

AMERICAN UNIVERSITY OF BEIRUT

Effect of Algal-Algal Interactions in Toxicity
Testing Bioassays

By

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AN ABSTRACT OF THE THESIS OF

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This study examines single and multispecies algal bioassays using copper toxicity. Three green (*S. subspicatus*, *S. quadricauda* and *A. angustus*) and one blue-green algae species (*O. prolifera*) were selected based on their morphology and availability. Single and multispecies toxicity bioassays were conducted based on cell density as per the standard toxicity testing, and on equivalent surface area to avoid the confounding effect on toxicity of metal binding sites. Cells were counted and differentiated using investigative microscopic imaging. Toxicity tests of single species based on cell density showed a higher copper sulfate toxicity on the blue-green algae *O. prolifera* (96h-EC₅₀=126 µg/L), followed by *S. subspicatus* (96h-EC₅₀=198 µg/L), *S. quadricauda* (96h-EC₅₀=359 µg/L), and *A. angustus* (96h-EC₅₀=1809 µg/L), while toxicity tests based on surface area showed increased copper toxicity with increasing algal surface area. Algae cell surface area of 47, 108, 742, 2348 µm² resulted in respective 96h-EC₅₀ values of 1123, 692, 190, and 126 µg/L as CuSO₄. In multispecies control bioassays, the growth of *A. angustus* was significantly (*p*-value<0.05) inhibited in the presence of *S. subspicatus*, *S. quadricauda* and *O. prolifera* in both cell density and surface area based tests. This was in part associated to nutrients limitation, including CO₂, as demonstrated by an increase in the culture pH. Toxic exudates released by *O. prolifera* could have also contributed to the growth inhibition of *A. angustus*. As compared to single species bioassays, *O. prolifera*, and *S. quadricauda* showed a decreased sensitivity to copper sulfate in both cell density and surface area based multispecies tests. However, for the algae specie with the smallest surface area, *S. subspicatus*, different toxicity trends were observed whereby the algae EC₅₀ value decreased from 1123 µg/L in single species to 461.6 µg/L in multispecies bioassays based on surface area, and increased from 198 µg/L in single species bioassays to 321 µg/L in the multispecies bioassays based on cell density. This difference in copper toxicity to the smallest specie could be explained by the shielding effect of larger cells in cell density tests that reduced copper toxicity to *S. subspicatus*. The results from this study demonstrate that single-species bioassays may over- or underestimate metal toxicity in natural waters and support the need to adopt multispecies toxicity testing based on surface area to avoid the confounding effect on copper toxicity of increased biomass for metal binding.

Keywords: Algae, Copper, Interaction, Surface area, Cell density, Multispecies

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Water resources across the globe are threatened by pollution due to discharge of wastewater into surface areas, chemical contamination from treated sewage, agriculture runoffs, and urban development. Additionally, the increase in human activities and technological advances in the agriculture field are primary causes to the progressive degradation of aquatic ecosystems (Turner et al., 2003; Jancula & Maršálek, 2011; Aguiar et al., 2014; Çelekli et al., 2016; Koff et al., 2016; Rodríguez-Gallego et al., 2017). The anthropogenic excess of nutrients, mainly nitrate and phosphorous in rivers and lakes, alters algae growth and enhance organic production. Such phenomenon is known as eutrophication whereby rapid propagation of algal cells lead to an increase in water pollution and in the rate of climate change (Smith et al., 1999; Wang et al., 2013; Gilbert, 2017; Chai et al., 2017; Tang et al., 2017). In this context, eutrophication is majorly affected by the urbanization-induced increase in nutrients in lakes and reservoirs and the excessive use of fertilizers and industrial activities (Wang et al., 2011; Aguiar et al., 2014; Ge et al., 2016; Gao et al., 2017). Also, this phenomenon amplifies the occurrence of harmful algal blooms, enriches the biogeochemical cycling of organic and inorganic contaminants and decreases light penetration, leading to key ecological stresses and fish kills (Smith & Schindler, 2009; Soerensen et al., 2016). Generally, aquatic ecosystems, located in agricultural catchment areas, tend to be more eutrophic than those in natural landscapes as a result of excessive application of fertilizers (Rodríguez-Gallego et al., 2017).

In the event of algae accumulation, scum can form on the surface (Figueiredo et al., 2004; Garlich et al., 2016). Algal blooms can also be harmful as some species produce toxic metabolites that pose a risk to human health, the environment and wildlife (Hardy et al., 2016). In this context, algae growth advances the deterioration of water quality, increases the cost of treatment, compromises the

public water supply, and delays its use for restoration (Garlich et al., 2016; Hardy et al., 2016). Furthermore, humans are exposed to harmful algal blooms through drinking water, recreation activities, aerosols and consumption of fish and shellfish (Hardy et al., 2016). Thus, the control of algal blooms constitutes a major concern in water management (Le Jeune et al., 2006).

Several measures can be taken to efficiently utilize water resources and manage the aquatic environment. Algae blooms prevention methods include water coagulation, i.e a water treatment process that involves the addition and rapid mixing of a coagulant to remove algae, reducing nutrient input into water systems to prevent eutrophication, altering the hydrophysical conditions to favor other phytoplankton over the cyanobacteria, use of barley straw and destratifying the water body by aeration or mechanical mixers (Barrington & Ghadouani, 2008; Qian et al., 2010; Lim et al, 2014; Tang et al., 2017). However, these methods were deemed unsuccessful due to socio-economic stressors (Barrington & Ghadouani, 2008). The use of chemical reagents offers an alternative for algae bloom control by inhibiting and/or eventually killing the algae. This proposed control mechanism is less expensive and gives faster results, sufficient efficiency, and produces low toxicity for non-target organisms (Watson & Yanong, 2006; Zhang et al., 2012; Garlich et al., 2016). One of the most commonly used algaecides is copper. For many years, copper was used as a reference toxicant to control algae in ponds, specifically blue-green algae, and to reduce the abundance of algae that causes off-flavor in catfish (Haughey et al. 2000).

Copper is an essential micronutrient for algae metabolism at very low levels. It functions as an enzyme co-factor and electron transport in the photosynthetic process (Manahan and Smith, 1973; Illinois state water survey, 1989; Haughey et al., 2000; Andrade et al., 2004; Watson & Yanong, 2006; Garlich et al., 2016). Lack of copper interferes with photosynthesis, respiration, protein synthesis, and decreases algal resistance and reproduction (Bossuyt & Janssen, 2004) . However, at high

concentrations, copper becomes toxic to algae and may be potentially detrimental to recipient aquatic ecosystems, with the cupric ion (Cu^{2+}) being the primary toxic form. Cupric ion affects the efficiency of photosynthesis and the chloroplast structure by decreasing the electron carrier rate and the lipids biosynthesis (Lobban et al., 1985; Cooke et al., 1993; Sunda & Guillard, 1996; Ma et al., 2003; Garlich et al., 2016). As copper sulfate enters the cells, it substitutes magnesium in the chlorophyll molecule and interrupts the activity of electron transfer associated to the water splitting system through photosystem II (Moore & Kellerman, 1905; Pandey et al., 1992; Haughey et al., 2000; Garcia-Villada et al., 2004; Dewez et al., 2005; Jancula & Maršálek, 2011). Consequently, exposing algae cells to high dosages of copper sulfate inhibit the electron transport of photosynthesis and affect the metabolic pathways causing harmful effects in algae structure and physiology, indeed, high copper concentrations hinder CO_2 fixation, affect adenosine triphosphate (ATP) production, delay nitrate uptake, decrease cellular mobility and organelles instability, and reduce intracellular potassium and sodium ions concentrations. It also causes disarrangement of the plasmatic membrane by destructing the chloroplast membrane of green plants and interferes with cell permeability on the binding of essential metals (DeFilipis, 1979; Sunda & Huntsman, 1983; Stauber & Florence, 1987; Barón et al., 1995; Bossuyt & Janssen, 2004; Garlich et al., 2016; Wang et al., 2017).

In addition, copper effectiveness was found to be dependent on the treated water chemistry. When dealing with high pH and hardness, copper is less toxic, owing to the precipitation of copper into its insoluble forms and the lower binding ability of copper on the algal cell membrane in the presence of calcium and magnesium (Cooke et al., 1993; Hullebusch et al., 2002). A study conducted by Wang et al., (2017), proved that in the Aquil culture media used, high levels of ethylenediamine tetra acetic acid (EDTA) may reduce the possibility of the metal binding with cell sites while complexing with free Cu^{2+} . In addition, copper toxicity is countered by strong chelating agents such as

EDTA or citrate which prevent the complexation and precipitation of ions in water (Brand et al., 1986; Raman & Cook, 1988; Garcia-Villada et al., 2004; Tsai, 2016). In its chelated form, copper application maintains the metal long enough in the water column to produce the desirable effect. Additionally, chelated copper has been reported to be more efficient in controlling algal growth and appropriate for utilization in highly alkaline and hard waters (Cooke et al., 1993). However, in its non-chelated form, copper is considered as an economical and effective algicide as it does not impact human health at the generally applied dosages (WHO, 1996; Garcia-Villada et al., 2004; Watson & Yanong, 2006).

Algae susceptibility to copper varies widely between species; toxicity of copper is species specific. It was proven that blue-green algae are the most sensitive to the effect of copper, whereas in other algae groups such as *Anabaena sp.*, copper toxicity is reduced through a decrease in the bioavailability of the metal caused by algal excretion of metal-binding compounds or the production of intercellular metal-binding peptides (Illinois state water survey, 1989; Cooke et al., 1993; Hullebusch et al., 2002; Bossuyt & Janssen, 2004; Wang et al., 2017). Common standard algae toxicity tests were developed and are used to determine the optimal dose of copper to inhibit the growth of algae.

Growth inhibition tests aim at determining the toxicity of a contaminant on the growth of algal species. In algae growth inhibition tests, the adoption of specific species as test organisms is required (OECD, 1984; EEC, 1992; Bagley & Hurst, 1998). The green algae *Selenastrum capricornutum* is the test organism of choice in toxicity testing for being a good quality specie available throughout the year and able to be identified to other species. Growth test methods measure the chronic toxicity of effluents after exposing algae population to a series of concentrations in a four day static test (EPA, 2002). However, limited studies conduct toxicity tests taking into consideration algae surface area.

In ecology, surface area of organisms proved to be highly significant because it can provide a new approach in analysis of microalgae. The initial toxicant loading can be determined by assessing the adsorption of metal ions to algal surfaces. It is dependent on the cell surface area and the nature of metal binding sites (Dahl, 1973; Franklin et al., 2004). Because algal surface is composed of complex and heterogeneous binding sites for metals and protons, it is capable of absorbing nutrients and bioaccumulating heavy metals, such as copper (Xia et al., 2016). Further, agitation and mixing rate of nutrients is affected by the size and shape of microalgae. In this context, cell size are linked to several processes, including “nutrient uptake, light affinity, photosynthesis, respiration, settling rates, physical transport and plant-herbivore interactions” (Vadrucci et al., 2013). With regards to nutrient uptake, a study showed that size-metabolism and surface uptake of nutrients are interrelated. In fact, productivity and surface area of algae are proportional to the uptake of nutrients. As for photosynthesis, according to Jahnke and Lawrence (1965), a greater efficiency of a vertically elongate structure in intercepting light is demonstrated by a greater exposed surface (Dahl, 1973; Vadrucci et al., 2013).

Other critical aspects in algal surface binding capacity are the concentrations and characteristics of proton-active carboxylic, phosphoric, hydroxyl and amine functional groups. The binding capacity of surfaces and the formation of biofilm are affected by these characteristics. When a biofilm is formed in the surface of the photobioreactor, the penetration of light is prevented causing inaccessible algal biomass for harvesting (Xia et al., 2016). According to Green et al., (1984), and Crist and co-workers, (1981), several functional groups on the algae cell wall, including carboxyl and amine functional groups, may be responsible for the metal uptake and involved in the coordination of metallic ions. Ndolomingo & Meijboom (2016) proved that higher surface area provides better

dispersion of proton-active sites. Also, diffusion of reactant becomes easier, thus, improving sites suitability for catalytic activity (Franklin et al., 2004; Ndolomingo & Meijboom, 2016).

Of the many groups, green algae are considered to be the most diverse (Zhang et al., 2016). Previous studies conducted on Canal 900 used for the irrigation of the South Bekaa agricultural lands in Lebanon showed the occurrence of unicellular and filamentous algae including *Scenedesmus sp.*, *Ankistrodesmus sp.*, and *Oscillatoria sp.* in the upper sections of the Canal (Salam et al., 2006). The green algae *Scenedesmus sp.*, one of the most common freshwater algae species, is known to cause a bad odor and taste to water. As this genus governs the aquatic marine environment, it constitutes the key producer of the ecosystem. Also, it poses a risk for biofuel production due to a reduction in triacylglycerols (TAG) accumulation under stress such as exposure to copper (Nayak et al., 1996; Lurling, 2003; LWQM & USAID, 2005; Chen et al., 2012; Breuer et al., 2012; Devi et al., 2012; Rocha et al., 2015). According to a study conducted by Hu et al., (2016), the effective concentration to inhibit 50% of algal growth compared to control (EC_{50}) for *Scenedesmus sp.* group is 0.13 ± 0.003 mg/L as Cu^{2+} . The green algae, *Ankistrodesmus sp.* is mostly found in wastewaters and sewage ponds throughout the year and it is known for its production of scum on water surfaces along with distinct odor and taste (Patil, 1991; Lananan et al., 2016). Furthermore, filamentous species such as *Oscillatoria sp.* are a type of blue-green algae commonly found in lakes and ponds (Sustainable Soil & Water Ltd, 2010). *Oscillatoria sp.* has been reported to deteriorate water quality by promoting the formation of scum at the water surface. As a result, it induces odors and alters the taste and color in water. This blue-green algae outcompetes the beneficial algae, which are an essential part of the fish's food chain. It produces algal biomass that accumulates undesired compounds (heavy metals), by consuming excessive nutrients, including nitrate and phosphorus. However, these heavy metals can induce a disturbance to the natural behavior of organisms, and may even cause death of organisms

(Illinois state water survey, 1989; Schrader et al., 1998; Venter et al., 2003; Sustainable Soil & Water Ltd, 2010; Çelekli et al., 2016).

To assess the risk of pollutants in aquatic ecosystems, numerous laboratory scale toxicity tests were developed. Algae inhibition toxicity tests, characterized by four-day static tests, were used to determine the effects of contaminants on the growth of a unicellular green-algal species (OECD guideline for testing of chemicals, 1984; Beelen & Fleuren-Kemila, 1999; EPA, 2002). In previous studies, most metal toxicity testing included single-species laboratory bioassays to evaluate the concentration at which the chemical is deemed environmentally unsafe or may affect the algae species (Swartzman et al., 1990; Wagner & Lokke, 1991; Aldenberg & Slob, 1993; Beelen & Fleuren-Kemila, 1999; Franklin, Stauber, & Lim, 2004; Yu, et al., 2007). In such toxicity tests, a single species is evaluated for its susceptibility to a specific chemical under controlled laboratory setup (temperature, light, nutrients, presence of grazers, and water quality). In many cases, these bioassays present relative sensitivities to various organisms and are highly reproducible. However, they lack environmental realism as they do not represent the interactions among organisms as part of complex communities. Thus, the resulting effect concentration from these single toxicity tests reflect the susceptibility of the monitored specie to direct chemical effects and exclude potential effects of other coexisting algal organisms (Franklin et al., 2004; De Laender et al., 2009). The interaction among species may be deleterious to microalgae when exposed to toxicants. Some microalgae produce secondary metabolites when under chemical stress and influence the growth and the development of other algae species (Metaxas & Lewis, 1991; Legrand et al., 2003; Gross, 2003; Granéli et al., 2008). These effects are hard to simulate in isolated tests (Kayser, 1979; Suter, 1983; Yu, et al., 2007; De Laender et al., 2009).

Conducting multispecies studies could present an efficient way to elucidate the precise toxicity mechanism in complex systems. It can affect the activity of the test chemical and provide a

more realistic appraisal of the response to toxic exposure (Suter, 1983; Franklin et al., 2004; Yu et al., 2007; De Laender et al., 2009). It also provides a means of identifying the combined effect of algal-algal interactions and the indirect influence of toxicants, which pose a challenge to simulate in single species studies (Swartzman et al., 1990; Franklin et al., 2004; Yu et al., 2007; Picone et al., 2016). Nevertheless, algal-algal interaction test is limited by the difficulty of counting cells, detection of toxicity endpoints on the targeted species, and separating one species from the other in mixed algal population (Franklin et al., 2004; Yu et al., 2007; Debenest et al., 2011).

Whether unicellular, filamentous or colonial, algae pose a direct threat to the environment. Suitable test species were chosen according to their availability, ability to grow rapidly under defined laboratory conditions, and ability to differentiate between them according to their morphology. Compared to unicellular algae, filamentous algae groups present distinctive characteristics. They have strong ability to remove different pollutants (Zhang et al., 2016). While algae can be directly detrimental as a consequence of excessive algal biomass, it can be indirectly harmful due to the algaecide treatments used in their control. It is therefore critical to determine the minimal dose of copper algaecide necessary to effectively treat algae while minimizing the drawbacks of excessive unneeded algaecide levels (Ferrier et al., 2005; Jancula & Maršálek, 2011; Garlich et al., 2016). This study aims at determining growth interactions, in single and multi-species bioassays, of *Scenedesmus subspicatus*, *Scenedesmus quadricauda*, *Ankistrodesmus angustus*, and *Oscillatoria prolifera* under control and copper toxicity conditions. The test species were selected for their previous appearance in irrigation channels in Lebanon and common occurrence in lakes and ponds. Their different representation of shape and size render them ideal and well-aligned with the study objectives. The effect of copper on algal mixtures and single species was determined to identify whether the interaction between different species affects contaminant toxicity. The difference between cell density

and surface area considerations in both single and multi-species bioassays was assessed. Accordingly, bioassays are conducted on the basis of: i) cell density, which is important for growth activity and sensitivity of algae when exposed to metal ions, and ii) equivalent surface area to determine initial toxicant loading, which is dependent on the binding sites of copper and the nature of cell surface area. Copper sulfate was used as an inhibitory agent on the four algal species.

CHAPTER 2

METHODOLOGY AND SCOPE OF WORK

2.1 Algae culturing and selection of suitable algae culture medium

Scenedesmus quadricauda (UTEX B 76 strain), *Scenedesmus subspicatus* (UTEX 2532 strain), *Ankistrodesmus angustus* (UTEX 189 strain), and *Oscillatoria prolifera* (UTEX B 1270 strain) (Figure 1) were selected for the present study based on the availability of monocultures and their previous occurrence in an irrigation water canal, and imported from the Culture Collection of Algae at the University of Texas at Austin, designated as UTEX. Imported algae species were provided on solid culture media and were transferred aseptically in the laboratory to BG11 and EPA aqueous culture media. Indeed, to select a suitable culture medium for multi-species toxicity tests, it was important to choose a medium that allows all species to sustain normal growth rates. According to the EPA algal toxicity method (EPA, 2002), an adequate growth of algae in a culture medium is attained if the mean algal cell density in the control flasks reaches approximately 1×10^6 cells/mL at the end of the test with a maximum coefficient of variation of 20% among replicates. Previous work conducted in Environmental Engineering Research Center at the American University of Beirut, showed that green algae species grew effectively in EPA medium while *O.prolifera* growth was better supported in BG-11 medium. Hence, both the EPA and BG11 culture media were originally used to prepare the different algal cultures. Algal growth was then monitored in both media to determine the most suitable culture medium for each algae specie and consequently, the culture medium to be used in multispecies toxicity bioassays. The composition of the BG11 and EPA culture media is presented in Appendix A.

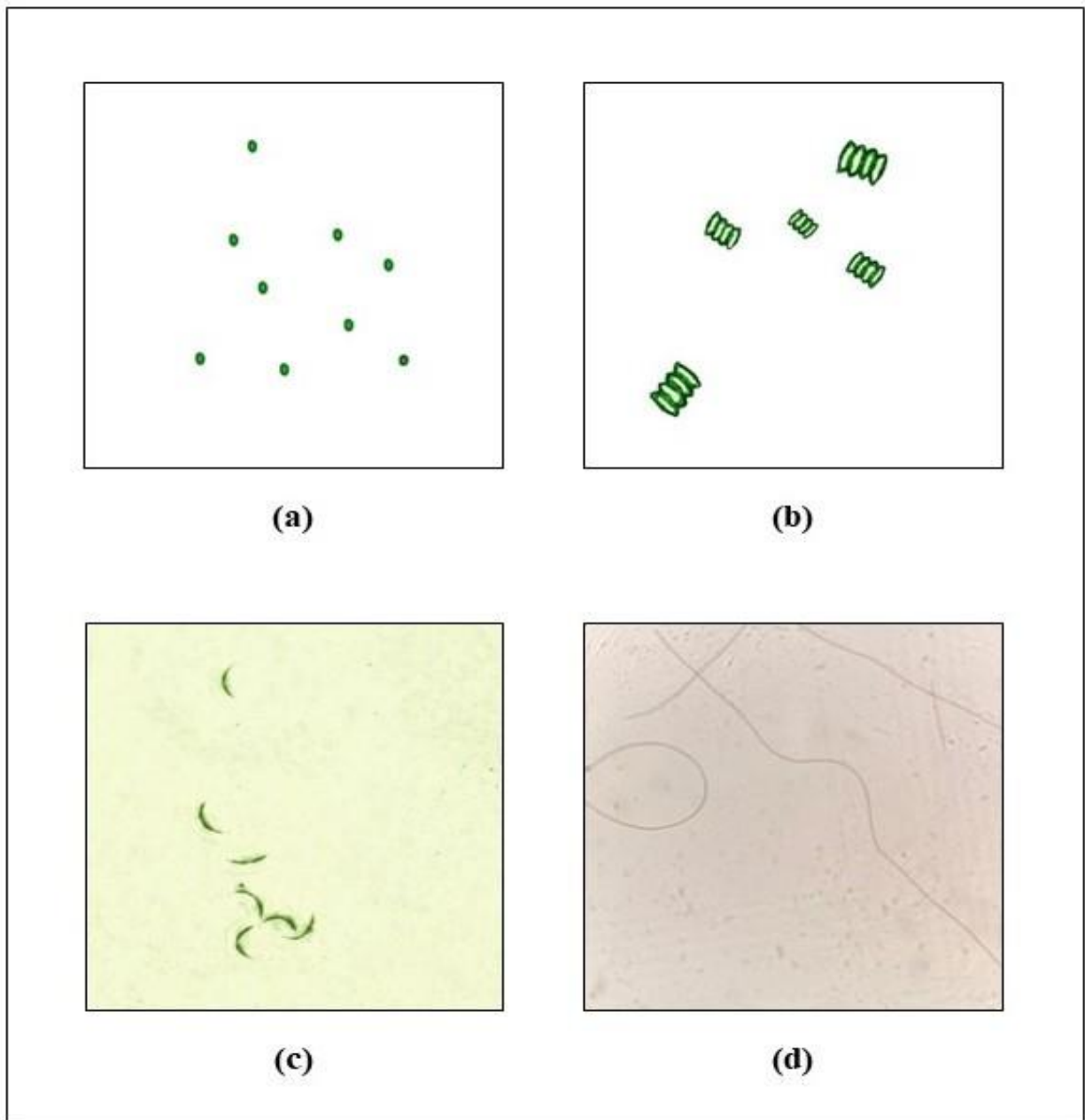


Figure.1 Algae species viewed under the microscope (eyepiece magnification: 40X), (a) *Scenedesmus subspicatus*,(b) *Scenedesmus quadricauda*, (c) *Ankistrodesmus angustus*, and (d) *Oscillatoria prolifera*

Algal cultures were maintained under an illumination intensity of 400 ± 40 ft-c at 25°C . Algae were grown in the laboratory for at least four weeks before being used in toxicity testing. This procedure provided enough time for the algae species to adapt to the culture medium and lab conditions. During this period, algal cultures were maintained through weekly transfer of aliquot samples from the stock cultures to fresh nutritive medium at each time the exponential phase of

growth was reached. This procedure ensured that sample species are healthy and ready to be used in the toxicity tests. Algal growth was monitored through a daily count using an inverted fluorescent microscope. The growth curves of the different algae species are presented in Appendix B. The results showed a higher growth rate for all species in BG11 medium which was adopted as culture medium in all toxicity testing.

2.2 Preparation of stock algaecide solution for the inhibition bioassays

Copper sulfate (CuSO_4), a commonly used algaecide, was acquired from Sigma Aldrich as Copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (209198 ACS reagents). Stock algaecide solution of 100,000 $\mu\text{g/L}$ as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ for treatments with copper sulfate pentahydrate was prepared one day prior to the experiments. Several concentrations of this algaecide were prepared from serial dilutions of stock solution to achieve the desired chemical dosages of 50, 100, 200, 350, 500, 1000, 1500, and 2000 $\mu\text{g/L}$ as CuSO_4 , which were used in toxicity testing. These corresponded to equivalent Cu concentrations of 20, 40, 80, 140, 200, 400, 600 and 800 $\mu\text{g/L}$, respectively.

2.3 Determination of algae species cells surface areas

A ZEISS inverted fluorescence microscope (Axiovert 200) was used to determine the surface area (SA) of each algae species. The cell dimensions (diameter, width, length) for selected species were determined by the organism approximate geometrical solid shape and by using the mean value of surface area of 65 cells (Franklin et al., 2004). Shapes could be represented as an oval for *S. subspicatus* ($\text{SA} = 4 \cdot \pi \cdot R_1 \cdot R_2$), where R_1 and R_2 represent the radius of the oval shape sp., a rectangle for *S. quadricauda* ($\text{SA} = 2 \cdot L \cdot W$), where L represent the length of the specie and

W its width, a crescent shape for *A. angustus* (determined by SA= area*2), and a filamentous length for *O. prolifera* (SA= area*2). The thickness was ignored as it was negligible. The geometrical representation of the different species along with their respective average surface area calculations are presented in Appendix C. The average surface area of each algae specie is presented in Table 1.

2.4 Experimental setup

Algae toxicity tests were conducted according to the EPA “Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms” (EPA, 1994). Before starting the test, all Erlenmeyer flasks and glassware were acid-washed in 10% concentrated HNO₃ (Nitric acid), then rinsed with distilled water several times to ensure their cleanliness.

Monoculture toxicity tests were conducted separately for the four species and the multi-algal toxicity bioassays were then performed using the four combined species. 250 mL Erlenmeyer flasks containing 40 ml of autoclaved BG-11 culture medium were used for toxicity tests. Two sets of experiments were carried out. In the first set, toxicity bioassays were conducted according to the EPA “Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms” (EPA, 1994). In the second set of experiments, the equivalent surface area of 10,000 cells/mL of the specie with the largest measured surface area was determined and used as the basis of toxicity testing conducted with each algae specie separately. As such, and knowing that the specie *Oscillatoria prolifera* has the largest average area/cell of 2348 μm^2 , the total algal surface area adopted in the toxicity testing was computed to 23,480,000 μm^2 . Thus, mono-algal toxicity tests conducted on *S. quadricauda*, *S. subspicatus*, *A. angustus* and *O.*

prolifera, were performed with an initial cell density equivalent to 23,480,000 μm^2 in each case (Table 1). Equation (1) represents the calculation for obtaining the required culture starter for each algae specie (cells/ml).

$$\text{Equation (1): Initial Starter (cells/ml)} = 23,480,000/\text{SA}_{\text{species}}$$

Table 1 Initial inoculum of each algae specie in mono-culture and multi-culture toxicity bioassays based on SA

Species	<i>S. subspicatus</i>	<i>S. quadricauda</i>	<i>A. angustus</i>	<i>O. prolifera</i>	Total surface area in 1 ml (μm^2)
Mean SA of 65 cells ($\mu\text{m}^2/\text{cell}$)	47	108	742	2348	
Initial inoculum in monoalgal toxicity bioassays (cells/ml)	499.57x 10 ³	217.4x10 ³	31.64x10 ³	10x10 ³	23.48x 10 ⁶
Initial inoculum in Multispecies toxicity bioassays (cells/ml)	122.34x10 ³	53.24x10 ³	7.749x10 ³	2.5x10 ³	5.87x 10 ⁶

For the multispecies toxicity test, in the case of the tests conducted based on cell density, the initial density was 10,000 cells/ml for all combined species, where 2500 cells/ml of each specie were cultured in each flask. As for the multispecies toxicity test based on algal surface area, the initial density used for each of the four algal species was equivalent to $\frac{1}{4}$ of the total defined surface area of the starting inoculum of 23,480,000 μm^2 ($23,480,000/4 = 5.87 \times 10^6$). Table 1 indicates the initial cell count in the test flasks for each specie in mono and multi-species toxicity testing based on surface area.

Control flasks with no added copper were prepared in triplicates for each set of toxicity bioassays. In the case of multispecies tests, two types of controls were prepared. The first type of control consisted of the combined species and was necessary to determine the inhibitory effect of copper on each algae specie in the multispecies cultures. The second type of controls consisted of each specie alone with an initial cell density equal to the specie's concentration in the algal mixtures, and

was necessary to allow a direct comparison of control growth rates in the presence and absence of other species. This would permit to determine the effect of algal-algal interactions on algae growth without the confounding effect of copper inhibition.

2.5 Algae Inhibition Tests

All toxicity tests were conducted according to the EPA 96-hour static chronic toxicity test and were performed in BG11 medium. Algae were exposed to copper concentrations ranging from 50 to 2000 $\mu\text{g/L}$ as CuSO_4 in test flasks, equivalent to 20 to 800 $\mu\text{g/L}$ as Cu. Test flasks were placed in an algae incubator, which was prepared using a high wooden stand (55 cm width, 150 cm length and 40 cm height) presented in Figure 1 in Appendix D. The stand is equipped with 5 daylight fluorescents (3 on the bench and 2 on the top (parallel) with a light intensity output of 400 ± 40 ft-c. To ensure constant environmental conditions, the temperature was checked using a digital thermometer every 48 hours and maintained at $25\pm 1^\circ\text{C}$. Laboratory temperature was fixed to 22°C , and under illumination, temperature was approximately 25°C . Specimens were manually shaken twice per day to enhance gas exchange and ensure the availability of CO_2 . A summary of test parameters for algae inhibition tests is shown in Table 2. Alkalinity, hardness, conductivity and pH were measured for each test flask at the beginning of the test in solutions of different copper concentrations (Appendix E). Also, pH and temperature were measured at the end of every 48-hour exposure period. Average of 3 replicates of each test solution concentration was chosen for pH and temperature measurements (Appendix F). Figure 2 represents a schematic representation of the experimental setup.

Table 2 Summary of test conditions for mono and multi--cultures toxicity tests

Number of total tests		7 single-species tests 2 multi-species tests
Test concentrations		8 concentrations ranging from 50 to 2000 µg/L as CuSO ₄ + Controls with no added copper
Duration of the test		96- hour
Initial cell density	Single-specie test based on cell density	10,000 cells/mL (± 10%)
	Single-specie test based on SA	Cell density/mL equivalent to a total surface area of 23.48*10 ⁶ µm ²
	Multi-specie test based on cell density	10,000 cells/mL (± 10%) of combined species
	Multi-specie test based on SA	Cell density/mL of each specie equivalent to a surface area of 5.87x 10 ⁶ µm ²
Temperature		25 ± 1°C
Light quality		Cool white fluorescent lighting
Light intensity		400 ± 40 ft-c
Photoperiod		Continuous illumination
Test flask size		250 mL
Test solution volume		40 ml
Number of replicate flasks per concentration		3
Agitation rate		Twice daily by hand
Algae culture medium		BG11

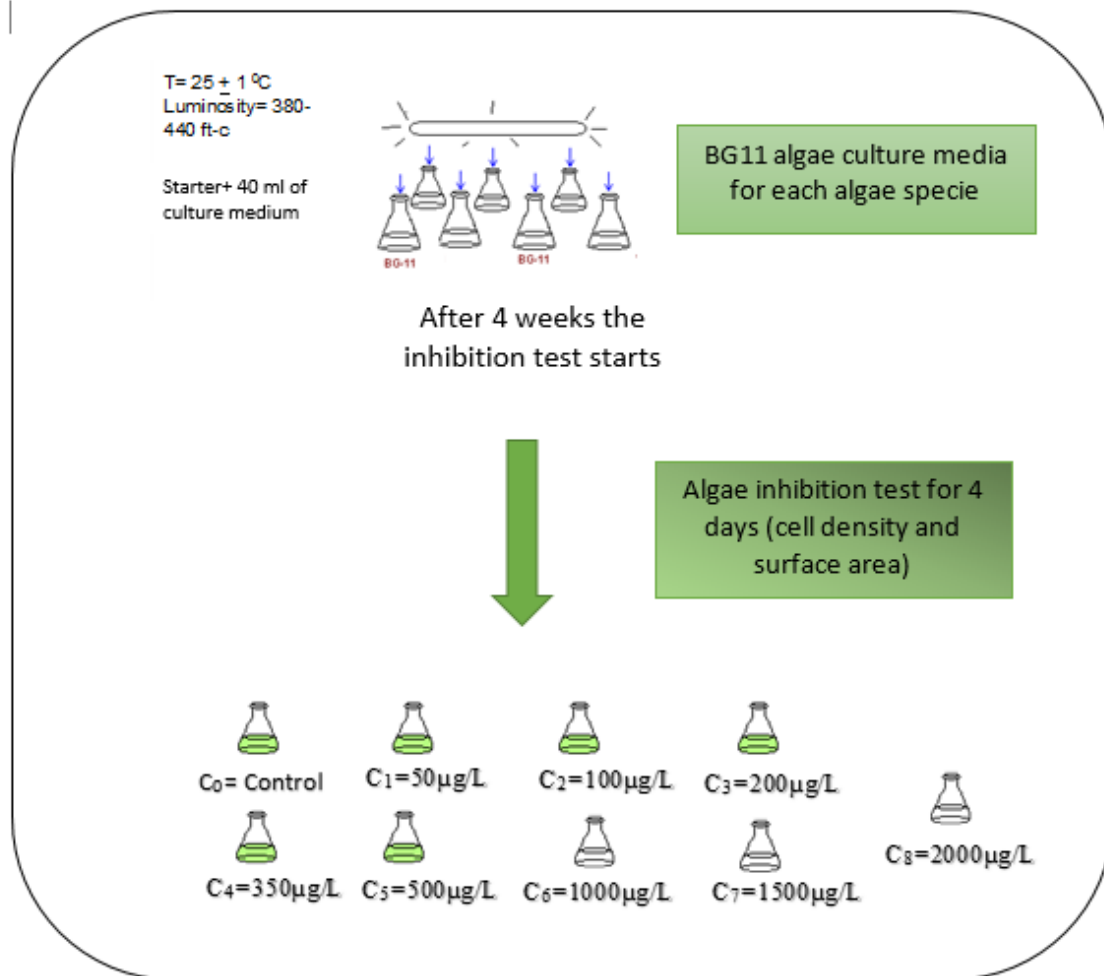


Figure.2 Schematic representation of the experimental set-up

2.6 Statistical Analysis

Copper toxicity was expressed as an EC_{50} value, which is the concentration of copper required to inhibit algae growth by 50% compared to the control at 96h, and determined for each specie alone and in the presence of other species. Data from replicates in single- and multispecies bioassays were pooled and a combined concentration-response curve was plotted using weighted linear regression analysis on probit transformed data. This value was computed with 95% confidence interval. Also, two-way Analysis of Variance (ANOVA) tests were used to evaluate statistically significant factors (cell count) of each specie. “Tukey Honest significant differences” multiple comparison (TukeyHSD)

was used to identify significance between control growth rates in single- and multispecies bioassays. The ANOVA and multiple comparisons analysis were fit using the R software (R Core team, 2015). The Student's *t*-test was used to identify significant differences between percent inhibition relatively to the control for each specie.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Effect of pH and temperature throughout the experiment

Water pH affects the growth of algal population since it is an important environmental factor (Danilov & Ekelund, 2001). When the pH is suitable for algae species, these species show a significant growth. Otherwise, the activity of these organism is inhibited or species die (Twist, Edwards, & Codd, 1998). Parameters including pH, alkalinity and hardness, measured at the beginning of each test show that the prepared test solutions were almost consistent (Appendix E). In all conducted monoculture and multi-culture toxicity tests based on cell density, the pH of the medium was increasing over 96 hours as algae growth increased. The pH variation was more remarkable in the case of *Scenedesmus species* as a single specie (pH increased by 1.5 units) and less accentuated in the case of *A. angustus* and *O. prolifera*. Small changes in pH cause little effect on copper speciation (Meador, 1991). pH variations reported in the conducted toxicity tests are tolerable according to the EU and EPA toxicity tests which allow pH deviation in the control flasks by a maximum of 1.5 units (European Commission, 1992; EPA, 1994). This increase in pH is due to the algal removal of carbon dioxide (CO₂) from water that increases the hydroxide (OH⁻) levels during photosynthesis (Ceci, 2015). According to Hullebusch et al.(2002) and Xue et al. (1998), copper treatment is less efficient when the density of algal biomass increases. Also, as algal blooms increase, the water pH increases favoring carbonate precipitation and copper complexation by organic ligands (Hullebusch et al., 2002; Xue et al., 1988). As for the temperature, it should not deviate by more than 3°C in the test flasks (European Commission, 1992; USEPA, 1994) and this conditions was achieved in all conducted experiments. Detailed average pH and temperature measurements are reported in Appendices F.

3.2 Algae inhibition test results and discussion

Algae inhibition tests were conducted according to the EPA “Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms” (EPA, 1994). Two sets of inhibition tests were conducted. In the first set, individual algae species were tested in mono-algal toxicity bioassays and in the second set of experiments, algae were combined and tested in multispecies cultures. In each case, two treatments were performed based on cell density and on equivalent surface area. All inhibition tests were run over a period of 96 hours. In each case, algae growth curves at each copper concentration were constructed by plotting the average cell count of three replicate samples measured at the end of each exposure day versus time. Algae growth curves of the different test species in mono and multi-species toxicity bioassays are presented in Appendix G. Copper toxicity on the different tested species was monitored through the % growth reduction of each specie relatively to its control. The % algae growth inhibition at the end of each exposure day of the toxicity tests are provided for each specie in Appendix H. The species % response to copper toxicity at 96 hours exposure period is presented in the subsequent sections. Copper toxicity was expressed as 96h-EC₅₀ value which represents the effective copper sulfate concentration inducing 50% reduction in algal growth rate after 96 hours of exposure. Table 3 shows EC₅₀ values for each specie alone and in the presence of other species.

Table 3 EC₅₀ values obtained by Probit analysis

Algal species	96-h EC ₅₀ (µg/L) ¹			
	Tests based on cell density		Tests based on surface area	
	Single species	Multispecies	Single species	Multispecies
<i>S.subspicatus</i>	198AG ²	321HS	1123BM	462NS
<i>S.quadricauda</i>	359CI	1695JT	692DO	1011PU
<i>A.angustus</i>	1809EK	235LV	190FQ	60RW
<i>O.prolifera</i>	126	454X	126	472X
<i>Mixed species</i>		389Y		462Y

¹EC₅₀: inhibitory concentration giving 50% reduction in algal growth rate compared to the control

²A to Y denote whether growth rates in multispecies tests in control for each species are significantly (p < 0.05) different from rates in mixed control (same letter means no significant [p > 0.05] difference).

3.2.1 Single Species Tests Based on Cell Density

EC₅₀ values obtained for *O.prolifera*, *S. subspicatus*, *S. quadricauda*, and *A.angustus* were respectively 126 µg/L, 198 µg/L, 359 µg/L, and 1809 µg/L as copper sulfate, and were significantly different (*p-value* <0.05)(Table 3).

O.prolifera. *O.prolifera* was the most sensitive to copper (EC₅₀= 126 µg/L) among all species in toxicity tests. At copper sulfate concentrations of only 100 µg/L, more than 60% growth inhibition occurred at 96h relatively to the control. Significant inhibition was observed at the highest copper sulfate dosages of 1500 and 2000 µg/L and exceeded 97%. (Table 4). It is a common knowledge that blue-green algae are more sensitive to the effects of copper than other algae groups, and consequently this type of algae is the most easily controlled by copper sulfate applications (Illinois state water survey, 1989). A study by McGuire et al. (1984) was run in the form of both laboratory and field tests on blue-green algal species in Lake Mathews controlled by copper sulfate. The tests indicated that copper sulfate at a dosage of 225 kg/ha (equivalent to only 2.17 µg/L copper sulfate, depth=46m) would provide an effective control of attached growth of blue-green algae in the Lake.

S. subspicatus. Copper sulfate proved to be significantly effective in inhibiting *S. subspicatus* except for the 50 and 100 µg/L dosages as compared to the control (*p-values* > 0.05). In fact, only 1.47% and 10.84% of the *S. subspicatus* cells were inhibited at the latter respective doses at 96h (Table 4). However, as copper concentration increased, the average cell concentration of *S. subspicatus* decreased significantly (*p-value* < 0.05) with growth inhibition reaching around 88.0% at 350 µg/L and exceeded 96% at higher dosages. *S. subspicatus* was less sensitive to copper sulfate than *O.prolifera* and reached a 96h-EC₅₀ value of 198 µg/L as copper sulfate (Table 3). In point of fact, *Scenedesmus sp.* has the ability to avoid metal toxicity. This is affected by biotic factors, including growth rate, cell wall and intracellular metabolism (Magdaleno et al., 2014; Levy et al., 2007). A study

by Le Faucheur et al. (2006) showed that the intracellular thiol content of *Scenedesmus sp.* is affected by the exposure of this specie to copper either by varying glutathione concentration or by producing phytochelatin. Another recent study by Kalinowska and Pawlik-Skowronska (2010) demonstrated that glutathione (predominant thiol peptide) in living cells is able to chelate directly certain heavy metals such as copper. This peptide that has a high metal binding affinity might have potentially contributed to the complexation of copper ions in this study that lead to the relative resistance of *S. subspicatus*.

Table 4 Induced % inhibition in single-species bioassays based on cell density

Prepared Test solution concentration (µg/L)	% Growth inhibition relatively to the control at 96 h			
	<i>S.subspicatus</i>	<i>S.quadricauda</i>	<i>A.angustus</i>	<i>O.prolifera</i>
0	0.00	0.00	0.00	0.00
50	1.47	6.10	2.60	16.48
100	10.84	16.82	7.16	60.20
200	28.33	32.65	14.84	66.63
350	88.02	37.54	18.49	71.06
500	96.39	45.53	25.52	80.10
1000	99.71	57.41	32.64	93.67
1500	99.71	63.30	40.28	97.89
2000	100.00	68.03	60.24	100.00

S. quadricauda. , The maximum attained growth inhibition for *S. quadricauda* was only 68% at 96 hours. Compared to *S. subspicatus*, CuSO₄ proved to be a less toxic algacide to *S. quadricauda* (EC₅₀= 359 µg/L). *S. quadricauda* may be producing and accumulating intracellular and extracellular metabolic products that play an important role in the protection of the algae against the copper toxicity stress factor. Wu et al. (1995) demonstrated that more tolerant algae species to copper accumulate more protein such as proline within their cells which could significantly lower toxicity of copper through chelation. Maršálek and Rojíčková (1996) demonstrated that a self-protective mechanism that

S. quadricauda uses under copper toxicity stress is the production of metal-chelating exudates that include carbohydrates and proteins among which proline that was detected in both the cell and test medium. Hence, complexation of copper by carbohydrates and proteins such as proline might have occurred inside and outside the *S. quadricauda* cells and contributed to the reduction of copper toxicity in this study. Starodub & Wong (1987) reported reduced toxicity of copper to growth of *S. quadricauda* (96h-EC₅₀ value of 250 µg/L as copper sulfate) that was caused by the production of algal extracellular products, which are capable of complexing with copper and reducing the free toxic cupric ion concentration in solution. Bringmann & Kuhn, (1980), also reached a threshold level of copper sulfate toxicity to *S. quadricauda* of 300 µg/L.

A.angustus. The attained *A.angustus* concentration at the lowest CuSO₄ dosage (50 µg/L) was statistically not different from the control (*p-value*= 0.69) and registered an inhibition percentage of 2.6 (Table 4). Concentrations of *A.angustus* at the highest dosage of 2000 µg/L were statistically lower than the control after 96 h of treatment but only reached 60% cells inhibition. *A.angustus* registered a 96h-EC₅₀ value of 1809 µg/L and was the most resistant specie to copper (Table 3). Vieira & Nascimento (1988) ran an Electron Paramagnetic Resonance (EPR) spectra of compounds excreted by *Ankistrodesmus densus* in cultures stressed with copper ions. The EPR spectra obtained were characteristic of complexes with and without superhyperfine lines suggesting the presence of nitrogenated bases and oxygenated compounds respectively coordinated with the copper ion. The single spectrum of superhyperfine nitrogen lines suggests the existence of a nitrogenated heteropolysaccharide (Vieira & Myklestad, 1986). The second observed spectrum is characteristic of oxygen ligands coordinated with the copper ion. This could explain the lower toxicity of copper on *A. angustus* in the present study.

3.2.2. Single Species Tests Based on Surface Area

The respective 96h-EC₅₀ for *S.subspicatus*, *S.quadricauda*, *A.angustus* and *O.prolifera* were 1123, 692, 190 and 126 µg/L as copper sulfate (Table 3). A trend between cell surface area of each species and their correspondent EC₅₀ could be deduced. As algae specie single cell increases in surface area, EC₅₀ decreases. Thus, the higher the algae cell surface area the more toxic copper may be to the specie.

O. prolifera. Having the highest cell surface area, *O. prolifera*'s initial concentration for cell density and surface area tests was the same (10*10³cells/ml). It thus showed similar sensitivity to copper sulfate in both tests.

Table 5 Induced % inhibition in single-species bioassays based on surface area (SA)

Prepared Test solution concentration (µg/L)	% Growth inhibition relatively to the control at 96 h			
	<i>S.subspicatus</i>	<i>S.quadricauda</i>	<i>A.angustus</i>	<i>O.prolifera</i>
0	0.00	0.00	0.00	0.00
50	2.20	16.19	1.90	16.48
100	5.92	20.00	41.44	60.20
200	9.38	22.04	39.70	66.63
350	14.91	60.41	46.24	71.06
500	17.50	62.04	53.10	80.10
1000	38.33	74.49	91.24	93.67
1500	65.87	77.28	95.07	97.89
2000	77.23	82.59	96.06	100.00

S. subspicatus. In the case of *S. subspicatus*, the initial inoculum in control bioassays based on cell density was 10*10³ cells/ml while an initial count of 499.57*10³ cells/ml was used in control bioassays based on surface area to provide an equivalent surface area to the largest specie (Table 1). *S. subspicatus* control cell concentration significantly increased (*p* <0.05) to reach 4,855,238 cells/ml at 96 h (Appendix G). Significant growth of the algae was also measured in the test samples with growth inhibition of only 38% being attained at copper

sulfate concentration of 1000 µg/L showing high tolerance of *S. subspicatus* to the algaecide. The algae % growth inhibition did not exceed 77.2% at the highest copper dosages (Table 5). The measured 96h-EC₅₀ for *S. subspicatus* of 1123 µg/L was significantly higher than EC₅₀ value measured in the test based on cell density for this specie (96h-EC₅₀ value of 198 µg/L as copper sulfate). According to Hullebusch et al.(2002) and Xue et al. (1998), copper treatment is less efficient when the density of algal biomass increases. Having a higher concentration of *S. subspicatus* in tests based on surface area, glutathione peptide production by *S. subspicatus* potentially increased, which contributed to the chelation of copper. Levels of free unbound metal ions in solution thus became sufficiently low and affected algal growth at only high concentrations.

S. quadricauda. As for *S. quadricauda*, the initial concentration was 217.4*10³ cells/ml and at 96 hours, this concentration significantly increased and reached 1,811,428 cells/ml in control flasks (Appendix G, Figure 3.2; Table 1). Under microscopic observations, *S. quadricauda* reductions of around 80% were observed at 96 h at the highest applied copper sulfate dose (2000 µg/L) (Table 5). The 96h-EC₅₀ value of *S. quadricauda* was 692 µg/L. The higher cells concentration used in the tests based on surface area might also have contributed to the high tolerance that *S. quadricauda* showed to copper sulfate. Under copper sulfate stress, this species could have produced and accumulated more intracellular and extracellular metabolic carbohydrates and proteins that played a significant role in complexing the metal ions which could no longer interact with the algal cells unless at high concentrations.

A.angustus. For *A.angustus*, the initial concentration was 31.64*10³ cells/ml. At 96 hours, these concentrations significantly increased and reached 6,716,667 cells/ml respectively (Appendix G, Figure 3.2; Table 1). Copper sulfate showed to be efficient through all dosages at

96h for the surface area tests of *A.angustus* (Table 3). For the same duration, *A.angustus*' cell concentration reached an inhibition percentage of around 96%. 96h-EC₅₀ for *A.angustus* was 190 µg/L. In the present test, *A.angustus* was sensitive to copper sulfate addition. *A.angustus*' photosynthetic apparatus might have been sensitive to the ion copper exposure that eventually inhibited the photosynthesis process of the specie. Shioi et al., (1978), assessed *Ankistrodesmus falcatus*' response to copper stress and suggested that copper ions inactivate electron transport between the oxidizing site of the reaction center of photosystem II and the electron-donating site of 1,5-diphenylearbazine. A more recent study done by Garlich et al., (2016), found that copper ion may also affect the chloroplast structure, by decreasing the electron transport rate and the lipids biosynthesis, affecting the photosynthetic efficiency of *Ankistrodesmus gracilis* as a consequence.

3.2.3 Comparison Between Algaecidal effects of Single Species Tests Based on Cell Density and Surface Area

96 h-EC₅₀ value of *S. subspicatus* was significantly ($p<0.05$) higher in tests based on surface area (EC₅₀= 1123 µg/L) than in tests based on cell density (EC₅₀= 198 µg/L) (Table 3). Similarly, *S. quadricauda* showed the same trend when comparing EC₅₀ results between the two test bioassays (EC₅₀= 692 µg/L in tests based on cell surface area and EC₅₀= 359 µg/L in tests based on cell density). Hence, as the initial cells concentration increased, EC₅₀ increased. To inhibit a higher cells' concentration of the same species, more copper is needed to be added to the medium. Dönmez et al., (1999), evaluated the influence of the alga concentration on the metal uptake for *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis sp.* The biomass concentration was varied from 0.25 to 2.0 g.L⁻¹ for all the species for 100 mg l⁻¹ of initial copper ion concentration. The results demonstrated that the algal concentration strongly affects the amount of metal removed from aqueous solution. In the presence of a high biomass concentration there is a very fast superficial adsorption

onto the cells that produces a lower metal concentration in solution than when the cell concentration is lower.

When *C. vulgaris* concentration was increased from 0.5 to 2.0 g.L⁻¹, copper ion uptake increased from 28.3 to 73.1 mg.L⁻¹. It was found that 32.4 mg.L⁻¹ of copper ions were adsorbed to 2.0 g.L⁻¹ of *S. obliquus* at the same initial metal ion concentration. The significantly higher initial inoculum concentration of the two tested *Scenedesmus* species in surface area-based toxicity bioassays contributed to the reduction of copper toxicity.

In contrary to *S. subspicatus* and *S. quadricauda*, *A. angustus* showed that with an increase in the initial cell concentration in the inhibition tests based on surface area as opposed to the ones based on cell density (31.64*10⁴ cells/ ml > 1*10⁴ cells/ ml), EC₅₀ significantly decreased from 1809 µg/L to 190 µg/L. A reason that might have induced such results is pH effect on CO₂ uptake by *A. angustus*. In toxicity tests based on cell density, the pH in the controls averaged 8.31 and increased to 9.31 in the controls based on surface area (Appendix F). Higher pH limits the availability of carbon from CO₂, which, in turn, suppresses algal growth. At higher pH, the carbon for algae is available in form of carbonates. Higher pH also lowers the affinity of algae to free CO₂ (Juneja et al., 2013). The limited availability of CO₂ due to increased pH and the inhibitory effect of copper on photosystem II process reported for *A. angustus* as previously discussed, are most likely responsible for reduced *A.angustus* growth in tests based on surface area.

3.2.4 Multispecies Toxicity Bioassays

Multispecies bioassays were conducted on the basis of cell density and surface area respectively.

- Tests based on cell density: the initial cell density of the four combined algal species counted 10,000 cells/mL.
- Tests based on surface area: a total surface area of the four combined species of 23,480,000 μm^2 was used. The combined surface area is equivalent to the surface area of 10,000 cell/mL of the largest tested algae *O. prolifera*.

In both cases, besides the multispecies controls, controls of each specie alone were prepared at the equivalent cell concentration used in multispecies bioassays.

3.2.4.1 Algal-algal interactions in multispecies control bioassays

To identify possible interactions between *O.prolifera*, *S. subspicatus*, *S. quadricauda*, and *A.angustus*, control growth (in the absence of copper sulfate) were compared for each specie alone (single species) and in the presence of the other species (multispecies) presented in Table 6.

Table 6 Average growth rate of each specie in control alone and when mixed with other species at 96 h

Test type in multispecies	Initial inoculum (cells/ml)	Average growth rate of specie in control (cells/ml/day)	Average growth rate in mixed control (cells/ml/day)
<i>S. subspicatus</i> cell density	2500	1.27 ± 0.001A ²	1.27 ± 0.006A
<i>S. subspicatus</i> SA	122.34*10 ³	0.54 ± 0.008B	0.51 ± 0.02C
<i>S. quadricauda</i> cell density	2500	0.96 ± 0.004D	0.95 ± 0.01D
<i>S. quadricauda</i> SA	53.24*10 ³	0.59 ± 0.01E	0.61 ± 0.01E
<i>A. angustus</i> cell density	2500	1.02 ± 0.006F	1.01 ± 0.01G
<i>A.angustus</i> SA	7.749*10 ³	1.28 ± 0.01G	1.11 ± 0.009H
<i>O.prolifera</i> cell density	2500	1.09 ± 0.01I	1.09 ± 0.02I
<i>O.prolifera</i> SA	2450	1.1 ± 0.01J	1.09 ± 0.01J

¹ SA=Surface area

² A to J denote whether growth rates in multispecies tests in control for each species are significantly (p<0.05) different from rates in mixed control (same letter means no significant [p > 0.05] difference).

Control growth rates based on cell density. In the multispecies test based on cell density, *A. angustus* control growth was reduced significantly at 96h (1.02 ± 0.006 cells/ml/d) in the presence of *S. subspicatus*, *S. quadricauda*, and *O. prolifera* compared to single species control tests (1.01 ± 0.1

cells/ml/d) (Table 6). Such results appear to be representative when two or more algal species are cultured together (Rijstenbil, 1989).

For *S. subspicatus*, *S. quadricauda*, and *O. prolifera*, similar growth was obtained for each species in the presence and absence of other algal species (p -values > 0.05). These species may have competed successfully with *A. angustus* for the nutrients' concentrations in the control medium and caused its lack of growth observed due to nutrient limitation. In fact, Kayser (1979) demonstrated that the main element controlling the growth of several species in multispecies cultures was nutrient competition.

Leao et al. (2009) showed that *Oscillatoria sp.* has allelopathic potential on green algae such as *C. vulgaris*. Thus, extra cellular toxins that *O. prolifera* produce could potentially explain the inhibitory pattern that *A. angustus* displayed in multispecies control tests. *Oscillatoria sp.* is in fact a blue-green algae that produces antitoxin-a under stress (Araoz et al., 2010). According to Chorus & Bartram, (1999), anatoxin-a may be involved in initial colonization causing inter-species interactions including allelophelic signaling. The green alga *Chlamydomonas reinhardtii* was reported to having been paralyzed by exposure to anatoxin-a (Kearns & Hunter, 2001).

Since *S. quadricauda* and *S. subspicatus*, control growth rates were not affected in multispecies bioassays compared to single species bioassays, *O. prolifera* did not have any allelopathic potential on them. In fact, in addition to multi-algal cultures, anatoxin-a is found to be produced in unialgal cultures, signifying that it is not necessarily only produced as a response to other organisms even if it may selectively adversely affect some algal species (Harland et al., 2013) which is the case of *A. angustus* in this study.

Control growth rates based on surface area. Similarly to the control cultures based on cell density, *A. angustus* control growth rates in tests based on surface area showed a significant decrease

(*p-value* < 0.05) in the presence of other species in the culture. Cell count at 96h dropped from 1.28 ± 0.01 cells/ml/d in single species control tests to 1.11 ± 0.009 cells/ml/d in the presence of other species (Table 6).

In addition, *S. subspicatus*, the algae with the smallest unit cell surface area, grew significantly poorer in the presence of other species than when alone. Similarly, Franklin et al. (2002) reported that *M. pusilla* that had the smallest cell size relatively to the other species, used in multispecies cultures presented a significant reduced algal growth in multispecies control bioassays based on surface area in comparison with single species control tests.

In addition to nutrients limitation and toxic effect of anatoxin-a secreted by *O. proliferata*, pH increase could have also contributed to the decreased growth of *S. subspicatus* and *A. angustus*. An increase by 1.4 in pH was observed in the multispecies controls compared to single-species controls with *S. subspicatus* over 96h. Similarly, an increase of 1.1 pH units was observed over 96h for *A. angustus* in the presence of other species (Appendix F). The growth retardation of *S. subspicatus* and *A. angustus* in multispecies controls compared to single-species controls may have been provoked by the increase in pH observed over 96h in the multi-algal controls mixture relatively to their respective single species controls. pH increase is due to algal removal of CO₂ from the medium which increases the hydroxide levels during photosynthesis (Ceci, 2015). This might have promoted the growth retardation of *A. angustus* and *S. subspicatus* which may be more sensitive to CO₂ limitation than the other species. In fact, although the control cultures of *S. quadricauda*, and *O. proliferata* showed an increased pH in multispecies controls relatively to their mono-species controls, these species control growth rates were not affected by the presence of other species in the mixture suggesting they were better competitors to CO₂ consumption.

3.2.4.2 Copper Sulfate toxicity in Multispecies Tests based on both cell density and surface area

The effect of copper on the growth of *S. subspicatus*, *S. quadricauda*, *A. angustus*, and *O. prolifera* in multispecies bioassays based on both cell density and surface area was measured as 96h-EC₅₀ values reported in Table 3. The 96h-EC₅₀ values were calculated based on the percentage inhibition of the growth rate compared to the controls of:

- Each specie alone to determine the 96h-EC₅₀ values of each specie in the multispecies mixture and,
- Combined species to determine the 96h-EC₅₀ values of the combined species in the multispecies bioassays.

In the following subsections the difference in copper toxicity in single and multispecies bioassays will be discussed to determine the effect of algal interactions on the metal toxicity.

A.angustus. In both multispecies toxicity tests based on cell density and surface area, *A. angustus* was the most sensitive to copper sulfate in the presence of other species in comparison to single species tests recording significant EC₅₀ (96h) drops from 1809 µg/L to 235 µg/L in bioassays based on cell density and from 190 µg/L to 60 µg/L in tests based on surface area (Table 3). The decreased EC₅₀ of *A. angustus* in multispecies bioassays was expected. Indeed, in addition to the copper stress, the significant (*p-value* < 0.05) inhibitory effect of the other micro-algal species on *A. angustus* growth in controls based on both cell density and surface area explain the specie's higher sensitivity to copper.

S. quadricauda. Copper was significantly less toxic to *S. quadricauda* in the presence of other species compared to single species bioassays in both tests run based on cell density and surface area (*p-value* < 0.05). The EC₅₀ value after 96h increased from 359 µg/L and 692 µg/L in single species bioassays based on cell density and surface area respectively, to 1694 µg/L and 1010 µg/L in the correspondent multispecies bioassays (Table 3). Decreased toxicity of copper to *S. quadricauda*

may be due to the decrease of free copper ions due to self-production of exudates. As discussed in the single species toxicity tests, *S. quadricauda* may be producing and accumulating intracellular metabolic products that play an important role in the protection of the algae against the copper toxicity stress factor. Carbohydrates and proteins that *S. quadricauda* is producing have potentially contributed to the complexation of copper and to the reduction of copper toxicity. Nonetheless, given the higher toxicity of copper to *S. quadricauda* in single species bioassays, a more eventual justification for the reduced copper toxicity on *S. quadricauda* in the presence of the other species would be the production and release of exudates by *S. subspicatus*.

O. prolifera. For *O. prolifera*, 96h-EC₅₀ values obtained in single and multispecies bioassays were significantly different (p -value < 0.05). EC₅₀ values increased from 126 µg/L in single species bioassays, to 454 and 472 µg/L in multispecies bioassays based on cell density and surface area respectively (Table 3). A potential explanation for the reduced toxicity of copper to *O. prolifera* in the presence of *S. subspicatus* and *S. quadricauda* is the production and extracellular release of algal exudates by these latter species in response to copper stress. These exudates may complex copper ions, making them less bioavailable to *O. prolifera* in multispecies bioassays.

S. subspicatus. Copper was significantly less toxic to *S. subspicatus* in the presence of other species compared to single species bioassays based on cell density. The EC₅₀ value after 96h increased from 198 µg/L in single species bioassays to 321 µg/L in the multispecies bioassays (Table 3). Decreased toxicity of copper to *S. subspicatus* may be due to the complexation of copper by the exudates produced by *S. quadricauda*. It is worth mentioning that a mutually profitable interaction between *S. quadricauda* and *S. subspicatus* may be concluded whereby, each species releases exudates that protect itself and the other species from copper toxicity.

However, in toxicity tests based on surface area, *S. subspicatus* showed to be less tolerant to copper in the presence of other species. EC₅₀ values decreased from 1123 µg/L in single species bioassays to 462 µg/L in the multispecies bioassays (Table 3). Quigg et al. (2006) reported that in surface area tests, small cells that have large surface area to volume ratios, are more sensitive to copper than larger species.

The difference in *S. subspicatus* sensitivity to copper between tests based on cell density and tests based on surface area could be explained by the positive effect that bigger algal cells have on smaller ones in the form of increased protection against copper toxicity. In multispecies toxicity tests based on cell density, the same initial inoculum was used for the different algal species (2500 cells/ml each). The different species present in the medium have different total surface areas each (*O.prolifera*, *A.angustus*, *S. quadricauda*, and *S. subspicatus* present respective single cell surface areas of 5,870,000 µm², 1,855,000 µm², and 270,000 µm² and 117,500 µm²). Having the same cell initial inoculum but different surface areas per specie, *O.prolifera* will occupy the biggest surface area followed by *A.angustus*, and *S. quadricauda*. The 2500 cell/ml of *S. subspicatus* will occupy the smallest surface area of the medium in the presence of the other algal species. This suggests that a faster copper uptake by active sites present on the cell membrane of bigger cells that occupy most of the medium solution area might have decreased copper uptake by the smallest algal cells and subsequently reduced copper toxicity to *S. subspicatus* in multispecies bioassays based on cell density (Dahl, 1973; Vadrucci et al., 2013). This shielding effect of bigger cells on smaller ones, suggests the necessity of conducting toxicity tests based on equivalent surface area rather than on cell density to avoid the confounding effect on copper toxicity of increased biomass for metal binding.

Combined algae species. In single species bioassays based on cell density, EC₅₀ values obtained for *O.prolifera*, *S. subspicatus*, *S. quadricauda*, and *A.angustus* were respectively 126 µg/L,

198 µg/L, 359 µg/L, and 1809 µg/L as copper sulfate. Nonetheless, for all species combined in the multispecies test based on cell density, 96h-EC₅₀ was reported to be 389 µg/L.

On the other hand, results of single algae bioassays based on surface area showed that respective 96h-EC₅₀ for *S.subspicatus*, *S.quadricauda*, *A.angustus* and *O.prolifera* were 1123, 692, 190 and 126 µg/L as copper sulfate. However, for the multispecies test based on surface area, 96h-EC₅₀ value of 425 µg/L was reported for all combined species. These results demonstrate that single-species bioassays may over or underestimate metal toxicity in natural water.

CHAPTER 4

CONCLUSIONS

The results from this study show the following main conclusions:

- Blue-green algae (*O.prolifera* in this study) showed to be the most sensitive to copper sulfate toxicity in single species bioassays.
- As surface area increases, copper sulfate toxicity increases for species in single-species bioassays mainly due to the increased metal binding sites.
- In multispecies controls, *A.angustus* was the most sensitive to the presence of other species in the absence of copper sulfate. Growth reduction in controls was namely associated to nutrients competition including CO₂ limitation and to the potential release of toxins by the blue-green algae, *O.prolifera*.
- Results from multispecies bioassays showed that copper sulfate toxicity to algae could increase or decrease in the presence of other species as compared to when the specie is alone. Hence, single-species bioassays may over or underestimate metal toxicity in natural waters, and it is thus recommended to conduct multispecies toxicity testing.
- Large algal cells could induce shielding effects on smaller ones decreasing copper toxicity in multispecies bioassays. Thus, it is recommended to conduct multispecies toxicity bioassays based on equivalent surface area to avoid the confounding effect on copper toxicity of increased biomass for metal binding.

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APPENDICES

Appendix A

Used culture medium

A. BG-11 medium ¹

<i>Ingredient</i>	<i>g l⁻¹</i>	<i>mM</i>
NaNO ₃	1.5	17.65
K ₂ HPO ₄ .3H ₂ O	0.04	0.18
MgSO ₄ .7H ₂ O	0.075	0.30
CaCl ₂ .2H ₂ O	0.036	0.25
Citric acid	0.006	0.03
Ferric ammonium citrate ²	0.006	0.03
EDTA (disodium magnesium))	0.001	0.003
Na ₂ CO ₃	0.02	0.19
Trace metal mix A5+Co	1 ml	
Deionized water	to 1 l	

pH after autoclaving and cooling: 7.4 , adjusted to 7.1 by using 1.0M HCl

Trace metals A5+Co	
<i>Ingredient</i>	<i>g l⁻¹</i>
H ₃ BO ₃	2.86
MnCl ₂ .4H ₂ O	1.81
ZnSO ₄ .7H ₂ O	0.222
Na ₂ MoO ₄ .2H ₂ O	0.390
CuSO ₄ .5H ₂ O	0.079
CoCl ₂ .6H ₂ O	0.04

¹ Available online at : <http://microbiology.ucdavis.edu/meeks/BG11medium.html>

B. EPA medium²

1. Five stock nutrient solutions were prepared using reagent grade chemicals as described in table A.B.1 below.
2. 1 ml of each stock solution was added in the order listed in Table B.1, to approximately 900 MILLI-Q water.
 - After the addition of each solution, flask was mixed.
 - After the dilution to 1 L, flask was mixed
 - pH was adjusted to 7.5 ± 0.1 , using 0.1N NaOH or HCl, as appropriate.
3. The medium was filtered through a 0.45 μm pore diameter membrane at a vacuum at a pressure of not more than one-half atmosphere (8 psi).
 - The filter was washed with 500 ml deionized water prior to use.
 - The medium was sterilized by autoclaving after it is placed in the culture vessels.

² Available online at: https://www.epa.gov/sites/production/files/2015-12/documents/method_1003_2002.pdf

Table A.B.1. Nutrient stock solutions used for the preparation of *Scenedesmus* algae culture medium according to the EPA method

<i>Stock solution</i>	<i>Compound</i>	<i>Amount dissolved in 500 mL Milli-Q water</i>
1. Macronutrients		
A	MgCl ₂ .6H ₂ O	6.08 g
	CaCl ₂ .2H ₂ O	2.20 g
	NaNO ₃	12.75 g
B	MgSO ₄ .7H ₂ O	7.35 g
C	K ₂ HPO ₄	0.522 g
D	NaHCO ₃	7.50 g
2. Micronutrients (in milligrams)		
	H ₃ BO ₃	92.8 mg
	MnCl ₂ .4H ₂ O	208.0mg
	ZnCl ₂	1.64 ² mg
	FeCl ₃ .6H ₂ O	79.9 mg
	CoCl ₂ .6H ₂ O	0.714 ³ mg
	Na ₂ MoO ₄ .2H ₂ O	3.63 ⁴ mg
	CuCl ₂ .2H ₂ O	0.006 ⁵ mg
	Na ₂ EDTA.2H ₂ O	150.0 mg
	Na ₂ SeO ₄	1.196 ⁶ mg

Source: United States Environmental Protection Agency, 1994. Short-term methods for estimating the chronic toxicity of effluents and receiving water to freshwater organisms. Third edition, EPA/600/4-91/002.

2. ZnCl₂ Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

3. CoCl₂.6H₂ O Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

4. Na₂ MoO₄.2H₂ O Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.

5. CuCl₂.2H₂ O Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock 2, micronutrients.

6. Na₂ SeO₄ Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

Note:

- Na₂SeO₄ was not included in the preparation of the micronutrients stock solution due to the lack of this nutrient and as it is not signaled in the preparation of algae culture medium according to other algae toxicity tests referred to.

Appendix B

Algae growth curves in EPA and BG11
culture medium

The graphs below represent a daily count of algal cells for two weeks in BG11 and EPA medium.

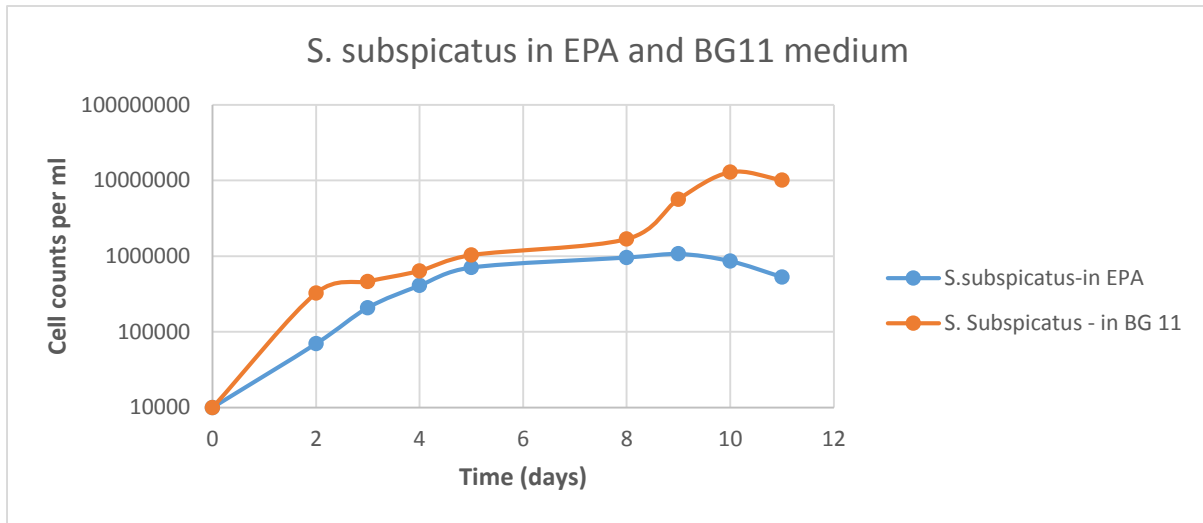


Figure 01 Logarithmic growth rate of *S. subspicatus* in EPA and BG11 medium

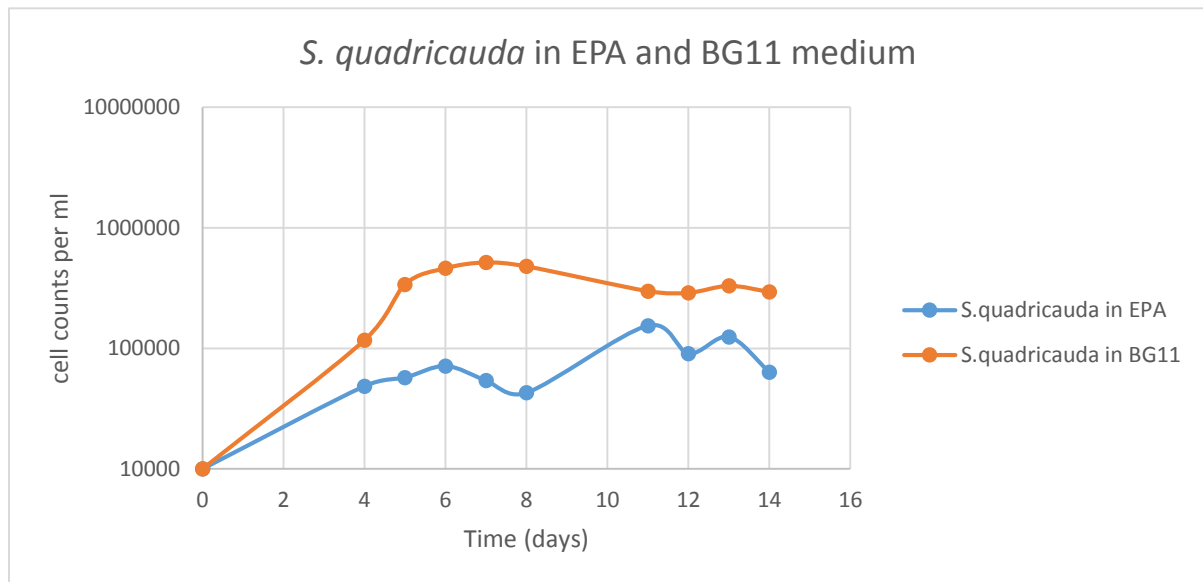


Figure 2 Logarithmic growth rate of *S. quadricauda* in EPA and BG11 medium

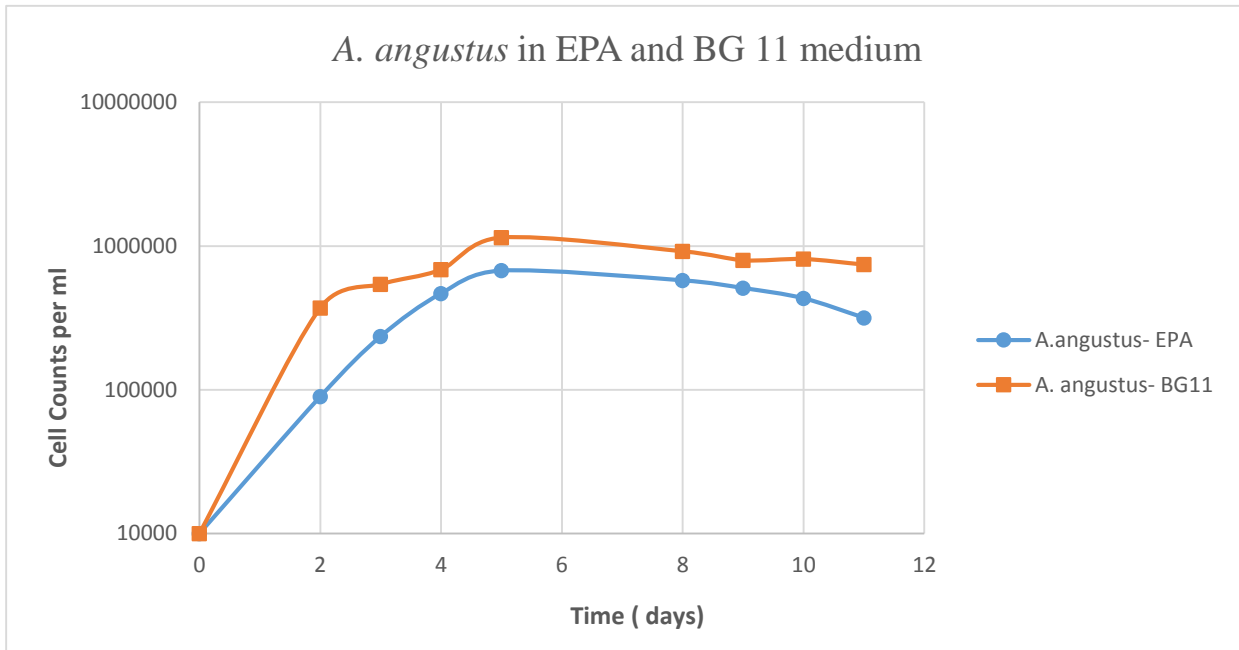


Figure 3 Logarithmic growth rate of *A.angustus* in EPA and BG11 medium

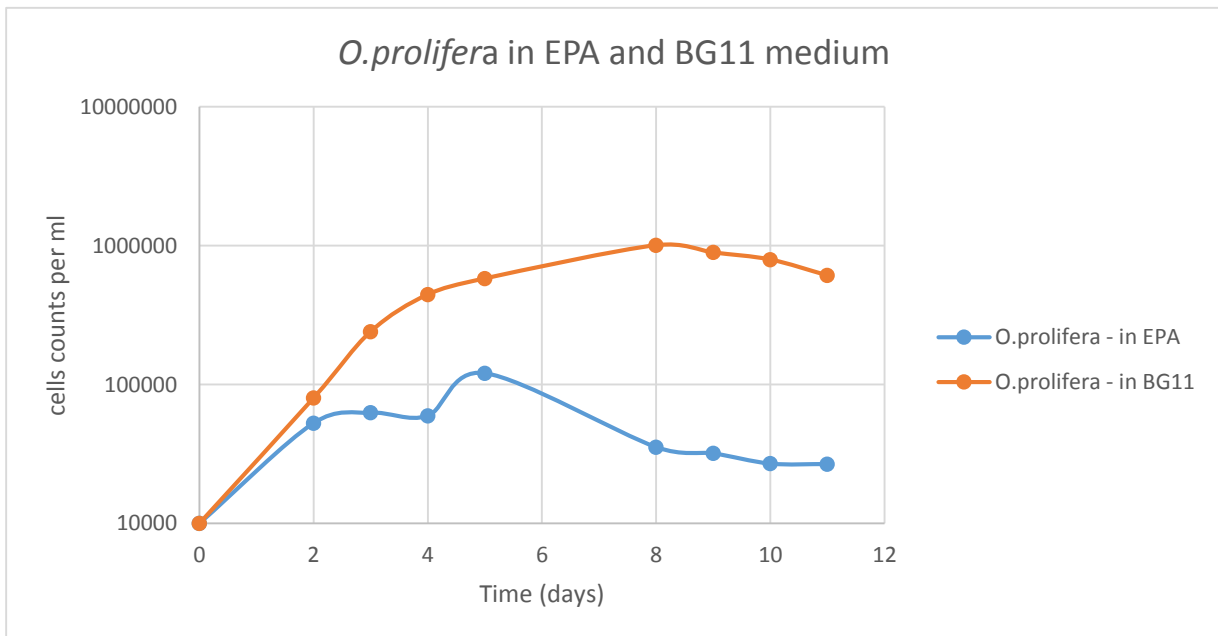


Figure 4 Logarithmic growth rate of *O.prolifera* in EPA medium and BG11 medium

Appendix C

Surface area (SA) calculations for each specie

C.1 Scenedesmus Subspicatus is oval

$$\text{Area} = \pi * R_1 * R_2$$

$$\text{Surface area} = 4 * \pi * R_1 * R_2$$

Table B.7 Calculations of surface area of 65 cells of Scenedesmus subspicatus

D₁³(μm)	D₂⁴(μm)	R₁⁵(μm)	R₂⁶(μm)	Area (μm²)	Surface Area (μm²)
4.78	3.85	2.39	1.925	14.4536824	57.8147296
4.48	2.75	2.24	1.375	9.676105373	38.70442149
4.18	2.32	2.09	1.16	7.616477229	30.46590892
4.41	3.52	2.205	1.76	12.19189277	48.76757108
3.32	2.23	1.66	1.115	5.814773843	23.25909537
4.05	2.59	2.025	1.295	8.238434035	32.95373614
4.1	2.8	2.05	1.4	9.016370916	36.06548366
4.41	2.85	2.205	1.425	9.871276817	39.48510727
4.61	2.95	2.305	1.475	10.68102232	42.72408929
3.37	2.32	1.685	1.16	6.140557001	24.562228
3.38	3.02	1.69	1.51	8.017030293	32.06812117
3.49	2.39	1.745	1.195	6.551084621	26.20433848
3.74	2.8	1.87	1.4	8.224689567	32.89875827
2.32	2.09	1.16	1.045	3.808238615	15.23295446
3.85	3.49	1.925	1.745	10.55300242	42.21200969
4.03	2.75	2.015	1.375	8.704175146	34.81670058
3.68	2.59	1.84	1.295	7.485786975	29.9431479
3.85	3.66	1.925	1.83	11.06704552	44.26818208
3.85	3.49	1.925	1.745	10.55300242	42.21200969
4.1	3.85	2.05	1.925	12.39751001	49.59004004
4.84	2.95	2.42	1.475	11.21391498	44.85565991
3.85	2.56	1.925	1.28	7.740884298	30.96353719
3.83	3.3	1.915	1.65	9.926647387	39.70658955
4.05	3.37	2.025	1.685	10.71950683	42.87802733
4.1	4.1	2.05	2.05	13.20254313	52.81017251
4.76	3.61	2.38	1.805	13.49596788	53.98387152
4.46	3.52	2.23	1.76	12.33012285	49.32049139
4.61	3.32	2.305	1.66	12.02067597	48.08270388

³ D₁ is diameter 1

⁴ D₂ is diameter 2

⁵ R₁ is radius 1

⁶ R₂ is radius 2

4.19	3.03	2.095	1.515	9.971179463	39.88471785
4.61	3.16	2.305	1.58	11.44136629	45.76546514
4.95	3.85	2.475	1.925	14.9677255	59.870902
4.89	3.74	2.445	1.87	14.36383285	57.4553314
3.89	3.89	1.945	1.945	11.88472355	47.53889419
6.05	4.78	3.025	2.39	22.71292949	90.85171795
5.58	3.95	2.79	1.975	17.31096092	69.24384368
4.95	4.05	2.475	2.025	15.74526968	62.98107872
5.71	4.15	2.855	2.075	18.61118758	74.44475032
5.98	5.91	2.99	2.955	27.75738481	111.0295392
4.67	4.53	2.335	2.265	16.61517669	66.46070675
6.06	4.76	3.03	2.38	22.65528126	90.62112505
6.64	5.14	3.32	2.57	26.80532516	107.2213006
3.89	3.16	1.945	1.58	9.654428384	38.61771353
3.85	2.93	1.925	1.465	8.859683982	35.43873593
4.29	3.37	2.145	1.685	11.35473687	45.41894747
4.02	2.7	2.01	1.35	8.524711666	34.09884666
3.25	2.9	1.625	1.45	7.40237769	29.60951076
1.1	1.1	0.55	0.55	0.950331778	3.801327111
5.51	5.5	2.755	2.75	23.80149134	95.20596537
5.34	4.78	2.67	2.39	20.0474452	80.1897808
5.32	3.85	2.66	1.925	16.08652518	64.34610073
5.13	4.22	2.565	2.11	17.00277068	68.01108272
5.39	3.38	2.695	1.69	14.30854082	57.23416328
3.2	2.95	1.6	1.475	7.414158662	29.65663465
4.1	4.05	2.05	2.025	13.0415365	52.16614601
3.68	3.68	1.84	1.84	10.63617609	42.54470435
4.84	3.77	2.42	1.885	14.33100321	57.32401283
3.49	2.75	1.745	1.375	7.537858873	30.15143549
4.43	2.79	2.215	1.395	9.70728568	38.82914272
4.82	4.1	2.41	2.05	15.52103851	62.08415402
3.68	3.32	1.84	1.66	9.595680601	38.3827224
5.32	3.49	2.66	1.745	14.58233062	58.32932248
4.67	3.61	2.335	1.805	13.24079202	52.96316807
4.53	4.31	2.265	2.155	15.33434936	61.33739745
4.27	3.2	2.135	1.6	10.7316805	42.92672202
4.46	3.95	2.23	1.975	13.83635944	55.34543778
5.49	5.25	2.745	2.625	22.63713856	90.54855426
Average area				12.40447268	49.61789073

C.2 *Scenedesmus quadricauda* is almost rectangle

Area = L* W

Surface area = 2*L*W ignoring the thickness because it is too small

Table B.8 Calculations of surface area of 65 cells of Scenedesmus quadricauda

Length (L) (μm)	Width (W) (μm)	Area (μm^2)	Surface area (μm^2)
29.84	20.24	603.9616	1207.9232
25.94	17.89	464.0666	928.1332
19.17	13.35	255.9195	511.839
21.26	15.01	319.1126	638.2252
26.43	17.45	461.2035	922.407
21.8	17.43	379.974	759.948
21.41	15.82	338.7062	677.4124
20.97	15.31	321.0507	642.1014
28.66	18.6	533.076	1066.152
23.57	17.59	414.5963	829.1926
26.94	17.21	463.6374	927.2748
22.4	17.1	383.04	766.08
27.78	20.55	570.879	1141.758
17.72	14.62	259.0664	518.1328
22.27	17.05	379.7035	759.407
29.17	19.68	574.0656	1148.1312
18.67	14.62	272.9554	545.9108
26.49	15.82	419.0718	838.1436
22.16	15.45	342.372	684.744
21.19	16.16	342.4304	684.8608
22.22	18.13	402.8486	805.6972
23.82	16.93	403.2726	806.5452
21.47	14.74	316.4678	632.9356
19.97	14.62	291.9614	583.9228
25.26	16.08	406.1808	812.3616
23.17	15.26	353.5742	707.1484
27.98	19.23	538.0554	1076.1108
18.7	14.51	271.337	542.674
21.35	14.68	313.418	626.836
18.27	16.08	293.7816	587.5632
19.86	12.14	241.1004	482.2008
22.48	15.07	338.7736	677.5472
18.54	13.54	251.0316	502.0632
18.32	12.13	222.2216	444.4432

21.51	12.35	265.6485	531.297
20.84	15.51	323.2284	646.4568
21.41	17.12	366.5392	733.0784
16.51	12.05	198.9455	397.891
24.73	16.58	410.0234	820.0468
27.3	17.7	483.21	966.42
24.29	15.11	367.0219	734.0438
15.75	15.39	242.3925	484.785
19.51	16.89	329.5239	659.0478
21.44	18.39	394.2816	788.5632
24.88	18.95	471.476	942.952
28.16	20.68	582.3488	1164.6976
29.73	16.22	482.2206	964.4412
29.19	14.8	432.012	864.024
23.8	15.28	363.664	727.328
24.09	17.6	423.984	847.968
21.03	17.84	375.1752	750.3504
25.2	21.2	534.24	1068.48
22.24	17.84	396.7616	793.5232
29.15	21.23	618.8545	1237.709
30.59	21.82	667.4738	1334.9476
23.85	14.39	343.2015	686.403
19.69	17.71	348.7099	697.4198
16.83	11.72	197.2476	394.4952
18.74	14.57	273.0418	546.0836
18.71	17.07	319.3797	638.7594
18.111	14.65	265.32615	530.6523
21.09	15.88	334.9092	669.8184
21.83	16.44	358.8852	717.7704
18.68	16.25	303.55	607.1
17.1	13.98	239.058	478.116
Average area		376.2345777	752.4691554

C.3 *Ankistrodesmus angustus* is a crescent shape

Area= perimeter of crescent

Surface area= 2* area

Table B.9 Calculations of surface area of 65 cells of *Ankistrodesmus angustus*

Area (μm^2)	Surface Area (μm^2)
108.45	216.9
41.53	83.06
40.66	81.32
33.67	67.34
34.73	69.46
30.92	61.84
28.28	56.56
38.88	77.76
43.6	87.2
49.57	99.14
26.63	53.26
46.35	92.7
51.88	103.76
92.42	184.84
62.82	125.64
55.59	111.18
66.06	132.12
46.67	93.34
48.89	97.78
56.02	112.04
59.59	119.18
78.8	157.6
67.03	134.06
32.26	64.52
28.7	57.4
50.09	100.18
41.49	82.98
48.63	97.26
48.75	97.5
57.05	114.1
42.77	85.54
33.14	66.28
33.37	66.74
64.74	129.48
39.34	78.68

35.9	71.8
114.85	229.7
66.49	132.98
43.12	86.24
52.11	104.22
32.55	65.1
89.26	178.52
52.71	105.42
45.5	91
40.05	80.1
87.64	175.28
73.75	147.5
125.73	251.46
43.01	86.02
38.92	77.84
53.45	106.9
45.31	90.62
39.13	78.26
39.63	79.26
68.82	137.64
60.1	120.2
57	114
48.04	96.08
42.9	85.8
55.92	111.84
37.73	75.46
53.98	107.96
99.78	199.56
43.08	86.16
79.49	158.98
48.75	97.5
35.22	70.44
95.68	191.36
Average surface area	108.09

C.4 *Oscillatoria prolifera* is filamentous

Area= perimeter of filamentous shape

Surface area= area * 2

Table B.10 Calculations of surface area of 65 cells of *Oscillatoria prolifera*

Area (μm^2)	Surface Area (μm^2)
6910.72	13821.44
289.19	578.38
892.41	1784.82
501.26	1002.52
216.38	432.76
1050.95	2101.9
133.61	267.22
282.63	565.26
1281.32	2562.64
337.19	674.38
218.48	436.96
270.45	540.9
336.95	673.9
521.25	1042.5
264.74	529.48
259.95	519.9
1127.41	2254.82
516.23	1032.46
3788.13	7576.26
972.95	1945.9
733.11	1466.22
450.93	901.86
646.54	1293.08
721.88	1443.76
1091.83	2183.66
767.51	1535.02
954.45	1908.9
1646.36	3292.72
504.6	1009.2
481.9	963.8
5651.38	11302.76
438.59	877.18
676.28	1352.56
1476.5	2953
486.77	973.54

874.16	1748.32
331.64	663.28
366.47	732.94
204.92	409.84
1291.73	2583.46
247.87	495.74
874.8	1749.6
1648.91	3297.82
1077.67	2155.34
2152.76	4305.52
297.14	594.28
845.36	1690.72
1593.11	3186.22
277.47	554.94
637.39	1274.78
1257.03	2514.06
1336.73	2673.46
431.24	862.48
541.31	1082.62
2905.51	5811.02
4172.49	8344.98
1411.56	2823.12
1322.12	2644.24
359.64	719.28
2534.1	5068.2
628.86	1257.72
1983.78	3967.56
1166.36	2332.72
2574.92	5149.84
4228.99	8457.98
1021.01	2042.02
Average surface area	2348.42

Appendix D

Wood stand

Test flasks were placed in an algae incubator, which was prepared using a high wooden stand (55 cm width, 150 cm length and 40 cm height).



Figure-1 wooden stand with 5 fluorescent lamps

Appendix E

Measurement of Alkalinity, Hardness,
Conductivity, pH and Temperature of
culture medium at the beginning of each
test

Table 11 Measurement of alkalinity, hardness, conductivity and pH of medium at the beginning of each test for each species

Parameters/ test type	pH unit at 25°C	Temperature (°C)	Alkalinity (mg/L)	Hardness (mg/L CaCO ₃)		Conductivity (µs/cm) at 25°C
				Calcium	Total	
<i>S. subspicatus</i> - cell density	7.1	25.5	19.8	41	162	2.59
<i>S. subspicatus</i> SA	7.1	25	23.8	47	163	2.41
<i>S. quadricauda</i> - cell density	7.1	26	18.4	53	176	2.55
<i>S. quadricauda</i> - SA	7.1	25	24.2	53	176	2.55
<i>A. angustus</i> - cell density	7.15	25.2	25.8	44	180	2.35
<i>A. angustus</i> - SA	7.1	26	22.2	44	169	2.35
<i>O. prolifera</i> - cell density and SA	7.1	26.1	19	43	190	2.48
Multispecies - cell density	7.1	25.9	19.6	42	187	2.46
Multispecies - SA	7.1	25.9	19.6	42	187	2.46

Appendix F

Algae growth, average pH and temperature
results

S. subspicatus- Cell density test

The initial pH was 7.1

Table 12 Measurements of the average pH at 25°C and temperature of 3 replicates at 48 and 96 hours for *S. subspicatus* inhibition test based on cell density

Cu Conc. (µg/L)	pH 48 h	T 48 h (°C)	pH 96 h	T 96 h (°C)
Control	8.13	22.2	8.335	21.5
50	8.05	22.5	8.14	22.2
100	7.98	23.4	8.35	22.8
200	7.96	23.5	8.29	22.6
350	7.91	23.4	7.75	23.4
500	7.74	22.8	7.66	22.8
1000	7.55	22.4	7.55	22.4
1500	7.35	21.7	7.45	21.6
2000	7.28	21.1	7.37	21.4

S. subspicatus- Surface Area test

The initial pH was 7.1

Table 13 Measurements of the average pH at 25°C and temperature of 3 replicates at 48 and 96 hours for *S. subspicatus* inhibition test based on SA

Cu Conc. (µg/L)	pH 48 h	T 48 h	pH 96 h	T 96 h
Control	9.8	23.5	10.2	23.6
50	9.4	23.5	10.02	23.3
100	9.15	23.1	9.33	23.6
200	8.89	22.9	9.17	24
350	8.75	23.6	8.89	23.6
500	8.42	23.9	8.66	23.7
1000	8.19	22.7	8.41	22.9
1500	8.07	23.5	8.29	23.8
2000	7.99	24.1	8.18	23.8

S. quadricauda- Cell density test

The initial pH was 7.1

Table 14 Measurements of the average pH at 25°C and temperature of 3 replicates at 48 and 96 hours for *S. quadricauda* inhibition test based on cell density

Cu Conc. (µg/L)	pH 48 h	T 48 h	pH 96 h	T 96 h
Control	7.6	23.9	8.1	23.9
50	7.51	23.6	7.84	23.8
100	7.45	23.6	7.63	23.7
200	7.32	23.7	7.58	23.6
350	7.27	23.9	7.44	23.7
500	7.18	24.1	7.41	24
1000	7.28	23.8	7.42	23.8
1500	7.25	23.5	7.38	23.8
2000	7.18	23.9	7.29	24

S. quadricauda- Surface Area test

The initial pH was 7.1

Table 15 Measurements of the average pH at 25°C and temperature of 3 replicates at 48 and 96 hours for *S. quadricauda* inhibition test based on SA

Cu Conc. (µg/L)	pH 48 h	T 48 h	pH 96 h	T 96 h
Control	9.3	21.3	9.7	23.7
50	9.06	23.2	9.3	23.8
100	8.5	22.8	8.9	23.6
200	8.2	23.7	8.37	23.8
350	7.92	23.5	8.16	23.5
500	7.78	23.7	7.94	23.6
1000	7.62	223.8	7.85	23.4
1500	7.56	23.6	7.62	24.1
2000	7.43	23.9	7.59	23.8

A.angustus- Cell density test

The initial pH was 7.15

Table 16 Measurements of pH at 25°C and temperature at 48 and 96 hours for A.angustus inhibition test based on cell density

Cu Conc. (µg/L)	pH 48 h	T 48 h	pH 96 h	T 96 h
Control	7.78	22.5	8.31	21
50	7.54	20.4	6.84	20.9
100	8.13	22	8.46	21.6
200	7.64	21.7	7.38	21.3
350	7.81	21.9	8.19	20.9
500	8.13	23.4	8.7	23
1000	7.8	23.2	8.61	24.4
1500	7.78	22.8	8.15	24.3
2000	7.51	22	7.59	23.3

A.angustus- Surface Area test

The initial pH was 7.1

Table 17 Measurements of the average pH at 25°C and temperature of 3 replicates at 48 and 96 hours for A.angustus inhibition test based on SA

Cu Conc. (µg/L)	pH 48 h	T 48 h	pH 96 h	T 96 h
Control	8.9	24.2	9.31	3.92
50	8.57	23.5	8.88	23.5
100	8.24	23.5	8.62	23.6
200	8.06	23.8	8.47	23.9
350	7.89	23.7	8.19	23.6
500	7.74	23.7	7.86	23.8
1000	7.66	23.5	7.74	23.5
1500	7.59	24.1	7.7	24
2000	7.37	23.7	7.4	23.8

O. prolifera - Cell density and Surface Area test

The initial pH was 7.28

Table 18 Measurements of the average pH at 25°C and temperature of 3 replicates at 48 and 96 hours for *O.prolifera* inhibition test based on cell density and SA

Cu Conc. (µg/L)	pH 48 h	T 48 h	pH 96 h	T 96 h
Control	7.71	23.6	7.97	24
50	7.55	23.6	7.84	23.9
100	7.48	23.5	7.8	23.6
200	7.42	23.7	7.73	23.7
350	7.32	23.6	7.6	23.7
500	7.26	23.5	7.48	23.4
1000	7.19	23.7	7.37	23.8
1500	7.12	23.8	7.25	23.7
2000	7.06	23.4	7	23.5

Multispecies test- Cell density

The initial pH was 7.1

Table 19 Measurements of the average pH at 25°C and temperature of 3 replicates at 48 and 96 hours for multispecies inhibition test based on cell density

Cu Conc. (µg/L)	pH 48 h	T 48 h	pH 96 h	T 96 h
Control Q ¹	7.1	23.7	7.7	23.7
Control S ²	7.21	23.5	7.65	23.6
Control A ³	7.14	23.5	7.69	23.7
Control O ⁴	7.19	23.9	7.54	23.8
Control	8.27	24	8.64	23.9
50	8.15	24	8.58	24.1
100	7.95	23.6	8.49	23.7
200	7.81	23.8	8.32	23.6
350	7.79	23.7	8.18	23.7
500	7.53	23.6	8.02	23.5
1000	7.47	23.9	7.95	23.8
1500	7.38	24	7.47	23.9
2000	7.31	24	7.35	23.8

¹Control of *S. quadricauda*

²Control of *S. subspicatus*

³Control of *A. angustus*

⁴Control of *O.prolifera*

Multispecies test – Surface area test

The initial pH is 7.1

Table 20 Measurements of pH at 25°C and temperature at 48 and 96 hours for multispecies inhibition test based on SA

Cu Conc. (µg/L)	pH 48 h	T 48 h	pH 96 h	T 96 h
Control Q ¹	7.4	23.7	7.43	23.7
Control S ²	7.29	23.5	7.36	23.6
Control A ³	7.26	23.5	7.59	23.7
Control O ⁴	7.29	23.8	7.62	23.7
Control	8.47	24	8.72	23.9
50	8.35	23.9	8.63	24.1
100	8.17	23.7	8.57	23.7
200	7.96	23.6	8.41	23.8
350	7.79	23.8	8.28	23.7
500	7.63	23.6	8.09	23.6
1000	7.57	23.7	7.85	23.7
1500	7.44	23.6	7.67	23.9
2000	7.33	24.1	7.52	23.8

¹Control of *S. quadricauda*

²Control of *S. subspicatus*

³Control of *A. angustus*

⁴Control of *O.prolifera*

Appendix G

Algal growth of specie over 96 h in single-species and multi-species toxicity test based on cell density and surface area

1. Single species toxicity bioassays- Cell density

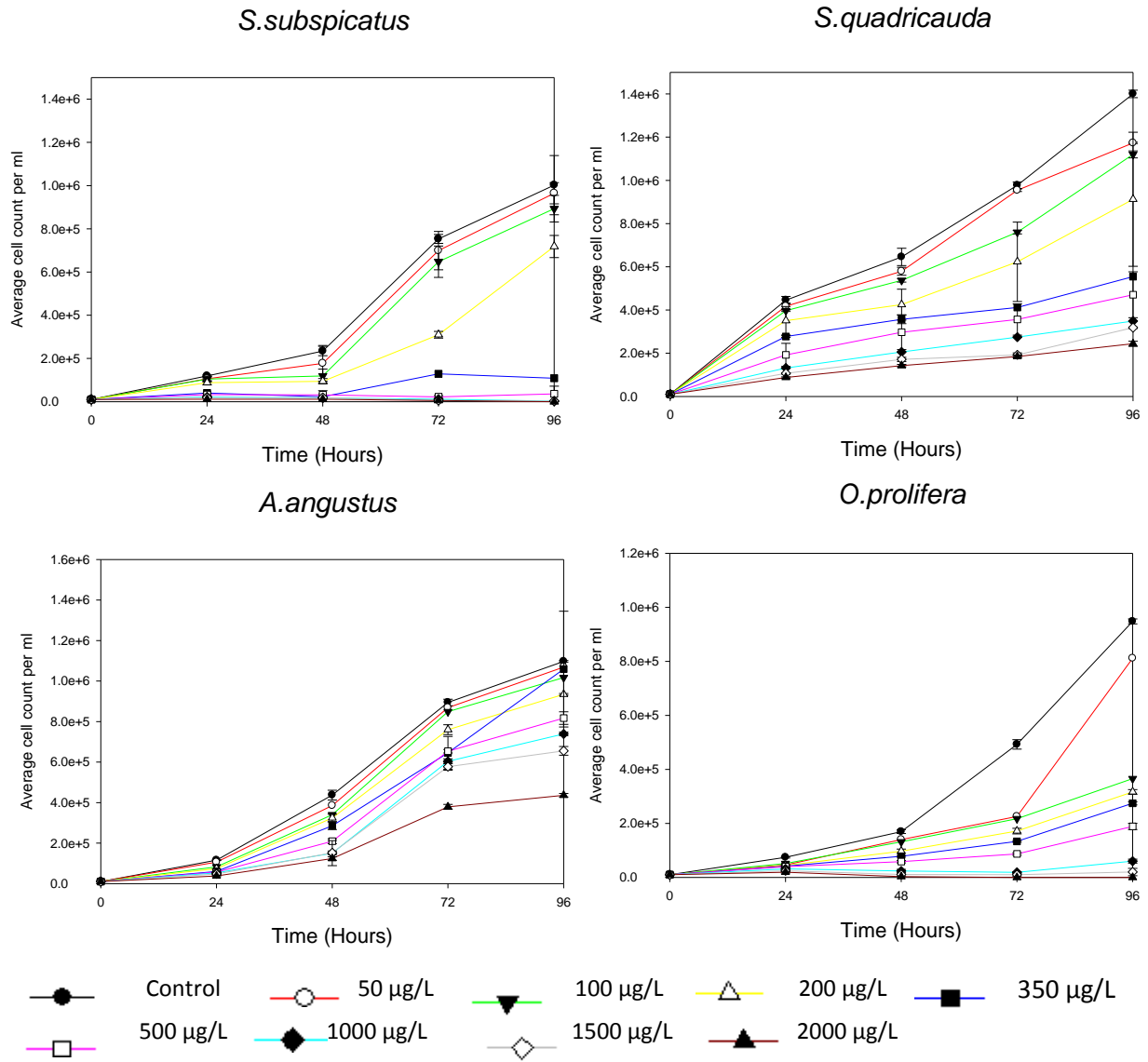


Figure 3.1 Algae growth of *S.subspicatus*, *S.quadricauda*, *A.angustus* and *O.prolifera* obtained over 96h in toxicity tests based on cell concentration, following addition of copper sulphate. Data points are average result of three replicate samples and error bars represent the standard deviation of the replicate results.

2. Single-species toxicity tests- based on Surface area

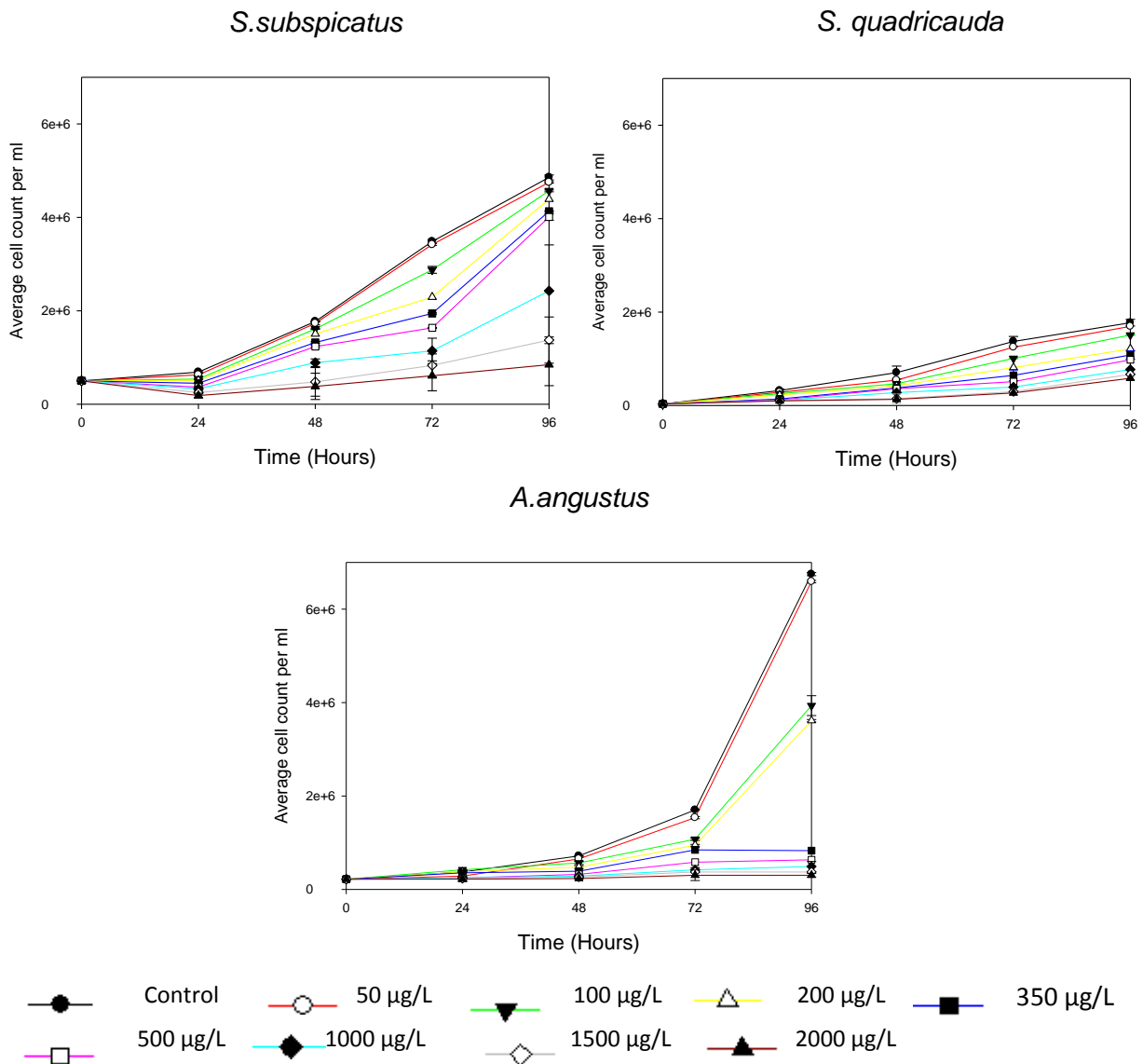
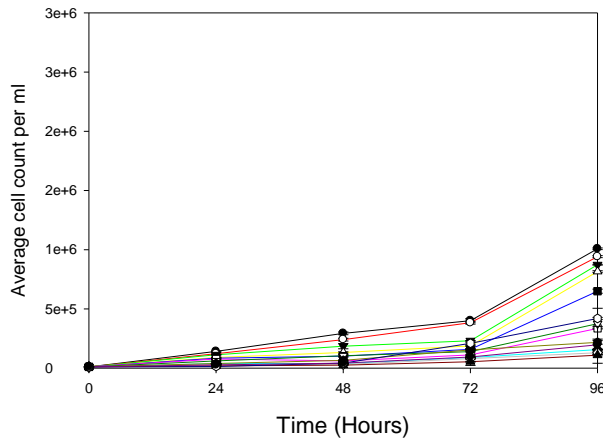


Figure 3.2 Algal growth of *S. subspicatus*, *S. quadricauda*, *A. angustus* and *O. prolifera* obtained, over 96h, in toxicity tests based on cell surface area, following addition of copper sulphate. Data points are average result of three replicate samples and error bars represent the standard deviation of the replicate results.

3. Multispecies toxicity bioassays- Cell density and surface area

Multispecies test- Cell density



Multispecies test- SA

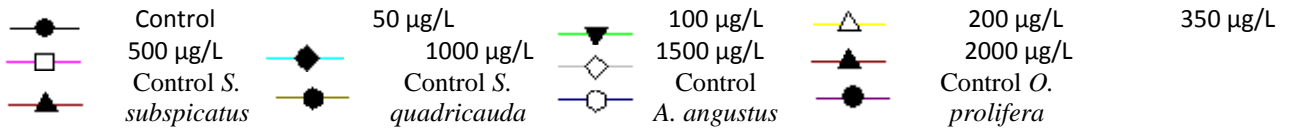
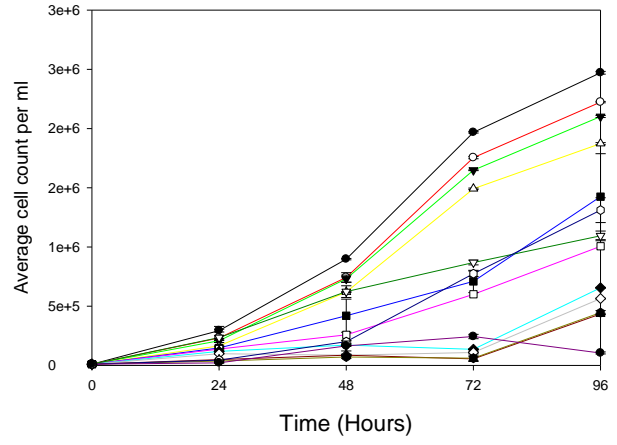


Figure 3.3 Algal growth of multispecies obtained in toxicity tests based on cell concentration and cell surface area, following addition of copper sulphate. Data points are average result of three replicate samples and error bars represent the standard deviation of the replicate results

Appendix H

% Growth Inhibition Relatively To The
Control For Each Specie In Single And
Multi-Specie Tests

S.subspicatus- Cell density- Single species tests

Table 21 % growth inhibition of *S. subspicatus* compared to the control in single-species tests based on cell density

<i>Cu</i> <i>conc.</i> ($\mu\text{g/L}$)	<i>24h</i>	% <i>Growth</i> <i>inhibition</i>	<i>48h</i>	% <i>Growth</i> <i>inhibition</i>	<i>72h</i>	% <i>Growth</i> <i>inhibition</i>	<i>96h</i>	% <i>Growth</i> <i>inhibition</i>
0	119048	0	234286	0	753333	0	1001905	0
50	111429	6.4	224286	4.268293	747143	0.821745	987143	1.473384
100	104762	12	119048	49.18699	647619	14.03287	893333	10.8365
200	87619	26.4	94286	59.7561	309524	58.91277	718095	28.327
350	37143	68.8	25714	89.02439	240000	68.14159	120000	88.02281
500	32381	72.8	31429	86.58537	21905	97.09229	36190	96.38783
1000	21429	82	17143	92.68293	11429	98.48293	2857	99.71483
1500	18571	84.4	18571	92.07317	5714	99.24147	2857	99.71483
2000	11429	90.4	11429	95.12195	7143	99.05183	0	100

S.subspicatus- SA- Single species tests

Table 22 % growth inhibition of *S. subspicatus* compared to the control in single-species tests based on SA

<i>Cu</i> <i>conc.</i> ($\mu\text{g/L}$)	<i>24h</i>	% <i>Growth</i> <i>inhibition</i>	<i>48h</i>	% <i>Growth</i> <i>inhibition</i>	<i>72h</i>	% <i>Growth</i> <i>inhibition</i>	<i>96h</i>	% <i>Growth</i> <i>inhibition</i>
<i>control</i>	685714	0.0	1771429	0	3483810	0	4855238	0
50	628571	8.3	1729524	2.4	3417143	1.9	4748571	2.2
100	538095	21.5	1611429	9.0	2876190	17.4	4567619	5.9
200	488571	28.8	1497143	15.5	2282857	34.5	4400000	9.4
350	448571	34.6	1321905	25.4	1940952	44.3	4131429	14.9
500	363810	46.9	1232381	30.4	1632381	53.1	4005714	17.5
1000	325714	52.5	934286	47.3	1297143	62.8	2994286	38.3
1500	231429	66.3	651429	63.2	971429	72.1	1657143	65.9
2000	214286	68.8	542857	69.4	791429	77.3	1105714	77.2

S. quadricauda- Cell density- Single-species tests

Table 23% growth inhibition of *S. quadricauda* compared to the control in single-species tests based on cell density

<i>Cu</i> <i>conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth</i> <i>inhibition</i>	48h	% <i>Growth</i> <i>inhibition</i>	72h	% <i>Growth</i> <i>inhibition</i>	96h	% <i>Growth</i> <i>inhibition</i>
<i>control</i>	445714	0.0	645714	0.0	977143	0.0	1400000	0.0
50	417143	6.4	579048	10.3	954286	2.3	1173333	16.2
100	397143	10.9	537143	16.8	760000	22.2	1120000	20.0
200	388571	12.8	465714	27.9	728571	25.4	1091429	22.0
350	277143	37.8	357143	44.7	411429	57.9	554286	60.4
500	222857	50.0	342857	46.9	397143	59.4	531429	62.0
1000	137143	69.2	208571	67.7	268571	72.5	357143	74.5
1500	106667	76.1	171429	73.5	192381	80.3	318095	77.3
2000	87619	80.3	142857	77.9	184762	81.1	243810	82.6

S. quadricauda- SA- Single-species tests

Table 24 % growth inhibition of *S. quadricauda* compared to the control in single-species tests based on SA

<i>Cu</i> <i>conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth</i> <i>inhibition</i>	48h	% <i>Growth</i> <i>inhibition</i>	72h	% <i>Growth</i> <i>inhibition</i>	96h	% <i>Growth</i> <i>inhibition</i>
<i>control</i>	337143	0.0	782857	0.0	1431429	0.0	1811429	0.0
50	281905	16.4	552381	29.4	1198095	16.3	1700952	6.1
100	251429	25.4	462857	40.9	1007619	29.6	1506667	16.8
200	220000	34.7	414286	47.1	814286	43.1	1220000	32.6
350	148571	55.9	382857	51.1	711429	50.3	1131429	37.5
500	112381	66.7	348571	55.5	497143	65.3	986667	45.5
1000	112381	66.7	264762	66.2	379048	73.5	771429	57.4
1500	104762	68.9	142857	81.8	295238	79.4	664762	63.3
2000	93333	72.3	125714	83.9	260952	81.8	579048	68.0

A. angustus Cell density- Single-species tests

Table 25 % growth inhibition of *A. angustus* compared to the control in single-species tests based on cell density

<i>Cu</i> <i>conc.</i> ($\mu\text{g/L}$)	<i>24h</i>	% <i>Growth</i> <i>inhibition</i>	<i>48h</i>	% <i>Growth</i> <i>inhibition</i>	<i>72h</i>	% <i>Growth</i> <i>inhibition</i>	<i>96h</i>	% <i>Growth</i> <i>inhibition</i>
<i>control</i>	115714	0.0	437143	0.0	894286	0.0	1097143	0.0
<i>50</i>	100000	13.6	381429	12.7	878571	1.8	1068571	2.6
<i>100</i>	82857	28.4	340000	22.2	861429	3.7	1018571	7.2
<i>200</i>	74286	35.8	324286	25.8	760000	15.0	934286	14.8
<i>350</i>	67143	42.0	295714	32.4	691429	22.7	894286	18.5
<i>500</i>	57143	50.6	209524	52.1	653333	26.9	817143	25.5
<i>1000</i>	53333	53.9	150476	65.6	603810	32.5	739048	32.6
<i>1500</i>	47619	58.8	152381	65.1	577143	35.5	655238	40.3
<i>2000</i>	38095	67.1	123810	71.7	379048	57.6	436190	60.2

A. angustus- SA- Single-species tests

Table 26% growth inhibition of *A. angustus* compared to the control in single-species tests based on SA

<i>Cu</i> <i>conc.</i> ($\mu\text{g/L}$)	<i>24h</i>	% <i>Growth</i> <i>inhibition</i>	<i>48h</i>	% <i>Growth</i> <i>inhibition</i>	<i>72h</i>	% <i>Growth</i> <i>inhibition</i>	<i>96h</i>	% <i>Growth</i> <i>inhibition</i>
<i>control</i>	372381	0.0	717143	0.0	1700952	0.0	6716667	0.0
<i>50</i>	364762	2.0	651429	9.2	1535238	9.7	6588889	1.9
<i>100</i>	360000	3.3	565714	21.1	1074286	36.8	3933333	41.4
<i>200</i>	346667	6.9	478095	33.3	944762	44.5	4050000	39.7
<i>350</i>	330000	11.4	371429	48.2	865714	49.1	3611111	46.2
<i>500</i>	280000	24.8	337143	53.0	562857	66.9	3150000	53.1
<i>1000</i>	212857	42.8	300000	58.2	465714	72.6	588571	91.2
<i>1500</i>	207619	44.2	249524	65.2	367619	78.4	331429	95.1
<i>2000</i>	180952	51.4	241905	66.3	240000	85.9	264762	96.1

O.prolifera- Cell density and SA- Single-species tests

Table 27% growth inhibition of *O.prolifera* compared to the control in single-species tests based on cell density and SA

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth</i> <i>inhibition</i>	48h	% <i>Growth</i> <i>inhibition</i>	72h	% <i>Growth</i> <i>inhibition</i>	96h	% <i>Growth</i> <i>inhibition</i>
<i>control</i>	74286	0.0	169524	0.0	492381	0.0	947619	0.0
50	50000	32.7	140000	17.4	240000	51.3	791429	16.5
100	47143	36.5	130000	23.3	201429	59.1	377143	60.2
200	43810	41.0	97143	42.7	171429	65.2	316190	66.6
500	41905	43.6	78095	53.9	133333	72.9	274286	71.1
350	40000	46.2	58095	65.7	86667	82.4	188571	80.1
1000	31429	57.7	23571	86.1	18571	96.2	60000	93.7
1500	24286	67.3	10714	93.7	10000	98.0	20000	97.9
2000	19048	74.4	2857	98.3	0	100.0	0	100.0

Multi-species test- Cell density

Table 28 % growth inhibition of mixed species compared to the control in multi-species tests based on cell density

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth</i> <i>inhibition</i>	48h	% <i>Growth</i> <i>inhibition</i>	72h	% <i>Growth</i> <i>inhibition</i>	96h	% <i>Growth</i> <i>inhibition</i>
0	140952	0.0	293333	0.0	400000	0.0	1007619	0.0
50	121905	13.5	240000	18.2	382857	4.3	940952	6.6
100	112381	20.3	184762	37.0	230476	42.4	872381	13.4
200	89524	36.5	131429	55.2	186667	53.3	815238	19.1
350	83810	40.5	100952	65.6	158095	60.5	649524	35.5
500	77143	45.3	60000	79.5	111429	72.1	334286	66.8
1000	38095	73.0	38095	87.0	89524	77.6	156190	84.5
1500	30476	78.4	30476	89.6	80000	80.0	129524	87.1
2000	19048	86.5	24762	91.6	53333	86.7	108571	89.2

Multi-species tests-Surface area

Table 29 % growth inhibition of mixed species compared to the control in multi-species tests based on SA

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth inhibition</i>	48h	% <i>Growth inhibition</i>	72h	% <i>Growth inhibition</i>	96h	% <i>Growth inhibition</i>
0	293333	0.0	897143	0.0	1967619	0.0	2470476	0.0
50	230476	21.4	742857	17.2	1754286	10.8	2222857	10.0
100	207619	29.2	731429	18.5	1647619	16.3	2100952	15.0
200	163810	44.2	619048	31.0	1491429	24.2	1872381	24.2
350	151429	48.4	500000	44.3	762857	61.2	1634286	33.8
500	137143	53.2	257143	71.3	602857	69.4	1014286	58.9
1000	118095	59.7	171429	80.9	135238	93.1	655238	73.5
1500	99048	66.2	85714	90.4	108571	94.5	563810	77.2
2000	49524	83.1	85714	90.4	55238	97.2	434286	82.4

S.subspicatus- Cell density- Multi-species tests

Table 30 % growth inhibition of *S. subspicatus* compared to the control in multi-species tests based on cell density

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth inhibition</i>	48h	% <i>Growth inhibition</i>	72h	% <i>Growth inhibition</i>	96h	% <i>Growth inhibition</i>
0	81905	0.0	175238	0.0	184762	0.0	413333	0.0
50	60952	25.6	150476	14.1	161905	12.4	384762	6.9
100	57143	30.2	146667	16.3	165714	10.3	354286	14.3
200	53333	34.9	87619	50.0	154286	16.5	306667	25.8
350	51429	37.2	60952	65.2	135238	26.8	238095	42.4
500	43810	46.5	55238	68.5	102857	44.3	156190	62.2
1000	32381	60.5	38095	78.3	78095	57.7	55238	86.6
1500	26667	67.4	32381	81.5	76190	58.8	15238	96.3
2000	19048	76.7	20952	88.0	19048	89.7	7619	98.2

S.subspicatus- SA- Multi-species tests

Table 31 % growth inhibition of *S. subspicatus* compared to the control in multi-species tests based on SA

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth inhibition</i>	48h	% <i>Growth inhibition</i>	72h	% <i>Growth inhibition</i>	96h	% <i>Growth inhibition</i>
0	234286	0.0	647619	0.0	868571	0.0	1093333	0.0
50	150476	35.8	575238	11.2	659048	24.1	878095	19.7
100	148571	36.6	584762	9.7	609524	29.8	769524	29.6
200	121905	48.0	466667	27.9	550476	36.6	689524	36.9
350	104762	55.3	411429	36.5	493333	43.2	645714	40.9
500	106667	54.5	369524	42.9	356190	59.0	400000	63.4
1000	104762	55.3	321905	50.3	72381	91.7	287619	73.7
1500	78095	66.7	259048	60.0	76190	91.2	182857	83.3
2000	45714	80.5	226667	65.0	66667	92.3	150476	86.2

S.quadricauda- Cell density- Multispecies tests

Table 32 % growth inhibition of *S. quadricauda* compared to the control in multi-species tests based on cell density

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth inhibition</i>	48h	% <i>Growth inhibition</i>	72h	% <i>Growth inhibition</i>	96h	% <i>Growth inhibition</i>
0	38095	0.0	62857	0.0	87619	0.0	116190	0.0
50	38095	0.0	51429	18.2	72381	17.4	102857	11.5
100	30476	20.0	47619	24.2	68571	21.7	100952	13.1
200	28571	25.0	41905	33.3	57143	34.8	89524	23.0
350	19048	50.0	34286	45.5	49524	43.5	78095	32.8
500	17143	55.0	31429	50.0	42857	51.1	74286	36.1
1000	11429	70.0	26667	57.6	38095	56.5	70476	39.3
1500	9524	75.0	22857	63.6	32381	63.0	60952	47.5
2000	7619	80.0	19048	69.7	26667	69.6	55238	52.5

S. quadricauda- SA- Multi-species tests

Table 33 % growth inhibition of *S. quadricauda* compared to the control in multi-species tests based on SA

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth inhibition</i>	48h	% <i>Growth inhibition</i>	72h	% <i>Growth inhibition</i>	96h	% <i>Growth inhibition</i>
0	36190	0.0	62857	0.0	80000	0.0	573333	0.0
50	26667	26.3	30476	51.5	74286	7.1	571429	0.3
100	26667	26.3	26667	57.6	70476	11.9	560000	2.3
200	24762	31.6	22857	63.6	45714	42.9	504762	12.0
350	17143	52.6	19048	69.7	32381	59.5	401905	29.9
500	17143	52.6	17143	72.7	32381	59.5	318095	44.5
1000	13333	63.2	7619	87.9	19048	76.2	308571	46.2
1500	7619	78.9	7619	87.9	13333	83.3	262857	54.2
2000	5714	84.2	5714	90.9	11429	85.7	213333	62.8

A. angustus- Cell density- Multi-species tests

Table 34 % growth inhibition of *A. angustus* compared to the control in multi-species tests based on cell density

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth inhibition</i>	48h	% <i>Growth inhibition</i>	72h	% <i>Growth inhibition</i>	96h	% <i>Growth inhibition</i>
0	13333	0.0	41905	0.0	59048	0.0	152381	0.0
50	9524	28.6	15238	63.6	47619	19.4	133333	12.5
100	9524	28.6	13333	68.2	30476	48.4	121905	20.0
200	5714	57.1	11429	72.7	22857	61.3	89524	41.3
350	7619	42.9	7619	81.8	19048	67.7	57143	62.5
500	3810	71.4	5714	86.4	17143	71.0	55238	63.8
1000	1905	85.7	3810	90.9	13333	77.4	7619	95.0
1500	0	100.0	1905	95.5	7619	87.1	1905	98.8
2000	0	100.0	1905	95.5	5714	90.3	0	100.0

A.angustus - SA- Multi-species tests

Table 35 % growth inhibition of *A. angustus* compared to the control in multi-species tests based on SA

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth inhibition</i>	48h	% <i>Growth inhibition</i>	72h	% <i>Growth inhibition</i>	96h	% <i>Growth inhibition</i>
0	42857	0.0	188571	0.0	773333	0.0	1310476	0.0
50	40476	5.6	127619	32.3	327619	57.6	655238	50.0
100	37143	13.3	123810	34.3	302857	60.8	596190	54.5
200	33810	21.1	112381	40.4	270476	65.0	554286	57.7
350	33428	22.0	81905	56.6	194286	74.9	472381	64.0
500	25714	40.0	80000	57.6	142857	81.5	422857	67.7
1000	22857	46.7	78095	58.6	97143	87.4	373333	71.5
1500	17143	60.0	74286	60.6	57143	92.6	316190	75.9
2000	9524	77.8	36190	80.8	43810	94.3	293333	77.6

O.prolifera- Cell density- Multi-species tests

Table 36 % growth inhibition of *O.prolifera* compared to the control in multi-species tests based on cell density

<i>Cu conc.</i> ($\mu\text{g/L}$)	24h	% <i>Growth inhibition</i>	48h	% <i>Growth inhibition</i>	72h	% <i>Growth inhibition</i>	96h	% <i>Growth inhibition</i>
0	20952	0.0	45714	0.0	93333	0.0	200000	0.0
50	17143	18.2	30476	33.3	80000	14.3	154286	22.9
100	17143	18.2	26667	41.7	51429	44.9	144762	27.6
200	17143	18.2	19048	58.3	45714	51.0	123810	38.1
350	9524	54.5	15238	66.7	38095	59.2	108571	45.7
500	9524	54.5	13333	70.8	32381	65.3	95238	52.4
1000	9524	54.5	11429	75.0	28571	69.4	80000	60.0
1500	5714	72.7	9524	79.2	26667	71.4	62857	68.6
2000	5714	72.7	7619	83.3	22857	75.5	60952	69.5

O.prolifera- SA- Multi-species tests

Table 37 % growth inhibition of *O.prolifera* compared to the control in multi-species tests based on SA

<i>Cu conc.</i> ($\mu\text{g/L}$)	<i>24h</i>	<i>% Growth inhibition</i>	<i>48h</i>	<i>% Growth inhibition</i>	<i>72h</i>	<i>% Growth inhibition</i>	<i>96h</i>	<i>% Growth inhibition</i>
0	20952	0.0	38095	0.0	97143	0.0	200000	0.0
50	15238	27.3	30476	20.0	78095	19.6	154286	22.9
100	11429	45.5	19048	50.0	45714	52.9	142857	28.6
200	9524	54.5	15238	60.0	40000	58.8	127619	36.2
350	7619	63.6	13333	65.0	34286	64.7	104762	47.6
500	5714	72.7	11429	70.0	26667	72.5	97143	51.4
1000	3810	81.8	9524	75.0	20952	78.4	85714	57.1
1500	1905	90.9	7619	80.0	20952	78.4	66667	66.7
2000	1905	90.9	7619	80.0	20952	78.4	59048	70.5

