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Original article

Effect of Copper on Marine Microalga *Tetraselmis suecica* and its Influence on Intra- and Extracellular Iron and Zinc Content

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Abstract In an aquatic environment, toxicity of metals to organisms depends on external factors (type of metal, exposure concentration and duration, environmental parameters, and water quality) and intracellular processes (metal-binding sites and detoxification). Toxicity of copper (Cu) on the marine microalga *Tetraselmis suecica* was investigated in this study. Dose-dependent (Cu concentration dependent) inhibition of growth and cell division, as well as, variation of intra- and extra-cellular Cu, Fe and Zn content was observed. *T. suecica* was sensitive to Cu; the 96 h EC_{50} (concentration to inhibit growth-rate by 50%) of growth rate (μ) (21.73 μM L^{-1}), cell division day^{-1} (18.39 μM L^{-1}), and cells mL^{-1} (13.25 μM L^{-1}) demonstrate the toxicity of Cu on this microalga. High intra- (19.86 Pg $cell^{-1}$) and extra-cellular (54.73 Pg $cell^{-1}$) Cu concentrations were recorded, on exposure to 24.3 and 72.9 μM L^{-1} of Cu.

Key words: copper, growth, intra-extracellular, *Tetraselmis suecica*

INTRODUCTION

Expansion of industrial sectors has amplified emission of pollutants in the ecosystems. Heavy metals are the most frequently recognized pollutants in aquatic environment. In a biological context, heavy metals are categorized into: essential and non-essential. Essential heavy metals could be toxic at elevated concentrations (Carfagna *et al*., 2013), for example, cobalt (Co), copper (Cu), zinc (Zn) and manganese (Mn) (Al-Hejuje, 2008). Metals could interfere with a wide spectrum of activities of living organisms, including metabolism and growth; keeping this in mind, the effects of heavy metals on aquatic organisms have been widely studied. Their influence on marine ecosystems, their possible transfer to food chain, and consequently the resulting human health problems have been conversed frequently (Nassiri *et al*., 1996; Volland *et al*., 2014).

Aquatic organisms take-up and accumulate metals, whether essential or not, with the potential to cause toxic injury. These metals could be classified into: 1) metal that could be detoxified, and, 2) metal that is metabolically available to satisfy essential needs or, in extreme circumstances, to interact in a way that manifests itself as a toxic response (Adams *et al*., 2011). Toxic effects of metals include: blocking functional groups of biologically important molecules (e.g., enzymes and transport systems for essential nutrients and ions), displacement and/or substitution of essential metal ions from biomolecules and functional cellular units, and, induction of cellular generation of reactive oxygen species (ROS; including superoxide anion, hydrogen peroxide, sin-

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glet oxygen, and hydroxyl radical) (Hossain *et al*., 2012). High ROS levels could oxidize proteins, lipids, and nucleic acids and thereby result in modification and inactivation of enzymes as well as disruption of cellular and organelle membrane integrity (Kaplan, 2013). In fact, heavy metals decrease the chlorophyll content, chlorophyll *a*/*b* ratio, phaeophytin levels, and increase the protochlorophyll levels and carotenoid/chlorophyll ratios of algae (Aggarwal, *et al*., 2011).

Copper (Cu) is a trace element essential for all living organisms, which acts as a structural element in regulatory proteins, and participates in electron transport in photosynthesis, mitochondrial respiration, oxidative stress responses, cell wall metabolism and hormone signaling (Čypaitė *et al*., 2014). It is particularly an essential micronutrient for growth, metabolism and enzyme activities of various algae, cyanobacteria and other organisms (Zhang *et al*., 2014). However, slight increase in endogenous Cu^{2+} concentrations (above optimum level) interferes with various metabolic pathways, causing inhibition of photosynthesis, respiration, ATP production, pigment synthesis, as well as, inhibition of cell division (Kumar *et al*., 2014). The toxicity of Cu is mainly due to the prevalence of two readily inter-convertible oxidation states, which makes it highly reactive; it can catalyze the formation of free radicals through Haber-Weiss reaction (Kanoun-Boulé *et al*., 2009).

Microalgae, forming the basis of most freshwater and marine ecosystems, are sensitive indicators of environmental change; they are widely used in risk assessment and development of environmental regulations (Levy *et al*., 2007). In the aquatic ecosystem, algae are considered as the best bio-indicators of heavy metals contamination (Al-Hejuje, 2008). They are responsible for the base production, and any change encountered in them would influence the higher trophic levels. Several studies elaborate the physiological effect of Cu on microalgae, for e.g. Kumar *et al*. (2014). In a physiological context, metal exposure could cause a concentration-dependent inhibition of growth, photosynthesis, respiration, nitrate uptake and nitrate reductase activity, as well as, a reduction in protein, carbohydrate, and photosynthetic-pigment levels, with a concomitant increase in intracellular levels of the test metals in algae (e.g. *Scenedesmus* sp.; Tripathi and Gaur, 2006).

The prasinophyceae *Tetraselmis* is a basic food organism in aquaculture; it is distributed throughout the world (Nassiri *et al*., 1996). It is an important source of antioxidants and is used in marine ecotoxicological testing (Lee and Hur, 2009). Nassiri *et al*. (1996) have discussed mechanisms of metal detoxification by *T. suecica*. However, this study evaluates the impact of Cu on the growth of *T. suecica* and its photosynthetic pigment (chlorophyll *a*) content, as well as, intracellular and extracellular intake of Cu, Fe and Zn.

MATERIALS AND METHOD

1. Sample collection and culture maintenance

Tetraselmis suecica was obtained from Korea Marine Microalgae Culture Center (KMMCC). They were cultured in artificial seawater medium, prepared by dissolving commercial sea salts (Coralife, Energy Savers, California, USA) in deionized water (salinity 15‰) enriched with Walne's medium (Walne, 1970). Unialgal cultures were maintained at 20°C for about 20 generations before use in experiments. During this period, cultures were illuminated with cool white fluorescent tubes that provided 130 µmol photonsm^{-2} s^{-1} of Photosynthetic Active Radiation (PAR; 16L : 8D).

2. Exposure

Copper stock solution was prepared using Copper II Chloride (Sigma, Cat. No 7447-39-4). The stock solutions were diluted with Milli-Q water to obtain the desired concentrations (0, 0.9, 2.7, 8.1, 24.3, 72.9 µM L^{-1} Cu). Toxicity of Cu was tested using three replicates; moreover, each set comprised a control with no copper. Long-term 96 h exposure experiments were carried out in sterile narrow necked polycarbonate bottles (1 L capacity, Nalgene) containing different Cu concentrations. Approximately 750 cells mL^{-1} of *T. suecica* was spiked into each bottle. The culture conditions and setup was similar to that mentioned above.

3. Specific growth rates

The specific growth rate (µ) was calculated as $\mu = (\ln C_1 - \ln C_0)/(t_1 - t_0)$, where C_1 and C_0 were the cell densities at time t_1 and t_0 ($t_1 - t_0 =$ days), respectively.

4. Chlorophyll *a* (Chl *a*) content

In order to determine the Chl *a* content, 100 mL of culture

was filtered (Whatman GF/F filters, 25 mm). The cells were then subjected to methanol extraction overnight, followed by centrifugation (5°C for 10 min at 5000 × g). The absorbance of the supernatant was measured between 200-800 nm. The concentration of the photosynthetic pigment was calculated based on the equation of Porra (2002).

5. Oxalate reagent preparation

An oxalate solution prepared according to Tovar-Sanchez *et al*. (2003) was supplemented with 0.5 mL of Hydroxylamine (1.44 M), 6.5 mL of Perchlorate (0.008 M) and 13 mL of 1,10 phenanthroline (0.055 M). The pH was adjusted to 8 with NaOH, followed by incubation in a water bath (50°C for 15 min). The solution was immediately transferred to a 250-mL Teflon separatory funnel while the solution was still hot; it was extracted twice using 6 and 4 mL of 1,2-dichloroethane ($C_2H_4Cl_2$). During each extraction, the mixture was vigorously shaken for 2 min and allowed to stand for 15 min for phase separation. The organic phase was discarded and aliquots of the reagent were collected. This oxalate reagent was transferred to an acid-washed polyethylene bottle. The bottle was left open for two days in a laminar flow unit, where it was periodically shaken to remove the excess volatile solvent.

6. Estimation of Intra- and Extra-cellular metal concentration

Quantification of intracellular Cu was carried out by filtering 100 mL of the sample through a 0.45 μm polycarbonate filter paper. The cells retained on the filter paper were thereafter rinsed with 20 mL oxalate reagent. This mixture was allowed to stand for 5 min after which the filtrate was discarded. The filter paper (containing the cells) was thereafter digested using a 3 : 1 mixture of concentrated HNO_3 and HCL, evaporated to dryness and re-dissolved in 2%HNO_3 to obtain a final volume of 30 mL. The metals Cu, Fe and Zn were analyzed using Inductively Coupled Plasma Mass Spectrometer (ICP-MS; Thermo-Elemental X7). Quantification of total Cu concentration was carried out using the aforesaid protocol, but, no oxalate reagent was used in this case. The extracellular Cu concentrations were determined by calculating the difference between the total and the intracellular Cu concentration. The impact of Cu on intra- and extra-cellular Fe and Zn content of this microalga was established using ICP-MS.

7. Statistical analyses

Data were expressed as mean ± 95% confidence interval. Significant differences between control and treated samples were determined using One-way analysis of variance (ANOVA), wherein values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Heavy metal-algae interactions suggest that initial metal binding to the algal cell occurs at the cell wall, which has a negatively charged external layer, surrounded by extracellular polymeric substances such as sulfated polysaccharides, glycoproteins, and lipids. Particularly initial copper binding at the cell wall may be to protein carboxylic and amino residues, rather than to thiol groups (Wilde *et al*., 2006). Thereafter, Cu accumulates and migrates through this layer to the plasma membrane, where it binds with physiologically inert sites, physiologically active sites (directly affecting cell membrane functions), or could be transported by facilitated diffusion or active uptake into the cell (Campbell *et al*., 2002). Once within the cell, Cu affects cell processes such as photosynthesis, enzyme activity, and cell division. Cu competes for binding sites of enzymes (e.g. urease, acid phosphatase and ATPase), and can also inhibit other enzymes of nitrogen metabolism and photosynthesis (Rai and Rai, 1997). In the cytoplasm, Cu could either inhibit enzymes such as esterase and β-D-galactosidase, or cause changes in intracellular pH. Cu is reported to oxidize glutathione (reduced form GSH to oxidized form GSSG) in the cytoplasm *Nitzschia closterium*, thereby causing disturbance of the GSH : GSSG ratio, and suppression of mitosis (Stauber and Florence, 1987). Cu also affects subcellular organelles such as the chloroplast and mitochondria; it causes structural alterations to thylakoid membranes, and also impacts chl *a* fluorescence and thereby photosynthesis (Wilde *et al*., 2006). However, in one study of Wilde *et al*. (2006), Cu concentrations were inhibitory to cell division but had no effect on other cell functions such as photosynthesis, respiration, ATP production, electron transport, and membrane

ultrastructure; they reported that the cells photosynthesized but were unable to divide, leading to an increase in cell size. On the other hand, Nassiri *et al.* (1996) evaluated Cu toxicity to *T. suecica*, stating that the presence of Cu in walls of a multilayered cell suggests that these structures constituted an additional adsorbing area for this element, and this helped reduce the free metal concentration in the medium.

1. Effect of Cu on algal growth rate

As growth reflects the proper functioning of various physiological and biochemical processes within the cell (photosynthesis and nutrient uptake), and can be easily monitored in laboratory, it has been used as a key indicator of toxicity of metals to microalgae. Growth inhibition in microalgae is directly related to the amount of metal ions bound to the cell surface or taken up intracellularly, besides correlating with the chemical nature of the metal at stake (Monteiro *et al.*, 2012).

Fig. 1a and b elucidate the effect of Cu on the growth of *T. suecica* in terms of growth rate (μ) and cell division. Although Cu serves as an essential micronutrient for several physiological processes at low concentrations, it is toxic at higher concentrations. In this study, a concentration dependent growth inhibition of *T. suecica* was evident; there was a slight increase in growth (upto 2.7 μM L^{-1} Cu in case of growth rate, cell division day^{-1} and cells mL^{-1}; Fig. 1) in the presence of lower Cu concentrations, nonetheless, higher concentrations of Cu was inhibitory (i.e. >20 μM Cu caused decreased growth of *T. suecica*); this could be explained by the fact that Cu^{2+} causes massive failure of many cellular processes and thereby influences algal growth (Li *et al.*, 2010). A few other reports elaborate a similar effect of Cu on *S. capricornutum*, *Chlorella* sp. (Franklin *et al.*, 2002a), *P. subcapitata* (Čypaitė *et al.*, 2014), *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (Zhou *et al.*, 2012). Rising Cu concentrations are also known to inhibit growth of *Scenedesmus incrassatulus* (Perales-Vela *et al.*, 2007). In this context, Zhang *et al.* (2014), studied Cu-spiked *Chlorella vulgaris* and described that high Cu concentrations caused substantial decrease in organic osmolytes (betaine and glycerol phosphocholine), which was an implication of Cu-induced redox imbalance; this was also accompanied by growth inhibition and photosynthesis impairments, which in turn revealed a clear relationship between Cu toxicity and redox homeostasis. According to Zhou *et al.* (2012), high Cu concentration decreased the photosynthetic pigments and destroyed algal cell ultrastructure. In another gene transcriptional study, Wei *et al.* (2014) reported several respiratory-related genes (nad5, SDH2, and cox3) and photosynthesis-related gene transcripts (psbD, petD, psaB and petF) of *Phaeodactylum tricornutum* to be strongly decreased after a 48 h exposure to 20 or 40 μM Cu. As per Wei

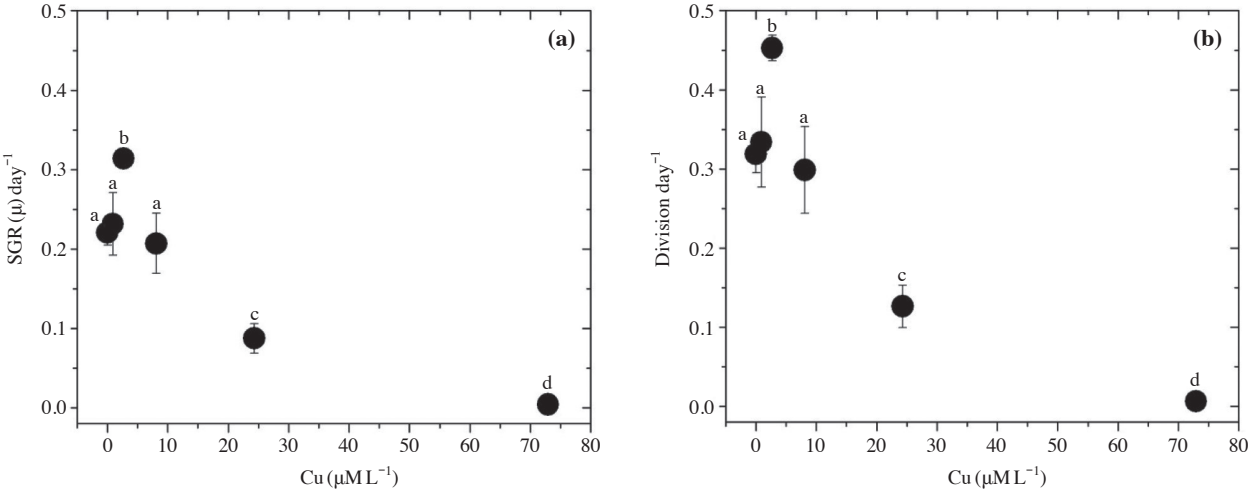

Fig. 1. Impact of Cu (96 h) on (a) Specific growth rate or SGR (μ) and (b) cell division of *Tetraselmis suecica*. Data points representing mean ± 95% confidence interval are shown.

Table 1. EC_{50} values for growth inhibition of various algae.

Period of exposure	Organism	EC_{50} ($\mu M\ L^{-1}$)	Reference
48 h	*Chlorella* sp.	0.30	Wilde *et al.* (2006)
	C. vulgaris	2.63	Qian *et al.* (2009)
	C. vulgaris	15.6	Abreu *et al.* (2014)
72 h	*Planothidium lanceolatum*	9.78	Levy *et al.* (2007); Sbihi *et al.* (2012)
	Dunaliella tertiolecta	8.34	
	Tetraselmis sp.	0.74	
	Emiliania huxleyi	0.31	
	Nitzschia closterium	0.28	
	Minutocellus polymorphus	0.009	
96 h	*S. obliquus*	0.79	Yan and Pan (2002); Lim *et al.* (2006)
	C. pyrenoidosa	1.07	
	C. lunula	3.15	
	T. suecica	20.46	
	D. tertiolacta	21.09	
	T. suecica	21.73	This study
120 h	*Isochrysis galbana*	22.03	Sbihi *et al.* (2012)

et al. (2014), this decrease in gene transcription could lead to a decrease in the net synthesis of proteins in the electron transfer chains; in turn, the cells would not over-express cellular redox proteins in response to Cu stress, but rather down-regulate or decrease their net synthesis due to toxic effects of Cu or to cope with a lower demand in cellular energy produced by the electron transport chain machinery due to slower growth rates at high Cu concentrations. This could probably explain the slow growth rates of *T. suecica* encountered in our studies at high Cu concentrations.

The effects of Cu on the growth of algae, depends on the species used, the composition of the culture medium and the experimental protocol used (Perales-Vela *et al.*, 2007). The effective concentration of heavy metal that causes 50% inhibition of algal growth (EC_{50}) is widely used as an index of toxicity (Regaldo *et al.*, 2013). After 96 h of exposure, the EC_{50} for growth rate and cell division day^{-1} of *T. suecica* were 21.73 and 18.39 $\mu M\ L^{-1}$ respectively; Table 1 compares the EC_{50s} of *T. suecica* obtained in this study with several other reports; this table testifies species dependent toxicity of Cu. The EC_{50} for growth rate and cell division day^{-1} of *T. suecica* obtained in our study were higher than the EC_{50s} of Yan and Pan (2002) but comparable with Lim *et al.* (2006). Rocchetta and Küpper (2009) observed 20-30 and 60-70% inhibition in the growth of *Euglena gracilis* on 96 h exposure to 10.07 and 49.89 $\mu M\ L^{-1}$ Cu^{2+} respectively. On another stance, Ouyang *et al.* (2012) observed that growth inhibition became weaker with the increase of exposure time in 5 $\mu M\ L^{-1}$ Cu exposed *C. vulgaris*, i.e. the percentage of inhibition (PI) were 85.5%, 67.8%, 55.05% and 38.3% after exposure times of 24, 48, 72 and 96 h. Even Zhang *et al.* (2014) observed growth inhibition in case of *C. vulgaris* exposed to 200 µM CuCl for 72 h.

Our study also evaluates the EC_{50} in terms of cell density; the 96 h EC_{50} for cell density for *T. suecica* was 13.25 $\mu M\ L^{-1}$. Most reports elucidate Cu induced reduction in growth rate (e.g. *Asterionella glacialis* and *Chlorella pyrenoidosa*; Pistocchi *et al.*, 1997), as well as, cell division and other biochemical composition of algae (e.g. *S. capricornutum*; Kim and Smith, 2001). Franklin *et al.* (2002a) report the cell density to influence inhibition of growth (cell division) rate; they observed that as the initial cell density increased from 10^2 to 10^5 cells mL^{-1}, the 72 h EC_{50} increased from 72.38 to 251.79 $\mu M\ L^{-1}$ for *Chlorella* sp. and from 103.86 to 267.52 $\mu M\ L^{-1}$ for *S. capricornutum*. Kebeish *et al.* (2014) too reported higher Cu concentrations (3 and 4.5 $\mu M\ L^{-1}$) caused a reduction in growth rate and cell density of *C. vulgaris*.

2. Effect of intra- and extracellular Cu concentrations on growth rate (µ)

Metal-algae interactions suggest that initial binding of a

metal to the algal cell wall takes place through the formation of coordination bonds between metals and the negatively charged amino and carboxyl groups of cell wall polysaccharides, glycoproteins, and lipids (Perales-Vela *et al*., 2007; Monteiro *et al*., 2012; Kumar *et al*., 2014; Kumar *et al*., 2015). However, the toxicity of metal to organisms is assumed to occur as the result of free metal ion reaction with the physiologically active binding sites and the accumulation at the binding sites is controlled by the free Cu concentration in aqueous phase (Ma *et al*., 2003).

Gonzalez-Davila *et al*. (1995) stated that Cu was bound to the cell wall of *Dunaliella tertiolecta* by two major binding sites, one with a high affinity for Cu and another with low affinity. Growth inhibition in microalgae is generally related to the amount of metal bound to the algal cell surface; particularly, in case of Cu, this inhibition is proportional to the amount of intracellular metal concentration (Wilde *et al*., 2006). Ma *et al*. (2003) reported that the extracellular Cu concentration level was a good indicator for measuring the toxic effects of Cu on alga growth in complex matrix. But, only few researchers have studied the relationship between intra- and extra-cellular Cu and algal growth inhibition. Fig. 2a and b demonstrates the relationship between intra- and extracellular Cu and growth rate (μ) of *T. suecica*; the intra- and extracellular Cu concentration required to inhibit the algal growth rate by 50% was 9.49 and 51 μM L^{-1} respectively ($p < 0.05$). Wilde *et al*. (2006) reported that growth inhibition of *Chlorella* sp. was independent of pH, and was related to both surface-bound and intracellular Cu; they observed 100-300 × 10^{-8} ng μm^{-3} and 30 × 10^{-8} ng μm^{-2} of intra- and extra-cellular Cu concentrations to respectively cause 50% growth inhibition. On the other hand, Ma *et al*. (2003) evaluated dissolved Cu, extracellular Cu, and intracellular Cu in *Scenedesmus subspicatus*; they observed that the concentration of intracellular Cu increased to 0.6-1.5 × 10^{-8} μM per cell when the growth inhibition reached ~50%. Franklin *et al*. (2000b) reported that when *Chlorella* sp. was exposed of 10 mg L^{-1} Cu, about 60% of the total cellular Cu was located intracellularly, while 40% was bound to the cell surface; however, at higher Cu concentrations (e.g. 640 mg L^{-1}), majority of cellular Cu (75%) was bound to the cell surface and only 25% was located intracellularly. Therefore, toxicity and growth rate inhibition, are correlated with both intra- and extracellular Cu, i.e. the more Cu bound at the cell surface, the more Cu penetrated the cell and the greater the toxicity. Our study complies with the report of Wilde *et al*. (2006) and Franklin *et al*. (2000b) in stating that toxicity of Cu and growth inhibition depends on the amount of intra- and extracellular Cu concentrations of the algae.

3. Effect of Cu on photosynthetic pigment (Chl *a*)

According to Lim *et al*. (2006) Cu ions initially affect the osmotic permeability of the outer cell membranes; however, when these Cu ions are transported into cytoplasm it affects the photosynthetic sites and uncouples the electron

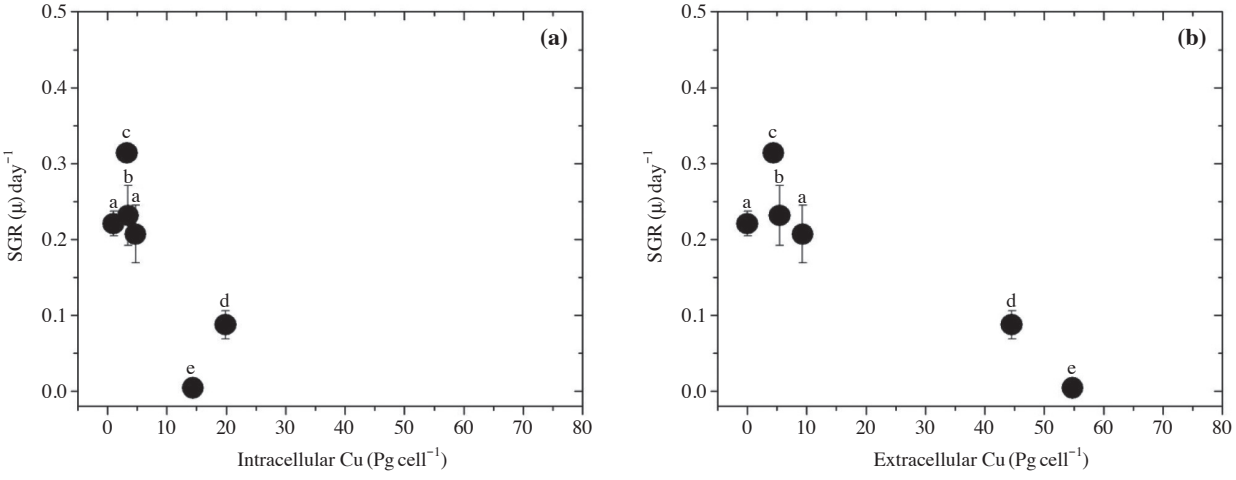

Fig. 2. Relationship between (a) Intracellular Cu and SGR inhibition, and (b) extracellular Cu and SGR inhibition of *T. suecica*. Data points representing mean ± 95% confidence interval are shown.

Table 2. Copper inhibits pigment content and growth of algae.

Reports	Findings
This study	Chl *a* content of *T. suecica* significantly decreased with increasing Cu concentrations
Perales-Vela *et al*. 2007; Veerapandiyan *et al*. 2014; Kumar *et al*. 2014	Cu inhibits photosynthetic pigments of algae and reduces their chl *a* content
Azeez and Banerjee (1986)	Cu toxicity induced decreased chlorophyll contents in two Cyanophytes, *Spirulina platensis* and *Anacystis nidulens*
Wei *et al*. (2014)	Chl *a* content of *Phaeodactylum tricornutum* was 0.02 and 0.01 Pg cell^{-1} in the presence of 40 and 60 µM L^{-1} Cu respectively; however, the reduction of chl *a* content was less pronounced as compared to the algal growth rate
Fargašová (2001)	10 day EC_{50} of growth for Cu exposed *Scenedesmus quadricauda* was 0.408 µM L^{-1}; a 33.8% reduction in the total chlorophyll at this concentration was evidenced
Fargašová *et al*. (1999)	10 day EC_{50} of *S. quadricauda* for chlorophyll accumulation was 0.613 µM
Qian *et al*. (2009)	At 0.5 and 1.5 µM L^{-1} Cu concentrations, 81.78 and 72.46% inhibition of chl *a* content was respectively observed in case of *C. vulgaris*.
Sunda *et al*. (2002)	Exposure to elevated Cu concentrations ([Cu^{2+}] = 1.0 nM and 3.2 nM; 11 days) caused a 67% decrease in the specific growth rate of *Emiliania huxleyi*, as well as, a 50% reduction in its Chl *a* concentration

transport to NADP in photosystem II. Cu disturbs the distribution of biochemicals such as proteins, lipids and free fatty acids in algae (Lupi *et al*., 1998; Lim *et al*., 2006); overall, Cu effects algal respiration and photosynthesis. At the lower concentrations (sub-µM), Cu^{2+} substitutes the central Mg^{2+} ion in the chlorophyll. However, at higher (µM or mM) concentrations, Cu^{2+} inhibits the synthesis of δ-aminolevulinic acid and the protochlorophyllide reductase (responsible for the final reductive step of chlorophyll biosynthesis), which leads to reduction in Chl content (Perales-Vela *et al*., 2007; Aggarwal *et al*., 2011; Kumar *et al*., 2014). Keeping in mind that Cu inhibits photosynthetic pigments of algae and reduces their chl *a* content (Perales-Vela *et al*., 2007; Veerapandiyan *et al*., 2014; Kumar *et al*., 2014), we investigated the Chl *a* content of *T. suecica*. Just like the other findings elaborated in Table 2, the Chl *a* content of *T. suecica* significantly decreased in the with increasing Cu concentrations (Fig. 3). Lim *et al*. (2006) also observed decrease in growth and chlorophyll content in case of Cu(I) oxide exposed *T. suecica*. Table 2 advocates that growth was more suggestively influenced by Cu rather than chlorophyll synthesis. According to Lim *et al*. (2006), when the concentration of Cu is increased, it binds to chloroplast membranes and other cell proteins causing reduction in chlorophyll pigments. In fact, higher concentrations of

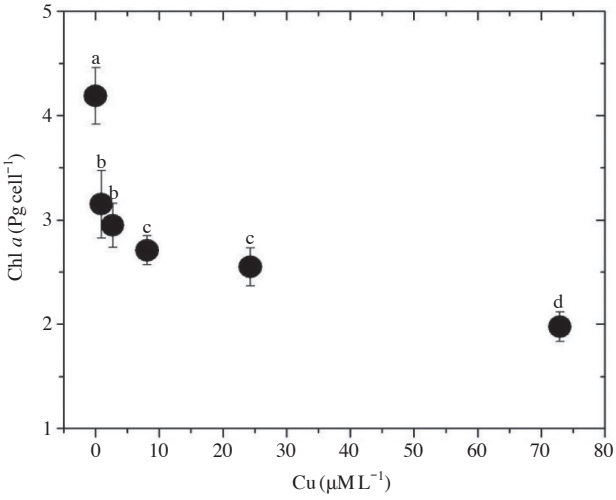

Fig. 3. Impact of Cu (96 h) on Chl *a* of *T. suecica*. Data points representing mean ± 95% confidence interval are shown.

Cu, cause irreversible damage to chloroplast lamellae, preventing photosynthesis and ultimately leading to cell death.

4. Intra- and extracellular accumulation of Cu, Fe and Zn

Mechanism of metal uptake and accumulation in microalgae involve: (1) passive absorption of metals to charged polysaccharides in the cell wall and intracellular matrix, and

(2) active metal uptake against large intracellular concentration gradients. Metals bound on the cell wall are transported across the plasma membrane, and the driving force of metal uptake is the presence of free chelating molecules in the algal cytoplasm. Phytochelatins are metal chelating molecules that bind to free metal ions (Monteiro *et al*., 2012; Zhang *et al*., 2014; Kumar *et al*., 2015). In our study, the toxicity of Cu on *T. suecica* increased with increasing Cu concentration (in the media); likewise, Franklin *et al*. (2002a) report concentration dependent increase in Cu toxicity in case of *Selenastrum capricornutum*.

1) Cu concentration in the medium influences the intra- and extracellular Cu content

In our study on *T. suecica*, intra- and extracellular Cu concentrations of *T. suecica* were dependent on the external dissolved Cu concentration (Fig. 4a). At higher Cu concentration (72.9 μM L^{-1}) a slight decrease in intracellular Cu content was recorded; similarly, Arredondo *et al*. (2006) also evidenced increase in intracellular Cu with increase in media Cu concentrations. Likewise, in a study of Özkoç *et al*. (2010), as the metal concentration and exposure time increased, the metal uptake into cells decreased but the amount of adsorbed metal on cell surfaces increased.

In our exposure studies, the intracellular Cu content of *T. suecica* ranged from 1.01 ± 0.21 to 14.39 ± 1.60 Pg $cell^{-1}$, while the extracellular Cu content ranged from 0 to 54.74 ± 7.29 Pg $cell^{-1}$. According to Levy *et al*. (2008), Cu (0.79 μM L^{-1}) exposed *P. tricornutum* had an intra- and extracellular Cu content was 0.062 and 0.1 Pg $cell^{-1}$, while Cu (7.86 μM L^{-1}) exposed *D. tertiolecta* had an intra- and extracellular Cu content of 0.59 and 5.7 Pg $cell^{-1}$. These values were lower than that obtained for *T. suecica* in this present study. Further, Levy *et al*. (2008), obtained 72 h IC_{50} values of 47 μg Cu L^{-1} for the green algae *Tetraselmis* sp., which had high intracellular Cu ($1.97 \pm 0.01 \times 10^{-13}$ g Cu $cell^{-1}$), suggesting that *Tetraselmis* sp. effectively detoxifies Cu within the cell. Uptake of Cu occurs through Cu(I) transport system, for e.g. in *Chlamydomonas reinhardtii* Cu is reduced from Cu^{2+} to Cu^{+} via a surface reductase (Sánchez-Marín *et al*., 2014).

On the other hand, Cu (5.35 μM L^{-1}) exposed *S. subspicatus* is reported to have an internal Cu content of 14.95 Pg $cell^{-1}$ (Ma *et al*., 2003); this was slightly higher than the internal Cu content of *T. suecica* obtained herein. After a 72 exposure to 8.5 μg Cu L^{-1}, Johnson *et al*. (2007) obtained intracellular ($66 \pm 17 \times 10^{-8}$ ng μm^{-3}) and extracellular ($21 \pm 7 \times 10^{-8}$ ng μm^{-2}) Cu concentrations in *Chlorella* sp. However, Franklin *et al*. (2002b) reported *Chlorella* sp. exposed (72 h) to 8.2 μg Cu L^{-1} to have intra- and extracellular Cu concentrations as 68×10^{-8} ng μm^{-3} and 25×10^{-8} ng μm^{-2}, respectively.

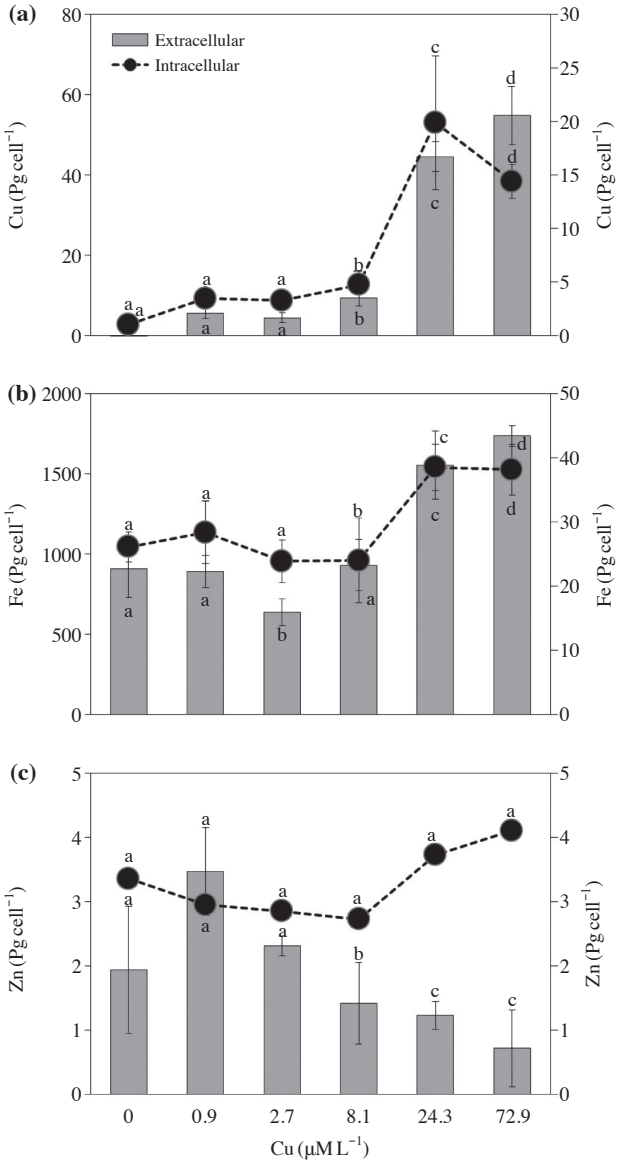

Fig. 4. Intra- and extra-cellular (a) Cu, (b) Feand (c) Zn content of *T. suecica* after 96 h of exposure to copper. Data points representing mean ± 95% confidence interval are shown.

2) Presence of Cu in the medium influences the intra- and extracellular Fe and Zn content

Copper (Cu), iron (Fe) and zinc (Zn) are essential mineral elements that exhibit important interactions and possible competitive inhibition of transport and bioavailability (Arredondo *et al*., 2006); therefore, it is essential to evaluate the effect of the presence of Cu on uptake of Cu, Fe and Zn. Knauer *et al*. (1997) investigated effects of free Cu^{2+} and Zn^{2+} ions on growth and metal accumulation in freshwater alga reporting that the growth of algae showed a high tolerance toward high intracellular Cu and Zn concentrations; they suggest that the cells may immobilize the metals intracellularly. In addition, they precisely mention that the freshwater algae they investigated (*S. subspicatus*, *C. reinhardtii*, *Chlorella fusca* and another *Chlamydomonas* culture) tolerated higher Cu^{2+} than marine algae. Thus, variation in intra- and extracellular metal content could occur based on the type of algal species (its mechanism, biochemical composition and functional groups), as well as, the metal concentrations used.

The Cu concentration dependent variation of intra- and extracellular Cu, Fe and Zn content of *T. suecica* obtained in this study is shown in Fig. 4b and c.

Iron (Fe) is undoubtedly the most versatile and important trace element for biochemical catalysis (Morel *et al*., 1991); it is required for chlorophyll synthesis. Although its precise role in the chlorophyll synthesis remains a mystery, Fe deficiency invariably leads to a simultaneous loss of chlorophyll and degeneration of chlorophyll structure (Aggarwal *et al*., 2011). In *Tetraselmis*, the presence of inducible cell surface Fe chelate reductase activity that operates at a rate commensurate with that of overall Fe uptake, suggests that, the process begins with a reduction of Fe(III) to Fe(II) (Hartnett *et al*., 2012). Blaby-Haas and Merchant (2012) state that Cu is required for Fe uptake. In our study on *T. suecica*, increasing media Cu concentration enhanced the intra- and extracellular Fe content. The intra- and extracellular Fe content respectively ranged from 26.10 ± 2.33 to 38.48 ± 3.61, and 908.96 ± 178.75 to 1734.2 ± 64.11 Pg $cell^{-1}$; notably, the internal Fe was much lesser than the external Fe content. However, when the Cu concentration increased from 0.9 to 24.9 μM, the intracellular Fe concentration increased from 3.45 to 19.87 Pg $cell^{-1}$. In a metal crosstalk, Blaby-Haas and Merchant (2012) mentioned that the reduced concentration of one metal ion can cause a secondary deficiency in another metal ion; they illustrate *Chlamydomonas*, stating about a Cu-dependent component of the high-affinity Fe transporter in algae. The presence of Cu could influence some metabolism of *T. suecica* which in turn caused the changes in the Fe content; however, this needs to be further investigated.

Zinc, another essential micronutrient, is required for many biological processes, and, acts as an important co-factor for enzymes (like carbonic anhydrase, superoxide dismutase and RNA polymerase) (Li *et al*., 2010; Monteiro *et al*., 2011). However, Cu and Zn have similar ionic radii, and, both bind strongly to oxygen- and nitrogen-containing ligands (Johnson *et al*., 2007); thus, it is possible that the uptake and bioavailability of Cu could influence Zn uptake in algal cells. The intra- and extracellular Zn content of the Cu exposed *T. suecica* ranged from 3.35 ± 0.54 to 4.10 ± 0.97, and, 1.96 ± 0.99 to 0.71 ± 0.60 Pg $cell^{-1}$ respectively. Though there was increase in Cu concentration 24.3 and 72.9 μM L^{-1}, the intracellular Zn content remained almost constant. Although there was an initial increase in the extracellular Zn concentration which rapidly declined under increasing Cu concentrations; however, exposure to high concentrations of Cu caused a remarkable increase in the intracellular Zn content of *T. suecica*. This reduction in metal content following addition of increasing second metal may be due to competitive inhibition; it probably indicated interaction at a single uptake site (Webster *et al*., 2011).

Exposure to Cu particularly causes ultra-structural changes in *Tetraselmis suecica*; this includes increased vacuolization of the cytoplasm (particularly larger-sized vacuoles are observed), the appearance of cells within multilayered cell walls, and the excretion of organic matter and an increased number of vacuoles (Nassiri *et al*., 1996; Levy *et al*., 2008). According to Levy *et al*. (2008), Cu exposed *T. suecica*, demonstrated high concentration of Cu in the organic matter and the vesicles; the Cu was found to be associated with sulfur and phosphorus within the vesicles. Moreover, high Cu concentrations also led to loss of their flagella, making the cells spherical; in addition intra-cytoplasmic granules were formed. Levy *et al*. (2008) reported the increase in organic matter in the medium led to greater aggregation of cells.

Overall, algal sensitivity to Cu is more likely to be related

to Cu internalization, rather than adsorption to non-specific surface binding sites (Levy *et al*., 2007); particularly, intracellular Cu concentrations are responsible for growth inhibition in microalgae (Stauber and Florence, 1987; Franklin *et al*., 2002a; Levy *et al*., 2007). Generally, the binding of Cu on the plasma membrane is a critical step before internalization of Cu and its toxicity. However, internal metal loadings do not always reflect differences in sensitivity, as organisms can bioaccumulate metals in a non-metabolically active form. Levy *et al*. (2007) have well-deliberated that, post-internalization, algae could either possess: (i) detoxification mechanisms (exclusion, internal sequestration and active efflux mechanisms), for e.g. Cu could be prevented from entering algal cells by release of exudates (that bind Cu in solution, reducing the bioavailable fraction of metal, thereby reducing its toxicity), or alternatively possess: (ii) physical exclusion mechanisms (such as reduced membrane permeability or alteration of the metal species at the cell surface). But, once the Cu is internalized, the produced cysteine-rich phytochelatins could bind to the excess Cu (making it less toxic through subcellular partitioning of metals to inactive sites). However, apart from the aforesaid internal metabolic mechanisms, Cu detoxification could also take place by cell wall sequestration (Nassiri *et al*., 1997), accumulation in thylakoid membranes (Soldo and Behra, 2005), and, via toxicant- or nutrient deficiency-induced production of proteins and antioxidant superoxide dismutase (Levy *et al*., 2007). Nevertheless, an efflux mechanism could also exist that would pump the metal back into solution as a potentially different, less toxic metal species.

CONCLUSIONS

Copper (Cu) at low concentration acts as a micro-nutrient, favoring several physiological processes in algae, but, when present at higher concentrations it could be toxic; it could be transferred and accumulated at higher trophic levels. In the aquatic environment, toxicity of Cu depends on external factors (exposure concentration and duration, environmental parameters, and water quality) and intracellular processes (metal-binding sites and detoxification). Microalgae, forming the base of freshwater and marine ecosystems, are sensitive indicators of environmental change and pollution; they are widely used in risk assessment. This study demonstrates Cu concentration dependent inhibition of growth and cell division, as well as, variation of intra-and extracellular Cu, Zn and Fe, content of *T. suecica*. *T. suecica* grown under low Cu concentrations manifested a slight increase in growth and cell division, however, exposure to higher Cu concentrations caused a tremendous decline in growth, cell division and chl *a* content. Evaluation of the intra- and extracellular Cu concentration, revealed that, a moderate increase in the intracellular Cu content was accompanied with notable decline in the growth rate and cell division, and this impact was more pronounced as compared to that of extracellular Cu content. The surface bound Zn content was reduced significantly, with increasing Cu concentrations. However, the intra- and extracellular Fe content of *T. suecica* was considerably constant under moderate amounts of Cu, and increased under high Cu concentrations. Cu, Zn and Fe, are important trace elements in algal metabolism; therefore, any change in their concentration would result in a change in physiology of the microalgae. Invariably, metals are transferred and accumulated at higher trophic levels, for e.g. grazing zooplanktons, crustaceans, or (via aquaculture feed to) aquaculture animals; therefore, any change in the physiology of the studied primary producer would definitely impact the diet supply to ecosystem. Thus, future research examining Cu uptake, its intracellular localization and detoxification (e.g. phytochelatins) in microalgae, could provide further insights into the mode of action of Cu.

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REFERENCES

Abreu, F.C.P.D., P.N.M.D. Costa, A.M. Brondi, E.J. Pilau, F.C. Gozzo, M.N. Eberlin, M.G. Trevisan and J.S. Garcia. 2014. Effects of Cadmium and Copper Biosorption on Chlorella vulgaris. *Bulletin of Environmental Contamination and Toxicology* **93**: 405-409.

Adams, W.J., R. Blust, U. Borgmann, K.V. Brix, D.K. DeForest, A.S. Green, J.S. Meyer, J.C. McGeer, P.R. Paquin, P.S. Rainbow and C.M. Wood. 2011. Utility of tissue residues for predicting effects of metals on aquatic organisms. *Integrated Environmental Assessment and Management* **7**(1): 75-98.

Aggarwal, A., I. Sharma, B.N. Tripathi, A.K. Munjal, M. Baunthiyal and V. Sharma. 2011. Metal Toxicity and Photosynthesis. *In*: Photosynthesis: Overviews on Recent Progress & Future Perspective, First 01/2011: chapter Metal toxicity and Photosynthesis. Ed. Itoh S, Mohanty P, Guruprasad KN, IK International Publishing House. New Delhi. pp. 16: 229-236. ISBN: ISBN978-93-81141-00-7.

Al-Hejuje, M.M. 2008. Effect of some heavy metals ions on the chlorophyll *a* pigment of *Nostoclinkia* and *Hapalosiphon aureus*. *Marsh Bulletin* **3**(2): 136-146.

Arredondo, M., R. Martínez, M.T. Núñez, M. Ruz and M. Olivares. 2006. Inhibition of iron and copper uptake by iron, copper and zinc. *Biological Research* **39**: 95-102.

Azeez, P.A. and D.K. Banerjee. 1986. Effect of copper and cadmium assimilation and uptake of heavy metals. *Bulletin of Environmental Contamination and Toxicology* **12**: 77-86.

Blaby-Haas, C.E. and S.S. Merchant. 2012. The ins and outs of algal metal transport. *Biochim Biophys Acta*. **1823**(9): 1531-1552.

Campbell, P.G.C., O. Errecalde, C. Fortin, V.P. Hiriart-Baer and B. Vigneault. 2002. Metal bioavailability to phytoplankton: applicability of the biotic ligand model. *Comparative Biochemistry and Physiology - Part C* **133**: 189-206.

Carfagna, S., N. Lanza, G. Salbitani, A. Basile, S. Sorbo and V. Vona. 2013. Physiological and morphological responses of lead or cadmium exposed *Chlorella sorokiniana* 211-8K (Chlorophyceae). Springer Plus. 2,147.

Čypaitė, A., J. Žaltauskaitė and J. Venclovienė. 2014. Assessment of chlorophyll-*a*, chlorophyll-*b* and growth rate in freshwater green algae *Pseudokirchneriella subcapitata* exposed to cadmium and copper. The 9th International Conference "ENVIRONMENTAL ENGINEERING" (Section: Environmental protection) 22-23 May 2014, Vilnius, Lithuania eISSN 2029-7092 / eISBN 978-609-457-640-9; pp. 1-7. Available from http://leidykla.vgtu.lt/conferences/ENVIRO_2014/Articles/1/009_Cypaite.pdf

Fargašová, A. 2001. Interactive effect of manganese, molybdenum, nickel, copper I and II and vanadium on the freshwater alga *Scenedesmusquadricauda*. *Bulletin of Environmental Contamination and Toxicology* **67**: 688-695.

Fargašová, A., A. Bumbálová and E. Havránek. 1999. Ecotoxicological effects and uptake of metals (Cu^{+}, Cu^{2+}, Mn^{2+}, Mo^{6+}, Ni^{2+}, V^{5+}) in freshwater alga *Scenedesmus quadricauda*. *Chemosphere* **38**: 1165-1173.

Franklin, N.M., J.L. Stauber, S.C. Apte and R.P. Lim. 2002a. Effect of initial cell density on the bioavailability and toxicity of copper in microalgal bioassays. *Environmental Toxicology and Chemistry* **21**: 742-751.

Franklin, N.M., J.L. Stauber, R.L. Lim and P. Petocz. 2002b. Toxicity of metal mixtures to a tropical freshwater algae (*Chlorella* sp.): the effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. *Environmental Toxicology and Chemistry* **21**: 2412-2422.

Franklin, N.M., J.L. Stauber, S.J. Markich and R.P. Lim. 2000. pH dependent toxicity of copper and uranium to a tropical freshwater alga (*Chlorella* sp.). *Aquatic Toxicology* **48**: 275-289.

Gonzalez-Davila, M., J.M. Santana-Casiano, J. Perez-Pena and F.J. Millero. 1995. Binding of Cu(II) to the surface and exudates of the alga *Dunaliella tertiolecta* in seawater. *Environmental Science & Technology* **29**(2): 289-301.

Hartnett, A., L.H. Böttger, B.F. Matzanke and C.J. Carrano. 2012. A multidisciplinary study of iron transport and storage in the marine green alga *Tetraselmis suecica*. *Journal of Inorganic Biochemistry* **116**: 188-194.

Hossain, M.A., P. Piyatida, J.A. Teixeira da Silva and M. Fujita. 2012. Molecular Mechanism of Heavy Metal Toxicity and Tolerance in Plants: Central Role of Glutathione in Detoxification of Reactive Oxygen Species and Methylglyoxal and in Heavy Metal Chelation. *Journal of Botany*, vol. 2012, Article ID 872875, 37 pages, doi:10.1155/2012/872875.

Johnson, H., M.S. Adams, J.L. Stauber and D.F. Jolley. 2007. Copper and zinc tolerance of two tropical microalgae after copper acclimation. *Environmental Toxicology* **22**: 234-244.

Kanoun-Boulé, M., J.A.F. Vicentea, C. Nabaisa, M.N.V. Prasad and F. Freitas. 2009. Ecophysiological tolerance of duckweeds exposed to copper. *Aquatic Toxicology* **91**(1): 1-9.

Kaplan, D. 2013. Absorption and Adsorption of Heavy Metals by Microalgae, in Handbook of Microalgal Culture: Applied Phycology and Biotechnology, Second Edition (Richmond, A. and Q. Hu, eds.). John Wiley & Sons, Ltd, Oxford, UK. doi: 10.1002/9781118567166.ch32

Kebeish, R., Y. El-Ayouty and A. Husain. 2014. Effect of copper on growth, bioactive metabolites, antioxidant enzymes and photosynthesis-related gene transcription in *Chlorella vulgaris*. *World Journal of Biology and Biological Sciences* **2**(2): 34-43.

Kim, M.-K. and R.E.H. Smith. 2001. Effect of ionic copper toxicity on the growth of green alga, *Selenastrum capricornutum*. *Journal of Microbiology and Biotechnology* **11**: 211-216.

Knauer, K., R. Behra and L. Sigg. 1997. Effects of free Cu^{2+} and Zn^{2+} ions on growth and metal accumulation in freshwater algae. *Environmental Toxicology and Chemistry* **16**: 220-229.

Kumar, K.S., H.U. Dahms, E.J. Won, J.S. Lee and K.H. Shin. 2015. Microalgae-A promising tool for heavy metal re-

mediation. *Ecotoxicology and Environmental Safety* **113**: 329-352.

Kumar, K.S., H.-U. Dahms, J.-S. Lee, H.C. Kim, W.C. Lee and K.-H. Shin. 2014. Algal photosynthetic responses to toxic metals and herbicides assessed by chlorophyll *a* fluorescence. *Ecotoxicology and Environmental Safety* **104**: 51-71.

Lee, H.J. and S.B. Hur. 2009. Genetic Relationships among Multiple Strains of the Genus *Tetraselmis* Based on Partial 18S rDNA Sequences. *Algae* **24**(4): 205-212.

Levy, J.L., B.M. Angel, J.L. Stauber, W.L. Poon, S.L. Simpson, S.H. Cheng and D.F. Jolley. 2008. Uptake and internalisation of copper by three marine microalgae: comparison of copper-sensitive and copper-tolerant species. *Aquatic Toxicology* **89**: 82-93.

Levy, J.L., J.L. Stauber and D.F. Jolley. 2007. Sensitivity of marine microalgae to copper: the effect of biotic factors on copper adsorption and toxicity. *Science of the Total Environment* **387**: 141-154.

Li, Y.X., S. Zhou, F.J. Zhao, Y. Liu, P.P. Fan and G.C. Wang. 2010. Physiological responses of *Porphyra haitanesis* to different copper and zinc concentrations. *Brazilian Journal of Oceanography* **58**(4): 261-267.

Lim, C.Y., Y.H. Yoo, M. Sidharthan, C.W. Ma, I.C. Bang, J.M. Kim, K.S. Lee, N.S. Park and H.W. Shin. 2006. Effects of copper (I) oxide on growth and biochemical compositions of two marine microalgae. *Journal of Environmental Biology* **27**: 461-466.

Lupi, F.M., H.M.L. Fernandes and I.S. Correia. 1998. Increase of copper toxicity to growth of *Chlorella vulgaris* with increase of light intensity. *Microbial Ecology* **35**: 193-198.

Ma, M., W. Zhu, Z. Wang and G.J. Witkamp. 2003. Accumulation, assimilation and growth inhibition of copper on freshwater alga (*Scenedesmus subspicatus* 86.81 SAG) in the presence of EDTA and fulvic acid. *Aquatic Toxicology* **63**: 221-228.

Monteiro, C.M., P.M.L. Castro and F.X. Malcata. 2012. Metal uptake by microalgae: Underlying mechanisms and practical applications. *Biotechnology Progress* **28**: 299-311.

Monteiro, C.M., S.C. Fonseca, P.M.L. Castro and F.X. Malcata. 2011. Toxicity of cadmium and zinc on two microalgae, *Scenedesmus obliquus* and *Desmodesmus pleiomorphus*, from Northern Portugal. *Journal of Applied Phycology* **23**: 97-103.

Morel, F.M.M., J.G. Rueter and N.M. Price. 1991. Iron nutrition of phytoplankton and its possible importance in the ecology of ocean regions with high nutrient and low biomass. *Oceanography* **4**: 56-61.

Nassiri, Y., T. Ginsburger-Vogel, J.L. Mansot and J. Wery. 1996. Effects of heavy metals on *Tetraselmis suecica*: Ultrastructural and energy-dispersive X-ray spectroscopic studies. *Biology of the Cell* **86**: 51-160.

Nassiri, Y., J.L. Mansot, J. Wéry, T. Ginsburger-Vogel and J.C. Amiard. 1997. Ultrastructural and electron energy loss spectroscopy studies of sequestration mechanisms of Cd and Cu in the marine diatom *Skeletonema costatum*. *Archives of Environmental Contamination and Toxicology* **33**: 147-155.

Ouyang, H.L., X.Z. Kong, W. He, N. Qin, Q.S. He, W. Yan, W. Rong and X.F. Liu. 2012. Effects of five heavy metals at sub-lethal concentrations on the growth and photosynthesis of Chlorella vulgaris. *Chinese Science Bulletin* **57**: 3363-3370.

Özkoç, H.B. and Z.S. Taylan. 2010. Assessment of various parameters of metal biology in marine microalgae *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*. *Fresenius Environmental Bulletin* **19**(12a): 2981-2986.

Perales-Vela, H.V., S. González-Morenom, C. Montes-Horcasitas and R.S. CañizaresVillanueva. 2007. Growth, photosynthetic and respiratory responses to sub-lethal copper concentrations in *Scenedesmus incrassatulus* (Chlorophyceae). *Chemosphere* **67**: 2274-2281.

Pistocchi, R.F., G.V. Balboni and L. Boni. 1997. Copper toxicity and carbohydrate production in the microalgae *Cylindrotheca fusiformis* and *Gymnodinium* sp. *European Journal of Phycology* **32**: 125-132.

Porra, R.J. 2002. The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls *a* and *b*. *Photosynthesis Research* **73**: 149-156.

Qian, H.F., J.J. Li, L.W. Sun, W. Chen, G.D. Sheng, W.P. Liu and Z.W. Fu. 2009. Combined effect of copper and cadmium on *Chlorella vulgaris* growth and photosynthesis-related gene transcription. *Aquatic Toxicology* **94**: 56-61.

Rai, P.K. and L.C. Rai. 1997. Interactive effect of UV B and Cu on photosynthesis, uptake and metabolism of nutrients in a green alga *Chlorella vulgaris* under simulated ozone column. *The Journal of General and Applied Microbiology* **43**: 281-288.

Regaldo, L., S. Gervasio, H. Troiani and A.M. danGagneten. 2013. Bioaccumulation and Toxicity of Copper and Lead in *Chlorella vulgaris*. *Journal of Algal Biomass Utilization* **2**: 59-66.

Rocchetta, I. and H. Küpper. 2009. Chromium- and copper-induced inhibition of hotosynthesis in *Euglena gracilis* analysed on the single-cell level by fluorescence kinetic microscopy. *New Phytologist* **182**(2): 405-420.

Sánchez-Marín, P., C. Fortin and P.G.C. Campbell. 2014. Lead (Pb) and copper (Cu) share a common uptake transporter in the unicellular alga *Chlamydomonas reinhardtii*. *Biometals* **27**: 173-181.

Sbihi, K., O. Cherifi, A. El Gharmali, B. Oudra and F. Aziz. 2012. Accumulation and toxicological effects of cadmium, copper and zinc on the growth and photosynthesis of the freshwater diatom *Planothidium lanceolatum* (Brébis-

son) Lange-Bertalot: A laboratory study. *Journal of Materials and Environmental Science* **3**(3): 497-506.

Soldo, D., R. Hari, L. Sigg and R. Behra. 2005. Tolerance of Oocystisnephrocytioides to copper: intracellular distribution and extracellular complexation of copper. *Aquatic Toxicology* **71**: 307-317.

Stauber, J.L. and T.M. Florence. 1987. The mechanism of toxicity of ionic copper and copper complexes to algae. *Marine Biology* **94**: 511-519.

Sunda, W., D.J. Kleber, R.P. Klene and S. Huntsman. 2002. An antioxidant function for DMSP and DMS in marine algae. *Nature* **418**: 317-320.

Tovar-Sanchez, A., S.A. Sanudo-Wilhelmy, M. Garcia-Vargas, R.S. Weaver, L.C. Popels and D.A. Hutchins. 2003. A trace metal clean reagent to remove surface-bound iron from marine phytoplankton. *Marine Chemistry* **82**(1-2): 91-99.

Tripathi, B.N. and J.P. Gaur. 2006. Physiological behavior of *Scenedesmus* sp. during exposure to elevated levels of Cu and Zn and after withdrawal of metal stress. *Protoplasma* **229**: 1-9.

Veerapandiyan, N., T. Lenin, P. Sampathkumar, A. Arokiasundaram and S.P.J. Sangeetha. 2014. Acute toxicity on growth and chlorophyll a' content of diatom *Odontellaaurita*. *International Journal of Science Inventions Today* **3**(6): 725-736.

Volland, S., E. Bayer, V. Baumgartner, A. Andosch, C. Lütz, E. Sima and U. Lütz-Meindl. 2014. Rescue of heavy metal effects on cell physiology of the algal model system Micrasterias by divalent ions. *Journal of Plant Physiology* **171**: 154-163.

Walne, P.R. 1970. Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera Ostrea, Crassostrea, Mercenaria, and Mytilis. *Fish Inves* **26**: 1-62.

Webster, R.E., A.P. Dean and J.K. Pittman. 2011. Cadmium exposure and phosphorus limitation increases metal content in the freshwater alga *Chlamydomonas reinhardtii*. *Environmental Science & Technology* **45**: 7489-7496.

Wei, Y., N. Zhu, M. Lavoie, J. Wang, H. Qian and Z. Fu. 2014. Copper toxicity to *Phaeodactylum tricornutum*: a survey of the sensitivity of various toxicity endpoints at the physiological, biochemical, molecular and structural levels. *BioMetals* **27**(3): 527-537.

Wilde, K.L., J.L. Stauber, S.J. Markich, N.M. Franklin and P.L. Brown. 2006. The effect of pH on the uptake and toxicity of copper and zinc in a tropical freshwater alga (*Chlorella* sp.). *Archives of Environmental Contamination and Toxicology* **51**: 174-185.

Yan, H. and G. Pan. 2002. Toxicity and bioaccumulation of copper in three green microalgal species. *Chemosphere* **49**(5): 471-476.

Zhang, L., X. He, M. Chen, R. An, X. An and J. Li. 2014. Responses of nitrogen metabolism to copper stress in *Luffa cylindrica* roots. *Journal of Soil Science and Plant Nutrition* **14**(3): 616-624.

Zhou, G.-J., F.-Q. Peng, L.J. Zhang and G.G. Ying. 2012. Biosorption of zinc and copper from aqueous solutions by two freshwater green microalgae *Chlorella pyrenoidosa* and *Scenedesmus obliquus*. *Environmental Science and Pollution Research* **19**(7): 2918-2929.