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RESEARCH

Effects of copper and cadmium mixtures on photosynthetic parameters of the freshwater microalga *Ankistrodesmus densus* (Chlorophyceae)

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Abstract

Metal discharges from anthropogenic activities are of great concern, and the effects of metallic mixtures in aquatic environments are still not fully understood. This study aimed to evaluate the effects of cadmium (Cd) and copper (Cu), isolated and combined, in the growth and photosynthesis – photosystem II (PSII) activity ($\Phi_{PSII} = F_v/F_M$), effective quantum yield, photochemical (qP) and non-photochemical quenching (qN and NPQ), and quenchings of regulated ((Y(NPQ)) and non-regulated ((Y(NO)) non-photochemical energy loss in photosystem II, in addition to the rapid light curves of the freshwater green microalga *Ankistrodesmus densus*. Both metals affected algal physiology and when combined most of the responses were antagonists, i.e., the observed effects were lower than those predicted for the mixture. The contaminants decreased the chlorophyll *a* production, photosynthetic activity, and increased heat dissipation, suggesting activation of the photoprotection mechanisms and with no evidence of photoprotection damage in the algae. Except for Y(NO), all evaluated photosynthetic parameters were sensitive indicators of the changes observed in microalgae under Cd and Cu exposure, isolated or combined. In addition, we suggest that algal protective mechanisms were efficient to decrease the impact of the combination of metals, reinforced by the increases in qN, NPQ and Y(NPQ), resulting mainly in antagonism in photosynthetic parameters.

Keywords Metal mixtures · Phyto-PAM · Photosynthesis · Photochemical energy · Quenching · Rapid light curves

Introduction

Aquatic ecosystems receive discharges of nutrients (e.g., nitrogen and phosphorus) and contaminants (e.g., metals, pharmaceuticals, pesticides) from natural and anthropogenic activities, which can cause acute and chronic effects on the aquatic biota, affecting its development, reproduction, and growth (Pinto et al. 2021). Generally, organisms are exposed to mixtures of several stressors, which may or may not interact. The prediction of mixture toxicity is based on the effects of each contaminant isolated, assuming the non-interaction between the different stressors. The concentration addition (CA) model assumes that individual contaminants present the same mode of action in the organism; thus the toxicity of the mixture is the sum of the relative effects of each contaminant (Olmstead and LeBlanc 2005); while the independent action (IA) model assumes different modes of action of the pollutants, thus the toxicity of the mixture is calculated by multiplying the effects of each contaminant (Loureiro et al. 2010). The CA and IA models indicate if there is a deviation from additivity (Jonker et al. 2005; Altenburger et al. 2013;

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Nys et al. 2017a) and they are applied based on their precision. Usually, different models are tested to evaluate which presents the best responses (Cedergreen et al. 2008; Nys et al. 2017b). If there are interactions between the contaminants, they can be: (i) less than additive (antagonism), i.e., the combination of the two stressors results in less damage than isolated compounds; or (ii) more than additive (synergism), when the combination results in more damage than the isolated stressors (Backhaus et al. 2004).

In the last decades there has been increasing concern, scientific discussion, and studies dealing with the effects of chemical mixtures on the environment (Kortenkamp and Faust 2018). A vast knowledge has been built regarding the effects of several chemical mixtures – e.g., pesticides, pharmaceuticals, metals – at different trophic levels (e.g., producers, primary and secondary consumers) and environments – mainly aquatic and terrestrial. Unfortunately, currently, except for Australia and New Zealand (ANZECC and ARMCANZ 2000), the regulatory agencies, such as CONAMA (Brazil), US EPA (USA), or the European Union (EU), do not have specific environmental mixture risk assessment directives. However, there are efforts to implement a mixture assessment factor in the EU (Kortenkamp and Faust 2018; COM 2020). Regulation is complex and challenging due to the different behaviors of the mixtures and responses of organisms (Kortenkamp 2009; Beyer et al. 2014), in addition to the inherent difficulties of replicating in the laboratory the multitude of compounds, species, and conditions in nature. The toxicological mode of action of the metals can be different according to the biological species, with complex interactions which are not fully understood, and review studies indicate the prevalence of antagonistic effects in metallic mixtures (Norwood et al. 2003; Vijver et al. 2011; Cedergreen 2014; Nys et al. 2017b; Martin et al. 2021).

Copper (Cu) is essential in the processes of photosynthesis and respiration in algae (Bossuyt and Janssen 2005; Lombardi and Maldonado 2011). At concentrations higher than required, it can affect chlorophyll synthesis, electron transport rate, and biochemical parameters (Prosnier et al. 2015; Baracho et al. 2019; Rocha et al. 2021a). Cadmium (Cd) has no known essentiality for freshwater algae, affecting their photosynthesis and biochemical composition (Rocha et al. 2020, 2021b). The range of metals concentrations found in aquatic ecosystems is vast, with values reecorded for Cd from < 1 µg L^{-1} in UK rivers (Neal and Robson 2000), 200 µg L^{-1} in Egyptian lakes (Mansour and Sidky 2002) and up to $\approx$ 300 µg L^{-1} in rivers under the influence of factories (Ivorra et al. 1999). For Cu, some values regcorded in the environment are 0.25 µg L^{-1} in Mediterranean coastal wetlands (Andreu et al. 2016), $\approx$ 150 µg L^{-1} in River Nidd (UK – Neal and Robson 2000), and up to 294 µg L^{-1} in Egyptian lakes (Mansour and Sidky 2002). The main sources of metal contamination are anthropogenic activities, such as agriculture, industry, and mining (Çelekli et al. 2016; Comber et al. 2023), releasing high amounts of metals in aquatic ecosystems, intentionally or not (e.g., when dam collapses occur).

The bioavailability of the metal ions to the organisms can vary according to the physicochemical properties of water, such as pH, hardness, presence of other toxicants, nutrients, and dissolved organic matter (Rocha et al. 2016). The free metal ions of Cd and Cu, Cd(II) and Cu(II), are considered the bioavailable metal fraction leading to effects on aquatic biota since they can be taken up by the organisms (Mendes et al. 2013). In algae, the light-harvesting complex has a series of pigments that transfer the light energy until reaching the special pair of chlorophyll in the photochemical reaction centers, where the central Mg(II) can be replaced by the free ions Cu(II) and Cd(II), resulting in unstable and non-functional chlorophylls (Küpper et al. 2002; Santos et al. 2015; Andresen et al. 2016). The oxygen-evolving complex, where the water oxidation occurs, has an inorganic cluster Mn_4CaO_5 (Vogt et al. 2015), whose formation starts with Ca(II) and Mn(II) (Vinyard et al. 2019), and Cd(II) and Cu(II) can compete and replace these divalent ions, affecting the functioning of this complex (Yachandra and Yano 2011). These replacements affect directly the algal photosynthetic efficiency.

Studies on the effects of metallic mixtures on microalgae have increased in the last years. Variable responses in the algal response to the mixtures were observed depending on the metal, dose, and species evaluated. Fettweis et al. (2021) observed that smaller species have stronger synergism in the binary mixtures of Ni, Cu, and Zn and that responses can vary from synergism to antagonism in the exponential growth phase. Other studies indicate that antagonism or synergism can occur in the same combination of metals (e.g., Al and Zn; Co and Cd; Co and Ni), and responses are related to the amount of metals in mixtures (Gebara et al. 2020, 2023; Reis et al. 2022, 2024a, 2024b), while the non-interaction of metals results in additivity (Cr and Mn – Alho et al. 2022). Considering (i) the ubiquitous co-occurrence of Cu and Cd in aquatic ecosystems, (ii) the worldwide distribution of the microalga *Ankistrodesmus densus* (John and Tsarenko 2002; Freitas and Loverde-Oliveira 2013; Barinova and Niyatbekov 2018), and (iii) that photosynthetic parameters response to the metal can be metal- or species-specific, being sensitive and well-suited endpoints to evaluate the effects of metals and nutrients on microalgae (Juneau et al. 2002; Mallick and Mohn 2003; Herlory et al. 2013; Rocha et al. 2021a, 2021b; Rocha and Melão 2024; Rocha et al. 2024), the present study evaluates the growth and photosynthetic responses of *A. densus* exposed to environmental concentrations of Cu and Cd, isolated and combined, contributing to a better understanding of the impacts of these mixtures in algal photosynthetic processes.

Material and methods

Algal cultures

The freshwater microalga *Ankistrodesmus densus* (Chlorophyceae) was obtained from the algae culture collection of the Botany Department at the Federal University of São Carlos (CCMA 3 UFSCar; São Carlos, SP, Brazil) and was kept under photoautotrophic conditions in modified WC medium (Guillard and Lorenzen 1972). Normally this medium also contains ethylenediamine tetraacetic acid (EDTA), But this was not added in our study, since it can contribute to the chelation of the metals, decreasing their bioavailability (Sunda and Huntsman 1998). Before exposure to the contaminants, the algae were acclimated for approximately 40 days to the medium without the addition of EDTA. Algae were exposed to the initial culture conditions: pH 7, light intensity of 130 µmol photons m^{-2} s^{-1}, photoperiod 12:12 h light:dark cycle, and temperature 22 ± 2 °C. The cultures were gently shaken three times a day. Media were sterilized by autoclaving for 20 min at 121 °C before inoculation and addition of the vitamins solution. Sterile conditions were used throughout the manipulation of algae.

Cadmium and Cu solutions were made by serial dilutions of $CdCl_2$ Titrisol 1000 mg L^{-1} (Merck) and $CuCl_2$ Titrisol 1000 mg L^{-1} (Merck), respectively, in ultrapure water (Barnstead Easy Pure II, Thermo Scientific, USA). Before the inoculation of the algae, 15 mL samples from the medium with Cd and/or Cu were taken, acidified with 1% HNO_3, kept cold (4 ºC) and measured as soon as possible by inductively coupled plasma – optical emission spectrometry (Thermo ICP-OES, iCAP 7000; Thermo Fischer Scientific, USA), and quantified using 228.80 nm (Cd) and 324.75 nm (Cu) emission lines. The quantification limits were 0.01 and 0.05 mg L^{-1}, while the detections limits were 0.00006 and 0.004 mg L^{-1} for Cd and Cu, respectively. Since the free ions correlates better with the metal toxicity, we calculated the Cd(II) and Cu(II) ions through the chemical equilibrium model MINEQL$^+$ 4.62.3 (Environmental Research Software, USA). This model estimated that approximately 98% of added Cd and 70% of added Cu at the beginning of the experiment was in the free form in the culture medium.

72 h-exponentially growing *A. densus* cells (initial concentration ≈ 5×10^4 cells mL^{-1}) were exposed for 72 h to four Cd concentrations: 40.00; 85.00; 150.00 and 180.00 µg Cd L^{-1}, to four Cu concentrations: 40.00; 60.00; 80.00 and 100.00 µg Cu L^{-1}. These values refer to the quantified metals. For control, we considered the value of 0 µg L^{-1} for Cd and 0.03 µg L^{-1} for Cu (present in the culture medium). The values of bioavailable metals are considered in this study. Thus, considering the bioavailable fraction, the concentrations tested were: Control – 0.00 µg Cd(II) L^{-1} and 0.02 µg Cu(II) L^{-1}; 39.20 (Cd1), 83.30 (Cd2), 147.00 (Cd3) and 176.40 (Cd4) µg Cd(II) L^{-1}; and 28.00 (Cu1), 42.00 (Cu2), 56.00 (Cu3) and 70.00 (Cu4) µg Cu(II) L^{-1} in the medium. The metals were tested isolated and the full factorial combination of the four Cd and four Cu concentrations, resulting in 16 mixture treatments (Fig. 1). These concentrations were defined after preliminary range-finding tests. In addition, these concentrations are environmentally relevant since Cd can be found in the range from 0 to 331 µg L^{-1} in the environment (Ivorra et al. 1999; Mansour and Sidky 2002; Smolders et al. 2003), while Cu can be found from 0 to 294 µg L^{-1} (Neal and Robson 2000; Mansour and Sidky 2002; Andreu et al. 2016). Toxicity tests were performed with three experimental replicates in 500 mL polycarbonate Erlenmeyer flasks containing 250 mL of sterile culture medium under the same conditions described above for algal culturing. The media containing metal(s) were kept at least 24 h before the metal quantification and algae inoculation, to allow the metal(s) to be in equilibrium with the media.

Samples (1 mL) were fixed with Lugol's solution and counted under an optical microscope using a Neubauer hemocytometer. Growth rate was calculated according to USEPA (2012): $\mu = [ln(d_f) - ln(d_i)]/t$; where: μ = specific growth rate (day^{-1}); d_f = cell density at the end of the experiment; d_i = cell density at the beginning of the experiment; t = exposure time (in days). At 72 h exposure, chlorophyll *a* (µg L^{-1}) was determined as described by Shoaf and Lium (1976) in 10 mL samples that were filtered onto cellulose ester membranes (0.45 µm pore size; Millipore) and extracted with dimethylsulfoxide (DMSO). After filtering the samples, the filters were placed in conical tubes where 5 mL of DMSO were added, shaken, left in the dark for

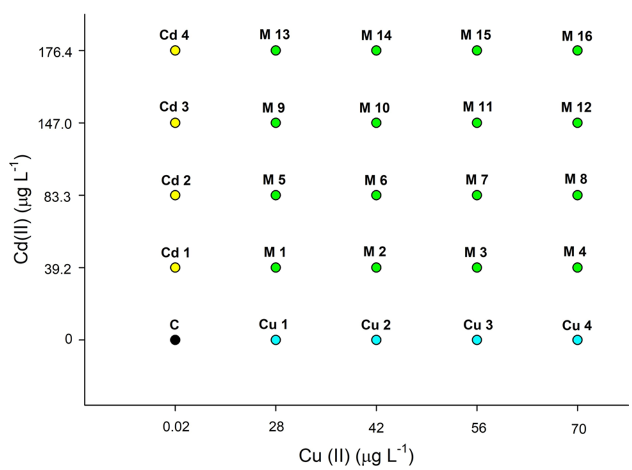


Fig. 1 Full factorial design of tests on *Ankistrodesmus densus* exposed to cadmium (Cd) and copper (Cu) mixtures for 72 h. The values presented refer to the bioavailable fraction of each metal [Cd(II) and Cu(II)] estimated through the chemical equilibrium MINEQL$^+$. The circle colors correspond to the control group (black), cadmium (yellow), copper (blue), and metal mixtures (green)

15 min, and absorption was measured in a spectrophotometer (HACH DR 5000, USA) at 664 and 647 nm. Chlorophyll *a* concentration was calculated as described by Jeffrey and Humphrey (1975).

PAM fluorescence measurements

At 72 h of metal exposure, 2.5 mL of culture was dark-adapted for 15 min for complete oxidation of PSII reaction centers. With the use of pulse amplitude modulated fluorometer Phyto–PAM I (Walz, Germany) equipped with an ED-101US/MP optical unit, we obtained the initial (F_0), the maximum (F_M), and the variable ($F_v = F_m - F_0$) fluorescence. F_0 was obtained with a low intensity modulated light, which does not induce photosynthesis (PAR 0), while F_m was obtained after a saturation light pulse (0.2 s, 2000 µmol photons m^{-2} s^{-1}), which provided the PSII activity ($\Phi_{PSII} = F_v/F_m$).

Subsequently, the samples were exposed to continuous actinic light (128 µmol photons m^{-2} s^{-1}), and new saturating light pulses were applied every 20 s for 10 min – to obtain the parameters of light-adapted samples. With the steady-state chlorophyll fluorescence (F_s) and maximum fluorescence in light (F'_m), were calculated the operational quantum yield ((($Y(II) = (F'_m - F_s)/F'_m$))). F'_0 was calculated as $F'_0 = ((F_0/(F_v/F_m) + F_0/F'_m))$ (Oxborough and Baker 1997), thus photochemical quenching (($qP = (F'_m - F_s)/(F'_m - F'_0)$)), non-photochemical quenching (($qN = 1 - [(F'_m - F'_0)/(F_m - F_0)]$)) were calculated (Juneau et al. 2002). The Stern–Volmer non-photochemical quenching (($NPQ = (F_m - F'_m)/F'_m$)) (Maxwell and Johnson 2000), as well as the quenchings of regulated (($Y(NPQ) = (F_s/F'_m) - (F_s/F_m)$)) and non-regulated non-photochemical (($Y(NO) = F_s/F_m$)) energy loss in PSII were calculated (Klughammer and Schreiber 2008).

Rapid light curves were obtained with a gradual increase in the intensity of photosynthetically active radiation (PAR; 0–1728 µmol photons m^{-2} s^{-1}) and light pulse intervals of 20 s, resulting in successive Φ'_M ((= Y(II)). The relative electron transport rate (rETR) was determined ($rETR = \Phi'_M \times PAR$; Ralph et al. 2002). The curve was fitted according to Jassby and Platt (1976), providing the initial slope (α), the maximum relative electron transport rate (rETRmax), which allowed the calculation of the saturating irradiance ($E_k = rETR_{max}/\alpha$; µmol photons m^{-2} s^{-1}).

Data analysis

The data were tested for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test). Statistically significant differences among treatments and controls were determined using two-way ANOVA and Dunnet's post hoc test at $p < 0.05$ (SigmaPlot 11, Systat). Each treatment had three experimental replicates ($n = 3$) and data are presented as mean ± SD.

To calculate the effects of the mixtures, we assumed the independent action model of the metals relative to control, due to a better prediction of this model to evaluate metallic mixtures at the species-level (Nys et al. 2018). The predicted effects were calculated using Eq. 1, while the observed effects in the mixture were calculated using Eq. 2. The synergy ratio of the mixtures (Eq. 3) was calculated for the endpoints that were significantly lower than the obtained values in the control or in the single exposure to metals (Cd or Cu), using the predicted and observed effects for sublethal endpoints (Gottardi et al. 2017). The synergy ratios below 1 indicate antagonism, equal to 1 indicate additivity, and higher than 1 indicate synergism.

$$Predicted = \frac{Cd}{C} \times \frac{Cu}{C} \quad (1)$$

where *Cd* refers to the mean value of the endpoint obtained under Cd exposure; *Cu*, under Cu exposure, and *C* refers to control.

$$Observed = \frac{Mix}{C} \quad (2)$$

where the *Mix* is the mean endpoint's value under the combination of Cd and Cu exposure.

$$Synergy\ ratio = \frac{Predicted}{Observed} \quad (3)$$

Results

Cadmium and Cu affected the algal growth rate linearly, i.e., higher metal concentration, lower growth rate (Supplementary Fig. 1 A). Regarding the algal growth in mixtures, it is possible to observe synergism in the combination of Cd1, Cd2 and Cd3 to Cu1 and Cu2 (i.e., M1, M2, M5, M6, M9, and M10), while the other combinations resulted in antagonism, especially at the highest metal concentrations (Table 1). The concentration of chlorophyll *a* decreased under Cd, Cu, and mixture exposure (Suppl. Fig. 1B). The decrease was lower in the lowest concentrations of Cd(II) (Cd1), and Cu (Cu1 and Cu2), isolated or in the mixture. The synergy ratio indicates that an antagonism occurred when the metals were combined, decreasing their impact on the chlorophyll *a* concentration, i.e., the observed value was higher than expected for predicted in their combination (Table 1; Supplementary Table 1); however, it is not possible to define which metal was responsible for this response.

The values for F_v/F_m and the synergy ratio are shown in Suppl. Figure 1 C and Table 1. It is possible to observe that F_v/F_m decreased significantly in almost all of the

Table 1 Synergy ratio of growth rate (GR), chlorophyll *a* (Chl *a*), and photosynthetic parameters of the freshwater microalga *Ankistrodesmus densus* exposed to Cd and Cu mixtures (M)

Treatment	Parameter						
	GR	Chl *a*	F_v/F_m	qP	Y(II)	$rETR_{max}$	α
M1	**1.11**	0.59	0.85	0.89	0.80	**1.17**	0.84
M2	**1.32**	0.57	0.85	0.90	0.82	**1.24**	0.80
M3	0.53	0.46	0.90	0.90	0.82	**1.22**	0.90
M4	0.43	0.62	0.86	0.86	0.75	**1.05**	0.85
M5	**1.13**	0.51	0.87	0.86	0.80	**1.10**	0.88
M6	**1.28**	0.53	0.86	0.85	0.76	**1.06**	0.91
M7	0.64	0.33	0.87	0.86	0.75	0.93	**1.14**
M8	0.46	0.50	0.86	0.83	0.73	0.86	0.96
M9	**1.23**	0.53	0.90	0.88	0.80	0.85	0.85
M10	**1.78**	0.46	0.88	0.91	0.80	0.93	0.96
M11	0.63	0.35	0.91	**1.01**	0.85	**1.01**	**1.10**
M12	0.33	0.54	0.88	**1.08**	0.82	0.85	0.93
M13	0.64	0.51	0.90	0.96	0.86	0.86	0.86
M14	0.35	0.38	0.90	0.99	0.87	0.87	0.90
M15	0.22	0.24	0.91	**1.02**	0.85	0.88	1.00
M16	0.35	0.34	0.89	**1.24**	0.88	0.80	0.94

F_V/F_m—PS II activity; qP – photochemical quenching; Y(II) – effective quantum yield; $rETR_{max}$ – maximum relative electron transport rate; α – efficiency in light capture. Values highlighted in bold indicated synergism. (< 1 – antagonism; > 1 – synergism).

concentrations tested in both metals and in the mixture, except for M1 and M2, indicating an apparent adjustment of the F_v/F_m in the lowest mixture combinations. The lowest value was detected at the highest Cu concentration. The synergy ratio was similar in all of the mixtures (0.85–0.91), indicating antagonism.

The photochemical (qP; Fig. 2A) and non-photochemical quenching (qN; Fig. 2B, and NPQ; Fig. 2C) were affected under Cd, Cu, and mixture exposure. The values of qP, which indicates the proportion of reaction centers that are open, decreased under all the concentrations of Cd or Cu exposure. The combination of the two lowest concentrations of Cd and Cu (M1, M2, M5, M6) did not affect qP ($p > 0.05$); while in the combination of the highest concentrations of metals (M11, M12, M15, M16), the effects were more deleterious than the isolated contaminants, indicating the synergism of the mixtures (Table 1). Regarding the non-photochemical quenching, qN and NPQ, there was an increase in both parameters in the presence of isolated or combined metals, with algae under Cu exposure presenting lower values (i.e., lower heat dissipation) than under Cd exposure.

The quenching Y(II) – the energy used in photochemistry; Y(NO) – energy passively dissipated; and Y(NPQ) – energy actively dissipated in the form of heat, are also shown in Fig. 2. It is possible to observe that both metals decreased significantly the Y(II) (Fig. 2D), and antagonism was observed in the mixtures (Table 1). In treatments M12 and M16, which have the two highest concentrations of Cd with the highest concentration of Cu, there is a more significant harmful effect of the two metals combined. The Y(NO) values in the treatments, except for Cu 4 and M16, were similar to control, indicating that algae were not dissipating more energy passively under metal exposure (Fig. 2E). The higher values of Y(NPQ) indicate that the algae activated the photoprotective mechanisms to dissipate energy in the form of heat, with no differences observed only in Cu1 and Cu2 (Fig. 2F).

The rapid light curves'parameters were also affected under metal exposure (Fig. 3), and the responses were not linear. To our knowledge, this is the first study dealing with rapid light curves of a freshwater microalga exposed to Cd and Cu mixtures. While Cd decreased the relative maximum rate of electron transport, the concentrations of Cu1 and Cu2 resulted in higher values in this parameter, with Cu3 not different from the control and Cu4 with lower values (Fig. 3A). The responses were not linear in the mixtures, i.e., in treatments M1 to M6, synergism was observed in $rETR_{max}$, and from M7 to M16, antagonism was observed. In treatments M8, M9, and M13-M15, Cu seems to mitigate the effect of Cd, while in the other mixtures, Cd appears to be responsible for the observed data. The α parameter (Fig. 3B) was affected at all concentrations tested, except for Cu3. In the mixtures, we observed synergism in M7 and M11, and additivity in M15 (Table 1), while antagonism was observed in the other mixtures. The values in M1 and M2 are more balanced by Cu. Mixtures with Cu4 were the most affected in any combination, especially with Cd2; however, not as harmful as predicted by the equations. Despite the changes

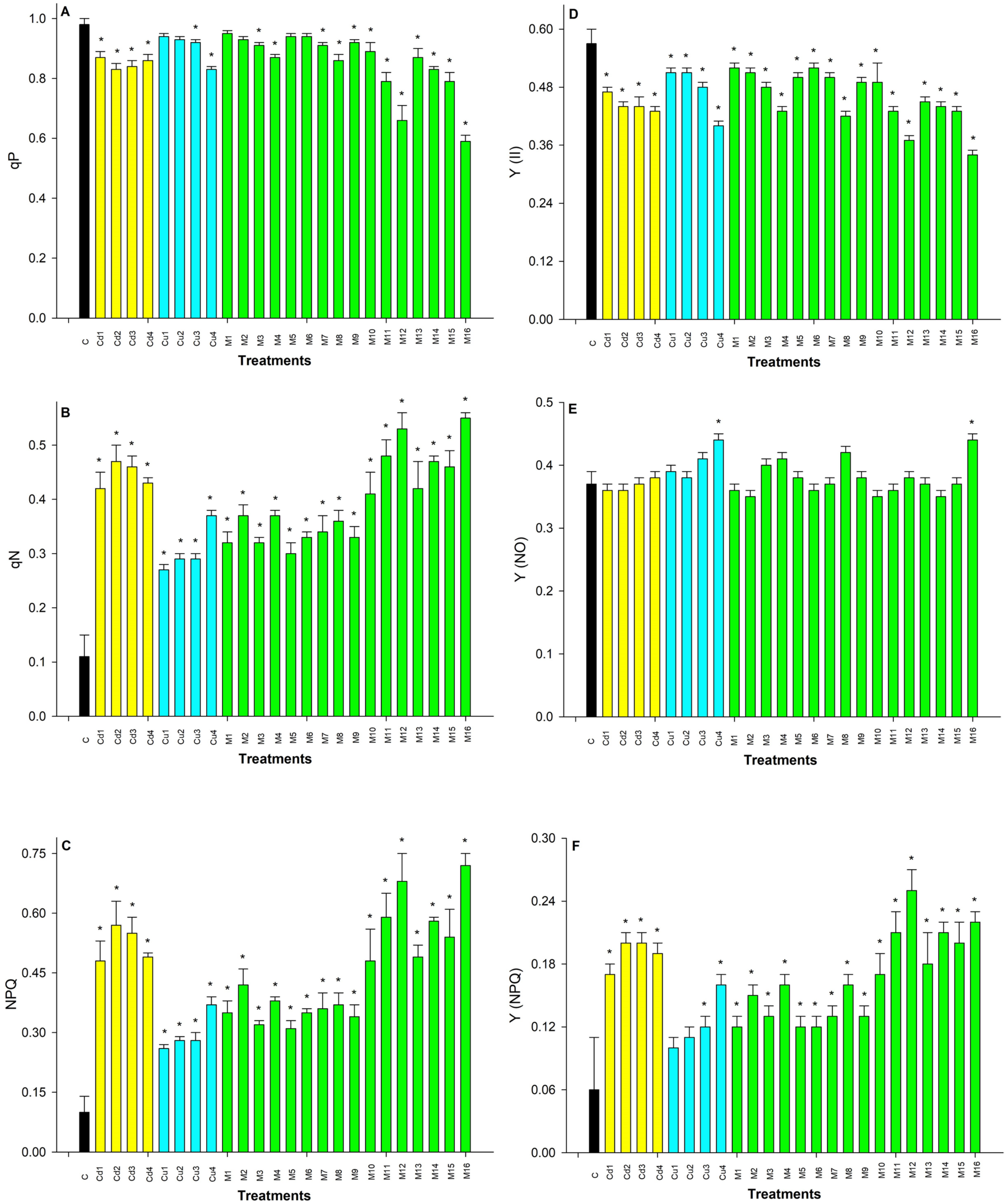


Fig. 2 Photochemical quenching (qP; **A**); non-photochemical quenching (qN; **B**); Stern–Volmer non-photochemical quenching (NPQ; **C**); effective quantum yield ((Y(II); **D**)); fractions of energy dissipated in form of heat and fluorescence ((Y(NO); **E**)); fractions of energy dissipated in form of heat by photoprotective mechanisms ((Y(NPQ); **F**)) of *Ankistrodesmus densus* exposed to cadmium (Cd, yellow bars), copper (Cu, blue bars), and Cd and Cu mixtures (M, green bars) for 72 h. C indicates control (black bars). * indicates values significantly different from control ($p < 0.05$; Dunnett post-test)

observed in $rETR_{max}$ and α, the saturating irradiance (E_k) was the least affected parameter from the rapid light curves (Fig. 3C). Values significantly different from control (Cu1, Cu2, M7, M9, M10, M13, M14, M16) were observed.

Discussion

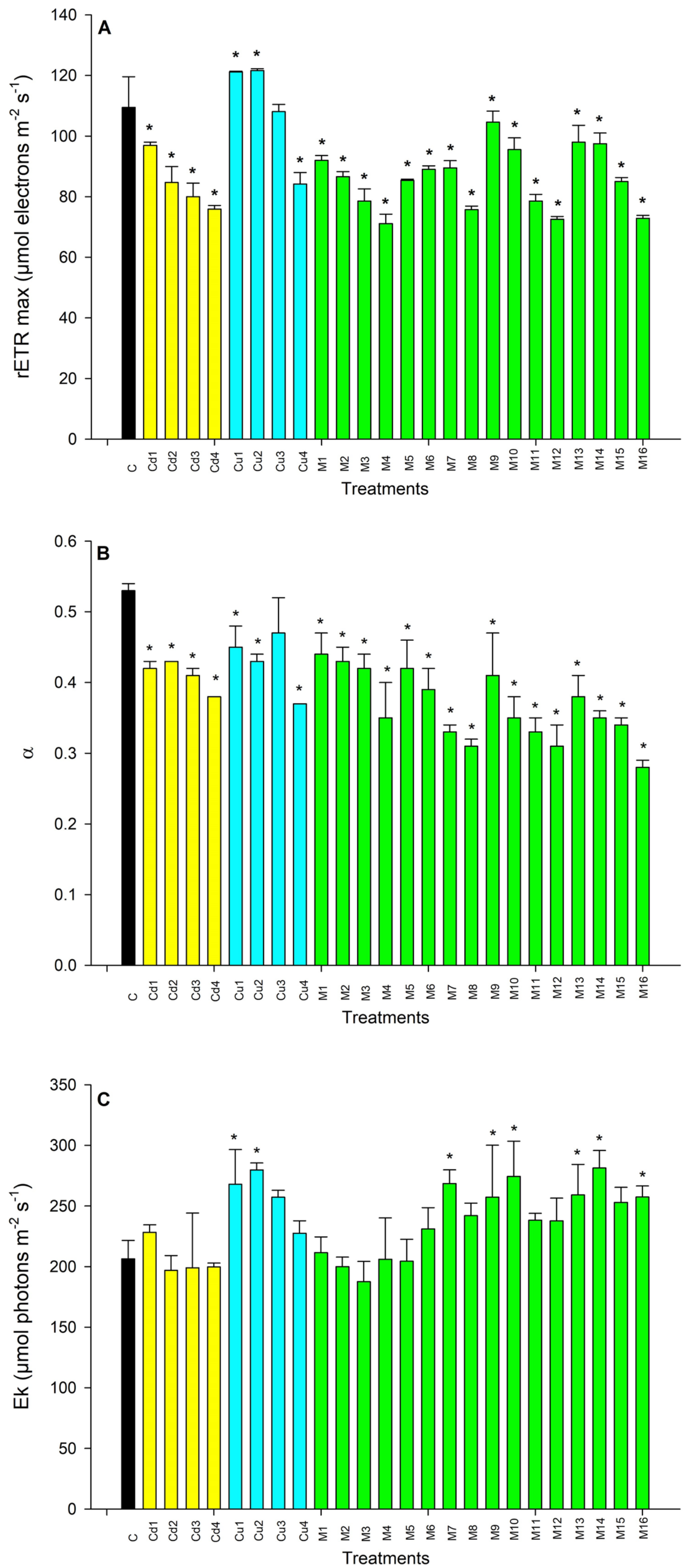
The growth rate was not a sensitive parameter in the metallic mixtures, and a stronger antagonism was observed in the highest combinations of Cd and Cu, reinforcing antagonism as the main response in organisms exposed to metallic mixtures (Norwood et al. 2003; Cedergreen 2014). This behavior could indicate competition between the two metals for the same binding sites since they have the same charge (2+). Our data differ from the study by Qian et al. (2009), where the authors observed synergism in *Chlorella vulgaris* exposed to similar concentrations of Cd and Cu combined. Regarding changes in chlorophyll concentration due to exposure to Cd or Cu, some authors report an increase in chlorophyll per cell on exposure to Cd (Echeveste et al. 2017; Rocha et al. 2020), others report stability concerning Cu (Rocha et al. 2021a) or a decrease in exposure to Cd (Chia et al. 2013 – by cell; Marchello et al. 2018; Rocha et al. 2021b – by volume). Evaluating the total metal(s) in the medium, we tend to consider that *A. densus* was more sensitive than *C. vulgaris* exposed to the same concentration range of both metals (Qian et al. 2009). In addition, in *C. vulgaris* there was synergism in the mixture of Cd and Cu in the production of chlorophyll (Qian et al. 2009), as for *A. densus,* antagonism was observed. However, even with similar concentrations of metals in both studies (present and Qian et al. 2009), the culture conditions (medium, photoperiod, and temperature) were different, most likely resulting in different speciation and bioavailability of metals, making it difficult to affirm which species was more sensitive categorically. The decrease in chlorophyll *a* can be related to the substitution of Mg(II) by the metals evaluated in the present study as suggested by Küpper et al. (2002), and the non-linearity of damages in the mixtures is most likely related to the competition between the two metals for the limited amount of binding sites in chlorophyll molecule. Our data highlight the challenges related to generalizations about algal response in presence of isolated or multiple stressors; however, the changes in growth and chlorophyll production impact the primary productivity, most likely affecting the higher trophic levels.

Decreased F_v/F_m values in the presence of the metals can indicate photosynthesis downregulation, as observed for *Scenedesmus obliquus* exposed to Cu (Dewez et al. 2005). Since the F_v/F_m values may be an indicator of photoinhibition (Malapascua et al. 2014) it can be inferred that the alga did not face photoinhibition in the tested conditions. In the present study, F_v/F_m was a sensitive parameter to evaluate the effects of Cd and Cu, isolated or in the mixture, to *A. densus*, diverging from the studies by Echeveste et al. (2017) for *Chlorolobion braunii*, and by Qian et al. (2009) for *Chlorella vulgaris.* It is interesting to highlight that our data suggest that increase in Cu in the mixtures drive the increase in antagonism, while increases in antagonism were more subtle with the increase of Cd concentration.

As higher values of qP indicate an optimized use of photochemical energy in carbon metabolism (Lombardi and Maldonado 2011); a decrease in qP indicates an incorrect functioning of PS II (Baracho et al. 2019) and the transport of electrons in the PS II (Dewez et al. 2005). In our study, the qP was a sensitive indicator of the toxicity of the metals, which can indicate a species-specific response in *A. densus* since in *C. braunii* qP was not affected by the presence of Cu (Echeveste et al. 2017); while Wang and Dei (2006) reported a lower sensitivity of qP in the presence of Cd or Cu compared to the Φ_{PSII} and Y(II) of *C. reinhardtii.* Regarding qN and NPQ, the values were higher than control, which indicates that there were no damages in photoprotection mechanisms, with no loss of reaction centers (Kalaji et al. 2014). For both parameters, the values in the mixtures were lower than in Cd exposure, except in the M16 for qN and NPQ; and M12 for NPQ. These data indicate a greater influence of Cu in this response since most values were closer to the isolated Cu, suggesting a true antagonism (Norwood et al. 2003). The highest values of qN and NPQ in mixtures were 5- and sevenfold the value of the control, respectively. Algae exposed to the isolated metals had qN and NPQ values 4- (Cd) or threefold (Cu) higher than the control. As observed for *A. densus* in the combination of phosphorus limitation and Cd exposure (Rocha et al. 2021b), both qN and NPQ showed a similar response, indicating that these parameters may present species-specific responses since qN was not a good indicator for Cu exposure in *C. reinhardtii* (Juneau et al. 2002), with the NPQ being suggested as a parameter more sensitive to energy dissipation (Ralph and Gademann 2005).

Our data indicate that *A. densus* did not have the photoprotective system damaged at the Cu concentrations evaluated, differing from the studies by Lombardi and Maldonado (2011) for *Phaeocystis cordata* and by Rocha et al. (2021a) for *Selenastrum gracile*, where Cu damaged the algal photoprotective mechanisms. In the present study, the Y (II) was more sensitive than the PS II activity (F_v/F_M). Such data differ from those observed by Lombardi and Maldonado (2011) in *P. cordata* exposed to Cu, where the authors observed a lower sensitivity of Y(II) to metal, and by Echeveste et al. (2017), where Cu did not affect the Y(II) of *C. braunii*. However, they are in agreement with previous studies with the species *S. gracile*, where Cd and Cu affected the Y(II) more than F_v/F_m (Rocha et al. 2020, 2021a). The values of Y(NPQ)

Fig. 3 Relative maximum electron transport rate ($rETR_{max}$; **A**); initial slope (α; **B**) and saturation irradiance (E_k; **C**) of *Ankistrodesmus densus* exposed to cadmium (Cd, yellow bars), copper (Cu, blue bars), and Cd and Cu mixtures (M, green bars) for 72 h. C indicates control (black bars). * indicates values significantly different from control ($p < 0.05$; Dunnett post-test)



in the treatments M1 to M10 were lower than observed in Cd, suggesting a higher influence of Cu in the activation of photoprotective mechanisms, mitigating the impacts of Cd, while from M11 to M16 Cd is suggested as the driving force to activate these defense mechanisms. However, the metals, alone or in the mixture, could not change the alga's efficiency in protecting itself from damage caused by excessive lighting, as marked by the stability of Y(NO) (Klughammer and Schreiber 2008). The data obtained in the present study and by Rocha et al. (2021b) indicate that the alga *A. densus* is efficient in activating photoprotection mechanisms under stress, as evidenced by the significant increase in Y(NPQ).

In studies by Baracho et al. (2019) with the alga *C. braunii* and by Rocha et al. (2021a) with the species *S. gracile*, the increase of Cu in the culture media resulted in a decrease in the electron transport rate, while Rocha et al. (2021b) also obtained a reduction in $rETR_{max}$ in *A. densus* exposed to Cd. While a decrease in ETR could indicate a lower primary productivity (Nicklish and Köhler 2001), the decrease in $rETR_{max}$ followed by an increase in NPQ indicates no damages in the functioning of Q_A acceptor in the presence of contaminants (Serôdio et al. 2006; Candido and Lombardi 2018). Yong et al. (2018) did not observe changes in the α parameter of *Scenedesmus quadricauda* exposed to Cu, while Baracho et al. (2019) observed a drop in α with an increase in Cu in the culture medium of *C. braunii*. Since the α indicates the efficiency in light capture, i.e., higher the α higher the efficiency, the data from the present study indicate that metals, isolated or in the mixture, can decrease the efficiency of light capture in microalgae. Regarding E_k, some treatments had higher values than control, which is unexpected as stress often decreases the E_k (Yong et al. 2018; Baracho et al. 2019; Rocha et al. 2021b). The changes obtained in E_k were not linear or related to a specific amount of Cd or Cu in the medium. Interestingly, the E_k data indicate that the drop in qP is not related to light stress, as the maximum saturation was higher than those observed in control. Based on these results and those obtained in the Y(NPQ), we can conclude that exposure to Cd and Cu, isolated or in the mixture, did not affect the photoprotection mechanisms of *A. densus.*

In the case of metal stress, qN (non-photochemical quenching) is generally the most sensitive indicator as it integrates the toxic effects of the contaminant in the electron transport process, while qP (photochemical quenching) is only indicative of open reaction centers in PS II (Herlory et al. 2013). However, when analyzing different species of algae exposed to other metals, we can observe that such statements cannot be so categorical. For example, in the present study, the most sensitive parameters to the tested metals were the NPQ and the Y(NPQ). In the case of *S. gracile*, the parameter that indicates the loss of light energy in passive dissipation ((Y(NO)) was the most sensitive in exposure to Cd (Rocha et al. 2020). In contrast, exposing the same species to Cu, the PS II activity (F_v/F_m) and effective quantum yield ((Y(II)) parameters were more affected than in the case of Cd, in addition to a non-linearity of photochemical and non-photochemical quenchings, indicating more significant damage to the photoprotective mechanism of the alga when exposed to Cu than to Cd (Rocha et al. 2021a). While for *A. densus*, the parameters qN, NPQ, Y(NPQ), and electron transport rate were more affected in exposure to Cd than to Cu. Such data reinforce the need for further studies involving different stressors and photosynthetic responses.

In summary, our data suggest that decrease in chlorophyll *a* can be a result of substitution of Mg (II) by Cu(II) and/or Cd (II) in the chlorophyll molecule, resulting in the formation of non-functional chlorophylls. Although a decrease (≈20%) in the F_v/F_m parameter (dark-adapted algae), with antagonism observed in mixtures, the photosynthetic parameters obtained after the light-adaptation ((qP, qN, NPQ, Y(II), Y(NO), Y(NPQ), $rETR_{max}$, and α)) were affected more, except for the E_k. This means that algae exposed to the metals could not harness the light energy as effectively as the algae without the stressors (control). The data suggest that the combination of Cd and Cu affected the efficiency of light capture (α parameter) and, consequently, the use of light energy, resulting in a drop in photochemical quenching and an increase in non-photochemical quenching, which indicates closure of reaction centers and increased heat dissipation caused by the stress of the mixture, in addition to a lower carbon metabolism (indicated by qP) and electron transport rate. Despite the significant changes observed in the parameters related to the photosynthetic efficiency—mainly the Y(II), qP, $rETR_{max}$, and α—the photoprotective mechanisms were able to prevent further damage to the photosynthetic apparatus of the microalga, as suggested by the value ≈3.7-fold higher in the Y(NPQ) and ≈ 7.2-fold higher in the NPQ in presence of the stressors, which is reinforced by stable Y(NO) values (indicative of loss of energy passively). While Y(NPQ) indicates the activation of photoprotective mechanisms, the NPQ indicates the light energy dissipated as heat, i.e., the light energy not used in photosynthesis.

The results obtained with the microalgae *A. densus* indicate that this is a sensitive species to evaluate the impacts of Cu and Cd in the photosynthetic machinery, with antagonistic responses suggesting a higher efficiency of protection in the presence of two stressors, which can be also related with intrinsic characteristics of competition between Cu and Cd for the limited available binding sites. However, other mechanisms can be also involved in the observed responses, e.g., the presence of Cu or Cd was responsible for changes in lipid classes and fatty acids profile of *S. gracile*, which is directly related to membrane fluidity and consequent metal internalization and cell damages (Rocha et al. 2020, 2021a). For a better understanding of algal response to multiple stressors, we suggest more studies evaluating other endpoints and biomarkers to detect more subtle changes in the physiology or metabolism of algae, which can provide a better understanding of the contaminants' toxicity mechanisms. These complementary analyses include, for example, enzymatic activity (Melegari et al. 2013), biochemical composition (Alho et al. 2020), reactive oxygen species and cellular complexity (Alho et al. 2019; Gebara et al. 2020). In addition, since the complex interactions between Cu and Cd are not still fully understood; the presence of other metals and the surrounding media, i.e., macronutrients such as phosphorus and nitrogen, can interfere in the metal internalization and toxicity (Webster et al. 2011; Rocha and Espíndola 2021), the analyses of metal uptake by algae is highly recommended, especially due to the competition of divalent ions Cd(II) and Cu(II) with Mg(II) in chlorophyll molecule (Küpper et al. 2002) and with Ca(II) in PS (II) (Wang and Dei 2006), which can directly impair photosynthesis.

Conclusions

Considering the amount of metal(s) that algae were exposed in the medium, isolated, Cd mainly increased the photosynthetic parameters qN, NPQ, Y(NPQ), and decreased $rETR_{max}$, while Cu decreased the growth rate, the photosynthetic parameters F_v/F_m, effective quantum yield, and qP, according to the amount of metals. Our data indicate that both metals alter the photosynthetic parameters similarly. In the mixtures, most of the damages to the photosynthetic apparatus were lower than the independent action model predicted, indicating antagonism. However; synergism was observed in qP in the mixtures with the highest concentrations of metals (Cd3, Cd 4, Cu3, and Cu4); and in $rETR_{max}$ occurred in the mixture treatments with the lowest Cd concentration (Cd1 and Cd2). It is also possible to conclude that photoprotective mechanisms were efficient under the tested concentrations of metals, isolated or combined, which is indicated by the increase of qN, NPQ, and Y(NPQ) parameters. The parameters Y(NO), Y(NPQ), and from the rapid light curves are sensitive endpoints that should be incorporated in the studies with the evaluation of photosynthesis under metallic mixture stress, especially due to the lack of studies evaluating these parameters in algae exposed to mixture of stressors.

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Data availability The data will be provided under request.

Declarations

Competing interests The authors declare no competing interests.

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