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Isolated and combined effects of cobalt and nickel on the microalga *Raphidocelis subcapitata*

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Abstract

Aquatic organisms are exposed to several compounds that occur in mixtures in the environment. Thus, it is important to investigate their impacts on organisms because these combined effects can be potentiated. Cobalt (Co) and nickel (Ni) are metals that occur in the environment and are used in human activities. To the best of our knowledge, there are no studies that investigated the combined effects of these metals on a freshwater Chlorophyceae. Therefore, this study analyzed the isolated and combined effects of Co and Ni in cell density, physiological and morphological parameters, reactive oxygen species (ROS), carbohydrates and photosynthetic parameters of the microalga *Raphidocelis subcapitata*. Data showed that Co affected the cell density from 0.25 mg Co L^{-1}; the fluorescence of chlorophyll *a* (Chl *a*) (0.10 mg Co L^{-1}); ROS production (0.50 mg Co L^{-1}), total carbohydrates and efficiency of the oxygen evolving complex (OEC) at all tested concentrations; and the maximum quantum yield (Φ_M) from 0.50 mg Co L^{-1}. Ni exposure decreased ROS and cell density (0.35 mg Ni L^{-1}); altered Chl *a* fluorescence and carbohydrates at all tested concentrations; and did not alter photosynthetic parameters. Regarding the Co-Ni mixtures, our data best fitted the concentration addition (CA) model and dose-ratio dependent (DR) deviation in which synergism was observed at low doses of Co and high doses of Ni and antagonism occurred at high doses of Co and low doses of Ni. The combined metals affected ROS production, carbohydrates, Φ_M, OEC and morphological and physiological parameters.

Keywords Mixture · Photosynthesis · Carbohydrates · ROS · Metal

Introduction

Contamination of aquatic environments by metals is a persistent problem as these compounds are not degradable and they can bioaccumulate and cause toxicity to organisms, which are exposed to a wide variety of metallic mixtures (Filová et al. 2021; Liu et al. 2017). Contaminants can have a different or similar mode of action, which, in a mixture, can result in antagonistic, synergistic or additive effects (Beyer et al. 2014).

Metals such as cobalt (Co) and nickel (Ni) co-occur in the environment (Dourado et al. 2017; Thompson et al. 2020). In the meta-analysis, the statistics of the average concentrations of Co and Ni in water bodies surface were 3.995 mg L^{-1} and 0.946 mg L^{-1} respectively (Kumar et al. 2019). According to WHO (World Health Organization - 2017) and USEPA (United States Environmental Protection Agency - 2009) guidelines, countries such as China, Pakistan, Bangladesh and Nigeria exceed the average values allowed for metals Co and Ni for drinking water

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(Kumar et al. 2019). In Brazil, these metals were quantified in the Paraopeba River at concentrations of 0.011 mg Co L^{-1} and 0.014 mg Ni L^{-1} after the Brumadinho dam collapse (Thompson et al. 2020) and in the Água Boa stream in concentrations of 0.253 mg Co L^{-1} and 0.296 mg Ni L^{-1} (Dourado et al. 2017).

Co is an essential metal for the synthesis of vitamin B12 and it can be found in soil, water, vegetation, rocks, and is regularly found associated with nickel (Pourkhabbaz et al. 2011). The uses of Co are diverse, and include such things as wear-resistant superalloys, battery electrodes, Ni-Cd batteries (which contain Co), chemicals (agricultural products, sewage effluents, mining and processing), paint, steel, cement, magnets, fertilizer, and catalysts in the removal of sulfur impurities from petroleum (Blust, 2011; Sridhar et al. 2020). Therefore, Co has already been found in high concentrations near mining and agricultural fertilizer runoff regions, and can be toxic to humans, animals and plants (Pourkhabbaz et al. 2011). Co has been quantified in surface waters in USA in concentrations of less than 0.0001 to more than 1 mg L^{-1}, with the highest values found in streams polluted by mining (Stubblefield et al. 2020). In view of this, some studies have evaluated the effects of the metal on organisms, reporting protein oxidation, DNA damage, ROS production, decrease of chlorophyll *a* and *b* synthesis and impairment of photosynthetic activity in microalgae (El-Sheekh et al. 2003; Novak et al. 2013; Rocha e Melão 2023); inhibition of active transport, leaf fall, inhibition of greening, stimulation of ROS production and formation of hydrogen peroxide (H_2O_2) radicals in plants (Mahey et al. 2020); decrease in fertilization rate, embryo survival and reduction in reproductive success in fish (Reinardy et al. 2013).

Ni is a metal that occurs naturally in the environment and the main sources of metal entry into the aquatic ecosystem are urban runoff, landfill leaching, municipal and industrial effluents (mining debris), natural sources, and soil or substrate disturbances (Wang et al. 2020). In addition, human activities and poor management of waste paints, electronic equipment, Ni-Cd batteries, cigarettes, stainless steel, jewelry, and coin-making alloys cause Ni pollution (García-García et al. 2018). Ni has already been quantified in polluted sites at concentrations from 2.2 to 118 mg L^{-1} (Dizge et al. 2009; Sanliyuksel Yucel et al. 2016) raising environmental concerns about its effects as high concentrations of Ni in the aquatic ecosystem can be toxic to organisms (Wang et al. 2020). Some effects of Ni are decreased cell density, ROS increase, change in the maximum rate of relative electron transport (Yong et al. 2019); pigment concentration reduction and change in enzymatic activity of superoxide dismutase – SOD (decreased), catalase – CAT (increased), glutathione peroxidase – GPx (increased) in microalgae (Martínez-Ruiz and Martínez-Jerónimo 2015). Ni also affected the reproduction and the size of males of *Danio rerio* (Alsop et al. 2014); and alterations in plant metabolism by inhibiting enzymatic activity, chlorophyll synthesis and electron transport (Sreekanth et al. 2013).

Algae have an essential environmental role as they are the main producers in the aquatic food chain, helping to maintain the structure, function and balance of ecosystems (Guo et al. 2020; Moreira et al. 2020). The green alga, *Raphidocelis subcapitata*, used as a test organism in this study, is globally distributed and widely used in ecotoxicological tests, as it has a fast life cycle, high sensitivity to xenobiotics and an easy cultivation in the laboratory. It is used in studies of the effects of metals on these organisms (Guo et al. 2020; Machado et al. 2015; Machado and Soares 2014). There is a lack of knowledge about the combined effects of Co and Ni on microalgae. Some authors assessed the effects of Co-Ni mixtures to other organisms, observing antagonistic effects for the oligochaete *Enchytraeus crypticus* (He et al. 2015), synergistic effects on *Escherichia coli* (Barabasz et al. 1990) and antagonistic, additive and synergistic effects, varying according to metal concentration, on *Pseudomonas fluorescens* (Nweke et al. 2018). Therefore, this research aims to evaluate the effects of isolated and combined effects of Co and Ni on cell density, chlorophyll *a* fluorescence (Chl *a*), cell size, cell complexity, ROS production, total carbohydrates and photosynthetic activity of the freshwater microalga *R. subcapitata*.

Material and methods

Algae culture and toxicity tests

The microalga *R. subcapitata* was obtained from the Department of Ecology and Evolutionary Biology (DEBE, Universidade Federal de São Carlos - UFSCar, São Carlos - SP, Brazil). It was cultivated in CHU12 medium (Chu 1942) (Table S1. Supplementary material) previously autoclaved (121 °C, 1 atm above standard pressure, 20 min) with pH adjusted to 7 ± 0.05. The algae cultures were maintained at 25 ± 1 °C, with a photoperiod of 12 h/12 h (light/dark), light intensity of ≅ 130 µmol photon m^{-2} s^{-1} (LED light) and periodic manual shaking.

Toxicity tests followed the same culture conditions. *R. subcapitata* in the exponential growth phase was inoculated at 5×10^4 cells mL^{-1}, in polycarbonate Erlenmeyer flasks (capacity 500 mL) containing 200 mL of the test solution. The algae were exposed to different concentrations of the metals Co (0.10; 0.25; 0.50 and 0.75 mg L^{-1}) and Ni (0.10; 0.15; 0.25 and 0.35 mg L^{-1}), isolated, in triplicate, during 96 h. The control group had no metal addition. Regarding mixture concentrations, we chose a partial

Table 1 Metal concentrations (mg L^{-1}) used in toxicity tests. Concentrations of Co1 to Co4 and Ni1 to Ni4 refer to isolated tests, while M1 to M23 refer to mixture tests and C = control group

Metal concentration (mg L^{-1})		
Treatment	Co	Ni
C	0	0
Co1	0.10	0
Co2	0.25	0
Co3	0.50	0
Co4	0.75	0
Ni1	0	0.10
Ni2	0	0.15
Ni3	0	0.25
Ni4	0	0.35
M1	0.063	0.063
M2	0.063	0.094
M3	0.063	0.156
M4	0.063	0.219
M5	0.125	0.031
M6	0.125	0.063
M7	0.125	0.125
M8	0.125	0.188
M9	0.188	0.031
M10	0.188	0.094
M11	0.188	0.156
M12	0.250	0.063
M13	0.250	0.125
M14	0.250	0.250
M15	0.313	0.031
M16	0.313	0.094
M17	0.375	0.063
M18	0.375	0.188
M19	0.375	0.250
M20	0.438	0.031
M21	0.500	0.125
M22	0.500	0.188
M23	0.500	0.250

fixed-ratio design (Cassee et al. 1998) to avoid treatments with high metal concentrations that could lead to mortality of algal cells, and the 23 combinations are described in Table 1. Stock solutions of Co and Ni were obtained from Titrisol standard solutions of $CoCl_2$ (1000 mg Cd L^{-1}) and $NiCl_2$ (1000 mg Ni L^{-1}) (Merck, Germany).

Metal determination

Metals were determined in stock solutions by inductively coupled plasma optical emission spectrometry (ICP OES, iCAP 7000 - Thermo Fischer Scientific, Madison, WI, USA). The results are presented in Table S2 (Supplementary material). Nominal concentrations did not vary more than 20% from measured (OECD - Organisation for Economic Co-operation and Development (2002)), thus the nominal concentrations are used. Limits of detection were 0.0001 mg Co L^{-1} and 0.005 mg Ni L^{-1}, while limits of quantification were 0.010 mg Co L^{-1} and 0.100 mg Ni L^{-1}.

Cytometry analysis

To analyze cell density, the samples (1800 µL) were fixed with formaldehyde buffered with borax (1% final concentration), left in the dark for 10 min and frozen in liquid nitrogen. Relative ROS assessment was done according to procedures recommended by Hong et al. (2009). Samples (495 µL) were collected and 5 µL of DCFH-DA (2′,7′-Dichlorofluorescein diacetate, CAS number 2044-85-1, Sigma Aldrich) was added obtaining a final concentration of 10 µM DCFH-DA. Then, the samples were incubated for 1 h in the dark, at 25 °C, and analyzed by flow cytometry.

Cell density and relative ROS were analyzed in FACS-Calibur flow cytometer (Becton & Dickinson Franklin Lakes, NJ, U.S.A.) equipped with a 15 mW Argon-ion laser (emission 488 nm). In each sample, we added 10 µL of 6 µm fluorescent beads (Fluoresbrite carboxylate microspheres; Polysciences, Inc., Warrington, PA, USA.) as a standard.

Cell density was determined according to Sarmento et al. (2008) procedures, using SSC-H (lateral dispersion) versus FL3-H (red fluorescence) parameters. FSC-H, SSC-H and FL3-H parameters correspond to the cell size, cellular complexity and chlorophyll *a* (Chl *a*) fluorescence, respectively, and were calculated as reported in Mansano et al. (2017). ROS were determined using FL3-H versus FL1-H (green fluorescence) parameters and calculated by the Eqs. (1 and 2) described by Hong et al. (2009). Then, the obtained data were analyzed by FlowJo software, version V10.0 (Treestar.com, USA).

$$FL1 - H_{relative} = \log(FL1 - H_{samples})/\log(FL1 - H_{beads}) \quad (1)$$

$$ROS_{relative}(\%) = (FL1 - H_{relative[treatments]}/FL1 - H_{relative[control\ group]})X100 \quad (2)$$

Total carbohydrates

Total carbohydrates were measured according to the methodology proposed by Liu et al. (1973) with the phenol-sulfuric acid reaction. The absorbance was read at 485 nm using a spectrophotometer (HACH DR 5000; HACH Company, Loveland, CO, EUA). Total carbohydrate values were calculated using a calibration curve (Fig. S1, Supplemental material), made with dextrose anhydrous

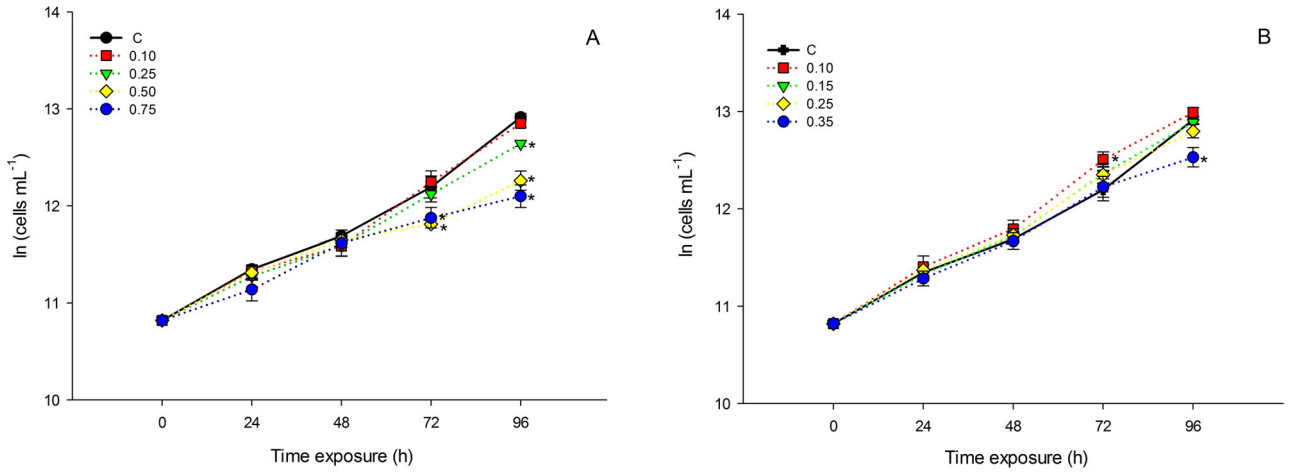

Fig. 1 Cell density of *Raphidocelis subcapitata* ((ln(cells mL^{-1})) exposed to Co (**A**) and Ni (**B**) during 96 h of exposure. The asterisk indicates a significant difference from the control group ($p < 0.05$). Metal concentrations are expressed in mg L^{-1}; C = control group

(Mallinckrodt Chemicals, USA) standard in 7 concentrations (5, 10, 20, 40, 100, 150 and 200 µg mL^{-1}).

Photosynthetic activity

The assessments of the maximum quantum yield (Φ_M) and the efficiency of the oxygen evolving complex (OEC) were performed using pulse amplitude modulated fluorometer (PHYTO-PAM® Fluorometer Analyzer, Heinz Walz, Germany). Samples (3 mL) were collected and kept in the dark for 15 min. Minimum fluorescence (F_0), maximum fluorescence (F_M) and Φ_M values (Genty et al. 1989) were provided by Phyto-PAM; and the efficiency of OEC was calculated by the ratio F_0/F_v (where $F_v = F_m - F_0$) (Kriedemann et al. 1985).

Statistical analysis

For statistically significant differences, normal data were analyzed by ANOVA (one-way analysis of variance) and Dunnett's post-hoc tests, while non-normal data were evaluated using Kruskal-Wallis test and Dunn's post hoc test. Statistical analyses were performed with SigmaPlot 12.0 software and significant differences were considered when $p < 0.05$. The inhibitory concentration (IC_{50}-96h) was calculated by nonlinear regression, with a sigmoidal three-parameter logistic curve in the SigmaPlot 12.0 software. ROS, SSC-H and FL3-H mixture data were normalized using log transformation.

Data from the cell density obtained in mixture toxicity tests were analyzed by CA (concentration addition) and IA (independent action) models using the MIXTOX tool (Jonker et al. 2005). Then, for both models, CA and IA, the deviations S/A (synergism/antagonism); DR (dose ratio-dependent) and DL (dose level-dependent) were modeled by adding the parameters "a" and 'b". In S/A deviation, the parameter "a" becomes negative to synergistic interactions and positive to antagonistic. In DR, the addition of a second parameter, b_{DR}, can indicate if the deviation from the reference model is controlled by the mixture composition. In DL, the "a" parameter indicates the deviation, $a > 0$ = antagonism; while $a < 0$ = synergism, and the b_{DL} parameter indicates at what dose level the deviation changes. More information on these deviation functions are available in Jonker et al. (2005). At the end of the analysis, the best fit was chosen by the maximum likelihood method and the best deviation was statistically identified.

Results and discussion

Single toxicity: algal growth

Our data showed that Co treatments inhibited the cell density of *R. subcapitata* after 72 h of exposure at 0.50 and 0.75 mg Co L^{-1}; while at 96 h, cell density was inhibited at 0.25; 0.50 and 0.75 mg Co L^{-1} (Fig. 1A). Cobalt is classified as a trace element necessary to various physiological and biochemical activities (Filová et al. 2021). Although, even though Co is an essential metal, in high concentrations (above essential concentration) it can be toxic to microalgae, and the level of toxicity may vary according to the species (Ghafari et al. 2018). Other studies also reported that Co decreased cell density to several algae species such as *Chlamydomonas reinhardtii* – 20 mg L^{-1} (Lustigman et al. 1995); *Chlorella vulgaris* (0.6 mg L^{-1} of Co) (Afkar et al. 2010); *Monoraphidium minutum* (1, 2 and 3 mg L^{-1} of Co^{2+}), and *Nitzchia perminuta* (2.5, 3.5 and 5 mg L^{-1} of Co^{2+}) (El-Sheekh et al. 2003).

Ni treatments increased cell density after 72 h of exposure at 0.10 mg L^{-1} and inhibition was observed after 96 h at the highest concentration tested (0.35 mg L^{-1}) (Fig. 1B). Some studies indicate that low metal concentrations can stimulate algal growth, and the response of microalgae cells to metals depends on several factors, such as nutrient availability, presence of organic compounds, cultivation conditions and strain tolerance (Miazek et al. 2015). In addition, metals can also increase cell density by acting as a nutrient at low concentrations (Napan et al. 2015). An increase in cell density after exposure to metals was previously observed to *Anabaena doliolum* (Shukla et al. 2009) and *Scenedesmus obliquus* (Napan et al. 2015) exposed to Ni; to *Dunaliella tertiolecta* exposed to aluminum (Al) and *Desmodesmus quadricauda* exposed to Cerium (Miazek et al. 2015).

On the other hand, we observed a decrease in cell density in algae exposed to Ni. Martínez-Ruiz and Martínez-Jerónimo (2015) pointed that this metal is able to enter the cell through different pathways, such as specific transporters or receptor channels and ion exchange causing toxic effects if present in concentrations above those needed. Previous studies have also observed a drop in cell growth of the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana*, *Dunaliella bioculata* and *Scenedesmus vacuolatus* exposed to Ni (1, 5 and 10 mg L^{-1}) (Haiduc et al. 2009). In addition, Osman et al. (2004) obtained results similar to ours, i.e., at low concentrations, the metal stimulated growth while at high concentrations it decreased the cell growth to *Nitzschia perminuta* (0.5 and 2 mg L^{-1}) and *Scenedesmus obliquus* (0.1 and 1 mg L^{-1}).

The IC calculation is important to determine the concentration of the compound that presents toxicity to organisms, construct sensitivity curves (SSD) using the values obtained in chronic toxicity tests, and thus, define the most sensitive species to contaminants to establish safe concentrations. (Golding et al. 2015; Mansano et al. 2017; Posthuma et al. 2002). In the present study, the IC_{50} was 0.38 mg Co L^{-1} and 0.40 mg Ni L^{-1}.

Other species of microalgae exposed to Co presented EC_{50} values: 4.1 mg Co L^{-1} for *Chlamydomonas acidophila* (Nishikawa and Tominaga 2001) and EC_{50}: 23.80 mg Co L^{-1} for *Spirulina platensis* (Sharma et al. 1987). Stubblefield et al. (2020) obtained an IC_{50} of 0.14 mg L^{-1} for the microalgae *R. subcapitata*, a slightly lower value in relation to our study, probably due to the different conditions of the toxicity test (OECD cultivation medium and continuous light). Regarding Ni, the value was close to that found by Filová et al. (2021), IC_{50}: 0.50 mg Ni L^{-1} for *R. subcapitata*. Other species of microalgae exposed to Ni obtained the following values, IC_{50}: 0.017 mg Ni L^{-1} for *Ankistrodesmus falcatus* (Martínez-Ruiz and Martínez-Jerónimo 2015); *Chlorella sp* EC_{50}: 2.41 mg Ni L^{-1} (Peters et al. 2018); *Desmodesmus spinosus* EC_{50}: 0.17 mg Ni L^{-1} and *Scenedesmus acuminatus* 0.189 mg Ni L^{-1} (Deleebeeck et al. 2009).

Table 2 Summary of the modeled data from reference model concentration addition (CA) to cell density of *Raphidocelis subcapitata* exposed to mixtures of Co and Ni during 96 h

Concentration addition (CA)				
	CA	S/A	DR	DL
Max	4.35	4.43	4.25	4.60
β Co	1.17	1.23	1.38	0.10
β Ni	3.17	2.83	4.20	1.82
IC_{50} for Co	0.29	0.42	0.38	0.38
IC_{50} for Ni	0.31	0.39	0.40	0.40
A	-	−2.23	−4.25	0.01
$b_{DR/DL}$	-	-	4.73	262.67
SS	9.74	5.93	4.45	6.77
r^2	0.74	0.84	0.88	0.82
χ^2 or F test	19.56	15.88	25.1	11.64
Df	-	1	2	2
p (χ^2/F)	1.16×10^{-7}	6.75×10^{-5}	3.6×10^{-6}	0.003

Data obtained using Jonker et al. (2005) methodology. *Max* Maximum response value, *β* Slope response of isolated compounds, *IC_{50}* Median growth inhibition concentration; a and *$b_{DR/DL}$* Function parameters, *SS* Sum of the squared residuals, *r^2* Regression coefficient; χ^2 or F test = statistical test, *df* Degrees of freedom; *p*(χ^2/F) Level of significance of statistical test, *CA* Concentration addition model, *IA* Independent action model, *S/A* Synergism/antagonism deviation, *DR* Dose ratio-dependent deviation, *DL* Dose level-dependent deviation. DR is in bold because it was the deviation that best fit the data

Mixtures

Growth

The results obtained from binary mixtures of Co and Ni in the cellular density of *Raphidocelis subcapitata* were presented in Table 2 (data from concentration addition (CA) model) and Table S3 of Supplementary Material (data from independent action (IA) model). In this study, we used the CA and the IA models to analyze the results. The CA model admits that the individual compounds have the same mechanism of action, acting on the same biological target, and consequently the biological response is proportional to the respective toxicities of the compounds. While the IA model admits that the isolated compounds have different mechanisms of action, therefore, their effects are independent from each other. Then, when the chemical mechanism of action is unknown, both models are tested and the one that best fits the data is used to explain the data (Mansano et al. 2017). Furthermore, Jonker et al. (2005) concluded that there may be patterns to which data deviate from the CA and IA models, i.e., no deviation, synergism or

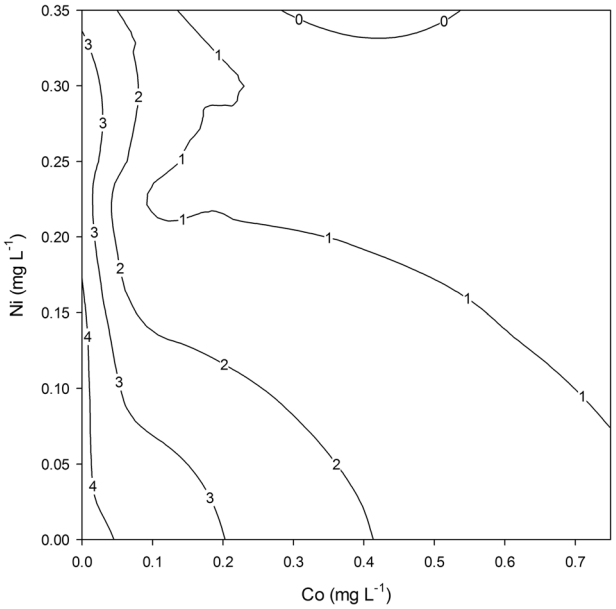

Fig. 2 Isobologram of the cobalt (Co) and nickel (Ni) mixtures, based on their effects on the cellular density of *Raphidocelis subcapitata*, following the reference model of concentration addition (CA) and dose-ratio dependent (DR) deviation. There was synergism at low doses of Co and high doses of Ni; while antagonism occurred at high doses of Co and low doses of Ni. The concave shape represents synergism while the convex shape shows antagonism (Alloatti et al. 2013)

antagonism (S/A), dose level-dependent (DL) deviation, and dose ratio-dependent (DR).

From our data, both models (CA and IA) were statistically significant, but the model that best fit the Co-Ni data was CA (Table 2). Results from IA model yielded a sum of the squared residual (SS) value of 9.26 ($p = 5.91 \times 10^{-8}$, $r^2 = 0.76$; Supplementary Material, Table S3). After adding the parameter "a", the SS value was 6.13 ($p = 2.81 \times 10^{-4}$; $r^2 = 0.84$). By adding the parameter $b_{DR/DL,}$ the model did not calculate the results of SS, r^2 and p. Data from the CA model yielded an SS of 9.74 ($p = 1.16 \times 10^{-7}$; $r^2 = 0.74$). By adding the parameter "a", the SS value decreased to 5.93 ($p = 6.75 \times 10^{-5}$; $r^2 = 0.84$). When the parameters b_{DR} and b_{DL} were added, the SS values were 4.45 ($p = 3.6 \times 10^{-6}$; $r^2 = 0.88$) and 6.77 ($p = 0.003$; $r^2 = 0.82$), respectively. Thus, based on these results and on the mixtures' isolobogram (Fig. 2), the CA model and DR deviation best fit our data as they presented the significant *p*-value, the smallest SS value and the largest r^2 value. The DR deviation indicates that there was synergism at low doses of Co and high doses of Ni; while antagonism occurred at high doses of Co and low doses of Ni ($a < 0$ and $b > 0$).

Based on that, synergistic effects at low concentrations of Co and high concentrations of Ni (around 0.25 mg L^{-1}), these results are a matter of concern given the concentrations already recorded for both metals in the environment. It is known that Co and Ni are widely used in industrial processes, due to their high durability and corrosion resistance. Consequently, their demand increases the waste generation, such as urban and industrial effluents, chemicals, and especially mining wastes (Alves et al. 2022). In the environment, metals have already been quantified in different concentrations: e.g., 0.01 mg Co L^{-1} - Paraopeba River (Thompson et al. 2020); 0.25 mg Co L^{-1} - Água Boa stream (Dourado et al., 2017) and 2.39 mg Co L^{-1} – Tanjero River (Aziz et al. 2012); while for Ni, 0.014 mg Ni L^{-1} - Paraopeba River (Thompson et al. 2020); 0.30 mg Ni L^{-1} - Água Boa stream (Dourado et al. 2017) and 1.08 mg Ni L^{-1} - Doce River (de Carvalho et al. 2017).

The surface of algae has several functional groups (carboxylic, sulfhydryl and phosphatic) that have high affinity for metal ions and function as binding sites for the transport of ions across the cell membrane. The absorption process occurs in two phases: passive transport (rapid absorption) followed by slow and facilitated transport to the cell cytoplasm. The metal coordination sites on the cell surface are not completely specific for a single nutrient or metal, so it is possible that there is competition for membrane transport and intracellular metal binding sites (Franklin et al. 2002; Starodub et al. 1987). This competition may explain the antagonistic metal interactions obtained in this study, since Co and Ni are both divalent metals and can compete for transport sites. The synergistic effect can occur when one metal facilitates the absorption of another, because exposure to metals can increase membrane permeability, leading to increased cellular uptake and toxicity; whereas competition for absorption, transport or sites of toxicity does not occur (Franklin et al. 2002; Starodub et al. 1987), that is, synergistic interactions cannot be explained solely in terms of metal-metal competition (Vijver et al. 2011). Franklin et al. (2002) evaluated the effect of a mixture of Cu, Cd and Zn on *Chlorella sp*. The authors argued that Cd increased membrane permeability or may have caused conformational changes to membrane proteins that consequently resulted in elevated copper entry into the cell. This could explain the synergistic effect observed and the fact that one metal facilitates the increase of another.

Metals are able to enter algal cells by active transport or endocytosis and affect their metabolism. Toxicity mechanisms are diverse and depend on the species of algae, the nature of the metals and environmental conditions. The main mechanisms are generally: displacement of essential cations from the specific binding site, damaging its function; direct binding to sulfhydryl groups (in proteins) altering their structure and function and generation of reactive oxygen species (Petsas and Vagi 2017). Polechońska and Samecka-Cymerman (2018) evaluated the effects of Co and Ni on *Hydrocharis morsus-ranae* (plant) and observed a lower decrease in biomass in plants exposed to Ni compared to plants exposed to Co and Ni, indicating

that Ni toxicity can increase with other metals, such as Co, Zn, Mn and Mo. The mixture of metals can change the toxicity of the compounds, i.e., they show different effects than their isolated forms (Batool and Javed 2015).

To the best of our knowledge, this is the first study that investigates the effects of Co and Ni mixtures in multiple endpoints of *R. subcapitata*. Therefore, we reinforce the importance of investigating the effects of metallic mixtures on organisms as their interaction can cause metabolic changes, interfere with transport, bioavailability, binding and excretion (Cedergreen 2014). Furthermore, our results indicated synergistic and antagonistic effects in the Co-Ni mixture, varying according to the mixture composition (CA model and DR deviation).

Morphology and Chlorophyll a (Chl a) fluorescence

Co and Ni single exposure did not significantly change the FSC-H (cell size) parameter (Fig. 3A), but metal mixtures led to an increase in cell size (Fig. 3A) in the treatments: M4; M8; M11; M14; M19; M21; M22 and M23. In previous studies, where microalgae were exposed to metals, an increase was observed in the cell size: Mn (0.4 and 0.8 mg L^{-1}) and Cr (0.2 mg L^{-1}) (Alho et al. 2022); Cu (1 mg L^{-1}) (Franqueira et al. 2000); Zn (0.0052–0.03 mg L^{-1}) and Al (0.4–1.0 mg L^{-1}) (Gebara et al. 2020). This increase may indicate damage to the cell division process, and is related to the uncoupling of cell growth and cell division (the cell is not able to complete the division) (Gebara et al. 2020; Mansano et al. 2017). Furthermore, metals can change membrane permeability, and the increase in cell size can occur due to increased permeability to Na^+ (Franqueira et al. 2000), or by interference with cell division and continued carbon fixation (photosynthesis) resulting in the accumulation of products of the photosynthetic processes and consequently increasing cell size (Franklin et al. 2001). Our results suggest that the mixed Ni-Co metals interfered with the cell division process, which caused an increase in the cell size in some treatments. Therefore, we highlight the fact that the isolated metals did not significantly interfere with this endpoint, reinforcing what was stated by Batool and Javed (2015). The authors pointed out that all combinations of metals Co, Cr and Pb showed synergistic interactions, that is, the effects of the mixture of metals were more toxic than the effect of isolated metals.

Co exposure led to an increase in SSC-H (cellular complexity) at 0.50 and 0.75 mg Co L^{-1}; while Ni exposure did not significantly change this parameter (Fig. 3B). Co-Ni mixtures did not change significantly in metal combinations M1 to M12. The increase was significant in combinations M13 to M23, except for M17. Cell complexity may be related to the internalization of metals by the microalgae (Gebara et al. 2020), and previous studies have already recorded an increase in the size and number of cytoplasmic vacuoles (Nishikawa et al. 2003; Aguilera and Amils 2005). Under stress conditions, the microalgae may have this action as a defense mechanism, to minimize or prevent the toxic effects of metals (Alho et al. 2022). Exposure to Zn, Al and Mn increased cell complexity for *R. subcapitata* cells (Alho et al. 2022; Gebara et al. 2020). Our data suggest that Co may activated this defense mechanism in treatments of 0.50 and 0.75 mg Co L^{-1}; as well as some treatments of the mixture, in which we observed an increase in cell complexity. Exposure to isolated Ni did not alter this parameter, which may indicate that another defense mechanism was activated, such as extracellular adsorption (Juarez et al. 2008).

Co exposure led to a decrease in FL3-H (Chl *a* fluorescence) at 0.10 mg Co L^{-1} and Ni exposure decreased the Chl *a* fluorescence in all concentrations tested (Fig. 3C). Combined metals showed significant increases and decreases in this parameter. Treatments that decreased fluorescence were M1 to M8, and M17; and treatments that led to an increase in Chl *a* fluorescence were M13 to M16; M18; M19; M21 and M22 (Fig. 3C). Chl *a* fluorescence is a good indicator of the physiological status of microalgae, as it provides information on energy in photosynthesis (absorption, distribution and utilization) (Franqueira et al. 2000). Therefore, the decrease in fluorescence may indicate some damage in the photosynthetic process, such as the inhibition of electron flow in the reaction center of photosystem II (PSII) on the donor side (Alho et al. 2022; Franqueira et al. 2000). However, the increase in fluorescence may suggest an attempt to maximize light capture, or an inhibition of electron transport may occur, but on the acceptor side (Alho et al. 2022; Franqueira et al. 2000). Čypaitė et al. (2014) showed that photosynthetic pigment production is a parameter that is more sensitive to the metal toxicity than algal growth and our data corroborate this affirmation, since exposure to Ni decreased cell density only at the highest concentration tested (0.35 mg L^{-1}), while Chl *a* fluorescence decreased at all concentrations of the metal.

ROS production

ROS generation by aerobic organisms occurs during metabolic processes such as photosynthesis and electron transport, in which mitochondria, chloroplasts and peroxisomes are natural production sites of ROS, as well as detoxification. Enzymatic activity and non-enzymatic defenses are essential to maintain the antioxidant defense system of aerobic organisms, which control ROS levels. Any imbalance in these defense mechanisms leads to increased ROS production and oxidative stress (Szivák et al. 2009). Our results showed an increase in ROS production at 0.50 mg Co L^{-1}, while in Ni exposure, there was a decrease in the

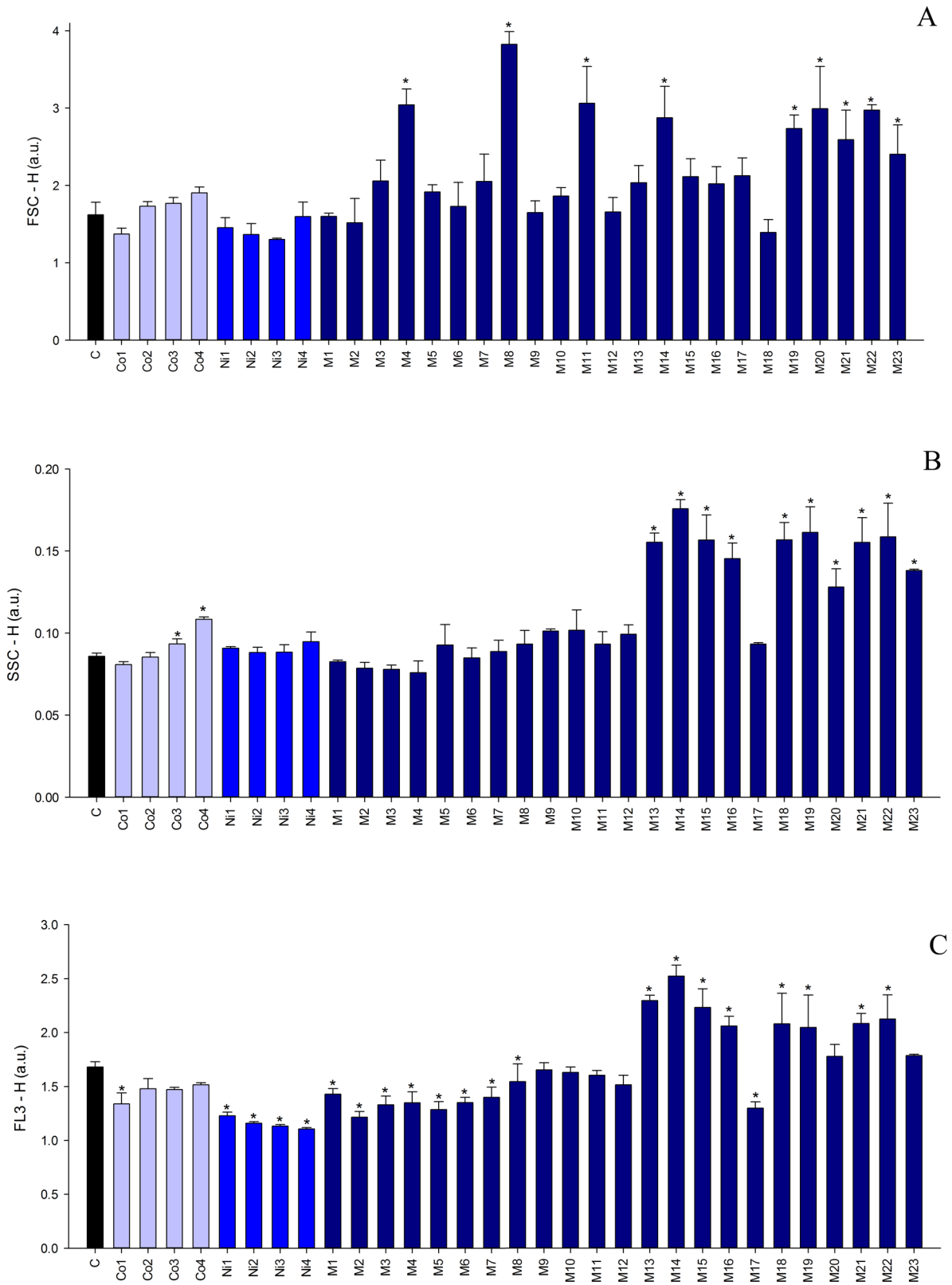

Fig. 3 Mean values of cell size (FSC-H) (**A**), cellular complexity (SSC-H) (**B**) and chlorophyll *a* fluorescence (FL3-H) (**C**) of *R. subcapitata* exposed to different concentrations of Co and Ni, isolated (Co1-Co4 and Ni1-Ni4) and combined (M1-M23) after 96 h of exposure. Asterisks (*) indicate significantly different values ($p < 0.05$) when compared to the control group (**C**)

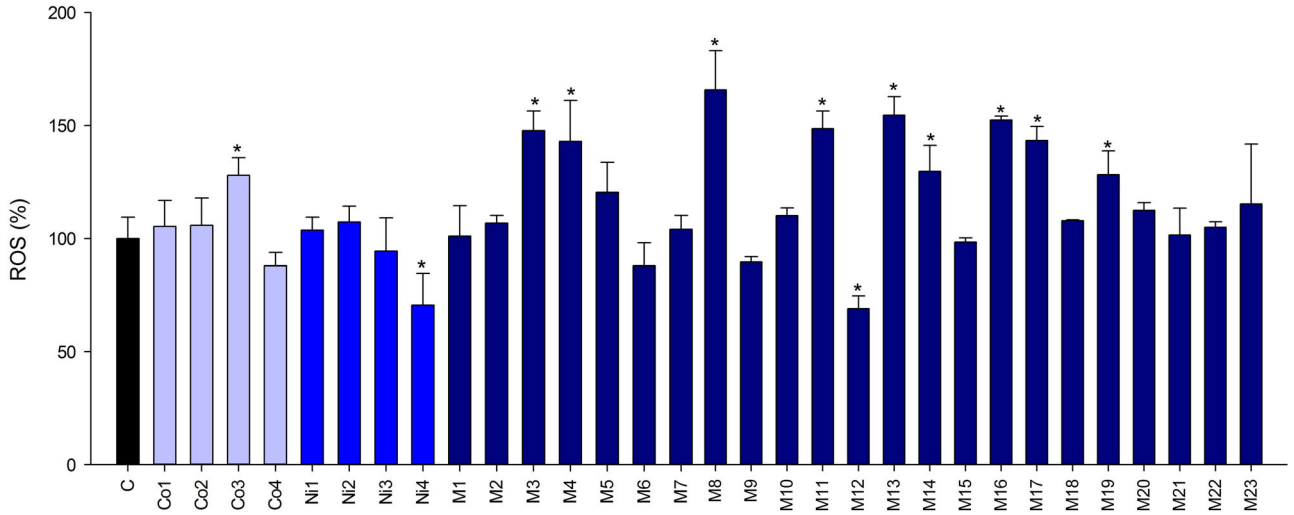

Fig. 4 Cellular reactive oxygen species (ROS) produced by *R. subcapitata* exposed to Co (Co1-Co4) and Ni (Ni1-Ni4), isolated and combined (M1-M23), at 96 h. Asterisks represent significant differences ($p < 0.05$) from the control group (C). Metal concentrations are described in Table 1

ROS at 0.35 mg L^{-1} (Fig. 4). In mixtures, we observed increased ROS production at M3, M4, M8, M11, M13, M14, M16, M17 and M19, and the ROS production increased with increasing Ni concentration in metallic combinations. In addition to that, only at M12 the ROS production decreased.

Environmental stress conditions such as UV radiation, high light intensity and exposure to contaminants such as metals and herbicides, can increase ROS production in cells, causing serious injury or cell death (Mei et al. 2007). Metals have the ability to inactivate antioxidant enzymes by binding to thiol groups or replacing ions, generating high levels of free radicals (Szivák et al. 2009). High ROS production can be extremely harmful to organisms, in addition to causing damage to the photosynthetic apparatus and cell death (Pinto et al. 2003; Sharma et al. 2012).

Co is categorized as Redox active, as are Cr, Mn, Fe and Cu. Such metals can damage cells by directly generating ROS by up-regulating Haber–Weiss and Fenton reactions (Mahey et al., 2020), then Co is capable of inducing oxidative stress through its reaction with hydrogen peroxide to produce hydroxyl radicals (Mei et al. 2007). Our results showed an increase in ROS production by Co single exposure and Co-Ni mixture, suggesting that they led cells to oxidative stress.

Ni is able to increase ROS production causing oxidative stress in aquatic organisms (Brix et al. 2017). The metal is categorized as a non-redox active metal, like Cd, Hg, Zn and Al which are capable of damaging cells and subcellular vitals through indirect accumulation of ROS by reducing glutathione, inhibiting antioxidant enzymes, binding groups of sulfhydryl proteins, activating ROS-producing enzymes (Mahey et al. 2020). Even though Ni is a metal capable of increase ROS production and cause oxidative stress, our data showed a decreased ROS production (Ni4) suggesting that *R. subcapitata* activated its antioxidant mechanisms and these were able to decrease the content of intracellular ROS (Alho et al. 2022), which possibly occurred at Ni4 and M12.

Total carbohydrates

Carbohydrates are important macromolecules for energy storage, they are present in the structure of the cell wall and are intermediaries in the respiratory and photosynthetic pathways (Martínez-Ruiz and Martínez-Jerónimo 2015). These macromolecules are important as alternative sources of energy, raw material and food storage material, which are used for structural modifications that adapt cells under conditions of metal exposure or other stress conditions (Chia et al. 2015). Our data showed that total carbohydrates significantly decreased after exposure to both metals, isolated, at all concentrations tested (Fig. 5). In the metal mixtures, carbohydrate synthesis decreased in M1; M2; M5; M9; M12; M17 and M20. On the other hand, the synthesis was increased in treatments M8; M11; M14; M15; M16; M18; M19; M22 and M23. Low concentrations of Ni combined with different concentrations of Co showed a predominant decrease in carbohydrate synthesis. Significant increases occurred in combinations in which Co and Ni concentrations were intermediate or high. We highlight this last effect as it differs from the effect observed in the exposure of isolated metals.

Carbohydrate synthesis can be altered by environmental stress and also by metals (Fawzy et al. 2020; Rocha et al. 2018). Alho et al. (2019) observed an increase in carbohydrate synthesis in *R. subcapitata* exposed to Cd, suggesting that this increase may function as a detoxification

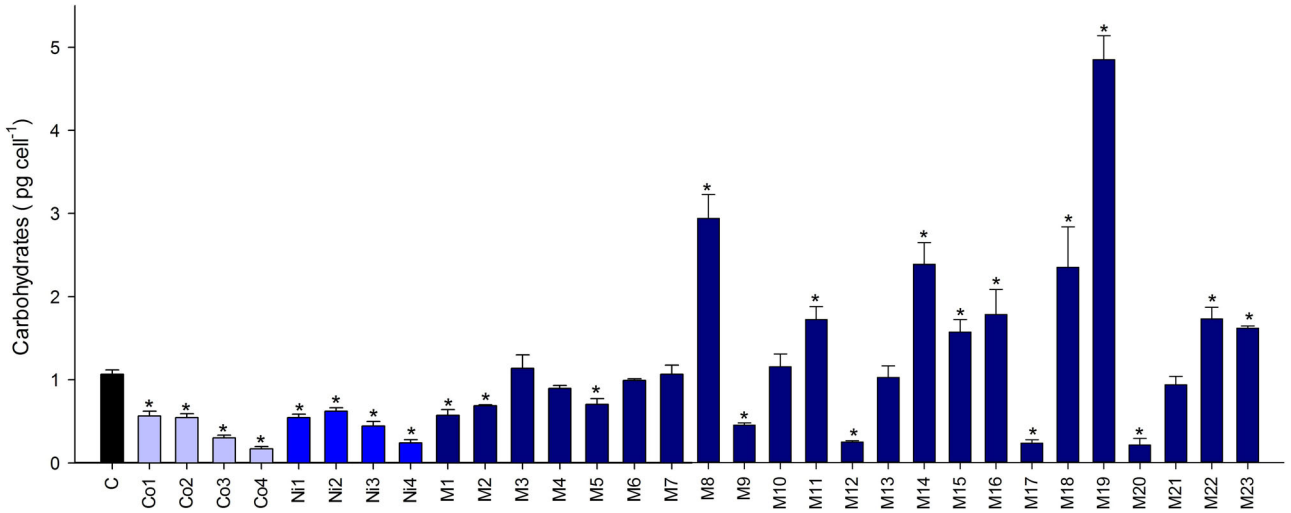

Fig. 5 Total carbohydrates (pg cell^{-1}) of *R. subcapitata* exposed to different concentrations of Co and Ni, isolated (Co1-Co4 and Ni1-Ni4) and combined (M1-M23), after 96 h of exposure. Asterisks (*) indicate a significantly different value ($p < 0.05$) when compared to the control group (C)

mechanism due to the interaction that occurs between metal cations and the negative charge of these biomolecules. Almost all of Co-Ni treatments increased total carbohydrates, suggesting an attempt to minimize the damage caused, activating this detoxification mechanism, or storing these molecules as an alternative energy reserve.

On the other hand, some authors have reported a decrease in the synthesis of this macromolecule under metal exposure (Elsalhin et al. 2016; Fawzy et al. 2020; Martínez-Ruiz and Martínez-Jerónimo 2015). Martínez-Ruiz and Martínez-Jerónimo (2015) observed changes in the external cellular structure of *Ankistrodesmus falcatus*, as well as a decrease in carbohydrate synthesis due to Ni exposure (0.005, 0.008 and 0.017 mg L^{-1}). The authors suggested that these results may be directly related as the cell wall provides protection to the cells, but at high metal concentrations it cannot fulfill this function as the metal possibly altered the structure of the cell wall. Furthermore, the decrease in the biomolecule may also indicate that there had been membrane depolarization.

Elsalhin et al. (2016) reported a decrease in carbohydrates in *Spirulina platensis* by exposure to Co (2.5 and 3 mg L^{-1}), suggesting that the reduction in synthesis may have been responsible for the inhibitory effect on photosynthetic activity. Fawzy et al. (2020) exposed *Synechocystis pevalekii* and *Scenedesmus bernardii* to Co, and reported the decrease in carbohydrates, highlighting the ability of metals to alter the metabolism of the organisms. Therefore, our results suggest that isolated metals and some mixture combinations altered the metabolism of *R. subcapitata*, which may indicate that there was a change in the plasma membrane (change in its structure or depolarization) resulting in a decrease in the synthesis of total carbohydrates.

Phyto-PAM analyses

Measurements carried out by the Phyto-PAM device are advantageous for investigations in aquatic toxicology as they are sensitive and can be obtained quickly. Maximum quantum yield (Φ_M) of photosystem II (PSII) indicates the ability of dark-adapted cells to convert light energy into chemical energy (Juneau et al. 2002). The F_0/F_v ratio, in which F_0 indicates the initial fluorescence and F_v the variable fluorescence, points to the efficiency of the oxygen evolution complex (OEC), where water photolysis occurs (breakdown of the molecule and production of oxygen)(Herlory et al. 2013). Our data showed that isolated Co exposure decreased Φ_M at 0.50 and 0.75 mg Co L^{-1} (Fig. 6A), while the F_0/F_v ratio was altered in all the tested Co concentrations (Fig. 6B). Ni exposure did not affect photosynthetic parameters Φ_M (Fig. 6A) and F_0/F_v (Fig. 6B). Metal mixtures affected these endpoints at the same combinations: M16, M17, M21, M22 and M23.

Plekhanov and Chemeris (2003) investigated the effects of Zn, Co and Cd on *Chlorella pyrenoidosa* and observed a decrease in photosynthetic activity for exposure to all metals. Isolated Co decreased Φ_M values, which may indicate a partial blockage or a decrease in the transport of electrons from PSII to photosystem I (PSI), which occurs due to the reoxidation of quinone A (Herlory et al. 2013; Mallick and Mohn 2003). The increase in F_0/F_v with increasing metal concentration suggests that Co may have displaced Mn (manganese), calcium and chloride ions, interfering with the photo-oxidation reaction of water (Alho et al. 2019; Echeveste et al. 2017). El-Sheekh et al. (2003) exposed *Monoraphidium minutum* and *Nitzschia*

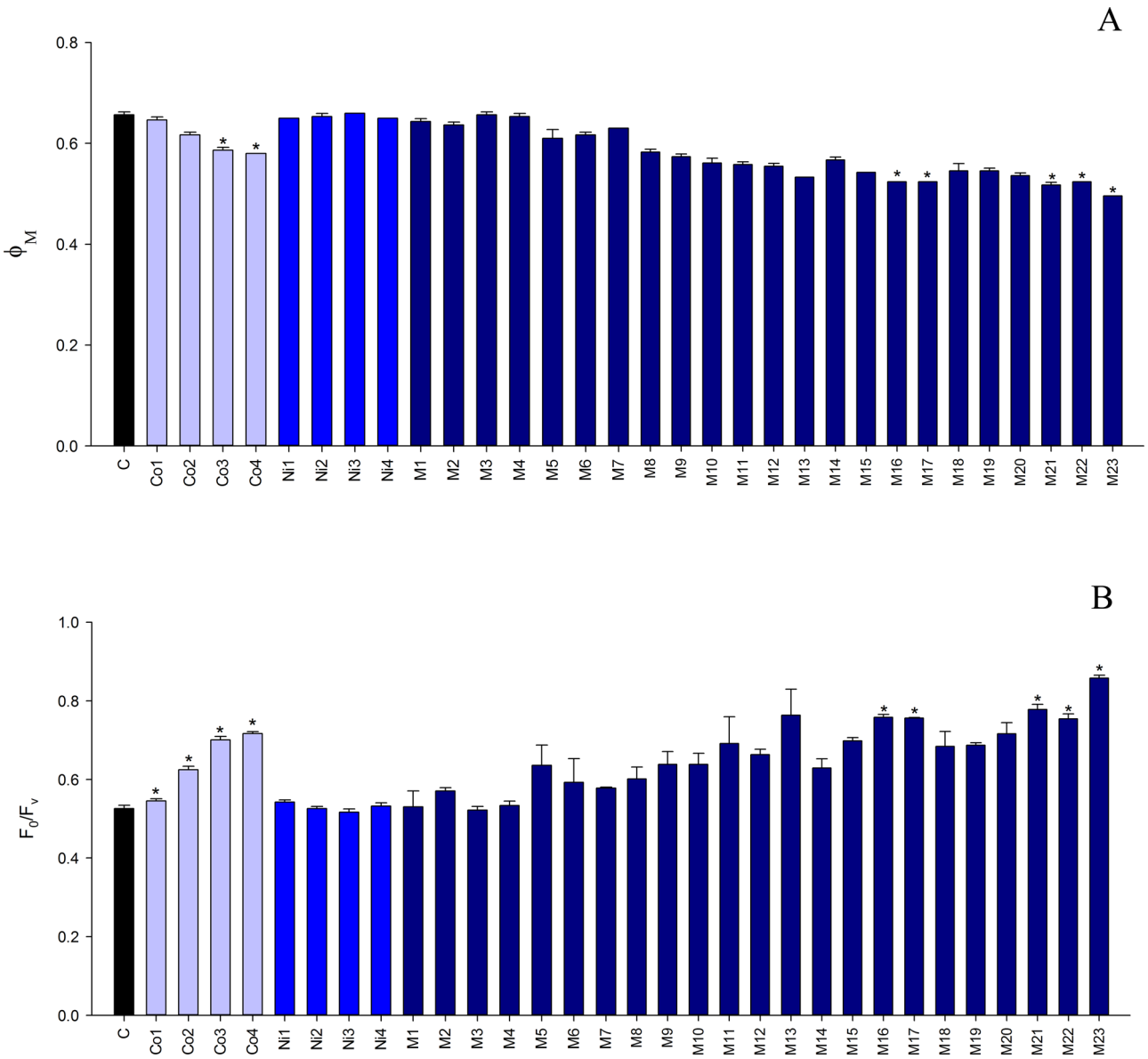

Fig. 6 Maximum quantum yield (Φ_M) (**A**) and measurement of the efficiency of Oxygen Evolving Complex (F_0/F_V) (**B**) of *R. subcapitata* exposed to Co(Co1-Co4) and Ni (Ni1-Ni4), isolated and combined (M1-M23), after 96 h of treatment. Asterisks (*) means the significant difference ($p < 0.05$) when compared to the control group (**C**)

perminuta to Co at different concentrations and observed that the site of action of the metal is located on the acceptor side of the PSII (P680) for both algae and there was a decrease in the amount of O_2 released as the metal concentration increased, interfering with electron transport. Begović et al. (2016) exposed *Lemna minor* to Co and observed the influence of the metal on the electron transport between the OEC and the PSII reaction centers, supporting the hypothesis of displacement of Mn by Co. Our results suggest that Co possibly interfered with electron transport, replacing Mn, altering the water photolysis reaction and consequently causing damage to photosynthetic activity.

Ni can replace magnesium (in the central atom of chlorophyll) forming an unstable molecule and consequently decreasing photosynthetic activity (Baumann et al. 2009). Furthermore, when interacting with the OEC, it can cause the depletion of two polypeptides, leading to the inhibition of electron transport. Therefore, it reaches the OEC, affects the PSII, and may interfere with the entire photosynthetic pathway (Martínez-Ruiz and Martínez-Jerónimo 2015). However, even though the metal is capable of causing these effects, the concentrations used in this study did not show significant damage to the photosynthetic parameters of *R. subcapitata* exposed to Ni, indicating that at these concentrations the metal has other toxicity pathways.

The significant effect of the mixture between Co-Ni occurred in the same combinations for Φ_M and F_0/F_v, suggesting that the mixture caused damage to the OEC and consequent interference in the photosynthetic process, leading to a decrease in Φ_M. These changes occurred in the combinations with the highest Co concentrations and intermediate and high Ni concentrations. Furthermore, we observed a decrease in Chl *a* fluorescence upon exposure of microalgae to isolated and combined metals (Fig. 3C). These results suggest that some damage may occur in the photosynthetic process and our data show that single and combined exposure affected photosynthesis (Φ_M and F_0/F_v). Besides, some mixture treatments showed an increase in fluorescence in combinations with higher concentrations of Co and Ni, suggesting an attempt to increase light capture, to minimize the damage to the microalgae physiology.

Other studies that investigated the effects of metal mixtures observed that isolated metals (Cd, Cu) decreased the Φ_M of *Elodea densa*, while combinations of metals, Ni-Cd; Ni-Cu; Ni-Zn; Mn-Ni; Mn-Cd; Mn-Cu and Mn-Zn did not significantly alter this parameter (Maleva et al. 2012). Dos Reis et al. (2022) observed that Co alone negatively affected the Φ_M and F_0/F_v of *R. subcapitata*, while the Cd-Co mixture did not affect these endpoints. Gebara et al. (2023) observed changes in the photosynthetic parameters (Φ_M and F_0/F_v) of *R. subcapitata* exposed to Zn and Al, in addition to synergistic (discreet) and antagonistic effects in the combination of metals. To the best of our knowledge, this is the first study that investigates the effect of Co-Ni metal mixture to photosynthetic parameters (Φ_M and F_0/F_v) in *R. subcapitata*. Therefore, we emphasize the importance of studying the effects of combined compounds, in order to increasingly understand the mechanisms of action of these compounds when they are combined.

Conclusion

Our results demonstrated that single Co and Ni altered the metabolism of *R. subcapitata* while Co-Ni mixtures showed that synergistic and antagonistic interactions can occur depending on the dose of metals (synergism at low doses of Co and high doses of Ni; and antagonism at high doses of Co and low doses of Ni). We emphasize the importance of investigating the effects of mixed metals on the metabolism of organisms as the isolated effect may differ from the effect of the mixture, in which it can be potentiated or not. The results can also guide future studies related to the mechanisms of action of combined metals and help the development of guidelines and risk analysis. In addition, we emphasize the relevance of studies with microalgae as they are organisms sensitive to contaminants and the basis of the trophic chain. Thus, any damage suffered by these organisms can reach higher trophic levels.

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Author contributions LLdosR: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft. CBdeA: Methodology, Validation, Investigation, Writing - Review & Editing. RCG: Validation, Formal analysis, Writing - Review & Editing. GSR: Methodology, Validation, Investigation, Writing - Review & Editing. EL: Resources and Funding acquisition, Writing - Review & Editing. AdaSM: Validation, Formal analysis, Writing - Review & Editing. MdaGGM: Conceptualization, Resources, Writing - Review & Editing, Funding acquisition.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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