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Growth dynamic on a co-cultivation of two Chlorophyta microalgae exposed to copper

Dinâmica de crescimento no co-cultivo de duas microalgas Chlorophyta expostas ao cobre.

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Abstract: Aim: Copper is an essential nutrient for the phytoplankton, but it can also act as a toxic agent, depending on its concentration. Considering the continuous increase of this metal in the natural aquatic ecosystems, understanding its actions in co-cultivation scenarios is of great relevance. Experiments with the combination of different species resemble more accurately the natural conditions, in contrast of results obtained in single-species tests, which cannot be directly used to describe observed effects on the environment. **Methods:** Therefore, growth parameters were investigated and compared on the co-cultivation of *Chlorella sorokiniana* and *Kirchneriella obesa* and their separate cultures exposed to three different free copper concentrations (control $6x10^{-9}$, intermediate $2x10^{-7}$ and high $1.5x10^{-6}$ mol.L^{-1} Cu^{2+}). **Results:** *C. sorokiniana* registered more cells in the control of the unialgal culture while *K. obesa* had higher cell density in the control of the co-cultivation. Growth rates decreased with the increment of copper in the unialgal conditions. However, both species maintained a high growth rate in the co-cultivation intermediate copper concentrations. Biovolume varied despite the cultivation method, being strongly related to the metal's concentration. The maximum photosynthetic efficiency decreased in higher copper. **Conclusions:** According to the results observed, no competitive exclusion occurred and both species were affected by copper in unialgal and co-cultivation conditions, with *K. obesa* being favored by the co-cultivation, which seems to have an attenuation effect on copper toxicity until intermediate concentrations. Ecologically, the results suggest that communities deal better with the toxic effects caused by intermediate copper concentrations than single-species cultures.

Keywords: Chlorophyta; community; metal; microalgae.

Resumo: Objetivo: Cobre é um nutriente essencial para o fitoplâncton, mas ele também pode agir como um agente tóxico, dependendo de sua concentração. Considerando o crescente incremento deste metal em ecossistemas aquáticos naturais, compreender sua ação em cenários de co-cultura é de grande relevância. Experimentos com a combinação de diferentes espécies assemelham-se com maior precisão as condições encontradas na natureza, contrastando com os resultados obtidos em testes unialgais, que não podem ser diretamente usados para descrever efeitos observáveis no ambiente. **Métodos:** Deste modo, parâmetros de crescimento foram investigados e comparados na co-cultura de *Chlorella sorokiniana* e *Kirchneriella obesa* e seus cultivos individuais expostos a três diferentes concentrações de cobre livre (controle $6x10^{-9}$, intermediário $2x10^{-7}$ e elevado $1.5x10^{-6}$ mol.L^{-1} Cu^{2+}). **Resultados:** *C. sorokiniana* apresentou mais células em seu controle unialgal enquanto *K. obesa* teve maior densidade celular no controle da co-cultura. As taxas de crescimento decairam com o incremento de cobre nas condições unialgais. No entanto, ambas as espécies mantiveram elevada taxa de crescimento na co-cultura em concentrações intermediárias do metal. O biovolume variou independentemente do método de cultivo, sendo diretamente relacionado à concentração de cobre. A eficiência fotossintética máxima decaiu sob elevado cobre. **Conclusões:** De acordo com os resultados observados, não houve exclusão competitiva e ambas as espécies foram afetadas pelo cobre em condições unialgais ou de co-cultura, sendo que *K. obesa* foi favorecida no cultivo em conjunto, que parece apresentar uma atenuação da toxicidade do cobre em concentrações intermediárias. Ecologicamente, os resultados sugerem que comunidades lidam melhor com efeitos tóxicos causados por concentrações intermediárias de cobre do que culturas isoladas.

Palavras-chave: Chlorophyta; comunidade; metal; microalga.

1. Introduction

Copper is an extremely used metal in various industries, ranging from civil construction to agricultural inputs. Its excessive use has generated a considerable amount of pollution that mainly ends up in water bodies (Shrivastava, 2009). According to the National Council for the Environment (CONAMA) (Brasil, 2005), the class 1 water bodies, intended for protecting aquatic biodiversity, should present a maximum dissolved copper concentration of $1.4x10^{-7}$ mol.L^{-1}. In rivers from England's countryside (in good water quality conditions), dissolved copper ranges from 10^{-9} to 10^{-7} mol.L^{-1} (Gardner et al., 2000) while rivers that receive mining cupric residues in China present detectable copper concentrations higher than 10^{-4} mol.L^{-1} (Liu et al., 2020), which are considered toxic to most aquatic organisms. Therefore, environmentally relevant dissolved Cu concentrations can be considered to be below 10^{-7} mol.L^{-1}. The continuous increase of copper in aquatic ecosystems may expose the phytoplankton to concentrations that can cause negative physiological alterations. Reduced photosynthetic yield, increased intracellular reactive oxygen species (ROS) and decreased chlorophyll *a* synthesis are some metabolic processes triggered by copper (Afkar et al., 2010; Knauert & Knauer, 2008; Perales-Vela et al., 2007; Pinto et al., 2003).

In the aquatic environments, copper is a natural component that can be found mainly in two distinct forms: particulate and dissolved. The particulate Cu correspond to the portion in which the metallic ions are associated with other suspended particulate matter, from inorganic (such as clay) to organic (such as decomposing matter) origins (Grassi et al., 2000). This forms colloidal structures that act as the main transporting mechanism of copper in aquatic ecosystems, carrying this metal along the water body or depositing it on the sediment. Most of the copper found in rivers and lakes are in this form (~70%), which reduces the bioavailability of Cu (Windom et al., 1991). The dissolved copper, on the free ionic form of Cu^{2+}, corresponds to the fraction of this metal that microorganisms can quickly interact (Lombardi et al., 2002). For this reason, on this study the free copper concentrations will be measured and used to evaluate the results.

The co-cultivation of microorganisms involves the growth of several species together, mixing bacteria, yeast, cyanobacteria and microalgae in order to create a more realistic scenario of the natural environment. These studies are widespread in the literature of biotechnological fields, either in bioremediation studies or in production of biofuels or biomolecules (Magdouli et al., 2016). The main idea behind co-cultivation is that these organisms all naturally coexist in the environment, already presenting some form of ecological relation. In the ecosystems, they can inhibit each other through allelopathic compounds or favor some other species by releasing metabolites in the media (Chiang et al., 2004; Dunker et al., 2013; Fergola et al., 2007; Nan et al., 2004). In experimental conditions, the co-cultivation may represent the combined work of these organisms enzymatic repertoire, allowing the degradation of complex substrates or increasing lipid production without biomass loss (Arumugam et al., 2014; Liu et al. 2018; Yen et al., 2015; Zhao et al., 2014). Stressful conditions associated with pollutants, nutrient starvation and light intensity are also being studied under co-cultivation conditions hoping to understand synergy mechanisms that allow communities to survive (Antunes et al., 2012; Barreiro & Hairston, 2013; Granéli & Johansson, 2003; Oncel et al., 2011).

The effects of growth inhibition between microalgae species and cyanobacteria are well documented. Many species of the *Chlorella* genus have been described as producers of chlorellin, an antibacterial mix of free fatty acids (Pratt & Fong, 1940; Spoehr & Milner, 1949). Other substances related with negative effects on phytoplankton growth that are produced by some microalgae or cyanobacteria are palmitic acid, linoleic acid and α-linolenic acid, causing damages to plasmatic membranes and affecting starch deposition on the chloroplasts (Klausner et al., 1980; Pinto et al., 2003). Nonetheless, there are resistant species that can even be stimulated by the presence of others in the same culture media or environment. These synergy mechanisms seems to be sensible and may vary according to specific interspecies relationships (Chiang et al., 2004; Magdouli et al., 2016).

There is a gap in the literature of works investigating metal effects on co-cultivation of microalgae. While bacterial presence on the absence of iron on microalgae cultivation is considerably explored (Kean et al., 2015; Rajapitamahuni et al., 2018) there are no works examining other metals, such as arsenic or copper. Therefore, this study aimed to observe the effects of three distinct copper concentrations on the population dynamics of the co-cultivation of *Chlorella sorokiniana* Shihira & R.W. Krauss 1965 and *Kirchneriella obesa*

West & G.S. West 1894. These species, which belong to different classes (Trebouxiophyceae and Chlorophyceae respectively), were chosen due their distinct features, such as presence of a dense mucilage in *K. obesa*, spherical shaped cells in *C. sorokiniana* versus sickle shaped cells in *K. obesa*, distinct copper sensitivities and growth rates. Previous works using the same genera of green microalgae in unialgal conditions exposed to copper reported the sensitivity of *Kirchneriella aperta* Teiling 1912, which suffered a considerable reduction in growth rate and chlorophyll *a* production, with EC_{50} = ~10^{-9} mol.L^{-1} free copper (Lombardi et al., 2002). The *Chlorella* genus seems to be copper resistant, with *Chlorella pyrenoidosa* H. Chick 1903 displaying a EC_{50} of 7.5x10^{-7} mol.L^{-1} of Cu (Yan & Pan, 2002) and *Chlorella sp.* (at pH 5.7) a EC_{50} of 5.5x10^{-7} mol.L^{-1} (Franklin et al., 2000). Despite being part of the *Chlorella* genus, *C. sorokiniana* has not been described in the literature as a chlorellin producer (Wilde et al., 2006; Zhao et al., 2014). Nonetheless, it grows faster than *K. obesa*, which is larger and produces mucilage, an important structure with ion retention properties that may postpone the toxic effects caused by copper (Lombardi et al., 2002).

2. Material and Methods

2.1. Experimental conditions

Both microalgae used were cultivated in previously sterilized (autoclave, 20 min) normal BG11 media (Rippka et al., 1979) in 1.0 L polycarbonate bottles containing 400 mL of media. The initial pH was adjusted to 7.0 and cultures were illuminated by LED (2700 ~ 3000 K) nourishing 180 µmol photons m^{-2} s^{-1} of photosynthetic active radiation (PAR) at the surface of the culture. Temperature was controlled to 24 ± 1 °C and light cycle adjusted for 12:12h. For the experiments, the BG11 medium was prepared without copper, which was later added in all treatments from a commercial mono-elemental pattern for AAS/ICP (1000 mg L^{-1}) (Sigma-Aldrich, Germany). The microalgae inoculum, cultivated at normal BG11, was only added to the experiment flasks' after the addition of the desired copper concentrations. The inoculum of *K. obesa* was obtained from the Microalgae Culture Collection (CCMA/ strain 345/ WCDM 835) of the Phycology Laboratory, Botanic Department of Universidade Federal de São Carlos (UFSCar) and the inoculum of *C. sorokiniana* was taken from the Microalgae Bank of the Algal Biotechnology Laboratory (strain 004), Botanic Department of UFSCar.

Before the start of the experiments, both microalgae in exponential growth phase (after 5 days of growth) had their maximum photosynthetic yield (Φ_m) measured in order to assess their health condition (Φ_m = 0.7 or above is considered healthy by Juneau & Harrison, 2005 and Lombardi & Maldonado, 2011). Only photosynthetic healthy inocula were used. Experimental cultures grew in 1.0 L polycarbonate bottles with 400 mL of BG11 medium. There were three experimental conditions (unialgal *C. sorokiniana*, unialgal *K. obesa* and co-cultivation) and all of them were exposed to three nominal copper concentrations (control 1.3x10^{-6}, intermediate 6.5x10^{-6} and high 6.5x10^{-5} mol.L^{-1} nominal copper) in triplicates for 6 days. The nominal concentration corresponds to a theoretical amount of a substance when preparing a solution. Therefore, all the results on this study were analyzed according to free copper concentrations, determined with an ion-selective electrode, corresponding more realistically to the bioavailable Cu present in the culture media. The control concentration represents the normal amount of copper used in the BG-11 formulation, the intermediate is a 5-fold increment and the high condition is a 50-fold increase. These concentrations were selected to represent a rising environmental gradient of copper and were based on values that can positively affect growth and photosynthesis of microalgae, as observed in the results of Lombardi et al. (2002). All experimental groups received an inoculum of 5x10^4 cells mL^{-1} but for the co-cultivation it was used a 1:1 ratio inoculum of each species, which represent a double initial cell abundance compared to single species cultures. All bottles used were previously washed with HNO_3 1.0 mol L^{-1} for 7 days in order to assure metal removal. Afterwards, they were rinsed with milli-Q water and autoclaved with culture medium. Only plastic or Teflon® materials were used, all previously washed for 24h in HCl 1.0 mol L^{-1} and rinsed in milli-Q water.

2.2. Copper determination

Free copper ions present in the culture media were determined prior to microalgae inoculation using an ion-selective copper electrode (Orion, model 94-29) and a double junction reference electrode. During the readings, temperature was maintained constant (22 ± 2 °C) and metallic buffers were used to extend the linear amplitude

of the calibration curve up to 10^{-10} mol L^{-1} Cu^{2+}. All metallic solutions used were kept in plastic recipients, which were previously washed in HNO_3 1.0 mol L^{-1} for 48h and rinsed in milli-Q water. To make the calibration curve, triplicates of 6 known copper concentrations (ranging from 10^{-5} to 10^{-7} mol L^{-1}) were used, plus the metallic buffer. Using the linear equation obtained, it was possible to estimate the free copper concentration of 50 mL samples of each experimental condition, which had their ionic strength adjusted to 0.02 mol L^{-1} using high purity $NaNO_3$ (Sigma-Aldrich, Germany), by reading their potential (mV). The free copper determinations were performed in a clean room with positive pressure given by horizontal filtered air flow. As expect due the presence of EDTA (chelating agent) in the BG11 medium, the nominal copper values used resulted in different final free copper concentrations (control $6x10^{-9}$, intermediate $2x10^{-7}$ and high $1.5x10^{-6}$ mol.L^{-1} Cu^{2+}). All results were discussed based on these estimated free copper concentrations, since this form of the metal correspond to the ionic form that is absorbed by cells (bioavailable) or complexed by molecules.

2.3. Population dynamics

Samples were taken daily for two purposes: cell count, performed manually in an optic microscope (Nikon Eclipse E200, Japan) and to determine the fluorescence *in vivo* of chlorophyll *a* (mg L^{-1}) using a fluorimeter (Turner Designs AU-10, Trilogy, US). A calibration curve was made considering the chlorophyll *a* extracted from a *C. sorokiniana* culture in exponential phase in several concentrations versus the *in vivo* chlorophyll *a* fluorescence of this same culture. The extraction of the pigment followed the protocol described in Jeffrey and Humphrey (1975). The curve was adjusted in its linear portion and the equation was used to calculate the chlorophyll *a* concentration of each sample.

Specific growth rates (μ, d^{-1}) were obtained as being the inclination of a linear regression of the exponential growth phase, traced as the natural logarithm of cell density (cells mL^{-1}) versus experimental time in days (Silva et al., 2018). Biovolume (μm^3) was calculated for both species in all experimental conditions at 72h of growth, measuring at least 50 cells under the optic microscope using a Fuchs-Rosenthal counting chamber. Each species had a specific equation for cell volume, as proposed by Hillebrand et al. (1999).

2.4. Photosynthetic measure

The maximum photosynthetic yield of the photosystem II (PSII) was determined daily using a Pulse-Amplitude-Modulation (PAM) chlorophyll fluorometer (Phyto-PAM, Heinz Walz GmbH, Germany), which applies a saturating light pulse in a 3 mL aliquot of the culture previously adapted to darkness (20 min). This previous acclimation without light is essential to maintain all reaction centers of the photosystem II (PSII) open, with the primary electron acceptor Q_A at its oxidized state. When the Phyto-PAM emits a short duration saturating pulse of light, the Q_A acceptors are all reduced, closing the reaction centers. At this moment, the maximum fluorescence is captured by the fluorometer, representing the maximum photosynthetic yield (Φ_m).

For unialgal cultures, the measurements represent the maximum photosynthetic yield of each species, using the logistic parameters determined by Juneau et al. (2002) for microalgae. The results presented for Φ_m of the co-cultivation represents a total fluorescence value emitted by the mixture of species.

2.5. Statistical analyses

The results were examined through a normality test (Shapiro-Wilk) in order to select accurate statistical analyzes based on the data's normal tendency. Afterwards, the results for copper per cell, growth rate and biovolume were analyzed through a simple ANOVA with a confidence interval of 95% to detect differences between the means of each copper concentration tested in relation to the control, using the Statistical Assistance software (ver. 7.7) for Windows.

The significantly statistical differences were marked with asterisks in all graphs, which were made using IgorPro 6.0.5 (WaveMetrics, USA). For copper per cell, each copper concentration (intermediate and high) had statistically noteworthy responses as the metal concentration increased. In the growth rate analyzes, the ANOVA showed that the highest copper tested generated statistical difference in both microalgae for both cultivation conditions. Additionally, *C. sorokiniana* and *K. obesa* also presented a significant result ($p < 0.05$) at unialgal conditions for intermediate copper. Finally, for biovolume, a similar trend happened, with the significant differences occurring at higher copper concentrations for both species and solely for *C. sorokiniana* unialgal cultivation at intermediate copper.

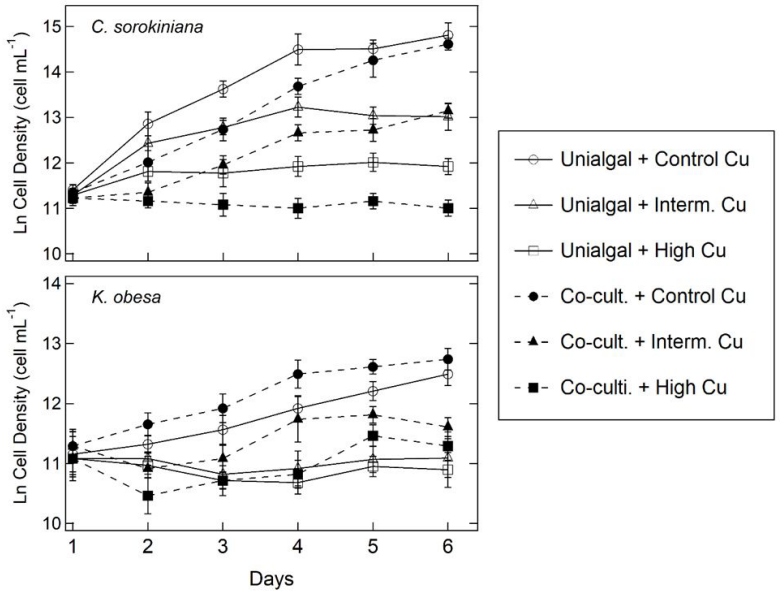

Figure 1. Population density (Ln cell mL^{-1}) of *Chlorella sorokiniana* (up) and *Kirchneriella obesa* (down) in unialgal conditions (continuous line, unfilled symbols) and co-cultivation (dashed line, filled symbols) as a function of experimental period in days with mean ± standard deviation. Copper concentration symbols: circle (control, $6x10^{-9}$ mol L^{-1}), triangle (intermediate, $2x10^{-7}$ mol L^{-1}) and square (high, $1.5x10^{-6}$ mol L^{-1}).

3. Results

The population density (cells mL^{-1}) of *C. sorokiniana* and *K. obesa* at co-cultivation and at the unialgal conditions as a function of experimental period (Figure 1) shows that *C. sorokiniana* reached higher cell number at the control of the single-species culture. The same trend was observed for the other copper conditions. *K. obesa* presented greater cell density at the co-cultivation with less copper (control, $6x10^{-9}$ mol L^{-1} Cu^{2+}). On the intermediate and high concentrations of the metal, the co-cultivation condition registered more *K. obesa* cells than its unialgal culture exposed to the same copper concentration.

In order to assess the average amount of copper each cell was exposed to at the moment of inoculation (day 1), it was calculated the free copper concentration per cell of each experimental treatment (Figure 2). It is noticeable that since the co-cultivation had more cells, each cell was potentially exposed to less copper than those in the unialgal cultures, especially in the intermediate and high copper concentrations, were the variation observed was statistically significant.

The increment of copper in the culture media caused an overall decrease in chlorophyll *a* concentrations (μg mL^{-1}), as observed in Figure 3. The highest value of chlorophyll *a* was obtained in the single-species cultivation of *C. sorokiniana* at the control, whereas all the other treatments

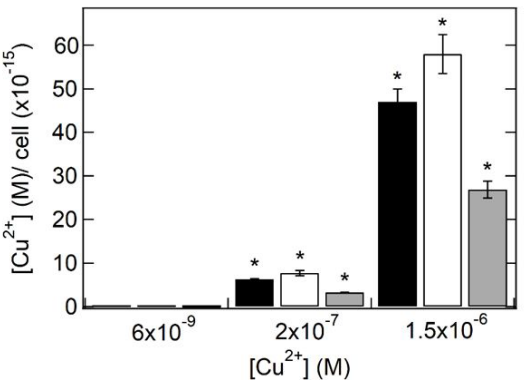

Figure 2. Free copper concentration per cell of *Chlorella. sorokiniana* (■), *Kirchneriella obesa* (□) and co-cultivation (■) as a function of the three copper concentrations tested. Error bars represent the mean ± standard deviation. The symbol * represents statistically significant difference ($p < 0.05$) regarding the control.

(both unialgal and co-cultivation) exposed to $1.5x10^{-6}$ mol L^{-1} Cu^{2+} (high copper condition) registered chlorophyll *a* concentrations lower than 0.1 μg mL^{-1}.

Copper has affected the growth rate of both species tested, causing a more intense reduction at the unialgal conditions (Figure 4). While in co-cultivation the intermediate copper concentration ($2x10^{-7}$ mol L^{-1} Cu^{2+}) did not caused a significant reduction ($p < 0.05$), at the single-species cultures there was a decrease of 55% and 65% on the growth rates of *C. sorokiniana* and *K. obesa* in relation to the

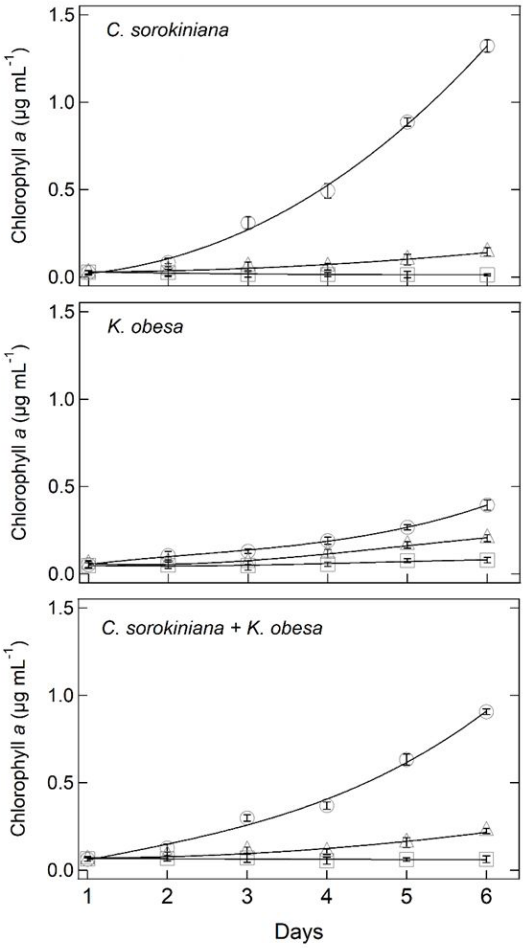

Figure 3. Chlorophyll *a* (µg mL^{-1}) of *Chlorella sorokiniana* (up), *Kirchneriella obesa* (middle) and co-cultivation (down) as a function of experimental period in days with mean ± standard deviation. Copper concentration symbols: ○ (control, 6x10^{-9} mol L^{-1}), △ (intermediate, 2x10^{-7} mol L^{-1}) and □ (high, 1.5x10^{-6} mol L^{-1}).

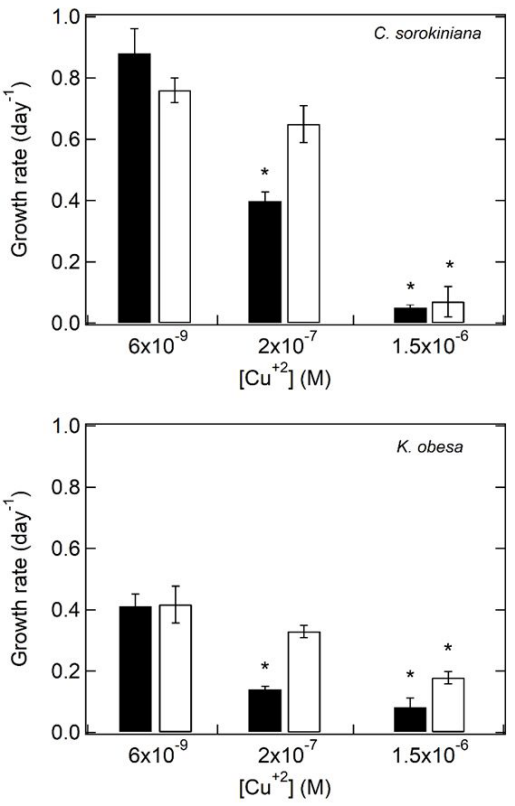

Figure 4. Growth rate (d^{-1}) of *Chlorella sorokiniana* (up) and *Kirchneriella obesa* (down) in unialgal conditions (black bars) and co-cultivation (white bars) as a function of the copper concentrations tested with mean ± standard deviation. The symbol * represents statistically significant difference ($p < 0.05$) regarding the control.

unialgal control. At the high copper concentration (1.5x10^{-6} mol L^{-1} Cu^{2+}) all treatments registered significant reductions on growth of unialgal (95% for *C. sorokiniana* and 80% for *K. obesa*) and co-cultivation conditions (90% for *C. sorokiniana* and ~60% for *K. obesa*).

The biovolume (Figure 5) increased significantly ($p < 0.05$) at the highest copper concentration tested in both single-species and co-cultivation conditions. Cell enlargement observed was of almost 3-fold in *C. sorokiniana* and ~30% in *K. obesa* when considering control versus high copper treatments.

The maximum photosynthetic yield (Φ_m) of all copper treatments on the single-species *K. obesa* culture registered values above 0.65 (Figure 6). In the co-cultivation, the control and the intermediate concentration (2x10^{-7} mol L^{-1} Cu^{2+}) presented a slight drop on the Φ_m during the experiment, but remained ≥ 0.6. The higher copper concentration tested caused significant reduction on the maximum photosynthetic yield on the co-cultivation and on the unialgal *C. sorokiniana* culture, with values < 0.6 on both conditions.

4. Discussion

Comparing growth patterns of the microalgae species tested, it is possible to observe that *C. sorokiniana* had higher cell concentration in unialgal conditions compared to co-cultivation for all copper concentrations tested, during most of the experiment. Conversely, *K. obesa* had lower cell count at unialgal compared to co-cultivation for all copper conditions tested. However, at the last day of the experiment, cell concentration was similar in both unialgal and co-cultivation conditions at the control copper, which resulted in similar growth rates for both species. It is common to observe in co-cultivation a limitation on growth, since it is believed that each species competes for light and nutrients, which takes them away from their optimum growth

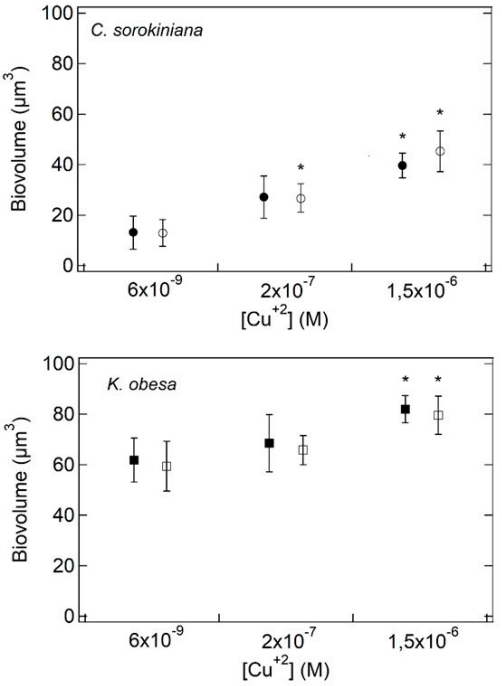

Figure 5. Biovolume (µm^3) of *Chlorella sorokiniana* (up) and *Kirchneriella obesa* (down) in unialgal conditions (black symbols) and co-cultivation (white symbols) measured after 72h of growth as a function of all three copper concentrations tested with mean ± standard deviation. The symbol * represents statistically significant difference ($p < 0.05$) regarding the control.

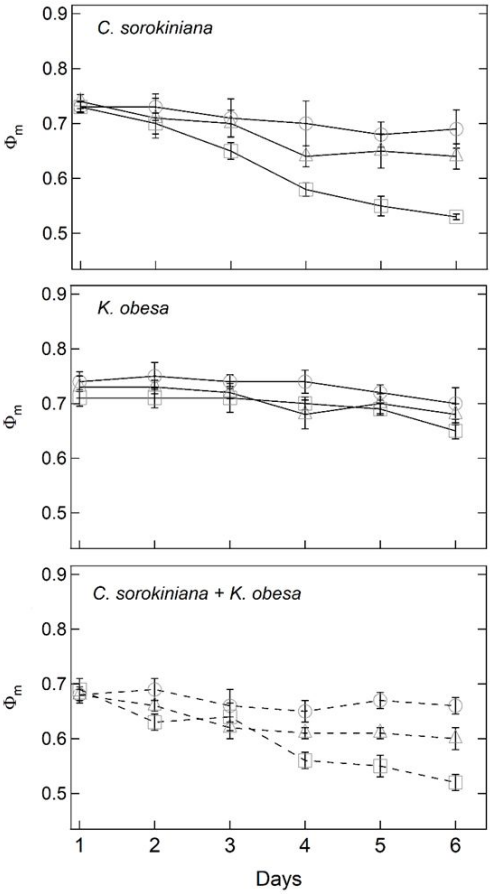

Figure 6. Maximum photosynthetic yield (Φ_m) of *Chlorella sorokiniana* (up), *Kirchneriella obesa* (middle) and co-cultivation (down) as a function of experimental period in days with mean ± standard deviation. Copper concentration symbols: ○ (control, $6x10^{-9}$ mol L^{-1}), △ (intermediate, $2x10^{-7}$ mol L^{-1}) and □ (high, $1.5x10^{-6}$ mol L^{-1})

conditions (Fergola et al., 2007; Nan et al., 2004). The culture medium used, BG11, is a nutrient rich medium. Additionally, since the experiment only lasted for 6 days, it is expected that nutritional limitations on growth were prevented. Another aspect discussed in the literature is the production of allelopathic agents that inhibit the growth of several microorganisms according to their specific sensibilities (Chiang et al., 2004; Zhao et al., 2014). The genus *Chlorella* is well known for its chlorellin production, particularly the species *C. vulgaris* Beyerinck 1890 (Pratt et al., 1944; Dellagreca et al., 2010). However, *C. sorokiniana* has not been reported as a chlorellin producer. In this study, *K. obesa* was not negatively affected by the presence of *C. sorokiniana*, leading to the conclusion that chlorellin was not produced, its concentration in the culture was not high enough to cause and impact on *K. obesa* or this species is resistant to this type of allelopathy. Many elements should be taken into account regarding these two species interactions, such as differences in metabolic activity, distinct cell sizes, presence of a mucilaginous capsule in *K. obesa* and a disparity in the growth rates of these species. Dellagreca et al. (2010) observed a stimulus in growth on the co-cultivation of *Pseudokirchneriella subcapitata* F. Hindák 1990 and *C. vulgaris* when exposed to filtered culture medium containing low amounts of free fatty acids and chlorellin (< 3.2 mg L^{-1}). Nonetheless, in higher concentrations of chlorellin (> 26 mg L^{-1}), both species were affected, with *P. subcapitata* showing an extreme sensibility. Microalgae in co-cultivation are capable of altering the metabolism and synthesis of biomolecules of each another through substances released extracellularly, especially in exponential and stationary growth phases. These substances may cause inhibition or stimulation on the species present, depending on the concentration of the biomolecules and species-specific sensibility (Dunker et al., 2013; Magdouli et al., 2016; Oncel et al., 2011).

Adding to this mixture and the stressful factor of a metal such as copper, the interspecific relations

of *C. sorokiniana* and *K. obesa* were reflected on their growth parameters. In the intermediate copper concentration ($2x10^{-7}$ mol L^{-1} Cu^{2+}) both species maintained their growth rates observed in the control ($p < 0.05$), with values higher than those observed in the unialgal cultures exposed to the same copper concentration. Sanders and Cibik (1988) argue that communities can thrive depending on their species composition and type of contaminant studied. In their research, they observed that arsenic was detrimental to the community, reducing cell sizes and the nutritional value of the plankton, while silver caused a positive swift in the overall diversity. On the present study, the maintenance of growth rates in intermediate and control copper concentrations for both species at co-cultivation shows that the combined metabolic capacity of the species tested may allow them to resist this toxic metal together at these specific concentrations. Many researchers investigate the absence of iron in co-cultivations, showing that the presence of siderophore-producing bacteria, which liberates iron-chelating compounds with high affinity in the medium, contributes to the survival and stimulates growth in microalgae (Kean et al., 2015; Keshtacher-Liebso et al., 1995; Rajapitamahuni et al., 2018). Conversely, results discussing the effects of other metals are scarce. Some of the stress agents commonly tested are light and nutrients. Antunes et al. (2012) tested the effects of *Cylindrospermopsis raciborskii* Seenayya & S. Raju 1972 (a producer of cylindrospermopsin) on *Ankistrodesmus falcatus* Ralfs 1848. In the co-cultivation, *A. falcatus* displayed reduction on biovolume and growth rate, which could be worsen by higher light intensity and medium alkalization. Oppositely, this study observed a reduction of the overall stressful effects of copper in intermediate concentrations of the co-cultivation in comparison to the single-species conditions. This may be related to the production of several extracellular agents with chelating functions, such as polysaccharides (Pistocchi et al., 1997) or metal-binding proteins (Sabatini et al., 2009), what may diminish the bioavailability of copper in the culture medium. At the highest copper concentration tested all growth parameters were affected, in co-cultivation and unialgal conditions, representing the toxic effects of copper in microalgae's metabolism.

It is important to highlight the differences in initial cell density between single-species cultures and co-cultivation (Figure 3) and how it may partially explain the results obtained. The greater amount of cells at the co-cultivation on the first day of experiment predicts that the cells are potentially exposed to less copper ions than unialgal cultures. Therefore, an attenuation effect on the metal's toxicity cannot be forwent. Despite this cell density difference, other factors may influence metal speciation and bioavailability in culture media, such as physicochemical aspects (pH and temperature), chemical and polarity affinity to other molecules and the presence of exudates with chelating properties, which have been described to be released by microalgae (Pistocchi et al., 1997; Sabatini et al., 2009).

The effects of copper in the physiology of microalgae are well-known in the literature for a great variety of species. The reduction on cell density and drop on growth rates are associated with direct effects of copper in the photosynthetic apparatus of the microalgae, whereas indirect effects include the production of ROS, damages to plasmatic membranes and intracellular ultrastructure (Angel et al., 2017; Jiang et al., 2001; Tripathi et al., 2006). The reduction on chlorophyll *a* observed in this study on both single and joint cultures of *C. sorokiniana* and *K. obesa* are most likely related to the toxic action of copper on the synthesis of pigments (Perales-Vela et al., 2007; Pinto et al., 2003). The increment in biovolume, which can be interpreted as an inability to divide and a survival strategy to copper due the alteration between the surface-volume ratio that reduces the cell exposure to copper ions (Echeveste et al., 2017; Machado & Soares, 2014), also expresses a consequence of the toxicity of copper rather than possible co-cultivation induced effects. Nevertheless, *K. obesa* had its growth favored at co-cultivation, which can be related to its greater cell size and presence of mucilage. These features may have given an extra protection to this species in the detriment of *C. sorokiniana*'s growth. Another possible scenario would be the accumulation on the extracellular environment of exopolysaccharides and metal-binding proteins from both species, helping both of them to resist the intermediate copper concentration together, maintaining their growth rates, differently from the single-species cultures were this copper concentration was enough to cause cell density and growth rate reductions.

In a general sense, it is argued that microalgae co-cultivation can have positive and negative effects on the culture and that these effects are intrinsically related to the species used and their possible ecological relations in natural environments

(Dunker et al., 2013; Fergola et al., 2007; Zhao et al., 2014). In this study, *C. sorokiniana* and *K. obesa* did not show antagonist relations at their joint growth condition. However, when analysing the maximum photosynthetic yield of the co-cultivation it is remarkable that the values observed were in general inferior to those obtained in the separate cultures, especially the control copper concentration. In this condition copper was not a stressful factor, so this small reduction on the overall photosynthesis performance can be attributed to a possible light limitation effect caused by self-shading due to the presence of more cells in the medium (Magdouli et al., 2016). As expected, copper reduced the maximum photosynthetic yield in all treatments exposed to $2x10^{-7}$ and $1.5x10^{-6}$ mol L^{-1} Cu^{2+}, with the co-cultivation and the unialgal culture of *C. sorokiniana* presenting the lower mean values (below 0.7 at the third day of experiment). Φ_m is a parameter that varies according to each species sensibility and, generally, shows the photosynthetic apparatus health in conditions > 0.7 (Juneau & Harrison, 2005; Lombardi & Maldonado, 2011). Therefore, copper affected the photosynthesis of these microalgae, reducing their maximum photosynthetic yields probably acting specifically in molecules such as plastoquinone and plastocyanin, both essential electron transporters between PSII and PSI and that have already been described as susceptible to damages mediated by copper (Knauert & Knauer, 2008; Mallick & Mohn, 2003; Mohanty et al., 1989).

C. sorokiniana and *K. obesa* were affected by copper in both culture condition tested (single-species or co-cultivation). The joint-cultivation caused distinct responses in these species, favoring the growth of *K. obesa* and reducing the toxic effects of copper in the intermediate copper concentration tested ($2x10^{-7}$ mol L^{-1} Cu^{2+}), in which the decrease in cell density and growth rates occurred less intensively than the reduction observed in the unialgal cultures ($p < 0.05$). Copper, without a clear relation with the separate or joint cultivation, altered the biovolume of both species. Additionally, it was observed that copper affects the photosynthesis of microalgae in a species-specific way, with some species being more sensitive to the damage mediated by copper than others. The effects detected in this study on the growth and photosynthesis parameters are responses induced by copper in unialgal cultures of *C. sorokiniana* and *K. obesa* and their co-cultivation, which did not present competitive exclusion between the species tested. Despite that, *K. obesa* was favored by the joint growth condition while *C. sorokiniana* suffered a reduction in cell density when compared to its unialgal growth. The presence of mucilage and different cell sizes may have been important factors related to the behavior observed in co-cultivation and could partially explain the results obtained. Additional research, testing other species and including more copper damage assessment parameters (such as production of antioxidants) can deepen the knowledge around copper effects in microalga communities. From an ecological point of view, this research strengthens the argument that in natural conditions, with species co-exiting, the community as a whole can deal differently with stressful factors such as metals concentrations, surviving and growing better than each individual species would separately.

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