Journal of Applied Phycology (2024) 36:155–166
https://doi.org/10.1007/s10811-023-03150-5

RESEARCH

Microalgae-bacteria interaction in alginate beads prevents the negative effect of copper oxide nanoparticles on the growth and metabolism of *Scenedesmus* sp.

Karen A. Alonso[1] · Francisco J. Choix[1,2] · Guadalupe V. Nevarez-Moorillón[1] · Oskar A. Palacios[1,3]

Received: 8 August 2023 / Revised: 15 November 2023 / Accepted: 16 November 2023 / Published online: 12 December 2023

Abstract

Owing to their extensive application, copper oxide nanoparticles (CuO NPs) has become common components in wastewater that arrives at wastewater treatment plants. Immobilization of microalgae and bacteria cells in alginate beads has been proposed as a potential tool for tertiary wastewater treatment processes to prevent the negative effect of biotic stress on immobilized microorganisms. Nonetheless, the effect of the emerging CuO NP contaminants on microalgae-bacteria interaction has not been evaluated. Thus, the aim of this study was to assess the effect of CuO NPs (1, 10, and 100 ppm) on the growth and metabolism of the microalga *Scenedesmus* sp. immobilized alone and concomitantly with the bacterium *Azospirillum brasilense*. Low CuO NP concentrations (1 ppm) induced higher growth rates for both microorganisms, regardless of their immobilization status (an average increase of 117.9% for *Scenedesmus* sp. and 73% for *A. brasilense*). In addition, microalga-bacterium co-immobilization enhanced the growth of *Scenedesmus* sp. in the control group (without NPs: 2.95 ± 0.32 cells $\times 10^4$ bead^{-1}) and in the presence of low (1 ppm: 8.69 ± 3.19 cells $\times 10^4$ bead^{-1}) and high NP concentrations (100 ppm: 2.39 ± 1.03 cells $\times 10^4$ bead^{-1}) compared with microalgae immobilized alone (without NPs: 1.79 ± 0.48 cells $\times 10^4$ bead^{-1}; 1 ppm: 6.82 ± 1.54 cells $\times 10^4$ bead^{-1}; 100 ppm: 1.32 ± 0.27 cells $\times 10^4$ bead^{-1}). Moreover, the interaction between *Scenedesmus* sp. and *A. brasilense* in the presence of 1 ppm CuO allowed for higher protein and carbohydrate contents than did the other treatments. The negative effect of the CuO NPs on the growth of the microorganisms was only observed at the higher concentration (100 ppm) when the microorganisms were immobilized alone. These results demonstrate that the use of immobilized cells can prevent the negative effects of emerging contaminants such as CuO NPs on microalgal growth. Moreover, the microalgae-bacteria interaction in the presence of CuO NPs allowed for identifying microalgae biomasses with high contents of the metabolites of interest.

Keywords Emerging contaminants · Metallic nanoparticles · Microalgae Growth-Promoting Bacteria · Stress mitigation · *Scenedesmus* · CuO

Introduction

Metallic nanoparticles (NPs) are currently commonly used and ultimately reach the environment (Markus et al. 2016). Metallic NPs can be present in concentrations ranging from 1,600 to 10,700 ng L^{-1} in influent wastewater and could be accumulated in water sludge from the sewage system (Cervantes-Avilés and Keller 2021). CuO NPs have optical, catalytic, semiconductor, and antimicrobial properties, making them viable for use in areas such as medicine, personal care products, semiconductor manufacturing, and antimicrobial products such as fungicides, bactericides, and herbicides (Otero-González et al. 2014; Shang et al. 2019). Owing to this wide application of CuO NPs, their

✉ Oskar A. Palacios
opalacios@uach.mx

[1] Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua (UACH), Circuito Universitario S/N, Campus Universitario II, 31125 Chihuahua, Chih., Mexico

[2] CONAHCYT, Facultad de Ciencias Químicas-Universidad Autónoma de Chihuahua (UACH), Circuito Universitario S/N, Campus Universitario II, 31125 Chihuahua, Chih., Mexico

[3] The Bashan Institute of Science, 1730 Post Oak Court, Auburn, AL 36830, USA

presence in wastewater arriving at wastewater treatment plants is expected. On the other hand, microalgae from the genera *Scenedesmus*, *Chlorella*, and *Chlamydomonas* have been used to remove inorganic compounds (N and P) from wastewater owing to their high capacity to accumulate lipids from this process (Wollmann et al. 2019). Moreover, the interaction between microalgae and bacteria is well known to enhance the degradation of organic and inorganic contaminants in wastewater owing to their metabolic complementation during their interaction (Li et al. 2023).

Nonetheless, changes in environmental conditions, such as nutrient deficiency, changes in temperatures and pH levels, and the presence of contaminants, are well known to affect the positive interaction between microalgae and bacteria, thus affecting the efficiency of the wastewater treatment process (Zhang et al. 2020; Rosero-Chasoy et al. 2021; Li et al. 2023). The presence of CuO NPs in wastewater at wastewater treatment plants has been reported to affect the biological process through the loss of cell viability in the microorganisms used for water treatment, even in the first 24 h of exposure (Miao et al. 2017). In microalgae, the presence of CuO NPs at concentrations of 5–200 ppm induces oxidative damage, with decrements in growth rate and production of photosynthetic pigments in *Nannochloropsis oculata* (Fazelian et al. 2019). Similarly, the presence of CuO NPs inhibited growth, reduced the photosynthetic apparatus, and increased the production of reactive oxygen species in the microalga *Isochrysis galbana* (Shoman et al. 2023). Moreover, the interaction between microalgae and bacterial cells can also be affected by the presence of metallic NPs such as CuO, as the motility of bacteria and production of exo-polysacharides, which are important in establishing physical interactions, are affected by NPs at gene expression levels (You et al. 2021).

Therefore, the use of immobilized microalgae and bacterial cells in alginate beads has been proposed as a potential tool for tertiary wastewater treatment processes with rapid nutrient removal, and the recovered beads can be used as soil inoculants (Covarrubias et al. 2012; Cruz et al. 2013; Lopez et al. 2013). Alginate beads maintain their stability for at least 96 h in real wastewater, but it has been reported that even only 48 h may suffice for successful nutrient removal (Cruz et al. 2013), allowing their removal and reuse as fertilizers for arid soils (Trejo et al. 2012). In addition, the immobilization of microalgae-bacteria cells for tertiary wastewater treatment protects against biotic stress caused by other microorganisms (Covarrubias et al. 2012). Co-immobilization of microalgae with microalgae growth-promoting bacteria (MGPB) such as *Azospirillum brasilense* improves the growth and metabolism of microalgae such as *Scenedesmus* and *Chlorella* (Choix et al. 2018). Specifically, the interaction between *Scenedesmus obliquus* and *A. brasilense* has been shown to promote growth and enhance CO_2 fixation in microalgae when they were co-immobilized in alginate beads (Choix et al. 2018; Barbosa-Nuñez et al. 2022). In suspension, their interaction showed a positive effect on the improvement of the response of microalgae against abiotic stress conditions (Pagnussat et al. 2020). All these advantages make the use of the interaction of immobilized microalgae and bacteria in alginate beads a valuable wastewater treatment strategy. Nevertheless, the effect of emerging contaminants such as metallic NPs, which are commonly found in wastewater that arrive in wastewater treatment plants, on this strategy must be evaluated to define the scope of the microalgae-bacteria immobilization technology.

Recently, the protective role of alginate beads against ZnO nanoparticles was demonstrated during the interaction between *A. brasilense* and *Chlorella sorokoniana* (Palacios et al. 2023). Nonetheless, although the interaction between alginate and NPs such as CuO or ZnO has already been demonstrated, the stabilization of CuO NPs by alginate particles has also been described to be lower than that of ZnO (Ekanayake and Godakumbura 2021). Although the use of microalgae and bacteria immobilized in alginate beads has been demonstrated as a successful strategy for wastewater treatment and the prevention of the abiotic stress caused by contaminants, the effect of CuO NPs, which are one of the major metallic NPs found in wastewater, on co-immobilized microalgae-bacteria has not been evaluated. In addition, the role of *A. brasilense* as a MGPB could allow better growth and metabolite production by *Scenedesmus* sp. even in the presence of emerging contaminants such as CuO NPs. Thus, we hypothesized that the immobilization of *Scenedesmus* and *Azospirillum* cells in alginate beads allows for a positive interaction, which can be observed in *A. brasilense* and *Scenedesmus* sp. growth and in the production of metabolites by *Scenedesmus* sp. despite the presence of CuO NPs. The objectives of this study were to assess the effects of three concentrations of CuO NPs on the growth of the microalgae *Scenedesmus* sp. and the MGPB *A. brasilense*, and to identify changes in the microalgae metabolite profile when immobilized in alginate beads alone or co-immobilized with the bacterium. In addition, we examined the effect of CuO NPs on microalgae and bacterial cell integrity using scanning electron microscopy (SEM).

Materials and methods

Microorganisms and initial inoculum

The unicellular microalga *Scenedesmus* sp. CC16 (isolated from Chapala, Mexico) and MGPB *Azospirillum brasilense* Cd (DSM 1843, Leibniz-Institute DSMZ, Braunschweig, Germany) were used. For the initial inoculum of microalgae, 10 mL of axenic culture (10^6 cells mL^{-1}) was inoculated into

90 mL of a sterile mineral medium (C30) (Gonzalez et al. 1997) and incubated at 27 °C ± 2 °C (the temperature commonly used in our laboratory for the maintenance of these microalgae since their isolation from Chapala Lake in 2016), with stirring at 140 rpm and continuous 90 µmol photons m^{-2} s^{-1} light intensity for 7 days. The bacterium was cultured in a BTB-2 medium and incubated at 35 °C ± 2 °C with constant stirring at 120 rpm for 24 h (Bashan et al. 2011).

CuO NPs

CuO NPs of < 50 nm particle size with a surface of 29 m^2 g^{-1} from Sigma-Aldrich (Cat. 544,868, Germany) were used for this study. The manufacturer provided the characteristics of the NPs.

Cell immobilization

For immobilization of the microorganisms, the procedure described by de-Bashan et al. (2004) was followed. Briefly, pellets from 20 mL of axenic cultures of either *Scenedesmus* sp. (10^6 cells mL^{-1}) or *A. brasilense* (10^9 cells mL^{-1}) were obtained and then 25 mL of sterile saline solution (0.85% NaCl) was added and mixed for 30 s. After this, the microorganisms were centrifuged at 6,000 × *g* for 5 min and the saline solution was discarded. They were then washed three times to eliminate the residual culture media from the microbial cells, resuspended in 20 mL of sterile saline solution (0.85% NaCl), and mixed with 80 mL 3000 cP 2% alginate solution (154,723, MP Biomedicals, USA). Beads (3 mm) were formed by dripping the alginate solution into a 3% (*w*/*v*) calcium chloride solution using a peristaltic pump under constant pressure to ensure a homogenous size. For beads containing both microorganisms, after washing the cultures in sterile saline solution (0.85% NaCl), each culture was resuspended in 10 mL of saline solution and then mixed with 80 mL of alginate solution before forming the beads. After immobilization, the beads were cured for 30 min in 3% (*w*/*v*) calcium chlorine and rinsed three times with sterile saline solution. For each treatment, 8 g of beads (on average, 167 beads to reach a cell concentration of 10^6 cells for each microorganism) of *Scenedesmus* sp. or *A. brasilense* immobilized alone or both microorganisms co-immobilized was added to the SGM (Synthetic Growth Media).

Experimental design and culture conditions

The experimental design consisted of the following treatments: *Scenedemus* sp. immobilized alone, *A. brasilense* immobilized alone, and *Scenedesmus* sp.-*A. brasilense* co-immobilized. The microorganisms in each growing condition were tested at three CuO NP concentrations (1, 10, and 100 ppm). The growth medium without NPs was considered as the control (0 ppm). Each treatment was replicated five times in 250-mL Erlenmeyer flasks containing 100 mL of volume. According to the treatment, 1, 10, or 100 ppm of CuO NPs was added to the SGM and sonicated in four 1-min cycles at 20 – 60 kHz and 4 °C with a Bioruptor Pico sonication system (Diagenode, usa) to obtain a homogenized medium with NPs. The incubation conditions consisted of stirring at 140 rpm and 27 °C ± 2 °C, with continuous 90 µmol photons m^{-2} s^{-1} light intensity during the 96-h duration of the experiment.

To verify the results, two independent experiments were conducted. The growth of the microalga and bacteria was determined every 24 h. In addition, the production of pigments (Chl*a*, Chl*b*, and β-carotenoids) and metabolite composition on Fourier-transform infrared spectroscopy (FTIR) were analyzed at 96 h. The data obtained from the two experiments showed similar trends and values; thus, they were analyzed together.

Counting of microorganisms

Three samples from each replicate (1 bead for immobilized or co-immobilized cultures) were collected during each sampling period. Alginate beads were dissolved by immersion for 30 min at 28 °C ± 2 °C in 1 mL citrate buffer (sodium citrate (110 mM), EDTA anhydrous (60 mM), and NaCl (300 mM)) to ensure the integrity of the cells (Chitemerere and Mukanganyama 2014) and at pH 8.0 under orbital agitation at 200 rpm at 25 °C ± 2 °C. Algae cells were counted under light microscopy using a Neubauer hemocytometer connected to an image analyzer (Image ProPlus 6.3, Media Cybernetics, USA). *Azospirillium brasilense* cells were stained with fluorescein diacetate (F7378, Sigma-Aldrich), as described by Chrzanowski et al. (1984). Then, the viable cells were directly counted under an epifluorescence microscope (Olympus BX41).

The growth rate (μ) was calculated in accordance with Oh-Hama et al. (1992) formula:

$$\mu = \frac{(\ln N_{t1} - lnN_{t0})}{t_1 - t_0}, \quad (1)$$

where N_{t1} is the number of cells at the sampling time, N_{t0} is the number of cells at the beginning of the experiment, t_1 is the sampling time, and t_0 is the initial experiment time.

Pigment production by Scenedesmus sp.

Samples of 3 g of beads were taken and dissolved in citrate buffer. Once dissolved, the cells were washed in a sterile saline solution (0.85% NaCl) and centrifuged at 6,000 × *g* for 5 min. The supernatant was discharged, and the pigments were extracted from the pellet with 100% methanol

overnight at − 20 °C. The pigment profiles were identified using the method described by Ji et al. (2018). The extract was analyzed at three optical densities (OD) of 665, 649, and 470 nm. The chlorophyll concentration was calculated using the following equations:

$$\text{Chl}a = (13.7 \times \text{OD}_{665}) - (5.76 \times \text{OD}_{649}) \quad (2)$$

$$\text{Chl}b = (24.96 \times \text{OD}_{649}) - (7.32 \times \text{OD}_{665}) \quad (3)$$

$$\text{Carotenoids} = ((1,000 \times \text{OD}_{470}) - (2.05 \times \text{Chl}a))/245 \quad (4)$$

Biomass characterization on FTIR

Samples of 3 g of beads were taken and dissolved in citrate buffer. Once dissolved, the cells were washed in a sterile saline solution (0.85% NaCl) and centrifuged at 6,000 × *g* for 5 min. The supernatant was discharged and the pellet was dried at 80ºC for 12 h. FTIR spectra from dry biomass were collected using an FTIR spectrometer CARY 630 (Agilent) equipped with an attenuated total reflection (ATR) accessory. Twenty scans were obtained per sample, with a spectrum range of 4,000 to 650 cm^{-1} and spectral resolution of 4 cm^{-1}. FTIR spectra were recorded in transmittance units (a.u.) according to the wave number (cm^{-1}), and the data were assessed using the Resolution-pro software (Agilent).

Visualization by SEM

At the end of the experiment (96 h), 2 g of beads from each treatment was taken, lyophilized, and pulverized in a mortar and pestle. The samples were visualized with a high-resolution (1 nm for high vacuum) scanning electron microscope (TESCAN-MIRA 3 LMU). Each sample was exposed to gold for 30 s at a power of 10 kV and then analyzed with a working plan of 15 mm and magnifications of × 5,000–20,000.

Statistical analysis

Statistical analyses of the effects of the three concentrations of ZnO NPs on the growth rate, maximum growth rate, and cell densities of the microorganisms obtained at different sample times, as well as the final pigment production, were performed using one-way analysis of variance, followed by a Tukey post hoc analysis. A Student *T* test for independent samples was performed to compare the growth rates of each microorganism alone and in interaction with the other microorganism. Significance was set at $P < 0.01$ using Statistica 8.0 (Tibco Statistica, USA).

Results

Effect of the CuO NPs on the growth of the Scenedesmus sp. immobilized alone

Scenedesmus sp. growing in the absence of NPs showed a constant μ (mean, 0.30 ± 0.22 day^{-1}) at all experiment times, with continuous increments in the number of cells to reach a maximum number of cells of 2.43 ± 0.15 cells × 10^4 $bead^{-1}$ at 96 h (Fig. 1). When CuO NPs were present and independently of the concentration tested, a higher μ was observed in the first 24 h and then decreased with time (Fig. 1b–d). The treatment with a lower NP concentration induced the highest μ and number of cells in the *Scenedesmus* sp. immobilized alone (1.66 ± 0.07 day^{-1} − 24 h and 7.60 ± 1.35 cells × 10^4 $bead^{-1}$ − 72 h; Fig. 1b). Nonetheless, the presence of 10 ppm of NPs induced a microalgal growth similar to that in the controls (without NPs). At this NP concentration (10 ppm), the constant increment in the number of microalgal cells reached the maximum number of cells at 96 h (2.66 ± 1.52 cells × 10^4 $bead^{-1}$; Fig. 1c). Finally, the higher NP concentration tested (100 ppm), which induced an increment in the μ of the *Scenedesmus* sp. in the first 24 h of incubation (0.95 ± 0.44 day^{-1}), reached also at this time the maximum number of cells (1.70 ± 0.18 cells × 10^4 $bead^{-1}$). Thereafter, the μ and number of cells in the *Scenedesmus* sp. constantly decreased (Fig. 1d).

Effect of CuO NPs on the growth of the Scenedesmus sp. co-immobilized with A. brasilense

The *Scenedesmus* sp. immobilized with *A. brasilense* without NPs showed a higher μ at 48 h of incubation (0.51 ± 0.16 day^{-1}) and then decreased with time (Fig. 2a). Under this treatment, the highest number of cells was obtained at 72 h (3.25 ± 0.85 cells × 10^4 $bead^{-1}$; Fig. 2a). In a form similar to the *Scenedesmus* sp. immobilized alone, when CuO NPs were present, independent of the concentration tested, a higher μ of the *Scenedesmus* sp. co-immobilized with *A. brasilense* was observed after 24 h (Fig. 2b–d). Co-immobilization of *Scenedesmus* sp. and *A. brasilense* in the presence of 1 ppm CuO NPs showed the highest μ and number of cells in the microalgae in the entire experiment, even higher than those in the microalgae immobilized alone in the presence of 1 ppm CuO NPs (Fig. 2b). At this low NP concentration, a higher μ was observed at 24 h of incubation (1.83 ± 0.71 day^{-1}), and a higher number of cells in *Scenedesmus* sp. was reached at 72 h of incubation (12.98 ± 1.89 cells × 10^4 $bead^{-1}$; Fig. 2b). When 10 ppm

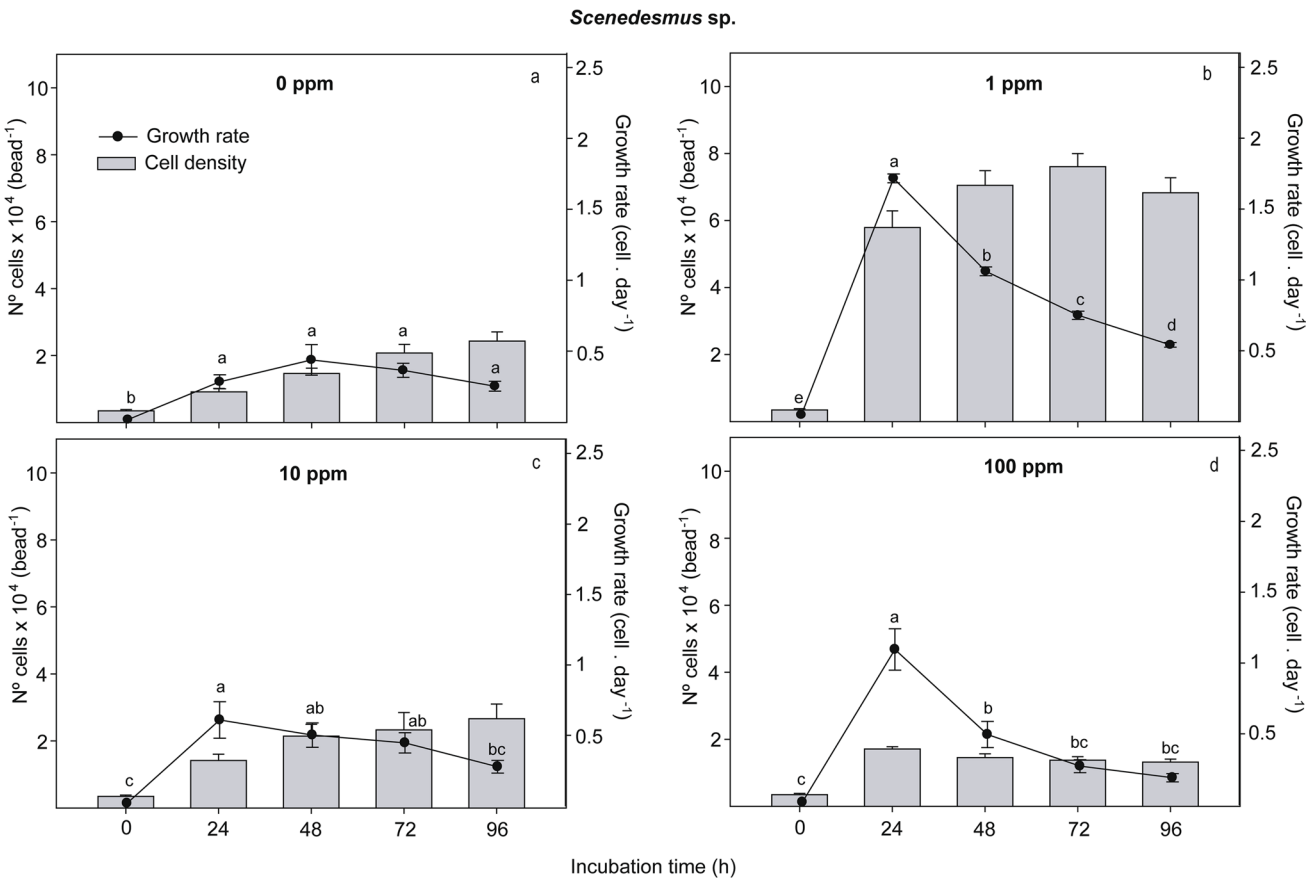

Fig. 1 Cell density (bars) and growth rates curves of *Scenedesmus* sp. immobilized alone and growing under different concentrations of CuO NPs (a- 0 ppm; b- 1 ppm; c- 10 ppm; d- 100 ppm). Different letters in each growth rate value differ significantly between sampling times in a same treatment using ANOVA and Tukey's post-hoc analysis at $P < 0.01$. Whisker lines represent standard error (SE). The absence of a line indicates negligible SE

of CuO NPs were added, the *Scenedesmus* sp. co-immobilized with *A. brasilense* showed a higher μ after 24 h of incubation (0.61 ± 0.37 day^{-1}), decreasing over time, and produced the highest number of cells at 72 h of incubation (2.41 ± 0.70 cells $\times 10^4$ bead^{-1}; Fig. 2c). The addition of NPs at a higher concentration (100 ppm) produced an increment in the μ of the *Scenedesmus* sp. co-immobilized with *A. brasilense* in the first 24 h of incubation (1.07 ± 0.69 day^{-1}). Thus, a similar number of cells was obtained between the microalgae and the controls without NPs (3.24 ± 1.62 cells $\times 10^4$ bead^{-1}), but the number was higher than that in the microalgae immobilized alone in the presence of NPs at the same concentration (Fig. 2d).

Effect of CuO NPs on the growth of A. brasilense

The growth of *A. brasilense* immobilized alone was similar to that of *A. brasilense* immobilized with microalgae in the absence of NPs (Fig. 3a). Compared with other NP concentrations, 1 ppm of CuO NPs induced a higher number of bacterial cells (independently of the presence of microalgae). Under these conditions, the growth of *A. brasilense* immobilized alone was constant during the experiment, but when microalgae were present, the number of bacterial cells decreased after 72 h of incubation (Fig. 3b). When 10 ppm of CuO NPs were present, the number of bacterial cells remained constant throughout the experiment when the microorganism was co-immobilized with microalgae. In the immobilized *A. brasilense* with 10 ppm of CuO NPs, the number of bacterial cells increased at 24 h and then decreased at 48 and 72 h (similar to the initial values, 0 h), to a final increment at 96 h of incubation (Fig. 3c). On the other hand, when 100 ppm of CuO NPs were present, a continuous reduction in the number of bacterial cells was observed when the microorganism was immobilized alone (Fig. 3d). At the same NP concentration, when the bacterium was co-immobilized with microalgae, the number of bacterial cells remained constant throughout the experiment (Fig. 3d).

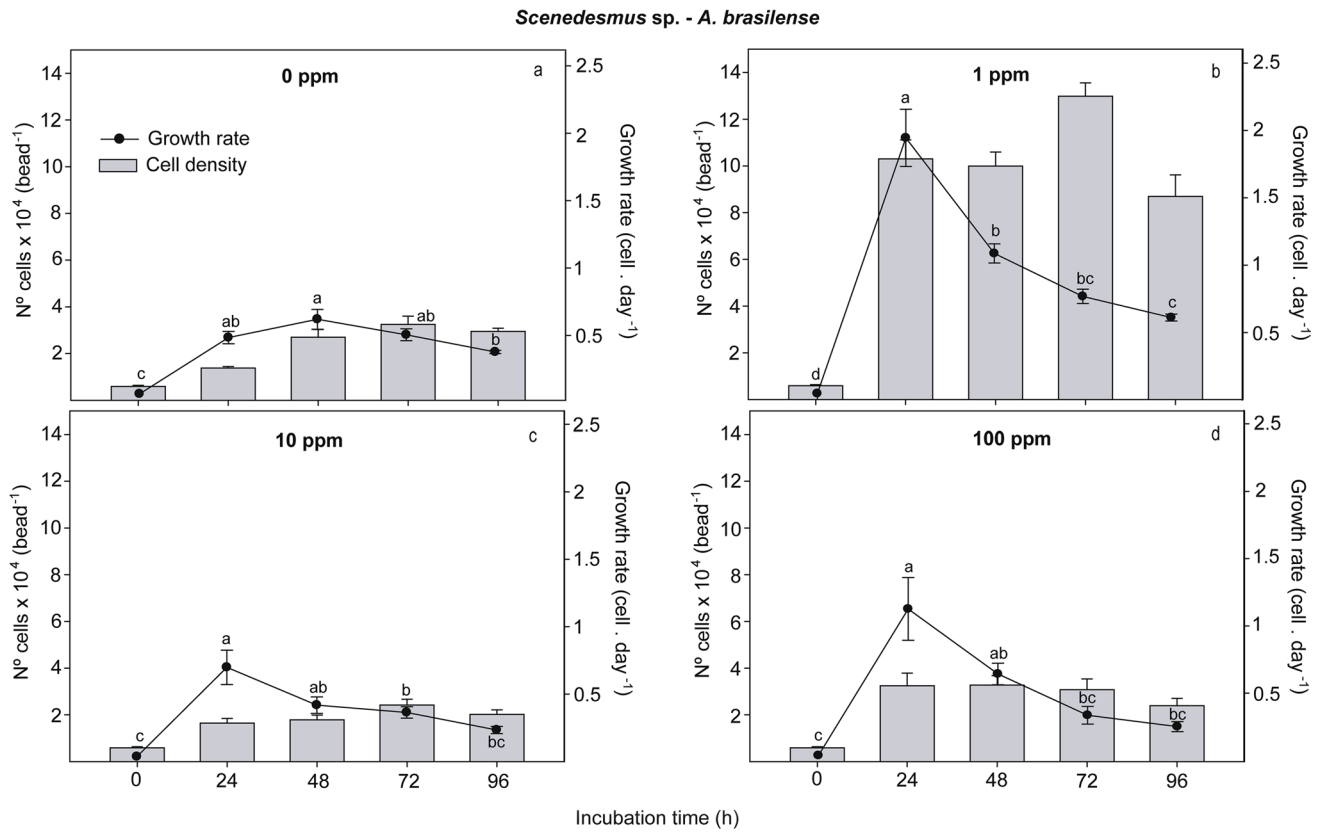

Fig. 2 Cell density (bars) and growth rates curves of *Scenedesmus* sp. co-immobilized with the MGPB *A. brasilense* and growing under different concentrations of CuO NPs (a- 0 ppm; b- 1 ppm; c- 10 ppm; d- 100 ppm). Different letters in each growth rate value differ significantly between sampling times in the same treatment using ANOVA and Tukey's post-hoc analysis at $P < 0.01$. Whisker lines represent standard error (SE). The absence of a line indicates negligible SE

Metabolites

The chlorophyll-*a* concentration in the *Scenedesmus* sp. immobilized alone showed a tendency to decrease with the increment in the CuO NPs. However, when the microalgae were co-immobilized with the bacterium, a higher chlorophyll-*a* content was found under the control condition (without NPs) and when 10 ppm CuO NPs were present (Fig. 4). The chlorophyll-*b* concentration remained constant when the *Scenedesmus* sp. was immobilized alone, independently of the NP concentration. The bacterial presence increased the chlorophyll-*b* content in the microalgae only in the presence of 1 ppm of NPs. Finally, the carotenoid contents did not change independently of the CuO NP concentrations and the presence of *A. brasilense* (Fig. 4).

The FTIR analysis of the microalgae samples revealed peaks at 1,020 cm^{-1}, which corresponded to the C–O–C bonds of carbohydrates, and peaks at 1,645 and 1,530 cm^{-1}, which corresponded to the C = O and N–H bonds of amide, respectively, which are associated with proteins (Choix et al. 2018). The FTIR spectra showed that 10 or 100 ppm of CuO NPs produced increments in protein and carbohydrate contents when the microalgae were immobilized alone, as compared with the controls (without NPs). On the contrary, the lower CuO NP concentration (1 ppm) produced decrements in protein and carbohydrate contents in the microalgae biomass compared with the controls (Fig. 5a). On the other hand, when the microalgae and bacterium were co-immobilized, the protein and carbohydrate concentrations in the microalgae biomass tended to increase according to the increment in the NPs tested (Fig. 5b).

Interaction CuO NP-alginate (SEM)

Analysis of the SEM scans revealed the affinity of the alginate surface to CuO NPs, allowing their agglomeration (Fig. 6a). Microalgal and bacterial cell integrity were observed when CuO NPs were incorporated in the experiment (Fig. 6b and c), and *Scenedesmus* sp. cells were covered by alginate.

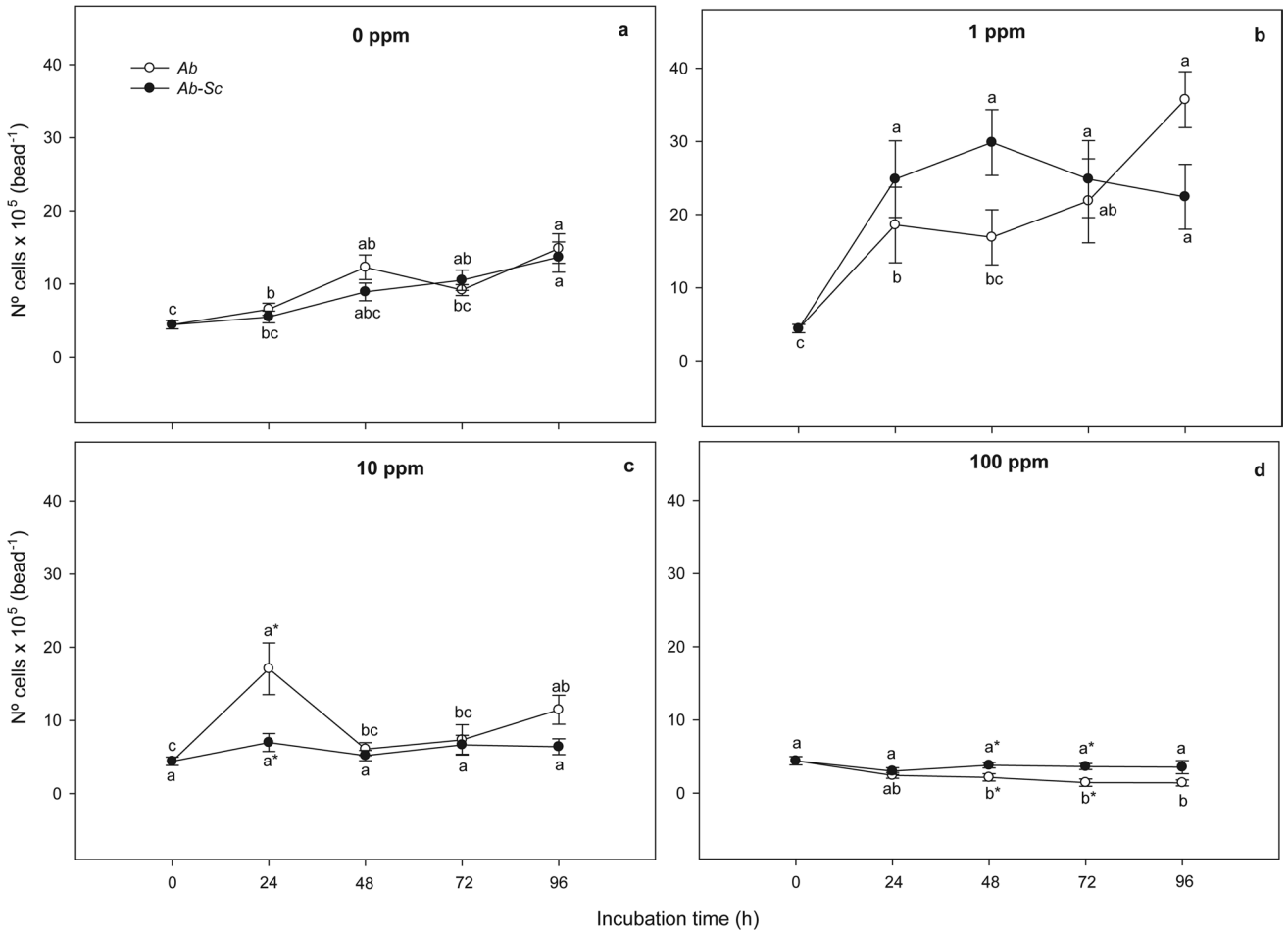

Fig. 3 Growth curves of *A. brasilense* immobilized alone and co-immobilized with the microalgae *Scenedesmus* sp. growing under different concentrations of CuO NPs (a- 0 ppm; b- 1 ppm; c- 10 ppm; d- 100 ppm). Different letters differ significantly between sampling times in the same treatment using ANOVA and Tukey's post-hoc analysis at $P < 0.01$. Asterisks indicate statistically difference between microalgae immobilized alone or co-immobilized with bacteria under the same sampling time used Student-T test for independent samples at $P < 0.01$. Whisker lines represent standard error (SE). The absence of a line indicates negligible SE

Discussion

The optical, catalytic, semiconductor, and antimicrobial properties of CuO NPs have allowed for their large number of applications in areas such as medicine, personal care products, semiconductor manufacturing, and antimicrobial products (Otero-González et al. 2014; Shang et al. 2019). Specifically, their use in wastewater treatment is related to their photocatalytic properties that promote the degradation of some pollutants at ambient temperatures and low costs (Sibhatu et al. 2022). CuO NPs in wastewater treatment plants can produce a pronounced loss in microbial cell viability, even during the first 24 h of exposure, with detrimental consequences (Miao et al. 2017). In microalgae, only 2 ppm of CuO NPs was necessary to induce a decrease in the growth of the microalgae *N. oculata*, and 116.98 ppm of CuO NPs was reported as the half-maximal effective concentration (Fazelian et al. 2019). Thus, the use of immobilized microorganisms in alginate beads has been proposed to prevent the negative effect of emerging contaminants such as ZnO NPs on microorganisms and to allow their interaction (Palacios et al. 2023). In our experiment, the growth of both microorganisms (immobilized alone or co-immobilized) was enhanced in the presence of 1 ppm of CuO NPs (117.9% for *Scenedesmus* sp. and 73% for *A. brasilense*). Metallic NPs (e.g., Co, Cu, Zn, Al, and Cr) at low concentrations may complement nutrient deficiencies (Vargas-Estrada et al. 2020), thus enhancing the growth of microalgae such as *Platymonas cordiforus*, *Chaetoceros curvisetus*, and *Skeletonema costatum* (Chen et al. 2018). CuO NPs at 0.1 and 1 ppm have been reported to enhance the growth of *Chlamydomonas reinhardtii* cultures (an average of 110%, 0.1 ppm; and 130%, 1 ppm) without adverse effects until after 72 h of incubation (Pedroso-Melegari et al. 2013). In addition, low concentrations of CuO NPs can stimulate bacterial growth, metabolism, and respiration

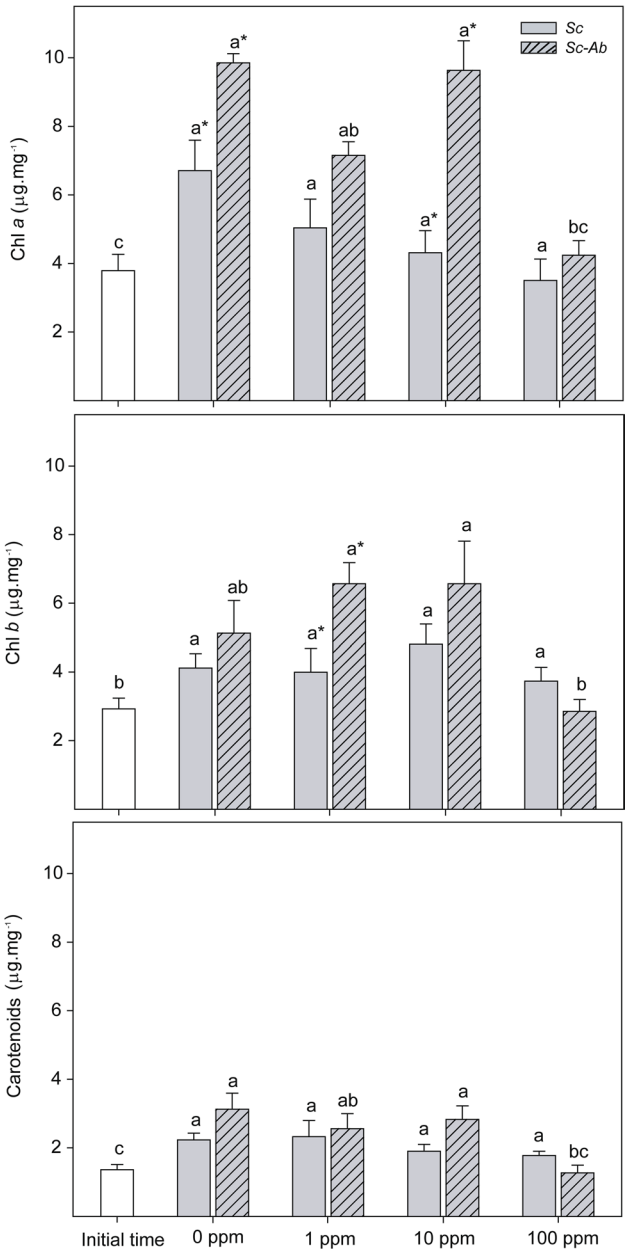

Fig. 4 Final Chlorophyll *a, b,* and Carotenoids content in *Scenedesmus* sp. immobilized alone and co-immobilized with the MGPB *A. brasilense* in presence of CuO NPs (0, 1, 10, and 100 ppm). For each subfigure, different letters differ significantly between nanoparticle concentration within microalgae treatment alone or with bacteria using ANOVA and Tukey's post-hoc analysis at $P < 0.01$. Bars with asterisks differ statistically between pigment concentration in the microalgae immobilized alone and co-immobilized with the bacterium at each NP concentration used Student-T test for paired samples at $P < 0.01$. Whisker lines represent standard error (SE)

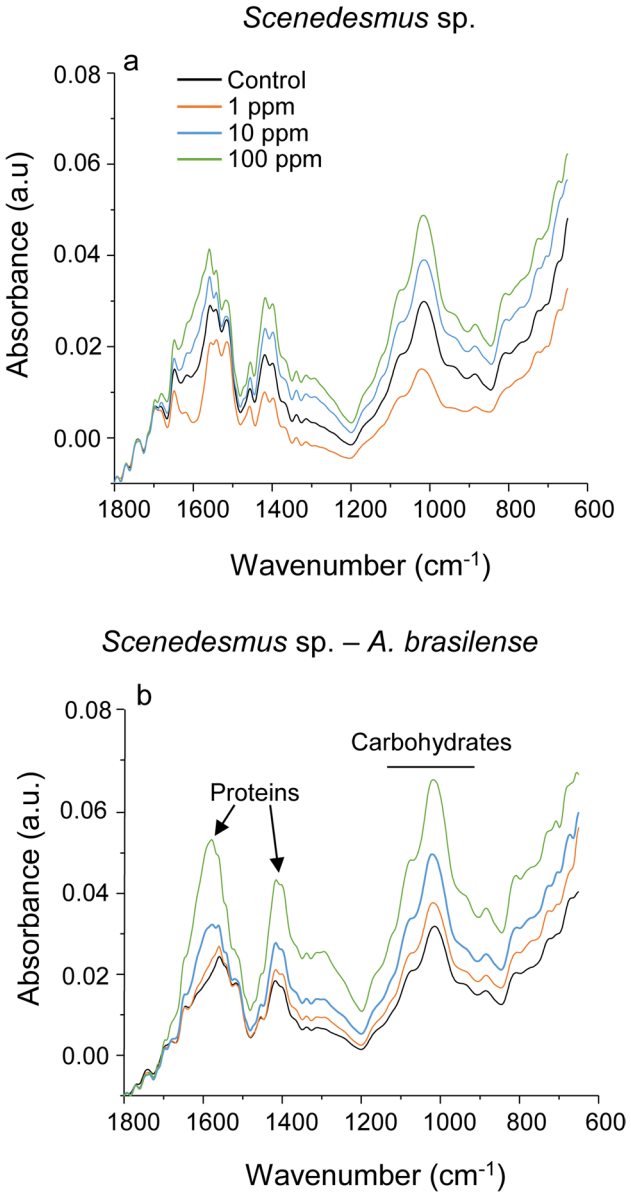

Fig. 5 Qualitative biomass characterization of *Scenedesmus* sp. immobilized alone (**a**) and co-immobilized with the MGPB *A. brasilense* (**b**) under different concentration of CuO NPs

(Jośko et al. 2019), which could explain the positive effect observed in the growth of *A. brasilense* cultured at 1 ppm of CuO NPs. The microalgae showed a higher number of cells when co-immobilized (8.69 ± 3.19 cells $\times 10^4$·bead^{-1}) with *A. brasilense* than when immobilized alone (6.82 ± 1.54 cells $\times 10^4$·bead^{-1}) at low NP concentrations. *Azospirillum brasilense* has been reported as a MGPB owing to its ability to produce the phytohormone indole-3-acetic acid (IAA), the vitamin riboflavin, and their degradation compound lumichrome, which enhance the growth and metabolism of microalgae (Palacios et al. 2022). In this sense, the production of IAA by *A. brasilense* has been reported as the growth promotion mechanism responsible for enhancing the growth of different microalgae genera under several conditions. For instance, the growth of *Chlorella vulgaris* was improved when interacting with wild-type strains of *A. brasilense* but

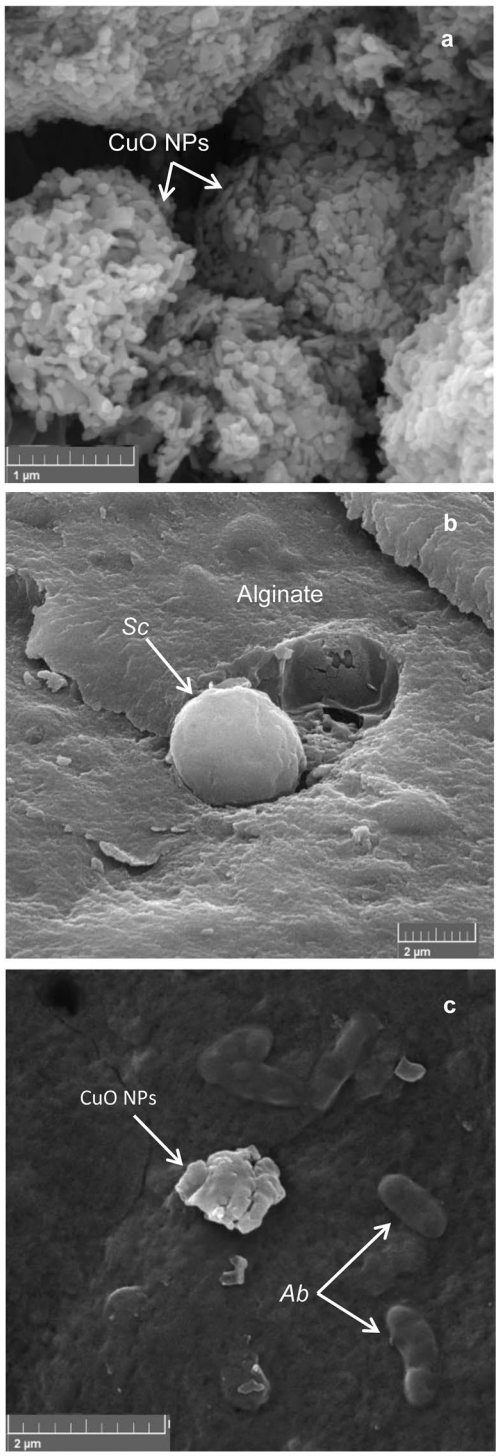

Fig. 6 Scanning electron micrographs of CuO NPs agglomerated in alginate surface (**a**), *Scenedesmus* cell (**b**), and *A. brasilense* cells (**c**), immobilized in alginate beads and growing in the presence of CuO NPs

not when interacting with an IAA-deficient mutant of *A. brasilense* (de-Bashan et al. 2008). IAA production by *Azospirillum* was identified as the main mechanism of interaction between this bacterium and microalgae such as *C. vulgaris* and *S. obliquus*, independently if they are growing in air or biogas atmosphere (Barbosa-Nuñez et al. 2022), under autotrophic or heterotrophic regimens (Palacios et al. 2016; Choix et al. 2023) or in the presence of different forms of abiotic stress, such as nitrogen depletion (Pagnussat et al. 2020), saline stress (Pagnussat et al. 2023), and copper stress (Peng et al. 2021).

Nonetheless, Cu ions have been reported to negatively affect IAA production by *A. brasilense* without affecting its growth (Kamnev et al. 2005). However, the physical barrier of alginate could prevent the negative effect of metallic NPs on IAA, as was recently observed at low concentrations of ZnO NPs (Palacios et al. 2023). At higher concentrations (10 ppm) of CuO NPs, growth promotion was not observed in microalgae immobilized with the bacterium (2.01 ± 0.67 cells $\times 10^4$ bead^{-1}; $\mu = 0.22 \pm 0.13$ day^{-1}) and showed a similar growth behavior to microalgae immobilized alone (2.66 ± 1.52 cells $\times 10^4$·bead^{-1}; $\mu = 0.22 \pm 0.11$ day^{-1}). These results indicate a possible negative effect on *A. brasilense* growth promotion mechanisms due to Cu diffusion at 10 ppm. Nonetheless, the effects of different CuO NPs on IAA production by *A. brasilense* immobilized in alginate beads have not been evaluated and requires elucidation. The production of other compounds, including riboflavin and lumichrome, has also been described as the growth promotion mechanism in *A. brasilense* (Palacios et al. 2022) and recognized as the mitigation stress mechanism for microalgae cultures under abiotic stress caused by high salinity (Lopez et al. 2019). The other growth promotion mechanisms (riboflavin and lumichrome) could prevent the algicide effect of high CuO NP concentrations (100 ppm), thus allowing for the growth of more cells in microalgae co-immobilized with the bacterium (2.39 ± 1.03 cells $\times 10^4$ bead^{-1}) compared with those immobilized alone (1.32 ± 0.27 cells $\times 10^4$ bead^{-1}), although the effect of these NPs on riboflavin and lumichrome production by *A. brasilense* must be quantified in subsequent studies.

The negative effects of CuO NPs on microalgal and bacterial growths have been related to the direct effect of metallic NPs on the cell wall. In addition, Cu ion released from copper NPs can induce the production of ROS and oxidative damage (Du et al. 2019). The effect of oxidative damage by metallic NPs in microalgae is commonly observed through the increment in photosynthetic pigments such as chlorophyll and accessory pigments such as carotenoids (Wang et al. 2020). However, in our study and independently of the CuO NP concentrations tested, the contents of chlorophyll *a* and *b*, and carotenoids did not show statistically significant differences in the *Scenedesmus* sp. The presence of *A. brasilense* enhanced the chlorophyll content in the *Scenedesmus* sp. independently of the CuO NP concentration, with this effect being higher for chlorophyll *a* at 10 ppm (without NPs:

46.9%; 1 ppm: 41.8%; 10 ppm: 123.3%; 100 ppm: 21% of chlorophyll *a* increment). This increment in pigment production could indicate that although IAA production could be affected by Cu ions, the production of lumichrome (degradation product of riboflavin) by *A. brasilense* is active, as it has been reported that lumichrome is the mechanism responsible for enhancing chlorophyll production in *C. sorokiniana* by *A. brasilense* (Lopez et al. 2019). On the other hand, the chlorophyll-*b* content was statistically higher when the *Scenedesmus* sp. was immobilized with *A. brasilense* at 1 ppm CuO NPs. Chlorophyll *b* is an antenna pigment involved in the transmission of light energy during photosynthesis (Dao et al. 2020). The increment in its content at 1 ppm CuO when the *Scenedesmus* sp. was co-immobilized with *A. brasilense* allowed a higher transmission of light, explaining the higher growth capacity in the microalgae when interacting with the bacterium at this NP concentration.

The productions of carbohydrates and proteins in *Scenedesmus* sp. immobilized alone increased at 10 and 100 ppm CuO NPs but decreased when the microalgae was cultured at the lower NP concentration (1 ppm). Higher carbohydrate production by microalgae is related to oxidative stress, as carbohydrates with high sulfate contents can capture ROS and act as effective free-radical scavenging ligands (Liang et al. 2020). The increment of carbohydrates at 10 and 100 ppm CuO could indicate oxidative stress for the microalgae. Moreover, the accumulation of proteins has been related to the inability of cells to divide and thus prepare an active defense of the cells against abiotic stress conditions (Romero et al. 2020), which could explain the higher accumulation of proteins at 10- and 100-ppm CuO concentrations with low cell growth. On the contrary, at the lower CuO concentration tested, the *Scenedesmus* sp. presented lower carbohydrate and protein contents than the controls. As mentioned earlier, low concentrations of metallic NPs can complement nutrient deficiencies and are therefore a beneficial growth condition, promoting better microalgae growth without carbohydrate and protein accumulation.

Similar to the *Scenedesmus* sp. immobilized alone, the co-immobilization of the microalgae with *A. brasilense* showed increments in carbohydrate and protein contents at 10 and 100 ppm, but with a markedly high increment in microalgae growth. Contrary to the microalgae immobilized alone, the microalgae co-immobilized with the bacteria at a 1 ppm CuO concentration showed higher carbohydrate and protein contents than the controls (without NPs). Although higher protein and carbohydrate levels have been related to microalgae mechanisms against abiotic stress (Liang et al. 2020; Romero et al. 2020), the interaction between microalgae and *A. brasilense* permits higher carbohydrate and total protein production and accumulation accompanied by higher microalgae growth. This effect of *A. brasilense* on microalgae has been reported to be related to a higher metabolic activity produced by the microalgae-bacteria interaction (Barbosa-Nuñez et al. 2022) instead of a response to oxidative stress.

Finally, the absorbent capacity of alginate can impede the CuO NP diffusion in the media, thus inhibiting their antimicrobial effect (Saravanakumar et al. 2020). In our results, the interaction between the alginate surface and CuO NPs was observed on SEM, and even microalgae and bacterial cell integrity were observed. This could explain the normal growth behaviors of both microorganisms (*Scenedesmus* and *Azospirillum*), even in the presence of 10 ppm of CuO NPs. However, at higher concentrations, the capacity of alginate to absorb CuO NPs could be saturated, with a negative effect on the final cell density of both microorganisms.

Conclusion

The co-immobilization of microalgae and bacteria cells in alginate beads allowed for the growth of both microorganisms in the presence of CuO NPs without affecting pigment production and enhancing the carbohydrate and protein accumulation by microalgae cells. Moreover, the low CuO NP concentration (1 ppm) showed a beneficial effect on the growth of both microorganisms, enhancing their growth (117.9% for *Scenedesmus* sp. and 73% for *A. brasilense*). In addition, the interaction between *Scenedesmus* sp. and *A. brasilense* at this NP concentration improved the cell density of the microalgae even more (239.5%) and pigment, carbohydrate, and protein contents. The co-immobilization of *Scenedesmus* sp. and *A. brasilense* mitigated the stress caused by the high CuO NP concentration (100 ppm), resulting in higher cell density in the microalgae (181%) when interacting with the bacterium. These results demonstrated that the use of immobilized cells can prevent the negative effects of emerging contaminants such as CuO NPs on the growth and metabolism of the microorganisms used in wastewater treatment. The use of immobilized microalgae and bacteria in alginate beads can be combined with the use of low concentrations of CuO NPs in wastewater treatment to eliminate organic and inorganic pollutants through the metabolism of the microorganisms and the heterogeneous photocatalytic properties of CuO NPs. Moreover, the interaction of microalgae and bacteria immobilized in alginate beads and in the presence of low NP concentrations could be a promising strategy to obtain microalgae biomass with high pigment, carbohydrate, and protein contents. Nonetheless, the effects of different CuO NP concentrations on the growth-promoting mechanisms of *A. brasilense* must be studied to elucidate the mechanism responsible for the positive effects observed in this study on the growth and metabolite production by *Scenedesmus* sp. when CuO nanoparticles are present.

Acknowledgements At Drs. José Roberto Ramos Ibarra, Martín Flores Martinez, and Sergio Oliva León by Scanning Electron Microscopy (SEM) service of CUCEI-UdG.

Author contributions Karen A. Alonso performed the experiments; Francisco J. Choix discussed experimental procedures for metabolites analysis and critically revised the final manuscript; Guadalupe V. Nevarez-Moorillón participate in the discussion of the results and statistical analysis; Oskar A. Palacios discussed the experimental setting, supervised the experiments, wrote the draft, and critically revised the article for intellectual content. All the authors read and approved the manuscript.

Funding This work was supported by Programa para el Desarrollo Profesional Docente – Prodep OF-20–8581.

Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Competing interests The authors have no relevant financial or non-financial interest to disclose.

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