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Sensitivity of two green microalgae to copper stress: Growth, oxidative and antioxidants analyses

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ABSTRACT

Depending on species, heavy metals, including copper (Cu), differentially affect algal growth and metabolism. Here, we aim to evaluate the differential responses of two green microalgae, *Chlorella sorokiniana* and *Scenedesmus acuminatus*, exposed to sub-lethal doses of Cu (25 and 50 μM, respectively) for 7 days. The changes in growth, oxidative damage markers, and antioxidants were analysed. We found that *S. acuminatus* could acclimatise during long-term exposure to Cu stress. *S. acuminatus* accumulated lower Cu content and showed a slight decrease in H_2O_2 levels when compared to *C. sorokiniana.* Cu stress induced membrane damage in the two microalgae species, however, this increase was slightly lower in *S. acuminatus.* To mitigate Cu stress impact, *C. sorkiniana* markedly increased proline, polyphenols, flavonoids, tocopherols, glutathione levels, as well as the activities of GST, APX, GR and SOD enzymes, which could explain less-stress sensitivity. On the other hand, *S. acuminatus* exhibited significant increases in proline, polyphenol, and tocopherol contents. Activity levels of POX, APX, GR and SOD enzymes, were also increased. These results suggest that the two microalgae differentially induced the antioxidant defence system to neutralise the oxidative damage induced by Cu stress. This study also provided new data for Cu tolerance and Cu removal abilities of two microalgal species, which commonly exist in surface water bodies, where low Cu uptake and efficient antioxidant defence system protected *S. acuminatus* against oxidative stress induced by Cu stress. This makes it feasible for treatment of Cu contaminated waters.

1. Introduction

Environmental levels of copper (Cu) and other heavy metals have been increasing due to human actions. For instance, the annual discharge of Cu into the ocean is 9×10^6 t per year (Pinto et al., 2003). Although, Cu is a trace element vital for the progress of photosynthetic species. Its high levels are phytotoxic to cells, may significantly inhibit growth, and may also lead to cell death (Yan and Pan, 2002; Sabatini et al., 2009). Cu stress decreased growth rate and pigment contents in microalgae (Sáeza et al., 2015; Machado and Soares, 2016), while increasing reactive oxygen species (ROS) generated through the interference of Cu ions in Fenton's reaction (Okamoto et al., 2001; Moenne et al., 2016; Machado and Soares, 2016; Olivares et al., 2016). Increased levels of ROS can rapidly attack nucleic acids, proteins and lipids, leading to permanent metabolic dysfunction and cell death (Gill and Tuteja, 2010; Pandey et al., 2015). Cu stress induced oxidative damages (*e.g.*, augmentation of protein and lipid peroxidation) in *Scenedesmus* sp. and *Spirulina platensis*-S5, were previously recorded (Sabatini et al., 2009; Choudhary et al., 2007; Tripathi and Gaur, 2004; Tripathi et al., 2006; Jara et al., 2014; Sáeza et al., 2015; Olivares et al., 2016). Several studies on the impact of Cu on microalgal growth have been done, however, its oxidative impact and the role of antioxidant defence mechanisms, to provide tolerance against excessive Cu stress in different microalgae species, is not well investigated.

Algae often minimise free radical damage by inducing an antioxidant defensive system (Li et al., 2006; Olivares et al., 2016), such as non-enzymatic (e.g., glutathione (GSH), tocopherols, ascorbate (ASC), and carotenoids), and enzymatic (e.g., superoxide dismutase (SOD),

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catalase, ascorbate peroxidase (APX)), which are known to be involved in protecting plants against toxic levels of heavy metals (Pinto et al., 2003; Sáeza et al., 2015; Machado and Soares, 2016; Moenne et al., 2016). For example, to reduce Cu stress, *Scenedesmus* sp. and *Spirulina platensis*-S5, significantly increased the intracellular proline level and SOD activity, respectively (Tripathi and Gaur, 2004; Choudhary et al., 2007). Significant increases in SOD activity and GSH content in *Scenedesmus vacuolatus* were recorded at high Cu levels (Sabatini et al., 2009), while induced antioxidant defence system was also observed for *Gonyaulax polyedra* under Cu metal stress, where Cu metal induced the activity of APX, and SOD enzymes, as well as GSH content (Okamoto et al., 2001).

The differential effect of time exposure of algae to Cu stress was previously observed (Tripathi and Gaur, 2004). SOD enzyme activity of *Scenedesmus* sp. was higher only during long-term, but not during short term of metal stress exposure. Moreover, differential responses of different microalgal species to heavy metals were reported (Yan and Pan, 2002). For instance, Sabatini et al. (2009) reported that growth of *S. vacuolatus* was significantly affected only at higher Cu concentrations (414 μM), while a lower dosage (210 μM) of Cu was lethal to *C. kessleri* after 7 days of metal exposure. Growth and photosynthesis of *Chlorella vulgaris* were also varied depending on the sub lethal concentrations and exposure time of different heavy metals (*e.g.,* Cu, Cr, Zn, Cd and Pb) (Ouyang et al., 2012).

Algae are generally exposed to elevated concentrations of heavy metals in polluted water bodies for a long period of time. Hence, there is a need to evaluate oxidative stress during long-term exposure to heavy metals. Meanwhile, the use of algae in bioremediation will depend upon their ability to survive potentially in toxic treatments. In this study, two algal species were selected due to their high prevalence in industrial waste water, which have also been reported as having high biosorption capacities for various heavy metals (Ali et al., 1999; Ramsundar et al., 2016; Petrovič and Simonič, 2016; Hamed et al., 2017). To improve the tolerance of algae to metal stress, and to maximize the potential application of algae in bioremediation, it is important to understand how algae will respond to heavy metals. Therefore, we studied the differential responses of two common green microalgae;*C. sorkiniana* and *S. acuminatus*, at oxidative stress levels and how their antioxidants defence systems are affected after long term exposure to Cu stress.

2. Materials and methods

2.1. Algal strains

The green microalgal species *Chlorella sorokiniana* and *Scenedesmus acuminatus* were obtained from the Culture Collection of Algae, Hochschule Bremen University, Germany, and were cultivated axenically in 200 ml of optimized culture medium (OCM) medium as described by Arroyo et al. (2011) in 500 ml Erlenmeyer flasks.

2.2. Effect of different copper concentrations on microalgal growth

The algae were grown under laboratory conditions in 100 ml of sterile optimized culture medium (OCM). The culture media were spiked with different concentrations of Cu, *i.e.*, control (0.32), 25, 50,100 and 400 μM in the form of $CuCl_2{\cdot}2H_2O$. The growth conditions were; 100 μmol photons $m^{-2}\,s^{-1}$ of light intensity with a photoperiod of 16/8 h (light/dark) at 25 ± 2 °C, agitated at 120 rpm in orbital incubator shaker (GFL-Gesellschaft für labortechnik mbH, D-30938 Burgwedel, Germany). The growth characteristics of *C. sorokiniana* and *S. acuminatus* were determined on basis of cell count and optical density at 700 nm. All reported data are the mean of three biological replicates.

2.3. Effects of sub-lethal doses of Cu on microalgal growth

C. sorokiniana and *S. acuminatus* were grown photoautotrophically during first cultural phase in a 2 L vessel containing 1300 ml OCM medium, inoculated by130 ml of an axenic culture, at the exponential growth phase, The cultures were kept at same growth conditions as aforementioned for 10 days. 250 ml of culture suspension was collected from each culture vessels and centrifuged at 5000 rpm for 15 min under sterilized conditions. The obtained microalgal pellets were placed into 500 ml culture flask units of Sixfors photobioreactor INFORSAG-CH4103 (Bottmingen, Switzerland), filled with OCM medium under either control or stressed conditions (sub-lethal dose of Cu concentration 25 and 50 μM for *C. sorokiniana* and *S. acuminatus* respectively). Cell counts at time zero were normalised in all treatment to 48×10^5 and 25 $\times10^5$ for *C. sorokiniana*, *S. acuminatus* respectively. All culture units were illuminated by tubular fluorescent lamps (PHILIPS Master TL-D 85 W/840). The light intensity at the surface of the culturing vessels was 100 μmol photons $m^{-2}\,s^{-1}$ with a photoperiod of 16/8 h (light/dark). Stirring speed, internal temperature, pH and dissolved oxygen were kept under control in all photobioreactor units (150 rpm, 25 ± 1 °C, pH 7, 90–100% dissolved oxygen).

2.4. Specific growth rate

After 7 days of incubation, cell density (number of cell ml^{-1}) was determined by using Zeiss Axiostar Plus light microscope (Carl Zeiss, Germany) and a Thoma Neu chamber with a depth of 0.1 mm (Paul Marienfeld GmbH & Co. KG, Germany). Cell suspension was diluted with OCM medium to give an appropriate cell concentration. The counting at least 25 squares was ensured and the error was less than 10% (Venrick, 1978). Specific growth rate μ were calculated using the equation described by Guillard (1973).

$$\mu = ln\,(N_{(t)}/N_{\mathbf{0}})\,(t{-}t_{\mathbf{0}})^{-\mathbf{1}}$$

Where N_t and N_0 are defined as the cell density (cells ml^{-1}) at times t (7 days) and t_0 at time zero, respectively. Obtained values were expressed as average ± standard error (S.E).

2.5. Total Cu content

Algal pellets were freeze-dried in a low freeze-drier and digested in HNO_3/HCL in a microwave oven and determined by mass spectrometry (ICP-MS, Finnigan Element XR, Scientific, Bremen, Germany). Samples were treated with10 ml of HNO_3 (10 mol/l) and 2 ml of HCl (10 mol/l). Lutetium (Lu) was added as internal standard. The dissolutions were cooled, and then the samples were filtered, transferred to volumetric flasks, and filled up with Milli-Q water. Before the ICPSFMS measurements, the samples were diluted with Milli-Q water. Optimization of the method was made with a standard solution of approximately 10 μg/l of Cu, coming from a standard dissolution that contained nearly 1 μg/l (Merck, Germany). Analysis of each sample was carried out in triplicate.

2.6. Chlorophyll content

Chlorophyll (*a* and *b*) and total chlorophyll content in the algal cultures were determined spectrophotometrically according to method based on Mackinney (1941). 5 ml of algal culture was centrifuged at 5000 g for 10 min; the supernatant was dried and re-suspended in 1 ml distilled water and 4 ml acetone. Samples were stored in the freezer overnight for complete chlorophyll extraction then, were re-centrifuged for 5 min. The optical density was measured at 645 nm and 663 nm using 80% acetone as a blank. The chlorophyll concentration (mg/l) of each algal extract was calculated using the following equation described by Mackinney (1941):