TOXICITY ASSESSMENT OF *ANABAENA SP.* FOLLOWING EXPOSURE TO COPPER OXIDE NANOPARTICLES AND SODIUM CHLORIDE

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Abstract. The widespread use of nanomaterials in recent years has caused rising pollution in surface and waste waters. It may be seriously affecting aquatic life such as cyanobacteria and thus, there is a compelling need to evaluate the potential effects of these substances in aquatic ecosystems. Copper oxide is one of the most common nanoparticles (CuO-NPs) used today. In this study, the responses of *Anabaena sp.* to CuO-NPs, salt stress and their combined effects (CuO-NPs+NaCl) were investigated. After 72 h exposure, MDA (malondialdehyde), proline, protein content, soluble sugar, total phenols and the activities of antioxidative enzymes such as peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and polypheno1 oxidase (PPO) were measured using spectrophotometry. Scanning electron microscopy (SEM) was used to image the results of exposure of cyanobacterial cells to nanoparticles. The effective concentrations (EC10, EC50, EC90), no observed effect concentration (NOEC) and percent inhibition (I %) was also calculated after 24, 48 and 72 hours. EC and NOEC data for CuO-NPs treatments were considerably higher than were those for NaCl treatments. CuO-NPs had a significant impact on the shape and morphology of *Anabaena sp.* cells and resulted in their swelling and enlargement. Modulations in enzymatic and non-enzymatic antioxidants were clearly evident in *Anabaena sp.* exposed to salt and CuO-NPs stress. Lipid peroxidation, measured in terms of MDA levels, increased with CuO-NPs and CuO-NPs+NaCl stress. Flavonoid and proline contents were also increased by CuO-NPs stress. Similarly, increases in POD and CAT activities were more pronounced in the CuO-NPs+NaCl treatments. On the other hand, APX activity enhancement was strongest in *Anabaena sp.* exposed to CuO-NPs.
Keywords: *antioxidant activity, aquatic ecosystems pollution, cyanobacteria, CuO-NPs, salinity, two combinational stress*

Introduction

Nanomaterials, products of nanotechnology, are an important part of numerous industrial and medical products and their use has increased dramatically in the past decade (Peralta-Videa et al., 2011). Being so widely used, it is likely that they will increasingly contaminate aquatic ecosystems (Klaine et al., 2008). Despite this obvious eventuality, the toxic ecological effects of nanomaterials have nevertheless not been easily evaluable; the mechanisms of toxicity of nanoparticles are not understood (Dhawan et al., 2009). The key physicochemical features of nanomaterials associated with toxicity which have been identified by toxicological researchers to date include factors such as aggregation, solubility, size, shape, elemental

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composition, contact area, porosity, surface ionic charge, and hydrodynamic diameter (Griffitt et al., 2008; Johnston et al., 2010, Peralta-Videa et al., 2011).

Biological responses of organisms are very much dependent on the physicochemical properties of nanoparticles. For example, nanoparticles are highly mobile in water and thus they can easily pollute aquatic ecosystems (Oukarroum et al., 2012). Nanotoxicity, as one of the most important aspects of nanotechnology, is induced by nanomaterials whose safety and toxicity characteristics are not clearly defined; and therefore must be determined to ensure public safety. In addition, high concentrations of salts, especially sodium chloride, are among the most important abiotic factors which can affect the survival of living organisms, including aquatic organisms such as cyanobacteria (Denizet al., 2011). The presence of toxic ions, mainly Na^+, creates distress due to osmotic potential and their effect on the uptake of inorganic nutrients (Deniz et al., 2011). This leads directly to inhibitory effects on growth, oxidative stress and finally death. It has been shown that high salinity inhibits photosynthesis, resulting in a decrease in the carbon pool of freshwater cyanobacteria (Srivastava et al., 2008) and lowering of the synthesis of compatible solutes (Ferjani et al., 2003). Furthermore, although nano metals and metal-oxides (Haulik et al., 2014) and salinity have harmful effects on cyanobacteria and plants individually, their effect cannot be directly extrapolated when two or more combinational stresses are induced (Suzuki et al., 2014). Algal cells, in contrast to human and animal cells, possess a cellulose cell wall, which acts as a barrier to particle uptake, prohibiting uptake of nanoparticles larger than 20 nm in size. Nevertheless, nanoparticles are capable of inducing pore formation, which may result in uptake of larger-sized particles (Navarro et al., 2008). Cyanobacteria are major organisms in aquatic productivity and significantly contribute to the availability of nitrogen in agriculture (Singh, 1961) and the evolution of oxygen-dependent respiration. *Anabaena*, as one of the significant cyanobacteria, is not only widely distributed across a wide range of salinities (Srivastava et al., 2011), able to cope with high osmotic potential by enhanced osmolyte synthesis (Hagemann, 2011), but also a preferred candidate for dinitrogen fixation (Singh, 1961). On the other hand, phytoplanktons are used as an early and direct warning signal because of their rapid response to stresses (Del Arco et al., 2014). However, not many studies have previouslybeen conducted on the combined effect of salt and CuO-NPs in cyanobacteria in general and *Anabaena* in particular, although their exposition to many kinds of stresses, individually, has been reported previously (Rai et al., 2013).

In the present study, the impacts of combined effect of CuO-NPs and salt stress were investigated on antioxidant activity and growth inhibition of *Anabaena sp.* collected from Guilan wetlands.

Materials and methods

Nanoparticles

CuO-NPs produced by US Research Nanomaterials Company, were used. The size of used CuO nanoparticles was 40 nm. A 2000 mg/l stock solution was used for preparing appropriate concentrations (*Table 1* and *Fig. 1*).

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Table 1. *CuO nanoparticle characteristics*

Purity	99%
Color	Black
Particle size	40nm
Specific area	20m2/g~
Morphology	nearly spherical
Bulk Density	0.79g/cm3
True Density	6.4g/m3

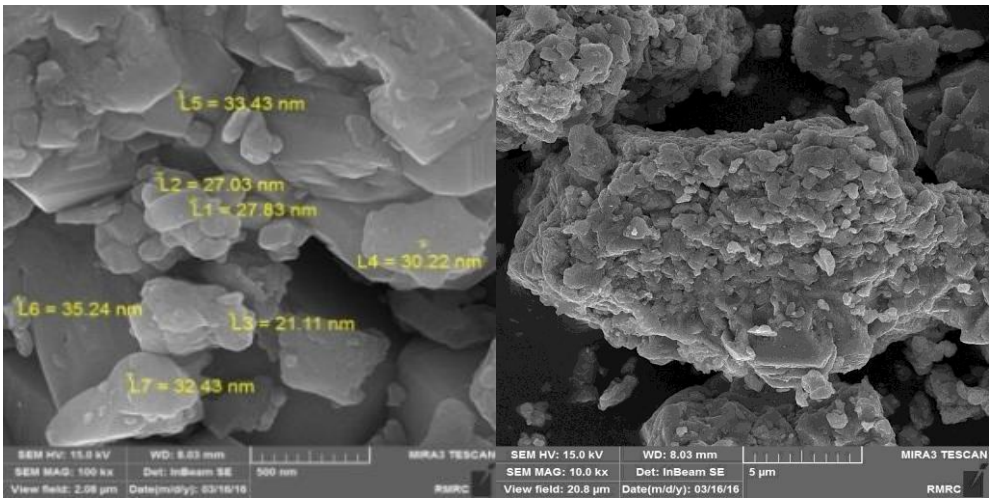

Figure 1. *SEM of CuO-NPs*

Release of Cu^{2+} From CuO-NPs

Stocks of nanoparticles at concentrations of 5, 10, 20 and 25 ppm were prepared with distilled water and the particles dispersed by sonication (Misonix Sonicator 3000, Iran) for 60 minutes. The pH was adjusted to 8.5 using NaOH. After 24 hours, 10 mL of the shaken up solution was taken from each flask, centrifuged 20 minutes at 4500 rpm and filtered through a 150 nm filter membrane. The content of Cu^{2+} was measured by atomic absorption flame spectrometer (Varian Spectra AA 220FS Varian Spectra Co. Australia).

Isolation of Anabaena sp. and preparation of culture medium

Cyanobacteria *Anabaena sp.* were collected from Guilan wetlands and isolated by repeated plating on solid Zander culture medium (Miller, et al., 1978). The pH of the culture medium was adjusted to 6.8, and the culture temperature was maintained at 24 ± 2 ˚C with aphoto period of 12 hours light/12 hours darkness. Light intensity was 60 µmol photon $m^{-2}S^{-1}$. Culture medium was sterilized by autoclaving at 121°C for 15 min and then refrigerated at 6°C.

Preparation of main treatments

The main stock of nanoparticles was prepared by 60 minutes ultrasonic treatment. Six treatments and one control with three replicates for each treatment and control were

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prepared. The concentrations of nanoparticles used in the experiments were 0, 5, 10, 15, 20 and 25 mg/l. 4×10^4 cells from the main stock of *Anabaena sp.* were added to 40 ml of the treatments by dilution of primary algal stock. This step was performed under completely sterile conditions. The test tubes were then stored at 24±2°C which was adjusted by a thermostat. All samples were maintained at constant light periodic condition (12 hours darkness and 12 hours light) for the 72 hours of the experiment.

Determination of growth rates in cyanobacteria

Growth inhibitory effects were studied according to the OECD201 method (Handy et al., 2012). At 24, 48 and 72 hours after the start of the experiment, aliquots of the solution were removed from the test tubes using Pasteur pipettes and the cells present were counted in a Neubauer chamber under an optical microscope. After counting and recording the data, the average number of cells in the top and bottom squares was calculated and then the number of cells was calculated using the following formula:

$$Cell\ density\ per\ ml = average\ cells\ counted\ in\ the\ large\ square \times 10^4 \times dilution\ factor$$

In each experiment, percent inhibition values were calculated using spectrophotometric data compared with the growth in control systems. EC values were calculated using linear regression analysis of transformed chemical concentration as natural logarithm data versus percent inhibition.

Experimental design and stress application

The EC_{30}doses of single and combined salt and CuO-NPs treatments were determined using the plate colony count method (Rai and Raizada., 1985). A 0.052 M solution of NaCl was used for salt treatment. In addition, 12.58 mg/L of CuO-NPs was used. For the NaCl+CuO-NPs study, the two doses were applied simultaneously. Biochemical evaluations were determined similarly 72 h after treatment. All experiments were done in triplicate.

Antioxidant assays

Assay for total phenolics

Measurement of total phenol was performed by method of Gao et al. (2000), using Folin- Ciocalteu reagent and a standard of gallic acid. For this purpose, 100 µl of extract (2 mg/ ml) was poured into a glass tube. Then, 200 µl Folin solvent and 2 ml of distilled water were added to the extract. 1 ml of sodium carbonate (21%) was added after 9 minutes to the glass tube in darkness. Finally, the samples were stored at room temperature in the dark for an hour. Blanks were prepared with 100 ml of solvent, instead of the extract. Absorptions were read at 765 nm. The total phenolic content was determined as gallic acid equivalents (mg GAE/g extract).

Assay for total flavonoids

Total flavonoid content was determined using a spectrophotometer according to Arvouet-Grand *et al.* (1994). 1 ml of extract solution with 2 mg/ml concentration was mixed with 1 ml of 2% aluminum trichloride ($AlCl_3$) methanolic solution. Absorbance of

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the reaction mixtures were measured at 415 nm against a blank after 10 min. Quercetin (QE) was employed as a standard reference and the total flavonoids content of the extracts was expressed as μg quercetin equivalents per gram of extract (μg QE/g extract).

Extraction and measurement of proline

Proline content in the samples was measured using the method of Bates et al. (1973). For this purpose, 1 g fresh biomass of Cyanobacteria was harvested and their cell walls were broken using 4 ml of 3% sulfosalicylic acid and sonication for 10 minutes (5 pulse seconds and 5 seconds rest). The resulting solution was centrifuged for 20 minutes at a speed of 13,000 rpm and immediately transferred to an ice bath. Finally, 4 ml of toluene was added to the reaction solution and absorbance was measured at 520 nm. Proline content in micrograms per gram fresh weight was calculated.

Total soluble protein

The measurement of total protein was conducted using 2 mg/ml extracts and Coomassie Brilliant Blue G-250 staining solution - in 95% ethanol and 85% ortho phosphoric acid (Bradford, 1976) using bovine serum albumin as a standard.

Lipid peroxidation content assay

Lipid peroxidation was measured in terms of the total content of 2-thiobarbituric acid (TBA)-reactive substances and expressed as equivalent of MDA (malondialdehyde), extinction coefficient 155 mM^{-1}, using the method of Heath and Packer (1968).

Estimation of enzymatic antioxidants

Measuring the activity of catalase (CAT)

The method of Aebi (1984) was used for investigation of catalase activities. For this purpose, 2.5 ml of 0.05 M phosphate buffer was mixed with 0.2 ml of 3% hydrogen peroxide and 0.2 ml enzyme extract. Absorbance was read at 240 nm for 2 minutes. The enzymatic activity of the enzyme was reported as units/mg of protein.

Measuring the activity of ascorbate peroxidase (APX)

APX activity was determined at an absorbance of 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 1 min in 1 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2, and 200 μl of enzyme extract (Nakano and Asada., 1981)

Measuring the activity of polyphenol oxidase (PPO)

Activity of polyphenol oxidase was assayed according to the method of Grigori (1993). 3 ml reaction solutions containing 2.5 ml phosphate buffer 50 mM (pH =7.2), 0.2 ml Pyrogallol 0.02 M and 300 Micro liters of enzyme extract were used. Changes in absorption were read at 430 nm for 2 minutes. The enzyme activity was calculated based on units of enzyme in 1 gram of fresh weight with extinction coefficient of 0.001 mM^{-1}/cm^{-1}.

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Measuring the activity of Guaiacol peroxidase (POD)

Kinetic activity of Guaiacol peroxidase was determined according to Kalir et al. (1984). First, 1 ml of the reaction solution containing 475 µl Guaiacol 45 mM, 475 µl hydrogen peroxide 100 mM and 50 µl enzyme supernatant was prepared. Changes in absorption due to Guaiacol oxidation were read by spectrophotometer at 470 nm for 2 minutes. An extinction coefficient of 26.6 mM^{-1}/cm^{-1} was used in the calculation of the enzyme activity. Peroxidase enzyme activity was calculated in Unit/gr FW. Each enzymatic unit is the amount of enzyme which increases 0.01% of the absorption at 470 nm per minute.

Electronic images

Scanning electron microscopy (SE-SEM, MIRA3 TESCAN) was used to investigate the effects of nanoparticles on the shape and size of cells in microscopic tissues of *Anabaena sp.* and to image the surfaces. Imaging of the surfaces of control and NP-treated cells was performed on cells exposed to the 10.93 mg/L NP concentration, which had affected 30% of the cells (EC_{30} = 10.93 mg/L).

Statistical analysis

Each treatment was replicated three times; the results were reported as the average of three parallel determinations of the mixture of three replicated samples. All statistical analyses were carried out using SPSS 16.0. One Way ANOVA with Duncan's test was used to determine significant changes in the results ($p < 0.05$).

Results

Release of Cu^{2+}

Suspensions of 0, 5, 10, 20 and 25 mg/l Cu^{2+} nanoparticles caused the release of 0, 0.017, 0.116, 0.249 and 0.310 mg/l of Cu^{2+} respectively, as assayed by atomic absorption flame spectrometry. After 24 hours, less than 0.31 mg/l Cu^{2+} was detected in our experiments (*Fig. 2*).

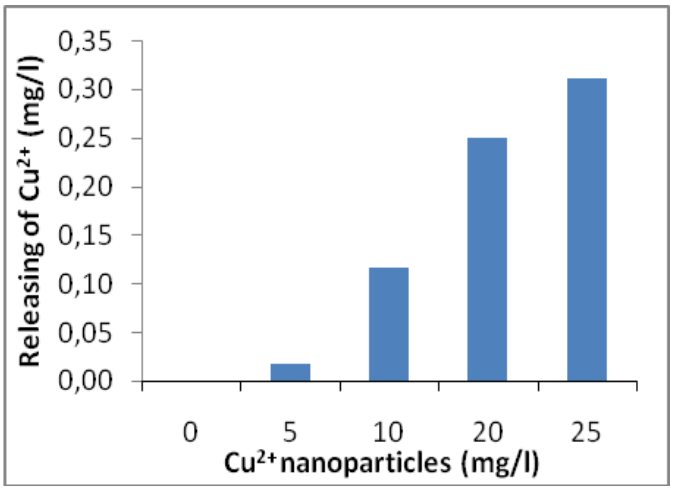

Figure 2. *Cu^{2+} release from N- CuO*

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Effects on growth rates

Anabaena growth in the presence of various concentrations of copper nanoparticles and salt is shown in *Figure 3*. The results indicate that cyanobacterial growth decreased by increasing the concentration of salt particles as compared with the control. The highest reduction occurred at 0.32 M NaCl and 25 mg/l CuO-NPs (*Fig. 3*). This was visually apparent, as seen by yellowing and fragmentation of filaments.

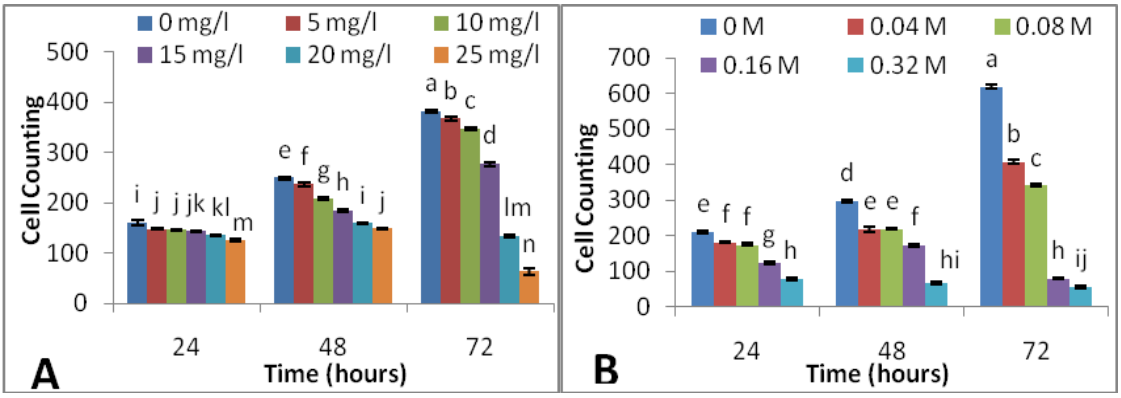

Figure 3. *Growth rate of Anabaena sp. after exposure to different concentrations of CuO-NPs (A) and NaCl (B).The data represents the average of three replicates ± standard error (SE). Different letters indicate significant differences among treatments according to Duncan's test with $p<0.05$.*

The results of 72-hour toxicity tests, the 24, 48 and 72-hour levels of EC_{10}, EC_{30}, EC_{50} and EC_{90} for CuO-NPs and NaCl toxicity are summarized in *Tables 2* and *3*. The range of EC_{30} values varied from 177.82 mg L^{-1} (24 h) to 12.58 mg L^{-1} (72 h) for CuO-NPs tests and from 0.114 M (24 h) to 0.052 M (72 h) for NaCl.

Table 2. *The values of EC_{10}, EC_{30}, EC_{50}, EC_{90} and NOEC for CuO-NPs*

Time (hours)	EC_{10} (mg/l)	EC_{30} (mg/l)	EC_{50} (mg/l)	EC_{90} (mg/l)	NOEC (mg/l)
24	13.7	177.82	1047.12	79432.82	104.71
48	8.91	18.19	29.51	97.72	2.95
72	7.76	12.58	17.37	38.9	1.73

Table 3. *The values of EC_{10}, EC_{30}, EC_{50}, EC_{90} and NOEC for NaCl*

Time (hours)	EC_{10} (M)	EC_{30} (M)	EC_{50} (M)	EC_{90} (M)	NOEC (M)
24	0.035	0.114	0.248	1.07	0.024
48	0.03	0.085	0.168	0.928	0.016
72	0.022	0.052	0.095	0.398	0.095

The effects on non-enzymatic antioxidants

The content of protein and soluble sugars

Protein content in all CuO-treatments showed a significant decrease compared to the control (*Table 4*). The percentage decreases of protein, relative to the control value were

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13, 44.8 and 41% in NaCl, CuO and NaCl+CuO-treated cultures, respectively. However cellular contents of soluble sugars after treatment with NaCl and CuO-NPs+NaCl increased significantly (*Table 4*).

Table 4. *Content of protein, proline, soluble sugars, total phenol, flavonoids and MDA in Anabaena sp. under NaCl, CuO-NPs, NaCl+CuO-NPs treatments. The data represents the average of three replicates ± standard error (SE). Different letters indicate significant differences among treatments according to Duncan's test with p<0.05.*

Column	Protein (mg/g FW)	Proline (mg/l)	Soluble Sugars (mg/l)	Total Phenol (mg/l)	Flavonoids (mg/l)	MDA (nmol/g FW)
Control	2.94± 0.25 a	6.27± 0.25 bc	15.65±0.45 b	13.5± 0.59 a	0.016± 0.0004 bc	6.25± 0.02 b
NaCl	2.6±0.34 ab	5.26± 0.02 c	63.47± 3.23 a	13.33± 1.24 a	0.015± 0.0002 c	9.89 ± 0.22 a
CuO	1.62± 0.39 b	10.21± 0.51 a	18.05± 0.1 b	13.6± 0.15 a	0.024± 0.0014 a	7.29± 0.29 b
NaCl+CuO	1.75± 0.12 b	7.42±0.44 b	65.96±1.83 a	13.87± 0.90 a	0.017± 0.0002 b	8.81 ± 0.63 a

The effect on malondialdehyde

Lipid peroxidation was assayed by measuring malondialdehyde (Zhanga et al., 2013). Changes of malondialdehyde in cyanobacteria *Anabaena sp.* under stress from copper nanoparticles, salinity and both stresses at the same time is shown in *Table 4*. Malondialdehyde content significantly increased under CuO-NPs and CuO-NPs+NaCl compared to control.

Proline content

Proline content in treatments with NaCl and NaCl+CuO-NPs showed no significant changes. However, in the CuO-NPs treatment, proline content increased 64.1 % at the 10.93 mg/l level of CuO-NPs (*Table 4*).

Total phenols and total flavonoids

Phenolic content did not differ significantly between the three types of treatment compared and with the control group. Flavonoid content significantly increased 63.1% in CuO treatment compared to the control, while there was no significant difference between control, NaCl and NaCl+CuO treatments.

The effect on enzymatic antioxidants

In this study, the enzyme activity of POD in treatment with CuO-NPs and NaCl was found to be significantly increased. In contrast, polyphenol oxidase (PPO), another enzyme that is involved in neutralizing ROS compounds and assayed in this study, was significantly reduced in all treatments compared to control. CAT activity showed a different behavior, with a significant increase compared to control (~600 %, $p < 0.05$) in treatment with NaCl+CuO as well as (~400 %, $p < 0.05$) in treatment with CuO, while it remained unchanged in NaCl treatments; and finally, the other peroxide scavenging enzyme, APX, was also found to be induced under CuO-NPs stress (*Table 5*).

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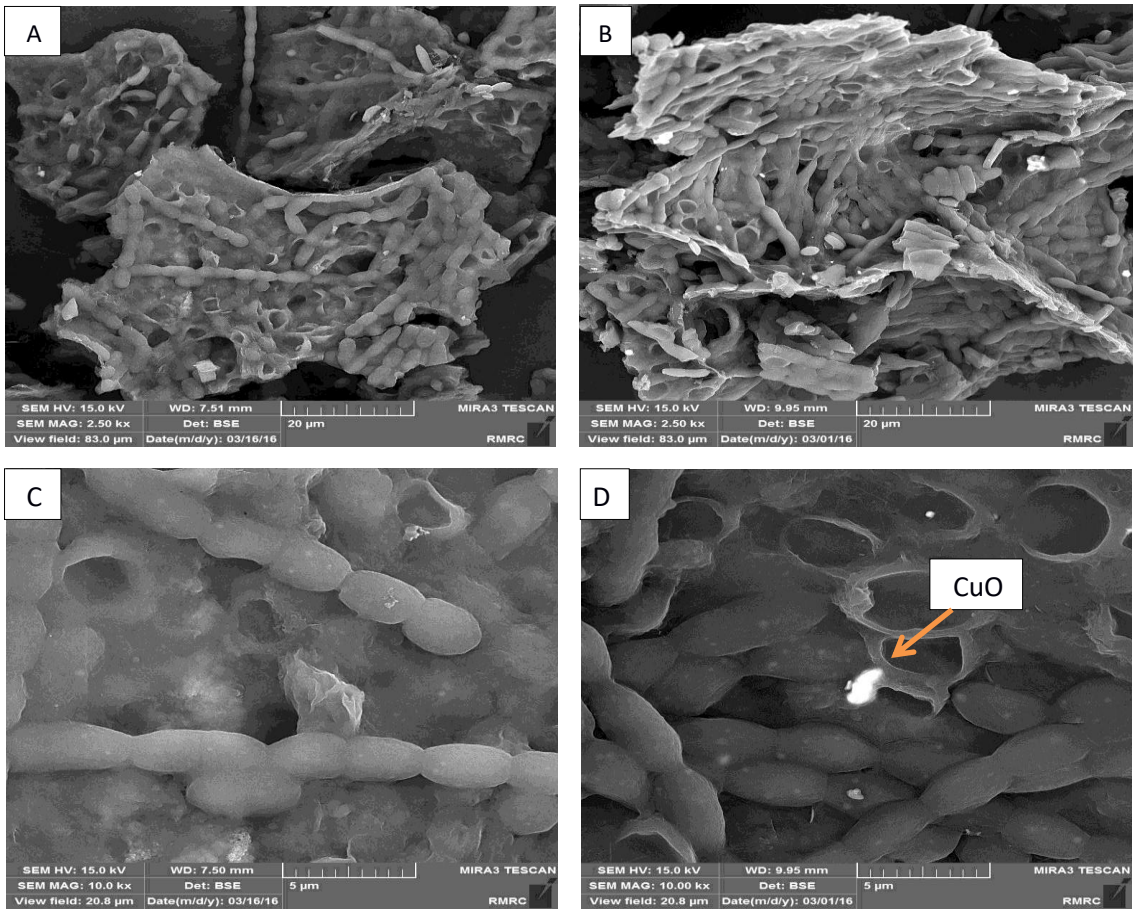
Table 5. *Enzymatic antioxidant content in Anabaena under NaCl, CuO-NPs, NaCl+CuO-NPs treatments. The data represents the average of three replicates ± standard error (SE). Different letters indicate significant differences among treatments according to Duncan's test with $p<0.05$.*

Factors / Treatments	POD (Unit/g FW)	PPO (Unit/g FW)	APX (Unit/g FW)	CAT (Unit/mg protein)
Control	0.008 ± 0.0006b	2200 ± 57.7a	0.446 ± 0.026c	0.0025 ± 0.0006c
NaCl	0.005 ± 0.0013a	1666.7 ± 33.3b	0.625 ± 0.026b	0.005 ± 0.0006c
CuO	0.009±0.0012a	1666.7 ± 33.3b	0.834 ± 0.015a	0.0095 ± 0.001b
NaCl+ CuO	0.016 ± 0.0013a	1000 ± 9c	0.521 ± 0.039c	0.0152 ± 0.0019a

Electron microscopy

CuO-NPs attached to the surface of the cyanobacterial cells in direct contact with them is demonstrated clearly by SEM images in *Figure 4*. Generally, electron microscopic images showed that the nanoparticles bound to each other and, more relevantly, to the surface of the cyanobacterial cells, altering the appearance of cells through disruption of direct physical interactions between the cells and increasing the number of lysed cells (*Fig. 4, A-E*).



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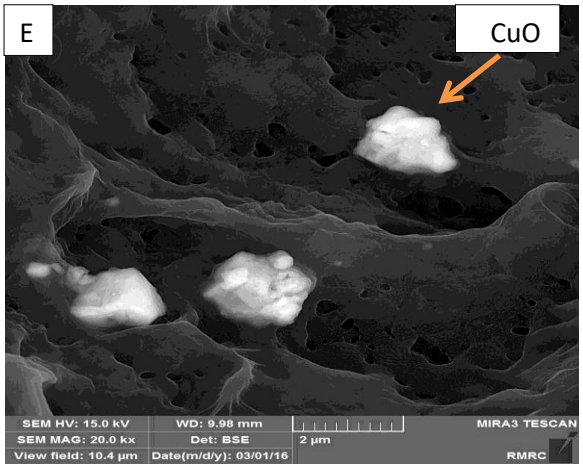

Figure 4. *SEM images of Anabaena sp. after 72 hours exposure to CuO-NPs at concentration of 10.93mg/l. Figures of A and C show control samples and figures B, D and E show Anabaena cells which treated with CuO-NPs.*

Discussion

As illustrated in *Figure 2*, small amounts of Cu^{2+}were released. This can be attributed to the aggregation of CuO-NPs and the slightly alkaline pH environment of the suspension (Wang et al., 2013). Cells cannot exclude Cu^{2+} and accumulate it in high quantity. Cu^{2+} becomes powerfully toxic for phytoplankton communities at concentrations between 0.1 and 1.0 mg/l (Deniz et al., 2011). The release of Cu^{2+} which is seen in our experiment is in this range too. Although algal cells are able to encase and detoxify Cu^{2+} but this can occur in restricted ranges (Toncheva et al., 2006). The decrease in the rate of cell division caused by metals has been primarily attributed to their binding to sulfhydryl groups which are important for regulating plant cell division (Visviki and Rachlin., 1991). Furthermore, The difference between effective concentrations ($EC_{10,30,50}$) in two treatments indicated that the degree of nanoparticle toxicity is also likely to depend on their nano structures and high surface to mass ratio as well as the nature of their constitutive element (Hernández Battez et al., 2010).This varies between algal species (Pendashte et al., 2013).The results of Shi (2011) have also indicated that CuO-NPs toxicity is three to four times higher than that of ionic Cu, because of the larger uptake of NPs-released Cu. Therefore, as it is shown in *Figure 2*, cell counting decreased during CuO-NPs treatment, compared to NaCl treatment.

As is shown in *Table 4*, percentages of proteins decreased in treated samples relative to control samples meaningfully. Rahman et al. (2011) observed similar findings in cyanobacteria under metal stress; Cu^{2+} treatment also resulted in reduction of protein content extensively. This decline may be due to production of ROS, which is known to damage protein, and therefore disturbs the cellular homeostasis. Similar observations were made, also, in *A. doliolum* exposed to cadmium and UV-B stressed (Bhargava et al., 2007). In contrast, adaptation to salt stress in cyanobacteria is generally accomplished by excretion of inorganic ions from the cells to balance osmotic potential; cells can also prevent denaturation of macromolecules via accumulation of some osmolytes, such as sucrose, trehalose, glucosylglycerol, glycine betaine, proline and/or glutamate (Allakhverdiev et al., 2005).

Lipid peroxidation is linked to the production of O_2^-. The presence of high amounts of transitional metals such as Cu (II) favors the enhanced generation of OH° from O_2^-

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through the Fenton reaction (Luna et al., 1994). Thus, the increased levels of MDA suggest that Cu (II) ions stimulated free radical formation in *Anabaena sp*.

Increases in proline concentration under heavy metal and salt stresses have been reported in some higher plants and in the cyanobacteria *Spirulina*, *Anabaena* and *Cylindrospermum* (Rahnama and Ebrahimzadeh., 2004; Chris et al., 2006; Choudhary et al., 2007). It seems that an adaptive mechanism via increase in proline is activated for neutralization of accumulated CuO-NPs and the acidity. Proline's chelating ability to bind metal ions can also be a defense mechanism for survival (Deniz et al., 2011).

It has been recently shown that accumulation of secondary metabolites, such as phenylpropanoids, including flavonoids, which has scarcely been reported in cyanobacteria, could be one of the strategies for cyanobacterial organisms to protect against cellular damage (Singh et al., 2014). Flavonoids, phenolic acids, and tannins, as well as some derivatives of flavonoids, are also compounds which have many different biological activities including antioxidant activity under stress conditions (Agati et al., 2011), in addition to some medicinal activities (Fresco et al., 2006). Although only scarce reports are available on total phenols and total flavonoid content under salt and nano particle stress tolerance responses in cyanobacteria, positive correlations with the accumulation of polyphenolics, enhanced antioxidant activity and tolerance to stresses have been reported in plant species (Korkina, 2007). As a result, there is no additive response of non-enzymatic antioxidant factors in NaCl+CuO-NPs combination treatment compared to their responses to single stresses. This may be due to their antagonistic effects (Rai et al., 2013).

During oxidative stress, POD is a very important enzyme able to remove the dangerous radical products of superoxide and hydrogen peroxide (Beier et al., 1991). The greater activity of POD in treated compared to control plants can be attributed to stress caused by the treatments (Cosio and Dunand, 2010). These results suggest that NaCl and CuO-NPs individually as well as NaCl+CuO-NPs can be very important stressors impacting plant physiology. Reduction of PPO in both NaCl and CuO-NPs-treated cells was more than other treatments. In our study, CAT activity significantly increased compared to control in all treatments of CuO-NPs. Free Cu^{2+} can catalyze the formation of highly toxic ROS such as hydroxyl radicals ($OH^{\bullet}$) from superoxide anions ($O2^{\bullet-}$) or hydrogen peroxide (H_2O_2) (Melegaria et al., 2013). Therefore, increase in CAT activity of treated samples compared to control very probably is a response to substantial ROS production. APX induction may be attributed to the *apx* operon induction by H_2O_2 production (Vranova et al., 2002). Therefore, it seems that H_2O_2 can be detoxified by a combined effort of POD, CAT and APX in *Anabaena sp*. That enhanced levels of most antioxidant enzymes, CAT, POD and APX, were observed for *Anabaena sp.* in this study indicates the protective role of these enzymes against copper-induced oxidative stress (*Table 5*). Finally, it can be noted that, except for CAT, which shows more activity in NaCl+CuO-NPs treatments compared to NaCl and CuO-NPs treatments, there were no additive activities in other enzymatic antioxidants.

CuO-NPs often increased cell aggregation by forming bridges between the cells in the areas where the nanoparticles had accumulated. The Cuo-NPs attached to the surface of the cyanobacterial cells and the direct contact is demonstrated clearly by the SEM images in *Figure 4*. Their external surfaces had begun to fracture compared with the surfaces of cells that were not in contact with these particles. The structure of the cell wall was probably affected by the metal ions (*Fig. 4E*). Similarly, others have found cellular ultrastructural changes in *Microcystis aeruginosa* which were exposed to Ag-

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NPs (1 mg l^{-1}) (Duong et al., 2016). After 48 h exposure with Ag-NPs the structure of *Microcystis* was changed; the cells were shrunken and distorted. Other studies on the impact of nanoparticles on other species have yielded similar results (Ayatallahzadeh Shirazi et al., 2015).

Conclusion

The outcomes of this study suggest that single stresses of NaCl and CuO-NPs can have inhibitory effects on *Anabeana sp.* in the used ranges, inducing severe damages in high concentrations. However, stress induced by these factors in combination did not result in elevated inhibitory effects, more than those observed in single stresses. This may be related to interactions between released ions from NaCl and CuO-NPs.

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