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Toxicity and Accumulation of Copper in *Nannochloropsis oculata* (Eustigmatophyceae, Heterokonta)

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Abstract: Algal communities play a crucial role in aquatic food webs by facilitating the transfer of essential trace elements and toxic compounds for a wide variety of organisms even to high trophic levels. Economically, algae can be utilized for the removal of heavy metals from industrial wastewater or other sources. In the present study, the toxicity and the ability of *Nannochloropsis oculata* (Heterokonta) to accumulate copper metal was investigated. *N. oculata* was cultured in $f/_2$-enriched sea water medium, which was supplemented with 0.04 μM (control), 0.10, 0.20, 0.30 and 0.40 μM Cu^{+2} and harvested after 3, 6, 9, 12 and 15 days. Changes in cell numbers; specific growth rate; chlorophyll-a concentration; copper accumulations and changes in algal cell ultra structure were monitored. Results revealed significant ($P \leq 0.05$) decreases in cell numbers; growth rate and chlorophyll-a content with increasing exposure time and concentration. There were significant ($P \leq 0.05$) increases in the accumulation of metal levels in algal tissue when the exposure time and concentration increased. The toxicity symptoms of Cu^{+2} were shown on *N. oculata* in the form of deformation and damage, disintegrated cell wall and death. It can be concluded that *N. oculata* is able to bind the copper in the culture media.

Key words: *Nannochloropsis oculata* · Growth rate · Copper accumulation · Chlorophyll-a · Ultra structure

INTRODUCTION

Members of the genus *Nannochloropsis* (Eustigmatophyceae) are widely distributed in the plankton of marine ecosystems, particularly in coastal waters [1, 2]. Algae are primary producers and affect the cycling of nutrients through marine, freshwater and aquaculture ecosystems [3,4]. They are useful organisms to assess metal contamination and bioavailability in aquatic systems, as they are sensitive to metal contaminants at environmentally relevant concentrations [5]. Copper is an essential micronutrient for plants and algae, being components of several proteins and enzymes involved in a variety of metabolic pathways [6], but it can also be a toxic element when applied in amounts higher than its particularly level. Algae are capable of regulating their internal cell environment and also their immediate surroundings, e.g. through the production of exudates with metal binding capacity [7,8]. Green algae appeared to be more tolerant to metals such as zinc, lead and copper than blue green algae and diatoms [9]. The microalgae, *Chlorella* sp. and *Scenedesmus* sp., investigated by Stokes and Hutchinson [10] are able to bioaccumulate up to 2400 mg Cu^{+2}/kg on a dry weight. Heavy metal accumulation by microalgae is well documented [11]. Uptake of metal into algal cells is considered to be a two-part process when the solution indirect contact with the cell, the diffusion layer, is at equilibrium with the surrounding bulk medium [12]. Firstly, fast metal adsorption to sites on the exterior of the cell membrane occurs, with the metal-binding sites consisting of both metabolically active sites at which copper may enter the cell and non-active sites. The second step in metal uptake is the internalization of metal across the cell membrane. It is hypothesized that metal internalization occurs via ion pores, channels or transporters in the algal cell membrane. Previous research has shown that inter-species differences in the sensitivity of microalgae to copper were not related to the adsorption of copper to a variety of different algal cell walls and surfaces [13]. In fact, it is known that algal cell walls, being porous, allow free passage of molecules and ions [14]. In addition, cell wall constituents provide an array of ligands with functional groups able to bind various metallic species. Copper was chosen as the contaminant of concern due to its high aquatic toxicity at environmentally relevant concentrations and increasing use as a replacement for tributyltin in antifouling biocides [5,15]. The aim of the

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present study was to document the influence of copper concentrations on the toxicity of the green alga (*Nannochloropsis oculata*). By monitoring the parameters of population growth including the number of cells, growth rate and the amount of chlorophyll-a. Also, measuring of copper accumulation and the change in cell ultra structure were investigated.

MATERIALS AND METHODS

Algal Culture: The culture of *Nannochloropsis oculata* was taken from National Institute of Oceanography and Fisheries, Alexandria, Egypt. Cultures were grown in $f/_2$-enriched seawater medium without sodium meta silicate in which copper concentration was 0.04 μM, pH was 8 and salinity was 25‰ [16], all solutions were autoclaved at 121°C and 15 psi for 20 min. The culture stock copper solution ($CuSO_4$. $5H_2O$) was prepared in bi-distilled water and sterilized by passing through Millipore membrane filters (0.22 μm), before supplementing to the culture medium, 250-mL Erlenmeyer flasks containing fresh $f/_2$-enriched seawater and the total experimental volume was 150 ml; copper was added to each test vessel containing culture media to gives the required range of copper concentrations of 0.04 μM (control), 0.10, 0.20, 0.30 and 0.40 μM, respectively. The microalgae were cultivated to the exponential growth phase for use; the initial cell density of *N. oculata* was set at 2×10^5 cell/ml throughout the experiment. Culture was kept illuminated with fluorescent lamps with photoperiod 24:0 h at 25±1°C under continuously controlled conditions. All flasks containing the *N. oculata* were shaken manually twice daily at set time. All the glass wares were washed with 20% HCl and rinsed thoroughly with distilled water, prior to use, so as to prevent the binding of metals to the wares.

Growth Measurements: Cell densities were determined after 3, 6, 9, 12 and 15 days. A sample of 0.5 ml was collected and preserved in Lugol's solution to monitor the growth of the algal species by direct counting the cell numbers using a haemocytometer under research microscope and growth curves were formed using the cell numbers. Growth rates were determined by using the equation $K = \log (N1 / N_0) (3.322 / t)$ [16], where N1 and N_0 were the measured final and initial cell densities, respectively at time t. For chlorophyll-a analysis, a 5 ml aliquot of the microalgae solution was filtered through a 0.45 μm filter paper. The filter paper and its contents were placed in test tubes, 5 ml of 90% methanol was added and the tube was heated in a water bath at 60-70°C for 2 min. Following centrifugation at 3500 rpm, the supernatant was with-drawn by using a Pasteur pipette and transferred to a 1cm cuvette of the spectrophotometer. The absorption measurement was done at 665 nm and the chlorophyll-a content was calculated according to Talling and Driver [17] as following:

$$\text{Chl.-a (mg/l)} = 13.9.D_{665}$$

Copper Accumulation: For the determination of cellular metal content, 10 ml of culture suspension was withdrawn at different time points of the experiment. The algal suspension was then centrifuged to harvest the cells, the pellet was transferred to 5 ml of digestion mixture containing HNO_3 (70%), H_2O_2 (30%) and de ionized water at a 1:1:3 ratios [18]. Digestion was performed on a hot plate at 80°C until the solution became colorless. The residue was dissolved in 2% (v/v) nitric acid. The samples were analyzed for Cu^{+2} metal content with an atomic absorption spectrophotometer (Shimadzu, AAS-6800).

All the experiments in this study were done separately in at least triplicate.

Toxicity Symptoms: After the exposure to Cu^{+2} the algae were harvested at the end of test duration (15 days). Toxicity symptoms of treated (0.40 μM) and control algae (0.04 μM) were observed using transmission electron microscopy (TEM).

Transmission Electron Microscopy (TEM): The algal cells were collected by centrifugation and were fixed with 5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 2 days at 4°C. After fixation, algal cells were harvested by centrifugation at 2000 x g for 1 min. the pellet was rinsed three times with 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% aqueous osmium tetroxide for 2 h. Samples were dehydrated through a graded ethanol series to acetone and embedded in Epon 812. Ultrathin sections were stained in 2% uranyl acetate and lead citrate and examined with a JIOL JEM.1010 transmission electron microscope in the regional center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Statistical Analysis: The mean values (with triplicate samples) of cell number and growth rate, chlorophyll-a content and metal accumulation were calculated and subjected to analysis of variance (ANOVA) using randomized block design and Least Significant Difference

method (LSD) on the SPSS for Windows program at a probability level of $P \leq 0.05$.

RESULTS

When *Nannochloropsis oculata* was exposed to different concentrations of copper (0.10, 0.20, 0.30 and 0.40 μM), a reduction in the maximum cell density; and chlorophyll-a concentration were observed after 9 days of incubation. Nevertheless, *N. oculata* control cultures (0.04 μM) grew well and had a maximum cell density (Fig. 1) and growth rate (Table 1) after 15 days of incubation. The results showed that, as the concentration of copper in the test solutions increased, cell density, algal growth rates decreased. The growth of *N. oculata* exposed to Cu^{+2} at every concentration were significantly decreased ($P \leq 0.05$) from those of controls. At high metal concentration (0.40 μM), algal growth was reduced by 31.3% than control after 15 days of incubation. The lowest growth was found in algae treated with Cu^{+2} at 0.40 μM as shown in Fig. 1.

The effects of Cu^{+2} on chlorophyll-a content of *N. oculata* at different concentrations and exposure times are shown in Fig. 2. There were significant decreases ($P \leq 0.05$) of chlorophyll-a content when the exposure time and metal concentration increased. Chlorophyll-a content of *N. oculata* exposed to Cu^{+2} at every concentration decreased significantly from controls after 9 days of exposure ($P \leq 0.05$). When the algal species exposed to higher copper concentration (0.40 μM), the lowest chlorophyll-a content were found and amounted 26.4 % as compared with control on day 15.

Copper accumulation by *N. oculata* at different concentrations and exposure times are shown in Fig. 3. There were significant increases ($P \leq 0.05$) of metals in algal tissue when the exposure time and metal concentration increased. At Cu^{+2} concentrations of 0.04 μM (control), 0.10, 0.20, 0.30 and 0.40 μM, the maximum Cu^{+2} accumulation in *N. oculata* were observed on day 15 (Fig. 3). The results showed that, the extent of toxicity increased with increasing concentration of the metals.

Table 1: Growth rates (μ) of *N. oculata* cultures at different copper concentrations

	Conc.				
Days	Control	0.10μM	0.20μM	0.30μM	0.40μM
3	0.53±0.03	0.48±0.01	0.44±0.02	0.4±0.02	0.35±0.02
6	0.29±0.03	0.28±0.01	0.24±0.02	0.2±0.02	0.19±0.02
9	0.10±0.01	0.1±0. 02	0.11±0.01	0.07±0.01	0.06±0.02
12	0.09±0.01	-0.05±0.03	-0.06±0.03	-0.05±0.03	-0.04±0.02
15	0.11±0.01	-0.05±0.03	-0.06±0.03	-0.01±0.01	-0.05±0.03

Values are means±SE. (n = 3)

Changes in Cell Ultra Structure: The cell ultra structure of *N. oculata* was examined in control cells (0.04 μM) and cells exposed to higher copper concentration (0.40 μM) after 15 days of incubation are shown in Fig. 4. *Nannochloropsis oculata* is a tiny unicellular alga, changes in cell shape are shown for the copper-treated cells in Fig. 4 (B, C, D and F), in all cases there were obvious changes in the fine structure of the cell wall and the surrounding confluent mucilage of the vegetative cells. These changes consisted mainly of a much darker staining areas of the cell walls with vacuolated cytoplasm (Fig. 4B). Transmission electron microscopy indicated that copper led to the most drastic morphometric changes, indicated by cell swelling, folding, shrinking and lyses (Fig. 4C) as well as cell damage (Fig. 4D). The main ultra structural changes were increased number and size of vacuoles in the cell, increased number and volume of starch grains and vacuoles as well as the presence of electron dense deposits in vacuole and membrane whorls (Fig. 4E and F) and a slight disappearance of gas vacuoles, in addition there were deformation and elongation in the nucleus.

DISCUSSION

Copper salts have been used as biocides for a long time, but their use has been limited in recent years due to concerns of heavy metal contamination. In the present study, there were comparatively important differences between the Cu^{+2} concentrations in *N. oculata* cells measured at the end of the 15 days. It was found that the rate of the cell division was lower in treated than in the control algal cells. Therefore, the inhibition of cell division and algal growth rate due to Cu^{+2} addition in the media was obvious . Lopez-Suarez *et al.* [18] carried out an experiment to determine the ability of *Chlorella vulgaris* to accumulate heavy metals in solution with Cu^{+2}concentration of 0.639 mg/l. They concluded that, metal concentrations in cells vary considerably both with species and environment [19]. Copper concentrations, for example, were 65 and 652 μg/g, in the marine microalgae *Dunaliella tertiolecta* and *Tetraselmis suecica*, respectively [20]. Yılmaz *et al.* [21] showed that, *Tetraselmis chuii* can accumulate copper in its cells, raising 9, 13, 42, 43 times as compared with the control when the culture medium containing 0.23, 0.32, 0.64, 0.96

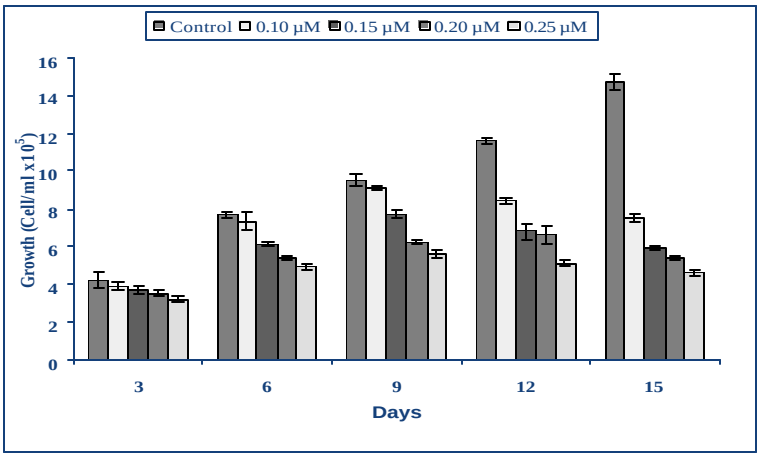

Fig. 1: Cell numbers (Cell/ml x10^5) of *Nannochloropsis oculata* cultures

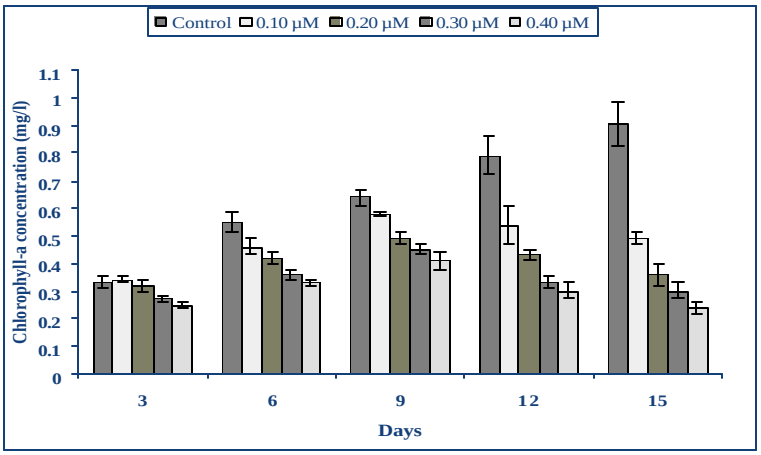

Fig. 2: Chlorophyll-a concentrations of *Nannochloropsis oculata* cultures

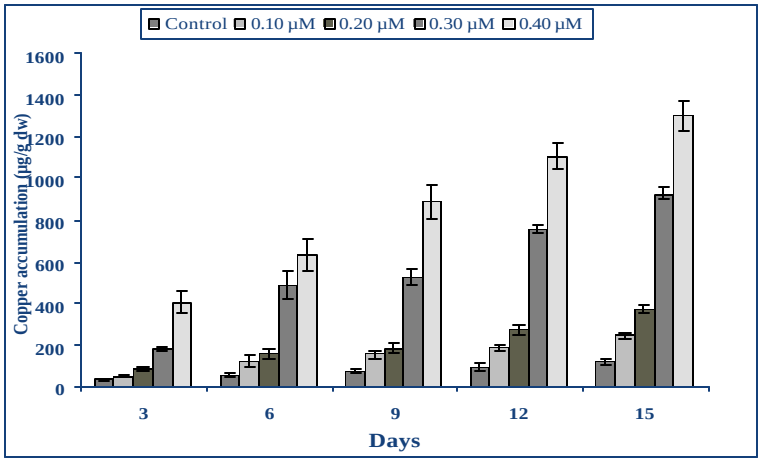

Fig. 3: Accumulation of copper in *Nannochloropsis oculata* from experimental groups

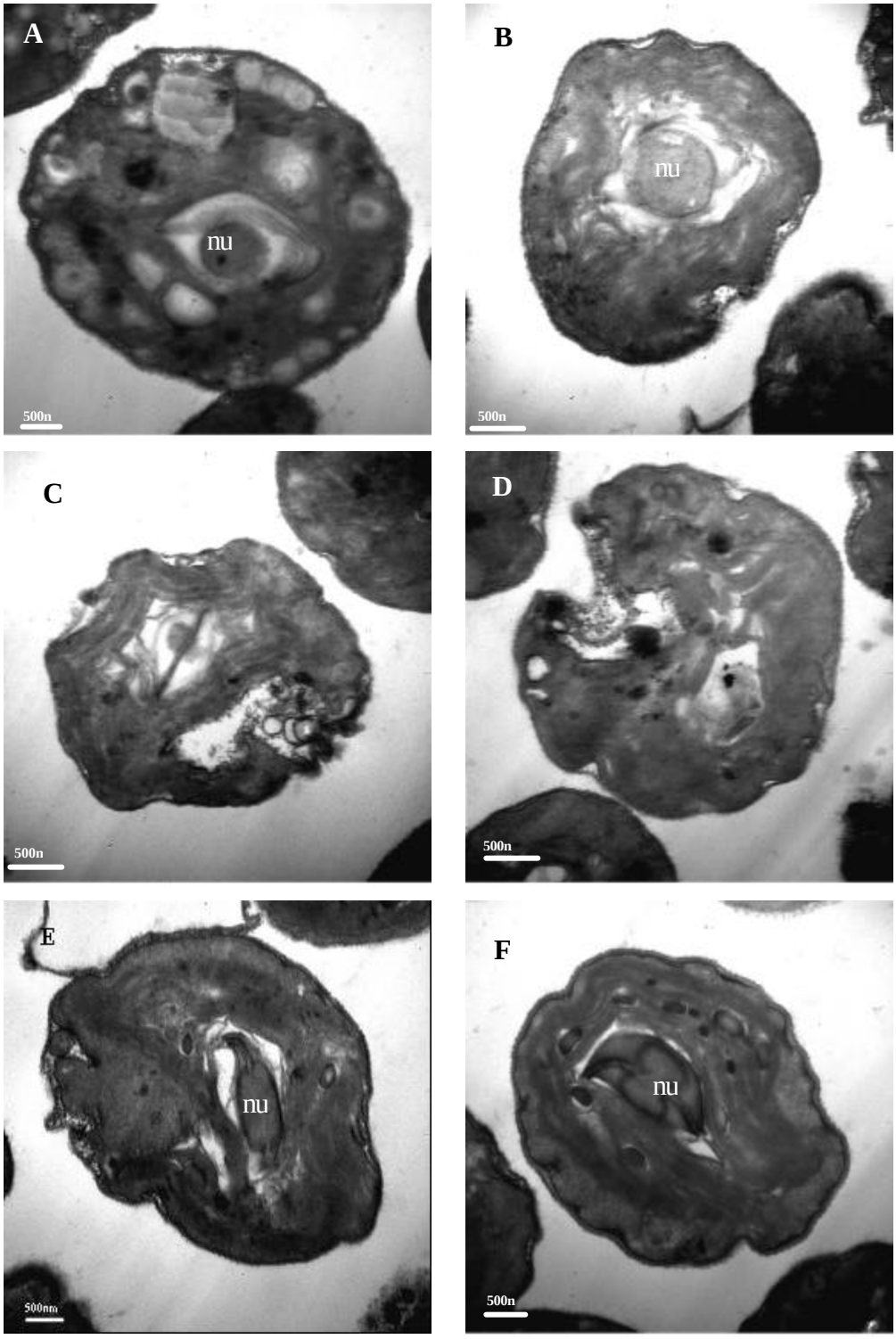

Fig. 4: (A)-Transmission Electron Micrograph of cells of *Nannochloropsis oculata* cultured at copper concentration 0.04 µM. Note the normal cell structure. (B, C, D, E and F)Electron micrographs of *Nannochloropsis oculata* cells exposed to 0.40 µM copper after 15 days of incubation. Note the changed in the cell shape. (nu = nucleus)

and 1.28 ppm Cu^{+2}, respectively. In the medium contained 0.20 and 1.00 mg/l Cu^{+2}, *Pavlova viridis* accumulated 125 and 599 μg/g dw, respectively [22]. Fargasova [23] studied the effects of Cd; Cu; Zn; Pb and Fe on the green alga *Scenedesmus quadricauda* and found that the toxicity for all the observed parameters increased with the concentration of these metals in the cultivation medium. *Nannochloropsis oculata* is a tiny unicellular alga about 3 mm in diameter belonging to Eustigmatophyceae (Heterokonta) [24]. Some changes in cell ultra structure were observed upon exposure to higher copper concentrations. *N. oculata* cells were larger in size and clumped together and an increased number of vacuoles. In this study, noticeable ultra structural alterations were observed after 15 days of culture and they were clearly discernible in the highest concentrations. However, there were obvious changes in the fine structure of the cell wall. The most obvious change upon copper exposure was the cell swelling and clumping, these results agree with Levy *et al.* [25] for *Phaeodactylum tricornutum.* These changes consisted mainly of a much darker staining of the cell walls. This could be attributed to the interaction of the heavy metal ions with a variety of organic ligands, which chelate the metal, thus rendering it non-toxic [26]. Metal sequestration in the cell wall [27] and wall anomalies caused by heavy metals have been described for some algae. In *Thalassiosira* iron and copper were concentrated in the cell wall but it was impossible to detect metals in other parts of the cell [28]. Further ultra structural changes may have been observed at higher copper exposure concentrations. The clumping of algal cells could be due to copper oxidation of intracellular thiols, which has been hypothesized to inhibit mitotic spindle formation and consequently inhibit cell division in the marine diatom *Nitzschia closterium* [29]. Thus algae continue to photosynthesize and fix carbon but cell division is impaired, leading to swollen and enlarged cells that clump together. Recent research by Stoiber *et al.* [30] supports this hypothesis. They have shown a decrease in the growth rate in the fresh water green alga *Chlamydomonas reinhardtii* after a 24-h copper exposure. Heavy metals from polluted aqueous systems may be removed by phytoplanktonic algae. It is concluded that this method, including the separation of the metal saturated algae from the medium, is an economic method for removing heavy metals from waste waters, resulting in high-quality re usable effluent water and valuable algal biomass, which could be used for different purposes for example bio fertilizer, living food or biogas [31,32]. As a result, *N. oculata* cells removed copper from the medium and harvested can be evaluated for reusing. On the basis of this study, it may be concluded that *N. oculata* cells that able to uptake relatively high concentrations of Cu^{+2} and promising to remove copper from the solutions.

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