Ecotoxicology and Environmental Safety 128 (2016) 30–35

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Influence of phosphorus on copper toxicity to *Selenastrum gracile* (Reinsch) Korshikov

Giseli S. Rocha [a,*], Ana T. Lombardi [b], Maria da Graça G. Melão [a]

[a] Departamento de Hidrobiologia, Universidade Federal de São Carlos (UFSCar), Rodovia Washington Luís, Km 235, CEP 13565-905 São Carlos, SP, Brazil
[b] Departamento de Botânica, Universidade Federal de São Carlos (UFSCar), Rodovia Washington Luís, Km 235, CEP 13565-905 São Carlos, SP, Brazil

ARTICLE INFO

Article history:
Received 22 September 2015
Received in revised form
3 February 2016
Accepted 4 February 2016
Available online 11 February 2016

Keywords:
Free copper
Orthophosphate
Acute toxicity
Chlorophyceae
Eutrophication

ABSTRACT

Microalgae need a variety of nutrients for optimal growth and health. However, this rarely occurs in nature, and if nutrient proportions vary, biochemical changes can occur in phytoplankton community. This may result in modifications of zooplankton food quality, affecting aquatic food chains. Our aim was to investigate the toxicity of copper (Cu) to *Selenastrum gracile*, a common freshwater Chlorophyceae, at different physiological status induced by varying phosphorus (P) concentration in culture medium. Phosphorus was investigated at 2.3×10^{-4}, 1.1×10^{-4}, 2.3×10^{-5}, 4.6×10^{-6} and 2.3×10^{-6} mol L^{-1} and Cu at six concentrations, ranging from 6.9×10^{-9} mol L^{-1} to 1.0×10^{-7} mol L^{-1} free Cu^{2+} ions. To guarantee the cells would be in a physiological status that reflected the external P concentration, they were previously acclimated up to constant growth rate at each P concentration. Phosphorus acclimated cells were then exposed to Cu and toxicity was evaluated through population density, growth rates and chlorophyll *a* content. Free Cu^{2+} ions concentrations were calculated through the chemical equilibrium model MINEQL^{+}. The results showed that higher Cu toxicity was obtained in P-limited than in P-replete cells, and that chlorophyll *a*/cell was higher in P-limited cells and excess Cu than in P-replete cells. This confirms the importance of microalgae nutritional status to withstand the negative effects of the trace metal.

1. Introduction

Phosphorus is an essential nutrient to microalgae and constituent of nucleic acids, adenosine triphosphate and phospholipid membranes. It has important role in enzymatic synthesis and energy transfer in photosynthesis. This nutrient can be limiting in natural freshwater aquatic environments (Cembella et al., 1982; Van Mooy et al., 2009) and in subtropical oceans gyres (Van Mooy et al., 2006), affecting physiological parameters in phytoplankton, such as chlorophyll synthesis, photosynthesis efficiency and molecules synthesis (Napoleon et al., 2013). Phosphorus limitation can affect RNA and DNA synthesis and reduce cell division and growth rate (Zhang and Hong, 2014; Wu et al., 2015).

The growth rate hypothesis suggests a strong positive relationship between available phosphorus and growth rate, but some considerations have to be made before assuming this is valid for the microalgae, such as their ability to store more P than needed or handle with a constant changing environment (Flynn et al., 2010; Giordano et al., 2015). The essentiality of P for microalgae is evidenced by the luxurious uptake and ability to store P, as well as the release of phosphatase enzymes to release inorganic P from organic bound phosphates (Fogg, 1973; Lai et al., 2011).

Phosphate in excess can be stored as polyphosphate granules that act as P reserve for the cells (Omelon and Grynpass, 2008; Rhee, 1973). Twiss and Nalewajko (1992) and Yu and Wang (2004) showed that such granules can be useful in metal detoxifying mechanisms, especially when metal cations combine with the polyphosphate granules, reducing the metals toxicity (Wang et al., 2013).

According to the literature, up to four cell duplications are achieved in phosphorus-scarce environments, so regular growth and physiological processes can be maintained for a period (Bhola et al., 2011; Reynolds, 2006). However, if the low P conditions persist, then intracellular biochemical modifications begin and higher carbohydrates and lipids are produced in opposition to lower proteins (Beardall et al., 2005; Ji and Sherrell, 2008). According to Bertilson et al. (2003) and Van Mooy et al. (2009), several physiological changes take place under prolonged P limitation, such as cell division processes, chlorophyll *a* synthesis and photosynthesis, in addition to an adjustment to lower P demand by the cells.

* Corresponding author.
E-mail address: swertsbio@gmail.com (G.S. Rocha).

http://dx.doi.org/10.1016/j.ecoenv.2016.02.007
0147-6513/

Copper (Cu) is a micronutrient to microalgae, supporting electron transfer in photosynthesis, enzymatic co-factor in respiration, iron metabolism and redox processes (Bossuyt and Janssen, 2004, 2005). However, concentrations above trace amounts can be toxic, decreasing growth rate, chlorophyll *a*, photosynthesis, and respiration (Baumann et al., 2009; De Schamphelaere et al., 2007) or affecting cell membrane permeability (Sivakumar et al., 2010). In the environment, Cu can be present as free ions or complexed with organic or inorganic ligands (Stumm and Morgan, 1996), so affecting its bioavailability.

Literature show that major nutrients affect metal toxicity in microalgae, and under limiting nutritional conditions, toxicity can be increased (Kaneko et al., 2004). However, because of P storage as polyphosphates, the exposure of a microalgae to a P limited condition does not guarantee that its metabolism reflect that condition. It is important to expose the cells for a period, defined by constant growth rates (Lombardi and Maldonado, 2011). In this study, we exposed *Selenastrum gracile* to various P concentrations for several generations up to constant growth rates, so certifying that the physiology of the microalgae would be responding to that specific P condition. It was only after this acclimation that Cu toxicity testing was performed. *S. gracile* was chosen as test organism because it is a common freshwater microalgae in aquatic tropical and subtropical ecosystems. The results we present are a contribution to the knowledge of the interactions among phytoplankton, copper and dissolved phosphorus.

2. Material and methods

The freshwater microalgae *S. gracile* (Chlorophyceae) 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 L.C. Oligo medium (AFNOR, 1980) at initial pH 7.0 under controlled conditions of light intensity (150 μmol m^{-2} s^{-1}), photoperiod (16:8 h light:dark cycle) and temperature (22 °C ± 2). Every 12 h, the cultures were gently shaken. All cultures were kept sterile through autoclaving for 20 min at 121 °C.

Phosphorus (P) was furnished as K_2HPO_4 in 5 different concentrations: 2.3×10^{-4} – control (C), 1.1×10^{-4} (50%), 2.3×10^{-5} (10%), 4.6×10^{-6} (2%) and 2.3×10^{-6} mol L^{-1} (1% of control). The P concentration in the control was that present in the L.C. Oligo medium. Before defining the P concentrations, preliminary tests were done with concentrations ranging from 100% (control) to 0.25% P, but at concentrations lower than 1%, the microalgae spent several days in lag phase so it was not possible to perform 120 h toxicity tests. For this reason, we used a P range between 1% and 100%. Microalgal cells acclimation was performed using semi-continuous cultures.

The algae in exponential growth phase were inoculated at initial cell density of 10^5 cells mL^{-1}, and after 4 days in exponential growth, we started the partial renewal of culture medium. This was performed every 72 h, keeping cell density around 2×10^5 cells mL^{-1}, the density present in mid exponential growth phase. Daily cell counts were performed every 24 h in Neubauer-Improved chamber to determine the growth rate, using the procedure described in Levasseur et al. (1993).

In the first days after the exposure to different P, there were no difference in growth rates between the control and the other concentrations. Considering the 4 duplications as advocated in the literature for P limited conditions, cells were kept under specific P concentration for several weeks, allowing the determination of 7 growth rates and at least 3 constant ones (not significantly different, $p > 0.05$). These acclimated cells were then used for the acute Cu toxicity experiments. In this condition, algae were considered acclimated and its metabolism reflecting the external P concentration, i.e., the P available in the culture medium. Growth rates obtained during acclimation are presented in Table 1. Dissolved orthophosphate content in culture media was determined employing the ascorbic acid method (APHA, 1995). No copper acclimation was performed.

Table 1
Growth rates (μ; d^{-1}) for the several P concentrations acclimations in *Selenastrum gracile* (Chlorophyceae). The last row refers to the mean growth rate (± SD) during the acclimation period. Rows with different superscript letters are significantly different ($p < 0.05$).

Treatment (P)	μ1	μ2	μ3	μ4	μ5	μ (mean ± SD)
Control	1.14	1.12	1.11	1.15	1.10	1.12 ± 0.03^{a}
50%	1.16	1.12	1.11	1.15	1.09	1.12 ± 0.03^{a}
10%	1.13	1.07	1.07	1.01	1.07	1.05 ± 0.05^{ab}
2%	1.00	1.05	0.99	0.99	1.00	1.00 ± 0.02^{b}
1%	0.96	0.98	0.83	0.88	0.92	0.91 ± 0.06^{c}

Sterile conditions were maintained throughout and only sterile materials were used to avoid culture contamination. Laboratory materials were washed with neutral detergent and kept for 7 days in 10% HCl for metal cleaning.

Free copper ions concentrations in culture media were obtained through the chemical equilibrium model $MINEQL^+$ 4.62.3 (Environmental Research Software, Hallowell, ME, USA).

2.1. Acute toxicity tests

Exponentially growing and P acclimated *S. gracile* cells were exposed for 120 h to several free copper (Cu^{2+}) ions concentrations: 0.7×10^{-8} (control), 1.2×10^{-8}, 3.0×10^{-8}, 4.8×10^{-8}, 6.1×10^{-8} and 10×10^{-8} mol L^{-1}. Copper solutions were made by serial dilutions of $CuCl_2$ Titrisol 1000 mg L^{-1} (Merck) in ultra-pure water (Barnstead Easy Pure II, Thermo Scientific, Dubuque, IA, USA). 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 cell mL^{-1}.

Cell densities were monitored every 24 h. Samples were fixed with acetic lugol and cells were counted under an optical microscope (Leica, DMLS) in an Improved Neubauer-Bright Line hemocytometer. Chlorophyll *a* was determined as described by Shoaf and Lium (1976) in 10 mL samples that were filtered through cellulose ester membranes (0.45 μm pore size-Millipore) and extracted with dimethylsulfoxide. Optical density was measured in a spectrophotometer (HACH DR 5000, USA) at 664 and 647 nm wavelengths. Blanks were performed using a clean filter submitted to the same extraction procedure. Chlorophyll *a* concentration (μg mL^{-1}) was calculated as described in Jeffrey and Humphrey (1975).

2.2. Data analysis

The copper concentration that reduces the number of cells to 50% of control (EC_{50}) was calculated using ICp 2.0 software (Environmental Protection Agency, Duluth, MI, USA) using cell density at 120 h exposure for each P concentration. In a preliminary study we observed that 120 h was better than the usual 96 h toxicity evaluation, because the cells were still in exponential growth and more data was acquired for growth rate calculation through linear regression.

Statistical analysis were based in one way ANOVA and Tukey's post-hoc at $p < 0.05$ with data obtained from three experimental replicate cultures. Data are presented as mean ± SD of the replicates ($n = 3$).