Journal of Applied Phycology (2025) 37:407–417
https://doi.org/10.1007/s10811-024-03398-5

RESEARCH

Impacts of phosphorus limitation in the toxicity of environmental concentrations of lead and zinc in *Raphidocelis subcapitata* (Chlorophyceae)

Giseli Swerts Rocha[1] · **Maria G. G. Melão**[2]

Received: 12 September 2024 / Revised: 17 November 2024 / Accepted: 18 November 2024 / Published online: 4 December 2024
© The Author(s) 2024

Abstract
Microalgal metabolism is affected by the surrounding environment and nutrients such as phosphorus (P) and nitrogen (N) are essential for optimal metabolism, as well as trace amounts of essential metals such as zinc (Zn); although in higher doses than required, Zn can be toxic. Lead (Pb) is a non-essential metal that can harm organisms from different trophic levels. In the environment, algae are exposed to several stressors simultaneously and adapt their metabolism. In the present study, we evaluated P limitation combined with environmental concentrations of Zn or Pb to the freshwater microalga *Raphidocelis subcapitata* regarding growth, pigments production, and photosynthetic parameters. Our results indicate that P limitation affected the growth, pigments production, relative maximum electron transport rate ($rETR_{max}$), and saturation irradiance; while Pb altered growth, pigments production, and maximum quantum yield; and Zn affected pigment production, photochemical and non-photochemical quenching, and $rETR_{max}$. However, the combination of metal and P limitation resulted in synergistic responses, i.e., higher damages than the isolated stressors, in growth, maximum and effective quantum yield, and in the rapid light curve parameters. On the other hand, antagonism, i.e., lower damages than isolated stressors, was observed in pigments production and non-photochemical quenching, suggesting that algae activated defense mechanisms to cope with both stressors simultaneously. In addition, our results indicate an algal metabolism adjustment to P limitation and highlight the importance of considering physicochemical water characteristics when defining regulations of acceptable levels of metals in aquatic ecosystems.

Keywords antagonism · photosynthesis · rapid light curves · synergism

Introduction

Algae require macro- (e.g., phosphorus – P, and nitrogen – N) and micronutrients (e.g., trace metals copper – Cu, zinc – Zn, cobalt – Co) for healthy metabolism. Phosphorus limitation affects cell division (Bhola et al. 2011), chlorophyll *a* production (Berdalet et al. 1994), photosynthesis and resistance to metals in microalgae (Rocha et al. 2021a). Physiological adjustments occur with a change in P availability (Touzet et al. 2007; Rocha and Melão 2024) to minimize the effects of nutrient limitation, although the mechanisms involved in this adaptation are still unknown (Van Mooy et al. 2009). Usually, P is taken up in concentrations higher than required by algae and is stored in form of polyphosphate bodies, which are important for metal detoxification and energy reserve (Fogg 1973; Solovchenko et al 2019), providing P during a few cell divisions under P starvation (Bhola et al. 2011; Solovchenko et al. 2019).

Metals are ubiquitous in the environment and their increases occur due to industrial activities, mining and agriculture (Nriagu 1988; Sharma and Dubey 2005; Harpprecht et al. 2024). Zinc is an essential metal for life, being co-factor of hundred enzymes and proteins with structural functions and modulates the antioxidant system (Maret

✉ Giseli Swerts Rocha
swertsbio@gmail.com

Maria G. G. Melão
dmgm@ufscar.br

[1] Escola Tècnica Superior d'Enginyeria Química, Departament d'Enginyeria Química, Universitat Rovira I Virgili, Av. Països Catalans, 26, 43007 Tarragona, Spain

[2] Departamento de Hidrobiologia, Centro de Ciências Biológicas E da Saúde (CCBS), Universidade Federal de São Carlos (UFSCar), Rodovia Washington Luís, Km 235, São Carlos, SP CEP 13565-905, Brazil

2013; Zheng et al. 2016); however its full role is still not understood (Maret 2013). As well as other essential metals such as Co and Cu, in higher concentrations than required, Zn are harmful to several organisms, e.g., algae, zooplankton, fish (Machado et al. 2015; Zheng et al. 2016; Gebara et al. 2021). On the other hand, Pb does not have any biological function, and toxicity is observed in different trophic levels, such as the impairment in physiological parameters in microalgae (Alho et al. 2019; Pascual et al. 2020; Guan et al. 2023) and alterations in metallothionein gene and in acetylcholinesterase activity in fish (Kim and Kang 2017). Levels of Zn in natural environments worldwide can reach up to 1000 µg L^{-1} (French river, Andres et al. 2000); while Pb can be found from 0.02 µg L^{-1} (Xiao et al. 2014) up to 150 µg L^{-1} in China (Gui et al. 2017), with environmental relevant concentrations ranging from 3–94 µg L^{-1} (Kataba et al. 2020). In Brazil, Zn can vary from 2 to 1125 µg L^{-1} (Rodgher et al. 2005), with a mean environmental concentration of 46.8 µg L^{-1} (Gebara et al. 2020); while Pb concentrations range from < 0.05 µg L^{-1} (Alves et al. 2014) to 260 µg L^{-1} (Dourado et al. 2017).

In the environment algae are most likely exposed to several stressors simultaneously. In our recent studies we obtained different interactions of P-acclimated *Raphidocelis subcapitata*, when exposed to aluminum (Al, non-essential metal; Rocha et al. 2024), where synergism (i.e., higher damage in the combination of the stressors) was observed; while when exposed to Co (essential metal, Rocha and Melão 2024) antagonism (i.e., lower damage than isolated stressors) occurred in the photosynthetic parameters. In the present study, we evaluated the combination of P limitation with environmental concentrations of an essential (Zn) and a non-essential metal (Pb), and assessed their effects in the growth, pigments production, and photosynthetic parameters (maximum and effective quantum yield, photochemical and non-photochemical quenchings, and rapid light curves) of P-acclimated freshwater microalga *R. subcapitata* to obtain information if alterations in these parameters can be related to the essentiality of metals.

Material and methods

The freshwater chlorophyte *Raphidocelis subcapitata* was obtained from the algae culture collection of the Botany Department at Federal University of São Carlos (São Carlos, SP, Brazil). Stock cultures were kept in LC Oligo medium (AFNOR 1980) at initial pH 7.0, under controlled conditions of light intensity (130 µmol photons m^{-2} s^{-1}), photoperiod (12:12 h light:dark cycle) and temperature (24 ± 2 °C). Cultures were manually shaken three times per day. Phosphorus was furnished as K_2HPO_4 in 2 different concentrations: 2.3×10^{-4} – control (C), which is the concentration in LC Oligo medium – and 2.3×10^{-5} mol L^{-1} (10% P). The algae were acclimated using semi-continuous cultures, with partial renewal of the medium performed every 72 h, keeping algae in mid exponential growth phase. Cells were kept under each P concentration for several weeks, allowing acclimation to this new scenario. These acclimated cells were then used for the acute metal toxicity experiments. Sterile conditions were maintained throughout and only sterile materials were used to avoid culture contamination. Laboratory materials were washed with neutral detergent and kept in 10% HCl for metal cleaning before tests.

Exponentially growing and P-acclimated *R. subcapitata* cells were exposed for 72 h to three Pb concentrations: 0.02; 0.03 and 0.04 mg L^{-1}; or three Zn concentrations: 0.03, 0.04 and 0.05 mg L^{-1}. These concentrations were chosen after preliminary range-finding tests, with concentrations up to 1 mg L^{-1} of each metal. In addition, these concentrations are of environmental relevance worldwide. Lead and Zn solutions were made by serial dilutions of $PbCl_2$ and $ZnCl_2$ Titrisol 1000 mg L^{-1} (Merck), respectively, in ultrapure water (Barnstead Easy Pure II, Thermo Scientific, USA). The metals were added to the medium 24 h before the beginning of the tests to allow the equilibrium between the metal and the medium. Fifteen mL samples from the medium with Pb or Zn 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 220.30 nm (Pb) and 213.86 nm (Zn) emission lines. The limits of quantification (LOQ) were 0.01 mg L^{-1} for both metals, while the limits of detection (LOD) were 0.002 and 0.0004 mg L^{-1} for Pb and Zn, respectively.

Toxicity tests were performed with three experimental replicates in 500 mL polycarbonate Erlenmeyer flasks containing 200 mL of sterile culture medium. Cells were inoculated providing initial cell density of approximately 10^5 cells mL^{-1}. Cell densities were monitored every 24 h. Samples were fixed with Lugol's solution and cells were counted in an Improved Neubauer-Bright Line hemocytometer. Ten mL samples were filtered through cellulose ester membranes (0.45 µm pore size—Millipore) and extracted with dimethylsulfoxide. Absorption was measured in a spectrophotometer (HACH DR 5000, USA) at 665, 649 and 480 nm wavelengths. Chlorophyll *a* (Chl $a = 12.47\ A_{665} - 3.62\ A_{649}$), chlorophyll *b* (Chl $b = 25.06\ A_{649} - 6.5\ A_{665}$), and total carotenoids ((Car = (1000 A_{480} – 1.29 Chl *a* – 53.78 Chl *b*) / 220)) were calculated as described by Wellburn (1994). Blanks were performed using a clean filter submitted to the same extraction procedure.

Chlorophyll fluorescence was measured using a pulse amplitude modulated fluorometer Phyto – PAM I (Walz, Germany) equipped with an ED-101US/MP optical unit. Samples were dark-adapted for 15 min to allow complete

oxidation of PS II reaction centers and the maximum quantum yield of photosystem II (PSII; $\Phi = F_v/F_M$) was obtained. Actinic light was turned on with photosynthetically active radiation intensity (PAR) of 128 µmol photons m^{-2} s^{-1}, and measurements of light-adapted samples were performed. From these measurements, we calculated the photochemical (qP) and non-photochemical quenching (qN and NPQ), in addition to the effective quantum yield ((Y(II)), the quantum yield of regulated ((Y(NPQ)) and non-regulated non-photochemical ((Y(NO)) energy loss in PSII as described in Gebara et al. (2023). Rapid light saturation curves were obtained as described by Rocha et al. (2021a, b). Briefly, the algae were exposed to a gradual increase of PAR (0–1780 µmol photons m^{-2} s^{-1}), in 20 s pulse intervals, providing effective quantum yield ((Y(II)) in different PAR; thus the electron transport rate (ETR) was calculated multiplying Y(II) x PAR. The ETR and PAR values were fitted according to Jasby and Platt (1976) equation, providing the initial slope (α) and the maximum relative electron transport rate ($rETR_{max}$; µmol electrons m^{-2} s^{-1}); and the saturating irradiance (E_k) was calculated as $Ek = rETR_{max}/\alpha$; µmol photons m^{-2} s^{-1}.

The data were tested for normality and homogeneity of variance. Statistically significant differences among treatments and controls were determined using one-way ANOVA and Fisher's post hoc at $p < 0.05$ (SigmaPlot 11, Systat). The data are presented as mean ± standard deviation (SD) of the replicates. Two-way ANOVA was applied to detect the effects of Pb and P, and Zn and P in the evaluated parameters.

To calculate the effects of the combination of the two stressors, we assumed the independent action model of the stressor relative to control. These calculations were done when the evaluated endpoint was significantly lower than the control. The predicted effects were calculated using Eq. (1), while the observed effects in the combination were calculated using Eq. (2), and the synergy ratio of the combination (Eq. (3)) was calculated using the predicted and observed effects (Gottardi et al. 2017). The synergy ratios below 1 indicate antagonism, equal to 1 indicate additivity, and higher than 1 indicate synergism.

$$Predicted = \frac{Metal}{C} x \frac{P}{C} \quad (1)$$

where *metal* refers to the mean value of the endpoint obtained under Pb or Zn exposure; *P*, under phosphorus limitation 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 metal (Pb or Zn) and P-limitation exposure.

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

where values lower and higher than 1 indicate antagonism and synergism, respectively.

Results

The data obtained for cell density, chlorophyll *a*, carotenoids and maximum quantum yield of *R. subcapitata* exposed to Pb (Fig. 1 A, B, C, D) or Zn (Fig. 1 E, F, G, H), isolated or in combination with P limitation are shown in Fig. 1. It is possible to observe that Pb slightly affected the cell density and maximum quantum yield, while Zn did not affect these parameters on control cells. In addition, Pb affected more the production of chlorophyll *a* and total carotenoids per cell than Zn. When any of the metals were combined with P limitation, the responses were synergistic in the cell density and in the maximum quantum yield. However, chlorophyll *a* and total carotenoids (pg $cell^{-1}$) had a tendency for antagonism in the combination of both stressors (Supplementary Tables 1 and 2). P limitation combined with Pb was more synergic (in cell density) or antagonistic (in chlorophyll *a,* and in total carotenoids, pg $cell^{-1}$) than when combined with Zn. Regarding chlorophyll *b* production, we can observe that there was no alteration in the pigments under Pb or Zn exposure, or P limitation compared to control P treatment (0.11–0.14 pg chl *b* $cell^{-1}$). However, the combination of P limitation and highest Pb concentration resulted in a pigment production about sevenfold higher (≈ 0.96 pg chl *b* $cell^{-1}$), while the combination of P limitation and the highest concentration of Zn resulted in the chl *b* twofold higher (≈ 0.26 pg chl *b* $cell^{-1}$), indicating a higher impact of combined stressors on chl *b* than on chl *a* production.

The changes in photochemical (qP) and non-photochemical quenching (qN and NPQ) are presented in Fig. 2. Phosphorus limitation or excess of Pb did not affect any of these parameters; while the highest Zn concentration decreased qN (Fig. 2E) and NPQ (Fig. 2F) values, suggesting damage to the photoprotection mechanisms of algae. The combination of P limitation and Zn excess was less harmful in qN and NPQ parameters than only Zn excess. On the other hand, under P limitation and Pb excess, qN and NPQ significantly increased, especially at the highest metal concentrations, indicating an efficient photoprotection mechanism, with higher heat dissipation. An interesting fact is that while Pb, Zn or P limitation alone did not affect qP, in combination P limitation slightly increased the qP at the lowest and intermediate concentration of Pb and Zn, while the highest concentration of Pb resulted in lower qP and the highest Zn concentration did not affect qP, when compared to control (i.e., with no P limitation and no metal added).

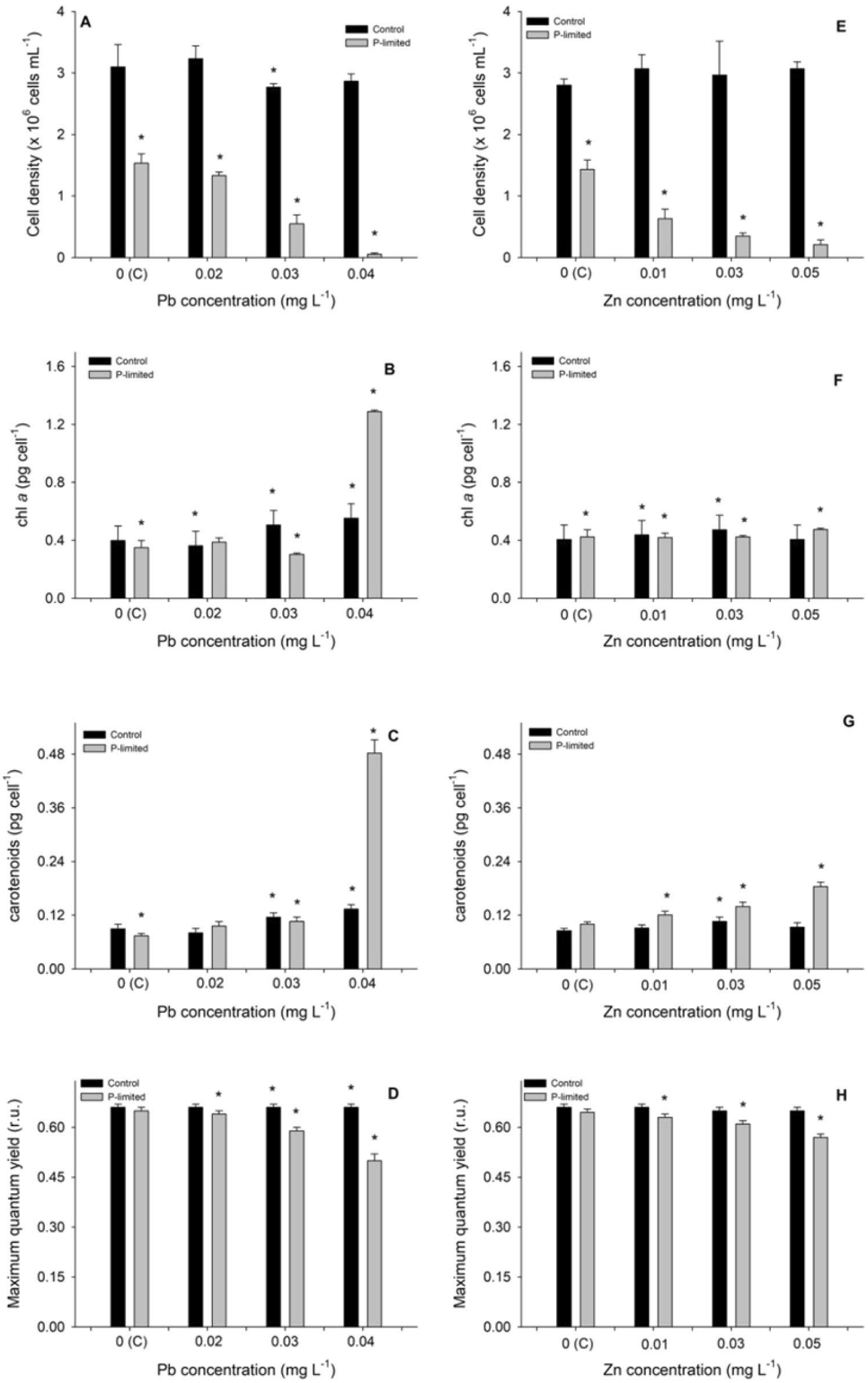


Fig. 1 Cell density, chlorophyll *a,* total carotenoids, and maximum quantum yield of *R. subcapitata* exposed to Pb (**A**, **B**, **C**, **D**) or Zn (**E**, **F**, **G**, **H**), isolated or combined with P limitation. C refers to control (i.e., metal added in the LC Oligo medium). * indicates values significantly different from control ($p < 0.05$)

The effective quantum yield – Y(II), and the quantum of non-regulated non-photochemical energy loss, i.e., Y(NO), were not significantly affected by the isolated stressors, i.e., P limitation or any of the metals. The quantum of regulated non-photochemical energy loss, i.e., Y(NPQ), decreased in the presence of excess of Zn, indicating the start of damages in the photosynthetic apparatus, i.e., the algae under Zn excess was not able to dissipate the excess of energy in form of heat (Fig. 3), reinforcing the qN and NPQ data. The combination of the two stressors significantly impacted the

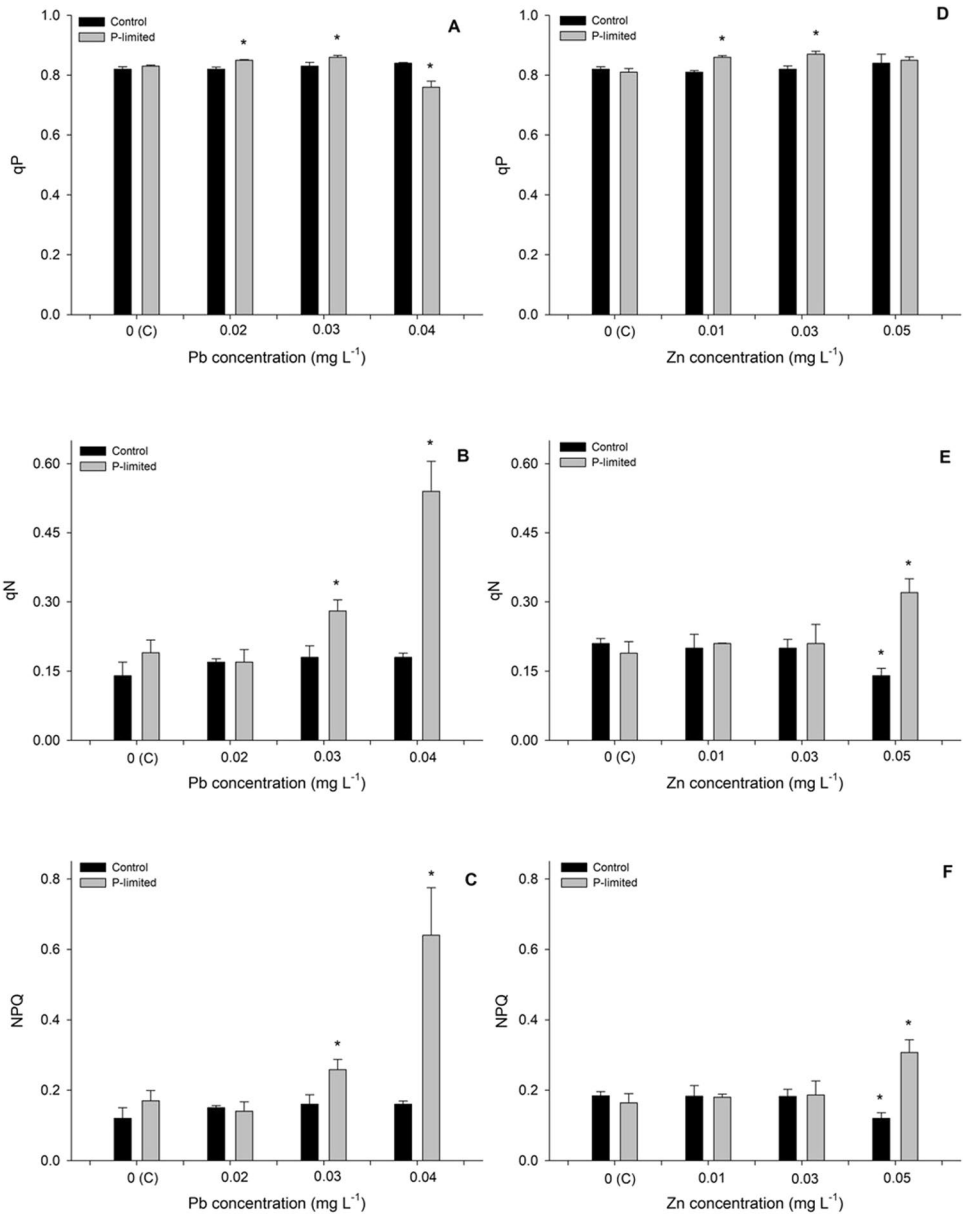


Fig. 2 Photochemical (qP) and non-photochemical (qN and NPQ) quenching of *R. subcapitata* exposed to Pb (**A**, **B**, **C**) or Zn (**D**, **E**, **F**), isolated or combined with P limitation. C refers to control (i.e., metal added in the LC Oligo medium). * indicates values significantly different from control ($p < 0.05$)

Y(II), especially under Pb exposure, resulting in a lower photosynthetic ability of algae in the presence of both stressors. On the other hand, the P limitation combined to both metals, especially Pb, indicate an efficient mechanism of defense in form of heat dissipation by active photoprotective mechanisms (Y(NPQ), in an attempt to decrease the damages to the photosynthetic apparatus.

The parameters from rapid light curves are presented in Fig. 4. The presence of Pb did not affect any parameter in control cells; however, combined with P limitation the effects were synergistic in $rETR_{max}$ and α (indicative of light use); and antagonistic in E_k (light saturation irradiance). This indicates that Pb affected the electron transport and the capacity of algae using light in photosynthesis, and it did not significantly affect the resistance to light with the increase of metal concentrations. Zn slightly affected the $rETR_{max}$ at the highest concentration, while synergism was observed in the combination with P limitation in all parameters evaluated, indicating the damage of this combination in the electron transport, use of light energy and light saturation. It is interesting to highlight that Zn combined with P limitation caused a stronger decrease in E_k compared to Pb and P limitation; while under Pb and P limitation, α was much more affected than in the combination of Zn and P limitation, indicating that Zn and P limitation combined result in a higher sensitivity to light, while in combination of Pb and P limitation, the efficiency in using light is more affected. Surprisingly, the only antagonistic response was obtained in E_k in the exposure of P-limited algae to Pb.

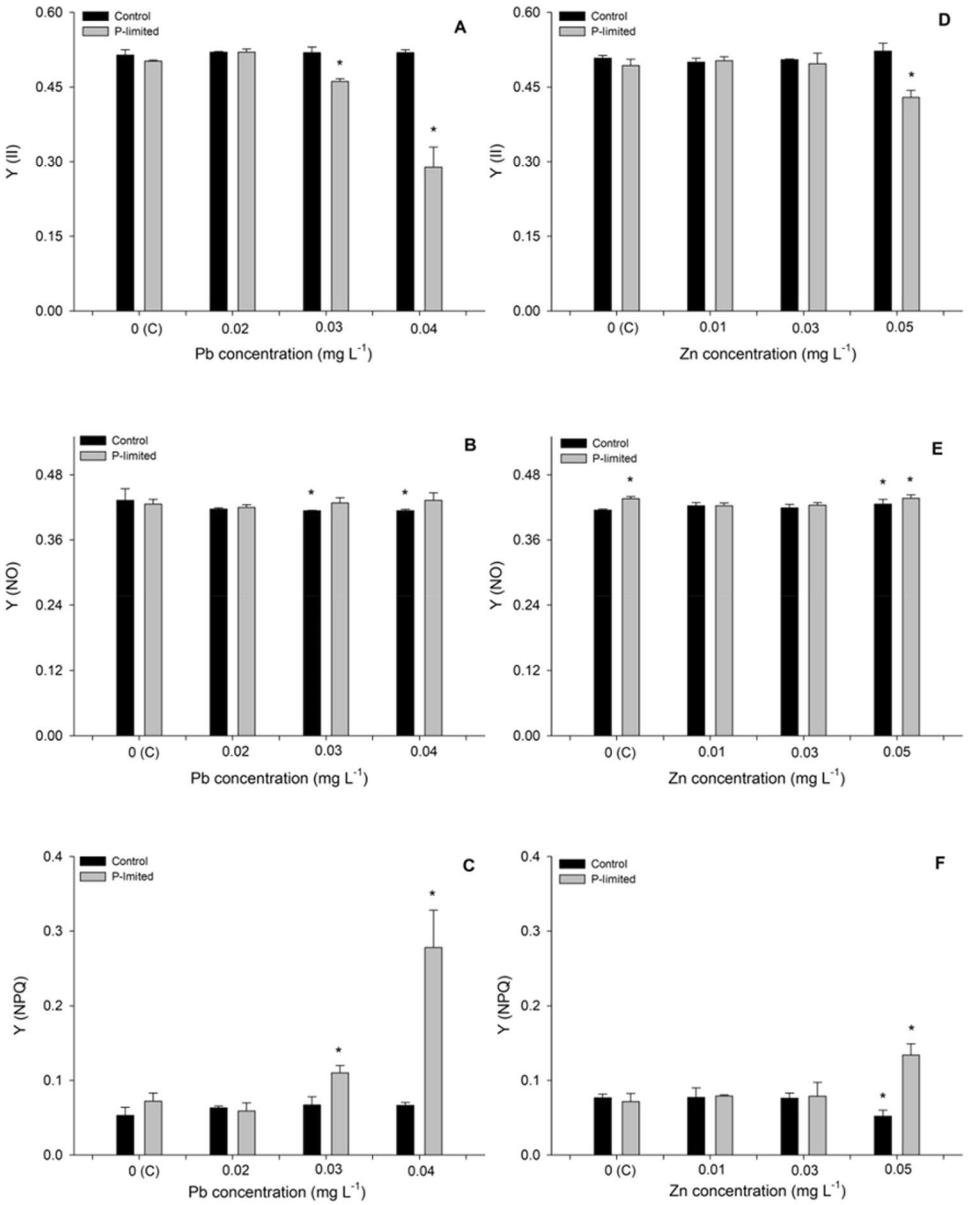


Fig. 3 Effective quantum yield – Y(II); quantum yield of non-regulated – Y(NO) and regulated – Y(NPQ) – non-photochemical energy loss in PSII of *R. subcapitata* exposed to Pb (**A**, **B**, **C**) or Zn (**D**, **E**, **F**), isolated or combined with P limitation. C refers to control (i.e., metal added in the LC Oligo medium). * indicates values significantly different from control ($p < 0.05$)

Discussion

The P limitation significantly affected the growth rate, mainly due to the role of P in cell division, corroborating previous studies (Ji and Sherrel 2008; Bhola et al. 2011). On the other hand, a decrease in pigments under P limitation would be expected (Lombardi and Wangersky 1991; Berdalet et al. 1994; Lai et al. 2011); however, P limitation did not significantly impact chlorophyll *a*, carotenoids or maximum quantum yield. We suggest that the highest impact of Pb in P-limited cells is a result of the non-essentiality of this metal, which can result in more damage to the algae. For both metals, the sensitivity of *R. subcapitata* according to synergism ratio was cell density > maximum quantum yield > chlorophyll *a* > carotenoids.

The impact of Pb on *R. subcapitata* already has been demonstrated by others (Alho et al. 2019), as well as the combination with P limitation in *R. subcapitata* (Pascual et al. 2020) and in *Chlamydomonas reinhardtii* (Guan et al. 2023). However, these authors used quite high and most likely unrealistic concentrations, reaching 5 mg Pb L^{-1}, which is 125 fold the highest concentration evaluated in the present study. For Zn, our results were less remarkable than those of Machado et al. (2015) and Gebara et al. (2020) where they observed a decrease in chlorophyll *a* and in growth in concentrations similar to ours; however, their exposure media was different (OECD medium and $CHU_{10/2}$, respectively) from ours, which could explain the differences observed since the toxicity of Zn and Pb can change according to medium composition (Pascual et al. 2020). Regarding

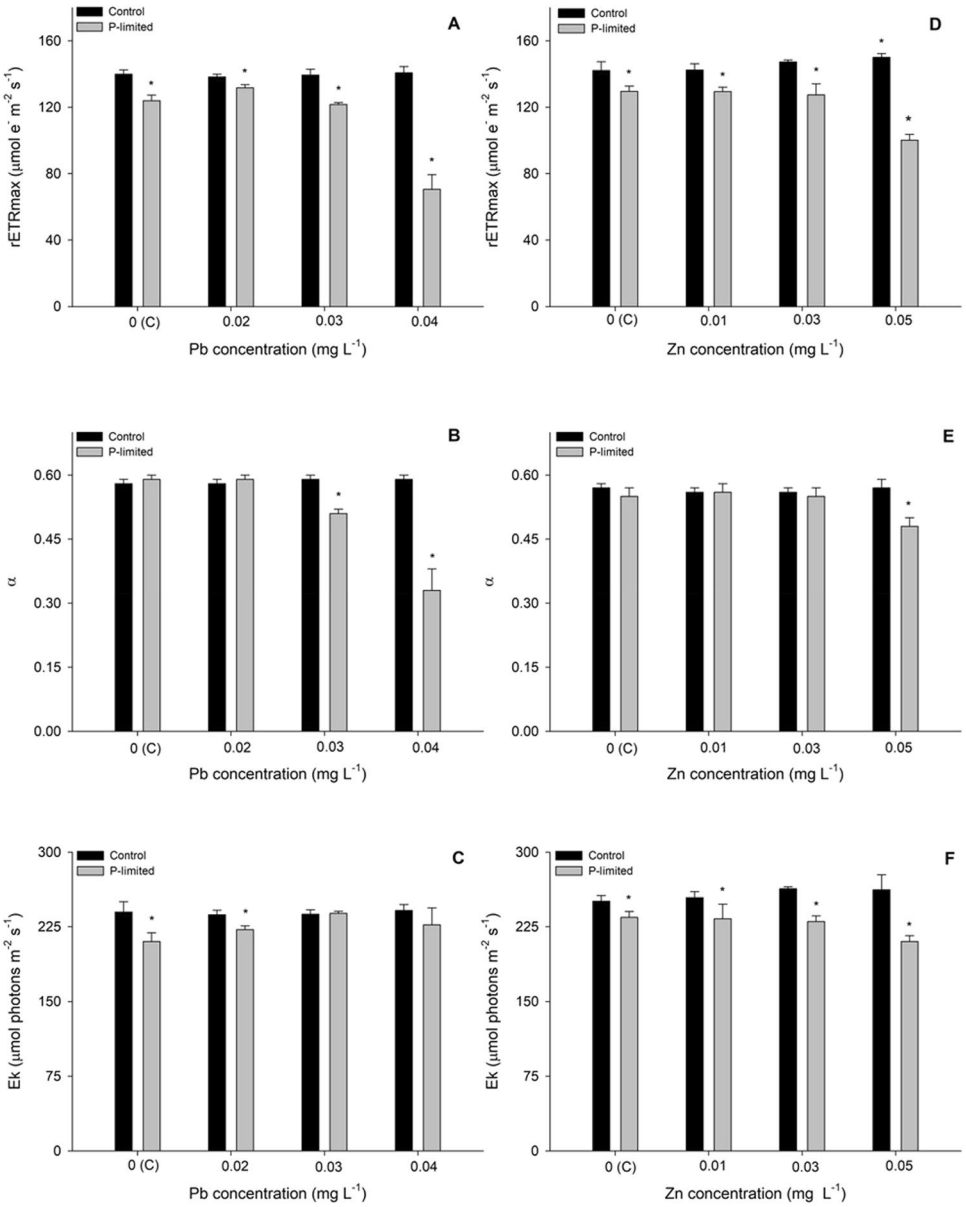


Fig. 4 Maximum relative electron transport rate ($rETR_{max}$), initial slope (α) and saturation irradiance (E_k) of *R. subcapitata* exposed to Pb (**A**, **B**, **C**) or Zn (**D**, **E**, **F**), isolated or combined with P limitation. C refers to control (i.e., metal added in the LC Oligo medium). * indicates values significantly different from control ($p < 0.05$)

the combination of P limitation and Zn, our data are in line with those obtained by Rodgher et al. (2020), where a higher toxicity of Zn was observed under P limitation. However, other authors had observed no influence of P in Zn toxicity (Kamaya et al. 2004) or a decreased Zn toxicity with less P (Gao et al. 2016). We suggest that methodological differences were responsible for the different observations. While in the present study and in Rodgher et al. (2020) the algae were previously acclimated to different P conditions, in the other cited studies there was no acclimation to different P concentrations before exposure to Zn, reinforcing the importance of P acclimation (Solovchenko et al. 2019).

Since metals and nutrients influence the production of antioxidant molecules (Coulombier et al. 2021) such as carotenoids (Cirulis et al. 2013), we suggest that the remarkable increase of total carotenoids per cell, is related to photodamage protection through the activation of microalgae antioxidant system (Singh et al. 2019) in an attempt to decrease the amount of reactive oxygen species (ROS), which can be generated by metals (Cirulis et al. 2013; Alho et al. 2019; Gebara et al. 2020), threatening the PS II. Due to their role in photoprotection and in light-harvesting in photosynthetic organisms (Pick et al. 2019), we suggest that the higher total carotenoids observed in P limitation combined to the highest Pb or Zn concentration are linked to the efficient photoprotection mechanism of the algae, supported by the increase in non-photochemical quenching (qN and NPQ) and in Y(NPQ), which indicates the activation of algae photoprotection mechanisms. On the other hand, a higher production of chlorophyll per reaction center, especially under

Pb exposure can be a compensation mechanism to maintain the photosynthetic activity, since Pb^{2+} can replace Mg^{2+} in the chlorophyll molecule, forming non-functional chlorophylls (Dao and Beardall 2016).

Decrease in qN and NPQ values under Zn exposure indicate damage to the photosynthetic apparatus and loss of reaction centers (Kalaji et al. 2014) in the presence of essential metal excess, corroborating previous results for other essential metals such as Co (Rocha and Melão 2024) and Cu (Juneau et al. 2002; Lombardi and Maldonado 2011; Rocha et al. 2021b). Interestingly, the combination of P limitation and excess of Zn on these parameters (qN and NPQ) was less harmful than only the excess of Zn, reinforcing our previous suggestion that algae acclimated their metabolism and were able to use the excess of essential metal in a beneficial way, increasing the heat dissipation to protect photosynthetic machinery (Rocha and Melão 2024). On the other hand, the combination of P limitation and Pb slightly decreased the qP, in a synergic way, and significantly increased the values of qN and NPQ, especially at the highest Pb concentration, indicating an effective defense mechanism of algae, increasing excess of light energy in form of heat, also corroborating our previous studies where *R. subcapitata* showed an effective response to stressors, reinforcing its use in ecotoxicology studies (Rocha and Melão 2024; Rocha et al. 2024). In the present study, qP was the least affected parameter when evaluating the light energy use. In addition, the increase observed in carotenoids at the highest metal (Pb and Zn) combined to P limitation followed by an increase in qN and NPQ values indicate that the xanthophylls cycle, which can be related to photoprotection in algae (Goss and Jakob 2010), may have an important role in the *R. subcapitata* defense mechanism; however, more studies are needed to confirm this relationship (i.e., NPQ and xanthophyll cycle) in this species since it can be species-specific. However, it is interesting to highlight that in combination with P limitation, Pb impacted more these parameters (total carotenoids, qN and NPQ) than Zn.

Compared to previous studies where in the evaluation of limitation of nutrients (P or N) combined to metal exposures, the decrease in the Y(II) seems to be the common response, with non-essential metals (Cd, Al, and Pb) affecting more this parameter than essential metals (Cu, Co, and Zn), in *R. subcapitata* and *Ankistrodesmus densus* (Rocha and Espíndola 2021; Rocha et al. 2021a, 2023; present study). On the other hand, Y(NPQ) has a tendency for increasing, indicating the activation of photoprotective mechanisms in the presence of two stressors; however, it is interesting to highlight that for *R. subcapitata*, the excess of essential metal (Co – Rocha and Melão 2024; or Zn – present study) combined to P limitation resulted in a better response from photoprotective mechanisms than only the excess of metal. The Y (NPQ) data for P limitation combined to Zn corroborate our previous studies where essential metals (Co and Cu) excess is suggested to be used in a beneficial way by the algae (Rocha and Espíndola 2021; Rocha and Melão 2024), while only under Co or Zn excess, damages were observed. For Y (NO) we could not find a clear pattern related to stressors or algal species since we observed increase (Rocha and Espíndola 2021; Rocha et al. 2021a; Rocha and Melão 2024); decrease (Rocha et al. 2023, 2024) or a non-remarkable change (present study) in this parameter.

Regarding the rapid light curve parameters, we could observe antagonism and synergism under P limitation and metal exposure (Pb and Zn), which is different from our previous studies with P limitation and non-essential metals exposure, where synergism was the only observed response (Rocha et al. 2021a – Cd; Rocha et al. 2024—Al) and it is similar to the response observed under P limitation and excess of essential metal (Co; Rocha and Melão 2024). For the parameters $rETR_{max}$ and α, the synergism observed under Pb or Zn exposure reinforce the data obtained for P-limited algae exposed to Cd (Rocha et al. 2021a) and Al (Rocha et al. 2024), being different from the antagonistic responses obtained under P limitation and Co exposure (Rocha and Melão 2024). To the best of our knowledge, this is the first study evaluating the rapid light curves in P-limited algae exposed to Pb and Zn, highlighting the importance of more studies with different metals to understand the algal responses to P limitation since there is no strict standard response to different metals, essential or not.

In summary, adaptations of growth and photosynthetic apparatus under P-limited conditions are unknown; however, acclimation results in adaptation (Bonachela et al. 2011; Napoleón et al. 2013). Since the increase in pigments can support photosynthesis, enhancing the antenna size and can be related to the amount fatty acids, which can alter the membrane fluidity and affect ETR (Wacker et al. 2015), and the dissipation of light energy in form of heat decreases the ETR (Wykoff et al. 1998; Petrou et al. 2008), we suggest that P limitation was the force driving the responses in the rapid light curve parameters as well in the activation of photoprotective mechanisms and pigments production, indicating that the combination of stressors were more intense than only one stressor.

Since the amount of P available directly impacts the availability of Pb, with more P reducing the Pb bioavailability through the formation of the mineral chloropyromorphite (Scheckel and Ryan 2002; Kopittke et al. 2008), we suggest that more P in the control medium was responsible for the chelation of Pb, decreasing its toxicity when compared to P-limited medium, where most likely there was more Pb available for the algae. In addition, previous studies have shown that a lower amount of P results in higher internalization of metals (e.g., Co, Cd, Fe, Pb, Zn) (Webster et al. 2011; Guan et al. 2023), probably due to the changes in algae

lipids classes and fatty acids under P limitation (Rocha et al. 2018). Based on this information and our results, we suggest that P limitation affected membrane conformation, resulting in higher metal internalization, facilitating direct access to the organelles, and affecting physiological processes such as growth and photosynthesis.

More studies are needed to understand the relationship and mechanisms involved in the algal acclimation to P. For example, P limitation + Co (Rocha and Melão 2024) resulted in antagonistic responses in all evaluated photosynthetic parameters, while P limitation combined to Zn (present study) has a higher tendency for synergism in photosynthetic parameters, reinforcing that responses can be metal-specific. The present study corroborates our previous observations indicating that combination of P limitation and Al (Rocha et al. 2024) or Co (Rocha and Melão 2024) does not affect the xanthophylls cycle in *R. subcapitata*. However, it is interesting to highlight that exposure only to excess essential metals (Co and Zn) resulted in more damages than non-essential metals (Al and Pb). On the other hand, acclimation to P limitation seems to result in an adaptation of algal metabolism, where the excess of an essential metal (Zn and Co) combined to P-limited algae resulted in the recovery of qN, NPQ and Y(NPQ) when compared with metal as only stressor. The increase in pigments under stress indicates an effective algal response to avoid higher damages to the photosynthetic apparatus.

Conclusions

Our results indicate the greater sensitivity of P-limited *R. subcapitata* to an essential and a non-essential metal, with synergistic response in several parameters evaluated (cell density, maximum quantum yield, qP, Y(II), $rETR_{max}$, and α) and antagonism in pigments production. In addition, our study reinforces the importance of considering the water characteristics to define metal limits of regulations. In Brazil, the amount of Pb allowed in freshwater is 0.03 mg L^{-1} and for Zn is 0.18 mg L^{-1} (CONAMA 2005). In our study, while the metals alone less severely affected the evaluated parameters; in P-limited algae all parameters were affected. The synergistic effects occurred in P limitation combined to Pb at a concentration slightly higher than accepted by the Brazilian legislation, while combination with Zn in concentrations much lower than accepted by the legislation, indicating how tricky is defining what amount of contaminant is acceptable in water since other factors interfere in the toxicity of the contaminant. Concerning the effects of essential or non-essential metal, our data indicate a slightly higher damage to P-limited algae exposed to a non-essential metal. Except for qP and Ek, where different responses were observed for P-limited algae exposed to Pb and Zn regarding synergism and antagonism, the other parameters had similar interaction (i.e., synergism or antagonism) in the general responses, which suggest that the essentiality of metal is not a determinant point in the effects observed in *R. subcapitata*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10811-024-03398-5.

Acknowledgements G.S.R. is grateful to Grant #2015/25436-1 (FAPESP) (São Paulo Research Foundation). MGGM thanks National Council for Scientific and Technological Development (CNPq) for research productivity grant #316064/2021-1.

Author contribution GSR: Conceptualization; Formal analysis; Investigation; Funding acquisition; Writing – review and editing. MGGM: Funding acquisition; Writing – review and editing.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. FAPESP (São Paulo Research Foundation)—Grant #201525436–1 (GSR), and CNPq (National Council for Scientific and Technological Development)—Grant #316064/2021–1 (MGGM).

Data Availability Data will be made available upon request.

Declarations

Competing interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

AFNOR (1980) Essais de eaux. Norme experimental T90–304. Determination de L'inhibition de croissance de *Scenedesmus subspicatus* par une substance. Association Française de Normalisation, Paris, p 6

Alho LOG, Gebara RC, Paina KA, Sarmento H, Melao MGG (2019) Responses of *Raphidocelis subcapitata* exposed to Cd and Pb: mechanisms of toxicity assessed by multiple endpoints. Ecotoxicol Environ Safety 169:950–959

Alves RI, Sampaio CF, Nadal M, Schuhmacher M, Domingo JL, Segura-Munoz SI (2014) Metal concentrations in surface water and sediments from Pardo River, Brazil: human health risks. Environ Res 133:149–155

Andres S, Ribeyre F, Tourencq JN, Boudou A (2000) Interspecific comparison of cadmium and zinc contamination in the organs of four fish species along a polymetallic pollution gradient (Lot River, France). Sci Total Environ 248:11–25

Berdalet JE, Latasa M, Estrada M (1994) Effects of nitrogen and phosphorus starvation on nucleic acid and protein content of *Heterocapsa* sp. J Plankton Res 16:303–331

Bhola V, Desikan R, Santosh SK, Subburamu K, Sanniyasi E, Bux F (2011) Effects of parameters affecting biomass yield and thermal behavior of *Chlorella vulgaris*. J Biosci Bioeng 111:377–382

Bonachela JA, Raghib M, Levin SA (2011) Dynamic model of flexible phytoplankton nutrient uptake. Proc Nat Acad Sci 108:20633–20638

Cirulis JT, Scott JA, Ross GM (2013) Management of oxidative stress by microalgae. Can J Physiol Pharmacol 91:15–21

CONAMA (2005) Brazilian Ministry of the Environment, National Environmental Council. Resolution n. 357/2005, March 17th. Brasília, DF, Brazil

Coulombier N, Jauffrais T, Lebouvier N (2021) Antioxidant compounds from microalgae: a review. Mar Drugs 19:549

Dao LHT, Beardall J (2016) Effects of lead on two green microalgae *Chlorella* and *Scenedesmus*: photosystem II activity and heterogeneity. Algal Res 16:150–159

Dourado PLR, Rocha MP, Roveda LM et al (2017) Genotoxic and mutagenic effects of polluted surface water in the Midwestern region of Brazil using animal and plant bioassays. Genet Mol Biol 40:123–133

Fogg GE (1973) Phosphorus in primary aquatic plants. Water Res 7:77–91

Gao C, De Schamphelaere KAC, Smolders E (2016) Zinc toxicity to the alga *Pseudokirchneriella subcapitata* decreases under phosphate limiting growth conditions. Aquat Toxicol 173:74–82

Gebara RC, Alho LOG, Rocha GS, Mansano AS, Melão MGG (2020) Zinc and aluminum mixtures have synergic effects to the algae *Raphidocelis subcapitata* at environmental concentrations. Chemosphere 242:125231

Gebara RC, Alho LOG, Abreu CB, Mansano AS, Moreira RA, Rocha GS, Melão MGG (2021) Toxicity and risk assessment of zinc and aluminum mixtures to *Ceriodaphnia silvestrii* (Crustacea: Cladocera). Environ Toxicol Chem 40:2912–2922

Gebara RC, Alho LOG, Mansano AS, Rocha GS, Melão MGG (2023) Single and combined effects of Zn and Al on photosystem II of the green microalgae *Raphidocelis subcapitata* assessed by pulse-amplitude modulated (PAM) fluorometry. Aquat Toxicol 254:106369

Goss R, Jakob T (2010) Regulation and function of xantophyll-cycle dependent photoprotection in algae. Photosynth Res 106:103–122

Gottardi M, Birch MR, Dalhoff K, Cedergreen N (2017) The effects of epoxiconazole and α-cypermethrin on *Daphnia magna* growth, reproduction and offspring size. Environ Toxicol Chem 36:2155–2166

Guan H, Jiang Z, Sun D, Wang Z, Sun Y, Huo H, Li Z, Tang L, Li Z, Zhang C, Ge Y (2023) Sufficient phosphorus enhances resistance and changes accumulation of lead in *Chlamydomonas reinhardtii*. Environ Toxicol Chem 42:1960–1970

Gui D, Yu RQ, Karczmarski L, Ding Y, Zhang H, Sun Y, Zhang M, Wu Y (2017) Spatiotemporal trends of heavy metals in Indo-Pacific humpback dolphins (*Sousa chinensis*) from the Western Pearl River Estuary, China. Environ Sci Technol 51:1848–1858

Harpprecht C, Xicotencatl BM, van Nielen S, van der Meide M, Li C, Li Z, Tukker A, Steubing B (2024) Future environmental impacts of metals: a systematic review of impact trends, modeling approaches, and challenges. Resour Conservat Recycl 205:107572

Jassby AD, Platt T (1976) Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. Limnol Oceanogr 21:540–547

Ji Y, Sherrell RM (2008) Differential effects of phosphorus limitation on cellular metals in *Chlorella* and *Microcystis*. Limnol Oceanogr 53:1790–1804

Juneau P, El Berdey A, Popovic R (2002) PAM fluorometry in the determination of the sensitivity of *Chlorella vulgaris, Selenastrum capricornutum* and *Chlamydomonas reihardtii* to copper. Arch Environ Contam Toxicol 42:155–164

Kalaji HM, Schansker G, Ladler RJ et al (2014) Frequently asked questions about in vivo chlorophyll fluorescence: practical issues. Photosynth Res 122:151–158

Kamaya Y, Takada T, Suzuki K (2004) Effect of medium phosphate levels on the sensitivity of *Selenastrum capricornutum* to chemicals. Bull Environ Contam Toxicol 73:995–1000

Kataba A, Botha TL, Nakayama SMM, Yohannes YB, Ikenaka Y, Wepener V, Ishikuza M (2020) Acute exposure to environmentally relevant lead levels induces oxidative stress and neurobehavioral alterations in larval zebrafish (*Danio rerio*). Aquat Toxicol 227:105607

Kim JH, Kang JC (2017) Effects of sub-chronic exposure to lead (Pb) and ascorbic acid in juvenile rockfish: antioxidant responses, MT gene expression, and neurotransmitters. Chemosphere 171:520–527

Kopittke PM, Asher CJ, Menzies NW (2008) Prediction of Pb speciation in concentrated and dilute nutrient solutions. Environ Pollut 153:548–554

Lai J, Yu Z, Song X, Cao X, Han X (2011) Responses of the growth and biochemical composition of *Prorocentrum donghaiense* to different nitrogen and phosphorus concentration. J Exp Mar Biol Ecol 405:6–17

Lombardi AT, Maldonado MT (2011) The effects of copper on the photosynthetic response of *Phaeocystis cordata*. Photosynth Res 108:77–87

Lombardi AT, Wangersky P (1991) Influence of phosphorus and silicon on lipid class production by the marine diatom *Chaetoceros gracilis* grown in turbidostat cage cultures. Mar Ecol Prog Ser 77:39–47

Machado MD, Lopes AR, Soares EV (2015) Responses of the alga *Pseudokirchneriella subcapitata* to long-term exposure to metal stress. J Hazard Mat 296:82–92

Maret W (2013) Zinc biochemistry: From a single zinc enzyme to a key element of life. Adv Nutr 4:82–91

Napoleón C, Raimbault V, Claquin P (2013) Influence of nutrient stress on the relationships between PAM measurements and carbon incorporation in four phytoplankton species. PLoS ONE 8:e66423

Nriagu JO (1988) A silent epidemic of environmental metal poisoning? Environ Pollut 50:139–161

Pascual G, Sano D, Sakami K, Nishimura O (2020) Effects of chemical interaction of nutrients and EDTA on metals toxicity to *Pseudokirchneriella subcapitata*. Ecotoxicol Environ Safety 203:110966

Petrou K, Doblin MA, Smith RA, Ralph PJ (2008) State transitions and nonphotochemical quenching during a nutrient-induced fluorescence transient in phosphorus-starved *Dunaliella tertiolecta*. J Phycol 44:1204–1211

Pick U, Zarka A, Boussiba S, Davidi L (2019) A hypothesis about the origin of carotenoid lipid droplets in the green algae *Dunaliella* and *Haematococcus*. Planta 249:31–47

Rocha GS, Espíndola ELG (2021) Copper affects photosynthetic parameters of N- or P-limited *Ankistrodesmus densus*. Environ Adv 4:100070

Rocha GS, Melão MGG (2024) Does cobalt antagonize P limitation effects on photosynthetic parameters on the freshwater microalgae *Raphidocelis subcapitata* (Chlorophyceae), or does P limitation acclimation antagonize cobalt effects? More questions than answers. Environ Pollut 341:122998

Rocha GS, Parrish CC, Lombardi AT, Melão MGG (2018) Biochemical and physiological responses of *Selenastrum gracile* (Chlorophyceae) acclimated to different phosphorus concentrations. J Appl Phycol 30:2167–2177

Rocha GS, Parrish CC, Espíndola ELG (2021b) Effects of copper on photosynthetic and physiological parameters of a freshwater microalga (Chlorophyceae). Algal Res 54:102223

Rocha GS, Parrish CC, Espíndola ELG (2023) Changes in photosynthetic parameters and lipid classes of N-limited *Ankistrodesmus densus* (Chlorophyceae) under cadmium exposure. J Appl Phycol 35:99–107

Rocha GS, Lopes LFP, Melão MGG (2024) Phosphorus limitation combined with aluminum triggers synergistic responses on the freshwater microalgae *Raphidocelis subcapitata* (Chlorophyceae). Chemosphere 352:141320

Rocha GS, Lombardi AT, Espíndola ELG (2021a) Combination of P-limitation and cadmium alters photosynthetic responses in *Ankistrodesmus densus* (Chlorophyceae). Environ Pollut 275 (C):11673

Rodgher S, Espindola EL, Rocha O, Fracacio R, Pereira RH, Rodrigues MH (2005) Limnological and ecotoxicological studies in the cascade of reservoirs in the Tiete River (Sao Paulo, Brazil). Braz J Biol 65:697–710

Rodgher S, Contador TM, Rocha GS, Espíndola ELG (2020) Effect of phosphorus on the toxicity of zinc to the microalga *Raphidocelis subcapitata*. An Acad Bras Ciênc 92:e20190050

Scheckel KG, Ryan JA (2002) Effects of aging and pH on dissolution kinetics and stability of chloropyromorphite. Environ Sci Technol 36:2198–2204

Sharma P, Dubey RS (2005) Lead toxicity in plants. Braz J Plant Physiol 17:35–52

Singh R, Upadhyay AK, Singh DV, Singh JS, Singh DP (2019) Photosynthetic performance, nutrient status and lipid yield of microalgae *Chlorella vulgaris* and *Chlorococcum humicola* under UV-B exposure. Curr Res Biotech 1:65–77

Solovchenko AE, Ismagulova TT, Lukyanov AA, Vasilieva SG, Konyukhov IV, Pogosyan SI, Lobakova ES, Gorelova OA (2019) Luxury phosphorus uptake in microalgae. J Appl Phycol 31:2755–2770

Touzet N, Franco JM, Raine R (2007) Influence of inorganic nutrition on growth and PSP toxin production of *Alexandrium minutum* (Dinophyceae) from Cork Harbour, Ireland. Toxicon 50:106–119

Van Mooy BAS, Fredricks HL, Pedler BE et al (2009) Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature 458:69–72

Wacker A, Piepho M, Spijkerman E (2015) Photosynthetic and fatty acid acclimation of four phytoplankton species in response to light intensity and phosphorus availability. Eur J Phycol 50:288–300

Webster RE, Dean AE, Pittman JK (2011) Cadmium exposure and phosphorus limitation increases metal content in the freshwater alga *Chlamydomonas reinhardtii*. Environ Sci Technol 45:7489–7496

Wellburn AR (1994) The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol 144:307–313

Wykoff DD, Davies JP, Melis A, Grossman AR (1998) The regulation of photosynthetic electron transport during nutrient deprivation in *Chlamydomonas reinhardtii*. Plant Physiol 117:129–139

Xiao J, Jin Z, Wang J (2014) Geochemistry of trace elements and water quality assessment of natural water within the Tarim River Basin in the extreme arid region, NW China. J Geochemi Explor 136:118–126

Zheng JL, Yuan SS, Wu CW, Li WY (2016) Chronic waterborne zinc and cadmium exposures induced different responses towards oxidative stress in the liver of zebrafish. Aquat Toxicol 177:261–268

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.