leaked out of the cells at this concentration, cells showed unspecific filling with La that covers the whole cell, however at extreme toxicity cells were alive, as cells own defensive response keep La away from highly sensitive cellular structures. This could explain the changing targets of La that bind in algal cells treated with sub-nanomolar to low nanomolar La., variation in response from toxic to sub-lethal to acute

toxicity. Analysis of metal binding to proteins or metabolites needs the use of a new system with high sensitivity that will allow the use of metalloproteomics in many fields of basic and applied research. HPLC-ICPMS provides a new system to analyze metal binding (lanthanum) under environmentally relevant metal ultra-trace (<ppt) concentrations of various analytes with a dynamic range. Our current study provides a new way for ultra-trace metal speciation and its analysis by using sector-field ICP-MS coupled with High-Resolution Size Exclusion and Reversed-Phase Liquid Chromatography (see Chapter 3 of this thesis) for more details. The initial experiments on metalloproteomics used the binding of lanthanum (La^{3+}) in green microalgae (*D. quadricauda*) as one of many application examples (Küpper et al., 2019). It was investigated using sf-ICP-MS coupled to high-resolution liquid chromatography on lanthanum binding to soluble proteins in microalgae treated with nano and sub-nanomolar La concentrations and showed that even at low environmental concentration La^{3+} binds to a soluble protein of approximately 20kDa in freshwater green microalgae of approximately 5 mg of fresh weight. This indicates the sensitivity of the technique that can detect such low levels in a few milligrams of samples. Detailed analysis of lanthanum binding soluble proteins is shown in (Chapter 4). In our present study on metalloproteomics, we showed at least 2 soluble peptides that have a high affinity for La at environmental concentration, the apparent size of 3kDa and >500kDa, respectively. These protein complexes are interesting for further analysis including purification and characterization. However, the >500kDa protein aggregates observed might be an aggregate of the 20kDa protein that was previously observed (Chapter 3) that was not found in our detailed metalloproteomic analysis. Our present results showed different target proteins at different La concentrations that indicate the different toxicity mechanisms in microalgae, very specific at low La but less specific at high La treatments. However, the following results confirmed our hypothesis that La can bind to proteins in algal cells exposed to natural conditions and concentrations. Environmental contamination is associated with the mining of metals, especially lanthanides in our present study as because of their increasing use in technologies worldwide, new reserves around the world have been explored through continuous mining, this raises concerns regarding remediation of highly polluted sites (Grosjean et al., 2020). Plant-based remediation techniques could help reducing the

possible risks of REE contamination through the use of REE-accumulating plants. Thus, in our present study we aimed to explore possible land plants that could be used for phytoremediation of REE from soils contaminated with them. In Chapter 5 we, in collaboration with our partners from the University of Sao Paulo, Brazil, investigated soils and plants REE content (mainly, light REE of the series; La, Ce, Nd, Pr, and Sm) for new hyperaccumulators. According to the USGS statistics, 2019, Brazil is the second-largest country for rare earth reserves, thus field studies were conducted in the ex-mining area for LREE using analytical techniques and possible extraction from the soils for bioavailability and their uptake and accumulation in aerial parts of plants. In a third study (Chapter 5), green remediation or botanical bioremediation approach was applied for REE remediation. This method can be applied to both organic and inorganic pollutants from soil, air, or water (Garbisu and Alkorta, 2001). Plants can concentrate REE that varies significantly from species to species, our results also showed significant variation among species belonging to the same family which is further explained in more detail in the below section in comparison with soils as an intricate system for bioavailability. The rising importance of REE phytoremediation was the main starting point for the third study in the present thesis. The total soil LREE concentration was analyzed in the soil samples using the WDXRF technique and their relation to concentrations that were bioavailable using ICP-MS to allow full quantification for soil LREE were performed. Total Ce was found in abundant concentration in all soil samples, as compared to La, Nd, Pr, and Sm. The total LREE found in our present study were comparatively higher as compared to the total concentration of LREE found in a recent study conducted in Poland measured mg/kg. Such higher concentrations could be dangerous for naturally grown plants, as we found that higher concentrations can affect plants negatively, and we also found that even low concentrations are detrimental for algal cells. Here we discuss the extractable concentration that plants can easily uptake via roots. The acetic acid method was employed to extract LREE from collected soil samples to understand bioavailability in our present study and we found that bioavailable LREE are quite less than total LREE, thus this explains that these metals are not easily bioavailable as many factors influence the available content. Following method reveals around 1% of the total content is available to plants. This value agrees with values previously reported by (Li et al.,

1998). The observed total LREEs distribution in soil profiles in the current study agrees with that displayed by the Oddo-Harkins rule, *i.e*., Ce>La>Nd>Pr>Sm (Zhenggui et al., 2001). This pattern follows the previous geochemical soil surveys. Poor positive correlation between total and extractable LREE were found in our present thesis, this explains the difficult dissolution of these elements and hence needs more complicated mechanisms. Our present field analysis indicates that LREE were not equally distributed with high availability of Ce was found and least for Sm that indicates low correlation coefficient for Sm with significance was greater than 5%, however, Ce shows the higher coefficient of determination at significantly lower than 5%. The inconsistent availability of LREE could be explained by an extraction procedure that is much more effective under low pH to mobilize bound oxides, sulfides, and organic matter, resulting in different bioavailability ratios of each lanthanide to plants. High observed bioavailability of Ce having a share of 37-40% than Sm as mentioned above was supported by Mittermüller et al. (2016) that explains the mobilization of REE in soils. Details of the results from that part of the study is explained in more detail in (Chapter 5). Below we are discussing now the major results associated with LREE in higher plants for phytoremediation. The experiments on the accumulation of LREE among land plants were started with a study among shoots of the hyperaccumulator *Dicranopteris dichotoma* grown at REE ores are extensively being studied (Wang et al., 1997) and relatively high concentrations mainly for LREE were found. In our present study, we screened potentially suitable plant species that can accumulate REE (specifically, Light Rare Earth Elements) from the contaminated mining area. In Chapter 5 we assess potential availability for LREE (Light Rare Earth Elements) to plants under natural conditions and by using ICP-MS we quantify the potential accumulators. We found plants grown under natural and uncontrollable conditions, for their LREE hyperaccumulation capacities. We found that in fern species REE concentrations were consistently higher than other collected plants from other families and thus showed strong accumulation capacity associated with them for REE. This indicates the high strong tolerance associated with fern leaves for REE. The concentrations of REE found in shoots are easily compared with rather good accumulators for REE found by Grosjean *et al*. (2020) in collected fern species that can accumulate in the range almost similar to what we have found. This first study in a

Brazilian mining site demonstrated that *Christella denata,* a fern belonging to the *Thelypteridaceae* family exhibits the highest concentration of LREE. Our recently identified fern species has the highest cerium content ranging from 115 up to 1872 mg/kg. Our results when compared with natural REE accumulators, like *D. dichotoma* grown in the ex-mining area in China we found lower levels in *C. dentata* shoots but appreciable concentrations in comparison with other harvested plants. Finally, a preliminary study indicates that our suggested plant species is found to be relatively a good hyperaccumulator for LREE Thus as the main conclusions it can be stated that *C. dentata*, has an enormous tendency for accumulation. However, the higher total LREE concentration found in soils in our present study does not show any concern to the plant, therefore the results do not confirm our second last hypothesis. Thus, it can be used as an indicator species.

6.2 CONCLUSIONS

In order to summarize the crucial messages of this work we will return to the combinations of null and working hypothesis that we summarized in Chapter 2. So in doing so we can state that:

According to our data we must **accept <u>null hypothesis 1</u>**. This is to say that the La under nanomolar environmental conditions in *D. quadricauda* does not show any beneficial effects in correlation with the cell growth process, rather showed acute toxic effects, therefore our extensive research under laboratory scale showed that **<u>working hypothesis 2</u>** was **verified** and La does inhibit the photosynthetic machinery in *D. quadricauda*; it causes toxic effect that ultimately lead to cell death.

Detailed analysis on La-binding proteins, indicates that out **<u>working hypothesis 3</u>** is **verified**, as La binds to a protein already at the lowest applied concentrations, with a change of the targets towards higher La concentrations. This leads to the conclusion that La in *D. quadricauda*, binds with high affinity to target proteins.

Regarding our **<u>working hypothesis 4</u>**, we **cannot accept** that high total content of LREE in soil has no detrimental effect on terrestrial plants, thus only viable content that plant can take up easily has role in growth. However, LREE showed no toxic effects on naturally grown plants.

6.3 FURTHER DEVELOPMENT IN FUTURE

This thesis is based on many new results on the development of studies concerning the physiology and influence of metals on freshwater aquatic green algae (*Desmodesmus quadricauda*) and metal accumulating plants (Metallophytes). Responses of heavy metal stress and its potential toxicity on microalgal species are currently used for the purification of industrial pollutants. Algae (micro- or macro) can serve as a primary producer in an aquatic food chain thus, the accumulation of metals leads to toxic effects for freshwater biota. Besides algae, plants serve a high capacity for metal remediation from soils because of their extensive root systems for metal uptake from contaminated soils and its accumulation in shoots. The first part of the present study involved *D. quadricauda*. (Chlorophyceae) that is being used as a model organism for the remediation of lanthanides specifically (lanthanum), followed by physiological responses, lanthanum uptake, and its compartmentation and metalloproteomics. The second part involves a field study for searching of suitable hyperaccumulators or possible accumulators and other plants grown in soils for LREE bioremediation by determining total and bioavailable contents and plant LREE content using analytical techniques. Thus, the present study circulates not only on aquatic biota (microalgae) but also around soil biota (plants) for developing remediation strategies for potential contaminants specifically rare earth from both ecosystems and its further biochemical and biophysical analysis. Thus, in the future, good accumulator plant species can be used as a model for LREE studies concerning more detailed research on Ln-based metalloproteins and so on. Therefore, the results presented in the publications that came out from the thesis were important to study scarce metals in aquatic and terrestrial ecosystems. Further work may include the detailed inhibition of photosynthesis that could be explained by the fact that divalent lanthanum ions inhibit Ca^{2+} channels, by blocking the channel, thus, therefore, resisting permeability of calcium ions across the membranes, as it is assumed that lanthanides used the same channel as Ca^{2+} channel for its transportation through the membrane. These cations could replace the functional Ca^{2+} dependent OEC (Oxygen Evolving Complex) with ionic radii similar to Ca yet showed higher affinity for Ca sites and oxygen evolution is suppressed, OEC can be protected or restored from this inhibition, induced by lanthanides when treated with Ca (Ono, 2000). Magnesium replacement by Ln^{3+} in chlorophylls could be another

possibility for indirect stress to photosystems in our present results. Thus, more accurate quantification of chlorophylls and their derivatives in the extracts of lanthanides treated cells need to be studied in the future to further *in vivo* studies. This involves, Mg-substitution by chromatography (HPLC) and well-defined photometric quantifications. Further studies concerning Mg-substitution *in situ* with lanthanides should mainly aim in finding the reasons for accessibility of the LHCII in algae or higher plants and aiming mainly the more direct evidence concerning the substitution of lanthanides with naturally occurring Mg in the PSIIRC during sun reactions, together with ROS (mainly singlet oxygen formation) with lanthanides substituted chlorophylls could be a more promising approach to understand the toxic effects associated with La (or generally lanthanides) and analyzing the in-depth effects of singlet oxygen formation by Mg-substitution and quenching found in the presence of lanthanides that ultimately influence the production of singlets in the cellular system under more relevant natural conditions. X-ray fluorescence tomography in shock-frozen hydrated samples was done to prevent element re-distribution. This reveals exactly in which cellular compartment or cellular structure, any particular lanthanide (La) accumulates. Since different cellular structures and compartments have different known functions in the metabolism of the cell, therefore in the future this technique will yield important insights into the mechanisms of REE effects and could clarify the substantial mechanisms by which these non-essential elements can so efficiently affect many metabolic processes in algae and plant cells. The toxic effect of lanthanum in microalgae could be explained by the loss of K from the cells at lethal concentrations, however, this was revealed by the additional peaks appearing in metalloproteomics and inhibition of photosynthesis. In this scenario, the unspecific effects associated with La toxicity need in-depth study in the future to understand the uptake mechanisms of lanthanides specifically through algal cell walls together with the identification of >500kDa protein aggregates that showed different responses with increasing La treatments in metalloproteomics.

Our present competitive study on LREE in plants does not show any detrimental effects, this could be explained by the extractable content that is available to living biota. Therefore, it is necessary to explain in the future by conducting further research on REE leached out from the mining area and available to plants and also to explain in

more detail the soil physical properties as the soil is an intricate system and other factors that particularly influence their mobility. The present BCR method doesn't explain the procedure for phosphates associated with lanthanides in soil materials. Thus, in future methods evaluating REE bound with phosphates should be explained, besides extraction procedure, soil parent material is also an important factor as the soil is an intricate system and soil-biota interactions are complicated. In the future, *Christella dentata* (a new potentially hyperaccumulator fern) can be used for REE compartmentation and speciation analysis may provide information to study the biochemical functions and also provide ideal material to study the long-term biological effects of REE on higher plants.

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Curriculum Vitae

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- Hendrik Küpper, Syed Nadeem Hussain Bokhari, Noelia Jaime-Pérez, Lyudmila Lyubenova, **Nermeen Ashraf,** and Elisa Andresen (2019). **Ultratrace Metal Speciation Analysis by Coupling of Sector-Field ICP-MS to High-Resolution Size Exclusion and Reversed-Phase Liquid Chromatography.** *Analytical Chemistry* 91(17), 10961-10969.

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PhD student international mobility grant from University of South Bohemia, České Budějovice 2019

SELECTED INTERNATIONAL CONFERENCE PARTICIPATION:

Participation in "8th INTERNATIONAL CONFERENCE ON BIOLOGICAL AND COMPUTATIONAL SCIENCES" C-BICS, 2020, Islamabad, Pakistan.

Participation in Chemistry of metals in biological systems 2019, Lisbon, Portugal for poster presentation on "Effects of Lanthanum in green microalgae".

Participation in Plant Biology conference 2017, Comenius University Bratislava, Slovakia for Poster presentation on "Genetic transformation of *Lycopersicum esculentum* using an abiotic stress tolerant gene".

SELECTED NATIONAL CONFERENCE PARTICIPATIONS:

Participation in KOROLID mini-symposium 2017, CAS, České Budějovice, Czech Republic for Poster presentation on "Genetic transformation of *Lycopersicum esculentum* using an abiotic stress tolerant gene".

Participation in Plant Biology conference 2019, University of South Bohemia, České Budějovice, Czech Republic for Poster presentation on "Effects of rare earth elements in green microalgae "*Desmodesmus quadricauda*".

Participation in USB conference of Doctoral Students, 2021, University of South Bohemia, České Budějovice, Czech Republic for Talk presentation on "Lanthanides in Microalga, soils and plant – current knowledge at biochemical and biophysical level".

INTERNSHIP:

Participation in foreign internship, Oct/Nov 2019, University of São Paulo, Centre of Nuclear Energy and Agriculture, Laboratory of Nuclear Instrumentation, Brazil for Rare Earth Elements in soils and plants.

I hereby declare that all the information provided by me is factual & correct to the best of my knowledge & belief.

Place: České Budějovice, Czech Republic

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Title: Beneficial and toxic effects of REE in algae and plants

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