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ELISE JOONAS

Evaluation of metal contaminant hazard on microalgae with environmentally relevant testing strategies

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Department of Botany, Institute of Ecology and Earth Sciences, Faculty of Science and Technology, University of Tartu, Estonia.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numerals:

I **Joonas, E.**, Aruoja, V., Olli, K., Syvertsen-Wiig, G., Vija, H., & Kahru, A. (2017). Potency of (doped) rare earth oxide particles and their constituent metals to inhibit algal growth and induce direct toxic effects. *Science of the Total Environment*, *593*, 478–486.

II **Joonas, E.**, Aruoja, V., Olli, K., & Kahru, A. (2019). Environmental safety data on CuO and TiO_2 nanoparticles for multiple algal species in natural water: filling the data gaps for risk assessment. *Science of the total viroment*, *647*, 973–980.

III **Joonas, E.**, Olli, K., Kahru, A., & Aruoja, V. (2021). Biodiversity and functional trait effects on copper toxicity in a proof-of-concept multispecies microalgal assay. *Algal Research*, *55*, 102204.

The participation of the author in preparing the listed publications is as follows (* denotes a moderate contribution, ** a high contribution, *** the leading role):

	I	**II**	**III**
Original idea		*	**
Study design	*	**	***
Data collection	***	***	***
Data analysis	***	***	***
Manuscript preparation	***	***	***

ABBREVIATIONS

ANW	nutrient-adjusted natural water
BEF	biodiversity and ecosystem functioning
BET	Brunauer-Emmett-Teller method for quantifying specific surface area
DI	deionized
DLS	dynamic light scattering
DOC	dissolved organic carbon
EC	European Commission
EC_{50}	median effective concentration of a substance that induces an adverse effect of 50% on the studied parameter after a specified exposure time
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EFSA PPR	European Food Safety Authority Panel on Plant Protection and their Residues
EU	European Union
Fv/Fm	effective quantum yield of photosystem II
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
MBC	minimal biocidal concentration
MRI	magnet resonance imaging
NOM	natural organic matter
NP	nanoparticle
OECD	Organization for Economic Co-operation and Development
REACH	European Union regulation (EC) No 1907/2006 on Registration, Evaluation, Authorisation and Restriction of Chemicals
REE	rare earth element
REO	rare earth element oxide
ROS	reactive oxygen species
SD	standard deviation
SR	species richness
SDG	United Nations Sustainable Development Goals
SSA	specific surface area
US EPA	United States Environmental Protection Agency
USGS	Unites States Geological Survey

1 INTRODUCTION

Chemical pollution is an exacerbating environmental concern that is even thought by some estimates to outpace other agents of global change, such as nutrient pollution or habitat destruction, due to the increasing diversification and quantities of chemical outputs (Bernhardt et al., 2017). Aquatic environments are particularly at risk, because they act as a sink for most environmental contaminants (Scown et al., 2010). Indeed, pollution is considered among the chief threats affecting freshwater and marine species alike, due to anthropogenic introduction of heavy metals, waste disposal, and fuel leaks among other sources (WWF, 2020, 2014). The organisms within waterbodies are directly exposed to these contaminants via their surrounding environment, which is why they are also the focus of REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) chemical safety assessments (ECHA, 2017a). Microalgae, the focus of my research, are relevant as primary producers within aquatic environments, accounting for ~50% of global primary production (Field et al., 1998). Microalgae are also often indicated as the most sensitive or among the more sensitive aquatic organism groups to contaminants next to zooplankton (Kahru and Dubourguier, 2010; Tebby et al., 2011; Weyers et al., 2000). Additionally, their short generation times are useful to detect rapid responses to various contaminants both in a laboratory setting and in using algal assemblages as a biomonitoring tool (McCormick and Cairns, 1994).

1.1 Microalgal ecotoxicological tests

According to the REACH regulation (European Commission, 2006), toxicity to freshwater algae must be quantified for all substances manufactured in or imported into the European Union in amounts exceeding 1 tonne per year. Algal toxicity quantification is also a requirement for the authorization of pesticide active ingredients (European Commission, 2013a) and formulated plant protection products (European Commission, 2013b). For this purpose, the standard algal growth inhibition test is usually conducted based on the Organization for Economic Co-operation and Development (OECD) test guideline 201 (OECD, 2011) or a similar guideline by the US Environmental Protection Agency (EPA, 1996). These types of standardized single-species study protocols are useful, because they are conducted in optimal conditions, in which it is possible to isolate toxicant effects from other stressors (Connon et al., 2012). For example, this type of testing has allowed for high-throughput efficient regulatory screening of the more than 23000 chemicals registered under REACH as of October 2021 (ECHA, 2021). However, single-species testing presents limitations in representativeness. One way to alleviate these innate weaknesses is conducting the monoculture test on multiple species separately, thus increasing certainty about the average sensitivity of algae towards a toxicant, which may be used in the derivation of species sensitivity distributions (SSDs) (EFSA PPR, 2013; Fettweis et al., 2021).

Alternatively, experimental setups with microalgae from multispecies experiments to mesocosms measuring various endpoints (Fig. 1) are also used for research and for refining ecotoxicological risk assessment in a regulatory context (EFSA PPR, 2013). Outdoor microcosms and mesocosms are studies conducted in realistic field conditions and may be constructed either with portions of samples from aquatic ecosystems, or are conducted in an enclosed section of natural waterbodies (OECD, 2006). Comprising of many species from several trophic levels, mesocosms and microcosms offer the possibility to evaluate stressor effects on multiple species simultaneously, evaluate recovery of the organisms from the exposure, detect indirect and sub-lethal changes in species richness and ecosystem function (Bernhardt et al., 2017; OECD, 2006; Wilson et al., 2004). These studies can generate different outcomes from single-species assays, since novel properties emerge with added complexity due to factors like species interaction or adaptation (Connon et al., 2012; Pomati and Nizzetto, 2013; Relyea and Hoverman, 2006). However, trade-offs are expected, as increasing ecological relevance is inevitably related to a decrease in assay reproducibility and specificity (Fig. 1) (Chapman and Maund, 1998; Connon et al., 2012; EFSA PPR, 2013).

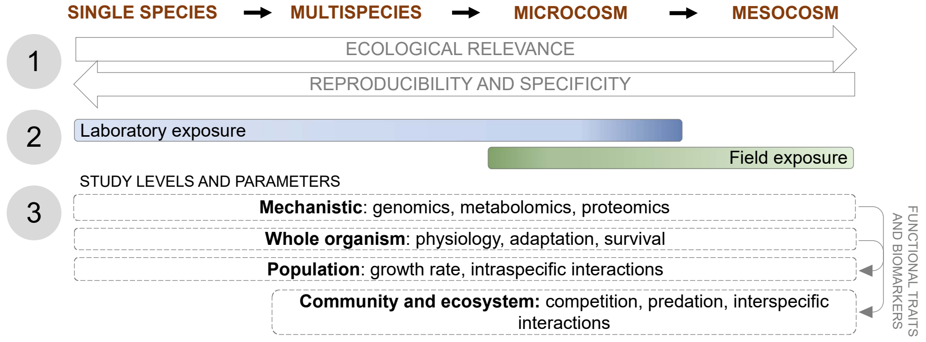

Figure 1 Microalgal toxicity test scope depending on study format. Pictured are 1) trade-offs in environmental relevance and reproducibility, 2) applicable exposure types for each format, 3) suitable parameters for study formats. Scheme partially adapted from Connon et al. (2012).

1.2 Biodiversity in ecotoxicological research

Toxicity testing and accompanying risk assessment is conducted to protect ecosystem structure, function and, more anthropocentrically, the ecosystem services provided. It has been generally established that higher species richness leads to more productivity and a more complete use of resources, decreases risk deriving from stochastic environmental changes, and protects ecosystems from invasive species (Clements and Rohr, 2009; Stravs et al., 2019). The provision of ecosystem services, such as primary production, energy flow and nutrient recycling, thus relies specifically on biodiversity, again establishing the importance of its protection (Clements and Rohr, 2009). According to the insurance hypothesis, biodiversity protects against ecosystem functioning declines during exposure to toxic stressors, thus making more diverse communities more resilient when facing chemical perturbations compared to less diverse communities (Viaene et al., 2013). This occurs, because the loss of sensitive species contributing to some ecosystem process can be compensated for by tolerant species that also perform the same function (Vinebrooke et al., 2004). In addition to this functional redundancy, the sampling effect may cause the positive stability-biodiversity relationship. Being more species-rich increases the likelihood of a community hosting a species that has a dominant effect on an ecosystem function or in coping with a toxicant stressor (Huston, 1997; Van den Brink et al., 2011).

1.3 Trait-based approaches in ecotoxicology

Traits are an organism's genetic, morphological, physiological, or life-history characteristics that influence its ecological functioning. Diversity in these functional traits, or functional diversity (FD), has been shown to better predict ecosystem functioning compared to taxonomic diversity (Cardinale et al., 2012; Fontana et al., 2014; Hillebrand and Matthiessen, 2009; Reiss et al., 2009; Suding et al., 2008). Trait-based approaches create mechanistic links between lower and higher levels of biological organization, because characteristics present at the sub-organism (molecular, organelle level) and organism level can be related to population, community or ecosystem level outcomes (Fig. 1). Trait-based approaches are broadly used in ecology and are also useful in ecotoxicology to understand, how toxicant-induced shifts in the presence of traits or their values at an organism or population level relate to changes in the ecosystem as a whole (Pomati and Nizzetto, 2013).

With the approach of functional traits, information can be gained about what kinds of species are more or less resilient to a certain toxicant. Traits may therefore be an easily measurable alternative or supplement to commonly used mechanistic toxicity assessment methods, such as genomics, proteomics and metabolomics (Connon et al., 2012; Van den Brink et al., 2011). Measuring stressor-related functional trait composition instead of species' composition offers benefits, because functional traits act as a common currency between different ecosystems. Using functional traits allows to compare stressor effects across large spatial scales encompassing highly variable populations, and therefore to uncover overarching change-driving mechanisms (Liess and Beketov, 2011; Pomati and Nizzetto, 2013; Salis et al., 2019; Van den Brink et al., 2011).

Ecotoxicologically relevant functional traits often include characteristics related to toxicokinetics (transfer and fate of toxicant in the organism) and toxicodynamics (toxicological outcomes), because these control the internal exposure and the sensitivity of the organism to that exposure, respectively (EFSA Scientific Committee, 2016). Relevant traits that explicitly increase a species' sensitivity to toxicants include a higher surface to volume ratio, a higher absorption rate of the toxicant from the environment, lower metabolism rate, possessing organs that accumulate the toxicant, having a lower growth rate, possessing particularly sensitive life stages that co-occur with toxicant exposure, possessing more high-affinity molecular receptors, and low reproduction rate (EFSA Scientific Committee, 2016). Single functional traits are often combined into multi-trait approaches, such as functional diversity indexes, which allow to account for many traits and their relative abundance or average values within populations, and therefore provide a more comprehensive view of trait variability in communities (Suding et al., 2008).

In the case of microalgae, high diversity despite a seemingly homogenous environment was already highlighted by Hutchinson (1961) and called the paradox of the plankton. One facet of microalgal diversity that has been garnering more attention and application during the past decades is functional or trait diversity. Diversity in microalgal functional traits is related to the niche differences deriving from the availability of resources and general environmental conditions (Litchman and Klausmeier, 2008). For phytoplankton, alongside cell shape, colony formation and pigment composition, cell size is a key functional trait, affecting cellular composition, metabolic rates, nutrient uptake and growth rates (Marañón, 2015; Pomati and Nizzetto, 2013).

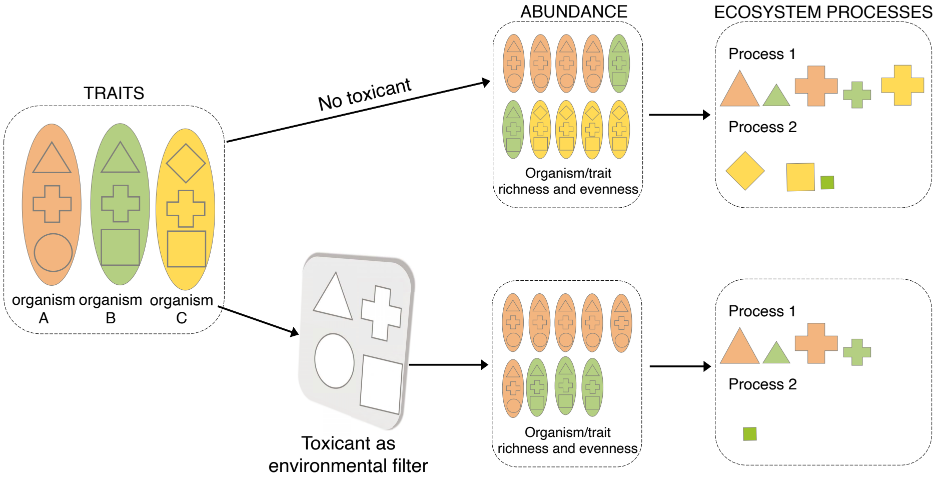

Figure 2 Filtering effect of toxicants on functional traits and consequences on ecosystem process rates compared to non-exposed organisms. Colours represent different species and shapes within the coloured ovals represent species' traits. Adapted from Reiss *et al.* (2009).

Exposure to pollutants decreases functional trait variance and may be thought of as an environmental filter for organisms possessing sensitivity-determining traits (Fig. 2). This concept has, for example, been realized for freshwater invertebrate communities as the SPEAR index, which has shown good results in correlating combinations of species' traits with their sensitivity to pesticides at much lower concentrations than usual community measures (*e.g.* principal response curves), and even allowing for identification of diffuse contamination in water bodies

(Liess and Beketov, 2011; Liess and Von Der Ohe, 2005). Furthermore, it is suggested by Pomati *et al.* (2017) that the capacity of a community to respond to environmental fluctuations is negatively affected by exposure to pollutants, in that case pharmaceutical and personal care products. This means that specific trait combinations are selected for under toxicant exposure, which may not be advantageous under other types of environmental stressors.

1.4 Aquatic hazard of metals: common and emerging contaminants

Many metals are essential elements for living organisms, meaning that deviance from an optimum to both lower and higher levels leads to adverse outcomes (Andersen, 2005; Walker et al., 2012). The vast majority of metal pollution is anthropogenic and is compounded by metals inherently not being degradable and having long residence times in soils and sediments (Walker et al., 2012). Metal pollution is also an intensifying problem, considering steadily rising global metal concentrations observed in rivers and lakes during the past 50 years (Li et al., 2019).

In this thesis, in addition to Cu, a commonplace and extensively studied metal, I focused on novel types of compounds that may be considered contaminants of emerging concern – the REEs (rare earth elements). According to Sauvé and Desrosiers (2014), the REE-doped particles and metal oxide nanoparticles (NPs) used in our studies may be considered true emerging contaminants, as they are new compounds that have not previously been known, at least in terms of the novel characteristics arising from their nano-scale size and use in particles consisting of multiple metals. REEs can be defined as contaminants of emerging interest or concern, because, while their existence was known beforehand, they are now being released in unprecedented amounts (Gwenzi et al., 2018; Sauvé and Desrosiers, 2014).

1.4.1 Rare earth elements: technologically critical novel contaminants

Rare earth elements, as defined by IUPAC, are 17 elements of the periodic table including yttrium and scandium in addition to 15 lanthanides, united by similar physicochemical properties (Navarro and Zhao, 2014). At concentrations around 0.8%, REEs are more abundant in the Earth's crust than Cu or Pb (Navarro and Zhao, 2014), but in contrast do not occur as distinct metal ores. Rather, all REEs are found together in usually either silicate, carbonate, oxide or phosphate mineral deposits (Balaram, 2019). China has up until now been the major global supplier and purifier of REEs, causing a volatile market and insecure supply with their monopoly (Navarro and Zhao, 2014). This situation, will, however, likely change by exploitation of new sources, including high-REE content deep-sea mud uncovered in the Pacific Ocean by Japan (Kato et al., 2011).

REEs are seen as indispensable for high-technology industries in particular (UNCTAD, 2014), even being called the 'vitamins of modern industry' (Balaram, 2019). They are crucial for uses in many sustainable technologies, including in the composition of permanent magnets in the engines of hybrid vehicles and wind turbines (Bonfante et al., 2021; Langkau and Erdmann, 2020). In a review of 44 life-cycle analyses of REEs, Bonfante et al. (2021) conclude that the use of these elements supports the UN Sustainable Development Goals (SDGs) by contributing to a greener economy, possessing the potential to support sustainable city and community development, supporting responsible consumption and production and showing potential for end-of-life recycling. Additionally, REEs have been used and directly introduced into the environment as fertilizers (Balaram, 2019) or as unintentional residues in phosphate fertilizers (Gwenzi et al., 2018). La-modified bentonite has even been directly used in bioremediation of eutrophic water bodies due to its phosphate-binding efficiency (Copetti et al., 2015).

These myriad uses for REEs have dictated surges in mining, as evidenced by global mining rates doubling from 124 000 tonnes/year in 2015 to 240 000 tonnes/year in 2020 (USGS, 2021a, 2016), in line with projections that predict further increases in REE demand (Alonso et al., 2012). For perspective, mining rates of Cu exceed those of REEs by nearly two orders of magnitude (Schüler et al., 2011; USGS, 2021b). In combination with REEs being mostly disposed of in landfills in current absence of relevant reuse and recycling (Binnemans et al., 2013; EPA, 2012), their environmental impacts are likely to significantly increase in the near future (Langkau and Erdmann, 2020).

These risks are already materializing, because increased REE levels from nanogram per litre (Bau and Dulski, 1996; Hatje et al., 2016; Klaver et al., 2014) to milligram per litre range (Kulaksiz and Bau, 2011; Migaszewski et al., 2016) have been detected in waterbodies worldwide compared to natural background levels. Especially, gadolinium (Gd) anomalies have been widely observed (Balaram, 2019) near densely populated areas in Central Europe and North America starting from mid-1990s, mostly due to release of gadolinium-based MRI contrast agents used in hospitals (Bau and Dulski, 1996). Since then, heightened Gd levels have been detected in rivers, lakes, estuaries and coastal waters, ground water, waste water, tap water, mine drainage and alluvial sediments (Gwenzi et al., 2018; Klaver et al., 2014; Kulaksiz and Bau, 2013; Rogowska et al., 2018). Irregularly high lanthanum (La) and samarium (Sm) additions have also been observed in the Rhine river in Germany as a result of release from a point source – a catalyst production facility (Klaver et al., 2014; Kulaksiz and Bau, 2013, 2011). Further environmental inputs are expected from multiple point and diffuse sources, such as research facilities, pharmaceutical and high-technology industries, mining and mineral processing, electronic waste, electrical equipment, recycling plants, fertilizers and livestock feeds (Gwenzi et al., 2018). Release from REE-rich mine tailings also occurs, as is the case for the copious waste generated as the by-product from the Estonian oil shale based electricity production (Gavrilova et al., 2005). REE contents were shown to reach up to 100 mg/kg of waste mass (Sørlie et al., 2004) and aquatic macrophyte species near oil shale industry sites show increased rates of REE accumulation (Blinova et al., 2021).

Trends of increased environmental inputs necessitate a better understanding of REE effects on living organisms. Even though REE mining is expected to generate more of an impact than these detected REE anomalies (EPA, 2012; Navarro and Zhao, 2014), the effects of REEs themselves should be examined as contaminants of emerging concern. Already, REEs have been shown to elicit adverse effects in different organism groups including plants (nutrition quality, root growth), animals (geno- and neurotoxicity), bacteria, soil organisms, and aquatic organisms at all trophic levels (Gwenzi et al., 2018). Being regarded as contaminants of emerging concerns, studying REE ecotoxicologial properites has steadily increased during the past 15 years, including research concerning microalgae (Fig. 3).

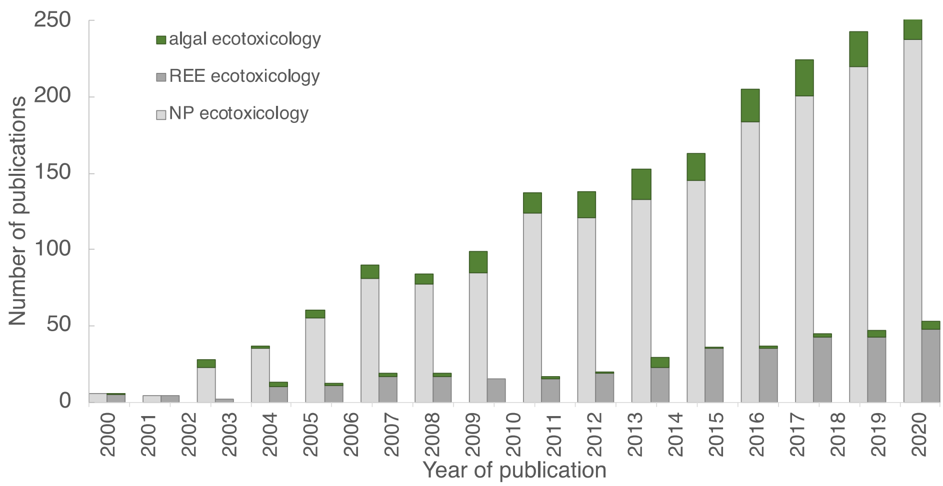

Figure 3 Publication numbers by year concerning the general ecotoxicity of rare earth elements (REE) and metal-based nanoparticles (NPs) and concerning algal ecotoxicology within the wider topic. Specific search terms used are presented in Table 1.

Table 1 Search terms used to retrieve publication numbers presented in Fig. 3 from Scopus (retrieved on 17th January 2022). General ecotoxicology and algae related search terms were used similarly for both material types.

Scope of search	Specific search terms used	
1. Search terms on general eco-toxicology and specific materials	TITLE-ABS-KEY (ecotoxic* OR “environmental* toxic*” OR “environmental* hazard*”)	
	AND TITLE-ABS-KEY (metal* AND (nano* OR nanopartic*))	AND TITLE-ABS-KEY (lanthanide* OR “rare earth” OR lanthanum OR cerium OR praseodymium OR neodymium OR samarium OR europium OR gadolinium OR terbium OR dysprosium OR holmium OR erbium OR thulium OR ytterbium OR lutetium)
2. Additional algae-related search terms	AND TITLE-ABS-KEY(alga* OR phytoplankton)	

1.4.2 Dissolved copper (II) contamination of water bodies

Cu is an essential nutrient for all organisms, as the functioning of many proteins depends on this metal in bacteria, fungi, animals and plants alike (Festa and Thiele, 2011). Cu, however, exhibits a biphasic dose-response relationship: while beneficial in minute amounts, it already becomes toxic at relatively low concentrations (Andersen, 2005). Cu is deemed a high risk active ingredient for aquatic organisms according to the risk assessment conducted by the European Food Safety Authority for different bactericidal and fungicidal Cu compounds, which are also used in organic farming (EFSA, 2018a). This is reflected in the algal EC_{50} values of Cu^{2+} that remain below 0.1 mg l^{-1} for $CuSO_4$ (ECHA, 2020). Other substances containing Cu, such as CuOH (lowest EC_{50} 0.022 mg l^{-1}), copper oxychloride (lowest EC_{50} 49.8 mg l^{-1}), Bordeaux mixture (mixture of copper $CuSO_4$ and CaO; lowest EC_{50} 0.07 mg l^{-1}) or CuO (lowest EC_{50} 0.045 mg l^{-1}) (EFSA, 2018b) have also been demonstrated to be highly toxic towards algae.

Cu is currently being newly mined at high rates and reserves are not yet depleted, with an estimated 870 million tonnes still in global reserves (USGS, 2021b). This trend indicates that more Cu will very likely be added from ores into the aquatic environment, aside other ecosystems. Cu enters surface waters from different sources in comparatively large amounts. Cu is released into the environment via factories that make products containing the metal, mining, combustion of waste and fossil fuels, domestic waste water, landfills, wood production, volcanoes or forest fires (Rehman et al., 2019). Direct inputs of Cu intentionally used for its toxicity to certain organism groups are occurring due to its widespread use in the composition of *e.g.* pesticides (EFSA, 2018b), for controlling cyanobacterial blooms (Le Jeune et al., 2007), and as antifouling agents (Backhaus and Arrhenius, 2012). Use in antifouling has led to rapid increases of Cu levels in newly opened marinas up to levels exceeding regulatory quality criteria (Biggs and D'Anna, 2012).

1.4.3 Metal nanoparticle contamination of water bodies

Nanoparticles (NPs) and nanomaterials are defined by their nanoscale (1–100 nm) size. A nanomaterial is either a natural, incidental or manufactured material that contains 50% or more nanoscale particles or has one or more external or internal dimensions in the specified size range (European Commission, 2011; ISO, 2017). More specifically, NPs are particles with all external dimensions in the nanoscale (ISO, 2017). NPs may emerge either naturally, for example as a result of forest fires or volcanic activity, or as a by-product of human activity, such as from car exhaust fumes or intentionally produced NPs (Turan et al., 2019). Natural nanoparticles also include the finer fractions of colloidal clays or oxide and hydroxide precipitates of various minerals (Al, Fe, Mn) and dissolved organic matter (humic and fulvic acids) (Batley et al., 2013).

The size of these materials and particles itself is what gives rise to the advantageous nanomaterial-specific electrical, thermal, optical, and mechanical qualities (Inshakova et al., 2020) and differentiates them from bulk materials with the same chemical composition. Nanomaterial use has permeated many sectors from agriculture, machine industry, construction, paint and pigment production, energy, cosmetics and personal care products, medicine, packaging and textile industries among others (Keller et al., 2013; Turan et al., 2019). Nanoparticles have also been shown to be promising materials during the COVID-19 pandemic, *e.g.* CuO NPs are being used in cloth masks and as antiviral coatings (Merkl et al., 2021; Pollard et al., 2021). An estimated growth of 16% to 2.3 million tonnes is expected from the global nanotechnology market between 2020 and 2028, further solidifying the position of nanomaterials as globally relevant industrial products (Inshakova et al., 2020). Metal oxides, which were used also in our research **(II)**, compose a large fragment of the overall nanomaterial market share, accounting for nearly ¾ of their global revenue in 2019 (Inshakova et al., 2020).

Demand for nanomaterials is projected to increase in the coming years for the energy and power sectors in particular, mostly due to NP use in solar cell manufacturing, as components of fuel cells and batteries (Inshakova et al., 2020). Nanotechnology is presumed to support the imperative move towards greener energy production, because of the intrinsically superior transport and storage properties of nanomaterials (Baxter et al., 2009). Nanotechnology can make fuel cell production more efficient and cost-effective, lead to improvements in the biofuel industry via nanocatalyst use, and support the wind energy industry via manufacturing highly durable light-weight materials (Ahmadi et al., 2019). Nanomaterials may, therefore, be considered a boon to sustainable energy production despite their energy-intensive production, as this may be compensated by their use in smaller amounts and their enhanced efficiency compared to their conventional counterparts (Kim and Fthenakis, 2013).

The effect of two metal oxide NPs was investigated in our research **(II)**. Focus was directed to the algal toxicity of TiO_2 NPs and CuO NPs, which both have been shown to occur in concentrations of 1 to 10 $\mu g\ l^{-1}$ in influent waters of wastewater treatment plants (Cervantes-Avilés and Keller, 2021). In a Danish risk assessment, the levels of these two NP types have previously been assessed as coming close to concerning environmental levels (Kjølholt et al., 2015). TiO_2 NPs are principally relevant due to their large production volumes reaching 10 000 t/year in Europe and 88 000 t/year globally compared to those of CeO_2 and ZnO NPs (1000 t/year in Europe, 10 000 and 34 000 t/year globally) or Ag NPs (<10 t/year in Europe, 450 t/year globally) (Janković and Plata, 2019; Keller et al., 2013; Piccinno et al., 2012).

Considering also the projected 8.7% increase in TiO_2 NP market size from 2019 to 2024 (Inshakova et al., 2020), an exacerbated release into the environment can be expected compared to past estimates (Keller et al., 2013; Kjølholt et al., 2015). CuO NPs, on the other hand, may constitute a great risk to microalgae as a result of its high toxicity (Aruoja et al., 2009; Bondarenko et al., 2016), despite not being the most widely applied nanomaterial (Kjølholt et al., 2015). Global newly mined Cu production was appraised at 20 million tonnes in 2020 (USGS, 2021b), which exceeds by five orders of magnitude the estimated Cu and CuO NPs production of 200 to 500 t/year (Janković and Plata, 2019; Keller et al., 2013).

While many data gaps remain in understanding the waste streams and environmental fate of nanoparticles in general (Part et al., 2018), Keller et al. (2013) estimate that 0.4–7% of all engineered nanomaterials will ultimately be deposited into the aquatic environment. NP discharge may occur through industrial point sources, effluents from wastewater treatment or via indirect soil runoff (Batley et al., 2013). Wastewater treatment plants are expected to capture most NPs within the sewage sludge, with the remainder being discharged and diluted in rivers or coastal waters and ultimately accumulating in benthic sediments (Batley et al., 2013). Predicted levels of TiO_2 in the aquatic environment range from the nanogram per liter range for Denmark (Gottschalk et al., 2015) to as high as 16 mg l^{-1} in the US (Nowack and Mueller, 2008). TiO_2 NPs are expected to enter surface waters by shedding from cosmetics and personal care products (use comprises ~ 60% of total production amount), weathering from paints and facades (24%), or from the incineration of and abrasion from discarded packaging (Mitrano et al., 2015; Nowack and Mueller, 2008; Scown et al., 2010). Cu-based engineered nanomaterials comprise a small part of global production, but are used in ways that result in direct introduction into the environment as intentionally toxic agents, *e.g.* pesticides or antifouling paints (Conway et al., 2015).

The environmental inputs and consequent hazard or risk posed by NPs to aquatic organisms has been widely studied during the past decade (Fig. 3), because the same characteristics that prove useful for their various applications are thought to induce adverse biological effects. When testing NP toxic effects, it is crucial to create NP-specific setups to avoid experimental artifacts and also to ensure the environmental realism of the experiments. Due to NPs forming colloids when dispersed in water, different considerations should be given to test setups compared to the standard soluble chemical testing, to provide quality data and insight into NP toxicity mechanisms (Djurišić et al., 2015). In addition to general recommendations of establishing representative controls (*e.g.,* soluble metal salts in the case of metal oxides), measuring dissolution rates and characterizing agglomeration, ECHA (2017b) recommends specific conditions for tests with microalgae. Microalgal tests should consider potential shading of light by NPs, mechanical effects (cell adherence), sedimentation should be prevented, fluorescence measurements should be preferred for biomass quantification, autofluorescence of the NPs should be measured separately, and measuring sub-inhibitive effects (membrane damage and oxidative stress) (ECHA, 2017b). These NP-specific requirements should be considered aside recommendations for environmentally relevant testing. Contrary to the appeal for further unification in testing, there are also calls for more alignment of nanomaterial ecotoxicological testing by choosing study conditions (concentrations, endpoints, duration, conditions) with realistic exposure scenarios (Holden et al., 2016). It is emphasized that results in culture media may need verification in natural water due to its complex composition (Zhang et al., 2016).

1.5 Aims of the thesis

The overall aim of this doctoral thesis was to evaluate the toxicity of major and emerging metal contaminants to freshwater microalgae and employ different non-standard test methods in parallel to the standard OECD 201 algal growth inhibition assay to improve on the environmental relevance of ecotoxicological testing and find mechanistic links for toxicity test outcomes.

The specific aims were:

1) To create new data on the toxicity of rare earth elements (REEs) and REE-doped particles as emerging contaminants in aquatic ecosystems.
2) To elucidate REE toxicity mechanisms to algae using the rapid cost-effective agar plating test in parallel with the standard OECD 201 algal growth inhibition test.
3) To examine the changes in algal photosynthetic efficiency as an algal toxicity parameter in different conventional laboratory algal strains under nanoparticle exposure.
4) To assess TiO_2 and CuO NP effects on algae in natural freshwater compared to standard freshwater medium.
5) To construct algal communities consisting of up to four conventional laboratory freshwater algal species to assess the utility of multispecies testing over standard monospecies assays.
6) To determine diversity effects in competitive multispecies assays under Cu stress and discern between selection and complementarity effects across toxicant levels.
7) To investigate the applicability of algal functional traits in informing about species sensitivities to toxicants and predicting community-level productivity outcomes.

2 MATERIALS AND METHODS

Algal toxicity of various metals was studied within the scope of this thesis: rare earth element (REE) ions **(I)**, (doped) rare earth element oxide (REO) nano- and microparticles **(I)**, CuO and TiO_2 nanoparticles **(II)**, and Cu^{2+} ions dissolved from $CuSO_4$ **(II, III)**. The experimental setup of each experiment is schematically represented in Fig. 4. In each study, experiments adhering to the standard OECD 201 guideline were conducted in parallel to alternative experimental approaches. Environmental realism of the experiments was augmented or additional endpoints were quantified as follows:

- The effect of REEs was studied in composition of doped REO particles that can act as toxicants both as particles and mixtures of dissolved metals **(I)**.
- More than one species was studied **(II)** or multispecies communities were assembled **(III)** to better assess interspecies variability and represent natural circumstances, respectively.
- Experiments were conducted in a natural water based medium to assess the appropriateness of laboratory experiments to represent field conditions **(II)**.
- Community diversity and functional traits on algal biomass production were measured as additional parameters in understanding the modes of action of metal toxicity and to allow for better extrapolation to field conditions **(III)**.
- Exposure to REEs was conducted in deionized water in an agar plating test **(I)** (Suppi et al., 2015).
- The physiological stress measuring parameter Fv/Fm was applied to more comprehensively characterize NP toxicity **(II)**.

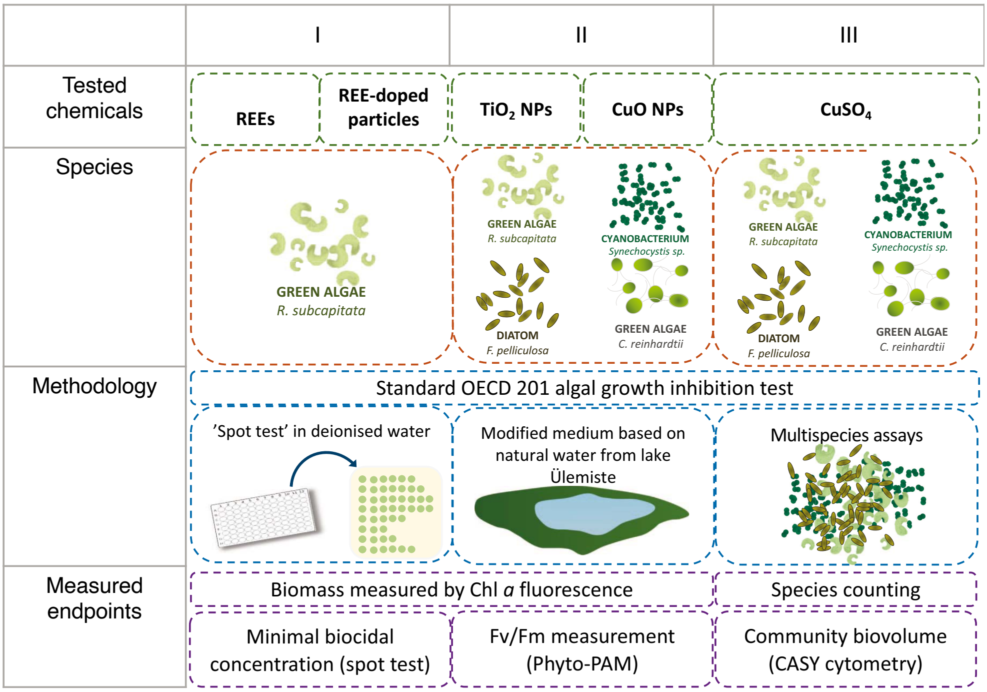

Figure 4 Overview of experimental setup of studies (I, II, III).

2.1 Algal toxicity tests

The OECD 201 experimental guideline (OECD, 2011) was used throughout **(I, II, III)** in a modified high-throughput setup (Fig. 5) using 20 ml scintillation glass vials with air-permeable caps as test vessels, as previously comprehensively described in Aruoja (2011). Four algal species, *Raphidocelis subcapitata*, *Fistulifera pelliculosa*, *Synechocystis sp.* and *Chlamydomonas reinhardtii*, were exposed to a concentration gradient of toxicants either individually **(I,II)** or in combination **(III)**.

Both the pre-experiment cultivation and experiments themselves were conducted with constant illumination of 4000–8000 lux (60–108 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 24 ± 2 °C. Exponentially growing algae were used to inoculate tests after a haemocytometric counting of algal cells. Algal biomass was measured every 24 h from samples exposed to an array of toxicant concentrations, up to the end of the experiments at 72 h **(I, II)**, or only at 72 h **(III)**. In multispecies assays (see section 2.4), samples needed to be ulatrasonicated (40W; S-450 Ultrasonifier, Branson Ultrasonics Corporation) to release cells from biofilm colonies of *F. pelliculosa* **(III)**.

2.2 Algal toxicity endpoints

Algal biomass was quantified by either chlorophyll *a* fluorescence measurement **(I, II)** or by cell counting using a CASY cytometer (Schärfe-System, Reutlingen, Germany) with a 150 µm capillary size **(III)**. Chlorophyll *a* fluorescence was measured (excitation 440 nm, emission 670 nm) after extraction in ethanol according to methods published in Aruoja et al. (2009) and described in brief in Fig. 5. This is referred to as a valid proxy to cell counting for algal biomass detection according to the OECD 201 guidance (OECD, 2011).

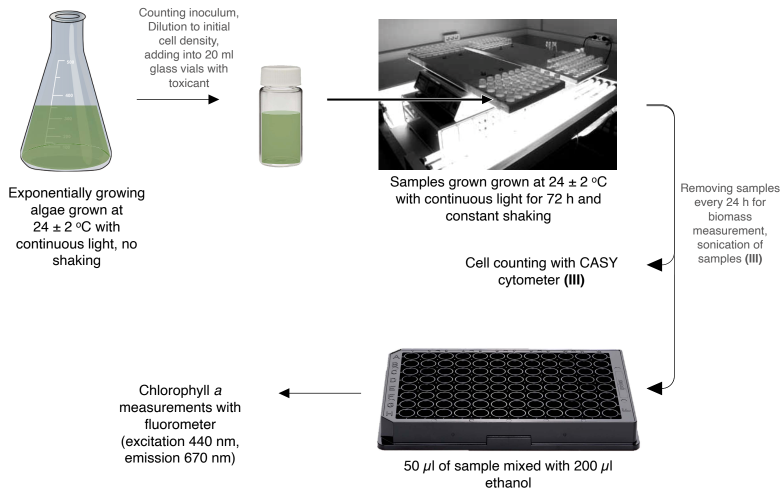

Figure 5 Algal growth inhibition test setup using a modified high-throughput version of the standard OECD 201 test. Algal cell density was assessed using Chl *a* fluorescence as a proxy **(I, II)** or using the CASY cytometer **(III)**.

In addition to chlorophyll *a* fluorescence, effective quantum yield of photosystem II (Fv/Fm) was measured using the PHYTO-PAM Phytoplankton Analyzer (Heinz Walz GmbH, Effeltrich, Germany) **(II)**. Fv/Fm indicates the efficiency of photosynthesizing organisms to convert light energy into chemical energy (Juneau et al., 2002), *i.e.* the efficiency of photosystem II open reaction centers in capturing excitation energy (Genty et al., 1989). The parameter is assessed by measuring the fluorescence yield of phytoplankton samples before (F_0) and after (Fm) a saturating light pulse (Consalvey et al., 2005). This calculation is possible because light energy that is absorbed by chlorophyll molecules is only used in three competing pathways: photosynthesis, dissipation as heat and re-emission as fluorescence (Macedo et al., 2008).

The agar plating test, as originally developed for bacteria and yeasts by Suppi et al. (2015), was used to assess the ability of the REE or REO-exposed algae to form colonies on toxicant-free nutrient agar after a 24 h exposure to the tested chemicals in deionized (DI) water (Fig. 6). In contrast to the conventional algal growth inhibition assay, the agar plating test allows to observe effects of toxicants without potential interactions from algal mineral medium components. The methodology is described in more detail in the relevant study **(I)**. Algal cells were exposed to REEs and REE-doped oxide particles in deionized water on 96-well transparent microplates. After 24 h of exposure, a 5 µl aliquot from samples was removed and pipetted onto agarized OECD 201 medium in translucent square Petri dishes. The samples were incubated at similar light and temperature conditions to the standard algal growth inhibition assay. The studied parameter was the minimal biocidal concentration (MBC), determined as the lowest concentration of a chemical that completely inhibited colony formation.

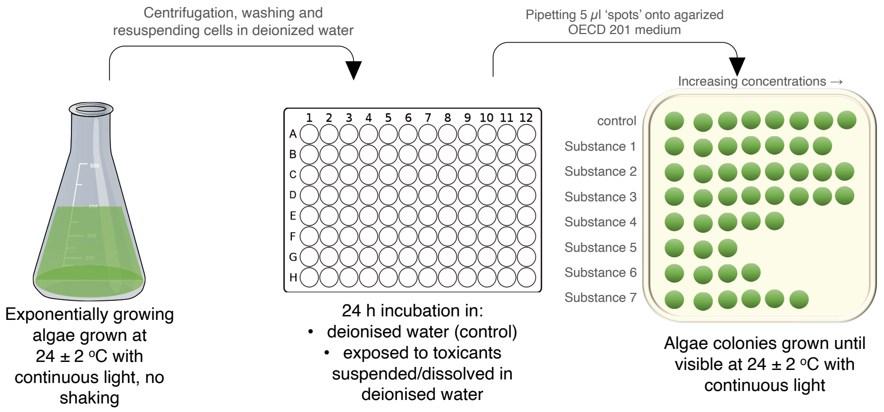

Figure 6 Setup of the agar plating test (Suppi et al., 2015), where algal ability to form colonies on agar medium after exposure to the toxicant in deionized water is used as the toxicity endpoint.

2.3 Tests in natural water

Growth medium based on natural lake Ülemiste (Estonia) water was tested in parallel to the standard OECD 201 mineral medium **(II)**. The water was collected in 2016 from a tap source in AS Tallinna Vesi water treatment plant and subsequently filtered (0.22 µm pore size) for microorganism and particle removal. Filtered water was stored at 4 °C until use in experiments. Chemical analysis of the natural water constituents was conducted in a certified laboratory at AS Tallinna Vesi. Lake Ülemiste water was enhanced with additional nutrients to provide a nutrient-sufficient growth medium for algae and allow comparison of natural DOC effect on experimental results. Major test media components of the OECD 201 medium and nutrient-adjusted natural water (ANW) are compared in Table 2.

Table 2 Comparison of physicochemical properties relevant to algal cultivation in the OECD medium and nutrient-adjusted natural water (ANW). The media contained other components that are not reported here. Dissolved inorganic nitrogen values are calculated as the sum of nitrogen in NO_3^-, NO_2^- and NH_4^+. Natural organic matter represents the dissolved organic carbon (DOC) content of the lake water. Ülemiste lake water measurements were conducted by the certified laboratories of Tallinna Vesi AS (Tallinn, Estonia). Measured data are presented as average values ± SD.

Parameter	OECD	ANW
pH	8.00	8.00
Conductivity (µS cm^{-1})	176 ± 2.6	545 ± 5.9
Natural organic matter (mg DOC l^{-1})	0	9.7 ± 0.12
Phosphate (mg PO_4^{3-} l^{-1})	0.36	0.36 ± 0.0006
Total phosphorus (mg P/l)	0.117	0.117 ± 0.00067
Dissolved inorganic nitrogen (mg N l^{-1})	3.93	4.81 ± 0.024
Chloride (mg Cl^- l^{-1})	16.46	27.46 ± 0.16
Calcium, Ca^{2+} (mg l^{-1})	4.90	76.90 ± 0.69
Magnesium, Mg^{2+} (mg l^{-1})	1.43	9.43 ± 0.07
Sodium (mg l^{-1})	13.70	20.13 ± 0.29
Potassium (mg l^{-1})	0.46	3.35 ± 0.13
Manganese (mg l^{-1})	0.12	7.72 ± 0.22
Iron (mg l^{-1})	0.013	21.81 ± 1.98
Cobalt (µg l^{-1})	0.00035	0.05 ± 0.0017
Copper (µg l^{-1})	3.73E-06	0.58 ± 0.019
Zinc (µg l^{-1})	0.0014	0.38 ± 0.017
Molybdenum (µg l^{-1})	0.0028	0.38 ± 0.01
Boron (B, µg l^{-1})	0.0323	0.0323

2.4 Multispecies assays

Toxicity of NPs to four algal species was studied individually **(II)**, but 2 to 4 species communities were also exposed to Cu^{2+} in a proof-of-concept study (Table 3) **(III)**. I propose that this approach is more environmentally relevant than testing in monoculture. *Raphidocelis subcapitata* (previously *Pseudokirchneriella subcapitata* or *Selenastrum capricornutum*) stock culture was obtained from Algal Toxkit F (MicroBioTests Inc., Nazareth, Belgium), *Fistulifera pelliculosa* (previously *Navicula pelliculosa*) from the Culture Collection of Algae of Göttingen University (SAG), *Chlamydomonas reinhardtii* and *Synechocystis sp.* strain PCC6803 from the Canadian Phycological Center (CPCC). Species' quantitative and binary functional traits (Table 3) were used to calculate functional diversity indexes **(III)**. Na_2SiO_3 was added to OECD 201 medium at a final concentration

10^{-4} M to ensure adequate growth conditions for the diatom *F. pelliculosa* monocultures **(II)** and to unify the test conditions for all algal monocultures and communities **(III)**.

Table 3 Functional traits of the species used in calculating functional diversity indices. Note the mixed use of both, binary (marked*) and continuous traits. Continuous trait data are presented as average values ± SD.

Trait	***C. reinhardtii***	***R. subcapitata***	***F. pelliculosa***	***Synechocystis sp.***
Greatest linear dimension (μm)	6.21 ± 0.32	5.43 ± 0.37	4.95 ± 0.28	2.35 ± 0.006
Log of cell volume (fl)	2.15 ± 1.32	1.98 ± 1.39	1.92 ± 1.29	1.02 ± –0.64
SSA (specific surface area, μm^{-1})	0.77 ± 0.15	1.79 ± 0.29	1.37 ± 0.21	3.89 ± 0.54
Motility*	1	0	0	0
Si content*	0	0	1	0
Colony formation*	0	0	1	0
Phycobilin content*	0	0	0	1
Chl *b* content*	1	1	0	0
Chl *c* content*	0	0	1	0
Growth rate (day^{-1})	2.5	1.7	1.7	2.2

2.5 Tested chemicals

The studies focused on assessing the algal toxicity effects of copper ions (Cu^{2+}) dissolved from $CuSO_4$, TiO_2 and CuO nanoparticles and four REEs (La, Ce, Pr and Gd) as both dissolved ions and constituents of doped REO micro- and nanoparticles (Table 4). The stock suspensions of particles were sonicated before each experiment (40W, 4 min; S-450 Ultrasonifier, Branson Ultrasonics Corporation, Danbury, CT) to ensure proper dispersion of the particles. Soluble salts of the metals and REEs contained in particles were tested in parallel to the particles. $CuSO_4{\cdot}5H_2O$ was tested in addition to nano-CuO **(II)** (no soluble salts were available for Ti). The soluble salts of all constituent metals and REEs were tested: $Ni(NO_3)_2{\cdot}6H_2O$ (Merck KGaA, purity 99.0%), $Co(NO_3)_2{\cdot}6H_2O$ (VWR, 98%), $Gd(NO_3)_3{\cdot}6H_2O$ (Sigma Aldrich, 99.99%), $Sr(NO_3)_2$ (Honeywell, 100%), $Mn(NO_3)_2{\cdot}4H_2O$ (American Elements, 100%), $La(NO_3)_3{\cdot}6H_2O$ (Treibacher Industrie AG, ≥95–100%), $Ce(NO_3)_3{\cdot}6H_2O$ (Treibacher Industrie AG, ≥95–100%), $Fe(NO_3)_3$ (Sigma Aldrich, 99.99%), $Pr(NO_3)_3{\cdot}6H_2O$ (Sigma Aldrich, 99.99%) **(I)**.

Table 4 Studied nano- and microparticles, all provided in powder form.

Study number	NP	Dopant	Primary size (nm)	Origin
I	CeO_2	–	38	CPT AS
	$Ce_{0.9}Gd_{0.1}O_2$	Gd	27	CPT AS
	$Gd_{0.97}CoO_3$	Gd	230	CPT AS
	$LaCoO_3$	La	590	CPT AS
	$LaFeO_3$	La	126	CPT AS
	La_2NiO_4	La	284	CPT AS
	$(La_{0.6}Sr_{0.4})_{0.95}CoO_3$	La, Sr	65	CPT AS
	$(La_{0.5}Sr_{0.5})_{0.99}MnO_3$	La, Sr	137	CPT AS
	$Ce_{0.8}Pr_{0.2}O_2$	Pr	23	CPT AS
II	CuO	–	22–25	Intrinsiq Materials Inc.
	TiO_2	–	< 5	CCMB

CPT AS – Ceramic Powder Technology AS (Norway)

2.6 Nanoparticle and rare earth oxide particle dissolution and characterization

Total reflection X-ray fluorescence (TRXF) was used to quantify solubilized metals using the Picofox S2 (Bruker AXS Microanalysis GmbH) **(I, II)**. Solubility of REEs and other metals contained in the doped particles was measured in deionized water after incubation for the same time period (72 h) at the same light and temperature conditions as the bioassays **(I)**. Subsequently, concentration of REEs and metals in the samples was quantified from quartz carrier disks after centrifugation of samples (20000 g for 30 min) and mixing with gallium internal standard using Picofox S2. As precipitation was noted in tests with REEs in the OECD 201 medium, the solubilized fraction remaining in the medium was also quantified. In this case, incubation was conducted in 5 media: deionized water, standard OECD 201 algal medium, OECD 201 medium without phosphates, OECD 201 medium without carbonates and OECD 201 medium without phosphates and carbonates. The incubation was also conducted at 72 h, after which samples were centrifuged (30 min at 16060 g) and measured using the same TXRF methodology as described above. NPs in were incubated in the two media (OECD201 medium and ANW) as well as in deionized water for 72 h at the conditions of the algal growth inhibition test **(II)**. After that, the samples were ultracentrifuged using a Beckman (USA) L8-55M Ultra centrifuge (40 min at 390000 g). Metal concentration was quantified in these studies with Spectra software (Bruker AXS Microanalysis GmbH). Hydrodynamic size and ζ-potential of the nano- and microparticles were measured using Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) after 24 h of settling of 100 mg l^{-1} dilutions made from NP stock suspensions **(I, II)**.

2.7 Statistical analyses and toxicity calculations

EC_{50} values based on either algal biomass yield or Fv/Fm measurements were determined from dose-response curves by the REGTOX software (Vindimian, 2001) using the Log-normal model **(I, II)**. For the EC_{50} calculations, results from all test repetitions were pooled and normalized by dividing the sample biomass values by average control biomass values.

ANOVA, linear regression, Pearson's correlation and Spearman's rank correlation calculations were conducted in R (R Core Team, 2015) throughout the studies. In addition to expressing micoralgal community diversity as species richness (*i.e.* species number), functional diversity indexes were calculated in the R environment **(III)**. Species diversity (D^1; *i.e.* the effective number of species; Jost 2006), the mean pairwise distances (MPD), and CWMs (community-weighted means of functional traits) were calculated using the function 'functcomp' from the R package 'FD' (Laliberté and Shipley, 2011; Lavorel et al., 2008). Using the same function, individual species were characterized by the relationship between species proportion in a community and the community biomass yield. Mean pairwise distance was calculated using the function 'mpd' from the package 'FD' (Laliberté and Shipley, 2011). The interspecies functional distances calculated using the function 'gowdis' from the 'picante' package (Kembel et al., 2010) were used as input for MPD calculations, with all traits having equal weight. Species diversity, expressed as the effective number of species – D^1 (Jost, 2006) at 72 h was expressed as the exponent of the Shannon diversity index, calculated with the 'diversity' function from the package 'vegan' (Oksanen et al., 2019).

3 RESULTS AND DISCUSSION

3.1 Algal toxicity of emerging contaminants

3.1.1 Metal particle toxicity

Behaviour of the particulate metal contaminants in our experiments depended both on initial particle characteristics (surface charge and functionalization, shape, material and size distribution), and the chemical properties of the test medium, similarly to what was proposed by Turan et al. (2019). Compared to their primary sizes (Table 4), the hydrodynamic sizes were usually larger when suspended in OECD 201 medium or deionized water (Table 5). This was expected, because physical transformations of (nano)particles in the environment include homo- and heteroaggregation (strong chemical or covalent bonding) and agglomeration (driven by weak forces, such as physical entanglement), which can lead to their sedimentation and deposition (Batley et al., 2013; ISO, 2017; Lowry et al., 2012; Turan et al., 2019). Aggregation and agglomeration are positively correlated with ionic strength (approximated by conductivity) and negatively influenced by stabilization induced from organic matter content (Conway et al., 2015) (Table 2). The hydrodynamic size distribution seen in the case of the non-functionalized CuO and TiO_2 NPs in our experiment, therefore, was determined by an interaction of these parameters.

To my best knowledge, adverse effects of doped REOs towards microalgae have not been studied before or subsequently to our publication **(I)**. Toxicity deriving from solely dissolved ion effects is unlikely in the case of doped REO particles, because the combined toxicity of dissolved ions did not fully explain the effect of REOs in all cases other than $LaNiO_4$ (**(I)**, Fig. 4), where dissolved nickel was the driver of toxicity. Other possible toxicity mechanisms include the physical entrapment of cells identified in our study, or the production of ROS, as described in Kurvet *et al.* (2017), also specified in Table 5. Blinova *et al.* (2018) demonstrated generally lower toxicity of the same particles to aquatic macrophytes *Lemna minor* and *Spirodela polyrhiza* (guideline OECD 221) compared to our results derived from the standard OECD 201 test with *R. subcapitata* (**(I),** Fig. 3).

Uniquely, these particles afforded the opportunity to research particle-specific effects combined with mixture effects of dissolved metal contaminants. This is relevant also, because REE exposure in the environment has already been shown to constitute of both the dissolved and particulate fraction (Klaver et al., 2014). It is difficult to provide relevant comparison for such cases for microalgae, since there is a general lack of data concerning such factors in aggregate in peer-reviewed ecotoxicological literature. REE mixtures have been shown to affect marine bacteria and microalgae synergistically, but these results were obtained using soluble REE salts (Romero-Freire et al., 2019).

Table 5 Physicochemical properties of particles used in algal toxicity experiments. Concentrations of dissolved metal ions were measured after at 72 h incubation at the conditions of the OECD 201 guideline. The ability of the (doped) rare earth oxides to abiotically produce reactive oxygen species is marked with an asterisk (*) (Kurvet et al., 2017). Dissolution data are presented as average values ± SD.

Particle	Test medium[a]	Hydrodynamic size (DLS, nm)	SSA (m^2/g)[b]	ζ-Potential (mV)	Dissolution at 72 h ($mg\ L^{-1}$)[c]
Publication I					
CeO_2	DI	280	31.1	−16.6	Ce 0.022 ± 0.01
$Ce_{0.9}Gd_{0.1}O_2$	DI	177	7.2	16.2	Ce 0.058 ± 0.06 Gd 0.018[d]
$Gd_{0.97}CoO_3$*	DI	166	3.4	18.8	Gd 0.022 ± 0.01 Co 0.014 ± 0.003
$LaCoO_3$*	DI	285	1.4	−17.5	La 0.072 ± 0.015 Co 0.009 ± 0.003
$LaFeO_3$*	DI	194	7	−1.8	La 0.102 ± 0.071 Fe 0.039 ± 0.007
La_2NiO_4*	DI	172	22	8.5	La 0.067 ± 0.01 Ni 0.198 ± 0.029
$(La_{0.6}Sr_{0.4})_{0.95}CoO_3$*	DI	147	36.1	166	La 0.127 ± 0.005 Sr 1.473 ± 0.009 Co 0.015[d]
$(La_{0.5}Sr_{0.5})_{0.99}MnO_3$*	DI	160	15	22.7	La 0.032 ± 0.005 Sr 0.230 ± 0.009 Mn 0.004[d]
$Ce_{0.8}Pr_{0.2}O_2$*	DI	266	3	−6.6	Ce 0.110 ± 0.047 Pr 0.033[d]

Particle	Test medium[a]	Hydrodynamic size (DLS, nm)	SSA (m^2/g)[b]	ζ-Potential (mV)	Dissolution at 72 h (mg L^{-1})[c]
Publication II					
CuO	DI	152 ± 2.03	n/a	16.6 ± 2.9	1.19 ± 0.021
	OECD 201	>1000[a]	n/a	−26.6 ± 4.8	0.034 ± 0.003
	ANW	>1000[a]	n/a	−19.7 ± 0.7	0.09 ± 0.0035
TiO_2	DI	277 ± 3.27	n/a	12.8 ± 0.6	0.004[e]
	OECD 201	728 ± 27.14	n/a	−22.9 ± 1.5	0.003[e]
	ANW	>1000	n/a	−18.5 ± 0.2	0.003[e]

[a] Merck Millipore MilliQ deionised water (DI), OECD Test Guideline 201 medium (OECD 201), nutrient-adjusted natural water (ANW).
[b] Brunauer–Emmett–Teller (BET) method was used to determine specific surface area (SSA).
[c] CuO and TiO_2 concentrations are based on incubation of 10 mg NP l^{-1}. Dissolution of all REOs was analysed by incubating at 100 mg REO l^{-1}.
[d] Derivation of SD was not possible.
[e] Measurements were below element's limit of quantification (LOQ) in TXRF spectroscopy.

Most pertinent studies in this area focus on microplastic particles in mixture with metal ions, with data pointing towards synergistic toxic effects of microplastics and Au for microalgae *Tetraselmis chuii* (Davarpanah and Guilhermino, 2019) or microplastics and Cd for fish *Cyprinus carpio* (Banaee et al., 2019). Additionally, TiO_2 NPs were shown to enhance Cd uptake of the crustacean *Daphnia magna* in Hartmann *et al.* (2010), however this increase in body burden did not appear to affect toxicity outcomes. In a study combining exposure of nano-CuO and nano-ZnO to microalgae *Scenedesmus obliquus*, additivity of effects was found and the toxicity was not found to stem from dissolved ion effects (Ye et al., 2017).

3.1.2 Non-standard endpoint measurements

In order to avoid interference by some methodological aspects inherent to the OECD 201 algal growth inhibition test, alternative endpoints were additionally measured **(I, II)**. First, it is well-documented that PO_4^{3-} removal from the medium constitutes a relevant part of growth inhibitory effects of REEs on algae and likely also other aquatic autotrophs (González et al., 2015, 2014; Herrmann et al., 2016; Stauber and Binet, 2000; Tai et al., 2010). In addition to the developed nutrient deficiency, this was an issue due to formation and sedimentation of REE-phosphate complexes, into which algal cells were captured **(I)**. Using the agar plate growth inhibition test developed by Suppi *et al.* (2015) helped to circumvent these indirect effects, because the 24-hour exposure of algae to REEs and doped REOs was conducted in deionized water. Algae remained viable in deionized water and it proved to be a suitable test environment due to not containing any components complexing REEs. The toxic effects are clearly seen also in this assay, where metals are fully bioavailable to the organisms. The ranges from the highest non-biocidal concentration (max-nBC) to lowest concentration that induced 100% inhibition of algal growth (minimal biocidal concentration MBC) can be supposed to contain the half-effective concentration EC_{50} in the agar plating test. Based on this approach, REE ion effects are similar between the two test procedures (Fig. 7). These values are in line with findings of other studies (**(I),** Table 2). The effects of doped REO particles appear to be somewhat mitigated in the agar plate assay compared to standard medium testing (Fig. 7).

Using endpoints other than those specified in the standard study procedure also proved to have benefits in deciphering particle toxicity mechanisms **(II)**. The endpoint Fv/Fm generally showed higher EC_{50} values, i.*e.* lower toxicity, compared to the standard biomass yield endpoint in studies with nano-CuO and nano-TiO_2 (Fig. 8). However, using the endpoint Fv/Fm afforded us to measure adverse effects earlier compared to Chlorophyll *a* autofluorescence based biomass endpoints, as also documented in Fai *et al.* (2007). This approach also proved useful to demonstrate that physical entrapment in TiO_2 nanoparticle agglomerates led to inhibition of algal growth, but did not significantly lower Fv/Fm, which may indicate low stress and a good physiological state of the cells. Therefore, evident benefits derive from diversifying the scope of measured endpoints, even in standard assays.

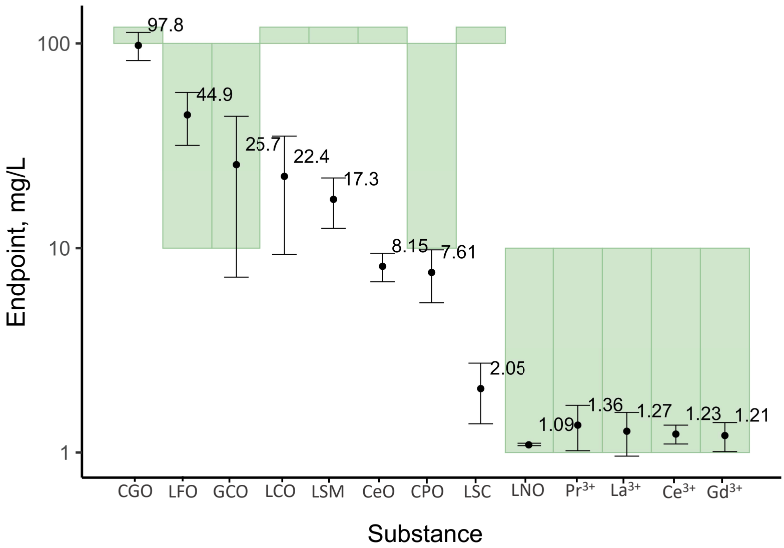

Figure 7 EC_{50} values based on the standard OECD 201 test (dots, error bars signify 95% confidence intervals) and range of maximal non-biocidal concentration (max-nBC) to minimal biocidal concentration (MBC) derived from the agar plating test (green rectangles). X-axis acronyms: $Ce_{0.9}Gd_{0.1}O_2$ (CGO), $LaFeO_3$ (LFO), $Gd_{0.97}CoO_3$ (GCO), $LaCoO_3$ (LCO), $(La_{0.5}Sr_{0.5})_{0.99}MnO_3$ (LSM), CeO_2 (CeO), $Ce_{0.8}Pr_{0.2}O_2$ (CPO), $(La_{0.6}Sr_{0.4})_{0.95}CoO_3$ (LSC), La_2NiO_4 (LNO).

3.2 Environmental realism in ecotoxicological testing

3.2.1 Nanoparticle testing in natural water

A multitude of factors will affect NP effects on microalgae in the natural environment. Non-uniform responses to chemically similar TiO_2 NPs have been observed for algae already in laboratory studies, as differences arise from varied exposure regimes and specific NP types used (Scown et al., 2010). Also, microalgae-NP interactions are expected to be affected by the production of exopolymeric substances algae produce (Dalai et al., 2013). Increased environmental realism was introduced into our studies by conducting assays in a natural water based medium (nutrient-adjusted natural water or ANW) and on four phyletically varied microalgal species' monocultures **(II)**. In almost all cases, algal toxicity was mitigated in ANW in these assays for both measured endpoints (biomass and Fv/Fm) compared to the standard OECD 201 medium (**(II)** Figs. 2 and 3). Toxicity of CuO NPs, which are generally inhibitive due to soluble ion shedding from particles (Aruoja et al., 2009), was lowered up to 30-fold in ANW due to Cu ions binding to natural organic matter (NOM) (**(II)**, Fig. 2). Aquatic behaviour of nano-TiO_2 is controlled by physical forces due to their low solubility (Table 5), which also means they will remain in the environment as a particle, but likely not preserve nanoscale size unless stabilized (Keller et al., 2013). NOM acts as a stabilizing factor, allowing more NPs to avoid agglomeration and sedimentation (Conway et al., 2015; Sousa and Teixeira, 2013; Thio et al., 2011), which is one of the main identified modes of action of TiO_2 NP algal toxicity (Aruoja et al., 2009; Cerrillo et al., 2016; Lin et al., 2012).

In our findings, the lower sensitivity of the Fv/Fm endpoint compared to biomass yield also changed in ANW, leading to a conversion of the two endpoint values (Fig. 8). This effect was more evident with the Cu^{2+} ion exposure and TiO_2 NPs, while CuO NPs did not exhibit such a marked shift. NP toxicity towards algae has been observed to be both decreased (Cerrillo et al., 2016; Huang et al., 2016; Lin et al., 2012; Van Hoecke et al., 2011) and increased (Akhil and Sudheer Khan, 2017; Jing et al., 2012; Wang et al., 2011) in natural waters containing NOM, again underlining the importance of examining NP effects in the context of natural conditions, to avoid misestimations of hazard. NPs remaining in suspension at a smaller size due to NOM content could lead to increased toxicity, due to an increase in specific surface area and concomitantly in higher reactivity (Turan et al., 2019). However, other water parameters such as ionic strength or pH also influence NP toxicity to a large degree (Sendra et al., 2017; Zhang et al., 2016), again by altering the surface properties, aggregate size, solubilization and photoactivity of NPs (Conway et al., 2015). These properties are indicated to be relevant in NP toxicity mechanisms in current literature, thus changes in these also modify test outcomes.

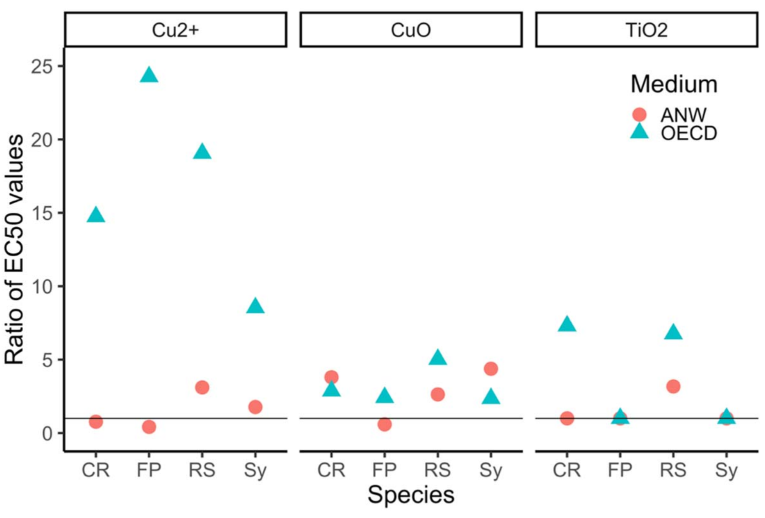

Figure 8 Ratio of soluble Cu^{2+} ions, CuO NPs and TiO_2 NPs EC_{50} values (mg metal/L) based on Fv/Fm to the standard E_yC_{50} (mg metal/L) based on biomass yield in two different media at 72 h. Plotted based on data from **(II)**. Letters on x-axis denote species: CR stands for *C. reinhardtii*, RS for *R. subcapitata*, FP for *F. pelliculosa*, Sy for *Synechocystis sp.*). Media used were the OECD 201 medium (OECD 201) and nutrient-adjusted natural water (ANW).

3.2.2 Multispecies testing

The prevailing single-species testing approach is often criticized for unknown representativeness of otherwise diverse and complex natural communities, which is thought to be an influential determinant of chemical stress susceptibility (Rubach et al., 2011). The expectation of Cu toxicity to individual species depending on diversity was not supported in our experiments. Individual species were mostly similarly inhibited by Cu^{2+}, whether tested in monoculture or in two- to four-species combinations (**(III)**, Fig. 1). This tendency cannot be considered universal, because both aggravation (Debenest et al., 2011; Franklin et al., 2004) and alleviation (Li et al., 2010) of toxicity has been previously observed in multispecies toxicity tests with microalgae. Furthermore, the direction of this influence may also depend on additional environmental factors, such as light intensity. Cheloni et al. (2019) demonstrated that *C. reinhardtii* was less sensitive to Cu in the presence of *Synechocystis sp.* in low light, but the effect was reversed at a high light intensity.

Community-level experiments can be thought of as a compromise between population and ecosystem levels that allow to consider species interactions and measure effects on biodiversity, therefore being generally considered appropriate for studying ecotoxicological effects (Clements and Rohr, 2009). However, to an extent, the observed lack of effect on individual species in our experiments could be an artefact of the standard experimental setup – the short duration of the study may not have been sufficient to establish interspecies relationships. In the case of mesocosm experiments that include multiple trophic level organisms and are conducted in realistic conditions outdoors, it is recommended to adapt the systems prior to toxicant exposure to ensure the acclimation of species and homogeneity between replicates (OECD, 2006).

While individual species inhibition did not depend on being part of a community, we observed a general increase in biomass production rates with more species richness (SR) at both levels of Cu^{2+} concentration and the toxicant-free controls (**(III)**, Fig. 2). In conditions lacking toxicant exposure, a positive biodiversity–ecosystem functioning (BEF) relationship has already been established for phytoplankton biomass production and resource use efficiency (Abonyi et al., 2018; Ptacnik et al., 2008; Striebel et al., 2009). Use of different functional diversity metrics have resulted in observations of both stimulating (Santos et al., 2015; Steudel et al., 2012; Vogt et al., 2010) and diminishing (Fontana et al., 2018; Pálffy et al., 2013) effects on algal biomass production

In our data (**(III)**, Fig. 2), in direct contradiction to SR, the functional diversity index MPD was correlated with a biomass production decrease, perhaps as a result of the microcosm experimental scale or use of too few species. This discrepancy in effects might also indicate that the selection effect dominated community-level functioning over resource use complementarity. This is evidenced in higher functional diversity, which is both dependent on trait numbers and evenness of their representation in a community, leading to lower bioproduction yields. A debate on which of these phenomena drives the widely observed positive BEF relationships has traditionally supported our outcome. This has been, however, challenged at least in grassland experiments by Pacala and Tilman (2002), who see selection effect dominance as a transient state due to an experimental bias to conclude on outcomes based on early-successional species.

Generally, biodiversity is thought to act as a buffer in conserving ecosystem function, even if a loss of species occurs. This was demonstrated in a microcosm experiment by Viaene et al. (2013), who observed changes in different biodiversity metrics (including SR) following linuron exposure, while phytoplankton biomass production remained at the same level. Additionally, despite a lowered diversity at higher Cu concentrations, the biomass production rates did not differ between communities in San Francisco Bay (Krett Lane, 1980). The idea is also formulated as the PICT (pollution-induced community tolerance) concept, that poses a shift in community structure takes place under toxicant stress due to dominance of tolerant and disappearance of sensitive species (Tlili et al., 2016).

Stability and faster recovery from disturbances are also characteristic of more diverse communities and ecosystems (Clements and Rohr, 2009). Increased resilience of microalgal biomass production to disturbances of more diverse communities has also been described for temperature and salinity stress (Steudel et al., 2012), fluctuating pH, temperature and light intensity, and predatory pressure (Cho et al., 2017). Higher-SR communities also had more stable productivity between different time periods and better recovery after introduction of grazers (Corcoran and Boeing, 2012). This tendency also applies for toxicant stress. In Cd-exposed samples, biomass production was positively affected by microalgal SR (Li et al., 2010). Also, both functional and taxonomic diversity (*i.e.* SR) were found to positively influence the rate of contaminant biotransformation by Stravs et al. (2019), indicating an enhanced remediation potential.

3.2.3 Mechanistic toxicity assessment with trait-based approaches

Species' identity influenced outcomes of community level biomass production. Average biomass production of 2-species communities appeared to significantly depend on the community containing certain species: *C. reinhardtii* at 0 and 0.01 mg Cu^{2+} l^{-1} or *R. subcapitata* at 0.02 mg Cu^{2+} l^{-1} (Fig. 9) **(II)**. These differences could have arisen from *C. reinhardtii* being the highest-yielding of the species in monoculture and *R. subcapitata* being the least sensitive to Cu toxicity (**(II)**, Fig. 3). No such differences were detected in the more competitive conditions of 3-species communities. Few generalizations can be made based solely on species identity, but a more mechanistic link is expected from using trait-based approaches. These approaches allow to connect (often phenotypical) characteristics of species to community- or ecosystem-level (Pomati et al., 2017; Pomati and Nizzetto, 2013), or even global scale processes (Litchman et al., 2015).

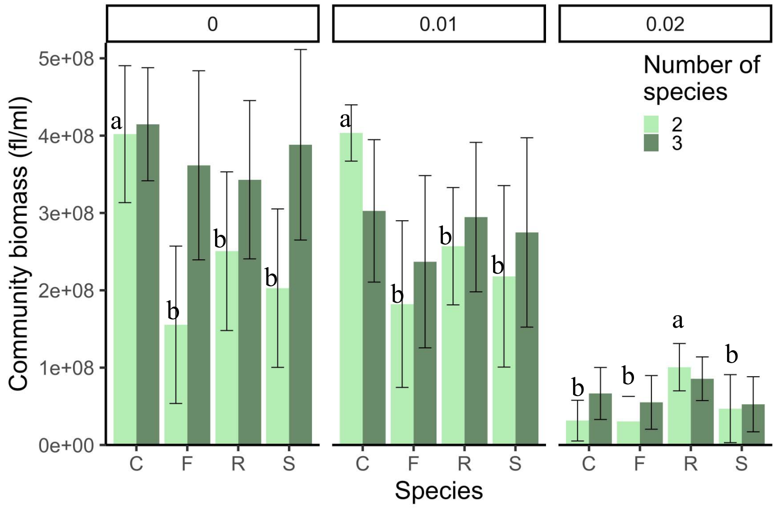

Figure 9 Average community biomass yield of 2- to 3-species communities containing a certain species (x-axis) at 0, 0.01 and 0.02 mg Cu^{2+} l^{-1}. Biomass values denoted with different letters are significantly different within the same species number and Cu^{2+} level (no significant differences found in 3-species communities). Letters on x-axis denote species: C stands for *C. reinhardtii*, R for *R. subcapitata*, F for *F. pelliculosa*, S for *Synechocystis sp*. Error bars indicate 95% confidence intervals.

Compared to species richness and the multiple trait functional diversity measure MPD, species traits related to size and growth rate were better predictors of community biomass production (**(III)**, Fig. 3). Bigger cells with lower specific surface area (SSA) exhibited higher bioproduction rates at all Cu exposure levels. To some extent this was expected, as size is a central functional trait in microalgae (Litchman and Klausmeier, 2008; Marañón, 2015) and generally for living organisms (EFSA Scientific Committee, 2016; Rubach et al., 2011). Cell size and diversity in sizes are considered among the fundamental macroecological properties of microalgal communites (Acevedo-Trejos et al., 2014).

Increased toxicity of Cu to *Synechocystis sp.* compared to other species (**(II)**, Fig. 3 and **(III)**, Fig. 1) could be due to a generally observed increased Cu sensitivity of cyanobacteria as a group (Le Jeune et al., 2006; Lopez et al., 2019; Miao et al., 2005; Zeng et al., 2010). Additionally, the small size and accompanying large SSA of *Synechocystis sp.* (Table 3) could have added to this predisposition. A smaller cell size results in efficient trace metal scavenging under normal conditions (Le Jeune et al., 2007), but contributes to increased vulnerability under toxicant stress (EFSA Scientific Committee, 2016). Therefore, a shift in community size structure benefitting larger-celled organisms is expected under Cu pollution. This has also been observed beforehand, for example as an increase in average cell size that followed triclosan exposure (Pomati and Nizzetto, 2013). However, to the contrary, size did not determine interspecies differences in Cu toxicity in marine microalgae according to Levy et al. (2007).

In addition to allowing to create more mechanistic links between simple algal traits and ecotoxicological outcomes, shifts in species traits may act as an orders of magnitude more sensitive detection device for toxicant effects in the environment compared to traditionally measured ones (Van den Brink et al., 2011). Trait-based approaches will also facilitate translating laboratory monoculture experimental data into outcomes in natural communities, and therefore predicting toxicant outcomes (Rubach et al., 2011). This is possible, because traits act as a common currency between species, leading even to suggestions to shift focus from species to only traits, which would allow to also account for intraspecies variability (Fontana et al., 2014). In our study, single traits had more predictive power than multi-trait indexes, which may suffer from trait autocorrelation or redundancy and inclusion of non-relevant traits, according to Van den Brink et al. (2011). They also highlight difficulty of use in biodiversity preservation, gaps in trait data availability and non-standard trait descriptions as weaknesses of trait-based approaches. On the other hand, functional traits are seen as a possible tool for the systemic detection of species vulnerable to different anthropogenic environmental changes (EFSA Scientific Committee, 2016).

3.2.4 Environmentally relevant and worst-case scenario testing

Testing methods used in this thesis **(I–III)** can be placed into categories along two parameters relevant to ecotoxicological test design: environmental realism and sensitivity of toxicity detection (Table 6). Testing in non-standard medium did not enhance assay sensitivity, but did reveal that NP and dissolved metal effects would likely be lowered in NOM containing natural water. Measuring alternative endpoints in addition to those specified in OECD guidance 201 did not affect environmental realism of the assay in the case of the parameter Fv/Fm **(II)**. However, this additional endpoint helped explain the role of heteroagglomeration in TiO_2 NP toxicity mechanism. Measuring the minimal biocidal concentration after exposure to REEs and doped REOs in DI water did not provide any improvements in terms of sensitivity and is not realistic, but did confirm another toxicity mechanism besides nutrient removal occurring for these contaminants. Testing different endpoints in various environmental conditions will therefore increase confidence that worst-case scenarios are covered in toxicant assessments.

Based on our data, the standard study design of testing single species in monocultures fell short based on both added realism and sensitivity in comparison to testing with multispecies artificial communities or multiple species in monoculture. Indeed, typical microalgal laboratory species were exposed to metal oxide nanoparticles as monocultures and an up to 50-fold difference in sensitivities (EC_{50} values) was detected within the same medium and endpoint type (**(II)** Figs. 2 and 3). If increased sensitivity is the aim, testing more species in monoculture is recommended as a simple standardized way to gain more knowledge about a toxicant's effects the tested organism group as a whole. In addition to suffering from inferior replicability, multispecies experiments did not improve upon experimental sensitivity, as inhibition rates of individual species did not differ from those in monocultures.

Table 6 Mapping of the environmental realism and assay sensitivity outcomes of used methodologies **(I, II, III)**.

	Less realistic		**More realistic**
Less sensitive	Single monoculture assay	Measuring Fv/Fm	Testing with natural water
	Agar plating test after exposure in deionized water	Measuring standard endpoints	Multispecies experiments Mixture testing with (doped) rare earth oxides
More sensitive		Multiple monoculture assays	Trait-based approaches

Using trait-based methods is a powerful tool to provide a mechanistic understanding of what kinds of species will be most affected by a certain toxicant. Defining most sensitive traits or trait combinations will likely ease extrapolation of study results from lab to field. Further recommendations for a more realistic experimental setup include testing at plausible toxicant concentrations (Holden et al., 2016) and considering multiple stressor effects (Côté et al., 2016; Vinebrooke et al., 2004), *e.g.* the effect of toxicant stress in tandem with nutrient enrichment, light intensity or temperature changes (Cheloni et al., 2016; Holmstrup et al., 2010; Salis et al., 2019).

4 CONCLUSIONS

1) Novel data was generated for microalgal toxicity of nine doped rare earth element oxide nano- and microparticles containing REEs (Ce, Gd, La, Pr) and dopant metals (Fe, Co, Ni, Mn and Sr).
   - In a first attempt to measure the toxicity of such particles, 72-hour EC_{50} values were found to range from 1 to 98 mg oxide l^{-1}.
   - Inhibitive effects were not solely related to metal dissolution from particles, with the exception of La_2NiO_4, where dissolved Ni ions were toxicity drivers. All oxide particles captured algal cells into agglomerates, likely leading to growth inhibition.
2) Solubilized rare earth elements (Ce, Gd, La, Pr) inhibited algal growth mainly indirectly in the OECD 201 standard test due to nutrient removal effects. The magnitude of inhibition was not alleviated during exposure in deionized water and the subsequent agar plating test, although direct effects likely prevailed in this assay.
3) TiO_2 nanoparticle toxicity was lowered up to a factor of 7 in the same OECD 201 assay when using the photosynthetic efficiency parameter Fv/Fm in comparison to the standard biomass yield inhibition. This indicated that even though biomass yield production was lowered for exposed algae, largely due to heteroagglomeration with nano-TiO_2 particles, the cells did not exhibit a high stress response.

   Therefore, the use of non-standard tests and endpoints can be recommended, because these proved useful to elude artifacts of the standard experiment and to gain a more multifaceted understanding of particulate metal contaminant toxicity.
4) In tests conducted in a natural water based medium containing natural organic matter, toxicity of CuO nanoparticles was mitigated up to 30-fold.
5) In tests with algal communities consisting of up to four algal species, it was found that:
   - The degree of inhibition by Cu^{2+} was not changed in multispecies microalgal tests compared to monoculture testing.
   - However, up to 50-fold differences were observed in algal species' sensitivities to metal nanoparticle toxicity when testing multiple microalgal species in monoculture.

   Thus, experimental sensitivity was generally lowered or unaffected in more environmentally relevant test conditions, such as a natural water based medium or a multispecies test. Even though not found in our studies, environmentally relevant testing approaches can be recommended to establish whether standard laboratory testing is truly worst-case. Testing multiple species for a comprehensive toxicity evaluation of a toxicant is recommended.

6) Biomass production increased with the number of species in multispecies assays. In this case, the sampling effect likely prevailed over resource use complementarity: multispecies samples more likely contained either the high-yielding *C. reinhardtii* or the Cu-resistant *R. subcapitata*, whose presence in 2-species communities was linked to higher community productivity.

7) A trait-based approach enabled to relate algal community attributes to biomass production and toxicity outcomes.

   - Cell size related traits were the most powerful predictors of community biomass production, exceeding a functional diversity index and species diversity.

Utilizing trait-based approaches more in ecotoxicological testing is recommended, because they may prove more universal and allow for mechanistic extrapolation from lab to field. Furthermore, testing with smaller-celled microalgae is recommended to enhance metal toxicity assay sensitivity.

5 SUMMARY

Waterbodies are particularly at risk of anthropogenic metal contamination, because they are the final depository for these non-degradable substances. Therefore, assessment of contaminant effects on aquatic organisms is crucial and required also in regulatory frameworks, such as the EU REACH regulation. Toxic effects on unicellular algae, the basis of aquatic food webs, are generally measured using the standardized monospecies growth inhibition assay. In this thesis, I summarized our research focusing on novel and particulate metal contaminant effects on microalgae using more environmentally relevant experimental approaches.

Natural background levels of many metals are exceeded due to mining and industrial waste generation. The focus of this thesis was on two types of materials: metal oxide nanoparticles and rare earth elements, both in the dissolved form and in the composition of doped rare earth element oxides. These materials are critical for sustainable energy production, but due to the concomitant increased production and emission levels, they need to be thoroughly characterized for potential toxic effects due to their high bioactivity and potential for multifaceted toxic effects, respectively. In parallel, the known algicide copper was used to evaluate the performance of novel multispecies assays. The tests were conducted on four microalgal species, either individually or in assemblages: *Raphidocelis subcapitata*, *Chlamydomonas reinhardtii*, *Synechocystis sp*. and *Fistulifera pelliculosa*.

Standard monoculture laboratory toxicity studies have the valuable feature of allowing for comparison across substances. However, as a trade-off, the monoculture assays are less representative of more complex field conditions, where multiple species and contaminants interact. In this thesis, different approaches were used to improve the environmental relevance of testing metal contaminant toxicity to algae. In our research, these methods did not enhance the sensitivity of tests and did not, therefore, represent a worst-case outcome, which is often relied upon in assessing contaminant risks. Metal nanoparticles were found to be less toxic in nutrient-adjusted natural water compared to standard mineral medium, due to the protective effect of natural dissolved organic matter that complexes Cu ions and interferes with TiO_2 nanoparticle agglomeration.

Furthermore, studying non-standard parameters proved useful to understand toxicity mechanisms and evade experimental artifacts. Various endpoints and experimental approaches were used to attain our research objectives, although the standard OECD 201 test was conducted in parallel in all experiments, with chlorophyll *a* fluorescence measured as proxy to biomass. Measuring the non-standard parameter Fv/Fm allowed us to observe the physiological stress response of phytoplankton cells captured in TiO_2 nanoparticle agglomerates. The lower inhibition indicated by Fv/Fm measurements compared to standard biomass production inhibition may indicate that mechanical effects thwarted algal growth, rather than direct toxicity. We found evidence of multifaceted toxicity mechanisms of rare earth elements by exposing algae in deionized water in addition to the standard mineral medium, in which nutrient depletion limited algal growth.

Adverse effects on algae were observed at similar levels in deionised water, but contrarily were caused by direct toxic effects.

Enhanced environmental realism was also the aim of conducting multispecies assays. Generally, algae proved indifferent to the presence of other algal species in their response to copper, because no differences in growth inhibition were detected for the same species in monoculture or in multispecies tests. No new information was therefore added compared to testing multiple species in monoculture, which I recommend for a more comprehensive analysis of toxicant effects. Nonetheless, cultures containing a mix of species produced more biomass even in Cu-contaminated water. This pattern likely arose due to the selection effect, meaning the multispecies assays more likely containing the highly productive (*C. reinhardtii*) or more toxicant-resilient (*R. subcapitata*) species. A predictable loss of experimental reproducibility was noted as an unfavourable attribute of multispecies assays.

We recommend focusing more on algal functional traits in ecotoxicological research, as these were the best predictors of biomass production ability and resiliency to toxicants based on our multispecies assays. Irrespective of copper levels, larger species with smaller specific surface areas produced more biomass. Therefore, testing smaller species (such as *Synechocystis sp.*) may be relevant to gain sensitivity in microalgal assays. Trait-based approaches are an effective mechanistic tool for applying data obtained in a laboratory setting to understand potential outcomes in the natural environment.

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SUMMARY IN ESTONIAN

Metalliliste saasteainete ohu hindamine mikrovetikatele keskkonnalähedaste katsestrateegiatega

Inimtekkeline keskkonnasaastus on toodetavate ainete aina kasvava arvu ja mahu tõttu hoogustuv keskkonnaprobleem. Eriti on ohustatud veekogud, kuhu enamik mittelagunevaid või aeglaselt lagunevaid saasteained lõpuks ladestuvad. Sestap keskendub veeorganismidele ka Euroopa kemikaalide registreerimise määrus REACH ((EÜ) nr 1907/2006), mis kohustab kvantitatiivselt hindama iga EL-is üle 1 tonni aastas toodetava või imporditava kemikaali mõju veeorganismidele. Nende katsete seas on vaja sooritada ka ainuraksete mikrovetikate kasvu pärssimise test. Mikrovetikatele põhjustatavaid negatiivseid mõjusid on vajalik hinnata, kuna tegu on veeökosüsteemide toiduvõrgustiku aluseks olevate ehk süsinikku siduvate organismidega, kes moodustavad ligi 50% üleilmsest primaarproduktsioonist.

Paljud metallid on elusorganismide elutegevuseks väikesel hulgal vajalikud, aga nende optimaalse koguse ületamisel on negatiivsed tagajärjed. Kuigi metalle leidub vees ja veekogu setetes ka looduslikult, on inimtegevuse tagajärjel aineringlus muutunud ja metallide sisaldus on tööstuse arenedes veekogudes ja eriti setetes anomaalselt tõusnud, kuna need ei ole lagunevad. Selles väitekirjas keskendusin ennekõike uudissaasteainete – metalliliste nanoosakeste ja haruldaste muldmetallide oksiidide osakeste – toksilisusele. Töötasime välja ka uudse toksilisuse testmetoodika katseteks mitmeliigiliste vetikakooslustega, kasutades toksikandina teadaolevalt algitsiidse toimega vaske.

Haruldased muldmetallid on 17 sarnaste füüsikalis-keemiliste omadustega elementi, mida leidub maakoores küll näiteks võrreldaval hulgal vase või pliiga, aga neist erinevalt mitte kontsentreeritud maakidena. Tehnoloogiakriitiliste ainetena on neil suur roll rohepöörde seisukohalt olulise jätkusuutliku energia ja elektrisõidukite tootmises, millega kaasnevad aga haruldaste muldmetallide kasvavad kaevandusmahud ning üha suurenevad kontsentratsioonid keskkonnas. Lisaks satuvad haruldased muldmetallid veekogudesse tahtlikult eutrofeerumise bioremediatsiooni eesmärgil või tahtmatu jääkainena fosfaatväetiste kasutamise tagajärjel. Kuna gadoliiniumi kasutakse magnetresonantstomograafia kontrastainena, on selle ebaharilikult kõrgeid sisaldusi täheldatud Euroopa jõgedes haiglatest allavoolu asuvates mõõtepunktides. Lisaks on punkreostuse allikateks haruldasi muldmetalle töötlevad tehased või nendega rikastunud maake ekspluateerivad kaevandused. Ka Eesti põlevkivitööstuses tekkivad jäätmed sisaldavad suhteliselt kõrges kontsentratsioonis haruldasi muldmetalle, mistõttu on täheldatud nende akumuleerumist piirkonna veetaimedes.

Vask on oma kõrgete kasutus- ja kaevandusmahtude tõttu suures mahus keskkonda sattuv saasteaine. Vaske rakendatakse ka veealustel pindadel pealiskasvu vältimiseks kasutatavates värvides, sealhulgas sihilikult vetikatega kattumise ennetamiseks. Selle kõrval toodetakse sünteetilisi metalli nanoosakesi väikesel hulgal, aga nende uuenduslike ainete bioaktiivsus on teisalt nanosuuruse tõttu kõrge. Metallioksiidide nanoosakesed moodustavad kolmveerandi kõigi nanoosakeste tootmismahust. Meie katsetes kasutatud TiO_2 ja CuO koosnevaid nanoosakesi peetakse kõige keskkonnaohtlikumateks vastavalt nende suurte tootmiskoguste (TiO_2) ja leostuvate ioonide kõrge toksilisuse tõttu (CuO).

Selle doktoritöö katsed sooritati lahustuva vase soolaga $CuSO_4$ **(III)**, CuO ja TiO_2 nanoosakestega **(II)** ning haruldaste muldmetallidega ja neid sisaldavate legeeritud metallioksiididega **(I)**. Töö peamiseks eesmärgiks oli nende materjalide kui saasteainete mõju hindamine mikrovetikatele realistlike keskkonnatingimustega kooskõlas olevate või toksilisusmehhanisme selgitavate katsemeetoditega. Töö täpsemad sihid olid:

- Kirjeldada esmakordselt erinevate metallidega (Co, Fe, Ni) legeeritud haruldaste muldmetallide oksiidide mõju mikrovetikatele. Nende uudissaasteainete toksiline mõju võib olla mitmetahuline, kuna ühelt poolt võivad nad mõju avaldada osakestena ja teisalt lahustunud ainete seguna.
- Selgitada haruldaste muldmetallide toksilisusmehhanisme, kasutades lisaks standardsele OECD 201 katsele ka deioniseeritud vees kokkupuute testi, millele järgnes agarsöötmel kasvatamine.
- Mõõta nanoosakeste mõju nelja vetikaliigi fotosünteesi efektiivsusele lisaks standardsele toksilisusnäitajale.
- Hinnata CuO ja TiO_2 nanoosakeste mõju mikrovetikatele looduslikul veel põhinevas söötmes.
- Moodustada kuni neljaliigilisi vetikakooslusi harilikest laboriliikidest, et võrrelda mitmeliigiliste katsete rakendatavust ning otstarbekust monokultuuride katsetega.
- Selgitada mitmeliigiliste katsete abil välja konkurentsisuhete mõju vase toksilisusele, eristades seejuures valimiefekti ja toitainete kasutamise komplementaarsust.
- Uurida mikrovetikate funktsionaalsete tunnuste rakendatavust liikide toksikandi tundlikkuse määramisel ja koosluse biomassi tootmise võime ennustamisel.

Püstitatud eesmärkide saavutamiseks kasutasime erinevaid katse ülesehitusi ning mõõtsime mitmeid toksilisusparameetreid. Paralleelselt sooritati kõigis artiklite katsetes **(I–III)** standardne OECD 201 test, kus mõõdeti biomassi klorofüll a fluorestsentsi kaudu. Samuti mõõtsime: vase toksilisuse mõju mitmeliigiliste koosluste biomassi tootlikkusele voolutsütomeetria abil **(III)**, TiO_2 ja CuO nanoosakeste efekti fotosünteesi efektiivsusele (Fv/Fm) PAM-fluoromeetriga **(II)**, lahustunud haruldaste muldmetallide ja nende legeeritud oksiidide osakeste mõju minimaalsele letaalsele kontsentratsioonile (deioniseeritud vees kokkupuutele järgneval agarsöötmel kasvatamisel) **(I)**. Laboris leitavate ja reaalsete metallioksiidide nanoosakeste mõjude võrdlemiseks kasutati standardse

OECD 201 mineraalsöötme kõrval ka Ülemiste järve veel põhinevat söödet, mille suurimaks omapäraks oli kõrgem lahustunud orgaanilise aine sisaldus **(II)**. Doktoritöö katsetes oli kasutusel järgnevad neli mikrovetikaliiki: rohevetikaid *Raphidocelis subcapitata* ja *Chlamydomonas reinhardtii*, tsüanobakterit *Synechocystis sp.* ja ränivetikat *Fistulifera pelliculosa*. Hariliku monokultuuris katsetamise kõrval moodustasime kuni neljaliigilisi kooslusi, et tuvastada potentsiaalseid Cu ioonide toksilisust vähendavaid või hoogustavaid mõjusid **(III)**. Sama katse tulemuste põhjal hindasime, kuidas vetikate funktsionaalsed tunnused (ehk elukäiku mõjutavad omadused) avaldavad mõju koosluse tasemel biomassi tootmise võimele toksilise stressi tingimustes.

Vetikate toksilisuskatsed sooritatakse harilikult monokultuurides OECD 201 juhenddokumendi alusel. Taoline katse ülesehitus ei võimalda küll looduslike koosluste keerukusega arvestamist, aga on standardiseerituse ja suhtelise lihtsuse tõttu laialt kasutatav. Looduslike koosluste keerukusega suuremaks arvestamiseks katsetasime monokultuuride kõrval kahe- kuni neljaliigilisi vetikakooslusi **(III)**, mis võimaldasid laboratoorsetes katsetes imiteerida looduslikke liikide vahelisi suhteid ja konkurentsi. Leidsime, et mitmeliigilise koosluse osaks olemine ei leevendanud ega süvendanud vase toksilisust võrreldes monokultuurides esinenud mõjuga. Samas varieerusid vetikakooslustega saadud katsetulemused enam kui monokultuurides.

Mõõtsime standardse biomassi kõrval ka teisi toksilisusparameetreid **(I, II)**. Leidsime, et kuna haruldased muldmetallide fosfaadid on lahustumatud, siis vetikate kasvusöötmesse lisatud lahustuvate soolade toksilisus vetikatele OECD201 testis tuleneb reeglina nende fosfaatidena sadestumisest ja sellest tulenevast vetikate toitainepuudusest **(I)**. Neid kaudseid vetikate kasvu inhibeerivaid mõjusid sai vältida, inkubeerides vetikaid haruldaste muldmetallide lahustes deioniseeritud vees ja külvates seejärel vetikarakud agarsöötmele, millele järgnes vetikakolooniate kasvu jälgimine. Selgus siiski, et haruldased muldmetallid olid ka selles testformaadis vetikatele sama toksilised kui standardkatses, ehkki mõju põhjusteks oli ilmselt rakumembraanide fosfolipiidide kahjustumine. Täheldasime ka, et TiO_2 nanoosakeste negatiivne mõju vetikate biomassi tootlikusele võis olla tingitud pelgalt mehaanilisest agregeerimisest vetikarakkude ümber **(II)**. Fotosünteesi efektiivsus, mõõdetud kui Fv/Fm, osutas nanoosakestega agregeerunud vetikarakkude üldiselt madalale füsioloogilise stressi tasemele.

Uurimistöös leidis kinnitust positiivne seos elurikkuse ja toksilisele stressile vastupanu võime vahel **(III)**. Nii kontrollkultuurides kui vasega kokkupuutunutes oli ilmne, et rohkemate liikide sisaldamine tingib koosluste kõrgemat biomassi tootlikkust, mille põhjuseks oli aga tõenäoliselt valimiefekt, mitte ressursside kasutamise komplementaarsus. See ilmnes ühelt poolt biomassi negatiivsest seosest funktsionaalse mitmekesisusega, mis on seda kõrgem, mida erinevamad liigid on võrdsemalt koosluses esindatud. Biomassi tootlikkust dikteeris tugevalt ka kindlate liikide olemasolu koosluses. Vaseta ja madala vase sisalduse juures olid keskmiselt oluliselt tootlikumad monokultuurina suurimat biomassi moodustanud rohevetikat *Chlamydomonas reinhardtii* sisaldanud kaheliigilised vetikakooslused. Kõrgemal vase kontsentratsioonil saavutasid suurima tootlikkuse kõige toksikandile vastupidavamat liiki *Raphidocelis subcapitata*'t sisaldanud kooslused. Kõige tugevam seos biomassi tootlikkusega oli koosluste katsetes aga funktsionaalsetel tunnustel. Olenemata vase kontsentratsioonist oli eelis rohkema biomassi tootmiseks keskmiselt suuremarakulisustel ja väiksema eripinnaga kooslustel.

Üldiselt nähtus uurimistööst, et keskkonnalähedased katsemeetodid ei ole sageli kõige tundlikumad ja ei pruugi seega esindada halvima juhu stsenaariumit, mida ettevaatusprintsiibist lähtudes riskide hindamisel rakendatakse. Näiteks kompleksseerib looduslikus vees sisalduv orgaaniline aine toksilisi vase ioone, vähendades nende biosaadavust ja seega ka toksilisust. Ülemiste järve veel põhinevas vetikasöötmes oli CuO ja ka TiO_2 nanoosakeste toksilisus nelja meie poolt uuritud vetikaliigi puhul oluliselt madalam võrreldes lahustunud orgaanilisi aineid mitte sisaldavad standardsöötmega. Siiski andsid katsed nelja vetikaliigi monokultuuridega väärtuslikku lisainfot, kuna liigid erinesid oma tundlikkuselt kuni 50-kordselt.

Doktoritööst selgus:

- Teadaolevalt esmakordselt uuritud 9 legeeritud haruldaste muldmetallide oksiidi olid mikrovetikatele erinevalt toksilised: EC_{50} ehk poolefektiivse kontsentratsiooni väärtused olid vahemikus 1 kuni 98 mg oksiidi l^{-1} kohta. Seda toksilisust ei saanud seletada pelgalt osakestest lahustuvate ioonide mõjudega – kõik oksiidiosakesed püüdsid vetikarakke ka aglomeraatidesse.
- Mittestandardsete testide ja toksilisusnäitajate kasutamine oli põhjendatud, et ammutada mitmekülgsemaid teadmisi metallide toksilisusest ja et vältida standardkatse nõrkusi:
  - Haruldaste muldmetalli soolade vetikate kasvu pärssivad mõjud tulenesid standardkatses toitainete eemaldamise kaudsest efektist. Deioniseeritud vees haruldaste muldmetallidega kokku puutunud ja hiljem agarile külvatud vetikate puhul ei saanud taoline segav mõju tekkida ja seega oli eeldatavasti suurem roll otsestel toksilisusel.
  - Fotosünteesi efektiivsuse parameeter Fv/Fm osutas TiO_2 nanoosakeste kuni 7-kordselt väiksemale toksilisusele võrreldes standardse biomassi tootlikkuse piiramisega. See võib tähistada, et kuigi vetikad olid mehaaniliselt piiratud, ei olnud nad füsioloogiliselt kõrges stressis.
- Katsete tundlikkus oli üldiselt kas madalam või ei muutunud keskkonnalähedastes katsetingimustes:
  - Ülemiste veel baasil valmistatud söötmes leevendus CuO nanoosakeste toksilisus kuni 30-kordselt võrreldes standardse mineraalsöötmega.
  - Mitmeliigiliste vetikakooslustega katsetes ei muutunud Cu iooni vetikaid inhibeeriv mõju võrreldes monokultuuridega. Seevastu võib soovitada suurema tundlikkuse saavutamiseks katsete läbiviimist monokultuuridega, kuna nelja kasutatud liigi tundlikkus nanoosakestele erines kuni 50-kordselt.

- Biomassi tootlikkus kasvas mitmeliigilistes kooslustes liikide arvu suurenedes. Selle põhjuseks võib pidada valimiefekti võimutsemist ressursside kasutamise komplementaarsuse üle: mitmeliigilised kooslused sisaldasid suurema tõenäosusega loomuomaselt kõrgema tootlikkusega *C. reinhardtii*'t või vasele vastupidavat *R. subapitata*'t. Nende liikide leidumine koosluses oli seotud oluliselt kõrgema biomassi tootlikkusega 2-liigilistes kooslustes.
- Tunnuste kasutamine võimaldas siduda vetikate omadusi koosluste biomassi tootlikkuse ja vase toksilisusega. Eriti tugevalt olid biomassi tootlikkusega mikrovetikate rakusuurusega seotud tunnused, isegi ületades liigirikkuse ning funktsionaalse mitmekesisuse mõjusid. Tunnusepõhine lähenemine võib seega olla võimas tööriist ökotoksikoloogias põhjuslike seoste avastamiseks ning laboris leitu otsesemaks ülekandmiseks reaalsetesse tingimustesse.

Leitud teadmistest johtuvalt soovitame mikrovetikate toksilisuse hinnangu tundlikkuse suurendamise meetodina nii metallide kui teiste toksikantide puhul viia läbi katseid mitme vetikaliigi monokultuuridega. Samuti võivad suurema eripinna tõttu olla ennekõike ohustatud väikeserakulised liigid, seega võiks olla põhjendatud nendele keskendumine. Eriti metalliosakeste puhul on kasulik kaudsete efektide ülehindamise vältimiseks ja toksilisusmehhanismi lahti mõtestamiseks mõõta ka mittestandardseid parameetreid. Lõppeks soovitame laialdasemalt ökotoksikoloogias rakendada tunnusepõhist metoodikat, mille najal oleks võimalik paika panna, milliste omadustega liigid on kindlale toksikandile tundlikumad, mis lihtsustab tulemuste ülekandmist laboritingimustest looduslikele.

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PUBLICATIONS

CURRICULUM VITAE

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Since 2019	Agriculture and Food Board of Estonia, Plant Protection and Fertilizers department, ecotoxicological risk assessor of plant protection products and pesticide active ingredients
2015–2019	National Institute of Chemical Physics and Biophysics, early-career researcher
2014–2015	Estonian Marine Institute, laboratory assistant

Education:

Since 2015	University of Tartu, *PhD* studies in botany and ecology (microalgal ecotoxicology)
2015	University of Tartu, *MSc* in aquatic ecology
2012	University of Tartu, *BSc* in ecology and biodiversity conservation
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Research interests:

Microalgal ecotoxicology of metal contaminants, metal particle toxicity, trait-based community ecology.

List of research papers:

Bondarenko, O. M., Heinlaan, M., Sihtmäe, M., Ivask, A., Kurvet, I., **Joonas, E.**, Jemec, A., Mannerström, M., Heinonen, T., Rekulapelly, R. and Singh, S. & Kahru, A. (2016). Multilaboratory evaluation of 15 bioassays for (eco) toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. *Nanotoxicology*, *10*(9), 1229–1242

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Joonas, E., Aruoja, V., Olli, K., & Kahru, A. (2019). Environmental safety data on CuO and TiO2 nanoparticles for multiple algal species in natural water: filling the data gaps for risk assessment. *Science of the Total Environment*, *647*, 973–980

Romero-Freire, A., **Joonas, E.**, Muna, M., Cossu-Leguille, C., Vignati, D. A. L., & Giamberini, L. (2019). Assessment of the toxic effects of mixtures of three lanthanides (Ce, Gd, Lu) to aquatic biota. *Science of the Total Environment*, *661*, 276–284.

Joonas, E., Olli, K., Kahru, A., & Aruoja, V. (2021). Biodiversity and functional trait effects on copper toxicity in a proof-of-concept multispecies microalgal assay. *Algal Research*, *55*, 102204.

Conference presentations:

Joonas E., Aruoja V., Olli K., Kahru A. 2016. Doped rare earth oxides and their constituent metals inhibit *Raphidocelis subcapuitata* growth via ion leaching, nutrient removal and agglomeration. **Platform presentation** at 'Biodiagnostics and assessment of environmental quality: approaches, methods, criteria and reference standards in ecotoxicology: International Symposium BIODIAGNOSTICS-2016'. 25–28 October 2016, Moscow, Russia.

Joonas E., Aruoja V., Olli K., Kahru A. 2016. Doped Rare Earth Oxides and Their Constituent Metals Inhibit Algal Growth by Metal Ion Leaching, Nutrient Removal and Agglomeration Around Algae. **Platform presentation** at EcoBalt conference, 9–12 October 2016, Tartu, Estonia.

Joonas, E.; Aruoja, V.; Olli, K.; Kahru, A. 2016. Toxic effects of (doped) rare earth oxides and their constituent metals on algae *Raphidocelis subcapitata*. **Poster presentation** at EUROTOX 2016, 4–7 September 2016, Seville, Spain.

Joonas, E.; Aruoja, V.; Olli, K.; Kahru, A. 2017. Toxic effects of (doped) rare earth oxides and their constituent metals on algae *Raphidocelis subcapitata*. **Poster presentation** at FMTDK scientific conference, 7–8 March 2017, Tartu, Estonia.

Joonas, E.; Aruoja, V.; Olli, K.; Kahru, A. 2018. Natural organic matter alleviates TiO_2 and CuO nanoparticle toxicity in four algal species. **Platform presentation** at FMTDK scientific conference, 7–8 March 2018, Tartu, Estonia.

Joonas, E, Romero-Freire, A., Muna, M. 2018. Multi-toxicity of lanthanides to aquatic biota. **Platform presentation** at COST Action TD 1407 conference "Technology Critical Elements in ecosystem and human health", 19–20 April 2018.

Joonas, E.; Aruoja, V.; Olli, K.; Kahru, A. 2018. Natural organic matter alleviates TiO_2 and CuO nanoparticle toxicity in four algal species. **Poster presentation** at SETAC Europe 28th Annual Meeting, 13–17 May 2018, Rome, Italy.

Joonas, Elise; Olli, Kalle; Aruoja, Villem; Kahru, Anne 2019. Artificial algal communities under Cu stress: effect of functional diversity on biomass production. **Poster presentation** at SETAC Europe 29th Annual Meeting, 26 to 30 May 2019, Helsinki, Finland.

Research grants and scholarships:

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Member of Estonian Society of Toxicology (from 2016)

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Improvement of skills:

Course EUROTOX “Principles of Toxicology”, Zagreb, Croatia, 4–8 April, 2016.

Workshop COST MODENA “Assessing the dose of nanomaterials in toxicological studies: Advanced approaches utilizing experimentation and modelling”, NICPB, Tallinn, 4–5 May 2016.

Course “Advanced course on analytical chemistry of TCEs” and workshop “Workshop on Environmental Concentrations, Cycling & Modeling of Technology Critical Elements”, Weizmann Insitute of Science, Rehovot, Israel, 15–19 January 2017.

Course “The Use of Trait Based Approaches in Community Ecology and Stress Ecology”, University of Coimbra, Coimbra, Portugal, 25–29 September 2017.

Course “Applied biostatistics in biological sciences using R”, Tartu, Estonia, October 29 to November 2 2018.

Course of the European Commission Better Training for Safer Food (BTSF) program ‘Evaluation and Authorization Procedures for Plant Protection Products – Eco-toxicological risk assessment on the environmental fate and behaviour in soil, air and water’, Mechelen, Belgium, 9 to 13 September 2019.

Course of the European Commission Better Training for Safer Food (BTSF) program “EFSA/ECHA Guidance to Identify Endocrine Disruptors”, Brussels, Belgium, 27 to 28 November 2019.

Course of the European Commission Better Training for Safer Food (BTSF) program “Evaluation and Authorization Procedures for Plant Protection Products – Eco-toxicological risk assessment for terrestrial and aquatic environment”, virtual attendance from 28 June to 2 July 2021.

Course of the European Commission Better Training for Safer Food (BTSF) program “Risk Assessment of Microorganisms used as Pesticides or Biocides”, virtual attendance from 22 to 26 November 2021.

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Metallide ökotoksilisus mikrovetikatele, metalliosakeste toksilisus, tunnusepõhine koosluste ökoloogia.

Teadusartiklid:

Bondarenko, O. M., Heinlaan, M., Sihtmäe, M., Ivask, A., Kurvet, I., **Joonas, E.**, Jemec, A., Mannerström, M., Heinonen, T., Rekulapelly, R. and Singh, S. & Kahru, A. (2016). Multilaboratory evaluation of 15 bioassays for (eco) toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. *Nanotoxicology*, *10*(9), 1229–1242

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Konverentsiettekanded:

Joonas E., Aruoja V., Olli K., Kahru A. 2016. Doped Rare Earth Oxides and Their Constituent Metals Inhibit Algal Growth by Metal Ion Leaching, Nutrient Removal and Agglomeration Around Algae. **Suuline ettekanne** konverentsil EcoBalt, 9.–12. oktoober 2016, Tartu, Eesti.

Joonas E., Aruoja V., Olli K., Kahru A. 2016. Doped rare earth oxides and their constituent metals inhibit *Raphidocelis subcapuitata* growth via ion leaching, nutrient removal and agglomeration. **Suuline ettekanne** sümpoosionil "Biodiagnostics and assessment of environmental quality: approaches, methods, criteria and reference standards in ecotoxicology: International Symposium BIODIAGNOSTICS-2016". 25.–28. oktoober 2016, Moskva, Venemaa.

Joonas, E.; Aruoja, V.; Olli, K.; Kahru, A. 2016. Toxic effects of (doped) rare earth oxides and their constituent metals on algae *Raphidocelis subcapitata*. **Posterettekanne** konverentsil EUROTOX 2016, 4.–7. September 2016, Sevilla, Hispaania.

Joonas, E.; Aruoja, V.; Olli, K.; Kahru, A. 2017. Toxic effects of (doped) rare earth oxides and their constituent metals on algae *Raphidocelis subcapitata*. **Posterettekanne** FMTDK konverentsil, 7.–8. märts 2017, Tartu, Eesti.

Joonas, E.; Aruoja, V.; Olli, K.; Kahru, A. 2018. Natural organic matter alleviates TiO_2 and CuO nanoparticle toxicity in four algal species. **Suuline ettekanne** FMTDK konverentsil, 7.–8. märts 2018, Tartu, Eesti.

Joonas, E, Romero-Freire, A., Muna, M. 2018. Multi-toxicity of lanthanides to aquatic biota. **Suuline ettekanne** COST Action TD 1407 konverentril "Technology Critical Elements in ecosystem and human health", 19.–20. aprill 2018, Tallinn, Eesti.

Joonas, E.; Aruoja, V.; Olli, K.; Kahru, A. 2018. Natural organic matter alleviates TiO_2 and CuO nanoparticle toxicity in four algal species. **Posterettekanne** konverentsil SETAC Europe 28th Annual Meeting, 13.–17. mai 2018, Rooma, Itaalia.

Joonas, Elise; Olli, Kalle; Aruoja, Villem; Kahru, Anne 2019. Artificial algal communities under Cu stress: effect of functional diversity on biomass production. **Posterettekanne** konverentsil SETAC Europe 29th Annual Meeting, 26.–30. mai 2019, Helsingi, Soome.

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Kursus "Advanced course on analytical chemistry of TCEs" ja seminar "Workshop on Environmental Concentrations, Cycling & Modeling of Technology Critical Elements", Weizmann Insitute of Science, Rehovot, Iisrael, 15.–19. jaanuar 2017.
Kursus "The Use of Trait Based Approaches in Community Ecology and Stress Ecology", University of Coimbra, Coimbra, Portugal, 25.–29. september 2017.
Kursus "Applied biostatistics in biological sciences using R", 29. oktoober kuni 2. november 2018.
Kursus Euroopa Komisjoni *Better Training for Safer Food* (BTSF) programmi raames "Evaluation and Authorization Procedures for Plant Protection Products – Eco-toxicological risk assessment on the environmental fate and behaviour in soil, air and water", Mechelen, Belgia, 9.–13. september 2019.
Kursus Euroopa Komisjoni *Better Training for Safer Food* (BTSF) programmi raames "EFSA/ECHA Guidance to Identify Endocrine Disruptors", Brüssel, Belgia, 27.–28. november 2019.
Kursus Euroopa Komisjoni *Better Training for Safer Food* (BTSF) programmi raames "Evaluation and Authorization Procedures for Plant Protection Products – Eco-toxicological risk assessment for terrestrial and aquatic environment", virtuaalne osalemine 28. juunist kuni 2. juulini 2021.
Kursus Euroopa Komisjoni *Better Training for Safer Food* (BTSF) programmi raames "Risk Assessment of Microorganisms used as Pesticides or Biocides", virtuaalne osalemine 22.– 26. november 2021.

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