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Effect of Copper on Growth and Enzyme Activities of Marine Diatom, *Odontella mobiliensis*

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Abstract The 72-h IC_{50}, **7-**d no observable effect concentration (NOEC), low observable effect concentration (LOEC), Chronic values were derived for copper on the growth of marine diatom, *Odontella mobiliensis*. The effect of copper was also studied on cell morphology, size, nitrate reductase and antioxidant enzymes (Catalase, Superoxide dismutase and peroxidase). The 72-h IC_{50} of 298.4 ± 28.3, NOEC of 15.6, LOEC of 29.6 and chronic value of 21.5 μg Cu L^{-1} were found in the present study. The chlorophyll *a* was significantly decreased with increasing concentrations of copper. The length of the cell (apical axis) was extended from 30.14 ± 5.98 μm at control to 71.4 ± 6.29 μm at 574 μg Cu L^{-1}, the spines were absent at 574 μg L^{-1} and the cell structure was entirely damaged at 926 μg Cu L^{-1}. The antioxidant enzymes viz. Catalase, Peroxidase activities and Melondialdehyde were increased whereas the Nitrate reductase and activity was reduced at 21.5 μg Cu L^{-1} during 7 days exposure.

Keywords Diatom · IC_{50} · Nitrate reductase · Antioxidant enzyme · Cell size · Morphology

Trace elements are one of the most common non-biodegradable pollutants among the diversified modern pollutants and affecting the organisms at different stages at elevated concentrations (Zhang et al. 2008). Some of the trace elements such as lead (Pb), copper (Cu), aluminium (Al), cadmium (Cd), boron (B), selenium (Se), chromium (Cr), manganese (Mn), cobalt (Co) and arsenic (As) are essential for organisms and can be toxic at more than the required level. Many of these have a direct influence on various physiological, bio-chemical processes and also bio-accumulated to reach toxic levels (Rietzler et al. 2001). Copper is an essential micronutrient for algae and being components of several proteins and enzymes which involved in metabolic pathways (Elisabetta and Gioacchino 2004) and toxic when it reaches above required level (Soldo and Behra 2000). It enters to the marine environment through river run-off, industrial, domestic activities, agricultural practices; copper mine drainages and anti-fouling paints (Srinivasan and Swain 2007). Microalgae are the most important and basic of food webs in marine and fresh water ecosystems (Li et al. 2006) and one of the first groups to be affected by metal pollution (Sampathkumar and Kannan 1998). Hence, a study on degradation and biotransformation of xenobiotics by phytoplankton is important to assess the environmental fate and risk of pollutants in marine ecosystems (Karthikeyan et al. 2010) to develop water quality criteria through toxicological studies and conduct of growth inhibition tests.

Such studies are mostly focused on freshwater green alga, *Selenastrum capricornutum* and the marine diatom, *Phaeodactylum tricornutum* (USEPA 2002). The effect of heavy metals on photosynthesis studied by the estimation of chlorophyll *a* concentration (Ferrat et al. 2003). In most cases cell density has been taken as a growth parameter for the toxicological studies (Franklin et al. 2002) because this only showed linear response with toxicant concentrations. Cell size and morphology was also affected by the oxidative stress of trace metal (Sabatini et al. 2009). The estimation of cell density using haemocytometer for chain forming species with different size (>30 micron) and shape

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are not more suitable because, the cells have not able to spread over the counting chamber of haemocytometer. However, flow cytometer is in use for determination of cell numbers and other cell parameters in recent years. It is more expensive and need much technical skills. So, a conventional counting method was developed and followed in the present study which is more easy and economic.

The physiology of algae can be affected even in no observable effect concentration (NOEC) of metals by means of cell density. The biochemical reactions have a vital importance in activity of the cell and are estimated by the enzyme activity assays. The uptake of nitrate by phytoplankton is a central issue in biological oceanography due to its importance to primary production and vertical flux of biogenic carbon. Among the enzymes present in algae, nitrate reductase (NR) is very important for nitrogen assimilation and it catalyzes the reduction of NO_3 to NO_2 (Vergara et al. 1998).

Trace elements at elevated level affect variety of processes in plants (Siedlecka et al. 2001). One of the major consequences is the enhanced production of Reactive oxygen species (ROS), which damage cell membranes, nucleic acids and chloroplast (Tewari et al. 2002). Accumulation of ROS may be the consequence of disruption of the balance between their production and the antioxidative system activity, composed of enzymatic antioxidants such as catalase (CAT), peroxidase (POD) and superoxide dismutases (SOD) (Li et al. 2006) and non-enzymatic scavengers, e.g. glutathione, carotenoids and ascorbates (Mallick 2004). SOD is the major O_2 scavenger and its enzymatic action results in H_2O_2 and O_2 formation. POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Bilkhina et al. 2003).

In stress condition, the free radical species (forms of active oxygen) may be increased, which will enhance the activities of these detoxifying enzymes. While in normal circumstances, the concentration of oxygen radicals remains low because of the activity of these antioxidative enzymes (Asada 1984). Malondialdehyde is a cytotoxic product of lipid peroxidation and an indicator of free radical production and consequent tissue damage. Thus, cell membrane stability has widely been utilized to study the effect of stress on plants. There are many reports concerning the response of the antioxidant systems in plants to metal stress (Mazhoudi et al. 1997), but studies on microalgae are very few (Elisabetta and Gioacchino 2004). So, the present study was carried out to investigate the effect of copper on the marine centric diatom, *Odontella mobiliensis* for the toxicity effects of copper on growth, photosynthetic pigment (Chl. *a*) concentration, cell size, morphology and enzyme activities viz. nitrate reductase (NR), superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), Malondialdehyde (MDA content).

Materials and Methods

The marine centric diatom, *O. mobiliensis* was collected from Vellar estuary, Southeast coast of India (Lat. 11°29′N; and Long. 79°46; E). It was isolated and maintained at Algal Culture Laboratory, CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai following the methods described in Andersen (2005). The strains were maintained with filtered natural seawater enriched with f/2 media recipe. The dissolved copper concentrations of seawater and test solution were analysed following the method of Grasshoff et al. (1999) in ICP-OES with the detection level of 1 μg L^{-1}. The culture was maintained at :Temperature at 25 ± 1°C, Salinity at 30‰, pH at 8.0 ± 0.3 and Light intensity at 4,500 ± 500 Lux with 12:12 h light and dark condition.

All the experiments were conducted in 250 mL conical flasks with 100 mL of 4–5 days aged exponentially grown algal cultures with the initial cell density of 1.8 ± 0.23 × 10^4 cells mL^{-1}. The standard growth inhibition test procedures were followed (OECD 2002; USEPA 2002). The range finding tests were conducted for 48 h before definitive test. The stock solution of copper was prepared in Milli-Q ultra pure water using its metallic salt of Copper chloride dihydrate (Merck, India (Pvt.) Ltd.). The definitive and chronic tests were conducted in triplicate experiments using different concentrations (52, 79, 127, 213, 335, 574 and 926 ppb) and (3.6, 6.2, 8.4, 15.6, 29.6, 61.7 and 97.8 ppb) of copper for 72 h and 7 days (each concentration were triplicate in every experiment).The cell density was estimated at every 24 h intervals. Growth rate and percentage of growth inhibition were calculated by the equations described in OECD (2002). Chlorophyll *a*, cell size and morphology were studied at end of the acute definitive test. The enzyme activities NR, SOD, CAT, POD and MDA content were assayed in the chronic concentration of copper (21.5 ppb) exposed for 7 days and compared with control.

The IC_{50} values were calculated by Probit analysis software; NOEC, low observable effect concentration (LOEC) and chronic values were calculated by Dunnett's method (USEPA 2002). Other data process and graphs were plotted using SPSS 11.0 and MS-Excel software.

The cell density was calculated by conventional method (100 μL) of *O. mobiliensis* culture was made up to 1 mL with using lugol's iodine solution. 10 μL of diluted sample was placed on a glass slide exactly at the meeting point of plus mark. The slide was mounted on the binocular microscope and the cells were counted. The results were expressed as cells mL^{-1} of culture. The growth rate was calculated using the following formula (OECD 2002),

$$\mu = \frac{N_X - N_O}{T_X - T_O}$$

Where, N_0—Number of cells in time zero, N_x—Number of cells in time x, t_0—starting time (0), tx—time X (in days; 3 and 7 days for acute and chronic test, respectively). The doubling time was calculated by the following formula,

$$\text{Doubling time} = \frac{N_O \times 2}{N_t} \times t$$

Where, N_0—Number of cells in time zero, N_t—Number of cell in time t, t—Time in hoursThe results were presented in doubling time (DT) in hours.

Cell size and morphology were observed under microscope with micrometers respect to control (Rajendran 1986). Percentage of growth inhibition was calculated by the following formula:

$$\text{Percentage of Growth Inhibition} = \frac{\mu_{\text{Control}} - \mu_{\text{Concentration}}}{\mu_{\text{Control}}} \times 100.$$

Chlorophyll was estimated by the modified method of Strickland and Parsons (1972). Five mL of acetone was added to 2 mL of algal culture and vortexed for 1 min and kept in refrigerator at 4°C for 24 h. Then the samples were centrifuged at 5,000 rpm for 10 min and the supernatant was read at 630, 645 and 660 nm using UV–Vis Spectrophotometer (Perkin-Elmer Lamda 25). Raw acetone was used as blank. 10 mL aliquots of algal cultures were collected by centrifugation at 12,000 rpm for 10 min. Proteins were analyzed in pellets based on the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard after re-suspending the cells in 0.1 N NaOH and sonicated for 5 min.

After 7 days of exposure to copper, the algal cultures were collected by centrifugation at 10,000 rpm for 15 min; pellet was resuspended in 500 µL of 0.1 M sodium phosphate buffer (pH 7), sonicated for 5 min and centrifuged at 12,000 rpm for 20 min. The centrifugation process was repeated until the clear supernatant was obtained and it was used as enzyme extract for all assays. Nitrate reductase activity assay was performed according to Chow et al. (2004). NR activity assay was carried out at 20°C in a 0.2 M phosphate buffer (pH 8.0), 6 mM KNO_3 and 0.5 mM $MgSO_4$ after the addition of 0.04 mM NADH. A sample without NADH was used as control for each treatment. The enzymatic reaction was stopped after 5 min by adding 1.4 mM $ZnSO_4$ and 43% v/v ethanol, after 5 min 9.6 mM sulphanilamide and 0.7 mM n-(1-naphtyl) ethylenediamine dihydrochloride were added and read at 543 nm. One unit of NR activity (U) is defined as the amount of enzyme required to produce 1 µmol of nitrite per minute at 20°C. Catalase activity was measured following the method of Aebi (1984) using H_2O_2 as substrate. The decay of peroxide was monitored by measuring the absorbance of reaction mixture (50 mM potassium phosphate buffer (pH 7.4) and 10 mM H_2O_2) for 30 s at 240 nm. Results were expressed as CAT U mg^{-1}. One CAT unit was defined as the enzyme amount that transforms 1 µMol of H_2O_2 per min. SOD was measured according to Beauchamp and Fridovich (1971) with a slight modification. The reactive mixture included 5×10^{-3} mol/L phosphorus buffer (pH 7.8); 13×10^{-3} mol/L methionine; 75×10^{-6} mol/L Nitrotetrazolium Blue chloride (NBT); 100 nmol/L EDTA; 2×10^{-6} mol/L riboflavin; and 0.1 mL enzyme preparation. For background measurement, instead of enzyme 0.1 mL phosphorus buffer was added to the mixture. The mixture was irradiated for reaction under a fluorescent light (25°C, 65 µE. m^2.s) for 20 min and the absorbance of reaction mixture was measured at 560. One unit of SOD activity (U) was defined as the enzyme dosage used for inhibiting the reactive starting velocity to 50% and was calculated as

$$\text{SOD activity} = \frac{OD_b - OD_s}{50\%\ OD_b} \times \text{diluted aliquot of sample.}$$

where, OD_b is the optical density (OD) value of background, OD_s is the OD value of the sample. MDA content was measured by thiobarbituric acid (TBA) reactive substances test (Draper et al. 1993; Janero 1990).To 1.5 mL of enzyme preparation 0.5% TBA with 20% trichloroacetic acid was added, and kept in water bath at 100°C. After 30 min they were refrigerated and centrifuged at 4,000 rpm for 10 min. The OD value of the supernatant was measured at 532 and 600 nm. The MDA content was expressed as µmol.$Cell^{-1}$ and calculated as follows:

$$\text{MDA content} = \frac{OD(532 - 600\ \text{nm})/155 \times V_1 \times S/A}{Nt \times V_2}.$$

where, OD532–600 nm/155 means ΔMDA (µmol/mL), V_1 (mL) is the volume of reaction mixture; V2 (mL) is the volume of the algal culture, S (mL) is the extract volume, A (mL) is the measured volume, and Nt (cells/mL) is the algal density at time (t). Peroxidase activity was determined following the method of Putter (1974). The rate of increase in absorbance at 436 nm was measured at 25°C. Enzyme activity was calculated with an extinction coefficient of 20 mM cm^{-1} for tetraguaiacol.

Results and Discussion

The cell density and average specific daily growth rate (μ) have indirect proportion to the concentration of copper, and the percentage of growth inhibition and doubling time have direct proportion to the concentration of copper after 72 h.

Minimum cell density, growth rate, high growth inhibition and doubling time were observed at higher concentration (926 μg Cu L^{-1}) (Fig. 1a, b). The calculated 72-h IC_{50} value was 298.4 ± 28.3 μg Cu L^{-1}. Mean NOEC, LOEC (15.6 μg Cu L^{-1},) (29.6 μg Cu L^{-1}) and chronic value (21.5 μg Cu L^{-1}) were derived in chronic tests based on measured copper concentrations (Table 1). Generally, direct comparisons of EC_{50} values are difficult because of the use of different species, initial cell densities and laboratory set-ups in respect of light illumination, temperature, composition of culture media and exposure time (Table 2). The toxicity variations were observed more than three orders of magnitude for 13 fresh water algae (Blanck et al. 1984). It is clear that the effects of Cu on the growth of algae, depends on the species used, the composition of the culture medium (i.e., phosphorus, nitrogen, EDTA) and the experimental protocol etc. (Cid et al. 1995). Growth inhibition in microalgae has also been related to intracellular copper concentrations (Franklin et al. 2002). However, biota may bioaccumulate metals in non-metabolically active forms, so internal metal loadings do not always reflect differences in sensitivity (Luoma and Rainbow 2005).

In the present study, chlorophyll *a* concentration decreased with increasing concentration of copper from 79 μg L^{-1} up to 335 μg L^{-1}. Maximum of 1.29 ± 0.79 and 1.59 ± 0.79 pg. $cell^{-1}$ were found in 52 μg L^{-1} and control, respectively. Minimum of 0.01 ± 0.04 pg. $cell^{-1}$ was found in 574 μg L^{-1} and chl. *a* was nil at 926 μg L^{-1} (Fig. 2). Similarly, Fargasova et al. (1999) also reported EC_{50} of 0.408 μM for *Scenedesmus quadricauda* exposed to copper for 10 days with reduced chlorophyll of 33.8% when compared to control. The chlorophyll *a* was significantly reduced with increasing concentrations of copper but it was slightly increased with lower copper concentration in the present investigation. According to Kupper et al. (2002) at lower concentrations Cu^{2+} took over the functions of Mg^{2+} which showed elevated level of chlorophyll concentrations, at the higher concentrations the chlorophyll level reduced because Cu^{2+} inhibits the synthesis of d-aminolevulinic acid and the protochlorophyllide reductase (Stiborova et al. 1986), peroxidative breakdown of pigments and membrane lipids by reactive oxygen species (Sandamann and Boger 1980) and prevention of chlorophyll to integrate in chloroplast photosynthetic membranes (thylakoids) (Caspi et al. 1999).

Cell length and diameter varied among different concentrations of copper. Maximum cell length and minimum diameter were observed in 335 μg Cu L^{-1} (72.19 ± 7.65 μm and 21.42 ± 2.38 μm) and followed by 574 μg Cu L^{-1} (71.4 ± 6.29 μm and 19.0 ± 2.38 μm length and diameter, respectively). Maximum diameter (30.14 ± 5.98 μm and 30.14 ± 3.63 μm) was found in control and 52 μg Cu L^{-1}, and minimum (33.3 ± 6.2) length was found in 926 μg Cu L^{-1}. Morphological changes were not noticed up to 335 μg Cu L^{-1} other than

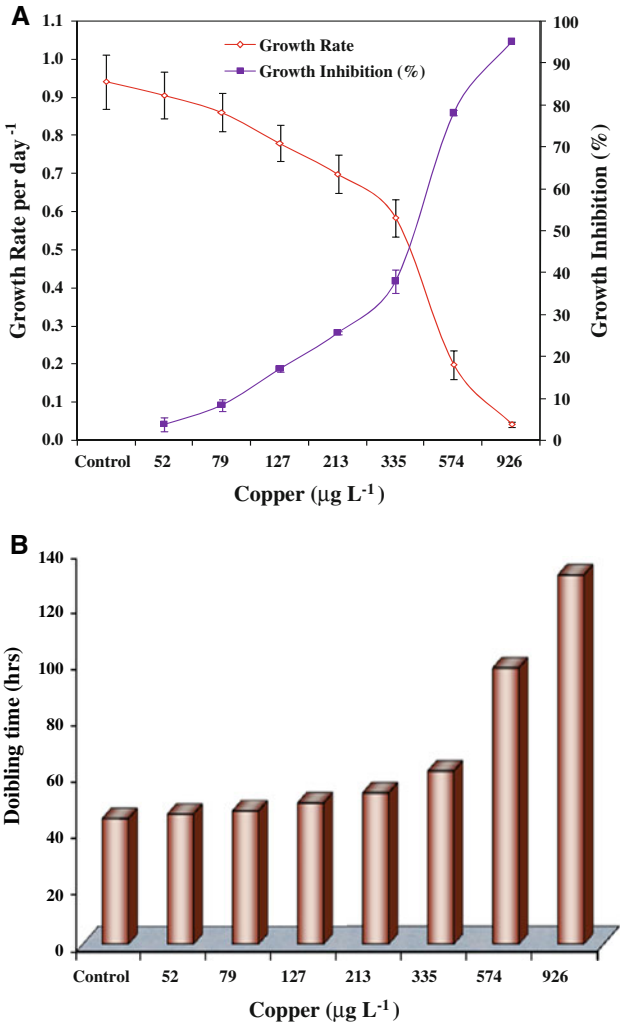

Fig. 1 **a**, **b** Dose–Response (growth rate, growth inhibition & doubling time) curve for the effect of copper (based on measured concentrations) on *O. mobiliensis* for 72 h exposure

Table 1 Nominal and Measured concentrations (Mean ± SD) of dissolved copper (μg L^{-1}) in test solution after 72 h and 7 days of exposure for acute and chronic test, respectively

Acute test		Chronic test	
Nominal concentrations	Measured concentrations	Nominal concentrations	Measured concentrations
61	52 ± 5.8	5	3.6 ± 0.3
98	79 ± 6.4	9	6.2 ± 0.4
156	127 ± 8.7	14	8.4 ± 0.6
250	213 ± 7.6	25	15.6 ± 0.5
400	335 ± 14.6	42	29.6 ± 0.8
640	574 ± 18.6	71	61.7 ± 0.2
1,024	926 ± 15.4	121	97.8 ± 0.4

Table 2 EC_{50} values of copper for different microalgae, obtained by different authors

Microalgae species	EC_{50} (ppb)	References
Aphanizomenon gracile	64	Luderitz and Nicklisch (1989)
Chlorella sp.	7–16	Franklin et al. (2001a, b)
Dunaliella tertiolecta	530	Levy et al. (2008)
Dunaliella tertiolecta	1,000	Franklin et al. (2001a, b)
Gonyaulax tamariensis	1,000	Anderson and Morel (1978)
Phaeodactylum tricornutum	100,000	Cid et al. (1995)
Phaeodactylum tricornutum	8	Levy et al. (2008)
Phaeodactylum tricornutum	20	Franklin et al. (2001a, b)
Rhodomonas salina	30	Moreno-Garrido et al. (1999)
Selenastrum capricornutum	7–17	Franklin et al. (2001a, b)
Tetraselmis sp.	47	Levy et al. (2008)
Isochrysis galbana	110–1,000	Wilson and Freeburg 1980
Isochrysis galbana	30–410	Ismail et al. 2002
Isochrysis galbana	910	Yap et al. 2004
Isochrysis galbana	4,200	Satoh et al. 2005
Skeletonema costatum	27	Ward and Boeri 1990
Odontella mobiliensis	298.4 ± 28.3	Present study

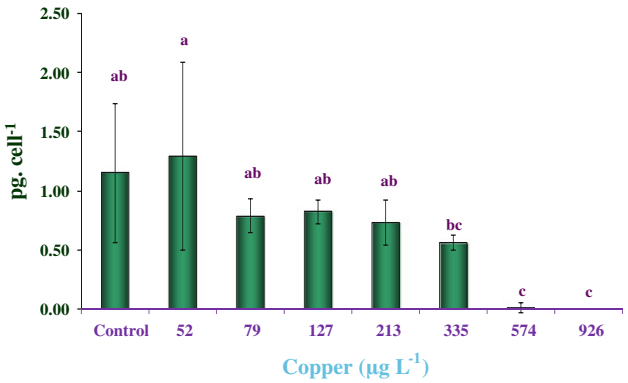

Fig. 2 Effect of Copper on chlorophyll *a* after 72 h exposure

enhancement of length but in the horn like structure at the apical part of the cells was visually modified and the entire cell shape was collapsed in 926 µg Cu L^{-1} after 72 h exposure (Figs. 3 and 4).

Odontella mobiliensis is the larger sized diatom than *Isochrysis galbana* (Satoh et al. 2005) and *Skeletonema costatum* (Ward and Boeri, 1990) but the sensitivity is lesser than *Skeletonema costatum* and greater than *Isochrysis. galbana*. It shows that the interspecies differences in copper sensitivity were not related to cell size, cell

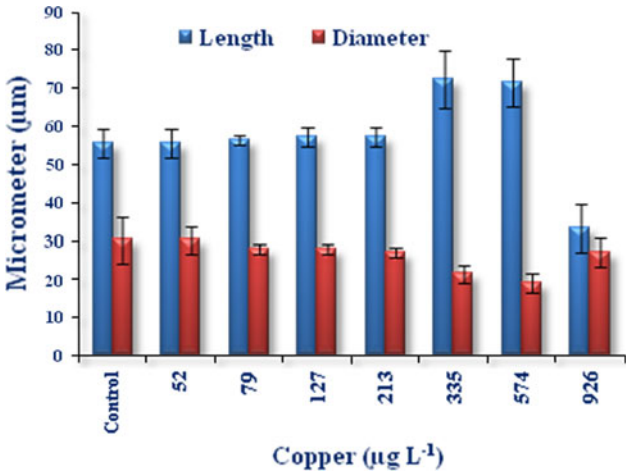

Fig. 3 Effect of Copper on cell size after 72 h exposure

wall type or taxonomic group. The differences in sensitivity may be due to differences in uptake rates across the plasma membrane, internal binding mechanisms and/or detoxification mechanisms between the different microalgal species (Levy et al. 2008). Quigg et al. (2006) reported that the cyanobacterium (*Synechococcus* sp.), is a most cu sensitive (i.e. 2–3 fold greater copper uptake) than eukaryotic algae such as *Tetraselmis levis* and *Emiliania huxleyi* (Levy et al. 2008). It is known that Cu had toxic effects on chromosomal morphology and mitosis cycle (Jiang et al. 2001).

In the present study, protein content was higher in copper treated sample than the control ($p < 0.05$) as reported earlier for *Spirulina* sp. and *Anabaena* sp. (Kumar et al. 2004). Increase in protein content under heavy metal stress in *Spirulina platensis* was also reported by Choudhary et al. (2007).

The nitrogen assimilation enzyme nitrate reductase activity was decreased under copper exposure (Table 3) as reported by Sharma et al. (1998). Trace elements play key roles in photosynthetic electron transport, participating in antioxidant enzymes such as ascorbate peroxidase and superoxide dismutase (Gueta-Dahan et al. 1997). In addition some transition metals are part of essential components of the photosystems or mobile electron carriers, such as the iron-containing cytochrome c and the copper containing plastocyanin (Raven et al. 1999). Although, many ROS generating processes are slow under normal conditions, toxic elements and xenobiotics can accelerate these processes (Torres et al. 2008). Higher levels of chloroplastic antioxidants would be critical for withstanding photo-oxidative stress elicited by a reduced energy utilizing capacity, resulting from trace elements and/or organic xenobiotic toxicity (Okamoto et al. 2001). Thus, algal tolerance to trace elements pollution in the environment is

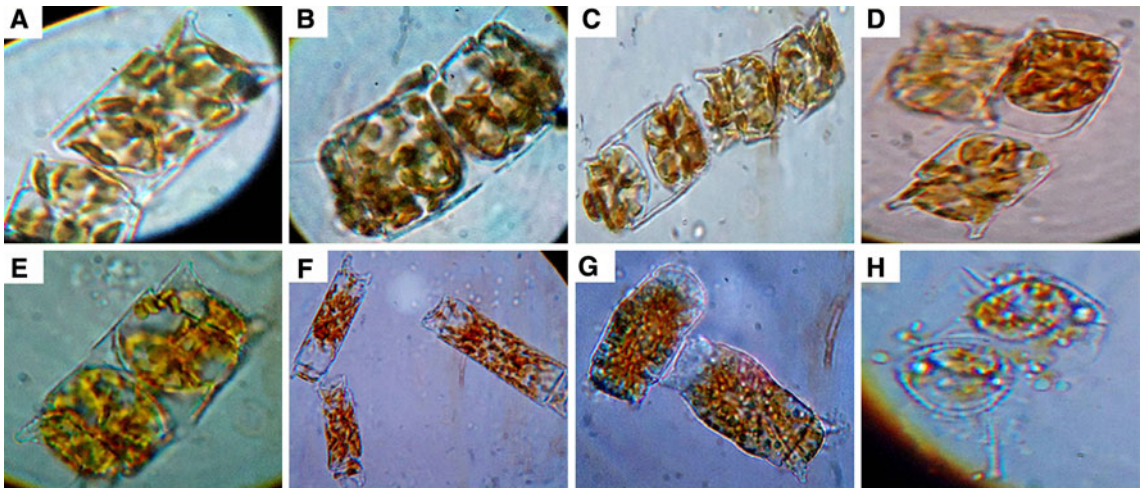

Fig. 4 Microscopic photographs of *O. mobiliensis* cells after exposure to different concentrations of copper for 72 h. **a** Control, **b** 52, **c** 79, **d** 127, **e** 213, **f** 335, **g** 574 and **h** 926 (μg Cu L^{-1})

Table 3 Protein, Nitrate Reductase and antioxidant enzyme activities in control and 21.5 μg Cu L^{-1} (based on measured concentration) *O. mobiliensis* for 7 days

	Protein (pg. $cell^{-1}$)	NR (U mg^{-1} protein)	MDA (μmol. 10^9 cells)	CAT (U mg^{-1} protein)	SOD (U 10^7 cells)	POD (U mg^{-1} protein)
Control	67 ± 5.8	0.11 ± 0.03	1.71 ± 0.02	127 ± 20.6	0.84 ± 0.11	44 ± 2.8
Test	84 ± 9.1	0.07 ± 0.01	2.16 ± 0.09	284 ± 37.8	0.72 ± 0.04	50 ± 4.7

The values in column are significantly different ($p < 0.05$)

likely to depend heavily on defense responses that prevent oxidative injury.

Significant increases in the activities of antioxidant enzymes (i.e. SOD and POD) were evident for *Skeletonema costatum* exposed to 2, 4-DCP for 96 h (Yang et al. 2002). Roy and Hanninen (1994) reported the induction of POD and SOD in the aquatic plant, *Eichhornia crassipes*, after exposure to PCP. Similarly in the present study the increased activities of catalase(CAT), peroxidase(POD) and malondialdehyde (MDA) contents were recorded more in treated samples than that of control ($p < 0.05$) (Table 3). It is evidenced that the ROS formed by metal stress and triggered *O. mobiliensis* to synthesis such enzymes for their survival.

Based on the results, the marine centric diatom, *O. mobiliensis* is more sensitive to copper. The mean NOEC, LOEC and chronic values were lower than the green algae higher than some diatoms from earlier report. Chlorophyll *a*, cell size and morphology were affected at higher concentration of copper. MDA, CAT and POD increased more in the copper treated samples than control, whereas NR and SOD were reduced. Protein content was significantly increased in the treated than the control samples. Further studies have to be carried out with other trace elements and common pollutants for the environment safety issues.

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References

Aebi H (1984) Catalase in vitro. Method Enzymol 105:121–126

Andersen RA (2005) Algal culturing techniques. Elsevier, Burlington. ISBN: 0-12-088426-7

Anderson DM, Morel FMM (1978) Copper sensitivity of *Gonyaulax tamarensis*. Limnol Oceanogr 23:283–294

Asada K (1984) Chloroplasts formation of active oxygen and its scavenging. Method Enzymol 10:422–429

Beauchamp CO, Fridovich I (1971) Superoxide dismutase improved assays and an assay applicable to acrylamine gel. Anal Biochem 44:276–287

Bilkhina O, Virolainen E, Fagerspedt KV (2003) Antioxidants oxidative damage and oxygen deprivation stress a review. Ann Bot 91:179–194

Blanck HWG, Wanberg DA (1984) Species dependent variation in algal sensitivity to chemical compounds. Ecotoxicol Environ Saf 8:339–351

Caspi V, Droppa M, Horvath G, Malkin S, Marder JB, Raskin VI (1999) The effect of copper on chlorophyll organization during greening of barley leaves. Photosynth Res 62:165–174

Choudhary M, Jetley UK, Khan MA, Zutshi S, Fatma T (2007) Effect of heavy metal stress on proline, malondialdehyde, and

superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. Ecotoxicol Environ Saf 66:204–209

Chow F, Oliveira MC, Pedersen M (2004) In vitro assay and light regulation of nitrate reductase in red alga *Gracilaria chilensis*. J Plant Physiol 161:769–776

Cid A, Herrero C, Torres E, Abalde J (1995) Copper toxicity on the marine microalga *Phaeodactylum tricornutum* effects on photosynthesis and related parameters. Aquat Toxicol 31:165–174

Draper HH, Squires EJ, Mahmoodi H, Agarwal JWS, Hadley M (1993) A comparative evaluation of thiobarbituric acid methods for determination of malondialdehyde in biological materials. Free Radic Biol Med 15:353–363

Elisabetta M, Gioacchino S (2004) Copper-induced changes of non-protein thiols and antioxidant enzymes in the marine microalga *Phaeodactylum tricornutum*. Plant Sci 167:289–296

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

Ferrat L, Pergent-Martini C, Romeo M (2003) Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality application to sea grasses. Aquat Toxicol 65: 187–204

Franklin NM, Adams MS, Stauber JL, Lim RP (2001a) Development of an improved rapid enzyme inhibition bioassay with marine and freshwater microalgae using flow cytometry. Arch Environ Contam Toxicol 40:469–480

Franklin NM, Stauber JL, Lim RP (2001b) Development of flow-cytometry based algal bioassays for assessing toxicity of copper in natural waters. Environ Toxicol Chem 20:160–170

Franklin NM, Stauber JL, Apte SC, Lim RP (2002) Effect of initial cell density of the bioavailability and toxicity of copper in microalgal bioassays. Environ Toxicol Chem 21:742–751

Grasshoff K, Ehrhardt M, Kremling K (1999) Methods of seawater analysis, 3rd edn. VCH publishers, Wainheins

Gueta-Dahan Y, Yaniv Z, Zilinskas BA, Ben-Hayyim G (1997) Salt and oxidative stress similar and specific responses and their relation to salt tolerance in citrus. Planta 203:460–469

Ismail M, Phang SM, Tong SL, Brown MT (2002) A modified toxicity testing method using tropical marine microalgae. Environ Monit Assess 75:145–154

Janero DR (1990) Malondialdehyde and thiobarbituric acidreactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 9:515–540

Jiang W, Liu D, Liu X (2001) Effects of copper on root growth, cell division, and nucleolus of *Zea mays*. Biol Plantarum 44: 105–109

Karthikeyan P, Jayasudha S, Sampathkumar P, Manimaran K, Santhoshkumar C, Ashokkumar S, Ashok prabu V (2010) Effect of industrial effluent on the growth of marine diatom Chaetoceros simplex (Ostenfeld 1901). J Appl Sci Environ Manage 14(14):35–37

Kumar S, Jetley UK, Fatma T (2004) Tolerance of *Spirulina platensis*- S5 and *Anabaena* sp to endosulfan an organochlorine pesticide. Ann Plant Physiol 18(2):103–107

Kupper H, Setlik I, Spiller M, Kupper FC, Prasil O (2002) Heavy metal-induced inhibition of photosynthesis targets of in vivo heavy metal chlorophyll formation. J Phycol 38:429–441

Levy JL, Angel BM, Stauber JL, Poon WL, Simpson SL, Cheng SH, Jolley DF (2008) Uptake and internalization of copper by three marine microalgae comparison of copper sensitive and copper tolerant species. Aquat Toxicol 89:82–93

Li M, Hu C, Zhu Q, Chen L, Kong Z, Liu Z (2006) Copper and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in the microalga *Pavlova viridis* (Prymnesiophyceae). Chemosphere 62:565–572

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193: 269–275

Luderitz V, Nicklisch A (1989) Response of phytoplankton to copper treatment with reference to species sensitivity. Int Rev Ges Hydrobiol 6:637–668

Luoma SN, Rainbow PS (2005) Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. Environ Sci Technol 39:1921–1931

Mallick N (2004) Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulgaris* response of the antioxidant system. J Plant Physiol 161:591–597

Mazhoudi S, Chaoui A, Ghorbal MH, Ferjani EE (1997) Response of antioxidant enzymes to excess copper in tomato *Lycopersicon esculentum* Mill. Plant Sci 127:129–137

Moreno-Garrido I, Lubia LM, Soares AMVM (1999) Oxygen production rate as a test for determining toxicity of copper for Rhodomonas salina Hill and Wehterbee (Cryptophyceae). Bull Environ Contam Toxicol 62:776–782

OECD (2002) Guidelines for the testing of chemicals, *Lemna* sp. growth inhibition test draft guideline, vol 221 Paris

Okamoto OK, Pinto E, Latorre LR, Bechara EJH, Colepicolo P (2001) Antioxidant modulation in response to metal induced oxidative stress in algal chloroplasts. Arch Environ Contam Toxicol 40: 18–24

Putter J (1974) Peroxidase. In: Hu B (ed) Methods of enzymatic analysis. New York, USA Academic Press, pp 567–1124

Quigg A, Reinfelder JR, Fisher N (2006) Copper uptake kinetics in diverse marine phytoplankton. Limnol Oceanogr 51:893–899

Rajendran K (1986) Studies on the effects of the heavy metals copper and zinc on two marine diatoms. *Thalassiosira fluviatilis* (hust) and streptotheca tamesis (shrubs). M. Phil., thesis Annamalai University, p 57

Raven JA, Evans MCW, Korb RE (1999) The role of trace metal in photosynthetic electron in O_2-evolving organism. Photosynth Res 2–3:111–150

Rietzler AC, Fonseca AL, Lopes GP (2001) Heavy metals in tributaries of Pampulha reservoir, Minas Gerais. Brazilian J Biol 61:363–370

Roy S, Hanninen O (1994) Pentachlorophenol uptake elimination kinetics and metabolism in an aquatic plant. Eichhornia crassipes Environ Toxicol Chem 13:763–773

Sabatini SE, Juarez AB, Eppis MR, Bianchi L, Luquet CM, Molina MCR (2009) Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. Ecotoxicol Environ Saf 72:1200–1206

Sampathkumar P, Kannan L (1998) Seasonal variations in physico-chemical characteristics in the Tranquebar-Nagapattinam region South-east coast of india. Poll Res 17(4):397–402

Sandamann G, Boger P (1980) Copper mediated lipid peroxidation processes in the photosynthetic membranes. Plant Physiol 66: 797–800

Satoh A, Vudikara LQ, Kurano N, Miyachi S (2005) Evaluation of the sensitivity of marine microalgal strains to the heavy metals Cu As Sb Pb and Cd. Environ Int 31:713–722

Sharma SS, Schat H, Vooijs R (1998) In vitro alleviation of heavy metal-induced enzyme inhibition by proline. Phytochemistry 49:1531–1535

Siedlecka A, Tukendorf A, Skorzynska-polit E, Maksymiec W, Wjcik M, Baszynski T, Krupa Z (2001) Angiosperms (Asteraceae, Convolvulaseae, Fabaceae and Poaceae: other than brassiacaceae). In: Prasad MNV (ed) Metals in the Environment. pp, Marcel Dekker Inc New york, pp 171–215

Soldo D, Behra R (2000) Long-term effects of copper on the structure of fresh water periphyton communities and tolerance to copper zinc nickel and silver. Aquat Toxicol 47:181–189

Srinivasan M, Swain GW (2007) Managing the use of copper-based antifouling paints. Environ Manage 39:423–441

Stiborova M, Doubravova M, Brezinova A, Friedrich A (1986) Effect of heavy metal ions on growth and biochemical characteristics of photosynthesis of barley *Hordeum vulgare* L. Photosysnthetica 20:418–425

Strickland JDH, Parsons TA (1972) A practical handbook of sea water analysis. 2nd ed. Bull fish res board of Canada bulletin 168: 310

Tewari RK, Kumar P, Sharma PN, Bisht SS (2002) Modulation of oxidative stress responsive enzyme by excess cobalt. Plant Sci 162:381–388

Torres MA, Barros MP, Campos SCG, Pinto E, Rajamani S, Sayre RT, Colepicolo P (2008) Biochemical biomarkers in algae and marine pollution a review. Ecotoxicol Environ Saf 71:1–15

USEPA (2002) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms, 3rd edn. Office of Water, Washington. EPA 821-R-02-014

Vergara JJ, Berges JA, Falkowski PG (1998) Diel periodicity in nitrate reductase activity and protein levels in the marine diatom *Thalassiosira weissfloggii* (Bacillariophyceae). J Phycol 34: 952–961

Ward TJ, Boeri RL (1990) Acute Static Toxicity of Nonylphenol to the Marine Alga Skeletonema costatum. Envirosystems Study 8970-CMA. Final Technical Report, Chemical Manufacturers Association Hampton NH, USA

Wilson WB, Freeburg LR (1980) Toxicity of metals to marine phytoplankton cultures. EPA-600/3-80-025 USEPA Narragansett RI: 110 p USNTIS PB80-182843

Yang S, Wu RSS, Kong RYC (2002) Biodegradation and enzymatic responses in the marine diatom *Skeletonema costatum* upon exposure to 2, 4-dichlorophenol. Aquatic Toxicol 59:191–200

Yap CK, Ismail A, Omar H, Tan SG (2004) Toxicities and tolerances of Cd Cu Pb and Zn in a primary producer (*Isochrysis galbana*) and in a primary consumer (*Perna viridis*). Environ Int 29: 1097–1104

Zhang E, Wang B, Wang Q, Zhang S, Zhao B (2008) Ammonia–nitrogen and orthophosphate removal by immobilized *Scenedesmus* sp isolated from municipal wastewater for potential use in tertiary treatment. Bioresour Technol 99:3787–3793