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Effects of α-Ag_2WO_4 crystals on photosynthetic efficiency and biomolecule composition of the algae *Raphidocelis subcapitata*

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Abstract The α-Ag_2WO_4 (hexagonal rod-shaped) is a multifunctional material with interesting physical and chemical properties, such as good electronic, photocatalytic, anticancer and microbicidal performance. Considering this, its use can contribute to the presence and accumulation of this compound in freshwater ecosystems. Therefore, the present study investigated the effects of α-Ag_2WO_4 on the freshwater Chlorophyceae *Raphidocelis subcapitata*, at the level of cell density, chlorophyll *a* (Chl *a*), total carbohydrate contents, and photosynthetic activity (maximum quantum yield and oxygen-evolving complex—OEC). The α-Ag_2WO_4 reduced cell density by ~48% already in the first 24 h of exposure at 31.76 μg L^{-1} (highest concentration). Moreover, at 31.76 μg L^{-1}, we observed a drastic reduction in the maximum quantum yield, and impact in the oxygen evolving complex at 24 h and 48 h. However, our results indicated a possible recovery of the photosynthetic activity in the surviving algal cells at 72 and 96 h. The contents of chlorophyll *a* (Chl *a*) and total carbohydrates decreased significantly (Dunnett's test, $p < 0.05$) at 4.11, 5.84, 10.55, and 10.67 μg L^{-1} treatments and increased significantly (Dunnett's test, $p < 0.05$) at the highest concentration (31.76 μg L^{-1}),

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which is possibly a mechanism for the algal cells to optimize the amount of energy to be used in the photosynthetic process and maintaining the integrity of the cell wall. This study contributes to clarifying how α-Ag_2WO_4 interacts with *R. subcapitata*¸ showing the toxicity mechanism of photosynthetic activity. This can help predict the fate and effect of these composites by providing a basis for their ecological risk assessment.

Keywords Silver tungstate · Toxicity · Chlorophyceae · Phyto-PAM · Photosynthetic efficiency

1 Introduction

Given the great applicability of alpha-silver tungstate (α-Ag_2WO_4) crystals (Assis et al., 2020; Cruz et al., 2020; Macedo et al., 2019; Nobre et al., 2019; Penha et al., 2020), mainly in photocatalysis (Macedo et al., 2018) and microbicidal activity (Assis et al., 2018, 2019; Foggi et al., 2017; Laier et al., 2020; Longo et al., 2014), its increase in natural ecosystems is expected. Increased concentrations of α-Ag_2WO_4 in the environment may occur due to the recovery of the semiconductor from the reaction mixture (consisting of the catalyst and the substance to be degraded) is not always possible, favoring the presence and accumulation in water bodies (Kumari et al., 2019; Matos et al., 2020). In addition, particles can be absorbed into the soil and carried to water bodies (Dewez et al., 2018) and are a source of ionic silver release into aquatic ecosystems, which can pose serious threats to their biota (Navarro et al., 2008).

Among the organisms that make up aquatic environments, phytoplankton contributes significantly to nutrient cycling (fixing carbon), oxygen production and is responsible for a large part of overall primary productivity (Baracho et al., 2019). As microalgae are at the base of aquatic food webs, any modification of the photosynthesis process through damage to their photosynthetic apparatus can affect higher trophic levels and, consequently, reach the entire ecosystem (Kahru & Dubourguier, 2010). Fast and relatively simple methods, such as the parameters obtained in Phyto-PAM and the chlorophyll *a* (Chl *a*) content, can indicate the physiological health in primary producers, i.e. algae and higher plants (Juneau et al., 2005). It is known that several environmental factors affect the physiological state of autotrophs by impairing photosynthesis or biochemical processes, and therefore the measurement of photosynthetic parameters is important and reliable to identify environmental stress (Juneau & Popovic, 2000; Rocha et al., 2021). Furthermore, macromolecules such as carbohydrates are essential in photosynthetic and respiratory processes (Martínez-Ruiz & Martínez-Jerónimo, 2015), energy storage, and the structural component of the cell wall (Markou et al., 2012). When microalgae are exposed to stressful conditions, changes often occur in the amount of carbohydrates (Rossi et al., 2018). Some studies show that different species of microalgae can alter the amount of carbohydrates when exposed to different types of contaminants (Alho et al., 2020; Huang et al., 2016; Silva et al., 2018). Thus, assessing the content of carbohydrates of *Raphidocelis subcapitata* provided relevant information about α-Ag_2WO_4 toxicity.

In this context, given the great applicability of α-Ag_2WO_4 combined with the lack of studies regarding its effects on the physiology and biochemical composition of microalgae in general; and considering the importance of these autotrophic organisms for aquatic ecosystems, our objective was to evaluate the effects of α-Ag_2WO_4 on photosynthetic activity, biological molecules and cell density of the Chlorophyceae *R. subcapitata*. This study contributes to clarifying and understanding how α-Ag_2WO_4 interacts with *R. subcapitata*¸ showing the toxicity mechanisms on photosynthetic activity, providing information that can help predict the fate and effects of these

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compounds. In addition, our study provides a basis for their ecological risk assessment.

2 Material and methods

2.1 Synthesis and characterization of α-Ag_2WO_4

The samples of α-Ag_2WO_4 were synthesized using the coprecipitation (CP) method in aqueous medium, according to Macedo et al. (2018). The hydrodynamic size, polydispersity index (PdI), and zeta potential of the particles were measured in exposure medium and in ultrapure water at 0, 24, 48, 72, and 96 h by dynamic light scattering (DLS) using Zetasizer Nano ZS90, Malvern. The results from 0 and 96 h are described in our previous study (Abreu et al., 2022). The results from 24, 48, and 72 h are presented in Table S1 (Supplementary material). Silver concentrations in α-Ag_2WO_4 test solutions used in the toxicity tests (data not shown) were determined by inductively coupled plasma mass spectrometry (ICP-MS PerkinElmer NexION 2000) (Abreu et al., 2022).

2.2 Algal cultures

The cosmopolitan freshwater microalga *R. subcapitata* (Chlorophyceae), which is recommended in international standards for ecotoxicological testing (OECD, 2011), was cultivated in CHU-12 culture medium (CHU, 1942) (Table S2, Supplementary material) at 25 ± 1 ºC, with a light intensity of ≅ 130 µmol photon m^{-2} s^{-1} LED light and 12 h/12 h of light/ dark photoperiod. The pH values were around 7 – 8.5 and did not vary by more than 1.5 units. The toxicity tests followed the same culture conditions. We used a bath sonicator (Ultra cleaner 1400 Unique, Brazil) for 30 min to disperse the α-Ag_2WO_4 in ultrapure water and immediately afterwards we prepared the test solutions. Exponentially growing *R. subcapitata* cells were inoculated (initial concentration of 1×10^5 cells ml^{-1}) and exposed to the concentrations of 0.00, 4.11, 5.84, 10.55, 10.67, and 31.76 µg L^{-1} of α-Ag_2WO_4 for 96 h in 500 mL polycarbonate Erlenmeyers containing 250 mL of culture medium. These concentrations were chosen based on preliminary tests results. The toxicity tests followed the OECD (201) guidelines (OECD, 2011), with 3 tests performed, each one with triplicates for the control and treatments.

Every day, 1.8 mL of samples was fixed with formaldehyde buffered with borax (1% final concentration) and the cells were counted in a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA) with a 15mW argon-ion laser (488 nm excitation), using 6 µm fluorescent beads as an internal standard (Fluoresbrite carboxylate microspheres; Polysciences, Warrington, Pennsylvania, USA). To identify the cells, we followed exactly the protocol described in Sarmento et al. (2008).

2.3 PAM fluorescence measurements

We utilized an amplitude modulated fluorometer (PHYTO-PAM, Heinz Walz GmbH, Germany), equipped with an optical drive ED- 101US/MP, to perform chlorophyll *a* fluorescence measurements. Daily, 3 mL of each sample was left in the dark for 15 min before measurements. The parameters F_0 (minimum fluorescence), F_M (maximum fluorescence) and Φ_M (maximum quantum yield) are provided by Phyto-PAM (Schreiber, 1986; Schreiber & Bilger, 1993). The efficiency of the oxygen evolving complex of PSII (F_0 / F_V, where $F_V = F_M — F_0$) was also determined by the fluorescence emission from algal cells acclimated to the dark (Kriedemann et al., 1985).

2.4 Determination of chlorophyll *a* and total carbohydrates

We determined the amount of chlorophyll *a* with dimethylsulfoxide (DMSO) according to the methodology described by Shoaf and Lium (1976). After extraction, we used Eq. (1) established by Jeffrey and Humphrey (1975) to quantify the content of chlorophyll *a* where E_{664} and E_{647} are the absorbance at 664 and 647 nm λ, respectively.

$$\text{Chl}a = 11.93E_{664} - 1.93E_{647} \quad (1)$$

Total carbohydrate quantification was determined based on the phenol–sulfuric reaction and anhydrous dextrose (Mallinckrodt Chemicals, USA) as a standard for the calibration curve, according to Liu et al. (1973). A spectrophotometer (HACH Company,

Loveland, CO, USA) was used for the reading at 485 nm.

2.5 Statistical analysis

The IC_{50} (inhibitory concentrations) based on cell density rates were calculated by nonlinear regression logistic curves using Statistica 7.0 software (Statsoft Inc, 2004). Data were tested for normality and homogeneity of variance. Statistical analyses were performed using the SigmaPlot software version 11.0 (Systat, 2008). Statistically significant differences among treatments and controls were determined using one-way ANOVA, followed by Dunnett's post hoc multiple comparison test. For non-normal data, the Kruskal–Wallis test and multiple comparisons with Dunn's test were performed. The statistical significance level was defined as $p < 0.05$. The data were obtained from three experimental replicate cultures and are presented as the mean ± SD of the replicates. Uniquely for the total carbohydrate data, we normalized these data using log transformation.

3 Results and Discussion

The results of the microparticle characterization are available in Fig.S1 and Table S1 (Supplementary Material). The α-Ag_2WO_4 particles were obtained with a hexagonal rod shape and orthorhombic structure (Fig. 1), and average transversal and longitudinal sizes of 0.23 and 1.22 µm, respectively. Overall, the zeta potential values averaged between -5.39 and -12.8 mV, indicating electrostatic instability (Kleiven et al., 2018, 2019; Lodeiro et al., 2017), because the aqueous solutions considered stable have values around ± 30 mV (Stensberg et al., 2011). The PdI values were higher than 0.22 ± 0.07, which indicated that the α-Ag_2WO_4 particles formed aggregates/agglomerates.

We observed significant changes in algae growth when in contact with α-Ag_2WO_4 particles (Fig. 2). At 24 h there was a difference (Dunn's test, $p < 0.05$) only between the control and the highest concentration (31.76 µg L^{-1}), with a ~ 48% reduction. On the other hand, at 48 h, all treatments entailed significant reductions (Dunnett's test, $p < 0.05$) in the cell density. Finally, at 72 h the 3 highest concentrations (10.55, 10.67 and 31.76 µg L^{-1}) caused significant reductions (Dunnett's test, $p < 0.05$) in cell density and at 96 h the 2 highest concentrations (10.67 and 31.76 µg L^{-1}) reduced (Dunnett's test, $p < 0.05$) the cell number when compared with the control. The IC_{50} based on relative growth rates (RGR), calculated according to Bao et al., (2011), in a previous work (Abreu et al., 2022) was 13.72 ± 1.48 µg L^{-1} and the IC_{50} based on the cell density was 14.9 ± 1.05 µg L^{-1}.

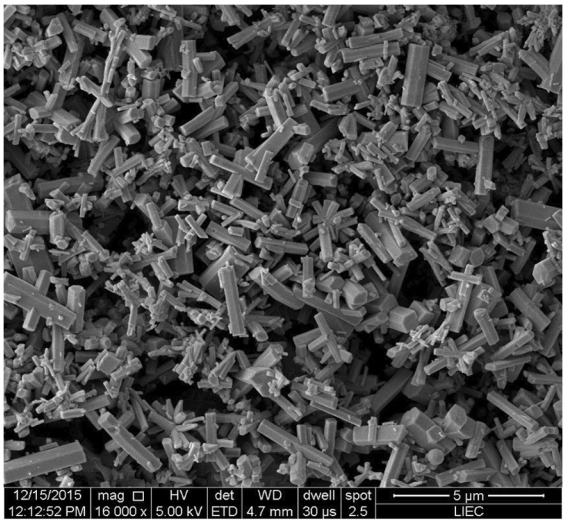

Fig. 1 Field emission scanning electron microscopy (FE-SEM) of the α-Ag_2WO_4 sample obtained by a Supra 35 VP, Carl Zeiss operated at 10 kV

According to previous studies, materials with silver in their composition are highly toxic to aquatic biota, especially for microalgae, inhibiting growth, forming reactive oxygen species, DNA damage, among others (Abreu et al., 2022; He et al., 2012; Huang et al., 2016; Lekamge et al., 2020; Odzak et al., 2017; Sørensen et al., 2016). Even at very low concentrations, dissolved silver can compromise photosynthesis and growth in phytoplankton (Navarro et al., 2008). This can help to explain the growth inhibition of *R. subcapitata* at the highest concentrations of α-Ag_2WO_4, which was probably due to the effects of the released silver ions into the medium and ROS production, as we observed in a previous study with α-Ag_2WO_4 (Abreu et al., 2022).

Regarding the photosynthetic activity, the maximum quantum yield, obtained via Phyto-PAM, indicates the amount of light used in photosynthesis, providing information about the physiology of the microalgae (Herlory et al., 2013). According

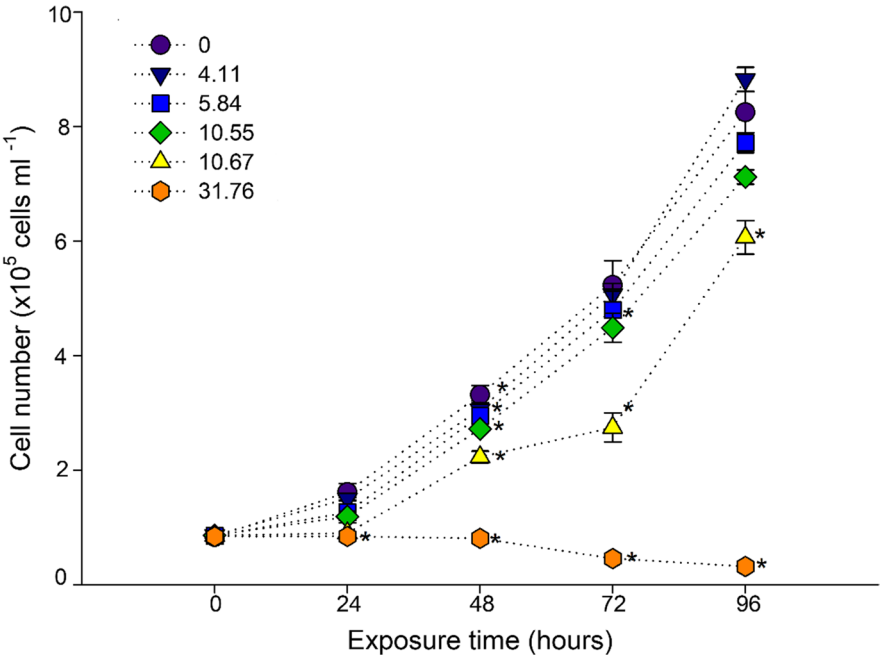

Fig. 2 Cell density (mean ± SD) of *Raphidocelis subcapitata* under α-Ag_2WO_4 -R exposure during 96 h. The concentrations are expressed in μg L^{-1}. Asterisks * represent a significant difference (Dunn's test, $p < 0.05$; Dunnett's test, $p < 0.05$) of treatments compared to the control group

to Dewez and Oukarroum (2012), the decrease in maximum quantum yield values indicates a reduction in the ability of PSII to perform primary photochemical reactions. The results of the maximum quantum yield (ΦM) are shown in Fig. 3. After 24 h and 48 h of exposure, there was a drastic reduction (Dunn's test, $p < 0.05$) of ~ 72% and ~ 78%, respectively, of this parameter at the highest concentration tested (31.76 μg L^{-1}). At 72 h, the concentrations of 10.67 μg L^{-1} and 31.76 μg L^{-1} of α-Ag_2WO_4 caused a significant reduction (Dunnett's test, $p < 0.05$) of ~ 4.5% and 35%, respectively, in the Φ_M, when

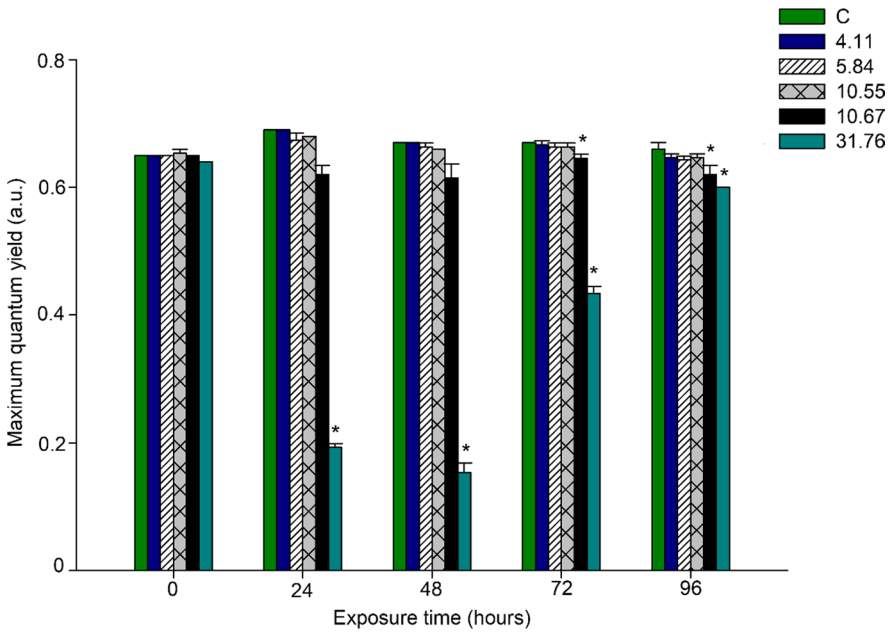

Fig. 3 Maximum quantum yield (mean ± SD) of *Raphidocelis subcapitata* after 24, 48, 72, and 96 h under α-Ag_2WO_4 exposure. Concentrations are expressed in μg L^{-1}, where: C = control group and asterisks * represent a significant difference (Dunn's test, $p < 0.05$; Dunnett's test, $p < 0.05$) of treatments compared to the control group

compared to the control. Finally, at 96 h there was a ~ 6% and 9% decrease (Dunnett's test, $p < 0.05$) in 10.67 and 31.76 µg L^{-1} concentrations, respectively. In light of this, our results indicate that the photosynthetic apparatus was affected, especially at the highest concentration of α-Ag_2WO_4 (31.76 µg L^{-1}), but this impairment was gradually reduced throughout the days of the experiment at this concentration, since the percentage of reduction of Φ_M diminished from the first to the last day of treatment.

We observed that the efficiency of the oxygen-evolving complex (F_0/F_v) was significantly affected at the highest concentration tested of α-Ag_2WO_4. In general, at 31.76 µg L^{-1}, F_0/F_v it increased 9.3 times (Dunn's test, $p < 0.05$) at 24 h and 11 times (Dunn's test, $p < 0.05$) at 48 h, when compared to the control (Fig. 4). At 72 h, the increase was around 2.7 times (Dunn's test, $p < 0.05$) and at 96 h it was about 1.3 times higher than in control cells. High values of $F_0/F_{v,}$ especially on the first two days of exposure to α-Ag_2WO_4, indicate that possibly water-splitting apparatus was damaged (Alho et al., 2019; Reis et al., 2021), which was already expected, due to the excellent photocatalytic property of α-Ag_2WO_4 (Macedo et al., 2018). The OEC constitutes the water splitting system, where the water molecule is broken down in the presence of light and this process is responsible for the production of oxygen (Mattoo et al., 1999). The composition of the OEC is basically formed by manganese atoms and proteins, which require the presence of chloride and calcium. Here, probably the silver ions released by the microcrystal have bound to chloride ions and this may have compromised the water-splitting apparatus mainly in 24 and 48 h. Therefore, we can assume that the water splitting apparatus was the main target of α-Ag_2WO_4, and the reduced maximum quantum yield was probably a consequence of the impacted OEC. Already in the last days of exposure, even with F_0/F_v values significantly different from the control, the not so high values indicate a recovery of the physiology of the algal cells that survived at the end of the ecotoxicity test.

Regarding Chl *a* content, we observed a decrease of ~ 41, 47, 52, and 43% (Dunnett's test, $p < 0.05$) at concentrations of 4.11, 5.84, 10.55, and 10.67 µg L^{-1} of α-Ag_2WO_4, respectively (Fig. 5A). This is probably a result of reactive oxygen species production, because the chloroplast is a site that favors ROS generation (Li et al., 2015), as recently observed in a study by our research group (Abreu et al., 2022). On the other hand, at the highest concentration of α-Ag_2WO_4 (31.76 µg L^{-1}), the amount of Chl *a* increased ~ 47% (Dunnett's test, $p < 0.05$), which is possibly a mechanism for the algal cells to optimize

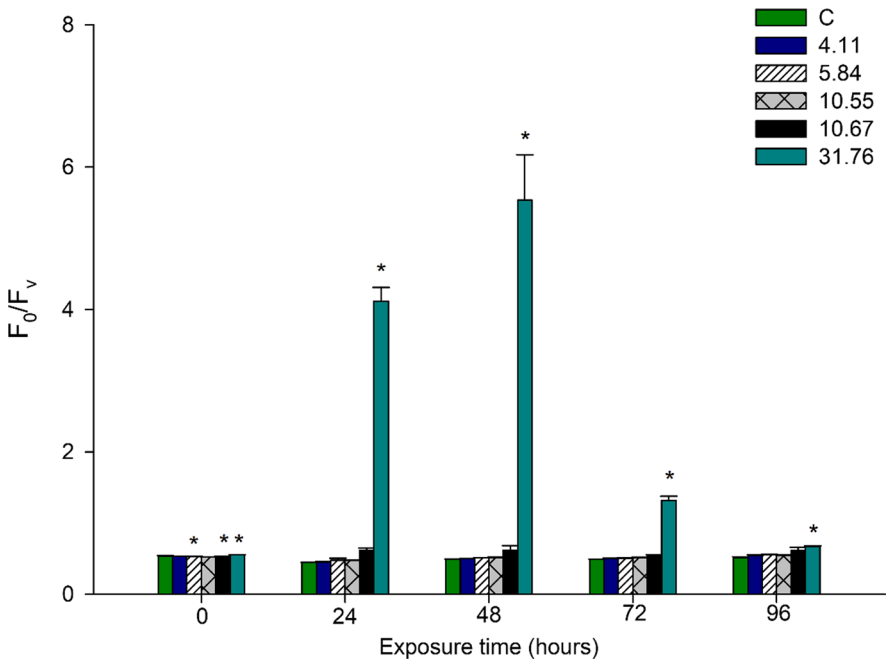

Fig. 4 Efficiency of the Oxygen Evolving Complex (F_0/F_V) (mean ± SD) of *Raphidocelis subcapitata* after 24, 48, 72, and 96 h under α-Ag_2WO_4 exposure. Concentrations are expressed in µg L^{-1}, where: C = control group and asterisks * represent a significant difference (Dunn's test, $p < 0.05$; Dunnett's test, $p < 0.05$) of treatments compared to the control group

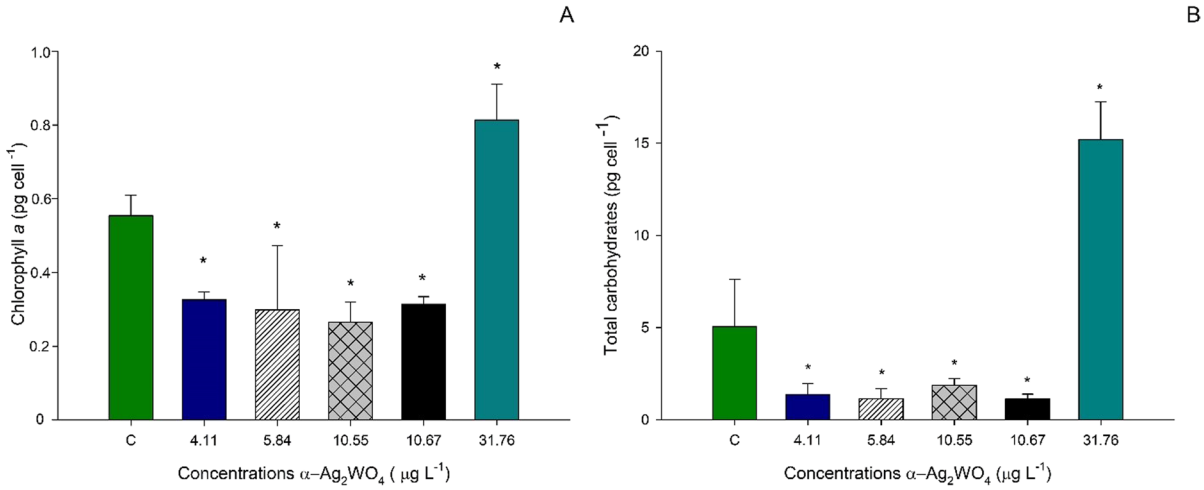

Fig. 5 Chlorophyll *a* content (mean ± SD) (A) and total carbohydrates (mean ± SD) of *Raphidocelis subcapitata* after 96 h exposed to α-Ag_2WO_4 (B). C = control group and asterisks * represent a significant difference (Dunnett's test, $p < 0.05$) of treatments compared to the control group

the amount of energy to be used in the photosynthetic process (Alho et al., 2020; Rocha et al., 2021; Silva et al., 2018; Wacker et al., 2015), in order to compensate for the stress caused by α-Ag_2WO_4 and maintain photosynthesis at high rates**.**

Following the same pattern as the Chl *a* content, the amount of total carbohydrates (Fig. 5B) decreased significantly ~ 3.6, 4.4, 2.7, and 4.5 times (Dunnett's test, $p < 0.05$) at concentrations of 4.11, 5.84, 10.55, and 10.67 µg L^{-1} and increased ~ 3 times (Dunnett's test, $p < 0.05$) at 31.76 µg L^{-1} of α-Ag_2WO_4. This is closely related to the higher production of Chl *a*, because the increased production of this pigment can enable greater amounts of CO_2 to be fixed and then converted into carbohydrates (Chia et al., 2015). Furthermore, under stress conditions, it is common that carbohydrate content in microalgae to increase (Rossi et al., 2018), which may be related to a protective mechanism of the algal cells, thus maintaining the integrity of the cell wall. This biomolecule has structural and storage functions, supplying the energy demand necessary for the maintenance of metabolism and cell wall structure (Markou et al., 2012), which explains why we observed higher carbohydrate content at the highest concentration (31.76 µg L^{-1}) of α-Ag_2WO_4.

Considering that the percentage of reduction of Φ_M values gradually diminished from 24 to 96 h at the highest concentration of α-Ag_2WO_4, and the F_0/F_V values indicated a gradually less severe impact at this same concentration between the beginning and the end of the experiment. This pattern can be a consequence of the increase in the Chl *a* and carbohydrate content that occurred at this concentration (31.76 µg L^{-1}) in the surviving cells. Probably an algal attempt to reduce the negative impacts of α-Ag_2WO_4, combined with the possible chelation of metals to dead cells, decreasing the metal available to the remaining cells.

4 Conclusion

Our results showed evidence of toxic effects of α-Ag_2WO_4 crystals on the photosynthetic activity of the microalga *R. subcapitata*, through a drastic reduction of the maximum quantum yield and loss of efficiency in OEC (increased values of F_0/F_v), mainly in the first hours of exposure. Besides the physiological aspects, we observed a reduction in the cell density and an increase in the biomolecules, such as Chl *a* and total carbohydrates contents at the highest experimental concentration of α-Ag_2WO_4, probably in an attempt to decrease the impacts of the α-Ag_2WO_4. At the end of the exposure, even with reduced cell number, the increased Chl *a* content possibly enabled the remaining cells to compensate for the stress caused by α-Ag_2WO_4 and maintain photosynthesis,

which is also corroborated by the maximum yield and OEC values, indicating the tendency to recover the physiological health. The parameters evaluated in this study were efficient and sensitive, with significant variations compared to the control group, which reinforces the importance of evaluating physiological, populational (cell density) aspects, as well as biomolecules contents (as Chl *a* and carbohydrate) in ecotoxicity studies. Therefore, identifying the targets of α-Ag_2WO_4 contributes to the elucidation of the mechanisms of action of this semiconductor on the microalga *R. subcapitata*. The changes observed in the microalgae in this study may be harmful in the long term, because as these are autotrophic organisms, impacts at the base of the food chain may pose threats to higher trophic levels. Thus, these data are useful for predicting and assessing risks caused by microcrystals.

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Author contributions This manuscript describes original work and is not under consideration by any other journal. All authors assume that they have read the final version of the manuscript and agreed with it. The contribution of each author of this work is described below:

CBA: co-developed the experimental design, carried out experimental tests and collected the data; performed statistical analysis; analyzed and interpreted the data and wrote the paper.

RCG: co-developed the experimental design, carried out experimental tests and collected the data; performed statistical analysis; analyzed and interpreted the data and reviewed the paper.

LLR: carried out experimental tests, collected the data and reviewed the paper.

GSR: carried out experimental tests and collected the data; performed statistical analysis; analyzed and interpreted the data and reviewed the paper.

LGOA: analyzed and interpreted the data and reviewed the paper.

LMA: performed the characterization of α-Ag_2WO_4 and reviewed the paper.

LSV: performed the characterization of α-Ag_2WO_4 and reviewed the paper.

MA: co-developed the experimental design; analyzed and interpreted the data and reviewed the paper.

ASM: co-developed the experimental design; performed statistical analysis, analyzed and interpreted the data and reviewed the paper.

EL: co-developed the experimental design; analyzed and interpreted the data and reviewed the paper. This author is also one of the sponsors, responsible for obtaining financial grant that supported this study (FAPESP CEPID-finance code 2013/07296-2).

MGGM: co-developed the experimental design; analyzed and interpreted the data and reviewed the paper. This author is also one of the sponsors, responsible for obtaining financial grant that supported this study (FAPESP 2018/07988–5; CNPq 316,064/2021–1).

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.No conflicts, informed consent, human or animal rights are applicable for this work.

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