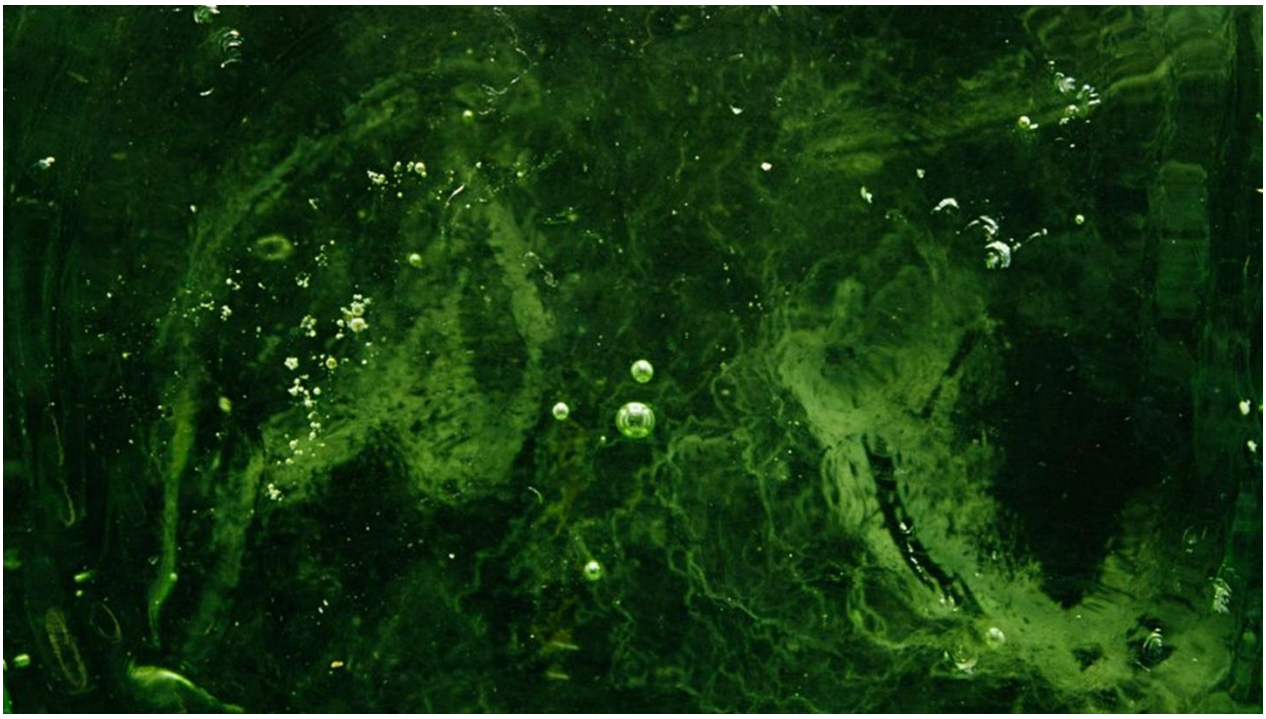
AALBORG UNIVERSITET

NEW FRESHWATER MICROALGA FOR MICROPLATE TOXICITY TESTING

CHLORELLA SOROKINIANA IN COMPARISON WITH THE STANDARD ALGAE SPECIES *RAPHIDOCELIS SUBCAPITATA*

Ina Kraus – Master thesis



July 2023

STUDENT REPORT

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ECTS:	60
Numbers of page:	xx

Submitted: 1st July 2023

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ACKNOWLEDGEMENT

First of all, I would like to express a big thank you to my supervisor Peter Roslev for the cooperation and help in form of knowledge as well as being alongside with advice throughout this thesis. Furthermore, I wish to thank Lana Flanjak for her kind support and for enabling a good start for me with her incredible knowledge.

The process of writing this project and studying abroad was rewarding, as well as challenging, and thanks to the people alongside me, I overcame many obstacles and grew not only in professional terms but also personally. I would like to extend my thanks to my research group that made me feel welcome and created a great team spirit. Especially to Helle Blendstrup who is the good soul in the department and who always helps all of us without hesitating.

I thank Aalborg University and the Head of the studies for approval of my thesis project. I am grateful for all the inspiring professors and lecturers who broadened my knowledge and have encouraged my curiosity. There I would like to point out Niels Iversen who led us through the challenging first months of studying abroad and who was always there to give great advice, study related as well as in other fields.

Finally, I thank my family and friends, in particular Konstantinos Stavridis, with whom I walked through those two years of Masters' education and build memories and a great friendship. I appreciate you all for providing support in different kind of ways and for always believing in me.

ABSTRACT

Microalgae build the base of the food chain as being the dominant primary producers but also secure water purification and the function of freshwater ecosystem. Discharged toxicants can cause harm to aquatic microorganisms and get the ecosystem out of balance. Algal toxicity tests find their application in classifying chemicals regarding their hazardous potential and elaborate water quality criteria. Further, they are used in biomonitoring and assessing effluents to detect hazardous substances and lead to possible steps to intervene. This study focused on improving the standard microplate algal toxicity test based on a new freshwater microalga. The species *Chlorella sorokiniana* was investigated due to its ability to be fast-growing. With a growth rate of 2.20 d^{-1} *C. sorokiniana* was examined to be faster growing than *Raphidocelis subcapitata* (formerly known as *Selenastrum capricornum* and *Pseudokirchneriella subcapitata*) with a growth rate of XX. The new alga species were assayed on their sensitivity with three quaternary ammonium compounds (BAC, DTAC, DDAC), four reference toxicants (K2Cr2O7, 3,5-DCP, ZnSO4, CuSO4), and two active compounds in herbicides (Glyphosate, pelargonic acid). The results show lower or similar EC_{50} values compared to the standard species. With the finding in this study, C. sorokiniana can be suggested as new, more sensitive species for microplate algal toxicity tests with a reduction of execution time to 48 hours instead of 72 hours.

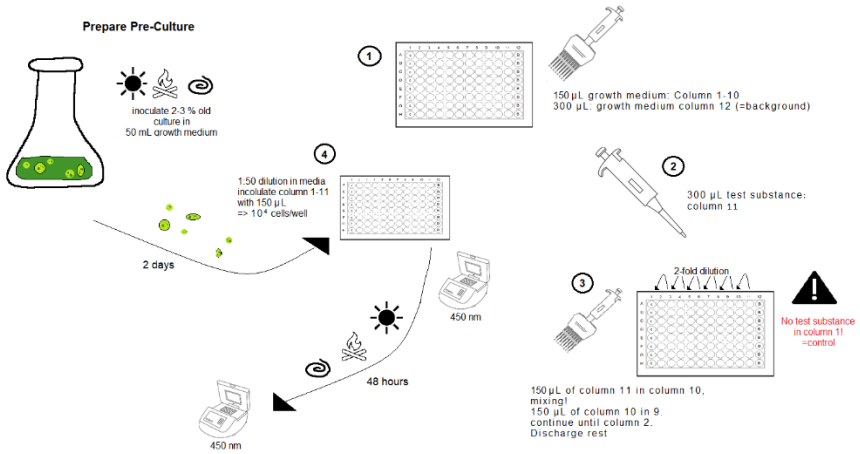

Figure 1: Schematic execution of the microplate algal toxicity test based on the new suggested version of the ISO standard test

LIST OF ABBREVIATIONS

CS:	*Chlorella sorokiniana*
RS:	*Raphidocelis subcapitata*
D*:*	*Dunaliella* sp*.*
EC_{50}:	effective concentration that causes 50 % inhibition of growth
EC_{20}:	effective concentration that causes 20 % inhibition of growth
CI:	Confidence Interval
SEM:	Standard error of the mean
QACs:	Quaternary Ammonium Compounds
BAC:	Alkylbenzyldimethylammonium chloride
DTAC:	Dodecyltrimethylammonium chloride
DDAC:	Didecyldimethylammoniumchlorid
3,5-DCP:	3,5-Dichlorophenol

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INTRODUCTION

Meeting our need for clean freshwater will be an increasing challenge in the future. Only 2.5 % of the water on earth is freshwater and anthropogenic activities are polluting freshwater systems due to e.g., farming, sewage discharge, and industrial processes (Regan, 2015). Polluted freshwater can cause harm to aquatic organisms, humans using it for drinking water or recreational activities as well as terrestrial organisms drinking the water. Microalgae build the base of the food chain as being the dominant primary producers but also secure water purification and the function of the freshwater ecosystem (Liu, et al., 2022) and are therefore of great importance. This is why contamination and the resulting disruption to those aquatic microorganisms can affect higher trophic levels and risk the ecological balance. Another concerning factor is that not only marine and freshwater organisms are exposed to an elevated risk, but humans may also experience decreases in fish catch or health risks due to the accumulation of pollutants in the food chain. As can be seen, it is ecologically significant to use microalgae for ecotoxicological assays because of its key role in aquatic ecosystems and its sensitivity to chemicals (Geis, et al., 2000).

Algae toxicity tests are broadly used to assess water quality. Many advantages can be seen by using the microplate technique after Blaise & Vasseur (2005) compared to other methods like the use of glass vessels. Some of those advantaged are for example high replicability and repeatability, minimal training for technicians as well as simple equipment, which is common in most laboratoriesTable 1). But as in every method, there are disadvantages, too. In particular, the test needs to run 72 hours plus a preliminary preparation of 2-5 days before the actual test can take place. And since time is a very precious variable, especially in ecotoxicity, this is a disadvantage that needs to be improved. Furthermore, sensitivity is a parameter that needs to be enhanced.

Another area in which algae species with high growth rates and biomass production are of high interest is the field of biofuels (Gim, et al., 2013). After producing biofuels from sugar and then using straws and bagasse as a possible source, microalgae promised to produce biofuel with higher efficiency (Ma, et al.,

2022). There the alga *Chlorella sorokiniana* started to raise attention in research because of its fast-growing and high biomass production.

This microalga *C. sorokiniana* was assayed of being a possible new species for microplate toxicity tests, using contaminants such as the quaternary ammonium compounds (QACs) and related bioactive compounds. In recent years, the use of those biocides has increased significantly, especially due to the Covid-19 pandemic. Biocides containing QACs are released into the environment and affect aquatic systems. To gain knowledge about the extent of the negative impact of QACs on aquatic phytoplankton is of high significance, which may be determined using representative freshwater microalgae.

OBJECTIVES

This study focused on developing a microplate algal toxicity test based on new freshwater microalga that is improving the standard method. By which parameters can the standard toxicity test be enhanced, and which algae species is qualified. In the first part of this study, a characterization of the new alga species takes place and the optimum conditions (e.g., algal growth medium, temperature) are determined. How can its characteristics, which made it chosen as a promising new species, be improved. This growth inhibition test assesses the sensitivity of a microalgae to substances, and the degree of sensitivity must be determined to figure out if the new species is suitable for ecotoxicity testing. These sensitivities should be set in context with different conditions, at which the test was performed. The focus lies on quaternary ammonium compounds and related bioactive compounds. Moreover, reference toxicants should be tested to be able to compare the results to those shown in the literature. Furthermore, it will be delved into the question of how and how much the freshwater species differs from the standard species *R. subcapitata*. Where are the two species similar and at which points are the new species improving the toxicity test.

The hypothesis is that the promising algal species *Chlorella sorokiniana* with high growth rates is a new species that can improve the microplate algal toxicity test by reducing procedure time without lowering but rather improving sensitivity.

THEORY

TEST ORGANISMS

In algal toxicity tests several test species are used. Guidelines like ISO and OECD proposed diatoms *P. tricormutum* and *S. costatum*, and green algae *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*) as well as *Desmodesmus subspicatus* (ISO, 2012) (OECD, 2011).

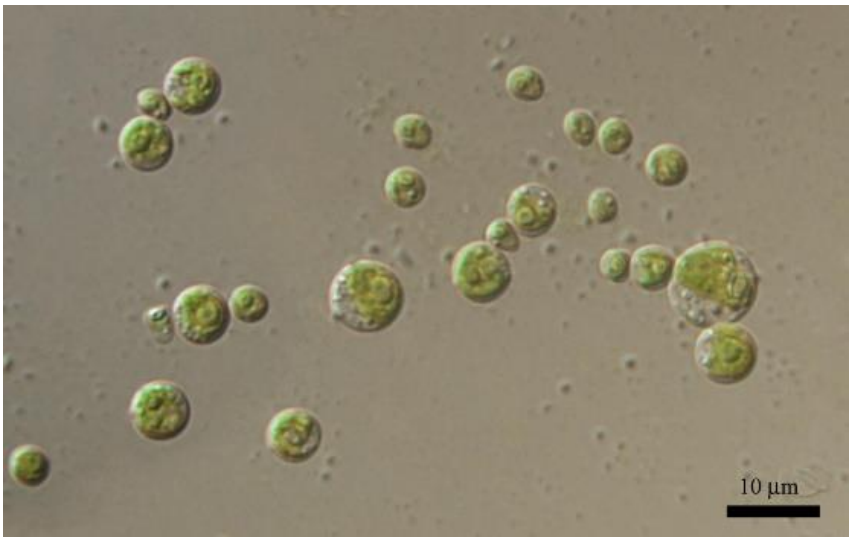

Figure 2: Chlorella Sorokiniana, (CCALA, 2023)

Chlorella sorokiniana Figure 2) is a green, unicellular, freshwater alga that was first isolated in 1953 by Constantine Sorokin (Sorokin & Myers, 1953). It is a subspecies that is thought to be a mutant of *Chlorella pyrenoidosa* (Lizzul, et al., 2018). The algae's optimum temperature range is 38 to 39 °C, whereas the maximum temperature is about 42 °C and the minimum lies between 15 and 20 °C. Furthermore, this species is distinguishable by larger cells than *Chlorella vulgaris*. The young daughter cells reach from 1.5 to 3 µm and the mother cell can reach up to 12 µm (Sorokin, 1967). *Chlorella sorokiniana* is able to grow mixotrophic and a preference for sugars like glucose or simple organic acids such as acetate was found in previous studies (Lizzul, et al., 2018). Light is the main source of energy in mixotrophic cultivation, nevertheless, organic, and inorganic carbon (CO_2) are assimilated at the same time (Gim, et al., 2013).

Researchers found a higher maximal growth rate compared to other Chlorella strains. Under conditions of 38 °C and 17.600 lumen/m^3 the number of cells doubles in 11.3 hours whereas *Chlorella vulgaris* doubles 9.9 times (Sorokin, 1967). According to Lizzul, et al. 2018, a doubling time between 4-6 hours at optimal growth conditions at 35-40 °C can be seen (Lizzul, et al., 2018).
With a cell number per mL of 10^4 algal cells at inoculation like it is suggested in ISO, and with a doubling time of 8 hours (average of Sorokin and Lizzul) the calculates time needed to reach the cell number required to make the test valid, is after 32 hours with cell number/mL of 160.000. That leads to the hypothesis that the execution time of the growth inhibition test by using C. sorokiniana can be reduced from 72 hours to at least 48 hours.
Chlorella sorokiniana has already aroused the interest of many researchers and companies to improve the process of producing biofuel and to gain more biomass in less time. *C. sorokiniana* produced, based on the results of Liu, et al. (2022), a higher amount of biomass per liter than Chlorella vulgaris, which is already well-represented in that field. The ability of fast-growing *C. sorokiniana* led to the assumption that algal toxicity tests could be executed in a shorter time span compared to those with the standard algae species.

ALGAL GROWTH AND RESPONSE TO POLLUTANTS

Algal growth can be divided intp several phases if grown under controlled light, temperature, and nutrient conditions. After inoculating active cells into a growth medium, the first phase is called a lag phase, followed by an exponential (log) phase, a phase of declining growth rate, a stationary phase, and a death phase (Figure 3). During the lag phase, the alga undertakes no cell division and the value of cells/mL stays constant. Reproduction of microalgae takes place in the second phase. Each algal cell doubles which leads to exponential growth and the growth rate can be calculated. The stationary phase is reached when nutrients become a limiting factor. Cell division ceases and cell density stays at one level. In the last phase, the death phase, microalga cells are dying due to the lack of nutrients. Understanding the growth of microalgae is important since it is required to use microalgal cells that are in the exponential phase to ensure a successful

toxicity test. The optimum growth rate is given at this point which allows the comparison of control and exposed algal cell densities (Blaise & Vasseur, 2005).

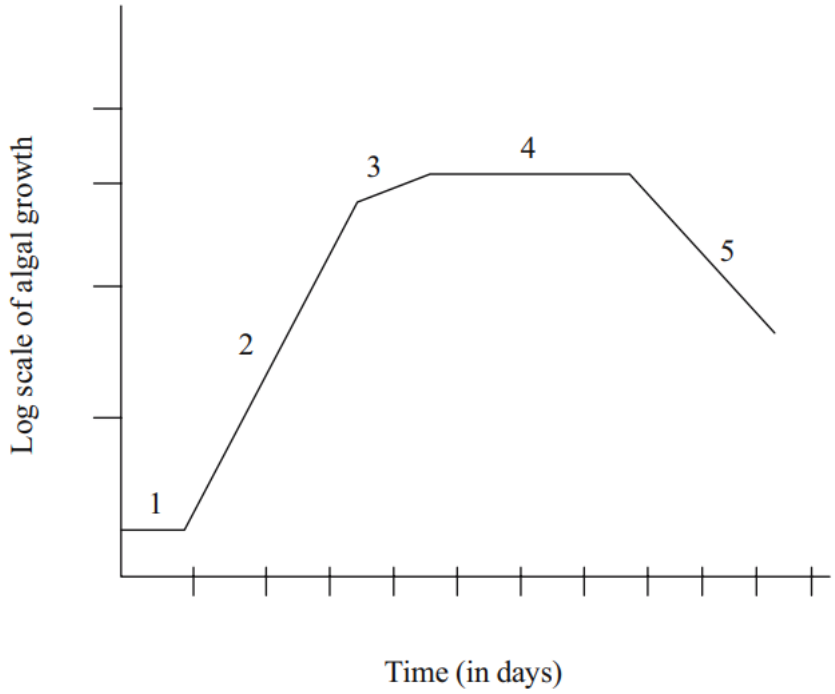

Figure 3: Growth phases of algal cultures: Lag (1), Exponential (2), Declining growth rate (3), Stationary (4), Death (5) (Blaise & Vasseur, 2005)

The life cycle of the standard species R. subcapitata is well understood. After inoculation in new medium, the cells grow exponentially for 3 days. There the maximum photosynthetic activity takes place, and the typical green colour occurs due to the big chloroplasts in the cells. After 5 days until 12 days the cells reach the stationary phase. The green colour decreases (decline in size in chloroplast) and decrease in chlorophyll content and photosynthesis activity. At day 12 the cells start to die due to the loss of cell membrane integrity. The colour of the culture now occurs yellow (Machado & Soares, 2022).

Microalgae can assimilate different carbon sources by using different mechanisms. Inorganic carbon is assimilated by the mechanism of CO_2-concentrating. The microalgae fixes dissolved inorganic carbon to biomass through photosynthesis. An example of a possible inorganic carbon source for microalgae can be sodium bicarbonate. Carbonic anhydrase converts HCO_3^- into CO_2, which then can be absorbed by the cells. It is shown that the maximum cell density can be increased. Some species (e.g., *C. vulgaris*) can uptake organic

carbon for cell metabolism. Pathways can be the Pentose Phosphate Pathway (PPP) and the Embden-Meyerhof-Pathway (EMP) (Ma, et al., 2022). Glucose is known to produce ATP, NADH, and NADPH in cells by taking the PPP pathway under heterotrophic conditions and the EMP under mixotrophic conditions. Not all microalgae species are able to use organic carbon for heterotrophic growth due to the lack of transporters. Moreover, some species can use combines carbon sources under mixotrophic conditions. Because of the synergy between chloroplast and mitochondria metabolism in mixotrophic growth, higher photosynthetic carbon fixation and more glyceraldehyde-3-phosaphase (supports ATP forming) can be achieved. Furthermore, acetate and glycerol are possible organic carbon sources. Glycerol diffuses into the microalgal cell whereas acetate goes through the cell passively by using transporters (Ma, et al., 2022).

Different toxicants have different influence on the structure and function of ecosystems and are organism or concentration dependent (sensitivity). Some toxicants in environmental environments target biological membranes of algae, which leads to leakage and reduction of full function and indirectly affect algal photosynthesis. Cell size of the organism can decrease, and reproduction may be stopped, leading to a declining algal biomass (Escher, et al., 2021).

Some toxic effects are species dependent and cell size, cell wall type, and thickness are factors that have an influence on the response of algae species to substances (Tato & Beiras, 2019).

Herbicides

Herbicides can initiate negative consequences for aquatic ecosystems, where they end up because of surface runoff from Agriculture. Herbicides are used as a useful tool for weed control but harm green algae. In natural ecosystems they can affect microalgae and due to their role as primary producers the whole functioning aquatic ecosystem (Chiellini, et al., 2020). Some herbicides have a mode of action of directly inhibiting the photosystem II. They block electron transfer followed by a lack of producing ATP. Consequently, loss of chlorophyll, slower growth, and death of cells occur (Escher, et al., 2021). Others inhibit cell division or lipid synthesis. Glyphosate is known to be an EPSP (5-enolpyruvylshikimic acid-3-

phosphate) synthase inhibitor. If this enzyme is hindered the biosynthesis of amino acids is (Annett, et al., 2014). Annett, et al. (2014) is suggesting a half-life range from 1.7 to 142 days. Furthermore, is toxicity of glyphosate is species-dependent (Annett, et al., 2014).

Pelargonic acid is a in nature widely occurring, saturated, fatty acid with nine carbons (Savage & Zorner, 1996). First isolated from leaves of *Pelargoinium* roseum it is used in weedkillers due to its herbicidal effects against weeds, algae, and mosses. For this active compound, a high risk to aquatic organisms was identified (Alvarez, et al., 2021). It was suggested that pelargonic acid is not damaging cell membranes directly. Its mode of action is moving through the cell membranes. The internal pH is lowered and ATP and Glucose-6-phosphase decline. Afterwards, membrane dysfunction can appear that is followed by leakage of the tissue (Savage & Zorner, 1996). A synergize effect with glyphosate was observed because it allows greater and more rapid uptake of glyphosate.

Heavy metals

Environmental contamination of aquatic systems with heavy metals be natural and anthropogenic. As sources can be named among others fossil fuel combustion, mining, municipal wastes, and sewage (Chiellini, et al., 2020). Heavy metals are present in aquatic ecosystems in low concentration and microalgae consume heavy metals as trace elements. But higher concentrations of those trace metals or other non-essential heavy metals are toxic for microalgae (Pinto, et al., 2003). Higher metal concentrations can be detected in coastal waters and waters where higher human activity takes place. Microalgae have formed strategies of self-protection against those toxicants (Immobilisation, exclusion, and chelation. The mode of action differs by metals. Some impact cell organelles or reduce chlorophyll content (Leong & Chang, 2020). Heavy metals further can cause damage to proteins as well as cause oxidative stress to the cell (Escher, et al., 2021).

MICROALGAE AS TEST SPECIES

Unicellular algae are abundant in aquatic ecosystems. They contribute a big part to carbon fixation and oxygen production. Moreover, as base of the food chain they keep the ecosystem functioning. With every adverse effect on a species in one trophic level another one is put in risk at the next trophic level. Therefore, is it important to start assessing toxic effects at the bottom to protect other species and the aquatic ecosystem (Franklin, et al., 1998). Additionally, are microalga more sensitive to toxicants than other freshwater organisms. Ranked by decreasing sensitivity: Alga ≥ Crustacea > Cnidaria > Mollusca > Chordata (Franklin, et al., 1998). This makes them highly valued test organisms.

Due to the shorter generation span of microalga a test on the toxicity of a chemical is of higher interest compared to other organisms like mollusks or chordata. A toxicity test with Daphnia takes 21 days whereas with alga results can achieved after 72 hours and is with 50-60 hours in the laboratory more time-consuming. The chronic test with bacterial bioluminescence lasts 22 hr. The study concluded that the algae test appears as the most sensitive one compared to tests based on response with Brachionus calyciflorus (2-Day Test), Microtox Chronic Toxicity Test (bacterial reagent: vibrio fischeri) and microcrustace (Radix, et al., 2000). But acute tests (15 min) give often results that are very far from chronic toxicity data. Furthermore, test with fluorescence can give results after 5 hours but sometimes in no toxic effect detectable after that short execution time (Radix, et al., 2000)

Toxic substances can be accumulated in algal cells. Hence algae can be used in wastewater treatment but also give a reason to worry about biomagnification along trophic levels. Even though heavy metal accumulation is not always related to toxicity it is important to consider (Pinto, et al., 2003). Biomonitoring here is important, so that heavy metal do not end up in high concentrations in e.g., fish for human consumption.

MICROPLATE TEST

A microplate algal toxicity test is carried out to determine the effects of a substance on the growth of microalgae. Unicellular organisms are exposed to a

liquid sample to measure the specific toxic effect. Instead of multiple glass vessels, a microplate with 96 wells is used which relates to advantages. Microalgae react to different concentrations of the test substance by reduction of growth so that growth inhibition is used as endpoint. The responses of the alga (growth inhibition) of with and without exposure to the test substance are compared (OECD, 2011). A common parameter worked with in literature is the EC_{50}. This value shows the effect concentration that led to 50 % of inhibition of a population. Microplate toxicity tests can be used for domestic and industrial wastewater, surface groundwater or leachates, and sediment interstitial waters (Blaise & Vasseur, 2005). It finds its application in biomonitoring, elaborates water quality criteria, and assesses effluents in order to regulatory statutes. Furthermore, it is possible to rank and screen chemicals regarding their hazardous potential.

Table 1: Advantages and disadvantages of Microplate toxicity testing

Advantages	**Disadvantages**
- high replicability and repeatability, perform high amount of analysis - minimal requirements for instrumentation and facilities - simple equipment, common in most laboratories - Good incubator space economy - minimal training for technicians - Small sample volume required - costs in glassware and reagents - (Tato & Beiras, 2019; Staveley & Smrchek, 2005; Blaise & Vasseur, 2005)	- exposure time may be too short for slow acting toxicants to have an effect - adsorption of substances in microplate walls - evaporation of volatile chemicals that can interfere with neighbour wells - cell density increases due to evaporation - (Tato & Beiras, 2019; Blaise & Vasseur, 2005)

Microplate toxicity testing is promising due to the simple and common equipment as well as non-demanding training for technicians (Table 1). Limited space is a widespread problem in laboratories that can be reduced with this test method. High replicability and repeatability allow a high amount of analysis. On top of that small sample volume, glassware and reactance are reducing costs. A downside can be well evaporation (Tato & Beiras, 2019). Under longer (>1 day) exposure, evaporation can occur which leads to increased cell density. Also, volatile chemicals can evaporate and interfere with neighbour wells. This cross-contamination may impact results (Blaise & Vasseur, 2005). Furthermore, substances can be adsorbed by microplate wells. At the moment, there is no evidence of increased adherence to plastic microplate wells compared to glass flasks. Still, it should be considered that adsorption on wells can occur with chemicals that show a higher affinity for plastic (Blaise & Vasseur, 2005).

With all advantages and disadvantage, the matter of time would be the main weakness of a microplate toxicity test. The duration of a chronic microplate toxicity test is 72 hours, set by the guidelines (OECD ISO). Moreover, an additional time of 2-5 days is needed before the toxicity test where the old culture must be inoculated in a new medium to ensure the algae cells are in an exponential phase. Therefore, the algae species *Chlorella sorokiniana*, as stated above, with a higher growth rate than other species, sounds promising to limit the number of hours needed to get a toxicity test result.

QUATERNARY AMMONIUM COMPOUNDS

Due to their strong cationic surface activity Quaternary Ammonium Compounds (QACs) are used as ingredients in detergents and disinfectants. Those surfactants are widely used in day-to-day products as personal care products, detergents, biocides, and phase transfer agent (Di Nica, et al., 2017). During the Covid-19 pandemic the use of disinfectants became a governmental principle in many countries to depress a spreading of the virus. Therefore, QACs became more detected in the environment. The release into the environment takes place due to the discharge of effluents and sludge from sewage treatment as well as from hospitals, laundry wastewater, and roof runoff (Zhang, et al., 2015).

For aquatic organisms like fish, daphnids, rotifer, algae and protozoan, and a lot of microorganisms QACs occur to have adverse effects. Their mode of action is penetrating their alkyl chain into microorganisms' membranes and altering the phospholipid bilayer. Therefore, they disrupt the membrane and lead to a leakage (Zhang, et al., 2015). Studies show that algae are more sensitive and higher affected by QACs than fish and crustaceans. QACs inhibit nutrient uptake by algae. The degree of toxicity decreases with length of alkyl chain (Zhang, et al., 2015).

MATERIAL AND METHODS

MICROALGAL STRAIN

The algae strain *Chlorella sorokiniana* (211-31) was purchased from the Culture Collection of Algae at Göttingen University, SAG. The strain is an axenic culture and arrived on an ESP agar. Further cultivation was proceeded in liquid freshwater medium based on ISO standard (see following chapter, p. 11) under steady illumination at 7000 lumens at 23 ± 2 °C.

The standard alga species *Raphidocelis Subcapitata* is in possession of AAU for generations. Cultivation is the same procedure as with *C. sorokiniana*. Furthermore, *Dunaliella* sp., which is in use for research at AAU for example about production of b-carotin, was subject of this study. It was cultivated in freshwater standard medium enriched with 3 g/L NaCL.

GROWTH MEDIUM

For toxicity tests with *R. subcapitata* and *C. sorokiniana* freshwater algal medium after ISO standard is used (ISO, 2012). All stock solutions were autoclaved except sodium hydrogen carbonate, which was prepared in double distilled autoclaved water. Autoclaving is used to sterilise equipment and media by suspend them to steam at 121 °C for around 30-60 min at a pressure of 103 kPa (15 psi/1.02 atm). By heating sodium hydrogen carbonate volatile carbon is produced with the thermal reaction (Radecki & Wesolowski, 1976):

$$2NaHCO_3 \rightarrow Na_2CO_3 + CO_2 + H_2O$$ *Equation 1*

With autoclaving the state of aggregation is changed and carbon dioxide leaves the solution. The carbon source for microalgae is therefore lessoned.

The final medium contains:

- Macronutrients: 15 mg/L NH_4Cl (N: 3.9 mg/L), 12 mg/L $MgCl_2 \cdot 6\ H_2O$ (Mg: 2.9 mg/L), 18 mg/L $CaCl_2 \cdot 2\ H_2O$ (Ca: 4.9 mg/L), 15 mg/L $MgSO_4 \cdot 7\ H_2O$ (S: 1.95 mg/L), 1.6 mg/L KH_2PO_4 (P: 0.36 mg/L)
- Fe-EDTA: 64 µg/L $FeCl_3 \cdot 6\ H_2O$ (Fe: 13 µg/L), 100 µg/L $Na_2EDTA \cdot 2\ H_2O$
- Trace elements: 185 µg/L H_3BO_3 (B: 32 µg/L), 415 µg/L $MnCl_2 \cdot 4\ H_2O$ (Mn: 115 µg/L), 3 µg/L $ZnCl_2$ (Zn: 1.4 µg/L), 1.5 µg/L $CoCl_2 \cdot 6\ H_2O$ (Co: 0.37 µg/L), 0.01 µg/L $CuCl_2 \cdot 2\ H_2O$ (Cu: 3.7 ng/L), 7 µg/L $Na_2MoO_4 \cdot 2\ H_2O$ (Mo: 2.8 µg/L)
- $NaHCO_3$: 50 mg/L (C: 7.14 mg/L)

Final mass concentration in brackets.

To determine the influence of different carbon sources on the growth of *C. sorokiniana* 10 g/L glucose, 2 g/L sodium acetate and 0.15 M glycerol were added to freshwater media.

TEST CHEMICALS

While examining experiments strict quality assurance were adhered to. Stocks and dilutions were prepared in double distilled and autoclaved water. In the following tTable 2, all chemicals, their CAS-number and suppliers are.

Table 2: Chemical name, formula, CAS-number, and provider of all chemicals used for algal toxicity tests

Chemical name	**Chemical formula**	**CAS-number**	**Suppliers**
Alkylbenzyldimethyl-ammonium chloride (BAC)	$C_6H_5CH_2N(CH_3)_2RCl$ (R=C_8H_{17} to $C_{18}H_{37}$)	63449-41-2	Sigma-Aldrich
Dodecyltrimethyl-ammonium chloride (DTAC)	$CH_3(CH_2)_{11}N(CH_3)_3Cl$	112-00-5	Sigma-Aldrich
Didecyldimethyl-ammonium chloride (DDAC)	$C_{22}H_{48}ClN$	7173-51-5	Sigma-Aldrich

Potassium dichromate	$K_2Cr_2O_7$	7778-50-9	Merck 4868
3,5-Dichlorophenol	$C_6H_4Cl_2O$	591-35-5	Sigma-Aldrich
Zinc sulphate heptahydrate	$ZnSO_47H_2O$	7446-20-0	Sigma
Copper(II)sulphate pentahydrate	$CuSO_4$ 5 H_2O	17829-58-2	Sigma
N-(Phosphonomethyl)glycine monoisopropylamine salt (Glyphosate)	$C_6H_{17}N_2O_5P$	38641-94-0	Sigma-Aldrich
Pelargonic acid (active compound in weedkiller AlgeFri)	$C_9H_{18}O_2$	112-05-0	Ecostyle

DEFINE GROWTHT CURVE

Before the start of the toxicity test the growth curves of the species were determined. Therefore, the exponential growth phase can be obtained, and growth rates be calculated. The measurements were made for all three species *R. subcapitata, C. sorokiniana* and *Dunaliella* in a period of 10 days.

To be able to examine growth of the different algae species after inoculation, cells were counted with a Multisizer Coulter Counter daily for 10 days. That method determines the occurring resistance between two electrodes as a particle enters the tube with a conducting fluid. The data can be plotted as a growth curve and different growth phases can be determined as discussed in the chapter about Algal growth and response to pollutants on page 4.

MICROPLATE TOXICITY TEST

The algal growth inhibition test with microplates was carried out based on the standard ISO 8692:2012 for water quality (ISO, 2012). The toxicity of a substance is determined by measuring the algal growth inhibition. The exponential growth phase of the algae species must be ensured to be able to calculate the growth rate. For that reason, a new culture is started 2-5 days before the test. The culture

was incubated in a 250 mL Erlenmeyer flask with 50 mL of growth medium under shaking at 100 rpm and continuous fluorescent cold light with an intensity of 7000 lux at 23 ± 2 degrees. Flasks are covered with a cotton plug, so that CO_2 is able to diffuse into the flask, but contamination of the culture is prevented, and evaporation is lessened. The cell density at inoculation is around $5x10^3$ – 10^4 cells/mL. An ideal microplate toxicity test should include a control group with no effect on algal growth (0% inhibition), two points below and above 50 % inhibitory growth effect, and a concentration that give an intense or total effect on the algae (90-100 % growth inhibition) (Blaise & Vasseur, 2005). Algae medium, a 2-fold serial dilution of the test substance, and algae cells are transferred in a microplate as illustrated in Figure 4. The algal culture is diluted in a relation 1:50 in the algal test medium, so 10^4 algal cells are inoculated per well. The endpoint in algal toxicity tests is the growth rate. Therefore, optical density, referring to the indirect biomass of the microalgae, is measured with a spectrometer at 450 nm (Termo Multiskan plate reader).

Test procedure briefly can be found in Table 3.

Table 3: Summary of test conditions for the standard and newly suggested algal microplate toxicity test

	(ISO, 2012)	Suggested new test
Test organism	Cells of *Raphidocelis subcapitata* from a culture in exponential phase (2-5 days after inoculating culture)	Cells of *Chlorella sorokiniana* from a culture in exponential phase (1-2 days after inoculating culture)
Type of test	Microplate (96 wells) toxicity test, 72h exposure	Microplate (96 wells) toxicity test, 48 h exposure
Well volume	150 µL media, 300 µL test substance, 150 µL algal inoculum	
Lightning	Continuous, vertical, cool white fluorescent light with an intensity of 7000 lux	
Temperature	23 ± 2 °C	

Initial algae density	10^4 algal cells/well	
Test medium	Freshwater culture media (ISO, 2012)	
Experimental configuration	8 control wells, 10 wells with 2-fold dilution of test substance, 8 wells with background	
Measurements	Absorbance (cell density) with a spectrometer at 450 nm at (24 h), 48 h and 72 h	Absorbance (cell density) with a spectrometer at 450 nm at (24 h), 48 h
Endpoint	Growth inhibition (absorbance as biomass indicator)	
Validity	Growth rate in the control group at least 0.9 d^{-1}	

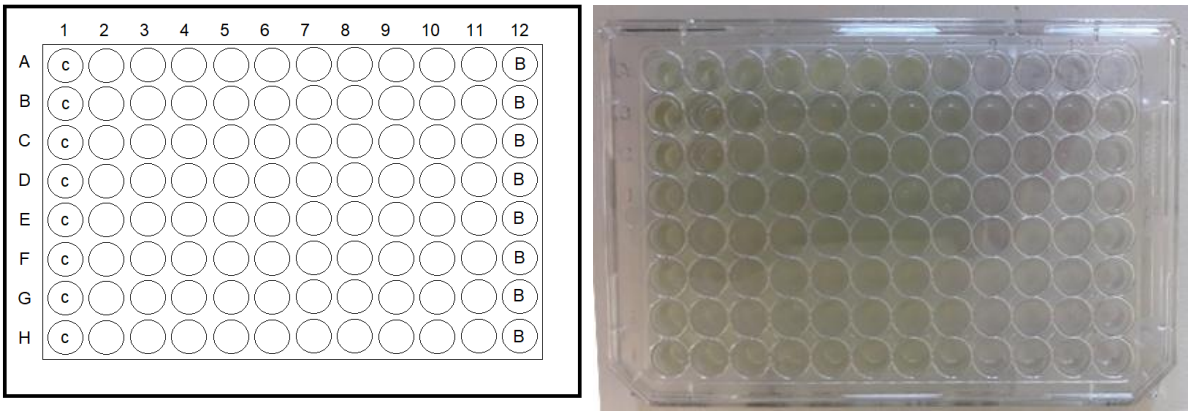

Figure 4: schematic experimental configuration of a 96-well microplate for algal toxicity testing (left) with c: control and B: background and a picture of a 96-well microplate (right)

Because the species *Chlorella sorokiniana* is a thermophilic species a test setting with higher temperature of 31 ± 2 °C was carried out to test the different response of the species under its optimal condition.

CALCULATIONS

For a growth inhibition test it must be assured that the cells are in exponential phase. Therefore, an exponential growth equation Equation 2) was fit to the data of cell numbers at a certain time (Nyholm, 1985).

$$Y = Y_0 \times \exp\left[\mu \times (t - t_0)\right] \qquad \text{Equation 2}$$

where:

Y is the cell number at the end of the exponential phase
Y_0 is the cell number at start of the exponential phase
μ is the maximum specific growth rate
t_0 is the time [d] at start of the exponential phase
t is the time [d] at the end of the exponential phase

Doubling time is a parameter often used in literature, which can be calculated with the received specific growth rate as can be seen in Equation 3. It helps to characterize microalga and compare with other species.

$$t_d = \frac{\ln(2)}{\mu} \times 24$$ *Equation 3*

where:
t_d is the doubling time
μ is the average specific growth rate

After 72 hours of starting the algal toxicity test the change and therefore inhibition of algae growth can be calculated (eq1.). The values can be plotted as percent growth inhibition (y-axis) versus logarithmic test concentration (x-axis). With this graph the EC_{50} values are determined. Other possibilities in measuring growth rate are through the chlorophyll content or fluorescence. Further endpoints used in literature are mortality rate, photosynthetic activity, enzymatic activity (Tato & Beiras, 2019).

To assess the toxicity of a substance the effect on the growth of microalgae can be determined. First average specific growth rate (Equation 4) over a test period is measured by using the concentration of the biomass, which is indirect gained from absorbance due to the assumption that absorbance is directly proportional to cell number. Absorbance was detected with the Termo Multiskan plate reader at 450 nm.

$$\mu_{0-t} = \frac{ln_{B_t} - ln_{B_0}}{t_t - t_0}$$ *Equation 4*

where:

$\mu_{0\text{-}t}$ is the average specific growth rate from start to time t
B_0 is the biomass concentration at the start of the measurements
B_t is the biomass concentration after a period
t_0 is the time [d] at start of the measurements
t_t is the time [d] at the end of the measurements in

Further the percent of growth inhibition is calculated in Equation 5.

$$I_\mu = \frac{\mu_c - \mu_T}{\mu_c} \times 100 \qquad \textit{Equation 5}$$

where:

I_μ is the percent inhibition of the specific growth rate in [%]
μ_0 is the growth rate in the control in [cells/d]
μ_T is the growth rate in the treatment in [cells/d]

DATA ANALYSES

The EC_{50} values were calculated by fitting data to inhibition model (Equation 7) and were performed using GraphPad Prism for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The data is represented with its mean values and a standard error (SEM). The 95% confidence intervals indicate that the probably of true data laying in the interval is 95%. It was used to compare fitted curves on similarity.

The model log(agonist) vs. response in GraphPad (Equation 7) was used in these experiments to gain the best fit of the data. Therefore, EC_{50} values, CI, and the Goodness of the Fit can be calculated. All R^2 values, describing the fit of the data to the model, are above 0.85 The model assumes a standard slope of 1 due to the slope when a ligand binds to a receptor.

$$Y = \frac{Bottom + (Top - Bottom)}{1 + (\frac{X}{EC_F})} \qquad \textit{Equation 6}$$

Top and Bottom referring to the minimum and maximum response of the species and would be set to 100 and 0 on a perfect dose response curve. In this experimental design the maximum of 100 cannot be crossed because no living cells are left at 100 % inhibitory effect. If stimulation takes place the values of the bottom can be negative. In GraphPad the parameter F can be constrained to the effective concentration that is look for. Log(agonist) (X) and response (Y) are the logarithmic concentration and the specific growth inhibition. Hence, the equation can be written as:

$$I_\mu = \frac{Y_{max}}{1 + (\frac{x}{EC_{50}})}$$ *Equation 7*

where:

I_μ	is the percent inhibition of the specific growth rate in [%]
Y_{max}	is the maximum response of the organism
x	is the concentration in [mg/L]
EC_{50}	is the effective concentration that causes 50 % growth inhibition

RESULTS

GROWTH OF MICROALGAE SPECIES

The different species *Chlorella sorokiniana*, *Raphidocelis subcapitata,* and *Dunaliella* sp. were grown at 23±2 °C in a 250 mL Erlenmeyer flask with ISO standard algal medium where the medium for *Dunaliella* sp. was enriched with 3 g/L NaCl. During the experiment, continuous light at an intensity of 7000 lux and shaking at 100 rpm was ensured. In order to identify growth as well as the different growth phases, a sample was taken daily for a period of ten days, and cell numbers were measured with a Multisizer Coulter Counter.

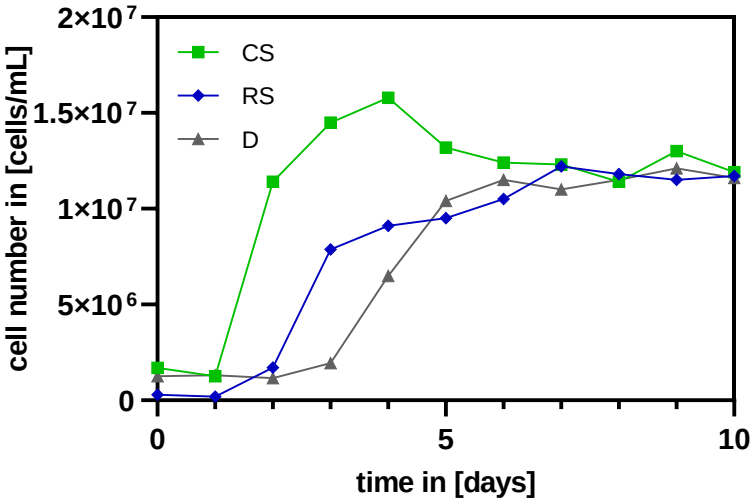

Figure 5: Growth of C. sorokiniana (CS, ■), R. subcapitata (RS, ♦) and Dunaliella sp. (D, ▲) based on the cell number per mL during 10 days after inoculation in freshwater medium (CS, RS) and in freshwater medium enriched with 3 g/L NaCl (D) at t= 0

One day after the inoculation *C. sorokiniana* showed an exponential growth with cell numbers from 1.26×10^6 cells/mL to 1.14×10^7 cells/mL 24 hours later (difference of 1.01×10^7 cells/mL). The data to exponential growth equation gives a maximum growth rate of 2.20 per day (t=1-2) and therefore a doubling time of 7.55 hours. The maximum cell number was reached after four days with 1.58×10^7 cells/mL. *R. subcapitata* increased the cell numbers exponentially from the first day after inoculation (1.71×10^6 cells/mL) to the third day (7.87×10^6 cells/mL) with an amount of 6.16×10^6 cells/mL. The growth rate here was 1.57 $^{d-1}$ (max: 1.86 d^{-1}) and the doubling time 10.62 hours. The maximum cell number detected was

1.22×10^7 cells/mL at t=5. To put the growth of the two species in perspective the growth of another species, *Dunaliella sp.,* was determined. Exponential growth became visible during the third day, where the culture grew 5.33×10^6 cells/mL in two days (t=3-5). In this study, *Dunaliella* reached a maximum specific growth of 0.83 per day and doubled it cells every 13.84 hours. The cell numbers of all three species were stabilized around 1.17×10^7 cells/mL. The growth curves based on the cell numbers are supported with measured absorbance on curtain time (Figure 6).

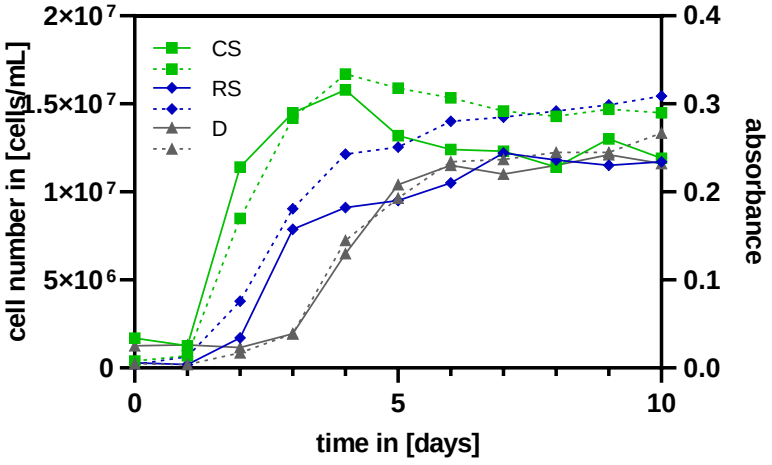

Figure 6: Growth of C. sorokiniana (CS, ■), R. subcapitata (RS, ♦) and Dunaliella sp. (D, ▲) based on the cell number per mL (solid line —) and on absorbance (dashed line ---) during 10 days after inoculation in freshwater medium (CS, RS) and in freshwater medium enriched with 3 g/L NaCl (D) at t= 0

The growth curves based on cell numbers and absorbance are similar as can be seen in Figure 6. This supports the assumption that absorbance is directly proportional to cell number and can be used in experiments to calculate growth rates. Therefore, are following experiments of algal growth inhibition based on the absorbance at 450 nm.

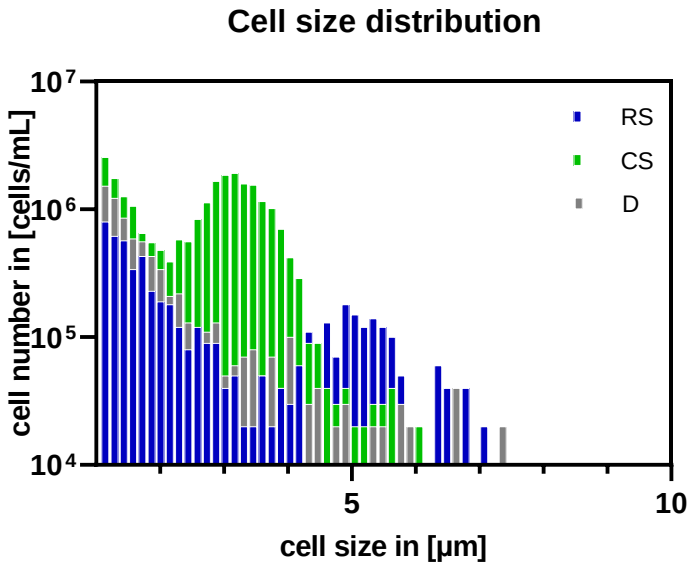

Figure 7: Cell size distribution for R. subcapitata (RS, blue), C. sorokiniana (CS, green) and Dunaliella sp. (D, grey). Data from the day with maximum cell number achieved, For C. sorokiniana after 5 days, R. subcapitata and Dunaliella after 2 days

The cell size distribution shown in Figure 7 was gained by data from the Multisizer Coulter Counter. Two days (RS, D) and five days (CS) after inoculation in new medium the maximum cell numbers per mL were detected. The mean cell sizes to this point of time appeared to be 4.184 µm, 3.215 µm and 3.501 µm for *R. subcapitata, C. sorokiniana* and *Dunaliella* sp., respectively. Dunaliella sp. showed a decrease in numbers of cells with increasing size, which was similar for R. subcapitata instead for and peek around 5 µm. C. sorokiniana appeared with more cells per mL in general and had a peek of cell numbers around 3-4 µm.

Growth rates are among others influenced by the growth media and the therefore contained nutrients for algae. Experiments with different media composition in terms of concentrations of nutrients and with different carbon sources were executed and are shown in fFigure 8 Figure 9.

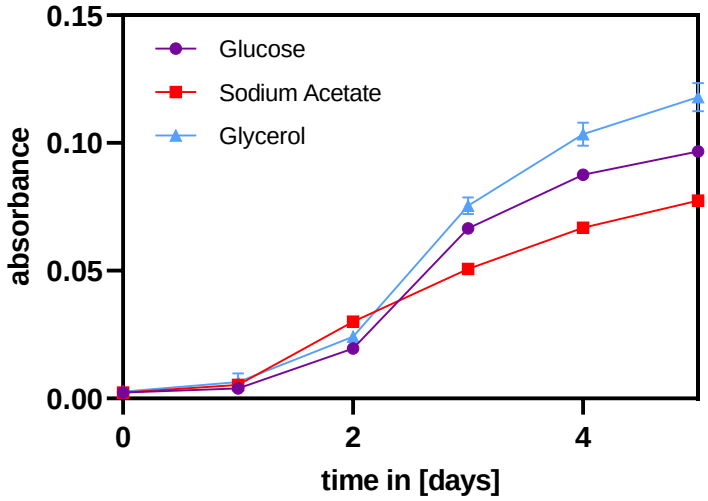

Figure 8: Absorbance at 450 nm for C. sorokiniana inoculated in media with different carbon sources Glucose (●), Sodium acetate (■) and Glycerol (▲), measured in a period of six days, number of replicates: 3, error bars represent SEM

C. sorokiniana showed the highest absorbance and therefore biomass in algal growth media with glucose with 0.118, followed by 0.097 in media with additional sodium acetate and 0.077 with glycerol. The access of CO_2 as a carbon during the experiment is given due to the exchange with air through a cotton plug. The cotton stopper is used to avoid contamination of the culture.

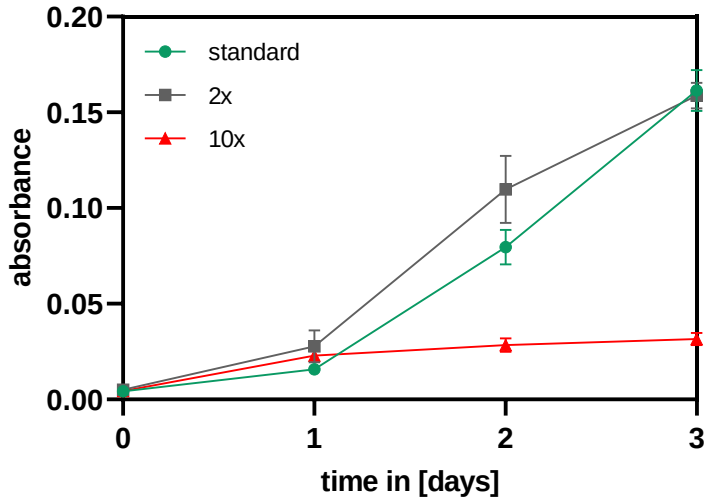

Figure 9: Absorbance of C. sorokiniana inoculated in media with different strength, standard based on the ISO growth media, double ISO (2x) and ten times ISO (10x) media of Macronutrients, Fe-EDTA, Trace elements and $NaHCO_3$ compared to the standard media. Two replicates for the standard and double media and n=4 for the ten times stronger medium, error bars represent SEM.

The growth of *C. sorokiniana* in the double concentration of the standard growth media was stimulated compared to the standard concentration. However, after three days, the values of the experiment with double concentration compared to the standard met the same values of 0.16. The alga in a ten times stronger medium showed growth during the first day but stayed at constant biomass at 0.031 afterward. The concentration of some elements was probably too high and switched from being essential to being inhibitory for the microalga. The results indicate that the alga never reached the exponential phase, which is a requirement to calculate growth rates. The medium with 10x stronger nutrients, therefore, was excluded from the following experiments.

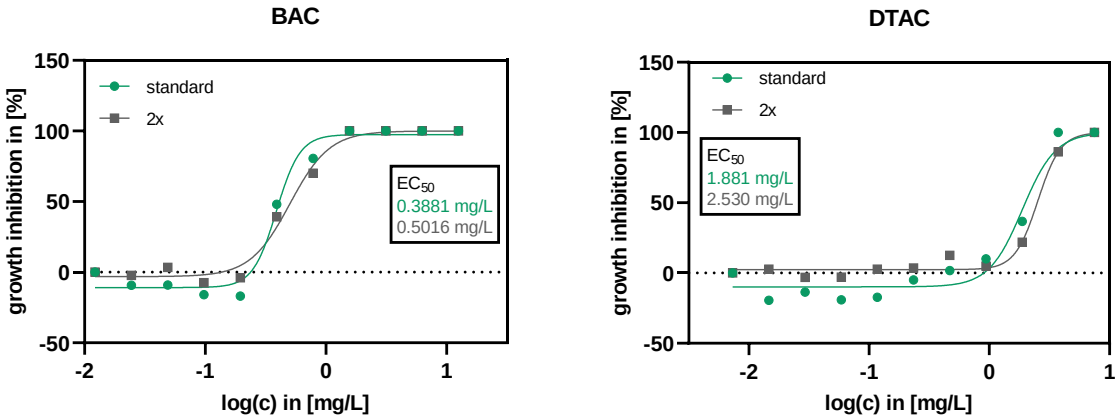

Figure 10: Growth inhibition curve of C. sorokiniana after 48 hours with quaternary ammonium compounds BAC (left) and DTAC (right) in ISO freshwater algae media with different strength (standard, 2x).

By testing the sensitivity of the alga whether it has grown in different strengths of standard media, higher sensitivity to BAC with EC_{50} values of 0.388 mg/L and 0.502 mg/L for standard and double concentrations of ISO freshwater algae growth media, respectively, was detected. Lower toxicity to DTAC for *C. sorokiniana* in growth media with double strength with an EC_{50} of 2.530 mg/L was measured compared to the toxicity test in ISO standard media, which gave an EC_{50} value of 1.881 mg/L.

In the standard of OECD (2011) it is suggested that with a slow-growing algae, the duration of the exposure should be prolonged as long as the validity criteria are adhered to. As *C. sorokiniana* is a fast-growing species, which revealed a

growth rate of 2.20 d^{-1}, which is higher than the standard species R. subcapitata as can be seen in Figure 6, in the following is discussed if a reduction of test time has a negative effect on the sensitivity of the alga.

EXPOSURE OF 72 AND 48 HOURS

To be able to determine whether the test duration can be decreased to 48 hours for *C. sorokiniana* instead of 72 hours algal toxicity tests with multiple substances were executed and the results of both times compared Figure 11 Figure 13).

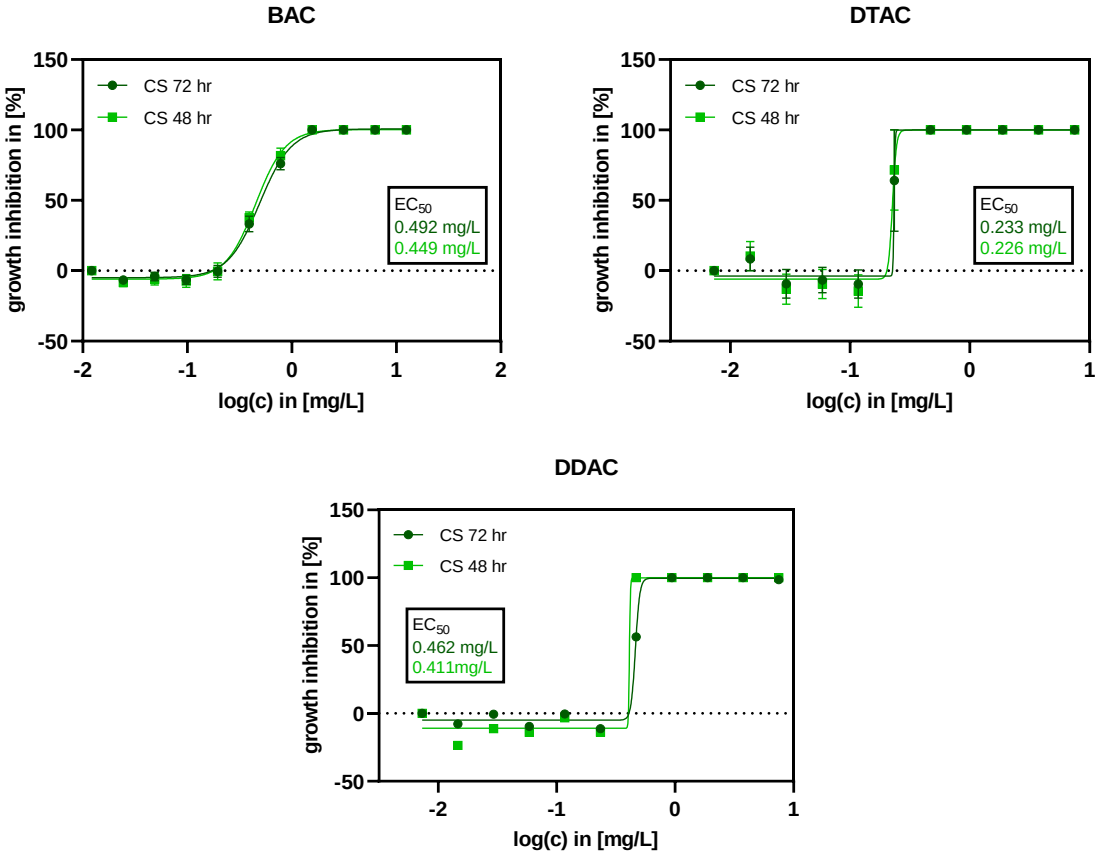

Figure 11: Growth inhibition of Chlorella sorokiniana (CS) after 72 (●) and 48 hours (■) of exposure to quaternary ammonium compounds BAC, DTAC and DDAC. n=4

EC_{50} values are evaluated and were 0.492 mg/L BAC after 72 hours and 0.449 mg/L after 48 hours. A 95 % confident interval was calculated of 0.454-0.533 mg/L and 0.412-0.491 mg/L for a duration of 72 and 48 hours, respectively, which gives an overlapping of 30.45 %. Toxicity tests with DTAC showed EC_{50} of 0.233 mg/L after 72 hours and 0.226 mg/L after 48 hours. With the quaternary ammonium

compound DDAC results of 0.462 mg/L and 0.411 mg/L as EC_{50} were measured after 72 and 48 hours, respectively.

In the following toxicity test with reference substances $K_2Cr_2O_7$, 3,5-DCP, $ZnSO_4$ and $CuSO_4$ after 72 and 48 hours are illustrated in Figure 12.

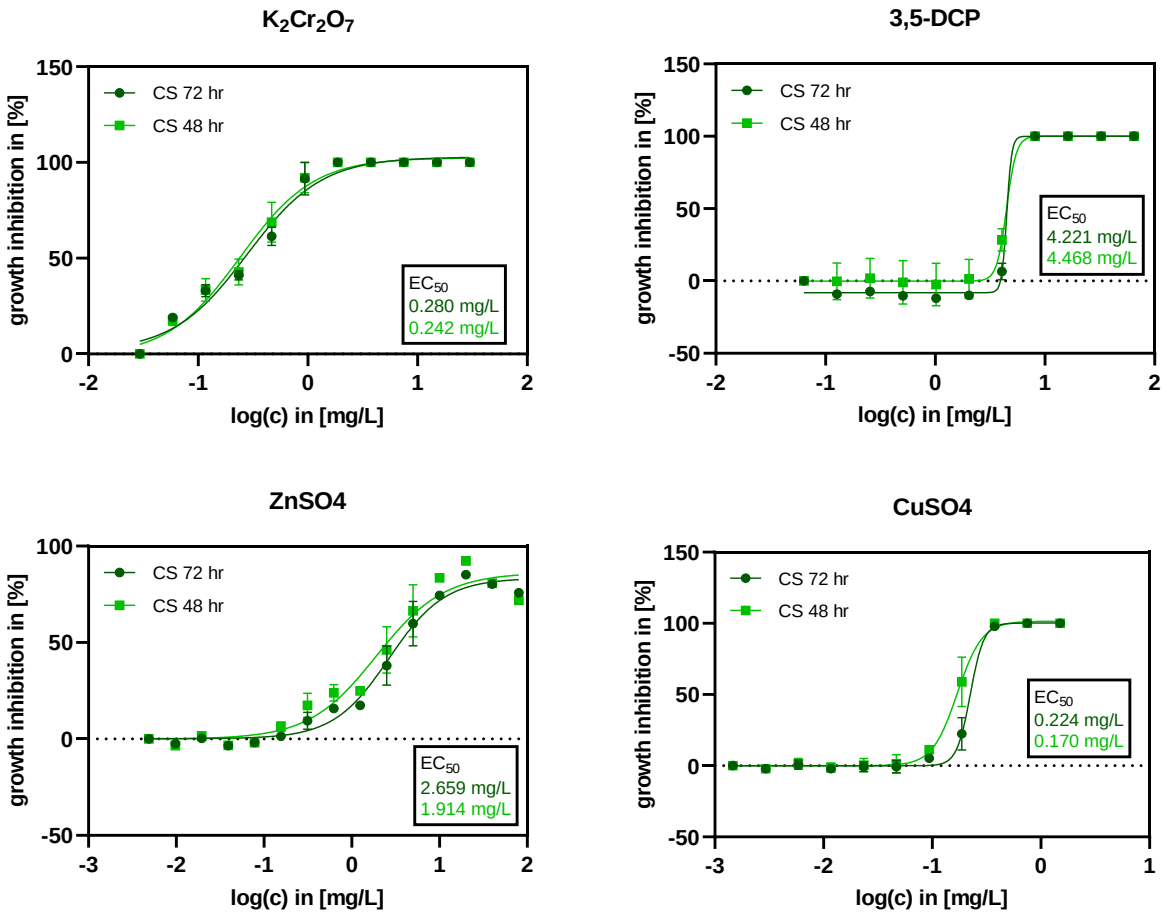

Figure 12: Growth inhibition of Chlorella sorokiniana (CS) exposed to reference substances $K_2Cr_2O_7$, 3,5-DCP, $ZnSO_4$ and $CuSO_4$ after 72 and 48 hours, data represent mean and SEM, n=2

The toxicological effects of $K_2Cr_2O_7$ were calculated EC_{50} values of 0.280 and 0.242 mg/L for *C. sorokiniana* after 72 hours and 48 hours, respectively. The 95 % CI of the EC_{50} for the 72-h test was 0.153 to 0.399 mg/L and for the measurement after 48 hours 0.125 to 0.348 mg/L, therefore showed an overlapping of 71.30 %. An EC_{50} of 4.221 mg/L after 72 hours and of 4.468 mg/L after 48 hours, which was 0.585 % higher than the toxicant 3,5-DCP on *C. sorokiniana* was assessed. Furthermore, an EC_{50} of $ZnSO_4$ was reached at 2.659 mg/L after 72 hours and 1.914 mg/L a day earlier. 51.45 % overlapping of the

EC_{50} values was derived from the 95 % confidence intervals of 1.918 to 3.737 mg/L (72 hr) and 1.091 to 3.279 mg/L (48 hr). The experimental run with $CuSO_4$ gave EC_{50} values of 0.224 mg/L and 0.170 mg/L (0.30 % lower) of *C. sorokiniana* after 72 and 48 hours, respectively.

Glyphosate (Figure 13) had a higher toxic effect on *C. sorokiniana* after 48 hours than 72 hours. With an EC_{50} of 0.239 mg/L (0.206-0.274 mg/L) the concentration needed for an inhibition of half the population was close to double of the concentration needed for after 48 hours (EC_{50}: 0.173 mg/L, 95 % CI: 0.146-0.208 mg/L).

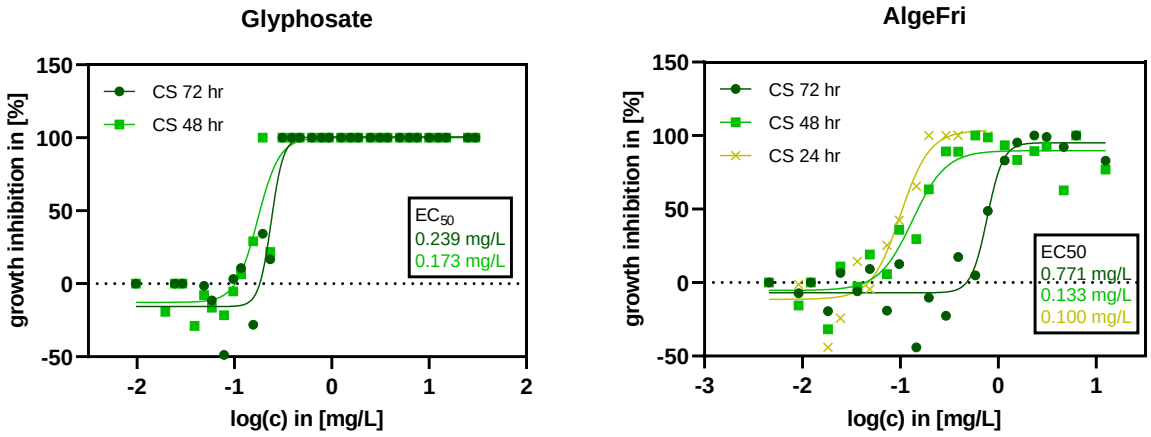

Figure 13: Growth inhibition of Chlorella sorokiniana (CS) exposed to Glyphosate (left) after 72 (●) and 48 (■) hours, data represented in mean, n=3 and the weedkiller AlgeFri with the active compound pelargonic acid (right) after 24, 48 (■) and 72 (●) hours of exposure, data represented in mean, n=3

Algal toxicity tests with weedkiller AlgeFri with the active compound pelargonic acid showed variable results during the measurements (Figure 13). After 72 hours calculated EC_{50} was 0.771 mg/L, after 48 hours 0.133 mg/L and after 24 hours 0.100 mg/L. 95 % confidence intervals of EC_{50} at 72 hours (0.627-0.958 mg/L) and 48 hours (0.087-0.188 mg/L) do not overlap whereas 48 and 24 hours (0.070-0.147 mg/L) overlap with 51.2 %.

The results of toxicity tests with *C. sorokiniana* after 24 hours were not used because the results of Figure 5 propose that the exponential growth was not reached at 24 hours. Except for AlgeFri to illustrate the difference in toxic response during the experiment. However, treatment with QACs, reference substances, Glyphosate, and pelargonic acid resulted in similar or lower EC_{50}

values for *Chlorella* at 48 hours compared to 72 hours. In the following, it was worked with the results of 48 hours in terms of *Chlorella sorokiniana.*

TEMPERATURE CONDITION

The species *C. sorokiniana* is describes as a thermophile species. Therefore, experiments (Figure 14Figure 16) were performed to examine the effect of temperature on the sensitivity of *C. sorokiniana* to toxicants.

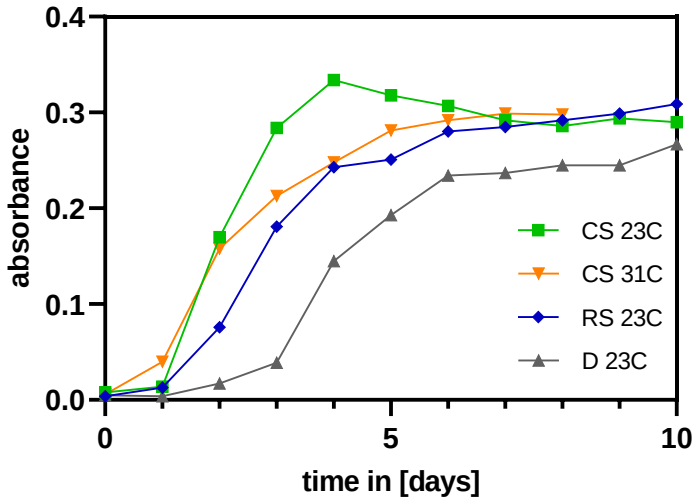

Figure 14: Growth of C. sorokiniana (CS), R. subcapitata (RS) and Dunaliella sp. (D) based on the cell number per mL during 10 days after inoculation at t= 0. Three growth experiments under ISO standard temperature of 23±2 °C with all species (CS, RS, D) and one experiment under 31±2 °C with C. sorokiniana (CS)

The absorbance of Chlorella sorokiniana under high-temperature conditions (31±2 °C) of 0.006 at tithe me of inoculation to 0.040 after 24 hours showed the entry of the exponential phase. A further exponential increase of biomass to 0.191 at 48 hours was given. The maximum growth rate of 1.90 d^{-1} was calculated for C. sorokiniana under 31±2 °C. Compared to the growth under the standard temperature of 23±2 °C high temperature Chlorella revealed an earlier start of the exponential phase from the day of inoculation. However, it did not reach the maximum biomass of Chlorella under standard temperature conditions. The curves of R. subcapitata and high temperature C. sorokiniana appeared similar yet started latter earlier in exponential growth.

The sensitivity of *Chlorella sorokiniana* to selected QACs under the two temperature conditions of 23±2 °C and 31±2 °C was determined with growth inhibition tests (Figure 15).

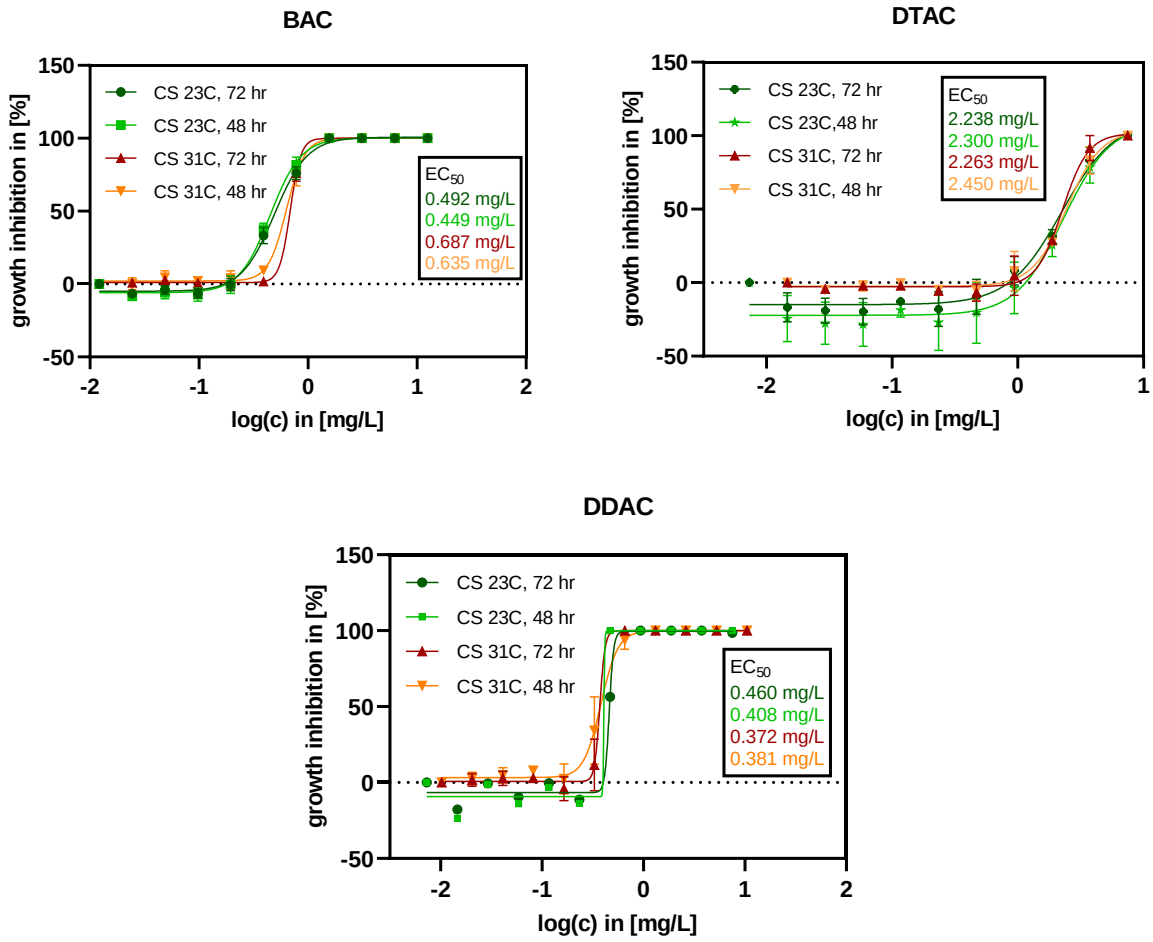

Figure 15: Growth inhibition of Chlorella sorokiniana (CS) carried out under 23±2 °C (green, n=4) and 31±2 °C (red, n=2), exposed to quaternary ammonium compounds BAC, DTAC and DDAC, measured after 72 (●, ■) and 48 hours (▲, ▼).

For BAC the EC_{50} values (72 hr: 0.492 mg/L, 48 hr: 0.449 mg/L) for a toxicity test at 23±2 °C are lower compared to those run at 31±2 °C (72 hr: 0.687 mg/L, 48 hr: 0.635 mg/L). The response of *C. sorokiniana* to DTAC was illustrated with values of EC_{50}. At 23±2 °C they were 2.238 mg/L and 2,300 mg/L after 72 and 48 hours, respectively. Effective concentrations with 50 % inhibitory effects on the population at 31±2 °C after 72 and 48 hours were 2.263 mg/L and 2.459 mg/L, respectively. *C. sorokiniana* showed lower sensitivity to DDAC at 23±2 °C

(EC_{50}=0.460 mg/L, 72 hr and EC_{50}=0.408 mg/L) compared to at 31±2 °C (EC_{50}=0.372 mg/L, 72 hr and 0.381 mg/L, 48 hr).

In Figure 16 toxicity tests with reference substances $K_2Cr_2O_7$, 3,5-DCP, $ZnSO_4$ and $CuSO_4$ at two different experiment setups are illustrated. *C. sorokiniana* was exposed to those toxicants under temperature conditions of 23±2 °C (72, 48 hr) and 31±2 °C (72, 48 hr).

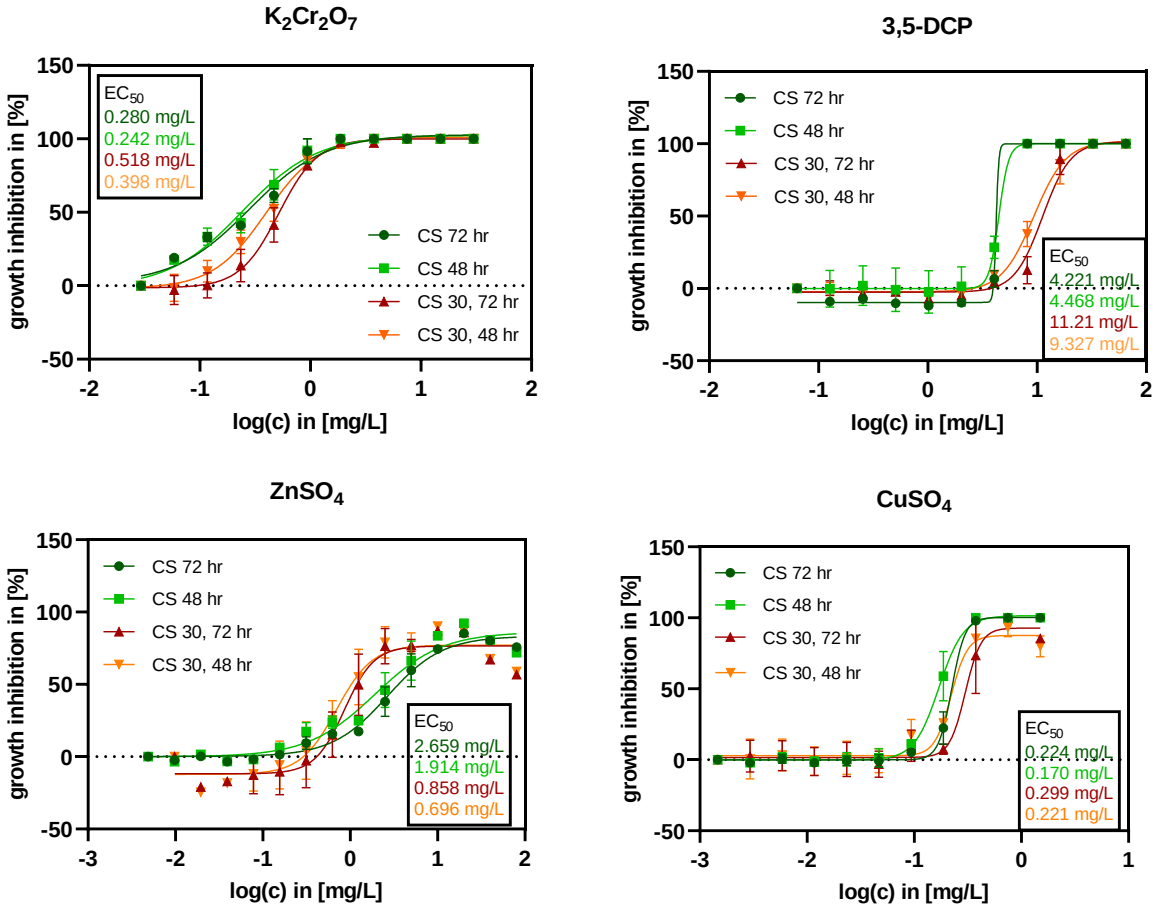

Figure 16: Growth inhibition of Chlorella sorokiniana carried out under 23±2 °C and 31±2 °C, exposed to reference substances $K_2Cr_2O_7$, 3,5-DCP, $ZnSO_4$ and $CuSO_4$, measured after 72 and 48 hours. Data represents mean and SEM, n=2

The growth inhibition values of *C. sorokiniana* at 23±2 °C after 72 and 48 hours as well as at 31±2 °C after 72 and 48 hours under exposure to $K_2Cr_2O_7$ were evaluated with EC_{50}, which are 0.280 mg/L, 0.242 mg/L, 0.518 mg/L, and 0.398 mg/L, respectively. Therefore, showed the test species had higher sensitivity grown under conditions of 23±2 °C. The results of toxicity tests with 3,5-DCP showed EC_{50} values of 4.221 mg/L, 4.468 mg/L at 23±2 °C (72 and 48 hr) and

11.21 mg/L, 9.327 mg/L at 31±2 °C (72 and 48 hr). As seen in Figure 16 the two different conditions present two different pathways of the growth curves based on the toxicity test with $ZnSO_4$. EC_{50} at conditions at 23±2 °C lay at 2.659 mg/L and 1.914 mg/L after 72 and 48 hours, respectively, and at 0.858 mg/L and 0.696 mg/L after 72 and 48 hours, respectively at 31±2 °C. With exposure to $CuSO_4$ EC_{50} is reached at 0.224 mg/L, 0.170 mg/L, 0.299 mg/L, 0.221 mg/L of *C. sorokiniana* after 72 and 48 hours at 23±2 °C as well as 72 and 48 hours at 31±2 °C, respectively.

COMPARISON WITH STANDARD SPECIES R. SUBCAPITATA

To determine if the aim of this study to improve the standard algal toxicity tests can be met, it is to examine how the suggested species respond to toxicants in terms of sensitivity compared to the standard species. Therefore, algal growth inhibition tests were performed with multiple substances for both species as seen in Figure 17 toFigure 19.

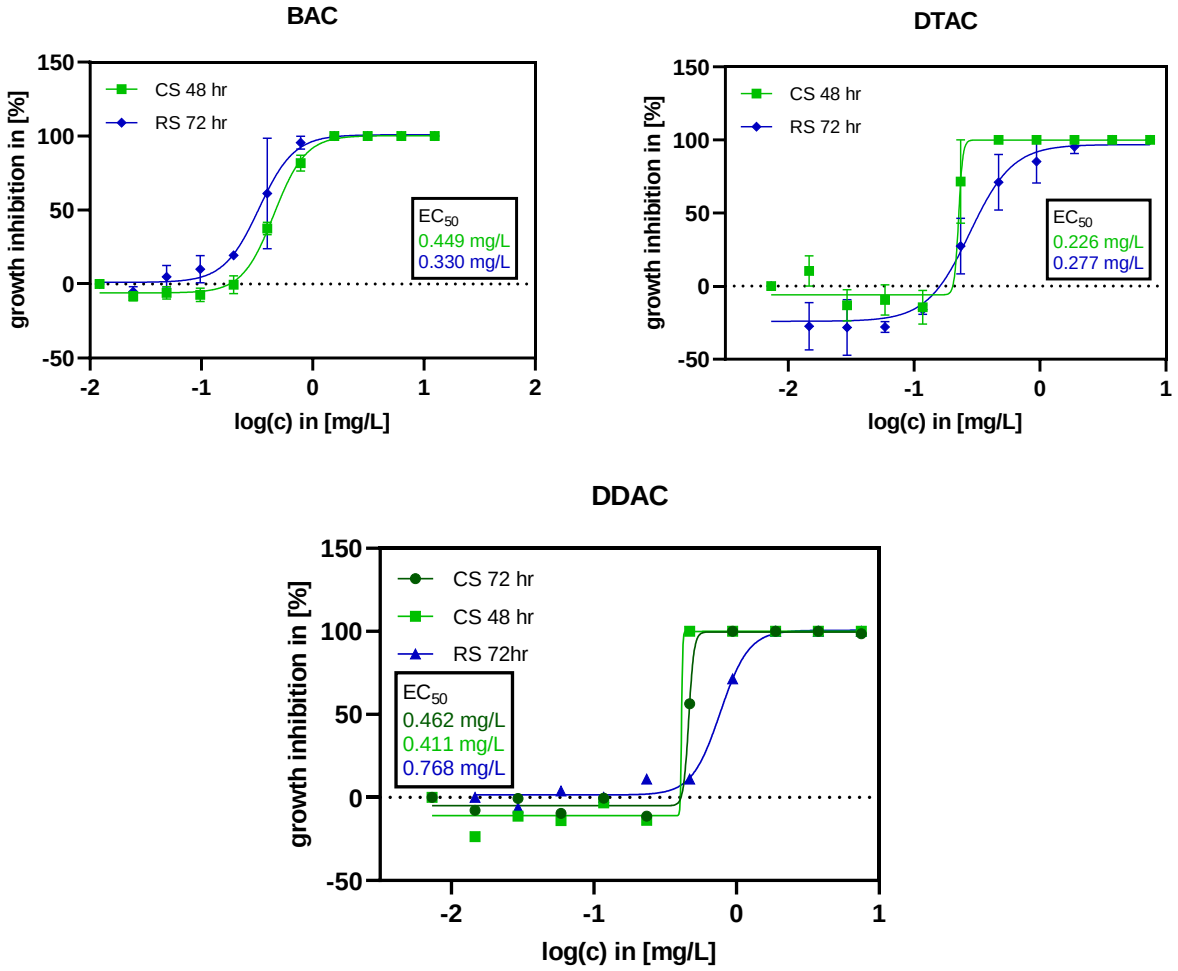

Figure 17: Nonlinear Fit of Growth inhibition of Chlorella sorokiniana (n=4) after 48 hours and R. subcapitata (n=2) after 72 hours of exposure to quaternary ammonium compounds.

EC_{50} value of the algal toxicity test with BAC for *C. sorokiniana* was 0.449 mg/L whereas for *R. subcapitata* it was 0.330 mg/L. Furthermore, were EC_{50} values of 0.226 mg/L for *C. sorokiniana* and 0.2768 mg/L for *R. subcapitata* if exposed to DTAC. At an effective concentration of 0.768 mg/L DDAC was the growth of *R. subcapitata* after 72 hours inhibited of 50 %, whereas for *C. sorokiniana* it appeared to be 0.411 mg/L DDAC.

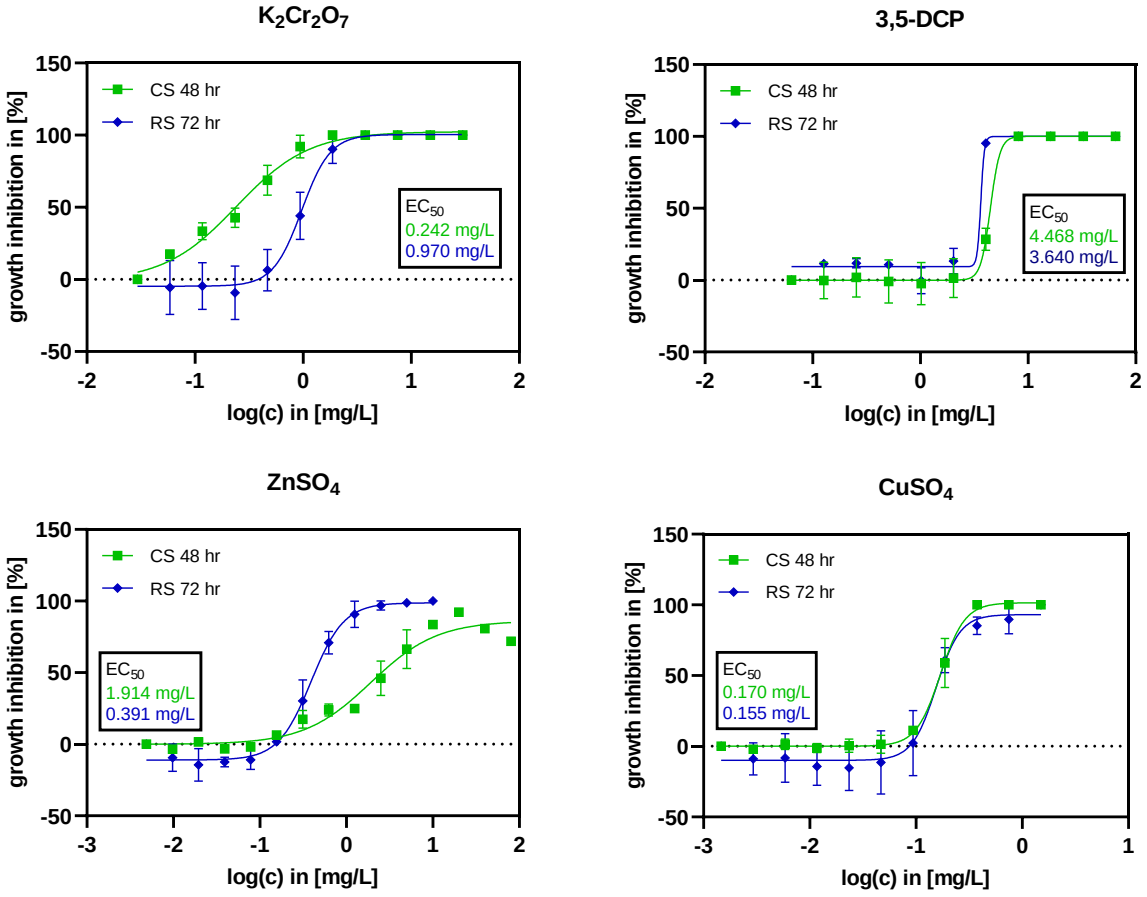

Figure 18: Growth inhibition of Chlorella sorokiniana after 48 hours (CS 48 hr) and R. subcapitata after 72 hours (RS 72 hr) of exposure to reference substances K2Cr2O7, 3,5-DCP, ZnSO4 and CuSO4. Error bars represent SEM, n=2

C. sorokiniana is more sensitive to $K_2Cr_2O_7$ with a EC_{50} of 0.242 mg/L than the standard test species *R. subcapitata* with an EC_{50} of 0.970 mg/L. In terms of 3,5-DCP responded *Raphidocelis* with a higher sensitivity (EC_{50}=3.640 mg/L) compared to *Chlorella* (EC_{50}= 4.468 mg/L). An EC_{50} of 1.914 mg/L $ZnSO_4$ for *C. sorokiniana* and 0.391 mg/L $ZnSO_4$ for *R. subcapitata* was calculated. The inhibition of the growth of *C. sorokiniana* did not reach 100 %. The growth inhibition curve of *C. sorokiniana* and *R. subcapitata* on $CuSO_4$ appears similar. Values of EC_{50} are 0.170 mg/L and 0.155 mg/L for *C. sorokiniana* and *R. subcapitata*, respectively.

Furthermore, algal toxicity tests were run with Glyphosate as active compound in Round Up© and the herbicide AlgeFri with the active compound pelargonic acid and illustrated in Figure 19.

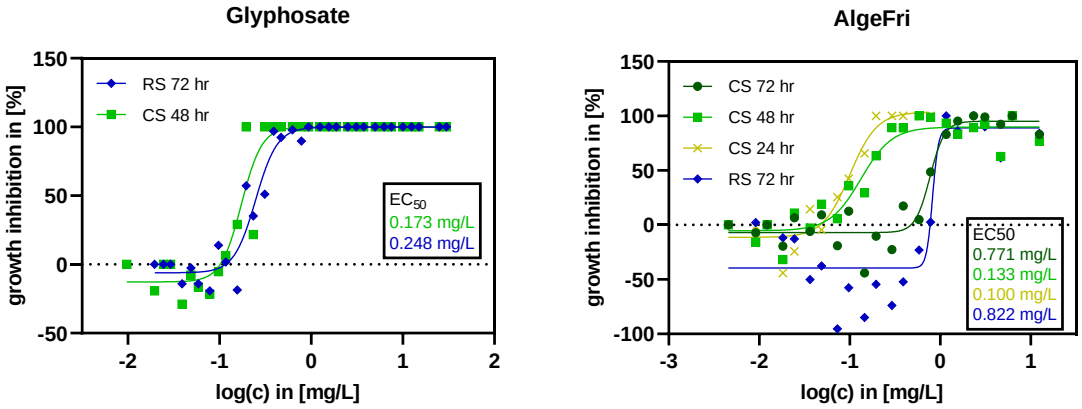

Figure 19: Growth inhibition of Chlorella sorokiniana after 48 hours (CS 48 hr) and R. subcapitata after 72 hours (RS 72 hr) of exposure to Glyphosate (left), data represents mean with number of replicates: 3 and the weedkiller AlgeFri with the active compound pelargonic acid (right) after 24, 48 and 72 hours of exposure, data represents mean with number of replicates: 2

Chlorella sorokiniana showed an adverse effect by exposure to glyphosate with an EC_{50} of 0.173 mg/L as well as *R. subcapitata* with EC_{50}= 0.248 mg/L. The toxic effect of the compound pelargonic acid in AlgeFri can be seen in the growth inhibition curve of *C. sorokiniana* and *R. subcapitata* in Figure 19. The herbicide showed different inhibitory effects throughout the exposure time. The longer the test period the less sensitive became the species *C. sorokiniana* (EC_{50}, time increasing: 0.100, 0.133, 0771 mg/L). This effect of increasing resistance can be seen with the standard species *R. subcapitata* as well. However, *Chlorella* (EC_{50}= 0.133 mg/L) is more sensitive after 48 hours than *Raphidocelis* with a measured EC_{50} of 0.822 mg/L after 72 hours.

DISCUSSION

GROWTH OF MICROALGAE SPECIES

Prior studies stated that *Chlorella sorokiniana* has a higher growth rate compared to other algae species. For example, compared Asadi, et al. (2019) *Chlorella sorokiniana* to *Chlorella vulgaris* and found higher specific growth rate, biomass productivity, and cell double time. The isolator of *C. sorokiniana,* Constantine Sorokin, determined a maximum cell doubling time of *C. sorokiniana* about 11.3 hours at 25 degrees Celsius (Sorokin, 1967). Whereas Lizzul et al (2018) found a doubling time between 4-6 hours at optimal growth conditions (35-40 °C).

In this study, *C. sorokiniana* revealed a maximum growth rate of 2.20 d^{-1} (t=1-2) with a doubling time of 7.55 hours. Therefore, *C. sorokiniana* had a higher growth rate than *R. subcapitata* with 1.86 d^{-1}. Also, Yamagishi (2017) found a specific growth rate of 1.95 per day for *R. subcapitata*. Calculated from data of cell numbers per mL counted by Multisizer Coulter Counter maximum cell doubling time of *R. subcapitata* is 10.89 hr. In similar results came Machado & Soares (2022) found a duplication time of around 10.4 hours. In terms of *Dunaliella* sp. the doubling time was 13.48 hours. In conclusion, the species can be ranked as the fastest-growing *Chlorella sorokiniana* > *Raphidocelis Subcapitata* > *Dunaliella sp*..

It is to mention that *Dunaliella* was not grown under its theoretical optimum conditions. The species can tolerate temperatures between 0 °C and 45 °C with its optimum around 32 °C (Khatoon, et al., 2020). Anyways, Khatoon, et al. (2020) found the highest specific growth rate at 0.53 day^{-1} and a maximum cell number of 6.95×10^6 cells/mL, which was exceeded in the present study with a maximum cell number of 1.17×10^7 cells/mL and a maximum growth rate of 0.83 d^{-1}.

Moreover, *C. sorokiniana* entered the exponential phase of growth one day after inoculation as well as *R. Subcapitata. Dunaliella* sp. started detectable exponential growth on day three. That led to the assumption that the standard and the new species *C. sorokiniana* can be used for the algal growth test 24 hours after inoculation. Additionally, it is to note that *Chlorella sorokiniana* reached a higher maximum cell number per mL compared to the other two species. That

supports the growing interest in the species in the field of biofuel and biomass production due to its high growth rate and cell production (Gim, et al., 2013)
Figure 7
The mean cell size of *R. subcapitata* on day two, where the maximum cell numbers occurred, was 4.184 µm. That is supported by the study of Yamagishi (2017), where this species grew to an average cell diameter of 5 µm. Cells of *R. subcapitata* grow to a bigger mother cell to divide by multiple fission into four daughter cells (sometimes two or eight daughter cells) (Yamagishi, et al., 2017). Normally cell division is followed by a slight decrease in mean cell diameter, which did not appear in the present experiment and instead followed by stabilization in size.
To assess the optimal growth conditions experiments with media enriched by different carbon sources were exhibited. Therefore, three freshwater media were prepared with either 10 g/L Glucose, 2 g/L sodium acetate, or 0.15 M glycerol. As other papers priorly stated *C. sorokiniana* grows better in media enriched with glucose, sodium acetate, or glycerol (Lizzul, et al., 2018). In this paper, a preference for glycerol is seen followed by glucose and sodium acetate (Figure 8). Gim, et al. (2013) found the highest increase in biomass for *Chlorella sp*. to be with glucose -enriched media with the most effective concentration of 0.2 % (Gim, et al., 2013). In Gims research glucose is ranked as the best carbon source for growth, but the researchers did not investigate the effect of sodium acetate or glycerol.
In IllustrationFigure 8 of this study, the early exponential phase was visible and changed to exponential growth on day two for all three carbon sources. Further research determined the effect of glucose and NaAc among others on the growth of *C. Sorokiniana*. Here it was found that with glucose followed by sodium acetate as a carbon source, the species grew more rapidly than with other carbon sources, which supports the data of this study. As glucose has sodium acetate a simple structure that makes it easy to be assimilated in the microalga. Therefore, they are able to give energy to the alga more easily (Hongjin & Guangce, 2009).
Chlorella sorokiniana displayed the highest absorbance and therefore biomass in growth media with added glucose with 0.118 (sodium acetate: 0.097, glycerol: 0.07) whereas *R. subcapitata* grew best in media with added glycerol (absorbance

0.08) (Appendix 2). The experiment with the heterotrophic growth of *C. sorokiniana*, illustrated in Appendix 3, showed the highest absorbance of 0.10 sodium acetate. Also, (Abiusi, et al., 2020) published results that indicate sodium acetate was a better carbon source than glucose in terms of biomass production. *Chlorella* can grow mixotrophic where light is the main source of energy, but organic and inorganic carbon is assimilated at the same time (Gim, et al., 2013). Abiusi, et al. (2020) concluded in their study that mixotrophic algal growth had better results in biomass productivity due to two energy sources available than under heterotrophic conditions. This statement is supported by evaluated data of the present study, where higher growth rates of *C. sorokiniana* were seen with added glucose than grown under heterotrophic conditions. Furthermore, the *Chlorella* grew faster than *R. subcapitata* with every added carbon source.

It emphasizes that-, if glucose or glycerol is added to an algal medium the risk of bacterial contamination is given.

Additionally, it is worth noting that the values of the absorbance appear lower than expected. A possibility is there were not enough generations achieved before starting the experiment. The alga arrived on an ESP-agar and was transferred into a freshwater algal medium. The alga has to adapt to the new environment and conditions first.

Nutrients are one limiting factor of algal growth, hence why an experiment was conceptualized to examine the effect of higher concentrations of nutrients in a standard growth medium. Thus, two growth media prepared, based on the ISO standard medium, but with double- and ten-times increased Macronutrients, FeEDTA, $NaHCO_3$, and trace metals prepared (see page 11). Stimulated growth of *C. sorokiniana* was seen in media with double the concentration. The alga in a ten times stronger medium showed similar growth during the first day as the other experimental setups but stayed at constant biomass afterward. The concentration of some elements was probably too high and switched from being essential to being toxic for the alga.

To answer the question with the medium of the above the algae is more sensitive to further toxicity tests. To both chemicals, BAC and DTAC, *Chlorella* was more sensitive in medium with standard concentration, than in medium with double strength. Therefore, the alga is more sensitive at its non-optimum conditions.

Abiotic factors, including pH, light, temperature, and nutrients can show an effect on the response to toxicants by microorganisms. For instance, can high concentrations of Ca and Mg lower the toxicity of metals, or the iron concentration affect Copper toxicity. Also, can the nutrient status have an effect on algal sensitivity to toxic substances. Their phosphor, nitrogen, and carbon metabolism play an important role. Nalewajko & Olaveson (1998) names the example of *Anabaena variabilis* which is able to grow under higher Cu concentration when loaded with phosphorus but not under limited phosphate conditions. A combination of stressors can be additive, synergistic or antagonistic and is unique for every species.

EXPOSURE 72 AND 48 HOURS

As the results showed above exponential phase of *Chlorella sorokiniana* was initiated after 24 hours, therefore the question was raised if the results of algal toxicity tests can be measured after 48 hours and are those reliable.

To all testes quaternary ammonium compounds (BAC, DTAC, DDAC) *C. sorokinia*na was more sensitive after 48 than 72 hours (Figure 11). Additionally, resulted in the growth inhibition tests with reference compounds in higher sensitivity after 48 hours compared to 72 hours except for 3,5-DCP, where the EC_{50} appeared slightly higher after 48 hours. For the active compounds in herbicides, glyphosate, and pelargonic acid, a similar case as with the majority of compounds, higher sensitivity after 48 hours, occurred.

It was noticed that the response of algae species varied after every measurement of one experiment. The active compound pelargonic acid is described to act rapidly. It damaged plants within 15-60 minutes and but re-growing cannot be prevented (Savage & Zorner, 1996). This may explain the lower toxic effect after longer exposure. The compound is highly active in the first hours but then loses its inhibitory effect and cells can grow unhindered.

Decrease with length of alkyl chain (Zhang, et al., 2015). BAC very long chain..

However, treatment with QACs, reference substances, Glyphosate, and pelargonic acid resulted in lower or similar EC_{50} values for *C. sorokiniana* at 48 hours compared to 72 hours. Also, Liu, et al. (2022) found apparent results after 48 hours by analyzing the toxic effects of flame retardances on *C. sorokiniana*. Therefore, can the use of results after 48 hours exposure to toxicants instead of 72 hours by applying test species *C. sorokiniana* be recommended.

TEMPERATURE CONDITION

Due to the circumstance that temperature plays an important role in the growth of microalgae, it should always be considered but can also be used as a tool to optimize microalgae growth (Khatoon, et al., 2020). The optimum temperature range for *C. sorokiniana* is 38 to 39 °C (Sorokin, 1967). In this study, it was investigated if the optimum temperature has an impact on the sensitivity of the species to toxicants. First growth curves without toxicants were determined to assess the growth under optimum conditions. Hence, a maximum growth rate of 1.897 d^{-1} was calculated for *C. sorokiniana* under 31±2 °C, which is lower than under 23±2 °C (2.20 d^{-1}). Nonetheless, the high-temperature version of *Chlorella* appeared with an earlier exponential growth phase under standard temperature. Further, algal inhibition growth tests were performed with the three QACS and four reference substances at two temperature conditions of 23±2 °C and 31±2 °C (Figure 15). Sometimes growing algae under optimum conditions can result in lower sensitivity to toxicants because there are not multiple stressors present that interact and weaken alga. The lack of these may follow in higher resistance compared to the condition that is in multiple parameters not optimal. This is indicated by present data of toxicity tests with different substances at 23±2 °C and 31±2 °C. *C. sorokiniana* has grown under less favoured conditions of 23±2 °C shows higher sensitivity to Quaternary Ammonium compound BAC and similar curves were obtained with DTAC and DDAC. Test runs with the reference toxicants $K_2Cr_2O_7$, 3,5-DCP, and $CuSO_4$ gave lower EC_{50} values for conditions under lower temperatures, whereas for $ZnSO_4$ the algae showed higher resistance. In general, the new test species *Chlorella* indicated a higher sensitivity grown under unfavourable conditions of 23 degrees Celsius.

Considering that *Chlorella* species are used as indicator species for zinc (Vander Wiel, et al., 2017), the toxicity test for zinc in this study with *C. sorokiniana* at 23±2 °C seems unrealistic. The toxic action of a metal is influenced by different water parameters. Organic matter concentration, hardness of water, and pH, among others, have an impact on metal bioavailability. Bioavailability is used to describe the rate and extent at which a toxicant (metal) accesses the site of action (Adams, et al., 2019). Researchers found with increasing pH value a lower concentration of toxicant is needed to reach EC_{50} which means the alga becomes more sensitive (Franklin, et al., 1998). The water used for dilution, and algal media growth used for the test could have changed the water parameters and therefore the way $ZnSO_4$ affected the algal cells. Nonetheless, the test procedure does not give the impression this reason is accurate, because all three experiments (CS 23C, CS 31C, RS 23C) were executed on the same day using the same material with a month difference between the replicates.

Even though multiple studies suggest an optimum temperature over 30 °C for *C. sorokiniana* and characterized it as a high-temperature strain, the study by Asadi, et al. (2019) disagreed. They compared the growth of algae species under different temperatures (25, 28, and 32 °C) and found that at 28 °C the maximum specific growth rate and biomass productivity can be reached for *C. Sorokiniana*. That indicates the optimum temperature for growth of *C. Sorokiniana* is 28 °C. The decline of growth rate after 28 °C in the study of Asadi, et al., (2019) may indicate why the growth rate in the present study is lower under high temperatures than standard temperature.

COMPARISON WITH STANDARD SPECIES R. SUBCAPITATA

Microalgae cells show a wide range of sizes and shapes. In literature, it is often stated that smaller cells should have higher sensitivity due to the higher cell surface. Levy, et al. (2007) found no clear evidence in their study to approve that statement. In their study, it appeared that there is no significant relationship between sensitivity to copper and cell surface area of different algae species. Furthermore, contrary to expectation the cell wall type does not influence sensitivity to copper. Coccolith-producing strains and species with cell walls were found to be less tolerant than coccolith-absent and naked species. Levy, et al.

(2007) suggests the uptake rates for algal species influence metal-sensitivity (Levy, et al., 2007). On the other side, some literature suggested that morphology correlates with sensitivity. The most sensitive are those with smaller diameters and microalgae with bigger cell sizes show higher tolerance. The correlation between cell size and the adverse effects of metals is seen in the study of Chiellini, et al. (2020) which examined the response of *Chlorella* species. In the present study, *R. subcapitata* is recognized with larger cells compared to. *C. sorokiniana.* The findings of Chiellini, et al. (2020) of a correlation of cell size and sensitivity was supported by examined data of this study. *C. sorokiniana* with smaller cells appeared overall as more sensitive than the bigger R. Subcapitata (Table 4).

In the paper of Zhang, et al. (2015) the toxic effect of QACs on C. vulgaris was examined. The EC_{50} values of BAC ranged from 0.11-0.203 mg/L which do not coincide with the EC_{50} values of *C. sorokiniana* and *R. subcapitata*. This can be explained because in the paper BACs with long alkyl chains were investigated and with an increase in length the EC_{50} values decrease (Zhang, et al., 2015) e. The present study, therefore, was due to the use of BAC with short alkyl chains, EC50 values higher than in compared paper. The trend of increasing sensitivity (lower EC_{50}) with longer alkyl chains of the QACs specie was demonstrated, in order: BAC (C8-18)> DDAC (C10) > DTAC(C12). Furthermore, the EC_{50} values calculated by this paper were 0.188 mg/L DTAC for *C. vulgaris* which are comparable with 0.266 mg/L on C. sorokiniana when considering that Zhang, et al. (2015) worked with 96-hour tests.

For visualisation EC_{50} and EC_{20} values were, for better visualisation, plotted in Figure 20 with values of the alga species *C. sorokiniana* against *R. subcapitata*.

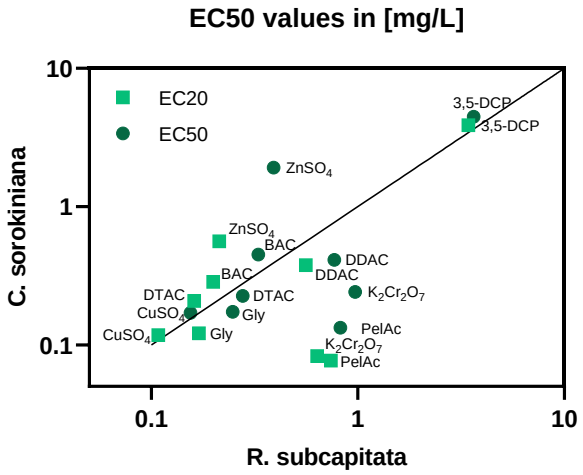

Figure 20: Values of EC_{50} of each tested substance on Chlorella sorokiniana after 48 hours (CS 48 hr) against R. subcapitata after 72 hours (RS 72), dashed line represents the zoomed in area to the right

The EC_{50} of $K_2Cr_2O_7$ estimated by Kusk, et al. (2018) based on the species *R. subcapitata* was 0.60 mg/L, whereas it was 1.19 mg/L in the ISO standard of 1997 what Kusk explains with their use of lower pH values. The evaluated EC_{50} value with 0.970 mg/L in the present study supports more the ISO standard which was worked with. Furthermore, Prince (2023) found the EC_{50} of Zinc on *Chlorella* sp. to be 112 microg Zn/L. Based on this the EC_{50} of $ZnSO_4 \times 7\ H_2O$, assessed in this study, is 0.493 mg/L. This value fits around the determined value of 0.391 mg/L of *R. subcapitata*. The inhibitory effect of Glyphosate in literature was found to be 7.8 mg/L with a lower 95 % CI border of 3.0 mg/L (Blaise & Vasseur, 2005). Both species *C. sorokiniana* and *R. subcapitata* in this study appeared with higher sensitivity than in the paper of Blaise & Vasseur (2005).

The species *Dunaliella* is more resistant to all test toxicants compared to *C. sorokiniana* and *R. Subcapitata*.

Table 4: determined EC_{50} and EC_{20} values of Raphidocelis subcapitata after 72 hours (RS 72 hr) and Chlorella sorokiniana after 48 hours (CS 48 hr) of microplate algal toxicity tests

	EC_{50} [mg/L]		**EC_{20} [mg/L]**	
Substance	**RS 72 hr**	**CS 48 hr**	**RS 72 hr**	**CS 48 hr**
BAC	0.330	0.449	0.199	0.286
DTAC	0.277	0.226	0.161	0.208

DDAC	0.768	0.411	0.559	0.377
$K_2Cr_2O_7$	0.970	0.242	0.636	0.083
3,5-DCP	3.640	4.468	3.434	3.873
$ZnSO_4$	0.391	1.914	0.213	0.562
$CuSO_4$	0.155	0.170	0.108	0.118
Glyphosate	0.248	0.173	0.170	0.121
pelargonic acid	0.822	0.133	0.740	0.077

Overall, responded *C. Sorokiniana* with higher sensitivity than the species *R. subcapitata* used in ISO standard toxicity test. The sensitivity to examined toxicants in order of declining toxicity: Pelargonic acid> CuSO4> Glyphosate> DTAC> K2CR2O7> DDAC> BAC> ZnSO4> 3,5-DTAC This and the fact that the execution time of the toxicity test can be reduced to 48 hours instead of 72 hours allows *C. sorokiniana* to be recommended as a new species for algal inhibition growth tests.

CONCLUSION

Anthropogenic activity is polluting ecosystems and especially freshwater should be under higher protection due to its important use as drinking water and the risk of disrupting the ecosystem`s balance. Toxicants discharged in freshwater can cause harm to aquatic microorganisms. Those microorganisms, in particular, microalgae build the base of the food chain as being the dominant primary producers but also secure water purification and the function of the freshwater ecosystem (Liu, et al., 2022). With the use of algal toxicity tests chemicals can be classified regarding their hazardous potential and elaborate water quality criteria. Further, they find its application in biomonitoring and assessing effluents in order to regulatory statutes to detect possible hazardous substances and lead to possible steps to intervene. This study focused on improving the standard microplate algal toxicity test based on new freshwater microalga. Therefore, the species *Chlorella sorokiniana* was investigated. Due to its ability to be fast-growing *C. sorokiniana* resulted in lower or similar EC_{50} values after 48 hours compared to 72 hours. Furthermore, the study revealed a higher sensitivity of the

Chlorella species to the majority of testes compounds than the microalga *R. subcapitata*, species used for ISO standard toxicity test. It can be concluded that *Chlorella sorokiniana* can be recommended as new test species for microplate algal toxicity tests.

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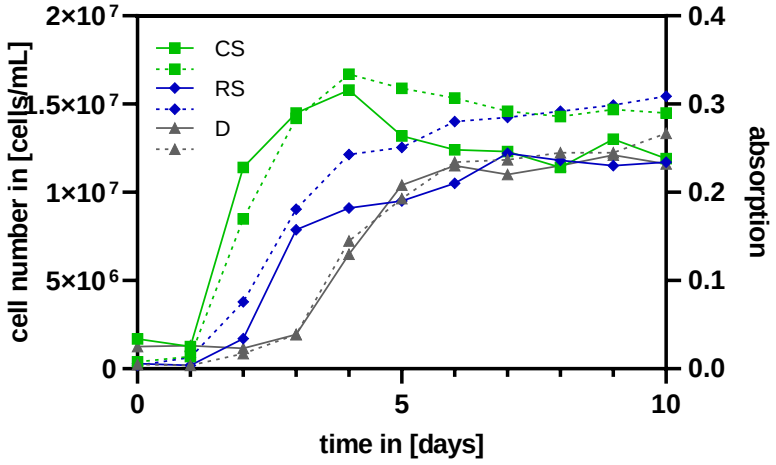
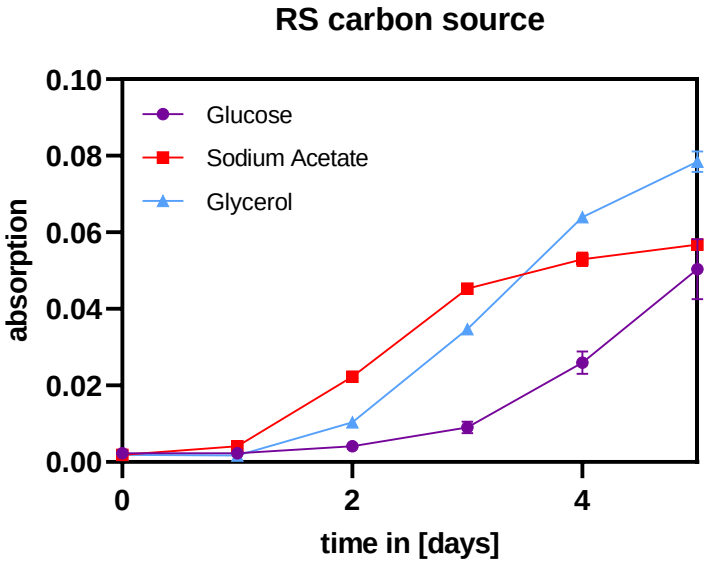
APPENDIX

Appendix 1: Absorbance of Chlorella sorokiniana (CS), Raphidocelis subcapitata (RS) and Dunaliella sp. (D) measured daily in a period of ten days with the inoculation at t=0.



Appendix 2: Absorbance of R. subcapitata inoculated in media with different carbon sources Glucose (●), Sodium acetate (■) and Glycerol (▲), measured in a period of six days, number of replicates: 3, error bars represent SEM



Appendix 3: Absorbance of C. sorokiniana under heterotrophic conditions inoculated in media with different carbon sources Glucose (●), Sodium acetate (■) and Glycerol (▲), measured in a period of six days, number of replicates: 3, error bars represent SEM