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Growth and saxitoxin production responses to copper ($CuCl_2$) exposure by the cyanobacterium *Raphidiopsis raciborskii*

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Abstract

Copper (Cu^{2+}) is an essential micronutrient for cyanobacteria, but it has a toxic effect above a certain threshold. The presence of Cu^{2+} in water is usually related to human activity due to it being used in pesticides, fertilizer, and algaecides. Previous studies observed that high Cu^{2+} concentrations stimulated toxin synthesis in cyanobacteria and microalgae. Furthermore, saxitoxins (STXs) can bind to Cu^{2+} transporters in microorganisms, decreasing the Cu^{2+} uptake and consequently reducing Cu toxicity. Therefore, considering the invasive capacity of the cyanobacterium *Raphidiopsis raciborskii* and its potential for STXs production, this study aimed to evaluate the effects of different Cu^{2+} concentrations in the production of STXs and growth of *R. raciborskii*. In acclimatized growth conditions, cultures of *R. raciborskii* strain (ITUC01) were exposed to four different copper concentrations for 20 days (0.8, 8, 80, and 800×10^{-3} μmol L^{-1} of $CuCl_2$). *Raphidiopsis raciborskii* growth and physiological responses were evaluated measuring the cell concentration, cell volume, biovolume, chlorophyll *a* levels, and STXs concentration. Comparing the lowest and highest Cu^{2+} concentration (0.8 and 800×10^{-3} μmol L^{-1}), it was observed that the increment of Cu^{2+} in the medium led to a reduced maximum growth rate (μ_{max}), cell concentration, biovolume, and chlorophyll *a* levels, while the cell volume increased. Despite the low cell concentration and biovolume in the highest Cu^{2+} condition, it was observed that the STXs volumetric concentration was significantly high on day 5, which is indicative of the fact that increased Cu^{2+} concentration might induce STXs production in early growth. In addition, our results revealed that STXs production was uncoupled with growth and a reduction of *R. raciborskii* toxicity from day 5 to 20 was observed. Therefore, the present study identified some of the survival responses of *R. raciborskii* in Cu-stressed condition and suggested that Cu^{2+} might be one of the factors that can affect *R. raciborskii* bloom toxicity.

Keywords Cyanobacteria · Copper · Saxitoxin · Ecophysiology

Introduction

Copper (Cu^{2+}) is an essential micronutrient in energy metabolism in cyanobacteria, identified as a component of electron carrier plastocyanin and cytochrome oxidase in photosynthesis and respiration, respectively (Baron et al. 1995). However, Cu^{2+} is harmful above a certain concentration and its cellular uptake must be regulated to maintain the intracellular concentrations below toxic levels (Baptista and Vasconcelos 2006; Huertas et al. 2014). Cu^{2+} requirements and toxicity vary from species to species, for instance, previous studies on cyanobacteria (*Synechocystis aquatilis*, *Microcystis aeruginosa*, and *Nostoc linckia*) observed toxic effects of Cu^{2+} above 0.3 μmol L^{-1} (Kumar et al. 1985; Shavyrina et al. 2001; Polyak et al. 2013; Iwinski et al. 2016; Wang et al. 2020).

The presence of Cu^{2+} in water is usually related to human activity due to its use in pesticides and fertilizers, and it is commonly used as an algaecide in lakes and reservoirs in order to control and prevent cyanobacterial blooms (Jones and Orr 1994; Hrudey et al. 1999). Cu^{2+} can affect different mechanisms in the cyanobacteria cells, such as an increase in reactive oxygen species (ROS) (Murphy and Taiz 1997; Ahad

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and Syiem 2018), inhibition of photosynthesis by disrupting the electron transport chain and photosystems, antenna complex damage (Küpper et al. 1998), and decreasing chlorophyll *a* levels (Wang et al. 2020). Despite these negative effects, earlier studies observed that repeated Cu^{2+} exposure of *S. aquatilis* and *M. aeruginosa* led to a Cu^{2+} tolerance due to selecting tolerant strains (Shavyrina et al. 2001; García-Villada et al. 2004).

Besides the toxic effects in cyanobacteria, metal pollution is related to increased cyanotoxins in water, with Cu^{2+} functioning as an important inducer of bloom toxicity (Moeller et al. 2007; Alexova et al. 2011; Martínez-Ruiz and Martínez-Jerónimo 2016; Facey et al. 2019; Long et al. 2019). One of the reasons for this correlation might be the cyanobacteria cell lysis caused by Cu^{2+}, which consequently increases the cyanotoxin concentration in water, as observed in several earlier studies (Jones and Orr 1994; Qian et al. 2010; Fan et al. 2013; Zhou et al. 2013). However, other studies identified Cu^{2+} as a regulator of toxin production, such as in the diatom *Pseudo-nitzschia multiseries*, in which a Cu-stressed condition (1.8 μmol L^{-1}) led to an increase by 20 times of extracellular domoic acid (DA) (Maldonado et al. 2002) and in *M. aeruginosa* under 0.5 μmol L^{-1} of Cu^{2+} led to 1.8 and 1.7 times higher intra and extracellular microcystins (MCYSTs), respectively (Chen et al. 2020). They also reported that MCYSTs might be controlled by an increased Cu^{2+} condition due to the expression modulation of genes *FueA* (ferric uptake regulator) and *mcyD* (microcystin synthetase genes) (Chen et al. 2020).

Toxins can function as organic ligands, complexing with metals and/or interacting with membrane proteins involved in trace metal assimilation (Humble et al. 1997; Wells et al. 2002; Moeller et al. 2007; Cusick et al. 2012; Facey et al. 2019; Chen et al. 2020). It was observed that the addition of saxitoxin (STX) in cultures of *Saccharomyces cerevisiae* (Cusick et al. 2012) and *Chlamydomonas reinhardtii* (Cusick et al. 2013), under 20 and 8 μmol L^{-1} of Cu^{2+} conditions, respectively, prevented the cellular uptake of Cu^{2+} ions, through the binding of STX molecules to Cu^{2+} membrane transporters, alleviating cellular toxicity (Cusick et al. 2013). A similar result was found in the diatom *P. multiseries*, which improved cell growth in response to the addition of toxin DA under a Cu^{2+}-stressed condition (Maldonado et al. 2002). Consequently, these facts led to the hypothesis that toxins may be produced in response to high metal concentration in order to decrease metal toxicity that affects cyanobacteria and microalgae, as a detoxification process (Maldonado et al. 2002; Baptista and Vasconcelos 2006; Cusick et al. 2012; Nogueira et al. 2012; Tonietto et al. 2014; Facey et al. 2019).

Saxitoxins (STXs), also known as paralytic shellfish poisoning (PSP), are potent neurotoxins that can be synthesized by cyanobacteria of several different genera (*Raphidiopsis*, *Cylindrospermopsis*, *Anabaena*, *Aphanizomenon*, *Lyngbya*, and *Planktothrix)* and marine dinoflagellates (*Alexandrium*, *Pyrodinium*, and *Gymnodinium*) (Hackett et al. 2013). STXs have an affinity for sodium channels, blocking them and interrupting sodium intake, which leads to muscle paralysis and death due to respiratory arrest (Wang et al. 2003). Generally, humans are affected by STXs when they eat clams, mussels, and oysters contaminated with this toxin (Anderson 1994).

Raphidiopsis raciborskii (Wołoszyńska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno (Aguilera et al. 2018) (basionym *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenaya and Subba Raju) is a potential STXs and cylindrospermopsin (CYN) producer. Previous studies reported that STXs production capacity in *R. raciborskii* is related to geographical origin which is predominantly in southern hemisphere regions, mainly tropical and subtropical regions, such as South America (Padisák 1997; Burford et al. 2016; Vico et al. 2020). Unlike the CYN production in *R. raciborskii*, which is coupled with growth as a constitutive process (Hawkins et al. 2001; Davis et al. 2014; Pierangelini et al. 2015; Willis et al. 2015; Orr et al. 2018), studies on STXs have suggested that their production is regulated by environmental factors (Kaebernick and Neilan 2001; Boopathi and Ki 2014; Burford et al. 2016). Earlier studies observed the effect of temperature (Castro et al. 2004; Mesquita et al. 2019), pH (Pomati et al. 2004), NaCl concentration (Pomati et al. 2004), light intensity (Carneiro et al. 2009; Mesquita et al. 2019), and Ca^{2+} and Mg^{2+} (Carneiro et al. 2011, 2013) on STXs production by *R. raciborskii*; however, there is no information about the effect of Cu^{2+}.

The *R. raciborskii* strain used in this study (ITUC01) was isolated from a subtropical reservoir, Itupararanga reservoir in São Paulo, Brazil, classified as mesotrophic, although it coexists with a Chlorophyceae, *Monoraphidium contortum*. There was a higher cell relative abundance of this species in winter, with 35.5% (15.3×10^3 cells mL^{-1}) (Cunha et al. 2012) and 83% (4.3×10^4 cells mL^{-1}) (Casali et al. 2017) in different years of the analyses. *R. raciborskii* bloom was also observed in a subtropical lake during late autumn, reaching 8.3×10^4 cells mL^{-1} (Everson et al. 2011). Vargas et al. (2019) associated the higher STXs production of this strain under limited phosphorus, as a survival adaptation in stressful conditions.

Therefore, in the present study, we evaluated the effects of different Cu^{2+} concentrations on STXs production and *R. raciborskii* growth. This cyanobacterium has gained considerable attention due to the frequent dominance in freshwater ecosystems in both tropical and temperate regions (Bittencourt-Oliveira and Molica 2003; Briand et al. 2004; Burford and Davis 2011). Considering the invasive capacity of *R. raciborskii* and its potential for STXs production, the findings of the present study may contribute to understanding

the dynamics of STXs synthesis and physiological response of *R. raciborskii* to toxic levels of Cu^{2+}, providing novel insights into Cu^{2+} regulation of *R. raciborskii* toxic blooms.

Materials and methods

Strain and cultivation

The non-axenic and STXs producer *R. raciborskii* (ITUC01) strain was isolated from the Itupararanga reservoir in São Paulo, Brazil (23° 36′ 42″ S and 47° 23′ 48″ W). This strain was deposited at the microalgae and cyanobacteria bank at the Biotoxicology Laboratory of Continental Water and Effluents (BIOTACE), São Carlos School of Engineering, the University of São Paulo, Brazil.

Raphidiopsis raciborskii was cultivated in modified ASM-1 Tris-buffered medium (Gorham et al. 1964) with 2.5 times higher nitrogen (N) concentration under 24 ± 0.5 °C, a photoperiod of 12 h/12 h and 60 μmol photons m^{-2} s^{-1} light intensity (fluorescent light 20 W—OSRAM). Transparent polycarbonate tubes of 50 mL (NALGENE) were used to cultivate cells in a volume of 20 mL. The culture medium was prepared in ultrapure water in polypropylene bottles prewashed with 1.0 M HCl to reduce trace metal contamination and prevent adsorption of metals on the surface of the bottles (Sunda et al. 2005).

Cu^{2+} ($CuCl_2$) concentration in the ASM-1 culture medium was modified to perform the experiments. The tested Cu^{2+} concentrations were as follows: 0.8×10^{-3} (Cu^{2+} concentration in the ASM-1 medium culture, as control), 8×10^{-3}, 80×10^{-3}, and 800×10^{-3} μmol L^{-1} (Table 1). For each concentration of Cu^{2+} tested, three biological replicates were analyzed for each time point (day 0, day 5, and day 20) to perform the STXs, chlorophyll *a*, and biovolume analyses.

Determination of growth parameters

Culture growth was measured using the following: optical density (OD_{750nm}) (Griffiths et al. 2011), biovolume (Hillebrand et al. 1999), and chlorophyll *a* (Chl *a*) concentration (Nusch 1980).

Table 1 Concentration of copper in each treatment.

Treatment Name	Copper Concentration (μmol L^{-1})
T0	0.8×10^{-3}
T1	8×10^{-3}
T2	80×10^{-3}
T3	800×10^{-3}

The OD_{750} was previously calibrated in relation to the dry biomass ($R^2 = 0.9842$, $y = 0.0038\ x + 0.0263$) and determined daily in a DR-4000 spectrophotometer (HACH Company, USA). Growth rates (μ) were calculated for each day and in the exponential phase of each treatment (μ_{max}), using OD_{750} measurements, according to Guillard et al. (1973).

Cell concentration, cellular volume, biovolume, and Chl *a* were measured on days 0, 5, and 20 (Fig. 1a). The biovolume was calculated multiplying the average of cell volume by cell concentration. A Fuchs Rosenthal chamber in the light microscope (Olympus BX51) was used to determine the cell concentration and the software Image-Pro Plus 4.5.1.20 was used to measure the length and width of the trichomes. The cellular volume was determined according to Hillebrand et al. (1999). Chl *a* was extracted using 80% ethanol, after filtration of 15 mL of the sample from days 0, 5, and 20, using Macherey glass fiber membranes (Nagel (GF-3) of 0.6 μm porosity) (Nusch 1980).

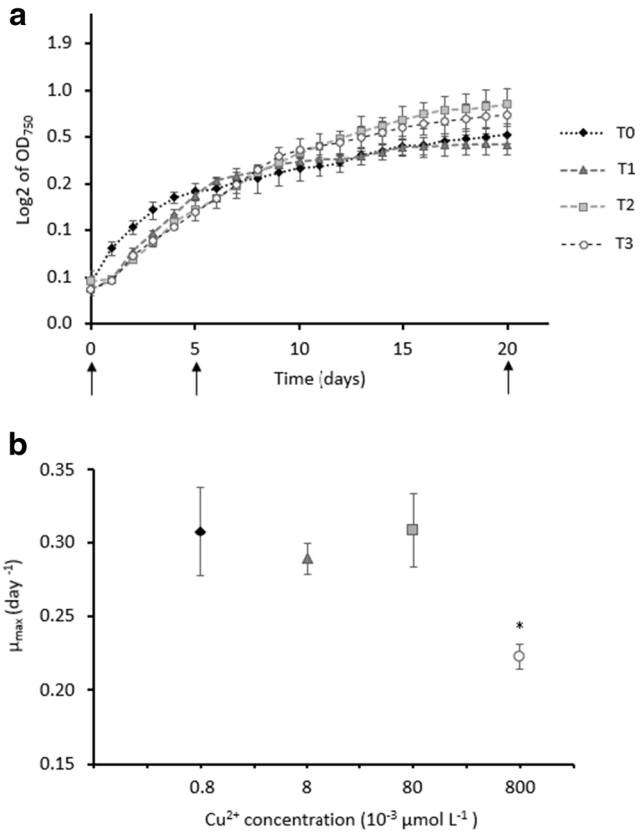

Fig. 1 Gowth curves of *Raphidiopsis raciborskii* cultures exposed to different Cu^{2+} concetrations for 20 days (Table 1) (**a**). Arrows indicate samplings for Chl *a*, cell concentration, cellular volume, and biovolume (**a**). Maximum growth rate (μ_{max}) calculated in the exponential phase of each treatment (**b**). The asterisk indicates the significant difference of T0, T1, and T2 ($p = 0.0045$, 0.0184, 0.0042, respectively) (**b**). Data presented as mean values and standard deviation ($n = 3$)

Saxitoxin determination

For the STX concentration measurements (μg L^{-1}), 2 mL of *R. raciborskii* culture was sampled on days 5 and 20 in each treatment and it was frozen at − 20 °C. The toxin extraction was performed by four freeze-thaw cycles and STXs were analyzed by the enzyme-linked immunosorbent assay (ELISA) using the commercially available saxitoxin plate kit (Beacon Analytical Systems Inc., USA) according to the manufacturer's instructions. The Atlantis microplate washer and Expert Plus Microplate Reader (ASYS Hitech, Austria) were used in this study.

The cross-reactivities for other STXs are 100% STX, 0.8% neosaxitoxin (NeoSTX), 18% decarbamoyl STX, 12% goniautoxin (GTX) 2 and 3, < 0.1% GTX 1 and 4, 0.4% decarbamoyl GTX 2 and 3, and 0.7% decarbamoil neo-STX. As STX was used as the standard, the concentration was expressed as STX affinity equivalents.

The specific rate of STXs production (μ_{STX}) and specific growth rate (μ_g) from the 5th to the 20th day were calculated according to Orr et al. (2018), using the volumetric STXs concentration in μg L^{-1} and biovolume in μm^3 mL^{-1}, respectively.

Data analysis

Statistical data analyses were performed using the Origin Pro 8.0 and GraphPad Prism software. A normality test was performed using the Kolmogorov-Smirnov test prior to performing statistical analyses. To compare the treatments, the ANOVA and Kruskal-Wallis tests were used for parametric and non-parametric data, respectively. After determining the difference between the data, the post hoc Tukey test was used for the parametric data and the Mann-Whitney test for non-parametric data to identify which treatments differed. The probability (p) value of ≤ 0.05 was used as the significance threshold.

Results

Effects of copper on cell growth parameters

Based on OD_{750} measurements of *R. raciborskii* cultures for 20 days (Fig. 1a), the growth rates (μ) were calculated in the exponential phase (μ_{max}) and for each day throughout the experiment (Fig. 1b; A.1 in Supplementary Material). It was observed that μ_{max} was significantly reduced 1.35 times in treatment T3, compared to T0 (ANOVA, $p = 0.028$, $n = 3$); however, no difference was observed between the μ_{max} of T0, T1, and T2 (Fig. 1b). The μ comparison of T0 between other treatments confirmed that Cu^{2+} significantly affected the early growth phase (specifically exponential phase) of

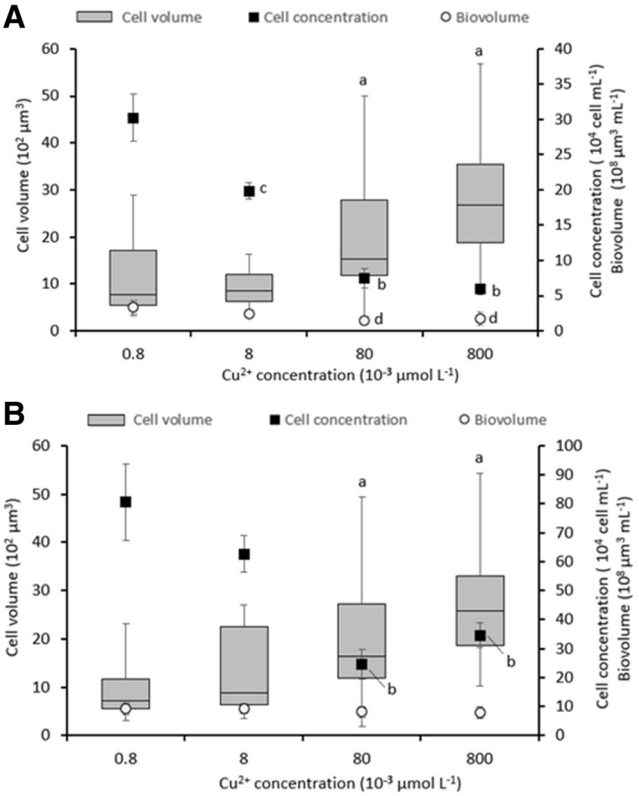

Fig. 2 Evolution of cellular volume, cell concentration, and biovolume of *Raphidiopsis raciborskii* exposed to different Cu^{2+} concentrations for 5 days (**A**) and 20 days (**B**). Cell concentration and biovolume are presented as mean values and standard deviation ($n = 3$). Cell volume is presented in box plots, based on mean values ($n = 30$). (a) Significantly different from T0 and T1 ($p \leq 0.03$). (b) Significantly different from T0 and T1 ($p \leq 0.01$). (c) Significantly different from T0 ($p = 0.0008$). (d) Significantly different from T0 ($p \leq 0.0465$)

R. raciborskii (A.1 in Supplementary Material). As expected, after the exponential phase, μ was reduced in all the treatments, probably due to other factors, such as nutrient limitation in the medium (A.1 in Supplementary Material).

Likewise, the high Cu^{2+} amount negatively affected *R. raciborskii* cell concentration, which was measured on days 5 and 20 (Fig. 2). The cell concentration in treatments T1, T2, and T3 was significantly lower than T0 on day 5, with a reduction of 1.5, 4, and 5 times, respectively (ANOVA, $p < 0.0001$, $n = 3$) (Fig. 2A). The same was observed on day 20, with a cell concentration reduction of 3.3 and 2.3 times in treatments T2 and T3, respectively, compared to T0 (ANOVA, $p < 0.0001$, $n = 3$) (Fig. 2B).

The effect of high Cu^{2+} concentration on the biovolume was observed only on day 5, which decreased 2 and 1.4 times in treatments T2 and T3, respectively, compared to T0 (ANOVA, $p = 0.0465$, $n = 3$) (Fig. 2A). Differently, the biovolume remained constant among the treatments on day 20 (Fig. 2B).

The cell volume was also affected by high levels of Cu^{2+}. The comparison with T0 revealed significant increased cell

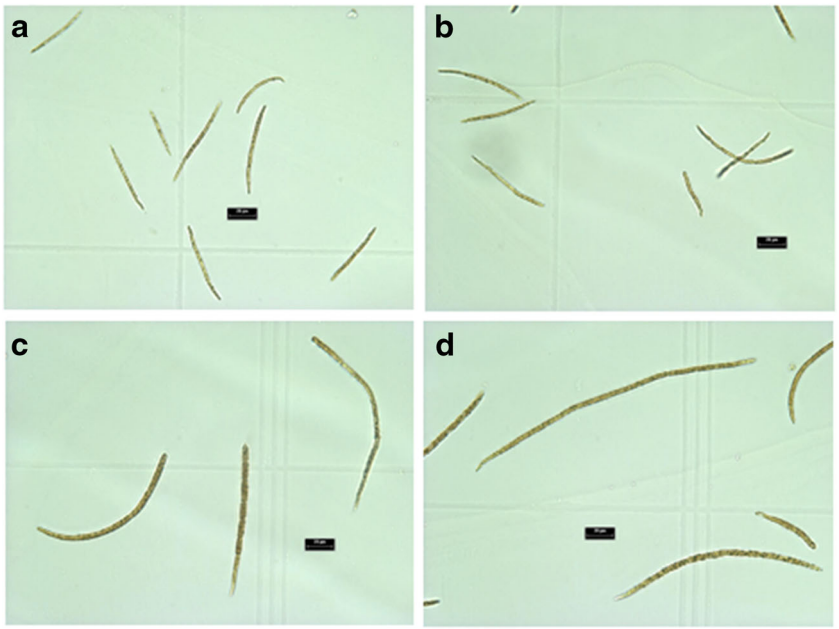

Fig. 3 Alteration of cellular volume in response to Cu^{2+} concentrations. *Raphidiopsis raciborskii* trichomes cultivated for 5 days exposed to different Cu^{2+} concentrations showed increased volume under high Cu^{2+} concentration. T0 (**a**), T1 (**b**), T2 (**c**), and T3 (**d**) (Table 1) (Scale bars = 20 μm)

volume in treatments T2 and T3, both on days 5 and 20. The enlargement observed on day 5 was 1.5 and 3.6 times in treatments T2 and T3, respectively (Kruskal-Wallis, $p < 0.0001$, $n = 30$) and 4.2 and 3.7 times on day 20, in treatments T2 and T3, respectively (Kruskal-Wallis, $p < 0.0001$, $n = 30$) (Fig. 2). Micrographs of *R. raciborskii* cells confirmed the increase in cell volume under the highest Cu^{2+} treatments (Fig. 3).

The Chl *a* levels were mostly affected in T3, which is the treatment with the highest Cu^{2+} concentration (Fig. 4). On day 20, T3 had low Chl *a* concentration, with a decrease of 7, 8.3, and 10 times compared to treatments T0, T1, and T2, respectively (ANOVA, $p < 0.0001$) (Fig. 4). In addition, T3 had unchanged Chl *a* levels over the time points (days 0, 5, and 20), while in the other treatments, an increase in Chl *a* concentration was observed on days 5 and 20 (ANOVA, $p < 0.0001$).

Effect of copper on STX production

The volumetric STXs levels, measured as the total concentration (intracellular plus extracellular STXs), were affected by different Cu^{2+} amounts and also by time. Except for the T0 treatment, the time significantly affected the volumetric STXs concentrations, which increased from the 5th to 20th day, with an increment of 1.89, 3.03, and 1.48, in treatments T1, T2, and T3, respectively (Fig. 5). The comparison among the treatments revealed that T0, T1, and T2 had the same STXs concentrations, while T3 had a significantly high STXs concentration. Treatment T3 had 1.5, 1.75, and 3.41 times more STXs on day 5 (compared to treatments T0, T1, and T2, respectively) and 1.52 and 1.67 times on day 20 (compared to treatments T0 and T1, respectively) (ANOVA, $p = 0.0002$) (Fig. 5). This increment of STXs under

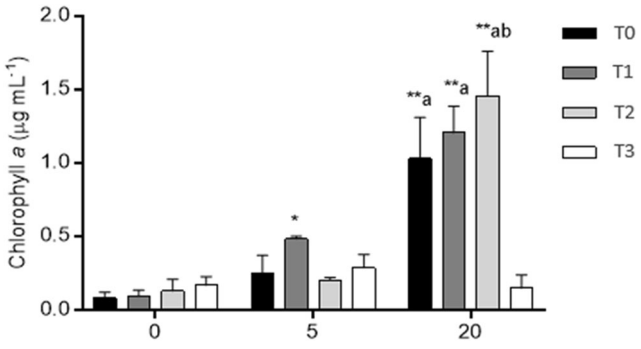

Fig. 4 Chlorophyll *a* concentration in *Raphidiopsis raciborskii* cells exposed to 4 different Cu^{2+} concentrations (Table 1). It was measured on days 0, 5, and 20 of the cultivation. Error bars indicate the standard deviation of the mean values ($n = 3$). The single asterisk indicates significant difference between the 0 and 5th day ($p = 0.019$). The double asterisks indicate significant difference between the 0, 5th, and 20th day ($p < 0.0001$). (a) Significantly different from T3 ($p < 0.0001$). (b) Significantly different from T0 ($p = 0.007$)

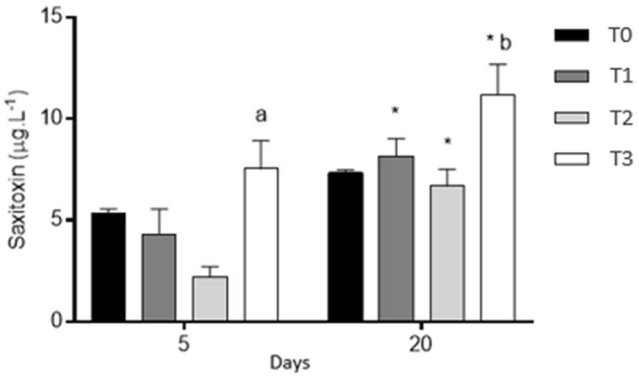

Fig. 5 Volumetric STX concentration (μg L^{-1}) measured on days 5 and 20 of *Raphidiopsis raciborskii* cultivation exposed to different Cu^{2+} concentrations (Table 1). Bars indicate standard deviation of the mean values ($n = 3$). The single asterisk indicates significant difference between days 5 and 20 ($p \leq 0.032$). (a) Significantly different from T0, T1, and T2 ($p \leq 0.049$). (b) Significantly different from T0 and T2 ($p \leq 0.024$)

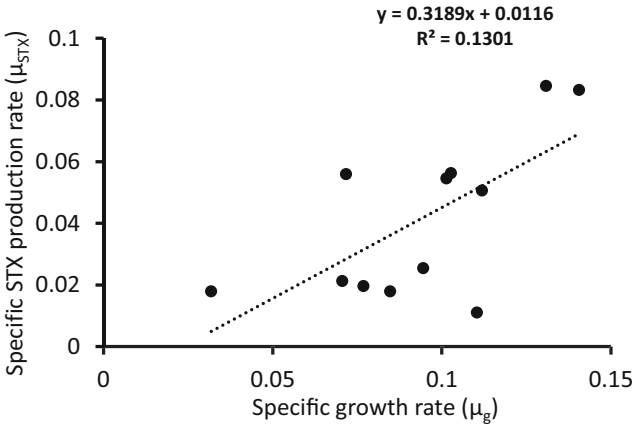

Fig. 6 Relation between specific growth rate (μ_g, day^{-1}) and specific rate of STX production (μ_{STX}, day^{-1}) of *Raphidiopsis raciborskii* under different Cu^{2+} concentrations. Plot of μ_g versus μ_{STX} from Cu^{2+}-exposed cultures in the time interval from day 5 to 20. The dashed line represents linear regression, and the coefficient of determination is indicated as R^2

high Cu^{2+} concentration was also observed in STXs cell quota (QSTXs). On day 5, treatment T3 had 7.1, 5.8, and 4.2 times more QSTXs than T0, T1, and T2 (ANOVA, $p = 0.0002$), indicating that perhaps individual cells were more toxic under higher Cu^{2+} concentration.

In addition, the ratios between μ_{STX} and μ_g were below 1 in all the treatments, such as 0.31, 0.48, 0.65, and 0.26 in treatments T0, T1, T2, and T3, respectively. The regression of μ_{STX} versus μ_g indicated a low correlation between the two variables ($R^2 = 0.13$) (Fig. 6) and the Pearson correlation analysis was not statistically significant (Pearson correlation = 0.36, $p = 0.2494$).

Discussion

One of the most well-known effects of Cu^{2+} toxicity on phytoplankton is the negative effect on growth. Previous studies reported that toxic Cu^{2+} conditions increased the amount of reactive oxygen species (ROS), decreased the activity of enzymes, and reduced the expression of genes involved in photosynthesis and oxidative phosphorylation (Murphy and Taiz 1997; Küpper et al. 1998; Ahad and Syiem 2018; Wang et al. 2020). Those molecular responses led to several types of cell damage, such as a decrease in photosynthesis and inhibition of cell division (Surosz and Palinska 2004; Nongrum and Syiem 2012; Hamed et al. 2017; Wang et al. 2020). In the present study, the highest Cu^{2+} level (T3) led to a reduction in cell concentration (5 times on day 5 and 2.3 times on day 20), μ_{max} (1.35 times reduction), and biovolume (1.4 times reduction on day 5), compared to T0 (Figs. 1 and 2). Similarly, a reduction in cell concentration was observed in *M. aeruginosa* cultures under Cu^{2+} concentration of 0.5 and 2.5 μmol L^{-1} (Zhou et al. 2013), whereas Polyak et al. (2013) identified that 80×10^{-3} μmol L^{-1} of Cu^{2+} was sufficient to inhibit *M. aeruginosa* cell growth. In a previous study carried out with *S. aquatilis*, under the condition of 0.8 μmol L^{-1} of Cu^{2+}, a 5-fold reduction in cell growth was observed when compared to the control, revealing the toxic effect of Cu^{2+} on this cyanobacterium (Shavyrina et al. 2001).

Metal toxicity in microorganisms can also cause morphological changes, which are considered indicators of the physiological state of a cell, such as the variation in cell volume (Long et al. 2001). Our experiments demonstrated an increase (3.6 times and 3.7 times on day 5 and 20, respectively) in *R. raciborskii* cell volume under a high Cu^{2+} concentration (T3) (Figs. 2 and 3). A similar result was found in a study with *M. aeruginosa*, which had the cell volume increased 2 times when exposed to the concentration of 80×10^{-3} μmol L^{-1} of Cu^{2+} (Polyak et al. 2013). There was also an increase in cell volume in microalgae such as *Phaeodactylum tricornutum* and *Pseudokirchneriella subcapitata* exposed to 7.44 μmol L^{-1} of Cu^{2+} for 96 h (Franqueira et al. 2000) and 1.3 μmol L^{-1} of Cu^{2+} for 72 h (Machado and Soares 2014). The increase in cell volume observed in these microalgae, compared to the control treatment, was approximately 1.49 and 1.7 times, respectively. It should be noted that the increase in cell volume in *R. raciborskii*, in the present study, occurred simultaneously with the reduction in cell concentration, which confirms that stressed cells have reduced cell divisions and consequently larger cell sizes (Abalde et al. 1995; Cid et al. 1995; Franqueira et al. 2000; Franklin et al. 2002; Polyak et al. 2013; Long et al. 2001).

A decrease in Chl *a* was described in previous studies compared with the control conditions in several microalgae species (*Chlorella* sp., *Scenedesmu* sp., *P. tricornutum*) exposed to 0.76 to 414 μmol L^{-1} of Cu^{2+} concentration (Cid et al. 1995; Sabatini et al. 2009; Hamed et al. 2017) and cyanobacteria (*M. aeruginosa* and *Nostoc linckia*) under 0.03 to 37 μmol L^{-1} of Cu^{2+} (Kumar et al. 1985; Polyak et al. 2013; Iwinski et al. 2016; Wang et al. 2020). Similarly, in the present study, a negative effect on Chl *a* levels was also observed, with a reduction in the condition with the highest Cu^{2+} concentration (800×10^{-3} μmol L^{-1}), compared to other treatments on day 20 (Fig. 4). Cu^{2+} causes a decrease in chlorophyll production (Wang et al. 2020) and inhibits photosynthesis by disrupting the electron transport chain replacing the magnesium atom (Mg^{2+}) in the chlorophyll molecules, which leads to damage to the photosystem and antenna complex (Küpper et al. 1998).

Despite the decrease in cell concentration and biovolume on day 5, in treatment T3 there was a significant increase in the volumetric concentration of STXs from 1.5 to 3.41 times. This result is an indication that high concentrations of Cu^{2+} can affect STXs levels in the early growth phase (Fig. 5). In addition, on day 5, a higher QSTX was also observed in treatment T3, with an increase of 7.1, 5.8, and 4.2 times compared with T0, T1, and T2, respectively. This result is an indication that the individual cells were more toxic under a high Cu^{2+} concentration. Maldonado

et al. (2002) also observed a 20-fold increase in the concentration of extracellular domoic acid (DA) under Cu-stressed condition (1.8 μmol L^{-1}) in the diatom *Pseudonitzschia*. They suggested that DA can complex to Cu^{2+} and play the role of a detoxification ligand, decreasing the bioavailability of Cu^{2+} in the culture. Interestingly, another study found that STXs, in addition to having affinity for sodium channels, also have affinity for Cu^{2+} transporters in the cell membranes of algae, cyanobacteria, and yeast. This study indicated that by adding STX to yeast (*S. cerevisiae*) and microalgae (*C. reinhardtii*) cultures, the Cu^{2+} uptake was inhibited in both species (Cusick et al. 2012, 2013). Recently, Chen et al. (2020) observed 1.8 and 1.7 times higher intracellular and extracellular MCYSTs, respectively, in *M. aeruginosa* under 0.5 μmol L^{-1} of Cu^{2+}. They also reported that MCYSTs might be controlled by an increased Cu^{2+} condition due to the expression modulation of genes *FueA* (ferric uptake regulator) and *mcyD* (microcystin synthetase genes) (Chen et al. 2020).

Although the physiological role of STXs is still unknown, these results corroborate with previous studies on STX-producing *R. raciborskii*, in which most of them reported more than 3 times higher STXs accumulation under stressful or less than ideal conditions (Castro et al. 2004; Pomati et al. 2004; Carneiro et al. 2009, 2011, 2013; Casali et al. 2017; Vargas et al. 2019). This implies that STXs might have a metabolic protective effect, which can provide survival advantages over other organisms. Ferrão-Filho and da Silva (2020) identified a toxic effect on *Daphnia* in a study of the interaction of *R. raciborskii* (producer of STXs) with this microcrustacean. Rangel et al. (2016) observed a reduction in the grazing of *R. raciborskii* producing STXs compared to the grazing of non-toxic *R. raciborskii* strain.

In contrast to what was previously observed about the production of CYN in *R. raciborskii* and MCYSTs in *M. aeruginosa*, we observed in the present study that the STXs production in *R. raciborskii* is not coupled with growth. Based on the calculations of STXs production dynamics using the first-order rate constants (μ_{STX} and μ_g), as described in Orr et al. (2018), we observed a ratio between μ_{STX} and μ_g lower than 1 in all the treatments, indicating that in this time interval (from day 5 to 20), the specific rates of growth were higher than the rates of STXs production. This result may also indicate a metabolic degradation of STXs (Jones and Negri 1997). Likewise, Vargas et al. (2019) also observed a ratio lower than 1 between μ_{STX} and μ_g in the same *R. raciborskii* strain (ITUC01) under oligotrophic and supereutrophic conditions in a period of 15 days, which supports our results. It should be noted that except for Vargas et al. (2019), most of the previous studies with *R. raciborskii* calculated the specific production rate of STXs using the cell quota concentration of STXs instead of the volumetric concentration, which can lead to misinterpretations (Orr et al. 2018).

In summary, our study has shown that Cu^{2+} concentration of 800×10^{-3} μmol L^{-1} significantly reduced the growth and chlorophyll *a* levels, while the cell volume increased in *R. raciborskii*. In addition, it revealed that STX production is uncoupled with growth and *R. raciborskii* toxicity decreased from day 5 to 20. However, on day 5, despite the low concentration of cells and the biovolume in 800×10^{-3} μmol L^{-1} of Cu^{2+}, it was observed that the volumetric concentration of STXs was significantly higher. This result is an indication that high concentrations of Cu^{2+} can affect the levels of STXs in the initial growth phase. Finally, in order to better understand the physiological role of STXs under Cu stress conditions, further studies on Cu^{2+} uptake and STX affinity for Cu^{2+} ions and Cu^{2+} transporters in *R. raciborskii* are necessary.

Conclusion

The exposure of *R. raciborskii* cultures to different concentrations of Cu^{2+} revealed important physiological responses to this stress condition. Growth parameters and chlorophyll *a* levels decreased under the highest Cu^{2+} concentration (T3), whereas the cell volume increased, which indicates that Cu-stressed cells have reduced cell division and consequently larger sizes.

In addition, although it was observed that *R. raciborskii* reduced the toxicity from day 5 to day 20, our results also revealed a significant increment of STXs volumetric concentration and QSTXs on day 5, in treatment T3. This result is evidence that high Cu^{2+} concentration (800×10^{-3} μmol L^{-1}) positively influenced the production of STXs in *R. raciborskii* in the initial growth phase.

This response to the high Cu^{2+} condition can be an advantage for toxic strains, as STXs may decrease Cu^{2+} bioavailability and inhibit Cu^{2+} uptake. Nevertheless, STX function is still unknown; therefore, further studies on Cu^{2+} uptake and STX affinity for Cu^{2+} ions and Cu^{2+} transporters in *R. raciborskii* are needed.

Finally, the results of the present study can help to understand the physiological responses, as well as the dynamics and regulation of STXs production of *R. raciborskii* under a toxic Cu^{2+} environment.

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