Assessment of the toxicity of inorganic and organic pollutants using the benthic alga *Closterium ehrenbergii*

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Contents

Abstract

In this thesis, *Closterium ehrenbergii* was selected as test species for the assessment of the toxicity of environmentally relevant pollutants towards a representative of benthic algae. Therefore, a miniaturized bioassay for toxicity tests on *C. ehrenbergii* was developed. Metals and pharmaceuticals were investigated to address both inorganic and organic pollutants of high concern. Established test procedures were questioned in order to identify potential drawbacks of existing standards. Finally, the knowledge gained from the tests with *C. ehrenbergii* was compared to existing data from standard species to highlight the benefits of non-standard species testing.

Chapter 1 outlines the dimensions of anthropogenic pollution, summarizes the key aspects of ecotoxicology, and highlights the essential role of algae in natural cycles. Chapter 2 presents a miniaturized method for toxicity tests on *C. ehrenbergii* applied to metals. The necessity of culture medium modification is demonstrated in order to avoid metal complexation and underestimation of metal toxicity. Chapter 3 deals with the toxicity of pharmaceuticals and highlights the relevance of extended test durations in order to avoid an underestimation of delayed adverse effects. Maximum photosynthetic quantum yield is shown to be a feasible additional endpoint for identifying adverse effects on photosynthetic efficiency. Chapters 2 and 3 both address the different modes of action of the test substances and analyze effects on quantitative and qualitative endpoints. Effect concentrations are based on cell growth and maximum photosynthetic quantum yield. Toxic effects are visualized based on the alteration of cell morphology and chlorophyll fluorescence. Chapter 4 synthesizes the findings of chapters 2 and 3 and discusses the knowledge gained from this thesis.

Future research is required to investigate whether the outcomes of this study also apply to standard species and procedures. In particular, the observed occurrence of delayed effects of pharmaceuticals highlights the need for further studies with standard species and extended test durations in order to question whether the current standard of short-term exposure sufficiently assesses the hazards of pharmaceuticals towards algae. In conclusion, this thesis has assessed the toxicity of thirteen inorganic and organic pollutants towards an important representative of benthic algae, demonstrated the benefits of investigations beyond recognized standards, and contributed to the understanding of the effects of anthropogenic pollutants on algae.

Chapter 1: General introduction

1.1. Environmental pollution

Pollution is defined as the introduction of harmful substances into the environment as a result of human activities (Nordberg et al., 2009). The sources of pollution are manifold but can in general be traced back to three major causes: (i) production, (ii) application, and (iii) disposal of anthropogenic compounds and excreta (Walker et al., 2012). Processes involved in these areas are among others the discharge of wastewater and exhaust air into the environment, the use of pesticides, the combustion of fuel for energy generation and transportation, and the incineration of waste. The history of humankind contains countless pollution events which in their entirety depict a continuum of human interference with natural cycles (Nriagu, 2019).

1.1.1. Pollution as a side effect of civilization and driver of biodiversity loss

Human civilization is unequivocally connected to environmental pollution. Historical evidence of freshwater contamination by untreated human feces dates back to the time of the first large settlements around 5000 years ago (George, 2015; Nriagu, 2019). At the same time, human ingenuity has long been seeking solutions to environmental problems. The invention of wastewater treatment plants and legislation for environmental quality standards are just two examples. Nevertheless, the impact of human activity on environmental integrity has reached an unprecedented extent since the industrialization era, giving rise to the term “Anthropocene” (DellaSala et al., 2018). The “Great Acceleration” since the 1950s refers to the explosive growth in population, consumption, and unsustainable technologies, that has influenced the earth system in such a universal manner that it is considered the marker for the beginning of a novel geological epoch (Ludwig and Steffen, 2018).

One component of the earth system, that has integrated the increasing changes since the Great Acceleration on a global scale, is biodiversity. According to the latest IPBES report on biodiversity and ecosystem services, around one million species are at risk of extinction and the rate of extinction is tens to hundreds of times higher than the average rate over the past 10 million years (IPBES, 2019). The causes for the decline in biodiversity are manifold but have dramatically increased their impact over the past 50 years. Alongside land use modification and climate change, pollution is one of the main drivers of biodiversity loss.

1.1.2. Main sources of pollution

Regarding aquatic ecosystems, pollution sources can be categorized in point and diffuse sources (Fent, 2013). Point sources are defined as traceable locations that emit contaminants into the environment. The most common type of point source is wastewater discharge, both municipal and industrial. In contrast, diffuse sources are more diverse and emit contaminants regionally dispersed into the environment. Examples of diffuse sources include agriculture, urban runoff, and the transportation sector.

Wastewater treatment plants were originally designed to reduce dissolved organic matter and nutrients such as nitrate, ammonium, and phosphate from urban sewage containing human excreta (Lofrano and Brown, 2010). However, the industrialization has led to an increasing complexity of sewage that meanwhile contains residues of a multitude of compounds from different chemical categories e.g. pharmaceuticals, personal care products, biocides, and metals, often referred to as micropollutants. Today, there are an estimated 350,000 chemicals (or mixtures of chemicals) on the world market (Persson et al., 2022) of which a certain proportion ends up in wastewater treatment plants. This complexity of the water matrix goes beyond the traditional purification targets of wastewater treatment plants and thus results in emissions of contaminants into surface waters. Therefore, urban wastewater is considered the dominant emission pathway for pharmaceuticals globally (Beek et al., 2016). In addition, considerable amounts of metals enter surface waters through wastewater treatment plants, especially due to stormwater overflows (German Environment Agency, 2017). Furthermore, an unknown number of metabolites and transformation products from active ingredients is formed both during the purification process and afterwards in the natural environment which potentially contribute to the pollution of waterbodies.

Agriculture shapes and influences the environment to a substantial extent. Today, more than 50 % of Germany's total area is used for agriculture (German Environment Agency, 2018). This is accompanied by the application of immense amounts of plant protection products i.e. herbicides, fungicides, insecticides, and others, which are summarized under the term pesticides. According to calculations of the Federal Environment Agency, the annual use of plant protection products in Germany amounts to 34,000 tons of active ingredients. These products are sprayed directly onto crops or soils and hence into the environment. Moreover, the spreading of liquid manure can be a relevant emission source of veterinary pharmaceuticals (Chopra and Kumar, 2018). Via spray drift and rainwater runoff, residues of pesticides and pharmaceuticals can be carried into nearby waterbodies and affect non-target organisms.

In addition, atmospheric deposition of contaminants is another pathway of a variety of chemicals into the environment. Combustion processes in the sectors of transportation, households, and industry can lead to inorganic emissions such as sulfur dioxide, but also to emissions of hazardous organic compounds such as polycyclic aromatic hydrocarbons (PAH), dioxins, and furans that can result from incomplete combustions (Fent, 2013). Fig. 1 summarizes the potential emission pathways of contaminants into aquatic ecosystems.

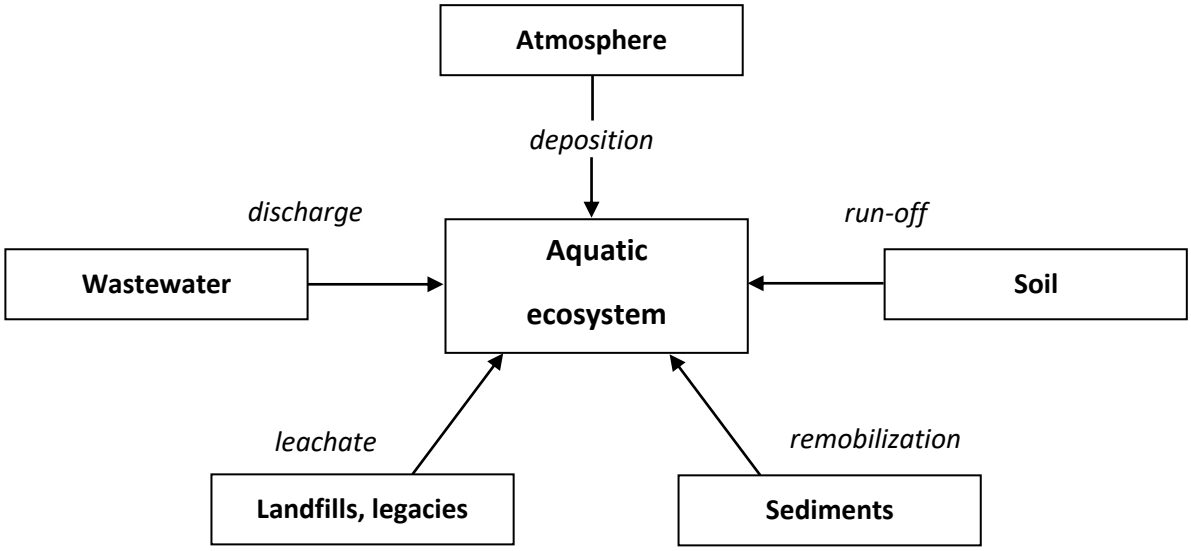

Fig. 1: Potential emission pathways of contaminants into aquatic ecosystems. Figure adapted from (Fent, 2013).

1.2. Ecotoxicology

The expanding impact of human activity on the environment led to an increase in environmental awareness and hence to the foundation of a novel science area which was named "ecotoxicology" for the first time at the Stockholm SCOPE environmental conference in 1969 (Truhaut, 1977). The main purpose of ecotoxicology is to determine hazardous effects of pollutants on the living environment (Walker et al., 2012). Therefore, ecotoxicology is a multidisciplinary science that connects concepts from toxicology, ecology, and environmental chemistry. Aspects such as bioavailability of chemicals or their fate in the environment as well as the interaction between direct effects on particular species and subsequent indirect impacts on ecosystems play important roles in ecotoxicological research. In this context, it is worth noting that hazardous effects can occur on all biological levels from the molecular to the ecosystem level. On the molecular level, pollutants can interact e.g. with genes or enzymes which may lead to a malfunction of metabolism or cell division and thus affect the organism level. Adverse effects e.g. on the reproduction or the survival of offsprings can affect the population level. The different sensitivities of organisms to chemicals can lead to alterations within a biocenosis. These alterations in connection with different mutual relationships between species and their abiotic environment can ultimately affect the composition of ecosystems and their functions such as biochemical cycles or energy fluxes. In the following sections, the main principles of ecotoxicology are summarized and complemented by current means of risk assessment and regulation procedures.

1.2.1. The principle of ecological representatives

As described above, the main purpose of ecotoxicology is to determine hazardous effects of pollutants on the living environment. Since the natural diversity of species is too large to be examined in its entirety, the principle of representatives is applied (Bláha and Hofman, 2020). A few test species that represent different levels of the trophic food web, and which are easily cultivated under laboratory conditions, are used as bioindicators in standardized bioassays. This reflects a compromise between the complexity of ecosystems and the feasibility of laboratory bioassays. Standardized test procedures for organisms from all levels of the food web are described in internationally recognized OECD or ISO guidelines. All guidelines have in common that the test protocols deliver comparable and reproducible toxicity data which are the basis for risk assessment and regulation of chemicals.

In the aquatic context, the ecological functions of decomposers, producers, and consumers are most often represented in the laboratory by bacteria, microalgae, microcrustaceans, and fish. Additionally, there are test procedures with aquatic macrophytes and other species belonging to the taxonomically diverse group of macroinvertebrates such as insect larvae and mollusks.

In addition to the aspect of trophic levels, different ecological traits of organisms within the same trophic level are another reason for the selection of test species. The testing of species with additional ecological traits can help to better understand the effects of pollutants under natural conditions. For instance, in case of microcrustaceans, the planktonic species *Daphnia magna* is the typically used standard test organism (OECD, 2004). However, if the chemical properties of a test substance under investigation suggest a considerable adsorption onto sediments, a sediment dwelling organism such as *Hyalella* sp. should be additionally investigated (EMA, 2006) as it represents species which are potentially affected by a different important exposure scenario via the contact to contaminated sediments.

In case of algae, the OECD guideline 201: “Freshwater Alga and Cyanobacteria, Growth Inhibition Test” (OECD, 2011) is the standard guideline for the testing of chemical toxicity towards algae and cyanobacteria. This guideline proposes five species of non-attached microalgae and cyanobacteria of which the planktonic chlorophyte *Raphidocelis subcapitata* is the most often used test species (Sharma et al., 2021). However, considering different ecological traits, there is no standardized test protocol for attached living benthic algal species, yet.

1.2.2. Determination of effect concentrations

An ecotoxicological principle is that independent of the test organism, the evaluation of a chemical’s toxicity is generally based on a concentration-response analysis (Walker et al., 2012). Beyond a certain threshold concentration, each chemical substance will exert adverse effects on living organisms that increase with its concentration. Effects of chemicals are assessed by the comparison of treatment groups

of organisms which are exposed to a dilution series of the test substance with a control group. Depending on the test organism and the test procedure, different measurement variables, so called endpoints, exist to determine the response of the organisms to the test substance. Regarding algae, the inhibition of growth rate is the standard endpoint for the evaluation of toxic effects. In order to determine comparable parameters, which are called effect concentrations, linear regression models such as Probit are commonly used to establish a quantitative concentration-response relationship. Plotted in a semi-logarithmic diagram, this relationship will typically result in a sigmoidal concentration-response curve. From the concentration-response relationship, several important parameters are derived. The EC_{50} value is the concentration at the inflection point of the sigmoidal function. In case of the algal growth inhibition test, the EC_{50} is the concentration of test substance that leads to an inhibition of growth rate of 50 % in comparison to the control. Likewise, the EC_{10} shows an effect of 10 %. The No-Observed-Effect-Concentration (NOEC) is the highest tested concentration that does not show a significantly different growth rate from the control group and the Lowest-Observed-Effect-Concentration (LOEC) is the lowest tested concentration that does show a significantly different growth rate. In addition to the numerical values of the effect concentrations, also the inclination of the concentration-response curve gives information on the toxicity of a test substance. The steeper the curve, the more sensitive an organism reacts to an increase in concentration and the range between NOEC and maximum effect becomes narrower. In contrast, the flatter the curve, the larger is the tolerance of an organism to an increase in concentration. Fig. 2 shows a typical concentration-response-curve and the relevant ecotoxicological parameters.

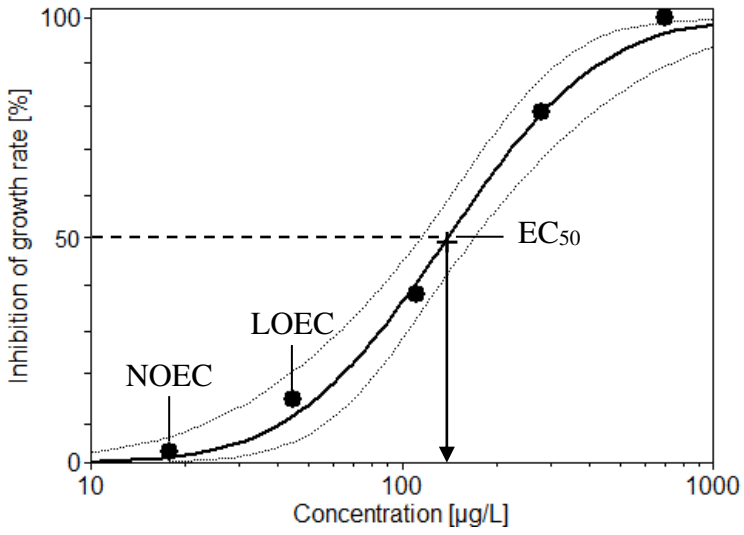

Fig. 2: A typical concentration-response curve showing the inhibition of growth rate as response to the exposure of a test substance. No-Observed-Effect-Concentration (NOEC), Lowest-Observed-Effect-Concentration (LOEC), and half-maximal effect concentration (EC_{50}) are indicated.

1.2.3. Environmental risk assessment

In the European Union, manufacturers as well as distributors of any kind of chemicals are required to evaluate the potential risks of their products prior to the marketing authorization. This regulation meets the precautionary principle in order to avert risks for the environment. Depending on the class of chemicals, different legal regulations exist. Pesticides are authorized by the European Food Safety Authority (EFSA, 2013), industrial chemicals are authorized by the European Chemicals Agency through the REACH regulation (EC, 2006), and pharmaceuticals are authorized by the European Medicines Agency (EMA, 2006). The basic principle of risk assessment is to compare exposure data with toxicity data. In case, the exposure of organisms to the chemical under investigation is likely to be higher than a safe threshold value, a potential risk exists and safety measures need to be taken or a product authorization may even be refused.

Exposure data are typically generated as part of the prospective ecotoxicology by modeling Predicted-Environmental-Concentrations (PEC) of chemicals before they are placed on the market. This applies both to chemicals that are intentionally introduced into the environment such as pesticides as well as to chemicals that enter the environment unintentionally such as residues of pharmaceuticals or industrial chemicals e.g. through wastewater treatment plants.

The toxicity data is typically generated in standardized bioassays. However, taking the complexity of ecosystems and the fact that hazardous effects of pollutants can occur on all biological levels into account, a tiered-approach from simple single-species tests (tier 1) to complex field studies (tier 4) is pursued to increase the complexity of the toxicity data. Thus, the tiered-approach improves the transferability of the information obtained in biotests to the real environment. For all substances under consideration, a base set of ecotoxicological data is required. If hazardous properties of a test substance are found, additional tests are necessary.

Due to their robustness, reproducibility, and comparability, a typical base set of tier 1 studies consists of standardized single-species tests from one species each of algae, *Daphnia*, and fish. The existing drawback of a limited transferability of the laboratory data to the ecosystem level is compensated for by the use of assessment factors (AF). Since natural ecosystems are highly diverse and only a few species are investigated in the laboratory, it is assumed that certain representatives of the ecosystem will be more sensitive than the few species tested. Therefore, the lowest toxicity value obtained in a set of biotests, e.g. EC_{50} value, is divided by an AF to extrapolate the Predicted-No-Effect-Concentration (PNEC). The value of the AF depends on the complexity of the test data but usually is in the range of 3 to 1000. The fewer species tested and the lower the complexity of the tests, the greater the uncertainty of the data and the higher the assessment factor. A risk quotient (RQ) is obtained by the comparison of PEC to PNEC values. If the RQ is larger than 1, a risk for the environment is indicated and higher tier

studies are required to reduce the degree of uncertainty of the data and to refine the risk assessment. Refinement is an iterative procedure in which the risks are reassessed after each tier.

At tier 2, additional laboratory tests are conducted and the approach of species sensitivity distribution is possible. In this approach, a relatively large number of species (> 5 vertebrates or > 8 invertebrates), including non-standard species, is tested to derive the HC_5 value which represents a threshold (hazard concentration) of the test substance at which 95 % of the species are assumed to be protected. Higher-tier experiments are possible in model ecosystems (tier 3) or field studies (tier 4). These studies are the most complex and have the potential to evaluate effects of pollutants under more realistic conditions as they consider abiotic and biotic interactions such as predator-prey relationships or recovery of effects after an initial dose of test substance. At the same time, higher tier studies may lack reproducibility as test substances underly influences such as degradation, sorption, or solar radiation, and they are most time-consuming and cost-intensive. The benefits and limitations of the different tiers of studies are graphically summarized in Fig. 3.

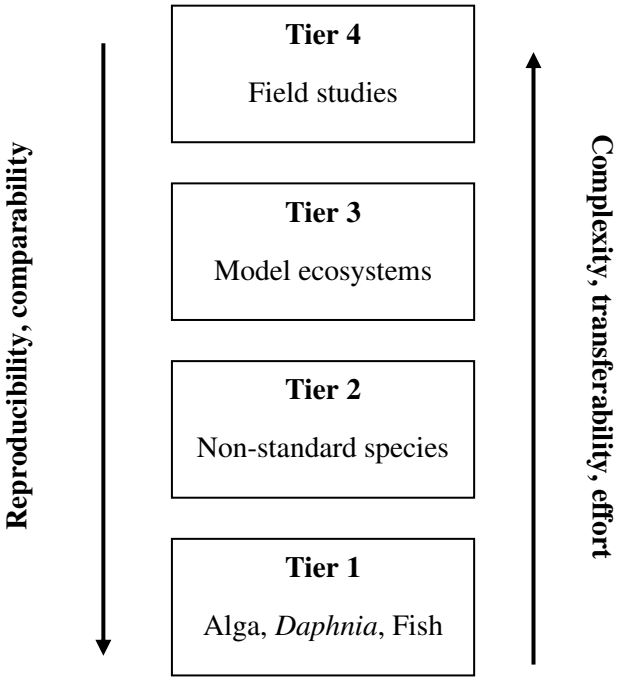

Fig. 3: Structure of the tiered approach indicating benefits and limitations of the different levels. Figure adapted from (Fent, 2013; van Gestel et al., 2019).

The tiered-approach is pursued to ensure that the final decision on the authorization of a chemical is based on scientific data which include all possible available information to both safeguard the environment and evaluate the chemical's risk appropriately. In case the RQ remains larger than 1 after the refinement, different possibilities exist depending on the class of chemicals. The marketing

authorization of a medicinal product will not be denied due to environmental risks. Precautionary measures such as indication of a potential risk by labelling the product and by including information in the package leaflet are the only consequences for this type of chemicals. For pesticides and industrial chemicals, risk mitigation measures such as engineering solutions may be imposed, or, in the final instance, the product authorization can be restricted or refused. Equations 1 and 2 summarize the required regulatory parameters.

$$PNEC = EC/AF \quad (1)$$

$$RQ = PEC/PNEC \quad (2)$$

RQ > 1: A risk for the environment exists.

RQ < 1: A risk for the environment is most likely not to be expected.

1.3. Algae

From the perspective of environmental risk assessment, algae stand for primary producers that are threatened by pollution through various pathways e.g. wastewater treatment plants and agriculture. However, from an ecological perspective, algae are by far more than non-target organisms in pollution scenarios. Under natural conditions, algae stand at the basis of global food webs, have major influence on biogeochemical cycles, and are bioindicators for the habitats they colonize. While algae generally depend on the availability of water, the habitats they colonize range from fresh and salt water, over hot springs, to snow fields and moist soils (Graham et al., 2016). From a taxonomic point of view, different definitions exist of the term algae. While current classification systems restrict the term algae to eukaryotes (Adl et al., 2019), cyanobacteria have traditionally been regarded as “blue-green-algae” due to their morphological similarities. Since cyanobacteria represent the single origin to all algal plastids (Büdel et al., 2024), here they are also included in the definition of algae. The general feature that connects this heterogenous assemblage of algal species is oxygenic photosynthesis.

1.3.1. The essential role of algae for biogeochemical cycles and biotic associations

Algal oxygenic photosynthesis converts inorganic carbon into biomass and simultaneously generates oxygen. Thereby, algae contribute to about half of the global oxygen production which is essential to most life on Earth today (Graham et al., 2016). The conversion of inorganic into organic carbon is a major driver of the carbon cycle. Algal organic carbon, i.e. biomass, serves as a food source for heterotrophic organisms and thus forms the basis of aquatic food webs. Herbivores depending on algal biomass range from microscopic protists such as flagellates, over mesograzers such as insect larvae to larger animals such as fish. In addition, decaying algal biomass is a food source for detritivores such as bacteria, fungi, and decomposing protists and the deposition of dead organic material into anoxic sea

and lake sediments contributes to long-term carbon sequestration. Moreover, the uptake of nitrate, ammonium, and phosphate, which are essential macronutrients for algal growth, influences the nitrogen and phosphorus cycles. However, excessive nutrient inputs into surface waters due to fertilizer runoff or the discharge of untreated wastewater can lead to harmful algal blooms as a result of eutrophication. "Dead-zones" of anoxic conditions are a possible consequence of decaying algal blooms that threaten aerobic organisms (Paerl et al., 2011).

1.3.2. Benthic algae

In addition to their roles in biogeochemical cycles and as food sources, benthic algae are of particular relevance for ecosystems as they shape the bottom of water bodies and create habitats that are colonized by other species (Gutowski and Foerster, 2009b). Together with non-photosynthetic bacteria and fungi, benthic algae (including cyanobacteria) form biofilms on rocks, sediments, dead wood, and other solid materials which act as microhabitats that provide shelter and food. In contrast to planktonic algae in marine or lake ecosystems, benthic algae may depict the majority of the algal flora and be the main contributors to primary production in lotic ecosystems since they are sedentary and not carried away by the current (Law, 2011). Phytobenthic growth and diversity both provides and depends on numerous biological, physical, and chemical functions and properties which are inter-related. The algal biomass provides food for herbivores while its growth is reduced by grazing. Nutrients are limiting factors for algal growth but at the same time inorganic forms of nutrients are converted into organic forms by algal uptake and thus are made available for higher trophic level species. The current velocity is a potential risk for being detached from the substrate while benthic algal structures reduce the velocity for other species providing shelter.

A major trait of the sessile way of life is that benthic algae integrate the influences of their habitats over time. In case of ecological stress e.g. due to pollution, the lack of mobility inevitably results in two options: tolerate or perish (Law, 2011). Therefore, benthic algae represent a fundamentally different ecological niche than planktonic algae, which makes them to important bioindicators of natural environments that should consequently be considered in the toxicity testing of pollutants.

From a legal perspective, benthic algae are recognized by the European Water Framework Directive (EC, 2000), which requires member states to monitor benthic algae in surface waters in order to determine their ecological status. However, although algae already are a fundamental part of ecotoxicological risk assessment, until today, benthic algae are not part of standardized test procedures. The OECD guideline 201 (OECD, 2011) proposes five species of non-attached microalgae and cyanobacteria of which the planktonic chlorophyte *Raphidocelis subcapitata* is the most commonly used test species (Sharma et al., 2021). Therefore, here, *Closterium ehrenbergii* is presented as a representative of benthic streptophyte algae and possible candidate species in ecotoxicity testing.

1.3.3. *Closterium ehrenbergii*: a benthic green alga

Closterium ehrenbergii is a unicellular streptophyte green alga. It belongs to the class of Zygnematophyceae which represents the closest relatives of embryophytes (Cheng et al., 2019). *C. ehrenbergii* lives attached to aquatic macrophytes and filamentous algae in both lotic and lentic freshwater ecosystems. It is a cosmopolitan that prefers a circumneutral pH and is considered a character species of meso- to eutrophic habitats, although it colonizes also oligotrophic water bodies (Gutowski and Foerster, 2009a). The morphological characteristics of *C. ehrenbergii* are shown in Fig. 4. The shape of the cell resembles a crescent moon and consists of two symmetric semicells, which is a typical feature of Desmidiales species, although it does not have a distinct constriction in the middle of the cell (Coesel, Peter F. M. and Meesters, 2007). Each semicell contains one chloroplast which is structured in longitudinal ridges and contains numerous scattered pyrenoids. The nucleus is located in the cell center. Vacuoles are located at the apices of the cells which contain crystals of barium sulphate that constantly move due to Brownian motion (Brook et al., 1980). The excretion of mucilage from pores in the cell walls at the apices enables *C. ehrenbergii* to attach to the substrate as well as to conduct a somersault-like movement (Graham et al., 2016). The cell size of this species is extraordinary and can measure up to 800 x 100 µm (Gutowski and Foerster, 2009a).

Fig. 4: Cells of *C. ehrenbergii* in the shape of crescent moons (A). The colorless nucleus is located in the center of the cell. Pyrenoids appear as scattered spherical structures (B). Crystals of barium sulphate are located in a vacuole at the apex (C). Chloroplasts are structured in longitudinal ridges. Visualized by chlorophyll fluorescence (D).

C. ehrenbergii replicates both asexually by cell division and sexually by conjugation and the formation of zygospores (Büdel et al., 2024). In order to perform conjugation, two complementary mating types from heterothallic strains are required, that identify each other by intercellular communication based on the excretion of mating-type-specific sex pheromones (Tsuchikane and Sekimoto, 2019). Under natural conditions, the formation of zygospores often occurs as a reaction to ecological stress such as desiccation or nitrogen shortage. Dormant zygospores can endure desiccation and may germinate again under more favorable conditions.

Closterium ehrenbergii has long been studied as a model organism in various fields of research such as influences of abiotic conditions on growth (Kasai and Ichimura, 1990), or reproduction mechanisms (Ichimura, 1983; Ichimura and Kasai, 1995; Lutman, 1911). There are also a few studies assessing the toxicity of single compounds such as copper (Wang et al., 2018) or chlorine (Sathasivam et al., 2016) to *C. ehrenbergii*. However, to date there has not been a systematic approach for assessing the toxicity of a wide range of environmentally relevant inorganic and organic pollutants to *C. ehrenbergii*, that follows the principles of OECD 201 and compares the results with commonly used planktonic test species in order to evaluate the potential benefits of the additional testing of this benthic species.

1.4. Scope of the thesis

To date, planktonic species form the basis of the ecotoxicological assessment of hazardous effects of pollutants on algae. Hence, knowledge gaps exist about the effects on benthic algae, despite the fact that those may play key roles in aquatic ecosystems. Therefore, the aim of this thesis was to investigate the toxicity of pollutants of high concern towards a benthic alga and to assess the potential knowledge gain from the testing of this additional species and of procedures that go beyond the common standard. To pursue this overall aim, the following objectives were defined:

(i) select a representative of benthic algae as test species,
(ii) develop a feasible bioassay for toxicity testing that meets the requirements of the test species and follows the principles of the OECD guideline 201,
(iii) question common standard procedures in order to determine potential limitations,
(iv) determine environmentally relevant inorganic and organic pollutants and assess their toxicity,
(v) evaluate photosynthetic quantum yield and cell morphology as additional endpoints to growth rate,
(vi) compare the findings from this thesis with existing data from planktonic standard species to identify potential knowledge-gains.

These objectives are addressed in the following chapters. Chapter 2 presents *C. ehrenbergii* as a benthic test species and describes a miniaturized bioassay for toxicity testing. The toxicity of six metals and the influence of the culture medium composition on the extent of the adverse effects of the metals were investigated. Effects were assessed both quantitatively by the inhibition of growth rate and qualitatively by the microscopic examination of cell morphology alteration. Chapter 3 deals with the chronic toxicity of seven pharmaceutical substances and the influence of exposure time on the extent of effects. The limitations of growth rate as the standardized endpoint in algal tests were evaluated in the context of delayed toxic effects. Furthermore, maximum photosynthetic quantum yield as well as cell morphology and chlorophyll fluorescence were studied as additional endpoints to growth. Chapter 4 synthesizes the findings of chapters 2 and 3 and discusses the knowledge gained from the studies.

Chapter 2:
A microplate-based bioassay for toxicity testing using the large benthic algal species *Closterium ehrenbergii*

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Abstract

Pollution of water bodies by metals has long been studied but still remains a threat to healthy ecosystems. While most ecotoxicological studies on algae are performed with planktonic standard species such as *Raphidocelis subcapitata*, benthic algae may depict the majority of the algal flora in rivers and streams. These species encounter different exposure scenarios to pollutants as they are sedentary and not carried away by the current. This particular way of life leads to an integration of toxic effects over time. Therefore, in this study, the effects of six metals on the large unicellular benthic species *Closterium ehrenbergii* were examined. A miniaturized bioassay with low cell densities of 10-15 cells/mL using microplates was developed. Through chemical analysis, metal complexing properties in the culture medium were demonstrated, that could lead to an underestimation of metal toxicity. Thus, the medium was modified by excluding EDTA and TRIS. The toxicity of the six metals ranked by EC_{50} values in descending order, was as follows: Cu (5.5 µg/L) > Ag (9.2 µg/L) > Cd (18 µg/L) > Ni (260 µg/L) > Cr (990 µg/L) > Zn (1200 µg/L). In addition, toxic effects on the cell morphology were visualized. Based on a literature review, *C. ehrenbergii* was shown to be partly more sensitive than *R. subcapitata* which suggests that it can be a useful addition to ecotoxicological risk assessment.

Keywords

Ecotoxicity; metals; *Closterium ehrenbergii*, miniaturized bioassay, culture medium modification.

1. Introduction

Environmental pollution on a large scale has been a major threat to healthy ecosystems since the beginning of the industrialization (Crutzen, 2002). Although much effort has been made to improve environmental protection, humankind faces ever new kinds of pollution. In many countries, ambitious goals such as the American Clean Water Act (US, 1972) or the European Water Framework Directive (EC, 2000) have been presented to counteract pollution and safeguard environmental integrity. However, a recent official report shows that 60% of European waterbodies have not reached good ecological and chemical quality yet (European Environment Agency, 2018). Furthermore, recent research has postulated that the safe operating space of the planetary boundary for novel entities (formerly known as chemical pollution) has already been exceeded since global production outstrips societies' capacities to conduct risk assessment and monitoring (Persson et al., 2022).

While metals represent one of the first groups of chemicals that were regulated politically (EC, 1976), these substances exhibit an ongoing risk to the environment until today. Since industrial direct discharge has dramatically diminished over the past decades, wastewater treatment plants are considered one of the main sources of metal emissions nowadays (German Environment Agency, 2017b). The separation efficiency depending on the metal and the technological status of the plant ranges from 20 to 90% (German Environment Agency, 2002), however stormwater overflows may lead to an untreated discharge into the connected river (German Environment Agency, 2017a). Further sources of emission are surface run-off from urban areas, historical mining sites and to some extent atmospheric deposition. Measured environmental concentrations vary considerably between different metals and locations. In extremely polluted rivers, metal concentrations can reach mg/L levels (Bhuiyan et al., 2011), but on global average, concentrations are within the range of µg/L (Li et al., 2020). Although the mass flow into surface waters has decreased over the past decades, metals persist as a significant source of pollution as they tend to adsorb onto sediments and may be remobilized under changing conditions (Liu et al., 2020).

At trace concentrations, many metals are essential for an intact metabolism of all kinds of living organisms as they are components of enzymes. At higher concentrations, however, many of these metals turn into toxic pollutants (Sunda et al., 2009). A common mode of toxic action is the formation of reactive oxygen species (ROS), leading to oxidative stress. ROS typically include hydroxyl radical ($^{\cdot}OH$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Sharma et al., 2012). Although cells have enzymatic and non-enzymatic protection mechanisms against ROS, such as superoxide dismutase, peroxidase, or glutathione (Wang et al., 2018), ROS may damage proteins, lipids, nucleic acids as well as entire cell membranes once the defensive capacity is exceeded, and hence lead to the destruction of the cells from inside (Pinto et al., 2003).

It is the task of ecotoxicological studies to determine hazardous effects of contaminants such as metals on non-target organisms. Algae are a large group of organisms that stand for such non-target organisms in several pollution scenarios (Sathasivam et al., 2016). Algae are considered a crucial part of ecosystems as they stand at the very beginning of the trophic food web and also play key roles in global biogeochemical cycles (Graham et al., 2016).

According to the OECD Guidelines for the Testing of Chemicals (OECD, 2011), five species of "non-attached microalgae and cyanobacteria" that are primarily planktonic are suggested as test organisms of which *Raphidocelis subcapitata* is one of the most commonly used test organisms (Sharma et al., 2021). In riverine ecosystems, however, benthic algae often play a more important role as they depict the majority of the algal flora. The main ecological difference between these two ways of life is that benthic algae are sedentary. They do not live free-floating but are attached to sediments, dead wood or macrophytes. Consequently, benthic species may be directly exposed to contaminated sediments and point sources of pollution. In contrast, planktonic species are carried away from the current and may benefit from natural dilution. Therefore, benthic species encounter different exposure scenarios, may develop adaption strategies, and eventually represent a valuable addition to ecotoxicological risk assessment of natural habitats (Law, 2011).

Closterium ehrenbergii is a large unicellular benthic green alga belonging to the Zygnematophyceae, a class of streptophyte green algae which represents the closest relatives of land plants (Embryophyta) (Cheng et al., 2019). It lives attached to filamentous algae and macrophytes in freshwater. Considering its ecological niche, *C. ehrenbergii* is a cosmopolitan species colonizing all ecoregions. It prefers a circumneutral pH and meso-eutrophic habitats (Gutowski and Foerster, 2009). With its extraordinary large cell dimensions of up to 800 × 100 µm, *C. ehrenbergii* is visible to the naked eye and ideally suited to be observed under low magnification using a stereomicroscope. Since there is no benthic green alga proposed by OECD 201 (OECD, 2011), *C. ehrenbergii* is presented here as a possible candidate species for ecotoxicity testing in a standardized and miniaturized manner using microplates.

The objectives of this study were to (i) evaluate the toxicity of six metals to *C. ehrenbergii*; (ii) investigate the impact of chelate-forming components in culture media on metal toxicity; and (iii) verify the applicability of a microplate-based bioassay using large unicellular algae for testing environmental pollutants.

To the best of our knowledge, this is the first attempt to measure and compare the toxic effects of metals on *C. ehrenbergii* in a microplate-based bioassay using the original (Ichimura, 1971) and a modified version of the commonly used C-medium.

2. Materials and Methods

2.1. Test chemicals

The test substances copper (as $CuSO_4 \cdot 5H_2O$), zinc (as $ZnSO_4 \cdot 7H_2O$), chromium (VI) (as $K_2Cr_2O_7$), silver (as $AgNO_3$), nickel (as $NiCl_2 \cdot 6H_2O$) (all purity ≥ 98%), and cadmium (as $Cd(NO_3)_2$ in 2% HNO_3, AAS-standard solution) were obtained from Carl Roth, Germany. For the preparation of stock solutions, metal salts were dissolved in deionized water to prepare metal ion concentrations of at least 100-fold higher than the highest test concentration and then filter sterilized (Rotilabo syringe filters, 0.22 µm PVDF). Stock solutions were stored in the dark at 5 °C. For each test, fresh dilutions were prepared from metal stock solutions using modified C-medium (C-modified). The concentration ranges of the final tests were determined by preliminary range-finding tests. All concentration data in the following refer to the nominal metals' dissolved speciation.

2.2. Test organism and culture conditions

Closterium ehrenbergii (strain SAG 134.80) was obtained from the Culture Collection of Algae at the University of Göttingen, Germany (SAG). Cultures were maintained in test tubes in C-medium (Ichimura, 1971), at 25 ± 1 °C and low light intensity of 23 µmol photons m^{-2} s^{-1} (cool daylight fluorescent tube) under a 14:10 h light:dark cycle. The culture medium was renewed every two weeks. A deviation from the standard culture conditions of OECD 201 (OECD, 2011) (40-60 µmol m^{-2} s^{-1} and continuous irradiation) was necessary because *C. ehrenbergii* conducts cell division only during the dark period (Lutman, 1911) and high light intensities damage the chloroplasts.

2.3. Modification of C-medium for metal testing

For studies with *C. ehrenbergii,* C (*Closterium*)-medium is the standard culture medium found throughout the literature (Sathasivam et al., 2016; Wang et al., 2018). However, some components are unsuitable for testing metal toxicity since they lead to metal chelation and thus influence metal bioavailability. Therefore, the original C-medium was modified (named "C-modified"). EDTA and TRIS were excluded, and ß-Na_2-Glycerophosphat$\cdot 5H_2O$ was substituted by KH_2PO_4 and NaCl. Since the medium without EDTA may not be autoclaved to avoid precipitation, all components were added aseptically to autoclaved deionized water. To achieve a final pH of 7.0, 160 µL/L NaOH 1 M were added to the medium after the macronutrient stock solution. In addition, a second modification of the medium (named "C-adjusted") was prepared using a 1:1 molar ratio of EDTA to iron, similar to the standard test medium OECD TG 201 (OECD, 2011). The three recipes, C-medium, C-modified, and C-adjusted, are shown in Supplementary Table A1.

2.4. Algal toxicity assay using microplates

An inoculum was taken from the exponential growth phase of the maintenance culture by collecting a tiny volume (approx. 50 µL) of dense algal suspension under sterile conditions with a glass Pasteur pipette from the bottom of the test tube. These cells were transferred into a small test tube, washed once with 5 mL of fresh C-modified medium and then used for the toxicity test. A preculture was not necessary. As test vessels, 24-microwell plates (PS) with tissue culture surface were used (Greiner Bio-One). One plate contained five test concentrations plus one control group with four replicates each. The tested concentration ranges of the six metals were as follows. Cu (1.0-16 µg/L); Ag (0.3-24.3 µg/L); Cd (3.75-60 µg/L); Ni (32-1250 µg/L); Cr (61.7-5000 µg/L); Zn (150-2400 µg/L). 10 to 15 cells were introduced into each well using a glass Pasteur pipette. Cells were counted at the beginning and end of the test under a stereo microscope (Zeiss Stemi 508) with 10x magnification. 1 mL of either C-modified medium (control group) or test substance dissolved in C-modified medium was added to each well. To minimize evaporation and simultaneously ensure a sufficient gas exchange, the microplates were sealed with Parafilm. The test conditions were equal to the maintenance culture conditions. The test duration was 72 h, and pH was measured (WTW Multi 9630 IDS) at the beginning and end of the test in each concentration and control group. For graphical representation of the test method, a video clip can be found in Supplementary File A2. In order to ensure the reliability of a test, validity criteria were established. Test results were only accepted if the following criteria were fulfilled: Growth rate of the control group > 0.47 1/d; coefficient of variation of the mean growth rate of the control group < 10%; cells of the control group morphologically intact during the whole test duration (determination at 200x magnification); all wells visibly free from contamination; clear concentration-response relationship with reasonably narrow confidence intervals present.

2.5. Data analysis

The growth rate (µ) and the inhibition of growth rate (I_μ) were calculated according to the following equations:

$$\mu = \ln(x_t/x_0)/t \quad (1)$$

$$I_\mu = (\mu_C - \mu_T)/\mu_C * 100 \quad (2)$$

where x_t is the absolute cell count after 72 h, x_0 is the cell count at the beginning of the test, μ_C is the growth rate of the control group and μ_T is the growth rate of the treatment group. No observable effect concentration (NOEC), lowest observable effect concentration (LOEC), 10% effect concentration (EC_{10}) and half-maximal effect concentration (EC_{50}) values were computed based on the nominal metal concentrations and the inhibition of growth rate using ToxRat Professional v. 3.3.0 software. To obtain quantitative concentration-response relationships, linear regression was performed based on replicate

values choosing either Probit (for Cu, Ni, Cd, and Cr) or Weibull (for Ag and Zn) function to achieve the best fitting model for each dataset. NOEC values were computed using Williams' test ($p < 0.05$).

2.6. Cupric-ion determination

To investigate the influence of the chelate-forming components of the original C-medium on the toxicity of copper towards *C. ehrenbergii,* copper was tested with both types of media (C-medium and C-modified), and the free cupric ion concentration was determined in both cases. In addition, the second modification of the medium (C-adjusted), which contained the same molar ratio of EDTA to iron as the OECD TG 201 medium, was also chemically analyzed on cupric ion chelation. For chemical analysis, a spectrophotometric method based on the reagent 1-(2-pyridylazo)-2-naphthal (PAN) was used. PAN forms a pink Cu-PAN complex with free cupric ions that can be quantified at λ_{max} 550 nm (Nozaki and Zhou, 1987).

3. Results and discussion

3.1. Sensitivity of *C. ehrenbergii* to metals

The presence of metal ions diminished cell growth rates in all experiments. EC_{50}, EC_{10}, NOEC and LOEC values are summarized in Table 1. Concentration-response curves are shown in Fig. 1. The toxicity of the six tested metals towards *C. ehrenbergii* ranked by EC_{50} values in descending order was as follows: Cu > Ag > Cd > Ni > Cr > Zn. Copper was found to be the most toxic metal (EC_{50} = 5.5 µg/L), and zinc was the least toxic one (EC_{50} = 1200 µg/L). Due to differences in the slope of concentration-response curves between the metals, the ranking order is not equal for EC_{50} and LOEC values. Silver for instance yielded a lower LOEC than copper (2.7 and 4.0 µg/L, respectively) but exhibited a less steep concentration-response relationship leading to a higher EC50 of 9.2 µg/L.

Table 1: Effect concentrations and confidence intervals of six metals on C. ehrenbergii after 72 h and growth rates and coefficients of variance of control groups.

Metal	NOEC [µg/L]	LOEC [µg/L]	EC10 [µg/L]	95% CI [a] [µg/L]	EC50 [µg/L]	95% CI [a] [µg/L]	μ_C [b] [1/d]	CV [c] [%]
Cu	2.0	4.0	3.5	3.0-3.9	5.5	5.0-6.0	0.53	2.4
Ag	0.9	2.7	2.8	1.8-3.7	9.2	8.0-11	0.49	5.0
Cd	3.8	7.5	3.5	2.5-4.4	18	16-20	0.52	7.5
Ni	80	200	90	65-110	260	230-300	0.52	7.7
Cr	190	560	540	500-580	990	940-1040	0.50	4.4
Zn	150	300	290	160-400	1200	990-1400	0.52	4.4

[a] 95%-confidence limit (according to Fieller`s theorem)
[b] Growth rate of the control group
[c] Coefficient of variance for the growth rate of the control group

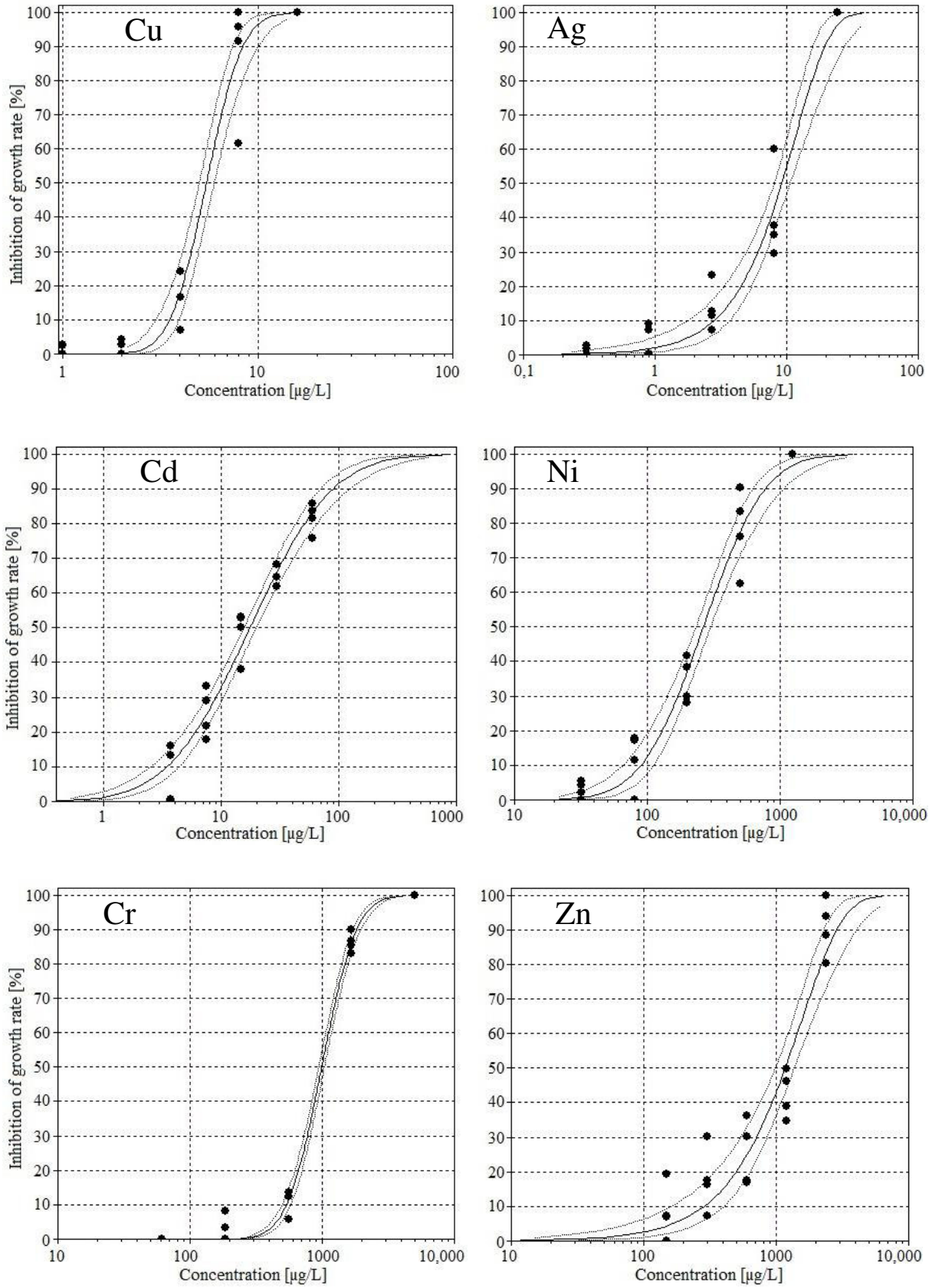

Fig. 1: Effects of six metals on the growth rate of *C. ehrenbergii* after 72 h. Tested in C-modified medium.

3.2. Applicability of the miniaturized test system using *C. ehrenbergii*

The miniaturized test system using *C. ehrenbergii* as a test organism demonstrated a high level of reliability. The growth rate of the control group was notably reproducible, showing a mean value of 0.51 1/d and an overall coefficient of variance (CV) of 5.7% over the six experiments (Table 1). Therefore, the validity criterion of OECD (2011) for "less frequently tested species", according to which a CV should not exceed 10%, could be fulfilled. For all tests, narrow 95% confidence intervals could be achieved (Fig. 1) demonstrating low fluctuations between replicates in treatment groups and hence the suitability of a microplate-based test system using *C. ehrenbergii* as a potential test organism for toxicity testing.

Although the tests were carried out without any pH buffer, the pH values did not vary more than 0.1 units during the tests (data not shown). Typically, the pH value increases over time during the exponential growth phase in algal assays without buffers due to the consumption of dissolved CO_2. However, this effect mainly depends on cell density (Sunda et al., 2009). In the standard test procedure of OECD (2011), initial cell densities of $2x10^3$ - 10^5 cells/mL are recommended leading to final cell densities of > 10^5 – 10^8 cells/mL after 72 h. In this study, due to the miniaturization of the test system and the cell dimensions of *C. ehrenbergii* very low cell densities of 10-15 cells/mL could be implemented at the test start leading to approx. 50-70 cells/mL after 72 h in the control group. These low cell densities did not lead to a substantial increase in pH allowing the omission of a pH buffer in the C-modified medium for metal toxicity testing.

3.3. Comparison with standard algae species and different test conditions

For the comparison of toxicity values of *C. ehrenbergii* with those of standard algae species, the ECOTOX database (USEPA/ECOTOX, 2022) was used to generate a comprehensive dataset. Selection criteria were single species, static laboratory tests with a test duration of 72 h and effects expressed as EC/IC50 on growth and population. *Raphidocelis subcapitata* was chosen as comparative species since this is one of the most commonly used alga species in ecotoxicity testing. The formerly known names *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata* were included in the query. As for test substances, data of all available metal salts were selected since the toxicity depends on the metal's ionic speciation rather than on the different compounds. Concentration values were converted to the ionic speciation of the metals if data were reported as compounds.

For nickel, only a single study was found with the selected filter criteria. Therefore, the filter criterium test duration was not specified for data collection of this substance, leading to a total of three studies. No published data were found at all for silver. Hence, instead of *R. subcapitata,* studies with any freshwater green algae and an unspecified test duration were retrieved, leading to four papers on silver.

In total, the database query resulted in a number of 39 studies, including 51 datasets that were used for the comparison of EC_{50} values. A compilation of the selected studies can be found in Supplementary Table A3. Fig. 2 shows the comparison of EC_{50} values between all six tested metals obtained in this study and in the literature review. *C. ehrenbergii* exhibited a higher sensitivity than *R. subcapitata* towards Cu and Cd but a lower sensitivity towards Cr and Zn. The EC_{50} value obtained here for nickel, is within the range of the literature data. Also, for silver the sensitivity of *C. ehrenbergii* is comparable to the range reported for six different freshwater green algae. Data are summarized in Table 2.

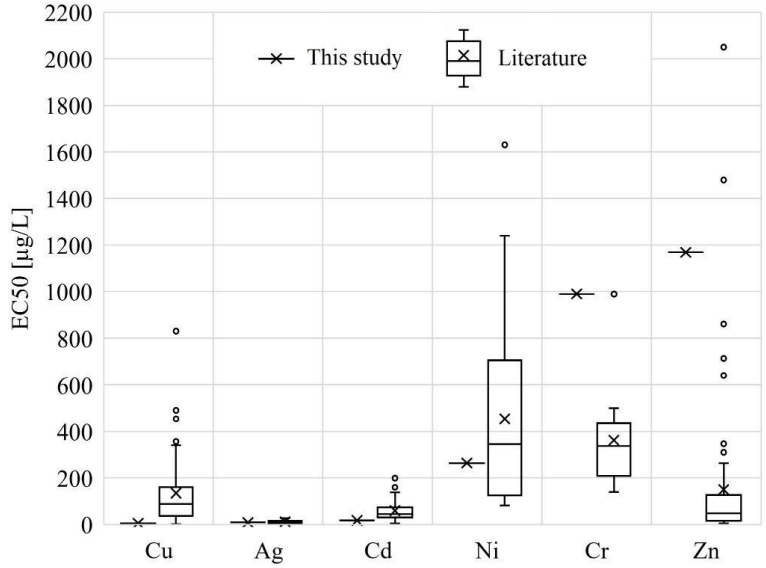

Fig. 2: Boxplots of EC50 values of six metals. Comparison between this study and the literature. A box represents the middle 50% of the available data from one dataset, ranging from the 25th to the 75th percentile. X is the mean and the cross line inside a box shows the median. Whiskers generally indicate the minimum and maximum values of a dataset. In addition, the maximum length of the whiskers is defined as 1.5 times the interquartile range above or below a box. Values outside this range are defined as outliers and are shown as circles. Literature data were derived from the ECOTOX database (USEPA/ECOTOX, 2022).

Table 2: Comparison of EC_{50} values of six metals (given as µg metal/L) between *C. ehrenbergii* (this study) and *R. subcapitata* (literature).

Metal	*C. ehrenbergii*	*R. subcapitata*[a]		
	Mean	Mean[b]	Range[b]	No. of Studies[c]
Cu	5.5	140	0.8 – 850	18
Ag	9.2	12	1.6 – 27	4
Cd	18	59	4.1 – 200	5
Ni	260	450	82 – 1630	3
Cr	990	360	140 – 990	9
Zn	1200	150	4.1 – 2100	13

[a] In case of silver, values represent data of six different freshwater green algae
[b] Data derived from ECOTOX database (USEPA/ECOTOX, 2022)
[c] The list of the selected studies can be found in Supplementary Table A3

Different test media compositions can affect metal bioavailability and hence toxicity. Both depend on various factors such as dissolved organic carbon (DOC), water hardness, and pH. Heijerick et al. (2005) reported that the presence of hardness-ions (Ca^{2+} and Mg^{2+}) acts protectively against Cu toxicity for *P. subcapitata*. The EC_{50} increased by a factor of 3 from 5.5 to 17 µg/L (toxicity decreased) when Ca or Mg concentrations were increased from 0.25 to 2 mM in standard ISO 8692 medium (ISO, 1989). The same effect was found for a shift of pH from moderately basic (pH = 8.0) to moderately acidic (pH = 5.8) culture medium (increase of H^+ ions). The basic medium exhibited a 3.5-fold higher Cu toxicity than the acidic medium (EC_{50} = 16 and 56 µg/L, respectively). This shift of toxicity values was attributed to the competition between Cu^{2+}, Ca^{2+}, Mg^{2+} and H^+ ions at the cell surface. The higher the concentration of competitive ions in the water, the lower the toxicity of cupric ions. Similar effects were reported for the toxicity of Zn towards *P. subcapitata* (Heijerick et al., 2002). Increasing concentrations of Mg, Ca, and Na resulted in a reduction of Zn toxicity by factors of 6.5, 1.7, and 2.1, respectively. Likewise, Deleebeeck et al. (2009) demonstrated a 9.5-fold decrease in Ni toxicity to *P. subcapitata* by a combined alteration of pH (7.8 to 7.0) and Mg concentration (0.12 to 3.0 mM) in the test medium expressed by an EC_{50} elevation from 96.3 to 914 µg/L.

The C-modified medium used in this study has higher concentrations of Ca (0.64 mM) and Mg (0.16 mM) in comparison to standard OECD TG 201 and ISO 8692 media, which both contain 0.12 mM Ca and Mg. The pH value of the modified C-medium is lower than that of the OECD standard medium (7.0 and 8.1, respectively). Despite these abiotic factors that could reduce metal toxicity in C-medium, *C. ehrenbergii* has shown a higher sensitivity towards Cu and Cd than *P. subcapitata* referring to mean EC_{50} values (Table 2). One possible explanation is that tests with lower cell densities exhibit higher sensitivities. Franklin et al. (2002) found that Cu toxicity towards *P. subcapitata* decreased 2.6-fold from an EC_{50} of 6.6 to 17 µg/L when the initial cell density was increased from 10^2 to 10^5 cells/mL. Similarly, Vasseur et al. (1988) reported decreased toxicity of Cd, Cu and Zn when inoculated cell densities increased from 2 x 10^4 to 3 x 10^6 cells/mL. EC_{50} values changed from 46 to 110 µg Cd/L, 10 to 280 µg Cu/L and 90 to 365 µg Zn/L. In this study, the initial cell density was 10-15 cells/mL.

In contrast, the sensitivity of *C. ehrenbergii* towards Zn and Cr is significantly lower than that of *P. subcapitata*. One common explanation for the different sensitivities to pollutants between algal species is the cell surface-to-volume ratio (S/V ratio) (Kent and Currie, 1995). In general, the higher this ratio, the higher the sensitivity. With decreasing cell size, this ratio increases. In smaller cells, also a minor intracellular pollutant concentration is necessary to evoke adverse effects, whereas a higher surface area leads to increased uptake. In order to compare the S/V ratios of *C. ehrenbergii* and *P. subcapitata*, equations by Hillebrand et al. (1999) were used to calculate biovolume and surface area. Since both species resemble bent double cones, the equation "monoraphidioid" was chosen. For *C. ehrenbergii*, a mean cell length and diameter of 450 µm and 70 µm were used for the calculation, resulting in an S/V ratio of 0.06 μm^{-1}. For *R. subcapitata*, according to the information stated in OECD

(2011), a mean cell length and diameter of 11 µm and 2.5 µm were used, resulting in an S/V ratio of 1.51 μm^{-1}. These results demonstrate a much higher S/V ratio of *R. subcapitata* which is attributed to its small cell dimensions.

The aforementioned considerations demonstrate that the toxicity of a substance is influenced by various abiotic and biotic factors, but ultimately remains species-specific. Therefore, it is worthwhile to increase the list of potential candidate species for toxicity testing to depict the natural environment in the best possible way.

3.4. Morphological alterations after metal exposure

In addition to the quantitative endpoint growth rate, the alteration of *C. ehrenbergii's* morphology after exposure to toxicants can be assessed under the light microscope. As a representative example of the six tested metals, Fig. 3 shows the comparison between cells of the control group and cells treated with 8.0 µg/L Cu. The abnormal cell morphology as a consequence of excess copper exposure in the treatment group is clearly visible. Cells have lost their symmetry due to incomplete cell division and chloroplasts have become granulated so that pyrenoids are not visible anymore. Around the cell core in the center of the cells, the chloroplasts have started to disintegrate due to the influence of ROS (Wang et al., 2018). This morphological analysis is feasible in *C. ehrenbergii* because of its large cell dimensions. The microscopic pictures were taken at 200x magnification and yet morphological details are well observable. In comparison, common standard species such as *Raphidocelis subcapitata,* which has cell dimensions of about 11 x 2.5 µm, are generally too small so that these morphological effects stay hidden even at the highest possible magnification of 1000x. The effects of the other five tested metals on the morphology of *C. ehrenbergii* are shown in Supplementary Figures A4-A8.

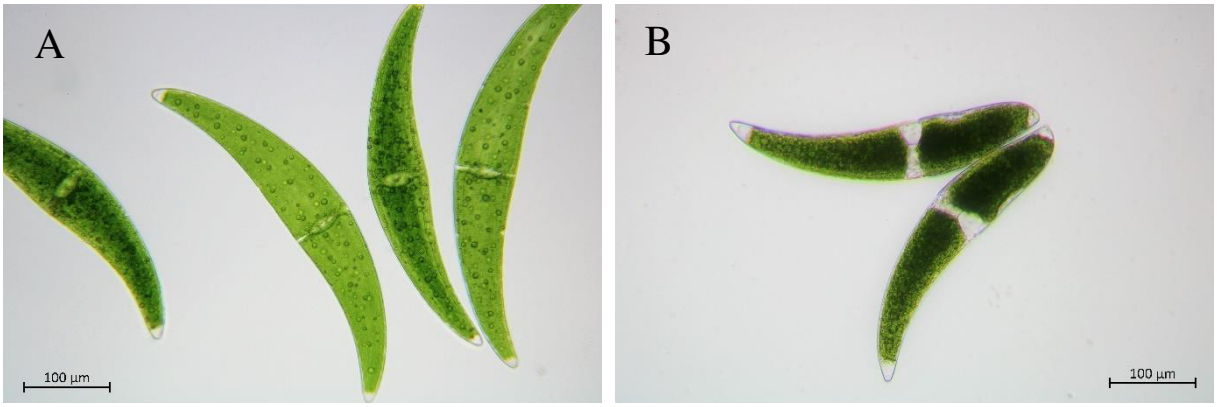

Fig. 3: Cells of *C. ehrenbergii* exhibiting normal cell morphology under control conditions (A) and abnormal morphology after 72 h of copper exposure c = 8.0 µg/L (B).

3.5. Metal complexation in original C-medium

It is well known that EDTA forms non-bioavailable metal chelates with divalent ions such as Cu^{2+} (Hsieh et al., 2004). Also, TRIS, which is mainly used as a pH buffer, may form complexes with metal ions (Ferreira et al., 2015). In terms of cellular uptake and hence toxicity, only metals in ionic speciation or as dissolved complexes with inorganic ligands (such as OH^-, Cl^-, and CO_3^{2-}) are relevant, and neither the total nor the organically complexed metal concentrations are biologically available (Sunda et al., 2009). In many culture media, including OECD TG 201 medium, EDTA is commonly added to prevent the precipitation of dissolved iron, especially during autoclaving (Andersen, 2009). However, Fe and other metal ions compete for the formation of chelates with EDTA, therefore, predicting the real concentration of free metal ions may be difficult.

To quantify the influence of the chelate-forming components of C-medium on the bioavailability of cupric ions, selective chemical analysis was conducted based on the chelating agent PAN. The Cu-PAN complex has a stability constant k smaller than that of the Cu-EDTA complex (Nakagawa and Wada, 1973), which makes it suitable to quantify only free cupric ions.

While the detection limit in the C-modified medium without EDTA was 5.0 µg/L Cu^{2+}, the pink Cu-PAN complex first started to appear at a Cu^{2+}-concentration of 400 µg/L in the original medium (Fig. 4). This comes in agreement with the ecotoxicological test results. These showed that the effect of copper on the growth rate of *C. ehrenbergii* started to occur at a very high concentration (LOEC = 400 µg/L) in case of the original C-medium (Fig. 5), which includes a molar EDTA:Fe ratio of 3.7:1. In contrast, in the modified medium the toxic effect of copper started to occur at a very low concentration (LOEC = 4.0 µg/L) in the absence of chelating agents (Fig. 1). Although markedly reduced, also the second modification of the medium (C-adjusted), including a molar EDTA:Fe ratio adjusted to 1:1, exhibited a chelating effect on cupric ions. In this version of the culture medium, a threshold concentration of 34 µg/L Cu^{2+} was found until which no free cupric ions were detectable (Fig. 4). These results demonstrate that the presence of chelate-forming components in C-medium trap cupric ions and render them biologically unavailable for *C. ehrenbergii*.

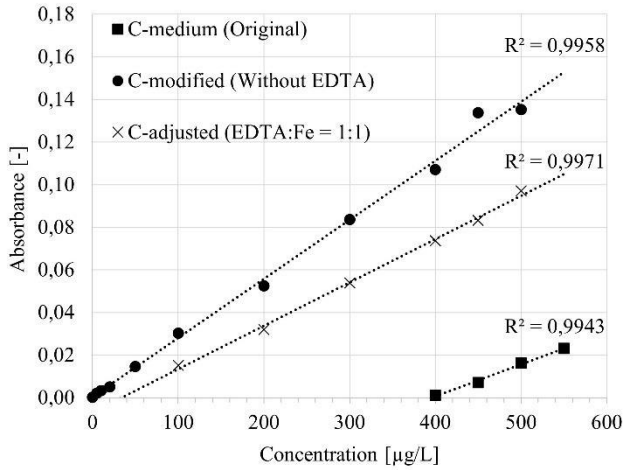

Fig. 4: Analytical cupric-ion determination in two different modifications of the C-medium.

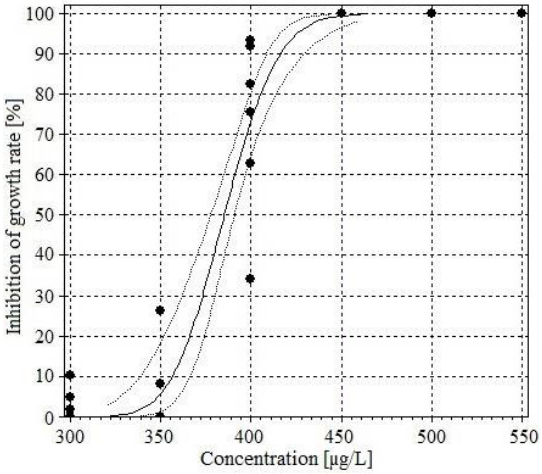

Fig. 5: Effects of copper on the growth rate of *C. ehrenbergii* after 72 h. Tested in the original C-medium.

These findings are of great importance because typically used media for algae cultivation and toxicity testing involve the addition of EDTA and pH buffers such as TRIS. Although the standard medium of OECD 201 takes the problem of metal chelation into account by adjusting the molar ratio of EDTA:Fe to approx. 1:1, in this study, tested metal ions were still found to be chelated to some extent at this ratio (Fig. 4). Furthermore, many other established culture media such as C-medium, Bold's Basal medium, or f/2 medium that may be necessary for the cultivation of non-standard species contain higher ratios of EDTA:Fe or include other complex-forming components (Andersen et al., 2009) and may therefore lead to underestimated toxicity values of metals. Wang et al. (2018) reported a 72 h EC_{50} value of 0.202 mg/L copper sulfate (= 80.4 µg/L Cu^{2+}) for *C. ehrenbergii* using non-modified C-medium that included EDTA and TRIS. Effects were still resolvable up to 400 µg/L Cu^{2+} while complete cell death already occurred at 16 µg/L in the present study and the EC_{50} value obtained here is 5.5 µg/L. Although considering EDTA-Cu complexation theoretically and hence stating estimated effective concentrations, Lam et al. (1999) reported a 72 h EC_{50} value of 4.01 mg/L Cu^{2+} for the freshwater green algae *Chlorella vulgaris* using Bold's Basal medium with an EDTA:Fe ratio of 9.6:1. In addition, Ebenezer and Ki (2012) reported a 72 h EC_{50} value of 12.74 mg/L for copper towards the marine dinoflagellate *Cochlodinium polykrikoides* using unmodified f/2 medium. Besides having an EDTA:Fe ratio of 1:1, this medium consists of filtered seawater that may contain unknown amounts of organic compounds which can also act as chelators (Harrison and Berges, 2009). Taking into consideration that all aforementioned species are unicellular, non-colony forming eukaryotic algae, the toxicity values mentioned above are several orders of magnitude higher than the ones in the present study, which suggests a strong influence of EDTA and other complex-forming agents on the toxicity of the tested metal.

4. Conclusions

C. ehrenbergii is a unicellular benthic alga that combines reproducibility and sensitivity under laboratory conditions. Due to its large cell dimensions, the absolute cell count is a feasible ecotoxicological endpoint in a miniaturized bioassay. Apart from a simple stereomicroscope, this method does not require any additional tools such as haemocytometer or particle counter. No surrogate parameters such as absorbance or chlorophyll fluorescence are required, allowing any laboratory to carry out the test method without special equipment. Hence, the test design is simple, reliable, and easy to implement.

C. ehrenbergii is susceptible to metal influence. The order of toxicity of the six metals in this study was Cu > Ag > Cd > Ni > Cr > Zn. The modification of C-medium has been shown to be of great importance for evaluating metal toxicity. Chelate-forming components such as EDTA and TRIS need to be excluded to avoid underestimation of toxic effects.

Finally, the approach of miniaturizing ecotoxicity tests with large algae has several advantages: (i) the amount of all kinds of chemicals such as toxicants as well as nutrients is reduced to a minimum; (ii) the test evaluation is non-invasive which makes the method fast; (iii) only one microplate is needed per test, so the experimental effort is low in comparison to the traditional test design using flasks or test tubes; (iv) in addition to cell count, the test organisms can be observed individually under higher magnification to visualize toxic effects on chloroplasts or cell membranes; and (v) the number of living organisms that are sacrificed by the test is dramatically reduced.

CRediT authorship contribution statement

A. Weber-Theen: Conceptualization, methodology, validation, formal analysis, investigation, writing original draft, project administration. **L. Dören:** Conceptualization, review and editing, project administration, funding acquisition. **S. Albaseer:** Methodology, investigation, review and editing. **T. Friedl:** Resources, review and editing, supervision. **M. Lorenz:** Resources, review and editing, supervision. **A. Gutowski:** Review and editing, supervision.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary tables and a video associated with this article can be found in the online version at: doi:10.1016/j.ecoenv.2023.114781

Supplementary Figures A4-A8. *C. ehrenbergii* after 72 h of metal exposure.

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Chapter 3:
Chronic toxicity of pharmaceuticals to the benthic green alga *Closterium ehrenbergii*

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Abstract

Pharmaceuticals in the environment have emerged to a topic of global concern. Since these substances are designed to be biologically active, hazardous effects on non-target organisms are frequently reported. Here, the effects of five pharmaceuticals, one radiocontrast agent, and one degradation product on the freshwater green alga *Closterium ehrenbergii* were evaluated after chronic exposure of 168 h. Growth and maximum quantum yield (F_V/F_M) were used as endpoints and complemented by the assessment of morphology and chlorophyll fluorescence. We found that the tested antibiotics Ciprofloxacin and Ofloxacin impaired chloroplast integrity, resulting in a reduction of F_V/F_M from 0.1 mg/L. The disintegration of chloroplasts at higher concentrations (c = 0.3 and 0.8 mg/L, respectively) was visualized by brightfield and fluorescence microscopy. In contrast, Sulfamethoxazole interfered with cell division, leading to malformation of cells from 0.8 mg/L. Furthermore, the antibiotics exhibited a latency period of 72 h after which they started to reveal their true effects. Therefore, the importance of long-term toxicity testing is outlined in order to avoid underestimation of toxic effects of pharmaceuticals. Based on the EC_{10} values obtained, the antibiotics were considered to meet the criteria for classification as toxic to aquatic life with long lasting effects. The other test substances were found to exert no effects on *C. ehrenbergii* or only at very high concentrations and were classified as nontoxic.

Keywords

Ecotoxicity; pharmaceuticals; algae; fluorescence; PAM.

1. Introduction

The presence of pharmaceuticals in the environment has emerged as a topic of global concern (Beek et al., 2016). The production and consumption of pharmaceuticals have tremendously increased over the past decades and are predicted to increase even further due to aging populations e.g. in Europe and a growing world population in general (Beek et al., 2016; Bunke et al., 2019; European Environment Agency, 2019). Pharmaceuticals represent a large group of chemicals that consists of over 3,000 compounds which belong to various therapeutic groups such as non-steroidal anti-inflammatory drugs, antibiotics, antidepressants, hormones, antihypertensive drugs, and many others (EC, 2018). Pharmaceuticals are considered one of the most concerning groups of environmental pollutants since they are designed to be biologically active and are continuously released into the environment, posing a threat to healthy ecosystems (Chopra and Kumar, 2018; Xin et al., 2021).

The main emission pathway of pharmaceuticals into surface water is via wastewater treatment plants (Beek et al., 2016). Traditionally, these plants were developed to efficiently decrease dissolved organic matter and nutrients from the wastewater (Lofrano and Brown, 2010). However, the applied technologies such as activated sludge treatment are not sufficient to completely remove pharmaceuticals from the wastewater (Shen et al., 2019). More advanced treatment technologies e.g. adsorption onto activated carbon or oxidation with ozone have shown promising results (Pistocchi et al., 2022), but it will take a long time to implement these on a widespread and global basis. Hence, more than 900 pharmaceuticals have been detected in the environment all over the world (German Environment Agency, 2021).

Concentration levels in surface waters typically are in the range of ng-µg/L (Liu et al., 2020). Since many pharmaceuticals such as the antibiotic Sulfamethoxazole meet the criteria of persistent, mobile, toxic (PMT) or very persistent, very mobile (vPvM) substances, they are distributed in the environment as they may pass natural and artificial barriers. As a consequence, pharmaceutically polluted ground and even bottled water has been reported with concentrations in the range of pg-µg/L (Chopra and Kumar, 2018; Huang et al., 2021). Furthermore, other emission sources such as large farming sites, aquaculture, hospitals, and manufacturing facilities can lead to locally elevated concentrations up to the range of mg/L (Fick et al., 2009; Okoye et al., 2022).

Although some countries such as the European Union member states have developed environmental quality standards to define maximum acceptable concentrations of certain substances in the environment (EC, 2013), there are no uniform regulations or legally binding threshold values for effluent concentrations of wastewater treatment plants (Braun et al., 2022; Xin et al., 2021). In addition to the active ingredients, metabolites and transformation products are formed during the degradation process of pharmaceuticals that may be more harmful to aquatic organisms than the parent substances (Ortiz de García et al., 2014).

In order to perform a reliable risk assessment of xenobiotics, real measured concentrations are necessary (German Environment Agency, 2020; Liu et al., 2020). However, analytical methods only existed for about one quarter of the known 3,000 pharmaceuticals until recently (Beek et al., 2016). This substantiates the postulated conclusion concerning planetary boundaries: The safe operating space for novel entities (formerly known as chemical pollution) has already been exceeded since global production outstrips societies' capacities to conduct risk assessment and monitoring (Persson et al., 2022).

Nonetheless, it is the task of ecotoxicological studies to evaluate hazardous effects of pollutants on non-target organisms. Algae depict such a group of non-target organisms which is exposed to pharmaceutical pollution due to the emissions from wastewater treatment plants. Algae play a key role for the environment as they stand at the basis of the food web and are involved in various biogeochemical cycles (Graham et al., 2016). Although it is acknowledged that the protection of algae is of major importance for the well-functioning of ecosystems, the large part of ecotoxicological studies on pharmaceuticals has been carried out with organisms of higher trophic levels (Xin et al., 2021). Furthermore, 60 % of the studies on the effects of antibiotics on algae have used the same species namely *Raphidocelis subcapitata* and over 90 % conducted short-term experiments with exposure times below 7 days (Sharma et al., 2021). This results in knowledge gaps concerning adverse effects on different algae species and the impact of long-term exposure, potentially leading to chronic effects.

Therefore, here, the effects of five pharmaceuticals, one contrast agent and one transformation product on the freshwater green alga *Closterium ehrenbergii* were evaluated in a miniaturized bioassay. Growth and maximum quantum yield were used as endpoints after 7-day exposure. In addition, alteration of morphology and chlorophyll fluorescence were assessed. *C. ehrenbergii* is a unicellular, cosmopolitan, streptophyte alga that lives attached to filamentous algae and macrophytes in rivers and streams (Gutowski and Foerster, 2009). Benthic algae encounter different exposure scenarios in comparison to planktonic algae such as *R. subcapitata* as they are sedentary and not carried away by the current (Law, 2011). Under natural conditions, this particular way of life leads to an integration of toxic effects over time which makes *C. ehrenbergii* a representative of a different ecological niche and a valuable candidate species for ecotoxicity testing. To the best of our knowledge, this is the first attempt to evaluate chronic effects of pharmaceuticals on the benthic freshwater green alga *C. ehrenbergii*.

2. Materials and methods

2.1. Selection of test substances

Due to the large number of pharmaceuticals present in the environment, a prioritization of test substances was necessary. Firstly, the database "Pharmaceuticals in the environment" (German Environment Agency, 2023) was used to identify substances with high environmental concentrations. From this

database, all pharmaceuticals with measured environmental concentrations (MEC) above 1 µg/L in treated effluents were selected. Secondly, the European watch lists with substances of greatest concern were used as indicators for substances of public interest (EC, 2022, 2020). Thirdly, the ECOTOX database was used to capture the current state of knowledge regarding ecotoxicological studies on algae with the pharmaceuticals under consideration (USEPA/ECOTOX, 2023). In order to close existing knowledge gaps, substances with few or no studies available were generally prioritized. Finally, physicochemical properties such as logKow < 3 (water soluble) and vapor pressure < 10 mm Hg (non-volatile) were taken into account to ensure the feasibility of the bioassay applied. Table 1 shows the selected pharmaceuticals including their therapeutic group as well as available information on measured environmental concentrations in effluents and on toxicity towards algae. A compilation of the data base queries can be found in Supplementary Tables A1 and A2.

Table 1: Selected test substances including the current state of knowledge on MEC and toxicity to algae.

Chemical	Category	No. of analytical studies[a]	MEC[a] [µg/L]			No. of ecotox studies with algae[b]	Reported toxicity[b] [µg/L]	
			Average	Median	Max		LOEC	EC_{50}
Ciprofloxacin	Antibiotic	161	450	0.2	31,000	5	0.1 - 20,000	17 - 40,660
Ofloxacin	Antibiotic	108	6.2	0.2	414	3	-	21 - 12,100
Sulfamethoxazole	Antibiotic	259	28	0.2	9,748	6	800	520 - 1,900
Metformin	Diabetes medication	27	5.8	2.2	92	1	-	320,000
Guanylurea	Transformation product	7	25	6.4	110	0	-	-
Gabapentin	Anticonvulsant	20	4.2	2.7	43	0	-	-
Iopamidol	Radiocontrast agent	27	170	3.2	3,353	0	-	-

[a]: Data derived from the database Pharmaceuticals in the environment (German Environment Agency, 2023)
[b]: Data derived from ECOTOX database (USEPA/ECOTOX, 2023). EC values selected are based on both growth rates and yield.

2.2. Stock solutions and dilutions

The test substances Ofloxacin (OFLO), Gabapentin (GABA), Iopamidol (IO), Metformin hydrochloride (MET) (all pharm. sec. standard), Ciprofloxacin (CIP) (> 98 %), and Guanylurea sulfate salt hydrate (GUA) (> 97 %) were purchased from Sigma Aldrich. Sulfamethoxazole (SULF) (> 98 %) was purchased from TCI. For the preparation of stock solutions, the test substances were dissolved directly in the algae culture medium, named C (*Closterium*)-Medium (Ichimura, 1971). Those substances with low solubility e.g. Ciprofloxacin, were stirred with a magnetic glass stirrer for 24 h in the dark to ensure a complete dissolution of particles. All stock solutions were stored in the dark at 5 °C for max. 12 days. For each test, fresh dilutions were prepared from the pharmaceutical stock solutions using C-Medium. The concentration ranges of the final tests were determined by preliminary range-finding tests. All concentration data in the following refer to the active ingredients (AI).

2.3. Test organism and culture conditions

Closterium ehrenbergii (strain SAG 134.80) was obtained from the Culture Collection of Algae at the University of Göttingen, Germany (SAG). Cultures were maintained in test tubes in C-medium (Ichimura, 1971), pH = 7.5, which is the standard culture medium for this species found throughout the literature (Juneau et al., 2003; Sathasivam et al., 2016). The composition of the C-medium can be found in Supplementary Table A3. The culture conditions were 25 ± 1 °C, low light intensity of 23 µmol photons m^{-2} s^{-1} (cool daylight fluorescent tube) and a 14:10 h light:dark cycle. The culture medium was renewed every two weeks. A deviation from the standard culture conditions of OECD 201 (OECD, 2011) (40–60 µmol m^{-2} s^{-1} and continuous irradiation) was necessary because *C. ehrenbergii* conducts cell division only during the dark period (Lutman, 1911) and high light intensities damage the chloroplasts.

2.4. Chronic algal toxicity assay using microplates

The test method has been described before for acute toxicity testing of metals (Weber-Theen et al., 2023). Here, it was slightly adapted for the assessment of chronic effects by extending the exposure time to 168 h. This was done due to microscopic observations of preliminary tests with antibiotics that revealed increasing effects on cell morphology over time starting first after 72 h.

An inoculum of the exponentially growing stock culture was collected aseptically using a glass Pasteur pipette. Approx. 50 µL of dense algal suspension were transferred into 5 mL of fresh C-Medium. These cells were directly used for the toxicity test. A preculture was not necessary since the test conditions were equal to the culture conditions.

As test vessels, 24 microwell-plates (PS) were used (Roth selection). On average, 10 ± 1 cells were introduced into each well using a glass Pasteur pipette. One plate contained 5 test concentrations and 1 control group with 4 replicates each. 1 mL of either pure C-Medium or test substance dissolved in C-Medium was added to each well. The microplates were closed with lids and sealed with Parafilm to ensure a sufficient gas exchange and simultaneously prevent evaporation.

The tested concentrations of the pharmaceuticals were as follows: CIP (0.16, 0.31, 0.63, 1.3, 2.5 mg/L); OFLO (0.13, 0.32, 0.80, 2.0, 5.0 mg/L); SULF (0.16, 0.80, 4.0, 20, 100 mg/L); MET (26, 64, 160, 400, 1000 mg/L); GUA (26, 64, 160, 400, 1000 mg/L). In case of IO and GABA, preliminary tests showed no toxic effects, hence limit tests were performed at 100 mg/L with 6 replicates each of control and treatment group. For each test substance, 3 independent experiments were carried out.

In order to determine growth rate and yield, cells were counted manually at test start, day 3 and day 7 using a stereo microscope (Zeiss Stemi 508) at 10x magnification. Due to the low cell number and the large cell size of *C. ehrenbergii* of up to 800 x 100 µm, no counting chamber was required. pH was

measured (WTW Multi 9630 IDS) at the beginning and end of the test in each concentration and control group.

Validity criteria were established to ensure the reliability of each test. Since *C. ehrenbergii* represents a non-standard test species that has a lower growth rate than e.g. *R. subcapitata*, validity criteria were defined that are based on OECD 201 but reflect the typical growth pattern of this species under the test conditions mentioned above. Test results were only accepted if the following criteria were fulfilled: growth rate of the control group after 168 h > 0.42 1/d (19-fold increase in cell density); coefficient of variation of the mean growth rate of the control group < 10 %; cells of the control group morphologically intact during the whole test duration (determination at 200x magnification); all wells visibly free from contamination; change of pH during incubation < 1.0 units, clear concentration-response relationship. The threshold value for the growth rate of the control group after 168 h was defined empirically as the average growth rate of approx. 30 experiments minus the standard deviation.

To visualize the different toxic effects of pharmaceuticals on *C. ehrenbergii*, microscopic photographs were taken at 200x using a Zeiss Primovert with Canon EOS 2000D. In addition, chlorophyll-fluorescence images were taken to visualize in particular the destruction of chloroplasts by antibiotics. For this purpose, a Keyence VHX-7000 digital microscope combined with an adjustable blue LED lamp (excitation wavelength = 450 nm) and a dichroic color filter (transmission > 630 nm) (Qioptiq) was used.

2.5. Photosynthetic efficiency

The maximum photochemical quantum yield of PSII (F_V/F_M) is a feasible parameter to examine the physiological state of the photosynthetic apparatus (Lee et al., 2021) and was assessed as second endpoint in addition to growth. The fluorometer Maxi-Imaging-PAM (I-PAM; Walz) was used to measure F_V/F_M after 3 and 7 days. The maximum quantum yield is defined as follows:

$$F_V/F_M = (F_M-F_0)/F_M \quad (1)$$

F_0 = minimal fluorescence yield of a dark-adapted sample which is induced by a pulsed measuring light with very low intensity that keeps all reaction centers of PSII open,

F_M = maximal fluorescence yield of a dark-adapted sample which is induced by a light pulse of very high intensity that is able to saturate all PSII reaction centers,

F_V = variable fluorescence yield defined as the difference between maximal and minimal fluorescence.

For the measurement of F_V/F_M, a microplate was placed into the I-PAM to adapt the cells to the quasi-dark conditions. After 15 minutes of dark adaptation, a saturating light pulse was addressed to the microplate. Within the software ImagingWin (Walz) it is possible to select individual areas of interest

(AOI) that fit to the size of the 24 micro wells. For each well (AOI) the F_V/F_M was assessed simultaneously during the saturation pulse. A mean value of the total AOI was not calculated since *Closterium ehrenbergii* is a large organism and due to the small amount of test organisms, there was no homogenous distribution of cells across the entire bottom of each well. Only those pixels (algae cells) that expressed a variable fluorescence were evaluated by the software, black background pixels were excluded.

2.6. Chemical Analysis

Test concentrations were analyzed at the beginning and at the end of the tests by a commercial analytical laboratory (Wessling GmbH, Germany). The analytical method applied was LC-MS/MS and the limit of quantification was 0.025 µg/L. Samples were pooled from four replicates of one concentration level of each test substance. Cells were removed from the samples by membrane filtration (0.45 µm PVDF) at the end of the test. A potential reduction of analyte due to the filtration was assessed analytically and proved to be negligible. Differences between nominal and measured concentrations were below 20 % for OFLO, SULF, MET, GUA, IO and GABA. Hence, calculations of effect concentrations were based on nominal concentration values. Only CIP showed a decrease of 25 % in concentration during the exposure time. Therefore, the geometric mean was used to calculate effect concentrations.

2.7. Data analysis

Toxic effects were evaluated both by the inhibition of growth rate (µ), which is based on the exponential increase in cell count during the test period, and by the inhibition of yield (Y), which is the absolute difference in cell count between the end of the test and the beginning of the test. Calculations were performed according to the following equations:

$$\mu = \ln(x_t/x_0)/t \quad (2)$$

$$Y = x_t - x_0 \quad (3)$$

$$I_R = (\mu_C - \mu_T)/\mu_C * 100 \quad (4)$$

$$I_Y = (Y_C - Y_T)/Y_C * 100 \quad (5)$$

where x_t is the absolute cell count after 72 or 168 h, x_0 is the cell count at the beginning of the test, μ_C is the average growth rate and Y_C the average yield of the control group, μ_T is the average growth rate and Y_T the average yield of the treatment group. The software ToxRat Professional v. 3.3.0 was used to compute 10 % effect concentration (EC_{10}) and half-maximal effect concentration (EC_{50}) values. Probit linear regression model was selected to obtain quantitative concentration-response relationships. William's test with $p < 0.05$ was used to determine significant differences of cell counts between treatment and control groups.

3. Results and discussion

3.1. Sensitivity of *C. ehrenbergii* towards pharmaceuticals

Five of the seven tested substances caused effects on the growth of *C. ehrenbergii*. The radiocontrast agent IO and the anticonvulsant GABA did not show any effects up to 100 mg/L. Both the diabetes medication MET and its transformation product GUA exerted effects at rather high concentrations. In addition, these effects were time-dependent. The E_RC_{50} of MET increased from 289 to 355 mg/L between day 3 and day 7. In contrast, the E_RC_{50} of GUA decreased significantly from 465 to 89.2 mg/L between day 3 and day 7.

The response of the algae towards the three tested antibiotics was particularly different from that towards the other pharmaceuticals. During the first 72 h, no concentration-response relationships were found for the tested ranges. The inhibition of growth rate was below 20 % in the highest tested concentrations. However, between day 3 and day 7 the effects of all 3 antibiotics increased remarkably so that concentration-response relationships were found. For CIP and OFLO, E_RC_{50} values of 3.25 and 5.73 mg/L were obtained after day 7. For SULF, even though the E_RC_{10} at day 7 was 1.31 mg/L, no E_RC_{50} was found up to 100 mg/L due to a very flat slope of the concentration-response curve. The results of the toxicity tests are summarized in Table 2. In addition to effect concentrations based on growth rates, EC_{10} and EC_{50} values are also given based on yield. Concentration-response-curves at day 7 based on both growth rate and yield are shown in Fig. 1. Additionally, concentration-response-curves based on section-by-section growth rates are shown in Supplementary Fig. 1.

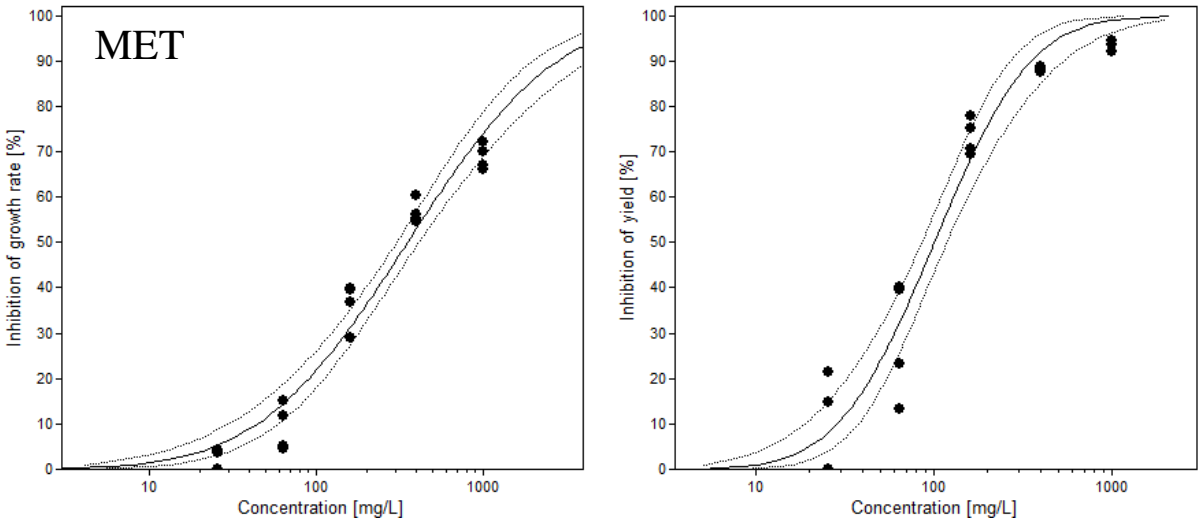

Fig. 1: Concentration-response curves showing the inhibition of growth rate (column 1) and yield (column 2) for MET, GUA, CIP, OFLO, and SULF after 168 h of exposure time.

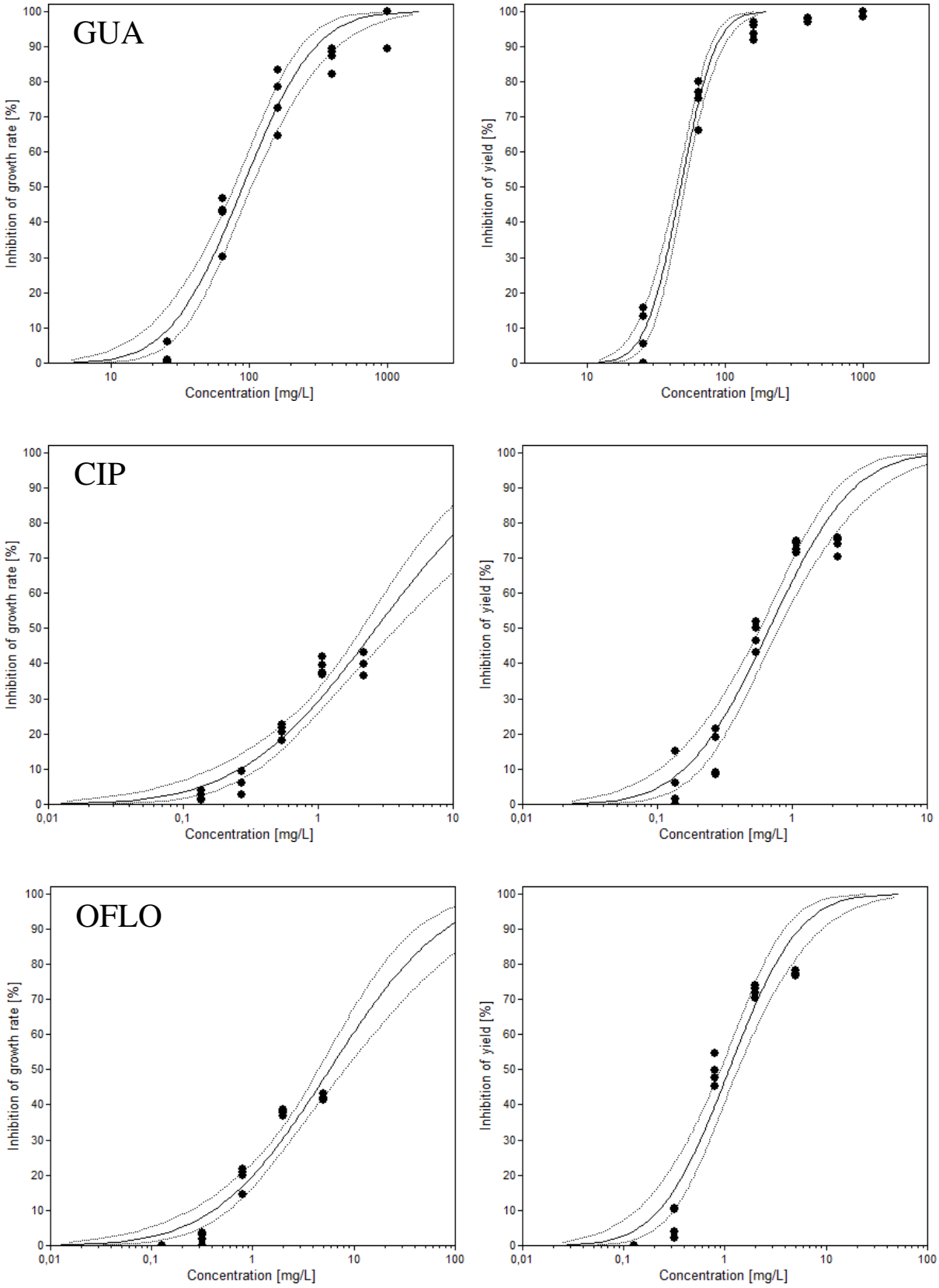

Fig. 1: Continued.

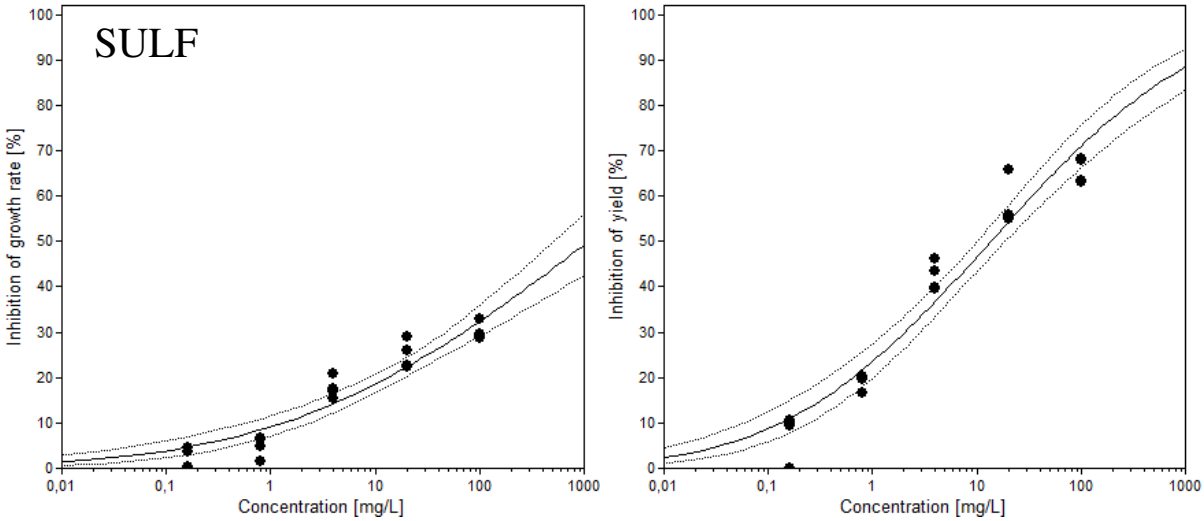

Fig. 1: Continued.

Table 2: Effect concentrations of the seven pharmaceuticals on *C. ehrenbergii* based on yield and growth rate after 72 and 168 h (given as mg AI/L).

Pharmaceutical	Exp. time [h]	Yield				Growth rate			
		E_YC_{10}	95 % CI[a]	E_YC_{50}	95 % CI	E_RC_{10}	95 % CI	E_RC_{50}	95 % CI
Ciprofloxacin	72	1.03	0.09 – 2.57	n. d.[b]	-	2.37	1.49 – 8.44	n. d.	-
	168	0.16	0.10 – 0.21	0.68	0.58 – 0.79	0.26	0.16 – 0.36	2.68	2.11 – 3.81
Ofloxacin	72	1.02	0.07 – 2.14	n. d.	-	4.38	2.82 – 9.86	n. d.	-
	168	0.22	0.13 – 0.31	1.10	0.90 – 1.34	0.41	0.24 – 0.58	5.73	4.45 – 8.14
Sulfamethoxazol	72	0.86	0.00 – 4.53	n. d.	-	25.9	3.64 – 419	n. d.	-
	168	0.13	0.06 – 0.25	13.6	10.0 – 18.8	1.31	0.57 – 2.35	n. d.	-
Metformin	72	21.6	15.0 – 28.5	126	109 – 145	38.3	28.7 – 48.3	289	258 – 326
	168	28.6	18.8 – 37.7	100	85.4 – 118	44.5	30.2 – 59.7	355	305 – 416
Guanylurea	72	83.9	60.6 – 106	294	258 – 335	135	108 – 160	465	425 – 509
	168	26.2	22.1 – 29.7	47.4	44.0 – 50.7	26.3	18.2 – 33.8	89.2	77.1 – 103
Gabapentin	72	n. t.[c]	-	n. t.	-	n. t.	-	n. t.	-
	168	n. t.	-	n. t.	-	n. t.	-	n. t.	-
Iopamidol	72	n. t.	-	n. t.	-	n. t.	-	n. t.	-
	168	n. t.	-	n. t.	-	n. t.	-	n. t.	-

[a] 95 %-confidence limits (according to Fieller`s theorem)
[b] n. d.: Not determined
[c] n. t.: Non-toxic

3.2. Underestimation of toxicity due to latency period of effects

Toxicity is considered a process in time and the influence of exposure time on effects can vary between test substances depending on the properties of the chemicals (Baas et al., 2010). Different modes of action as well as different chemical properties affecting e.g. the uptake velocity can influence time dependency of effects. This is also reflected in the results from this study. While MET and GUA as well as six metals studied in our previous study (Weber-Theen et al., 2023) showed concentration-dependent effects on *C. ehrenbergii* already after 72 h, a latency period of effect occurred during the same exposure time in case of the tested antibiotics. However, due to the increase in effects over time, concentration-response relationships of CIP, OFLO, and SULF were found after 168 h. Furthermore, although a concentration-response relationship was found for GUA after 72 h, also these effects significantly increased over time. Fig. 2 depicts the absolute cell counts over time and demonstrates the time dependency of effects. In case of OFLO and SULF, significant differences ($p < 0.05$) between the cell counts of treatment and control groups were only found at the highest test concentration after 72 h. For CIP, no significant differences were found after 72 h. In contrast, after 168 h, significant differences in a concentration-dependent manner were found for all antibiotics. For MET and GUA, significant differences were found both after 72 and 168 h, whereas the effect of GUA at 64 mg/L showed a similar latency to that of the antibiotics.

In some cases, a lag phase in cell growth of the control group may occur at the beginning of the test duration e.g. due to too short adaptation durations of precultures (OECD, 2011) which may potentially lead to a delay in the detection of effects. However, this is unlikely here since the test conditions were equal to the culture conditions in this study and no preculture was necessary. Moreover, the quality criteria which were derived during method development ensure that the cell growth of the control groups was not delayed at the beginning of the test, but exponential. Data showing a typical growth curve of *C. ehrenbergii* under the test conditions described above can be found in Supplementary Table A4. Furthermore, since concentration-dependent effects already occurred after 72 h both for MET and GUA as well as for metals studied previously (Weber-Theen et al., 2023), insufficient cell growth of the control groups at the beginning of the test can be excluded as a reason for the delayed effects of the antibiotics. In contrast, Eberius et al. (2002) state that growth stagnancy of the treatment groups at the end of a test may be interpreted as an important sign for large but retarded ecotoxicological or chronic effects.

If these lagged effects are evaluated based on the average specific growth rate, a false basic assumption is made. The function of growth rate requires constant exponential growth during the whole test period. Hence, the inhibition of growth rate requires an evenly reduced exponential growth from the beginning of exposure, but this was not the case here. CIP, OFLO, SULF, and GUA showed an increasing inhibition of growth rate over time and especially the antibiotics exhibited a latency period where growth was not significantly affected. Also Sharma et al. (2021) state that the toxicity of antibiotics is strongly

exposure-time-dependent. This leads to a serious underestimation of toxicity when effects are only evaluated after short-term exposure and also when effect concentrations are calculated based on growth rate after long term exposure where a latency period of effects occurred. Even if a population nearly stopped growing at all between day 3 and day 7, which was the case e.g. for CIP and OFLO at 2.2 and 5.0 mg/L, respectively, mathematically, the average specific growth rate at day 7 would only be inhibited by approx. 40 %. However, such an evenly reduced average growth rate does not reflect the real growth situation observed in the bioassays as growth was unaffected at first and almost came to halt after 72 h at the mentioned concentrations. Therefore, two additional possibilities for stating effect concentrations were compared that are described by OECD 201 (OECD, 2011). The first is to assess the difference in absolute cell count which avoids the assumption of constant exponential growth rates of the treatment groups throughout the test period and compares the total population sizes (yield). The second is to assess the section-by-section growth rates, i.e. between day 3 and day 7. Here, both options were applied, and the results were found to coincide for CIP, OFLO, and GUA. The 168 h E_YC_{10} and 72-168 h E_RC_{10} values for CIP are 0.16 and 0.20 mg/L. The respective EC values for OFLO are 0.22 and 0.29 mg/L. These values are approx. 30-50 % lower than the corresponding 168 h E_RC_{10} values. The 168 h E_YC_{50} and 72-168 h E_RC_{50} values for CIP are 0.68 and 0.67 mg/L and the respective EC values for OFLO are 1.10 and 1.16 mg/L. These values are approx. 75-80 % lower than the corresponding 168 h E_RC_{50} values. For GUA, the 168 h E_YC_{10} and 72-168 h E_RC_{10} values are 26.2 and 32.4 mg/L which are 0-20 % higher than the corresponding 168 h E_RC_{10} value. The 168 h E_YC_{50} and 72-168 h E_RC_{50} values are 47.4 and 46.9 mg/L which are approx. 50 % lower than the corresponding 168 h E_RC_{50} value. For SULF and MET, the results of the two additional endpoints were found to be less consistent. The 168 h E_YC_{10} and 72-168 h E_RC_{10} values for SULF are 0.13 and 0.33 mg/L which are 75-90 % lower than the corresponding 168 h E_RC_{10} value. The 168 h E_YC_{50} and 72-168 h E_RC_{50} values are 13.6 and 53.4 mg/L and cannot be compared to the corresponding 168 h growth rate value because no E_RC_{50} was determined due to a very flat slope of the concentration-response-curve. For MET, the 168 h E_YC_{10} and 72-168 h E_RC_{10} values are 28.6 and 48.5 mg/L which are approx. 35 % lower and 10 % higher, respectively, compared to the corresponding 168 h E_RC_{10} value. The 168 h E_YC_{50} and 72-168 h E_RC_{50} values are 100 and 448 mg/L. These values are approx. 70 % lower and 25 % higher, respectively, compared to the corresponding 168 h E_RC_{50} value. The comparison data of all three endpoints evaluated are shown in Supplementary Table A5. It should be noted here that due to the mathematical basis of the different approaches, effect concentrations based on yield are generally lower than those based on growth rate and this should not be mistaken for differences in toxicity (Eberius et al., 2002). However, the concept of evaluating toxicity on the basis of effects on average specific growth rate is intended to be independent from test duration (OECD, 2011) which was not the case for the pharmaceuticals investigated here. The effects were strongly time-dependent. Therefore, the additional endpoints are considered valuable to better reflect the real growth situation in the bioassays, especially in case of latency periods of effect. Moreover, for all substances where the effects increased over time, i.e. CIP, OFLO, SULF, and GUA,

the section-by-section growth rates resulted in significantly lower EC_{50} values compared to those based on the average specific growth rates. This substantiates an underestimation of toxicity in case of latency periods of effects when EC_{50} values are only calculated based on average growth rates. Nevertheless, further research is required to investigate whether similar patterns of time dependency may also occur in other algal species and to evaluate whether the additional endpoints are also applicable across species.

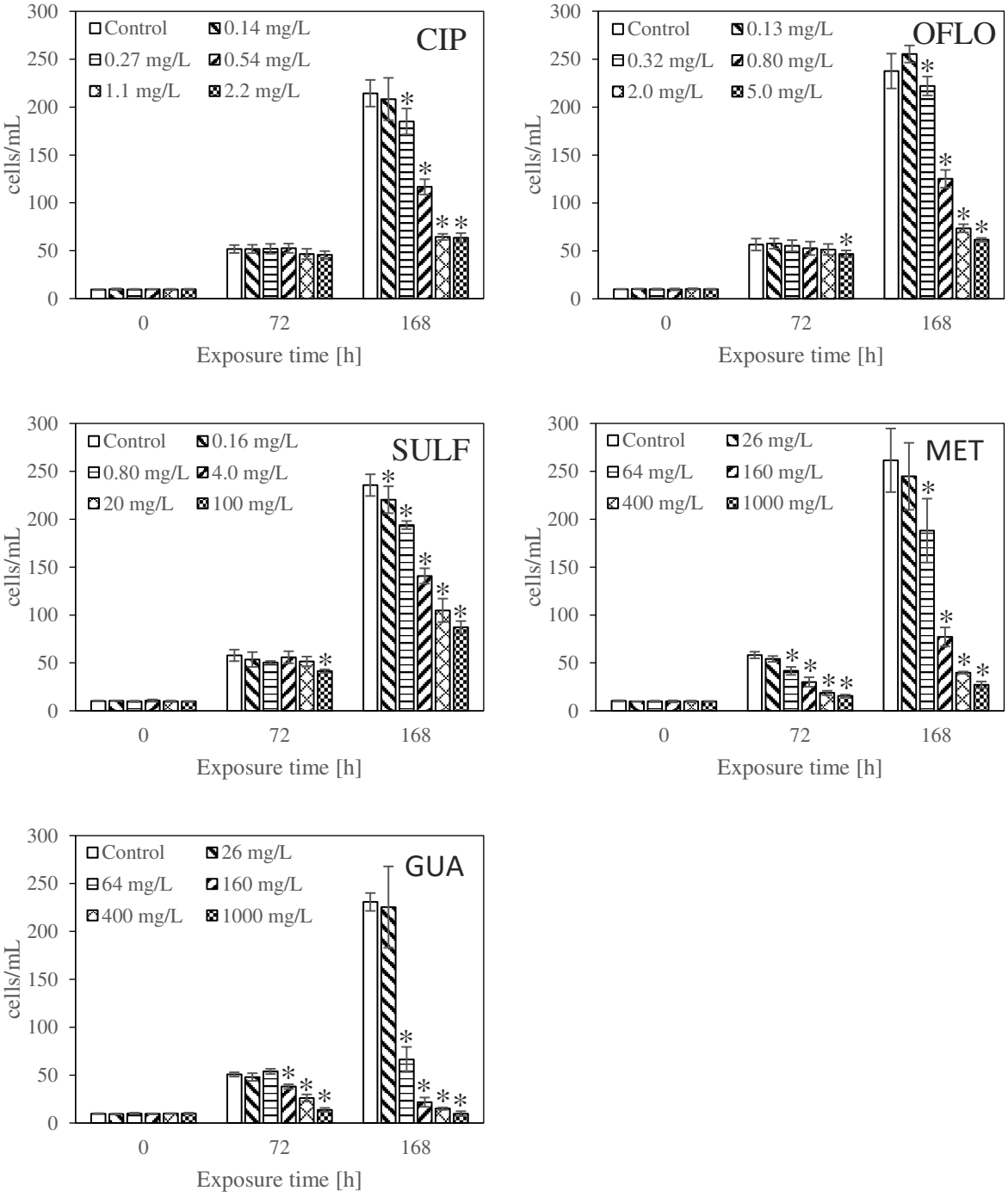

Fig. 2: Cell counts of *C. ehrenbergii*, exposed to five test concentrations of CIP, OFLO, SULF, MET, and GUA after 72 and 168 h. The plots illustrate a latency period of inhibition of 72 h for antibiotics compared to MET and GUA. Error bars indicate the standard deviation of cell counts. The asterisk (*) indicates a significant difference between cell counts of treatment and control groups (Williams' test with $p < 0.05$).

3.3. Comparison with toxicity data from the literature

Literature data comparing the effects of exposure time on the toxicity of antibiotics to algae are scarce as most studies followed standard test procedures with exposure times of only 3 to 4 days (Sharma et al., 2021). For the frequently used green alga *Chlorella vulgaris* a rather high E_RC_{50} of CIP was reported which slightly decreased from 38.0 mg/L at day 2 to 30.7 mg/L at day 4 (Geiger et al., 2016). Robinson et al. (2005) investigated the effects of several antibiotics on *R. subcapitata* but reported toxicity data only after 72 h. The E_RC_{50} found for CIP was 18.7 mg/L and that for OFLO was 12.1 mg/L. These literature data of E_RC_{50} values for CIP and OFLO obtained with standard test species are significantly higher than those found in this study with *C. ehrenbergii*. The reported E_RC_{50} values for CIP are about 7 to 14 times higher compared to the 168 h E_RC_{50} value and about 28 to 57 times higher compared to the 72-168 h E_RC_{50} value found in this study. The reported E_RC_{50} for OFLO is about 2 times higher than the 168 h E_RC_{50} and about 10 times higher than the 72-168 h E_RC_{50} from this study. Both species variation in sensitivity and longer exposure time may be possible reasons for the lower toxicity values obtained here. For SULF, which was the least toxic antibiotic in this study, no comparison data about the effects of exposure time were found in the literature. A 72 h E_YC_{50} of 1.90 mg/L was reported for *R. subcapitata* (Yang et al., 2008) that is about 7 times lower than the 168 h E_YC_{50} of this study.

In the field of ecotoxicology, both eukaryotic algae and prokaryotic cyanobacteria are commonly investigated. In the ECOTOX database, which was used to compile studies on pharmaceuticals, there is no differentiation between those two taxonomically heterogeneous groups and cyanobacteria are displayed along with eukaryotic algae. The substantially lower EC values in the range of µg/L for antibiotics in Table 1 correspond to tests with cyanobacteria. This is comprehensible since bacteria are the target organisms of antibiotics. For CIP and OFLO, 120 h E_RC_{50} values of 17 and 21 µg/L were reported from tests with *Microcystis aeruginosa* (Robinson et al., 2005).

In addition, Fan et al. (2022) reported time-dependent effects of CIP on the submerged aquatic plant *Vallisneria natans*. During an exposure time of 56 days, the inhibition of growth increased. High concentrations of 5 mg/L and above exhibited hazardous effects from day 7. However, the lower the concentration, the longer the latency period until effects occurred. 2.5 mg/L affected growth from day 14, 1.25 mg/L from day 28, and 0.25 mg/L first at day 42.

For MET, only one study was found in the literature that reported a 72 h E_RC_{50} of > 320 mg/L for *Desmodesmus subspicatus* (Cleuvers, 2003). No precise value was stated by the author since the effect of the highest test concentration was below 50 % inhibition. Therefore, no detailed comparison is possible with the data obtained in this study. However, the 168 h E_RC_{50} found here was 355 mg/L and thus also > 320 mg/L.

For GUA, IO, and GABA no literature data were found for the comparison of effect concentrations.

3.4. Influence of pharmaceuticals on photosynthetic efficiency

From the seven tested pharmaceuticals, only two were found to influence F_V/F_M in a concentration-dependent manner. CIP and OFLO affected photochemistry at lower concentrations than growth which reflects in lower EC_{10} values. For both antibiotics, inhibition of quantum yield was 20 % greater than inhibition of cell yield at the second lowest treatment concentration, i.e. at 0.3 mg/L. Furthermore, as shown in Table 3, the inhibition of F_V/F_M strongly increased over time as it was the case with growth as well. The EC_{10} values of CIP and OFLO decreased from 1.41 and 2.84 mg/L, respectively at day 3 to 0.08 and 0.14 mg/L at day 7. In addition, no EC_{50} values could be obtained at day 3 as the effects were too weak. In contrast, at day 7, EC_{50} values of 0.91 and 1.08 mg/L were obtained for CIP and OFLO, respectively. These EC_{50} values are in the same range as the E_YC_{50} values at day 7 (Table 2). SULF inhibited F_V/F_M by 16 % at day 7 from 4 mg/L, while an increase in concentration did not result in an increase in inhibition. Also MET and GUA only reached an inhibition of 10-20 % between 160 and 1000 mg/L. IO and GABA did not affect F_V/F_M. Hence, EC_{10} and EC_{50} values based on F_V/F_M were only obtainable for CIP and OFLO.

Table 3: Effect concentrations of CIP and OFLO on *C. ehrenbergii* based on F_V/F_M after 72 and 168 h (given as mg AI/L).

Pharmaceutical	Exp. Time [h]	F_V/F_M			
		EC_{10}	95 % CI	EC_{50}	95 % CI
CIP	72	1.41	0.71 – 11.6	n. d.	-
	168	0.08	0.04 – 0.12	0.91	0.74 – 1.15
OFLO	72	2.84	1.90 – 4.59	n. d.	-
	168	0.14	0.07 – 0.22	1.08	0.84 – 1.39

3.5. Analysis of different modes of action and impact on cell morphology

Different modes of action were found between the two groups of antibiotics investigated (fluoroquinolones and sulfonamide antibiotics) that both increased their intensity over time (Table 2 and Table 3). CIP and OFLO belong to the group of fluoroquinolones that were developed to treat Gram-positive and Gram-negative bacterial infections. Their mode of action is to inhibit DNA-gyrase and hence bacterial cell division (Shariati et al., 2022; Smythe and Rybak, 1989). Although green algae are eukaryotic organisms, they possess cell structures, such as circular chloroplast DNA, that originate in prokaryotes due to their endosymbiotic evolution (Graham et al., 2016). These structural similarities may likely be the reason for the antibiotics to exert effects on green algae, although they are primarily non-target organisms. In fact, gyrase which mainly is present in bacteria, has also been found in plants, e.g. *Arabidopsis thaliana*, and was shown to be the target of CIP (Evans-Roberts et al., 2016).

Here, CIP and OFLO targeted the chloroplasts of the green alga *C. ehrenbergii*, leading to disintegration of the organelle structure starting at 0.3 and 0.8 mg/L, respectively at day 7 (Fig. 3 B). As a result, the maximum photochemical quantum yield (F_V/F_M) decreased by up to 68 and 77 % in the highest test concentrations (Fig. 3 D). This happened due to an increase in F_0 and a decrease in F_M. The photochemical reaction to the fluoroquinolones therefore differs from that of PSII inhibitors such as diuron or atrazine. These PSII inhibitors typically bind to the D1 protein in the reaction center of PSII and suppress electron transfer after plastoquinone Q_A. Hence, even under very weak actinic conditions, the absorbed light energy is converted into fluorescence, leading to an increase in minimum fluorescence F_0 while under dark conditions, there are no differences of both F_0 and F_M between control and diuron treated samples (Zharmukhamedov and Allakhverdiev, 2021). In addition, Escher et al. (2006) reported that the inhibiting effect of diuron on the effective quantum yield of *Chlorella vulgaris* was independent of exposure time from 1 to 25 hours and related only to toxicokinetics. In contrast, the effects of CIP and OFLO were strongly time-dependent, and the reduction of F_V/F_M is rather correlated with the overall destruction of chloroplasts than with the targeted inhibition of electron transport. This is in line with the findings that CIP impaired chloroplast replication leading to chlorotic leaves (Evans-Roberts et al., 2016). Furthermore, the observation of chlorophyll fluorescence of single cells under high magnification clearly revealed the impact of the antibiotics on chloroplast integrity resulting in dark spots showing the lack of chlorophyll (Fig. 3 B). In addition, a lamellar structure of chloroplasts could be observed in untreated cells using fluorescence microscopy, which was not recognizable anymore after treatment with CIP (Fig. 3 A and B). Interestingly, although Liu et al. (2011) reported an inhibition of about 30 % in efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A, a significant reduction of F_V/F_M was not found up to 2.5 mg/L CIP after 96 h for *R. subcapitata*. Since F_V/F_M was found to be significantly inhibited from 0.3 mg/L after 168 h in this study and the effects were shown to increase over time (Table 3), this highlights the importance to examine different test organisms and the need for further research on extended exposure durations to standard algal species in order to investigate whether similar delayed effects may occur.

SULF belongs to the group of sulfonamide antibiotics and is also effective against Gram-positive and Gram-negative bacteria. Its mode of action is to inhibit dihydropteroate synthase, an enzyme that takes part in the synthesis of folic acid (Borsetto et al., 2021). Dihydropteroate synthase does not only occur in bacteria, but also in the mitochondria of plants and algae (Tabatabaei et al., 2019). This organelle, just like chloroplasts, originates in bacteria due to the endosymbiotic evolution of algae (Graham et al., 2016). Hence, the inhibition of folic acid synthesis was shown to be the target of sulfonamides in green algae (Eguchi et al., 2004).

Here, SULF led to malformations due to incomplete cell divisions from 0.3 mg/L at day 7 (Fig. 3 C). Normally, *C. ehrenbergii* is structured in two symmetric half-cells with the cell core localized in the center (Fig. 3 A). During cell division, the cell separates at the center, leading to one original half-cell

and a smaller second half-cell which then regrows until it reaches symmetry again (Lutman, 1911). At this step, the effects of SULF take action and inhibit the normal regrowth of the second half-cell leading to asymmetry. This may likely be attributed to the lack of folic acid, which is also known to inhibit growth and root formation of plants (Tabatabaei et al., 2019). As a result, over time the whole population of algae consisted of malformed cells since this effect was irreversible and continued with each cell division. Eventually, cells died during the process of cell division which could be observed starting from 0.8 mg/L (Fig. 3 C). Moreover, these effects increased over time. In contrast to CIP and OFLO, SULF did not primarily damage chloroplasts which is also reflected in a minor inhibition of F_V/F_M of only 16 % at 100 mg/L (Fig. 3 E). However, in addition to malformation of cells, an alteration of chloroplast appearance in the form of granulation was observed (Fig. 3 C). This may be attributed to the formation of reactive oxygen species (ROS) as it was shown that exposure to SULF significantly increased the activity of superoxide dismutase (SOD) and catalase (CAT), enzymes involved in the cellular protection mechanisms against ROS (Zhang et al., 2021). The deposition of starch granules inside of algal cells was also shown to occur in connection with ROS after treatment with the antibiotic Tetracycline and is considered a cellular defense mechanism (Xu et al., 2019).

The other tested substances, i.e. MET, GUA, IO, and GABA, were not found to have such specific effects on chloroplasts or cell division as the antibiotics. IO and GABA did not influence cell morphology at all. The chloroplasts of cells treated with MET ($c \geq 64$ mg/L) became slightly paler at day 7 than those of the control group. In contrast, at the same concentration the transformation product GUA exerted stronger effects than its parent substance MET leading to granulation of chloroplasts and cell death. Micrographs showing the described effects on cell morphology can be found in Supplementary Fig. A2.

As transformation products are less frequently studied than AI, the stronger effects of GUA compared to MET are an important example to highlight that this large group of chemicals should not be neglected although the effects occurred at relatively high concentrations here. However, other studies on the ecotoxicity of transformation products reported environmentally relevant EC_{50} values in the range of µg/L. 1,4-benzoquinone was found to exhibit toxicity towards various freshwater organisms at concentrations 3 to 4 orders of magnitude lower than that of its parent substance Paracetamol, one of the most widely used analgesics (Ortiz de García et al., 2014).

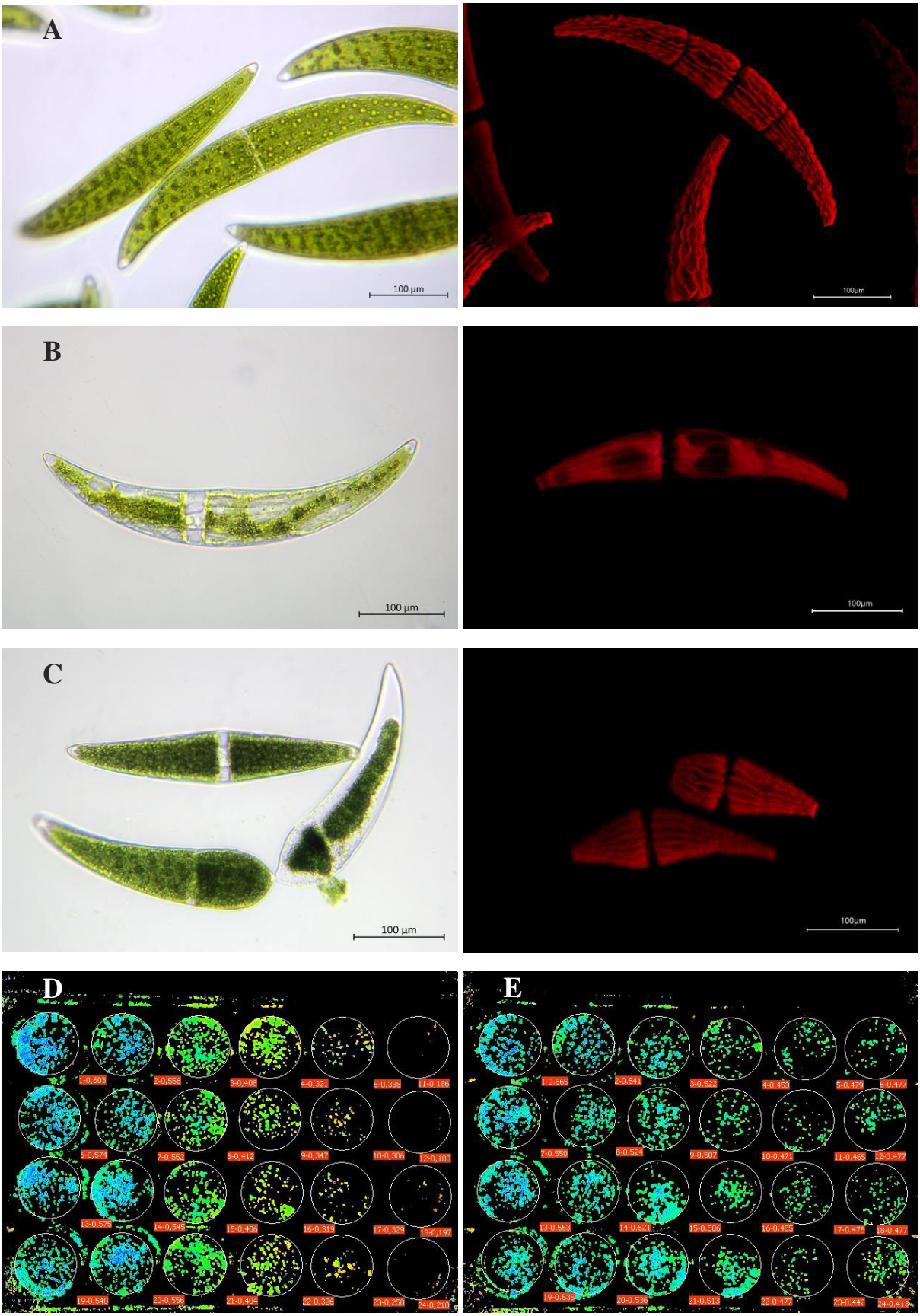

Fig. 3: Comparison between *C. ehrenbergii* under control conditions (A) and under influence of 0.3 mg/L CIP (B) and 0.8 mg/L SULF (C) after 168 h. First column shows bright field micrographs and second column chlorophyll fluorescence. Last row shows the effects of CIP (c = 0.14-2.2 mg/L) (D) and SULF (c = 0.16-100 mg/L) (E) on F_V/F_M measured with the I-PAM.

3.6. Evaluation of pharmaceutical risk for the environment

To evaluate a potential risk for the environment, the tested substances were classified according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (United Nations, 2021). Based on the EC_{10} values after 168 h, substances were considered "very toxic to aquatic life with long lasting effects" ($EC_{10} \leq 0.1$ mg/L), "toxic to aquatic life with long lasting effects" ($EC_{10} \leq 1$ mg/L), or nontoxic ($EC_{10} > 1$ mg/L). The E_YC_{10} was chosen for the classification due to the latency period of antibiotics. Since effects on growth rate are required to be independent from exposure time (OECD, 2011), the E_RC_{10} was considered inappropriate for classification here. Fig. 4 shows the classification of the tested substances. Since IO and GABA did not show any effects on *C. ehrenbergii*, no EC_{10} values were obtained. Therefore, they could not be included in the diagram, but are considered nontoxic.

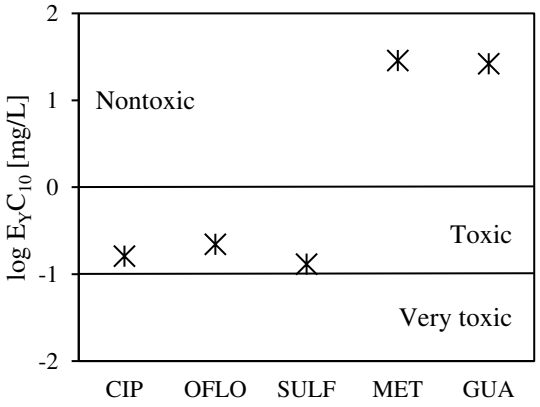

Fig. 4: Classification of test substances into three toxicity categories according to GHS based on E_YC_{10} values obtained with *C. ehrenbergii* after 168 h.

The risk assessment compares toxicity data with MEC data and is therefore different at each location. For pharmaceuticals, MECs can vary significantly between countries, especially because of the technological state of wastewater treatment plants and the emissions of production sites (Beek et al., 2016). Since urban wastewater is considered the main emission pathway of pharmaceuticals globally (Beek et al., 2016), chronic exposure of the aquatic environment is generally assumed (EMA, 2006). The concentrations detected in surface waters are typically in the range of ng-µg/L (Liu et al., 2020). Likewise, the global median concentrations in treated effluents of the tested substances in this study are in the range of 0.2 to 6.4 µg/L (Table 1). Since the effect concentrations obtained here are mostly within the range of mg/L to the upper range of µg/L (Table 2), a risk from the seven tested substances for *C. ehrenbergii* is unlikely in most environmental situations. However, several studies revealed locally elevated concentrations of antibiotics up to the range of mg/L in connection to production facilities. Over the past decades, large parts of the pharmaceutical production sector have gradually shifted to countries with lower production costs, mainly to Asia (Beek et al., 2016; Bjerke, 2022; Larsson, 2014). MEC values of 14, 4.1, and 1.3 mg/L CIP, OFLO, and SULF, respectively have been reported from

effluents of production facilities in India, Pakistan and Taiwan (Fick et al., 2009; Hussain et al., 2016; Lin and Tsai, 2009). From the data obtained in this study, a clear risk for the aquatic environment exists at such elevated concentration levels in case of chronic exposure. Moreover, even in the European Union there are no uniform regulations on the concentrations of pharmaceuticals in effluents (Braun et al., 2022). Consequently, equal environmental protection goals and monitoring strategies should exist on a global scale to safeguard environmental integrity and thus also the basis for human health.

4. Conclusions

In this study, the effects of five pharmaceuticals, one contrast agent and one transformation product were assessed after 168 h on growth and maximum photosynthetic efficiency of the benthic green alga *C. ehrenbergii*. The contrast agent IO and the anticonvulsant GABA did not exert any effects up to 100 mg/L. The influence of exposure time was found to be of major importance for the realistic evaluation of pharmaceutical toxicity towards *C. ehrenbergii*. While the diabetes medication MET and its transformation product GUA were both found to exert minor effects after 72 h (E_RC_{50} = 289 and 465 mg/L), only GUA significantly increased its toxicity after 168 h (E_RC_{50} = 89.2 mg/L). Furthermore, the tested antibiotics CIP, OFLO, and SULF exhibited an even stronger time dependency of effects than GUA which resulted in a latency period of 72 h, during which no concentration-dependent effects were found for the tested ranges. In contrast, after this period of time, effects significantly increased and concentration-response relationships were found after 168 h. CIP and OFLO affected the chloroplasts of the green alga, leading to the disintegration of this cell organelle and an inhibition of maximum photosynthetic efficiency from 0.3 mg/L. SULF interfered with cell division, leading to malformation of cells from 0.8 mg/L. *C. ehrenbergii* was shown to be a valuable test species that can be assessed in a standardized way. Especially its large cell dimensions are helpful to visualize toxic effects on the morphology as it was demonstrated for the antibiotics CIP, OFLO, and SULF. The photosynthetic efficiency F_V/F_M was shown to be a useful additional endpoint that can help to identify specific modes of action of substances that affect the photosynthetic apparatus of algae. The endpoints yield and section-by-section growth rate were shown to better reflect the real inhibition pattern of substances that exhibit a latency period in the bioassay. Therefore, we draw four conclusions: (i) for the assessment of pharmaceutical toxicity to *C. ehrenbergii* it is necessary to investigate longer-term exposure; (ii) further research is needed to investigate the value of longer-term exposure to standard algal species; (iii) EC values based on additional endpoints to growth rate may be valuable for the assessment of studies which show latency periods of effects, although further research is needed to investigate the applicability across species, and (iv) the assessment of transformation products should not be neglected as they may be more toxic than the parent substances.

CRediT authorship contribution statement

A. Weber-Theen: Conceptualization, methodology, validation, formal analysis, investigation, writing original draft, project administration. **L. Dören:** Supervision, review and editing, project administration, funding acquisition.

Declaration of Competing Interest

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

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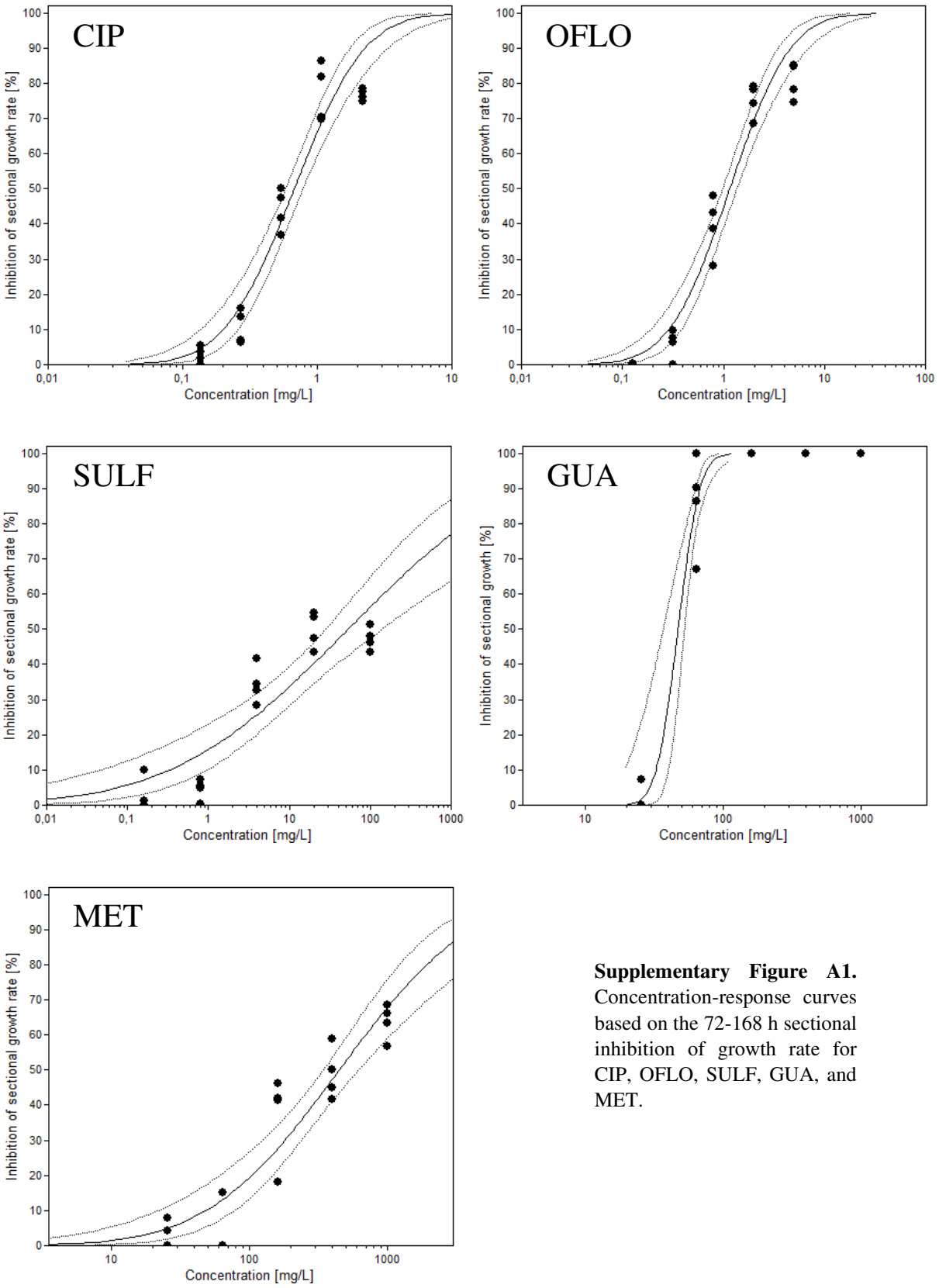
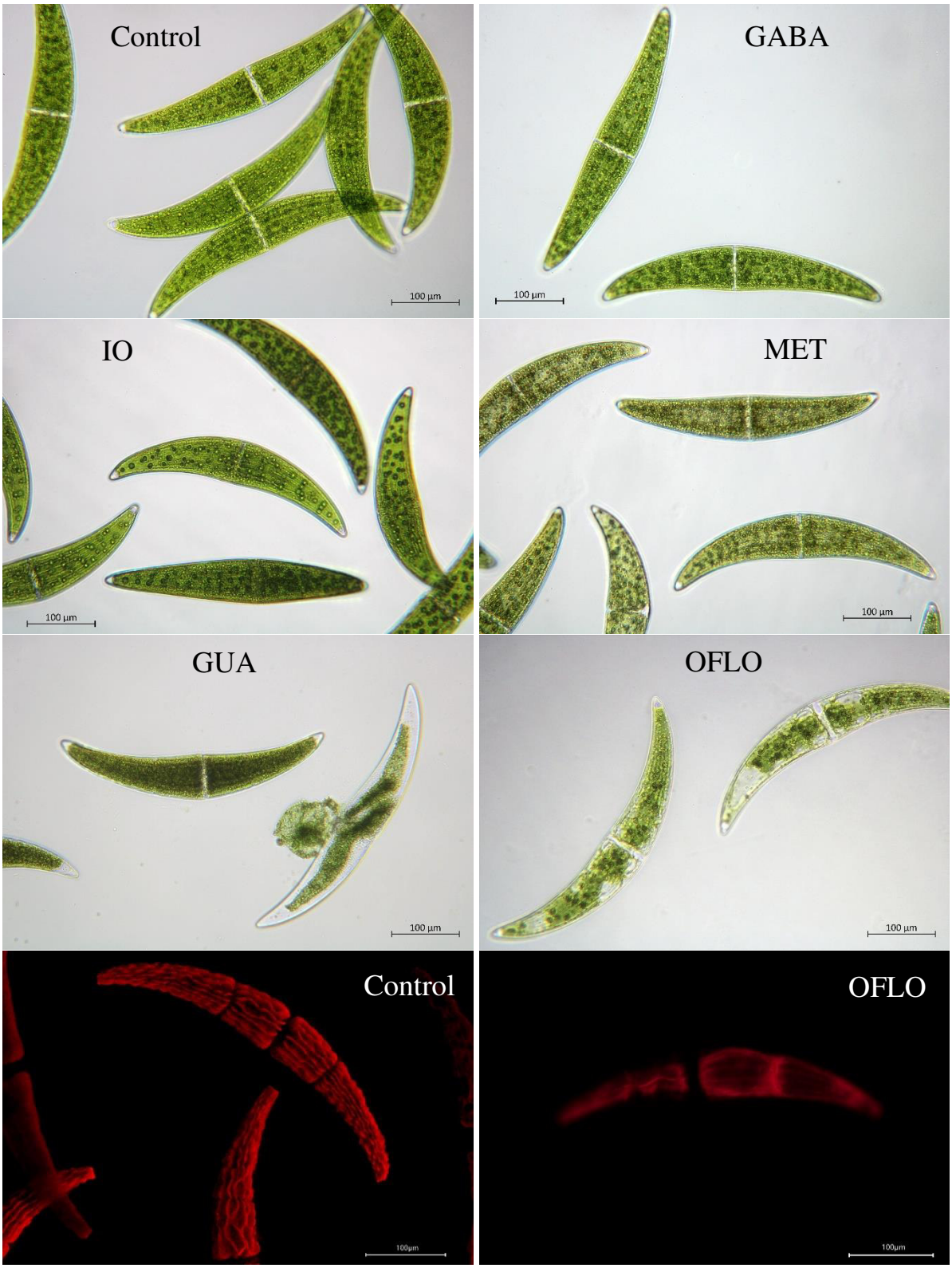
Appendix A. Supporting information

Supplementary tables associated with this article can be found in the online version at: doi:10.1016/j.ecoenv.2024.116025

Appendix A1



Supplementary Figure A1. Concentration-response curves based on the 72-168 h sectional inhibition of growth rate for CIP, OFLO, SULF, GUA, and MET.



Supplementary Figure A2. Brightfield micrographs of C. *ehrenbergii* after 168 h of exposure to GABA (c = 100 mg/L), IO (c = 100 mg/L), MET (c = 64 mg/L), GUA (c = 64 mg/L), and OFLO (c = 0.8 mg/L) in comparison to the untreated control group. GABA and IO did not influence cell morphology. MET influenced chloroplasts so that they became slightly paler than the control cells. GUA as transformation product from MET exerted stronger effects than MET at the same concentration. Chloroplasts became granulated and cell death occurred. OFLO significantly damaged chloroplasts so that they partly disintegrated. Additional chlorophyll fluorescence images show darker parts of the OFLO treated cell that indicate a lack of chlorophyll in comparison to the control group.

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Chapter 4: General discussion

This chapter aims to synthesize the findings of the previous chapters, in order to demonstrate the relevance and the knowledge gained from the testing of metal and pharmaceutical toxicity towards the benthic non-standard species *C. ehrenbergii*. Therefore, the test organism, test method, and test substances are discussed as well as modifications made to existing standard procedures and additional endpoints used for the toxicity assessment.

4.1. Relevance of *C. ehrenbergii* as an ecotoxicological test organism

Benthic algae play key roles in rivers and streams (Law, 2011) but simultaneously face exposure to pollutants from various emission sources such as wastewater discharge (chapter 1.1). *C. ehrenbergii* has been selected as an ecotoxicological test species that represents benthic algae. When it is affected by a pollution source under natural conditions, *C. ehrenbergii* does not benefit from dilution in the same way as planktonic species, which are carried downstream by the current. Therefore, *C. ehrenbergii* represents a different ecological niche than commonly assessed planktonic species such as *R. subcapitata*. The test substances assessed in this thesis were metals and pharmaceuticals both of which enter lotic ecosystems to a substantial extent through wastewater treatment plants. Due to its cosmopolitan distribution throughout freshwater habitats worldwide, *C. ehrenbergii* is a test organism that is likely to be exposed to the tested substances from this study and others in the real environment. In conjunction with its benthic way of life, this demonstrates the relevance of *C. ehrenbergii* as a model organism for ecotoxicity testing.

4.2. Feasibility of a miniaturized bioassay for toxicity testing with *C. ehrenbergii*

Due to the large cell dimensions of *C. ehrenbergii*, the cells of this species can be counted individually under the stereomicroscope in order to determine growth rates. In addition, toxic effects that alter cell morphology such as chloroplast integrity or cell symmetry, can be examined in detail with higher magnification. Taking these benefits of the cell dimensions together, a miniaturized bioassay using 24-well microplates and low cell densities of approx. 10 cells/mL at test start was developed. In comparison to commonly used test vessels like test tubes or flasks, the microplates combine several advantages. An entire test series including five test concentrations, one control group, and four replicates each can be assessed in a single microplate which reduces the experimental effort. Furthermore, an equal light distribution during algal growth inhibition tests is important to ensure similar growth conditions for all

treatment and control samples. In comparison to 24 test tubes or flasks, the size of a single microplate is very small leading to a significant reduction of inhomogeneities of light distribution inside a climate chamber. In addition, the evaluation of both quantitative and qualitative endpoints i.e. growth rate and morphology can be performed in a non-invasive manner. The microplates enable the counting of cells for the determination of growth rates under a stereo microscope without opening the test vessel. Therefore, in contrast to standard procedures with minuscule planktonic species, no counting chamber or surrogate parameter such as absorbance is needed for the evaluation of growth rate since the cells are counted directly. This reduces a potential source of inaccuracy in the endpoint evaluation. Furthermore, *C. ehrenbergii* has shown little fluctuations between replicates leading to coefficients of variance of the control groups well below 10 % (Table 1, chapter 2) which meets the requirements of OECD 201. As a result, narrow confidence intervals in the dose-response relationships were obtained, indicating high statistical precision of the test method using *C. ehrenbergii*.

4.3. Determination and toxicity assessment of environmentally relevant pollutants

The number of chemical substances that potentially enter aquatic ecosystems and affect organisms is large. Today, there are an estimated 350,000 chemicals (or mixtures of chemicals) on the world market (Persson et al., 2022). Therefore, a prioritization process was necessary for the selection of test substances. Since pollutants can generally be classified in two groups, namely inorganic and organic ones, this thesis aimed to address both groups.

4.3.1. Metals

Metals were selected as inorganic pollutants that are known to be of high concern for aquatic organisms (Arambawatta-Lekamge et al., 2021; Hsieh et al., 2004) and are released into the environment around the world through various emission sources (Li et al., 2020). Although at trace concentrations, many metals are essential for the metabolism of all kinds of organisms, at elevated concentrations they can turn into toxic pollutants as they can lead to reactive oxygen species (ROS) or impair the intact functioning of enzymes (Sunda et al., 2009). In addition to their environmental relevance, metals combine several beneficial aspects as test substances for a novel test method. In the form of metal salts, they are water soluble and bioavailable, they do not adsorb onto the surface of microplates and ecotoxicological data from different test species already exist for the comparison of results. At the same time, this thesis was the first to assess a variety of six metals to *C. ehrenbergii* in a miniaturized bioassay using microplates.

The results of the toxicity tests showed that all tested metals affected cell growth in a concentration-dependent manner. Hence, concentration-response curves for the inhibition of growth rate were obtained after 72 h confirming the applicability of the test method. The toxicity of the six metals in descending order, ranked by E_RC_{50} values, was as follows: Cu (5.5 µg/L) > Ag (9.2 µg/L) > Cd (18 µg/L) > Ni (260 µg/L) > Cr (990 µg/L) > Zn (1200 µg/L). Furthermore, all metals were found to alter cell morphology. Chloroplasts became granulated or even started to disintegrate due to the influence of ROS.

In order to evaluate the potential risks from the tested metals for the environment, a classification of toxicity was conducted according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (United Nations, 2021). Due to the short exposure time of 3 days, the tests were considered acute. Hence, the following categories were applied based on 72 h E_RC_{50} values: "very toxic to aquatic life" ($E_RC_{50} \leq 1$ mg/L); "toxic to aquatic life" ($E_RC_{50} \leq 10$ mg/L); "harmful to aquatic life" ($E_RC_{50} \leq 100$ mg/L). The tested metals Cu, Ag, Cd, Ni, and Cr were classified as very toxic to aquatic life, Zn was classified as toxic to aquatic life.

Based on a literature review, *C. ehrenbergii* was shown to be more sensitive than *R. subcapitata*, towards Cu and Cd but less sensitive towards Cr and Zn (Fig. 2, chapter 2). For Ni, the toxicity data of both species were in the same range. For Ag, no literature data of *R. subcapitata* were available but compared to six different freshwater green algae, the toxicity data of *C. ehrenbergii* were also within the same range. These findings are in line with one of the ecotoxicological main principles that toxicity is not a substance property but instead is species specific which demonstrates the value of additional test species.

4.3.2. Pharmaceuticals

Regarding organic pollutants, pharmaceuticals were selected as an emerging group of contaminants that is of particular concern for the environment due to the biologically active nature of these substances. Since more than 3000 pharmaceutical agents exist (Beek et al., 2016), and over 700 active ingredients or transformation products were already reported in the environment of European Union member states (German Environment Agency, 2023), an iterative prioritization process for the selection of test substances was conducted. In order to close existing knowledge gaps and to select substances of high environmental relevance, the focus was placed on those substances with high measured environmental concentrations of > 1 µg/L in treated effluents and few or no available ecotoxicological studies. Additionally, the European watch lists with substances of greatest concern (EC, 2022, 2020) were considered to identify particularly relevant substances. Finally, the antibiotics Ciprofloxacin (CIP), Ofloxacin (OFLO), and Sulfamethoxazole (SULF), the diabetes medication Metformin (MET), its transformation product Guanylurea (GUA), the anticonvulsant Gabapentin (GABA), and the radiocontrast agent Iopamidol (IO) were selected. This thesis was the first attempt to investigate the effects of the selected pharmaceutical substances on *C. ehrenbergii*.

The test results revealed that the toxicity of some pharmaceuticals was strongly time-dependent. Especially the antibiotics but also the transformation product increased the intensity of effects over time. Therefore, the test duration was extended from 72 to 168 h in order to avoid an underestimation of toxic effects. After 168 h, clear concentration-response relationships were obtainable for five of the seven tested substances. IO and GABA did not show any effects on growth or morphology of *C. ehrenbergii* up to 100 mg/L. The toxicity of the other five pharmaceuticals ranked in descending order by 168 h-E_YC_{10} values, was as follows: SULF (0.13 mg/L) > CIP (0.16 mg/L) > OFLO (0.22 mg/L) > GUA (26.2 mg/L) > MET (28.6 mg/L). Since inhibitory effects on average growth rate are required to be independent from test duration (OECD, 2011), but the effects of several pharmaceuticals on *C. ehrenbergii* were shown to increase over time, the effect concentrations were given based on cell yield instead of growth rate. Furthermore, CIP and OFLO were found to impair chloroplast integrity and SULF interfered with cell division, leading to malformation of cells.

In order to evaluate the potential risks from the tested pharmaceuticals for the environment, the test substances were classified based on the E_YC_{10} values according to the GHS (United Nations, 2021). Due to the extended exposure duration of 7 days, the pharmaceutical tests were considered chronic. Hence, the following categories were applied: “very toxic to aquatic life with long lasting effects” ($EC_{10} \leq 0.1$ mg/L); “toxic to aquatic life with long lasting effects” ($EC_{10} \leq 1$ mg/L); nontoxic ($EC_{10} > 1$ mg/L). The antibiotics CIP, OFLO, and SULF were classified as “toxic to aquatic life with long lasting effects”. The other test substances were classified as “nontoxic”.

In comparison to literature data from standard species, *C. ehrenbergii* was shown to be significantly more sensitive to CIP and OFLO. The EC_{50} values of CIP from this study were up to 57 times lower than those of *C. vulgaris* and 28 times lower than those of *R. subcapitata*. The EC_{50} of OFLO was up to 10 times lower than that of *R. subcapitata*. In contrast, the EC_{50} of SULF was about 7 times higher than that of *R. subcapitata.* Regarding the other tested pharmaceuticals, only one study was found for MET which reported a comparably low toxicity towards *Desmodesmus subspicatus*.

In conclusion, the findings from this thesis have shown that *C. ehrenbergii* was partly more sensitive towards both inorganic and organic pollutants than commonly assessed standard species which highlights the value of this representative of benthic algae in the hazard assessment of pollutants in order to depict the natural diversity of species in the best possible way.

4.4. Modifications to standard procedures

The toxicity of a test substance may be influenced by the test conditions that are applied during a test (Gomes and Juneau, 2017). These conditions include among others the composition of the culture medium in which the algae grow as well as the exposure time towards the test substances. One objective of this thesis was to investigate the potential influences of the culture medium composition and of the test duration on the outcomes of the toxicity tests with *C. ehrenbergii*.

4.4.1. Culture medium composition

C-medium is the standard culture medium for studies with *C. ehrenbergii* (Ichimura, 1971; Wang et al., 2018). However, as shown in chapter 2 the composition of algal culture media can have a significant impact on the toxicity of the test substances. In case of metals, chelate forming agents such as EDTA and TRIS, which both are components of the C-medium, can form stable complexes with the ionic speciations of metals and thus render them non-bioavailable (Ferreira et al., 2015; Sunda et al., 2009). Hence, if metal toxicity tests were carried out with an unmodified C-medium, the metal toxicity was shown to be significantly underestimated. Therefore, a modified version of the C-medium, named C-modified, was developed for the toxicity tests with metals, which excluded EDTA and TRIS. As a result of the exclusion of chelate forming components from the culture medium, copper toxicity was found to occur at 100-fold lower concentrations (LOEC = 4.0 µg/L) than in the original version of the medium (LOEC = 400 µg/L) (Fig. 5). These findings were supported by chemical analysis which revealed that the original composition of the C-medium was able to trap cupric ions up to a threshold value of 396 µg/L (Fig. 4, chapter 2). Furthermore, in the C-modified medium, the sensitivity of *C. ehrenbergii* to an increase in copper concentration was found to be high, which resulted in a very small concentration range of 2.0 – 16 µg/L Cu between NOEC and complete cell death. In contrast, the respective concentration range in the original medium was significantly larger (350 – 450 µg/L Cu), indicating interactions between the chelating agents and the cupric ions even beyond the analytically determined threshold concentration of complexation. Fig. 5 shows the comparison of the concentration-response curves from the copper toxicity tests conducted with the original and the modified version of the C-medium.

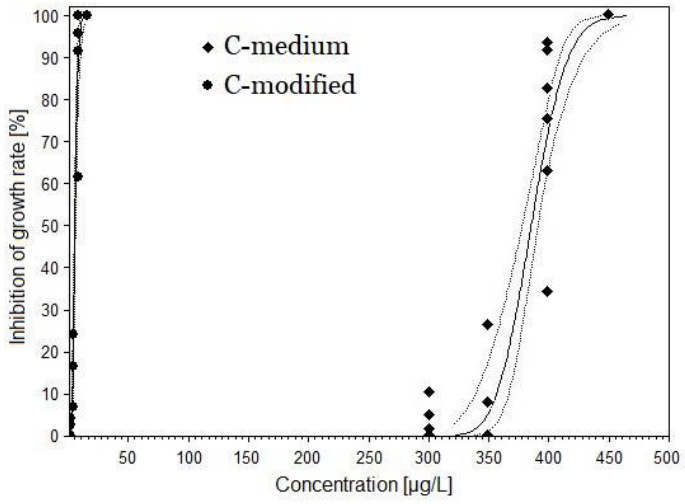

Fig. 5: Comparison of concentration-response curves from *C. ehrenbergii* after copper exposure for 72 h obtained with two versions of the C-medium.

These findings are of great importance, since EDTA and TRIS are common components of many algal culture media (Andersen et al., 2009) which may be necessary for the testing of non-standard species and therefore have the potential to lead to an underestimation of toxic effects. Wang et al. (2018) used the original non-modified version of the C-medium and reported resolvable effects of Cu on the growth of *C. ehrenbergii* up to c = 400 µg/L, which is in accordance with the findings of the complexing properties of the C-medium from this thesis. Furthermore, several studies that used various unmodified media such as Bold's Basal medium or f/2 medium, also reported a very low copper toxicity towards algae with EC_{50} values within the range of mg/L (Ebenezer and Ki, 2012; Lam et al., 1999) which suggests a strong influence of chelating agents on the outcomes of these studies. In addition, also the standard test medium of OECD 201 contains EDTA (OECD, 2011). Although it is briefly stated that a "modification of the growth media may be necessary for certain purposes, e.g. when testing metals", no further details on modifications are given. Hence, until today, studies are published on metal toxicity that use culture media containing chelating agents. As an example, Arambawatta-Lekamge et al. (2021) reported very high EC_{50} values for Cu and Cd of 342 and 4229 µg/L obtained from a standard green alga *Chlorella vulgaris* using the original OECD 201 medium. Since these values are several orders of magnitude higher compared to both the EC_{50} values from this thesis and the literature review on metal toxicity towards *R. subcapitata* (Table 2, chapter 2), a strong influence of EDTA on these reported toxicity data may be assumed. Consequently, the findings from this study have demonstrated the need for the exclusion of complexing agents from culture media in order to conduct a realistic assessment of metal toxicity.

4.4.2. Test duration

According to OECD 201, the standard test duration for algal growth inhibition tests is 3-4 days (OECD, 2011). However, as shown in chapter 3, the duration of exposure can be of major influence on the toxicity of certain test substances. Extended test durations may be necessary in order to avoid an underestimation of toxicity if effects first occur after a latency period. In contrast to the studied metals as well as to MET and GUA, the tested antibiotics, CIP, OFLO, and SULF did not show concentration-dependent effects after 72 h. However, the intensity of effects strongly increased over time leading to severe influences after 168 h on cell growth, morphological integrity, and in case of CIP and OFLO also on the maximum photosynthetic quantum yield. An inhibition of growth that only starts to occur at the end of a test, can be interpreted as an important sign for large but retarded ecotoxicological or chronic effects (Eberius et al., 2002).

Since most ecotoxicological studies on algae stick to the standard test duration of 3-4 days, literature data on the comparison of antibiotic effects over time are rare. Geiger et al. (2016) reported a slight increase in the toxicity of CIP towards *Chlorella vulgaris* from 48 to 96 h as the E_RC_{50} value decreased

by 20 % from 38.0 to 30.7 mg/L. Additionally, Fan et al. (2022) reported a strong time dependency of effects in correlation with the exposure concentration. The lower the concentration of CIP, the longer the duration until effects occurred on the growth of the submerged plant *Vallisneria natans*. While high concentrations of 5 mg/L and above affected growth from day 7 onwards, the lowest concentration of 0.25 mg/L first exerted significant effects at day 42. These studies support the findings from this thesis which therefore suggests that the increase in toxicity over time towards aquatic phototrophic organisms may be a characteristic feature of the tested antibiotics.

Unlike pharmaceuticals, the tested metals did not show a latency of effects. Dissolved metals are small cations that can bind to specific receptors on cell membranes from where they can be actively transported into the cells via transport proteins (Monteiro et al., 2012; Sunda et al., 2009). Once inside the cells, excess metal concentrations may cause reactive oxygen species (ROS) that can lead to oxidative stress and damage biomolecules such as proteins, lipids, or nucleic acids (Pinto et al., 2003; Sharma et al., 2012). As a result, toxic effects of the tested metals were already present after 24 h of exposure. The rapid induction of oxidative stress due to metal exposure is well known and can be observed by the increase in cellular enzymatic defense mechanisms. Wang et al. (2018) reported a significant increase in superoxide dismutase (SOD) activity in *C. ehrenbergii*, which was already detectable after 6 h of copper exposure.

In contrast, antibiotics are large organic molecules with complex modes of action. It is assumed that the uptake of organic pollutants into algal cells occurs mainly by diffusion through cell membranes, whereby the uptake rate depends primarily on the lipophilicity of the chemical (Li et al., 2024; Sun et al., 2017). Passive diffusion generally results in slower uptake rates compared to the active transport via membrane proteins, such as in the case of metals. However, further pathways of antibiotic uptake such as facilitated-diffusion and active energy dependent uptake are considered possible, as the mechanisms of uptake may vary across species and substances and are not fully elucidated, yet (Sutherland and Ralph, 2019). The tested antibiotics CIP, OFLO, and SULF are designed to bind to enzymes and inhibit their activity. CIP and OFLO target gyrase, a topoisomerase involved in DNA replication which is found in chloroplasts (Evans-Roberts et al., 2016; Shariati et al., 2022; Smythe and Rybak, 1989). SULF targets dihydropteroate synthase which takes part in the synthesis of folic acid and is present in the mitochondria of algae (Eguchi et al., 2004; Tabatabaei et al., 2019). The inhibition of enzyme activity may potentially result less rapidly in adverse effects on cell growth or morphology than the occurrence of oxidative stress. Antibiotics may compete with natural substrates for binding sites on enzymes and cells may have reservoirs of the inhibited enzymatic products that first need to be depleted before adverse effects develop. In conjunction with a possible slower uptake rate of the antibiotics, these may be reasons for the delayed occurrence of effects of CIP, OFLO, and SULF on *C. ehrenbergii*.

Further research is necessary to investigate whether a similar increase of effects over time may also occur in standard species beyond 3-4 days of exposure. However, as the standard test procedure requires

continuous illumination of the algae during the toxicity tests, extended test durations may result in additional stress for the cells. Diniz et al. (2021) reported a strongly reduced growth rate of *R. subcapitata* in a long-term experiment (μ_{16d} = 0.07 1/d) that was about 13 times lower than required by the validity criteria of short-term growth inhibition tests for this species (μ_{3d} > 0.92 1/d) (OECD, 2011). Moreover, after an acclimatization period with continuous illumination of 20 days, the growth function of the freshly inoculated cells was not exponential but linear during the course of 16 days of investigation. These findings are supported by unpublished data from this thesis which showed that both growth rate and photosynthetic efficiency of the control groups of *R. subcapitata* significantly decreased by approx. 50 % between day 3 and day 7, indicating unfavourable test conditions in case of an extended test duration (Keim and Weber-Theen, 2023). This may be one reason why the large part of published studies of antibiotic toxicity towards algae investigated only short-term exposure (Sharma et al., 2021). Hence, for the investigation of long-term effects of antibiotics on standard algae, a modification of the light regime may be necessary.

4.5. Evaluation of additional endpoints

In addition to the evaluation of inhibitory effects on growth rate, two further endpoints were assessed in this thesis. Alterations of cell morphology and impacts on photosynthetic efficiency were investigated in order to extend the knowledge gain from the toxicity tests and to draw conclusions about potential modes of action.

4.5.1. Morphology

The observation of morphological changes of organisms after exposure to pollutants can help in elucidating the target sites of specific substances. Due to the large cell dimensions of *C. ehrenbergii*, this test organism was shown to be ideally suited for such observations. In addition, the use of microplates enabled an efficient and regular microscopic monitoring throughout the test duration. Thereby, the development of effects over time could be observed.

Metals were found to lead to a granulation and darkening of chloroplasts. As a consequence, the typically scattered pyrenoids of *C. ehrenbergii* were no longer visible. Furthermore, incomplete cell divisions and cell death resulted as effects of metal exposure (Fig. 3 and Supplementary Fig. A4-A8, chapter 2). The deterioration of chloroplasts as well as the accumulation of starch granules have been reported as effects of oxidative stress caused by metals and other pollutants (Wang et al., 2018; Xu et al., 2019). In addition to the metals, also the antibiotic SULF and the degradation product GUA lead to comparable effects on chloroplasts (Supplementary Fig. A2, chapter 3), indicating oxidative stress after exposure to these substances.

Alongside oxidative stress, SULF was found to impair the regrowth of the second half cell during cell division, leading to malformations in *C. ehrenbergii*. Due to the inhibition of dihydropteroate synthase, SULF can cause a lack of folic acid which is an essential metabolite for the synthesis of DNA (Tabatabaei et al., 2019). Hence, the number of malformed cells increased over time and eventually, cell death was observable during the process of cell division.

Furthermore, specific morphological effects also occurred after exposure to CIP and OFLO. These antibiotics targeted the chloroplasts, leading to an increasing disintegration of the cell organelle over time. As their mode of action is to inhibit DNA gyrase, which is found in chloroplasts (Evans-Roberts et al., 2016), the effects increased with each cell division due to an impaired chloroplast division. Fig. 6 demonstrates the increasing effects of CIP at c = 0.3 mg/L over an exposure time of 7 days.

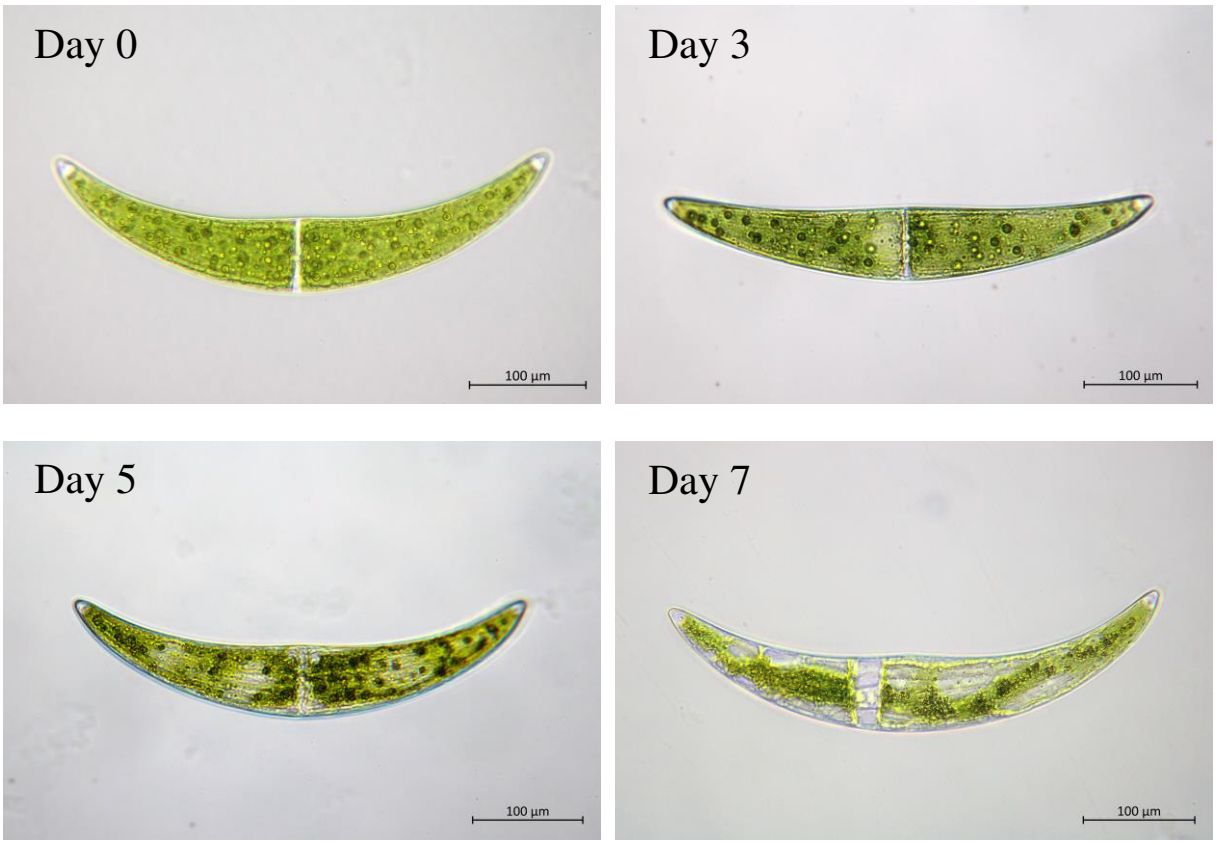

Fig. 6: Morphological alteration of *C. ehrenbergii* after exposure to 0.3 mg/L CIP for 7 days. The chloroplast is affected and increasingly disintegrates over time.

The possibility to assess morphological changes after chemical exposure is a particular benefit of *C. ehrenbergii* over commonly used standard species. The cells of *R. subcapitata* are about 50 times smaller than those of *C. ehrenbergii* so that effects on morphology generally remain hidden even at the highest magnification. Therefore, the evaluation of this additional endpoint in *C. ehrenbergii* contributes to the understanding of the effects and target sites of pollutants on algae.

4.5.2. Photosynthetic efficiency F_V/F_M

The assessment of the maximum photochemical quantum yield reflects the photosynthetic efficiency of energy transfer to the reaction centers of PS(II) and is a valuable method to gain insight into the functioning of the photosynthetic apparatus (Juneau et al., 2003; Lee et al., 2021). It provides an additional quantitative endpoint that can potentially be more sensitive than growth rate as it detects physiological changes that may not necessarily lead to direct effects on cell division. Here, F_V/F_M was found to be significantly reduced in *C. ehrenbergii* after exposure to the antibiotics CIP and OFLO. The inhibition of photosynthetic efficiency started to occur at lower antibiotic concentrations in comparison to cell yield. This was reflected in lower EC_{10} values obtained from F_V/F_M for CIP and OFLO (0.08 and 0.14 mg/L, respectively) compared to those of cell yield (0.16 and 0.22 mg/L, respectively). The other tested pharmaceuticals did not reduce photosynthetic efficiency in a concentration-dependent manner and reached maximum inhibition values of 10 – 20 %. These findings are in line with the morphological investigations that revealed the disintegration of chloroplasts due to the influence of CIP and OFLO, while the other tested substances did not primarily damage chloroplasts. Hence, the assessment of photosynthetic efficiency was shown to be a feasible additional endpoint, that can help to identify substances that specifically target the photosynthetic apparatus of algae.

4.6. Future prospects

The present thesis evaluated the toxicity of thirteen different inorganic and organic pollutants towards *C. ehrenbergii* and contributed to the understanding of interactions between test conditions and effects of pollutants on algae during experimental hazard assessment.

In view of the large number of known pollutants present in the environment, further studies are required as environmental risks of many substances still remain unassessed (Persson et al., 2022). The lack of algae-based ecotoxicological data on many pharmaceuticals and their metabolites leads to particular knowledge gaps about this highly relevant group of pollutants. Moreover, the occurrence of delayed antibiotic toxicity to *C. ehrenbergii* was identified as a topic of major interest for further investigations. Since to date, little knowledge exists on the influence of time on the toxicity of antibiotics to standard algal species (Sharma et al., 2021), there is need for future studies with extended test durations in order to evaluate whether current means of short-term testing can sufficiently assess antibiotic toxicity to algae.

Culture medium composition and test duration were shown to significantly affect the outcomes of the toxicity tests. Various other abiotic factors such as light and temperature are known to potentially influence the toxicity of test substances (Gomes and Juneau, 2017). However, these parameters are rarely varied in toxicity testing due to the need for comparability between studies. Hence, to date, no

studies are known that compared different temperatures or light intensities in the assessment of pollutant toxicity to *C. ehrenbergii*. Nevertheless, species underly seasonal changes of light and temperature under natural conditions. Therefore, further studies on the impacts of abiotic conditions could help to elucidate the role of environmental influences on pollutant toxicity towards *C. ehrenbergii*.

In addition, the possibility to study sexual reproduction in *C. ehrenbergii* makes this species a promising test organism for the investigation of another group of emerging contaminants, called endocrine disruptors (ER). ER are substances that can influence the endocrine system, leading to hormone imbalances in humans and animals. Since algae do not have an endocrine system, most ecotoxicological studies on ER focused on higher trophic level species such as invertebrates, fish, or amphibians (Wagner et al., 2017). However, the life cycle of *C. ehrenbergii* may involve the interaction with hormone-like substances as conjugation and zygospore formation are regulated by the excretion of mating-type specific sex-pheromones (Tsuchikane and Sekimoto, 2019). Hence, the inhibition of zygospore formation has been reported (Ciniglia et al., 2005) after exposure to Triclosan, a widely used antimicrobial agent with endocrine-disrupting properties (Wang and Tian, 2015). However, to date the assessment of interactions between ER and algae remains a largely unexplored area of research which highlights the importance for further studies in this field.

Chapter 5: Summary

To date, planktonic species form the basis of the toxicity assessment of pollutants on algae. Therefore, knowledge gaps exist on the effects of pollutants towards algal species with different ecological traits (Sharma et al., 2021; Xin et al., 2021). Benthic algae are a group of organisms that plays key roles in lotic ecosystems (Law, 2011). Due to their sedentary way of life, benthic algae are not carried away by the current in contrast to planktonic species. Therefore, they may be the main contributors to primary production in rivers and streams. In addition, benthic algae form microhabitats that are colonized by higher trophic level species. A major consequence of the sedentary way of life is the integration of effects over time. When exposed to emissions of pollutants, benthic algae do not benefit from natural dilution in the same way as planktonic species. In view of the ecological relevance of benthic algae and the existing knowledge gaps on ecotoxicological studies with this group of organisms, this thesis aimed to select a representative of benthic algae for the assessment of the toxicity of environmentally relevant pollutants.

Closterium ehrenbergii was selected as the test species for this thesis. *C. ehrenbergii* is a large unicellular, benthic, green alga that can be reliably cultivated under laboratory conditions. In order to perform toxicity tests with this species, a miniaturized test method using microplates was developed. The test design enables to conduct an entire test, containing a dilution series of five test concentrations and one control group with four replicates each in a single microplate. The test conditions of the standard algal growth inhibition test (OECD, 2011) were adopted to the requirements of *C. ehrenbergii*. Since this species conducts cell division only during the dark period and high light intensities damage its chloroplasts, the light regime was set to 14:10 h light dark cycle and low light intensity of 23 µmol photons m^{-2} s^{-1}. A particularly low cell density at test start of approx. 10 cells/mL was found to be suitable for this test method. Due to the large cell dimensions, the cells can be counted directly inside the microplate using a stereomicroscope. Therefore, no surrogate parameters are necessary for the determination of growth rates. In addition, morphological alterations can be observed under higher magnification. The miniaturized bioassay using *C. ehrenbergii* combines several benefits over traditional test designs with test tubes or flasks. The endpoint evaluation is non-invasive which makes it fast, the amount of chemicals used is reduced to a minimum, the experimental effort is comparably low, and the investigation of morphological alterations can help to elucidate target sites of test substances.

Metals and pharmaceuticals were selected as test substances of high concern for the ecotoxicological assessments with *C. ehrenbergii*. Both groups of chemicals are released into the environment due to anthropogenic pollution and hence are found in surface waters all over the world (Beek et al., 2016; Li et al., 2020). Metals are known as harmful substances for various aquatic organisms (Arambawatta-Lekamge et al., 2021; Hsieh et al., 2004) and pharmaceuticals are considered one of the most concerning

groups of pollutants due to their biologically active nature (Chopra and Kumar, 2018). *C. ehrenbergii* was found to be partly more sensitive towards both metals and antibiotics than commonly used standard species such as *R. subcapitata* and *C. vulgaris*. This highlights the value of this organism as an additional test species in ecotoxicity testing of pollutants.

The toxicity of the six tested metals in descending order, ranked by E_RC_{50} values, was as follows: Cu (5.5 µg/L) > Ag (9.2 µg/L) > Cd (18 µg/L) > Ni (260 µg/L) > Cr (990 µg/L) > Zn (1200 µg/L). For the assessment of metal toxicity, the modification of the commonly used C-medium was shown to be necessary in order to avoid metal chelation and hence an underestimation of toxicity. EDTA and TRIS were excluded from the culture medium due to their metal complexing properties (Ferreira et al., 2015; Sunda et al., 2009). Performing the tests without the pH buffer TRIS was shown to be suitable as it did not affect the pH during the toxicity tests which likely is a benefit of the low cell densities applied. By chemical analysis, the original composition of the C-medium was found to trap cupric ions up to 396 µg/L. Therefore, the effect concentrations of the copper toxicity tests were up to 100 times lower in the modified version of the medium that excluded chelating agents. Until today, studies on metal toxicity are published that use unmodified culture media which contain chelating agents such as EDTA (Arambawatta-Lekamge et al., 2021; Wang et al., 2018). Therefore, the findings from this study are of great importance in order to avoid underestimations of metal toxicity.

The duration of exposure was found to be of major importance for the assessment of pharmaceutical toxicity towards *C. ehrenbergii*. The antibiotics CIP, OFLO, and SULF exhibited latency periods of 72 h until they started to develop their true effects. Therefore, the test duration was extended to 168 h. After longer-term exposure, the antibiotics significantly affected growth and morphology of *C. ehrenbergii*. CIP and OFLO impaired chloroplast integrity while SULF interfered with cell division leading to malformation of cells. The assessment of the maximum photochemical quantum yield F_V/F_M confirmed the morphological observations revealing the inhibition of photosynthetic efficiency as a result of chloroplast disintegration after exposure to CIP and OFLO. The other tested pharmaceuticals exerted only minor (GUA and MET) or no effects (IO and GABA) on *C. ehrenbergii*. The toxicity of the five pharmaceuticals that affected cell growth, ranked in descending order by 168 h-E_YC_{10} values, was as follows: SULF (0.13 mg/L) > CIP (0.16 mg/L) > OFLO (0.22 mg/L) > GUA (26.2 mg/L) > MET (28.6 mg/L). The occurrence of delayed antibiotic toxicity to *C. ehrenbergii* was identified as a topic of major interest. Therefore, future research is required to investigate whether the outcomes of this thesis also apply to standard species and to evaluate whether common short-term exposure tests sufficiently assess the hazards of pharmaceuticals towards algae.

In conclusion, this thesis selected *C. ehrenbergii* as a representative of benthic algae, developed a miniaturized bioassay, and assessed the toxicity of thirteen highly relevant inorganic and organic pollutants. The investigations beyond recognized standards contributed to the understanding of effects of anthropogenic pollutants on algae.

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