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Effect of phosphorus on the toxicity of zinc to the microalga *Raphidocelis subcapitata*

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Abstract: The aim of this study was to evaluate the effect of phosphorus (P) on the toxicity of zinc (Zn) for the alga *Raphidocelis subcapitata*. P was provided in three concentrations: 2.3 x 10^{-4} mol L^{-1}, 2.3 x 10^{-6} mol L^{-1} and 1.0 x 10^{-6} mol L^{-1}. Algal cells were acclimated to the specific P concentrations before the start of the experiment. The chemical equilibrium software MINEQL* 4.61 was employed to calculate the Zn^{2+} concentration. After acclimated, the algal cells were inoculated into media containing different Zn concentrations (0.09 x 10^{-6} mol L^{-1} to 9.08 x 10^{-6} mol L^{-1}). The study showed that besides the reduction in algal growth rates, phosphorus had an important influence on the toxicity of zinc for microalga. The inhibitory Zn^{2+} concentration values for *R. subcapitata* were 2.74 x 10^{-6} mol L^{-1}, 0.58 x 10^{-6} mol L^{-1} and 0.24 x 10^{-6} mol L^{-1} for the microalgae acclimated at P concentrations of 2.3 x 10^{-4} mol L^{-1}, 2.3 x 10^{-6} mol L^{-1} and 1.0 x 10^{-6} mol L^{-1}, respectively. Ecotoxicological studies should consider the interaction between metal concentrations and varying P values to provide realistic data of what occurs in phytoplankton communities in environments.

Key words: Algal density, free zinc, green algae, nutrient.

INTRODUCTION

Eutrophication and the introduction of toxic materials such as metals are considered major types of degradation of aquatic systems (Wang & Dei 2006, Dirszowsky & Wilson 2016, Albano et al. 2018). Ecological concern about phosphorus is caused by its important role in biological metabolism and the low concentrations of this element in water bodies (Wetzel 2001). Phosphorus controls the biological productivity of algae in the greater part of the aquatic environment (Grossman 2000, Hessen et al. 2002, Esteves 2011). However, agro-industrial and domestic sewage discharges may insert excess P into freshwater and marine ecosystems, resulting eutrophication (Zeng & Wang 2009, Schindler et al. 2016). The potential effects of eutrophication on aquatic environments include changes in the biodiversity, drinking water treatment problems and a reduction in recreational use (Tundisi & Tundisi 2008, Pereira et al. 2010, Costa et al. 2014).

Some metals, such as zinc, are essential nutrients for prokaryotic and eukaryotic organisms. Zn is a component of many enzymes and guarantees biological stability of the genetic material and of biological structures, such as the ribosomes and cytoplasmic membranes (Eisler 1981). Currently, zinc is employed in coating other metals, inorganic medicines, and the manufacture of aesthetic products and non-corrosive alloys interfering (Tsushima et al. 2010). This element is one of the most important metals in the economic improvement of China (Xueyi et al. 2010), Korea (Jeong & Kim 2018) and Poland (Lewicka & Burkowicz 2017). In 2017,

the U.S. Geological Survey (2018) estimated the global zinc mining production as 13 million tons, with 5% increase compared to the previous year. Due to concern about the potential effects of zinc on the biota of aquatic systems, zinc is receiving attention from the regulatory environmental agencies in European Union and Japan (Bodar et al. 2005, Van Sprang et al. 2009, Naito et al. 2010, Tsushima et al. 2010).

Zinc is an essential element for phytoplankton, being required in both photosynthetic processes and energy storage (Cao et al. 2015). However, values above trace amounts can be dangerous, modifying cell volumes and inhibiting the growth rate and photosynthesis of microalgae (Guanzon Jr et al. 1994, Mallick & Mohn 2003, Machado et al. 2015). On the other hand, the presence of phosphorus in the aquatic metabolism is of concern to ecologists because this element can regulate algal productivity, causing eutrophication and affecting biological diversity. In the natural aquatic environment, organisms are continually confronted with simultaneous physical and chemical perturbations (Chia et al. 2017, Van Regenmortel et al. 2017, Kong et al. 2018, Roy et al. 2018). As a consequence, chemical compounds interacting with each other in antagonistic and synergistic manners can alter their toxic action to microalgae (Bere et al. 2012, Zhang et al. 2015, Mansano et al. 2017).

Studies about the interactions between metallic ions and nutrients have highlighted the influence of nutrients in culture media on the sensitivity of phytoplankton to metals. The toxicities of arsenic (Wang et al. 2013), cadmium (Zeng & Wand 2009, Webster et al. 2011), copper (Serra et al. 2010, Rocha et al. 2016) and chromium (Qian et al. 2013) to cyanobacterium and green microalgae have increased in response to the reduction of phosphorus in the environment. To the contrary, a decrease in the toxicity of Zn to green microalgae under limiting phosphate conditions was described by Gao et al. (2016). The variable data obtained from research about the interaction between phosphorus and metal exposure to algae could be related to the different procedures adopted in the experiments (Gao et al. 2016), including the absence of metabolic acclimation of algae to changes in the phosphorus concentration. According to the literature, excess P in the environment is incorporated in the algae as polyphosphate vesicles, which reduces the toxicity of metallic ions by way of detoxifying processes when the ions bind to the polyphosphates (Jensen et al. 1982, Twiss & Nalewajko 1992). Hence, previous studies about the interaction between phosphorus and metal exposures have reinforced the need for acclimation processes when the algae are exposed to different supplies of phosphorus, to assure that their metabolisms express that condition (Lombardi & Maldonado 2011, Chia et al. 2017, Rocha et al. 2018).

The intensification of eutrophication in freshwater systems justifies the need for studies about the interaction between metals and phosphorus, due to the influence of these elements on the physiology of autotrophic microorganisms (Reynolds 2006, Chia et al. 2013a, Gormley-Gallagher et al. 2016). Ecotoxicological tests are helpful tools to estimate the combined toxic effects of chemicals in aquatic systems. Of the unicellular algal species, the Chlorophyceae *Raphidocelis subcapitata* (previously named *Pseudokirchneriella subcapitata*) is one of the most suitable for carrying out toxicity tests, due to its high sensitivity to a variety of contaminants, adequately maintainable in laboratory conditions (Wei et al. 2006, Yan et al. 2015, Lewis & Thursby 2018) and for being a characteristic species of oligotrophic and eutrophic freshwater systems (Blaise & Vasseur 2005).

The main objective of this study was to evaluate the effects of various phosphorus/zinc combinations on the toxicity of the metal on the green alga *Raphidocelis subcapitata*. The choice of the phosphorus concentrations tested reflected the natural conditions of freshwater aquatic environments, including low and high concentrations of the element (Zeng & Wang 2009, Chia et al. 2017). In this research, the microalga *R. subcapitata* was exposed to three phosphorus concentrations for several generations up to constant growth rates, to guarantee that their physiology would reflect the nutrient concentrations (Rocha et al. 2016). Thus, the toxicity tests with zinc were carried out after acclimation of the microalgae. The results generated in this work represent an important contribution to research related to the effect of zinc on aquatic systems with different trophic states, with emphasis on the physiology of autotrophic microorganisms.

MATERIALS AND METHODS

Algal culture and acclimation experiments

The green alga *Raphidocelis subcapitata* was obtained from the algal culture collection of the Botany Department of the Federal University of São Carlos (São Carlos, SP, Brazil). Stock cultures of the *R. subcapitata* were maintained in L.C. Oligo medium (AFNOR 1980). The microalga was grown in 100 mL sterile culture medium with a photoperiod of 16:8 h light: dark cycle, light intensity of 60 µmol m^{-2} s^{-1} and temperature of 23 ± 1 °C.

The toxicity tests were conducted with the green algae acclimated to the three nominal phosphorus concentrations. The nutrient was provided as dibasic potassium phosphate (K_2HPO_4): 2.3 x 10^{-4} mol L^{-1} (Control, L.C. Oligo medium), 2.3 x 10^{-6} mol L^{-1} and 1 x 10^{-6} mol L^{-1}. These P values were selected after preliminary experiments, where we did not verified algal growth in values lower than 1 x 10^{-6} mol P L^{-1}. Acclimation of the green algae was carried out by transferring algal cells in the exponential growth phase with an initial density of 5 x 10^5 cells mL^{-1}, into semi-continuous cultures with specific phosphorus concentrations. The algal cells were maintained for 5 weeks under these conditions, keeping the algal cell density at 5 x 10^5 cells mL^{-1}. Algal cells were collected every 168 h and the number of cells in the cultures quantified using a Neubauer-Improved Chamber to determine the growth rate (Rocha et al. 2016, Chia et al. 2017). The growth rate values were evaluated according to Fogg (1975). The algal cells were maintained under distinct phosphorus concentrations for 35 days, allowing for the finding of at least three constant growth rate values (statistically similar values, $p>0.05$). Under this condition, the microalga was considered acclimated and its metabolism expressed the P values in the culture medium (Rocha et al. 2016). The experiments were carried out with 3 replicates per treatment. The acclimated algal cells were employed in the toxicity tests with zinc, but the microalgae were not acclimated to the metal.

All the laboratory materials used in the culture and acclimatization experiments were washed with 10% HNO_3 for 7 days and then rinsed with deionized water before use.

Toxicity tests

Phosphorus acclimated and exponentially growing *R. subcapitata* cells were exposed for 96 h to a range of zinc concentrations. Based on the results of the preliminary toxicity tests, seven metal concentrations were prepared: 1 (Medium L.C. Oligo - Control); 1.53; 3.06; 6.12; 12.2; 24.5; 48.9 and 97.9 x 10^{-7} mol Zn L^{-1}. Zinc was provided as $ZnCl_2.4H_2O$ (Titrisol, Merck, Germany). As the bioavailability and toxicity of essential metals to microorganisms are influenced by the free metal concentrations (Meylan et al. 2004), the chemical equilibrium software MINEQL$^+$ 4.61 (MINEQL$^+$ version 4.61, 2009) was used to calculate the Zn^{2+}

and $Zn_3(PO_4)_2$ concentrations under different P conditions. The P concentrations in the growth media were determined using the ascorbic acid method (APHA/AWWA/WCF 1995).

The experiments were carried out in 250 mL polycarbonate flasks containing 100 mL of medium, to which suitable volumes of metal were added. The green algae cells were inoculated into the flasks to provide initial cell densities of 10^5 cells mL^{-1}. The toxicity tests were carried out with three replicates per treatment and the environmental conditions used for the experiments were the same as those delineated for the algal cultures (ABNT 2011). After 96 h of metal exposure, 2 mL were taken from each test flask, fixed with acid Lugol's iodine solution (ABNT 2011) and used to determine the cell density. The cells were counted using an Improved Neubauer-Bright Line hemocytometer under an optical microscope (Carl Zeiss, Standard model 25). The cell density (cell mL^{-1}) was used to obtain the 50% inhibitory concentration for zinc.

Data analysis

The growth rate was analyzed using the One-way ANOVA and Tukey's post hoc test to identify significant differences between the control treatment and the metal concentrations. Statistical analyses were carried out with a significance level of 0.05 using the BioEstat 4.0 program (Ayres et al. 2007). The Trimmed Spearman-Karber method was used to estimate the Zn concentration that reduces the cell density to 50 % of control treatment (IC 50) (Hamilton et al. 1977).

RESULTS

Metal speciation (MINEQL$^+$ 4.61) showed that approximately 93% of the total zinc added remained available to the algae in the test media with the three P conditions tested, corresponding to free initial zinc ion concentrations of 0.09; 0.14; 0.28; 0.57; 1.13; 2.30; 4.54; and 9.08 x 10^{-6} mol L^{-1}. The chemical equilibrium model predicted that 1% of the total Zn added precipitated as $Zn_3(PO_4)_2$, and in this study, there was low zinc precipitation by phosphorus in the L.C. Oligo medium. The values of the nutrient (P) measured in the algal culture medium were 2.1 x 10^{-4}, 2.2 x 10^{-6} mol L^{-1} and 0.8 x 10^{-6} mol L^{-1}.

The results of the algal acclimation are shown in table I. When the *R. subcapitata* inoculum was added to a culture medium containing 2.3 x 10^{-4} mol P L^{-1}, the algal growth rate was not significantly different during the acclimation period for that treatment. In relation to the treatment with 2.3 x 10^{-6} mol P L^{-1}, the algal cells were considered acclimated after 28 days. The acclimation of the microalgae to 1.0 x 10^{-6} mol P L^{-1} took 36 days. A significant decrease in the algal growth rate was verified with a limitation of phosphorus in the culture medium (Tukey test, $p<0.05$).

In the algal toxicity tests, the values for the coefficient of variation amongst the replicates of the control treatments were less than or equivalent to 20 %, complying with the acceptability criteria proposed by the ABNT guidelines (ABNT 2011). Table II shows the results for the algal density values verified after the exposure of *R. subcapitata* to Zn. The algal cells acclimated to the highest phosphorus value showed the highest cell density (4.49 x 10^6 cells mL^{-1}) for the control treatment in comparison with the medium (2.24 x 10^6 cells mL^{-1}) and low (1.77 x 10^6 cells mL^{-1}) P concentrations (Tukey test, $p<0.05$), thus more phosphorus contributed to higher algal cell densities. A decrease in algal cell numbers was verified as the Zn concentrations increased in the test medium for the three P concentrations tested. The algal cells acclimated to 2.3 x 10^{-4} mol L^{-1} P showed the

Table I. Mean growth rate values (days^{-1}) for *R. subcapitata* in the acclimation experiments. Values with the same letters are not statistically different (Tukey's Test, p>0.05). Values are means of three replicates (± Standard deviation).

Generation	2.3 x 10^{-4} mol P L^{-1}	2.3 x 10^{-6} mol P L^{-1}	1.0 x 10^{-6} mol P L^{-1}
1	0.68^{a} ± 0.04	0.60^{a} ± 0.02	0.16^{c} ± 0.01
2	0.67^{a} ± 0.08	0.46^{b} ± 0.03	0.15^{c} ± 0.04
3	0.59^{a} ± 0.02	0.42^{b} ± 0.03	0.06^{d} ± 0.01
4	0.64^{a} ± 0.02	0.42^{b} ± 0.02	0.03^{d}± 0.00
5	0.60^{a} ± 0.02	0.46^{b} ± 0.03	0.04^{d} ± 0.02

highest algal densities for all the phosphorus/zinc combinations tested (Tukey test, p<0.05).

Figure 1 shows the results for the IC 50 based in cell density at 96 h Zn exposure. The zinc IC 50 values for *R. subcapitata* were 0.24 x 10^{-6} mol L^{-1}, 0.58 x 10^{-6} mol L^{-1} and 2.74 x 10^{-6} mol L^{-1} of free Zn^{2+} for the species acclimated at concentrations of 1.0 x 10^{-6} mol L^{-1}; 2.3 x 10^{-6} mol L^{-1} and 2.3 x 10^{-4} mol L^{-1} of P, respectively, indicating that metal toxicity to the microorganism was reduced by the addition of phosphorus to the test medium (Tukey test, p<0.05).

DISCUSSION

Interactions amongst environmental components control the growth and maintenance of algal communities. In this study, the chlorophyta *R. subcapitata* showed a reduction in density as a function of the decrease in P and increase in Zn in the test medium. Similarly, the cell densities of *Chlamydomonas reinhardtii* (Webster et al. 2011), *Selenastrum gracile* (Rocha et al. 2016) and *Chlorella* sp. (Ji & Sherrel 2008, Chia et al. 2017) significantly declined in response to phosphorus limitation in the growth medium. Zinc values above those considered essential can cause oxidative stress (Hamed et al. 2017), inhibition of photosynthesis and cell division in green microalgae (Omar 2002). In the present study, after exposing *R. subcapitata* for 96 h to metal values of 0.14 x 10^{-6} mol Zn^{2+} L^{-1} (10^{-6} mol P L^{-1}) and 2.27 x 10^{-6} mol Zn^{2+} L^{-1} (2.3 x 10^{-4} mol P L^{-1}), the algal density was reduced. The data obtained corroborate those of Muyssen & Janssen (2001) and Canli (2005) who showed inhibition of the growth of *Pseudokirchneriella subcapitata* in the presence of 0.6 x 10^{-6} mol L^{-1} and 10^{-6} mol L^{-1} of dissolved Zn, respectively.

The present study showed that besides the reduction in the algal growth rates, phosphorus had an important influence on the toxicity of zinc to *R. subcapitata*. The combination of a limited P supply and the presence of Zn was in the additive form, which implies that the two treatments inhibited the algal density more than their isolated effects. The IC 50 values for Zn^{2+} varied from 0.24 x 10^{-6} mol L^{-1} with limited nutrient to 2.74 x 10^{-6} mol L^{-1} with high supply of P. The value of 96 for the IC 50 registered for the treatment with the lowest P value tested (10^{-6} mol L^{-1}) was 88% lower than the value obtained in the treatment with the highest P supply (2.3 x 10^{-6} mol L^{-1}). The results of the present study agree with data in the literature about the interaction of metals and P for phytoplankton. Chia et al. (2017) showed that the density of *Chlorella vulgaris* decreased with increasing Cd concentrations

Table II. Mean algal density values (10^6 cells mL^{-1}) after 96 h of exposure to the phosphorus and zinc treatments. Values with the same letters are not statistically different (Tukey Test, p>0.05). Values are means of three replicates (± Standard deviation).

Zn^{2+} (10^{-6} mol L^{-1})	Phosphorus (mol L^{-1})		
	2.3 x 10^{-4}	**2.3 x 10^{-6}**	**1 x 10^{-6}**
0.09 (Control)	4.49 ± 0.9[a]	2.24 ± 0.05[d,h]	1.77 ± 0.07[h]
0.14	-	2.16 ± 0.17[d]	1.16 ± 0.2[i]
0.28	-	1.75 ± 0.08[e]	0.75 ± 0.13[j]
0.57	3.90 ± 0.1[a,b]	1.13 ± 0.01[e,l]	0.62 ± 0.05[j,l]
1.13	3.88 ± 0.1[a,b]	0.12 ± 0.05[f,m]	0.59 ± 0.01[j,m]
2.27	2.55 ± 0.04[b]	0.06 ± 0.02[f,g,k]	0.05 ± 0.06[k]
4.54	0.42 ± 0.01[c]	0.05 ±0.01[f,g]	-
9.08	0.08 ± 0.01[c]	0.01 ± 0.0[g]	-

-data not shown.

and decreasing P concentrations (2.3 x 10^{-4} to 6.0 x 10^{-7} mol L^{-1}). Rocha et al. (2016), evaluating the effect of P concentrations on copper toxicity for the chlorophyta *Scenedesmus gracile*, found that algal cells acclimated to low P values (2.3 x 10^{-6} mol L^{-1}) were more sensitive to the metal. Qian et al. (2013) reported the inhibitory effect of chromium on the growth of *Chlorella vulgaris* with limitation P values (10^{-9} mol L^{-1}). According to the authors, the tolerance of microalgae to metal toxicity with a high P supply is probably because microorganisms supplied with this nutrient resist metal toxicity better in comparison with algal cells under severely limited P conditions. In a phosphorus-rich environment, it has been reported that microalgae incorporate phosphorus as polyphosphate granules and these granules can bind metals, protecting the microorganisms from metal toxicity (Twiss & Nalewajko 1992, Rai et al. 2001, Lavoie et al. 2016). Also high phosphorus levels may alter the biochemical composition of the microalgae, promoting the synthesis of protective molecules such as phytochelatins (Hashemi et al. 1994, Wang et al. 2015).

The growth of *R. subcapitata* was simultaneously regulated by phosphorus and zinc. It has been reported that under limited phosphate conditions, algae can produce the alkaline phosphatase enzyme, which plays an important role in the use of dissolved organic phosphate by phytoplankton (Rengefors et al. 2003, Ji & Sherrel 2008, Huang et al. 2009). According to Ji & Sherrel (2008), Zn is a necessary cofactor for APase activity. Thus the cellular uptake of zinc probably tends to increase when the algae manifest high APase activity, which could improve the access of metal into the cell and induce toxic cell alterations to the membrane system and photosynthetic apparatus (Cao et al. 2015).

Concerning the comparison of the present data with that found in the literature, a variability of the effects of zinc on microalgae under different phosphorus conditions was found. Kamaya et al. (2004) observed no effects of P (0.6 x 10^{-6} – 6 x 10^{-6} mol L^{-1}) on Zn toxicity to *Selenastrum capricornutum* after 72 hours exposure. Gao et al. (2016) verified increasing zinc toxicity to *P. subcapitata* with a high cell P quota (1.7% P cell dry weight) in 24 h toxicity

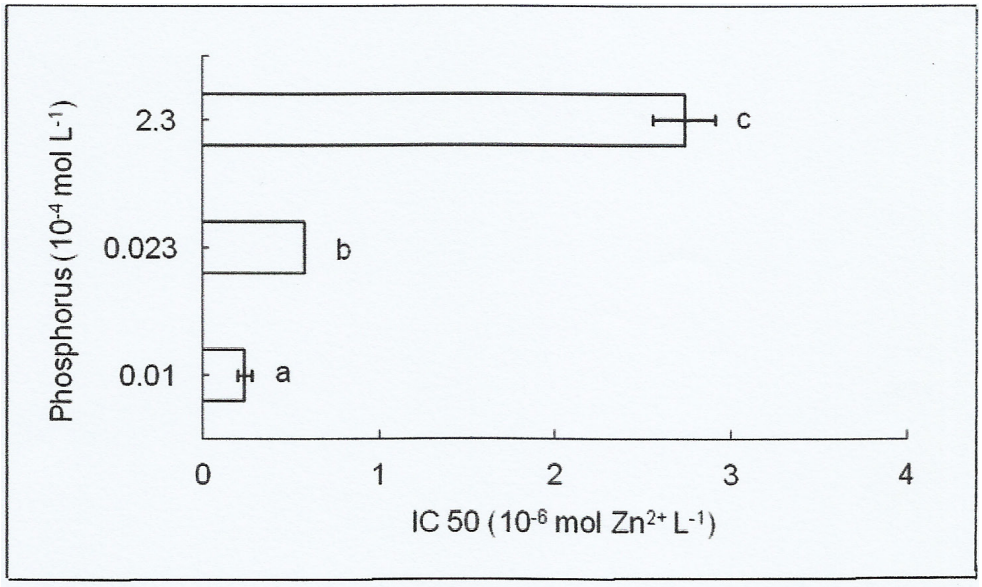

Figure 1. IC 50 values for the *R. subcapitata* acclimated to the phosphorus concentrations. Bars with the same letters are not significantly different (Tukey's test, p>0.05). Values are means of three replicates (± Standard deviation).

tests. The chemical equilibrium model MINEQL$^+$ was used in the present study to calculate the free Zn ion concentrations and little zinc precipitation was credited to the phosphorus. In the present research, the evaluation of Zn toxicity to the microalgae was based on the free metal ion concentrations, while the authors mentioned above using nominal metal concentrations (Kamaya et al. 2004) and lower metal exposure times in their experiments (Kamaya et al. 2004, Gao et al. 2016). Before the beginning the toxicity tests, the *R. subcapitata* cells were acclimated to the specific phosphorus treatment for four weeks. The acclimation period was necessary to guarantee that the physiology of the algae would reflect the nutrient starvation condition in the test medium and reduce the polyphosphate reserve (Rocha et al. 2016, Chia et al. 2017). Thus it was considered that the methodological differences amongst the studies could lead to different data and conclusions to the combined effect of the Zn and P supplies to green microalgae.

Many culture media have been employed to cultivate microalgae in ecotoxicological studies. The growth media used in this study was L.C.Oligo, which is recommended by the Brazilian standards (ABNT) for ecotoxicological assays with microalgae, and is considered to be cost effective with other culture media (Chia et al. 2013b). The present research showed that phosphorus limitation in a synthetic culture medium resulted in greater sensibility of *R. subcapitata* to metals. In the natural environment, phosphorus is the first element to regulate biological productivity and the least available in comparison to other nutrients (Bolier et al. 1992, Wetzel 2001). High production rates in laboratory-grown microalgae cultures depend on an appropriate supply of vitamins, trace elements and macronutrients, as well as other conditions, such as light, pH and temperature stability (Mostert & Grobbelaar 1997). Defined culture media are more appropriate in terms of the standardization of algal culture and testing conditions. However, standard algal toxicity bioassays using artificial

culture media under controlled conditions may have little environmental applicability since the complex chemistry of natural waters differs from that of algal culture media (Ward et al. 2002). Besides, artificial culture media contain nutrients in higher quantities than are found in natural aquatic systems. In ecotoxicological studies, the choice of an algal culture medium requires a compromise between standardization on one hand and its ability to predict the real process, for a better understanding of algal responses to metals in environments with different trophic levels.

Ultimately, it is essential to consider that the methods employed in risk assessments for the regulation of metals may not be adequate for the protection of aquatic species. For example, Conama Resolution 357 established a limit of 2.75 x 10^{-6} mol Zn L^{-1} for the protection of aquatic biota in Brazilian aquatic ecosystems. However, this Brazilian guideline does not take into consideration the influence of phosphorus on metal toxicity to establish safe limits of zinc for aquatic life. It was demonstrated here that zinc values below the limits defined by the Brazilian guidelines (CONAMA 2005) in aquatic environments with a low supply of phosphorus may well upset phytoplanktonic communities. Ecotoxicological investigations should consider the association between metal concentrations and different phosphorus conditions to provide more realistic data of what directly occurs in phytoplankton communities and indirectly in herbivorous consumers that depend on the microalgae in natural waters.

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