Research Article

Effects of copper oxide nanoparticles on the *Chlorella* algae in the presence of humic acid

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Abstract
Utilization of nano compounds has greatly increased the discharge of these compounds into the environment, and has increased the possible risk to human health and environment. The aim of this study was to investigate the possible toxic effects of copper oxide nanoparticles on the *Chlorella* algae in the presence of dissolved and surface-bound humic acid. *Chlorella* cells were left to grow in the culture media containing copper oxide nanoparticles and HA in different treatments. After specified contact times, the number of algae and Reactive Oxygen Species were daily measured. The results showed that the copper oxide nanoparticles and HA (individually) had a concentration-dependent toxicity on *Chlorella* algae and these compounds with high concentrations increase the amount of intracellular ROS and decrease the growth rate of *Chlorella* algae. The LC_{50} of copper oxide nanoparticles and HA was 2.7 mg/l and 25 mg/l respectively. Dissolved HA in mixture with copper oxide nanoparticles intensified the nanotoxicity by reducing the relative growth rate and increasing the reactive oxygen species. The ROS values reached to 315% and 340% in the experiments with 16 mg/l CuONP + 16 mg/l dissolved HA and 16 mg/l CuONP + 32 mg/l dissolved HA (respectively) compared to the blank controls. The relative growth rate scaled up with increasing the HA content of the complexes of CuONP–HA (surface-bound HA).

Keywords Humic acid · Copper oxide nanoparticles · *Chlorella* algae · Reactive oxygen species

Abbreviations

ROS	Reactive oxygen species
HA	Humic acid
CuONP	Copper oxide nanoparticles
ROS	Reactive oxygen species
RSM	Response-surface method

1 Introduction

In recent years, concerns about the potential environmental risks of the usage of Nano materials is increased, and the need to assessing and evaluating the potential risks of this technology in the environment is unavoidable [1]. Nano scale copper oxide is applicable in the industries such as solar cells, ceramic pigments, rotating magnets, and also it can be used as scum [2]. Considering the concerns about the toxic effects of copper oxide nanoparticles on different organisms, various studies have been done to investigate their harmful effects on various aquatic organisms [3–6].

Algae as a primary producer have a very important role in aquatic ecosystems and can be used as a model for assessing the potential toxicity of nanoparticles in aquatic systems [7]. *Chlorella vulgaris* (a non-mobile cell) is an ideal organism for cellular studies because of its high reproduction rate [8]. In order to evaluate the nanoparticles toxicity in the environment, especially natural waters, it needs to observe the physico-chemical properties of water [9].

Natural organic matters with different characteristics in the aqueous environments may have interactions with

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other pollutants in water and affect their toxicity [2]. For example, it is reported that natural organic matters have an effect on the cumulative behavior of nanoparticles and its effect is by neutralizing surface charges [10]. On the other hand, natural organic matters stabilize metal nanoparticles in suspension and increase their bioavailability [11].

Some investigations have shown that these matters reduce the toxicity of nanoparticles, but another group of studies has argued that organic matters increase the toxicity of nanoparticles on different organisms. Therefore, the effects of a natural organic matter on the toxicity of nanoparticles seem to be complex and require special investigations [12]. This study aimed to explore the influence of dissolved HA as a natural organic matter on copper oxide nanoparticles (CuONP) toxicity in *Chlorella* algae using 2 experimental methods.

2 Materials and methods

2.1 Copper oxide nanoparticles and HA

CuONP, with the particular average size of 40 ± 10 nm, specific area of 20 m^2/g, purity of 99%, bulk density of 0.79 g/cm^3 and true density of 6.4 g/cm^3 was supplied from Nanosany corporation company in Iran. The company claimed that the nanoparticle was purchased from US Research Nanomaterials, Inc., and presented the elemental analysis and SEM and TEM images of nanoparticles which are shown in Fig. 1 and Table 1. The toxic metals are important because of probable releasing and interfere in the experiments. The toxic metals in the used nanoparticle were very low (vs. Cu) and possible releasing of them from 16 mg/l nanoparticle (maximum used concentrations of CuONP in the experiments) was negligible.

In order to the following applications, stock solution of 100 mg/l was prepared. The solution was placed in an ultrasonic bath for half an hour and placed on a stirrer to allow particles to remain disperse [4]. HA was prepared from Sigma Aldrich Company and stock solution of 1000 mg/l was prepared. 4 g of NaOH were dissolved in 500 ml distilled water, then 1 g of HA was added to the solution, and the volume of the solution was adjusted to 1 l. It was then placed on a stirrer for 24 h at room temperature to be homogeneous. After adjusting the pH of the solution at 7, the solution was filtered and kept in the refrigerator. This stock solution was completely stable for 15 days and daily solutions were prepared from it [13].

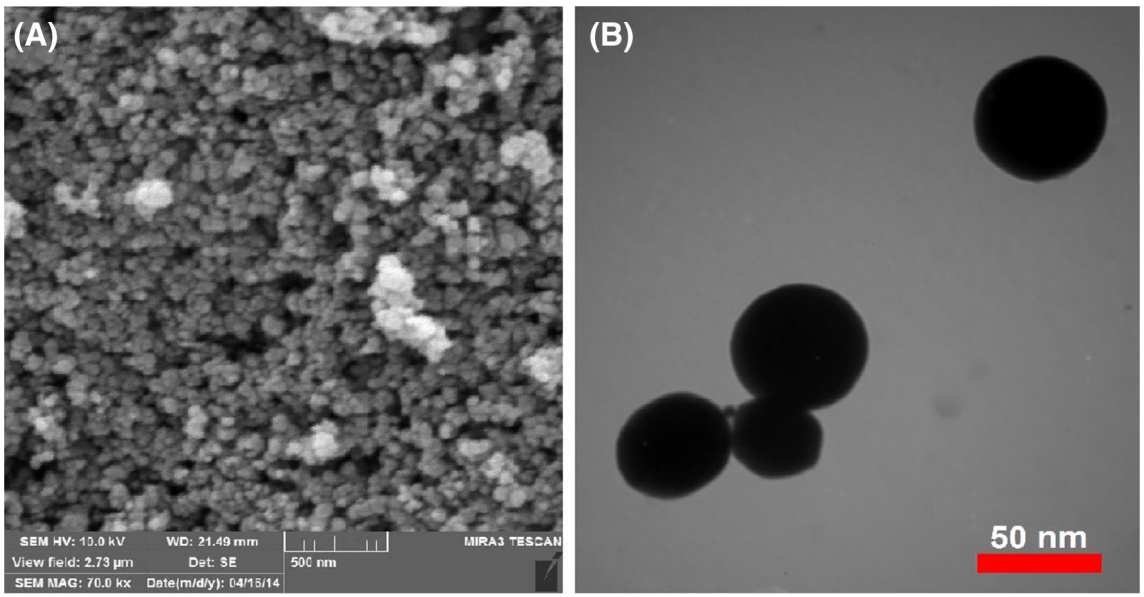

Fig. 1 SEM (**a**) and TEM (**b**) images of CuO nanoparticles

Table 1 The elemental analysis of CuO nanoparticles. Adopted from www.us-nano.com (the manufacturer of CuONP)

Elements (ppm)											
Ba	Cd	Co	Zn	Sr	Ca	K	P	Mg	Fe	Pb	Mn
0.75	2.5	6.4	195	2.3	400	300	300	75	87	90	3.5

2.2 Preparation of the CuONP–HA complexes

Different initial solutions with different concentrations of HA were prepared and CuONP with a concentration of 5 g/l were added to each of them and placed in a shaker at 110 rpm and 25 °C for 3 days. The resulting mixture was then centrifuged at 3500 rpm for 30 min. The resultant precipitate was washed with ultra-pure water, freeze-dried, gently grounded and stored as CuONP–HA complex.

In the initial solutions, the concentrations of HA were 0–10–50–100–500–1000–1500 mg/l, so the final compositions with different ratios of HA to the nanoparticle were named 0; 0.2; 1; 2; 10; 20; 30% complexes [12].

The adsorption of HA on the CuONP and released Cu were determined by measuring residual HA and dissolved Cu in the solution at pH = 7. After centrifugation of CuONP–HA complexes in (3500 rpm, 30 min), the residual HA in the supernatant were determined using a UV spectrophotometer (Hack DR5000) at 254 nm and Cu was measured by GF-AAS method (graphite furnace atomic absorption spectrophotometer) (Varian 2400). The amount of adsorbed HA on the nanoparticles was calculated by mass balance method. All of the samples and blank (without CuONP) were measured in triplicate. The tests of adsorption of HA on the CuONP showed that HA can be adsorbed on the CuONP.

The results of GF-AAS analysis demonstrated that Cu was released into the solution. The concentration of released Cu in the solutions with different concentrations of HA, was 0.15% (in the absence of HA) to 5% (in the presence of 1500 mg/l of HA). The amount of dissolved Cu released from CuO NPs was markedly increased by the presence of HA.

2.3 Algal growth assays

The vials *containing Chlorella* algae cells were prepared from the National Center for Genetic Reserves of Iran. This study was carried out according to the OECD guidelines and BBM medium (BOLD'S BASAL MEDIUM (MODIFIED) was prepared and used for development of *Chlorella* algae [14].

Algal growth rate test was conducted in bath cultures in different flasks according to the standard procedure (OECD guidelines, 2006) using fresh water *Chlorella volgaria*. A cabinet with laminar air flow was prepared and was pre-sterilized with ultraviolet lamp for test performances. It should be noted that the required materials to prepare the culture medium were first sterilized under pressure of 0.1 MPa for 20 min and the control flasks were used as a comparative criterion. The tests were started in exponential growth phase and, the initial number of algal cells was about 1×10^5 cells/ml in each culture.

The growth response of Cholorella exposed to each of tested substances was determined by measurement of relative growth rate (after counting the cells) and reactive oxygen species (ROS). The cells were counted using a hemocytometer and the number of algae per ml was calculated according to the OECD guideline. Algae cells were counted at 24-h intervals and the relative growth rate (G) was calculated using the following equation.

$$G = \left[\frac{N}{N_0}\right] * 100$$

G = relative growth rate, N = Number of cells counted, N_0 = Primary Cell Number.

Experimental K The growth constant coefficients (K) of algae for each group were calculated using the first order kinetic model equation in different time intervals and the mean of values of K are reported.

$$K = \mathrm{Ln}(N/N_0)/_t$$

K = growth constant coefficient.

Plotting method K The values of K were calculated by plotting Ln (N/N_0) against the time (plotting method), and the linearity of the plot was tested using the regression coefficient (R^2).

2.4 Measurement of reactive oxygen species (ROS)

The role of metallic nanoparticles in generation of reactive oxygen species (ROS) is previously well known [15].

Using the intracellular oxidation of 2′,7′-dichlorodihydroflurescein diacetate (H2DCF-DA), the amount of intracellular ROS was determined by fluoromet'ric method. Algae were incubated for 4 days in BBM medium in the control group and other treatment groups. Then, 50 ml of the above treated suspensions were separated and centrifuged (1000 rpm, 15 min). The supernatant was discarded and the suspended algae was washed with BBM medium twice and re-fed to 50 ml. Then 10 µl of H2DCF-DA was added to the medium for 30 min in the dark. It was repeatedly washed with BBM medium. The fluorescence intensity was measured with spectrofluorimeter using an excitation of 495 nm and an emission of 530 nm. Finally, relative ROS was calculated [12].

2.5 Experimental design

Step 1 In order to investigate the individual effect of CuONP and HA (independent variables), these compounds were added individually to culture medium in a series of

experiments (one-factorial method) and after determined time intervals, the relative growth rate and ROS were measured as dependent variables.

Step 2A In order to determine the effect of CuONP in mixture with dissolved HA, and identification of interactions between them, response-surface method (RSM) was used using Design Expert®6.0.6, (state-Ease Inc., Minneaoolis, USA) software. In statistics RSM explores the relationship between several depended variables and one or more independent variables. After entering the data (concentration levels of CuONP and HA and time intervals) the software presented a series of different experiments (treatments) with different concentration of CuONP and HA and time using D-optimal method. The software also presented the statistical analysis such as analysis of variance and regression methods.

Each experiment in both experimental methods was conducted in 3 replicates, dependent variables were measured, and the results were analyzed.

Step 2B In order to test the validity of the results, a series of experiments were conducted with fixed concentration of CuONP and different concentrations of HA.

Step 3 A series of bath experiments were conducted with 2 mg/l of CuONP–HA complex with different preloaded concentrations of HA, in order to find out the influence of surface-bound HA on the nanotoxicity. The concentration of CuONP–HA complex was constant (2 mg/l), because the aim of the tests was to find the effect of increasing of HA loading on the nanoparticles. Therefore, our experiments were performed with different complexes which were loaded by different concentrations of HA.

Each experiment was conducted in 3 separate flasks (triplicate) in all of the steps.

3 Results

3.1 The results of one factorial experimental design

Adding different concentrations of copper oxide nanoparticles individually (1–16 mg/l) to the algae culture medium showed that the relative growth rate was reduced significantly in the flasks with more than 4 mg/l of nanoparticles ($p < 0.05$) (Fig. 2). The relative growth rate of *Chlorella* algae was plotted against different concentrations of nanoparticles after 96 h of exposure and the IC_{50} of the nanoparticle was determined as 2.7 mg/l.

The kinetic models and obtained growth constant coefficients (K) for *Chlorella* algae calculated for all treatments are shown in Table 2. The K values after 96 h were significantly lower than the control treatment in all tests and decreased with increasing concentration of CuONP from 0.66 to − 0.29. The pattern of decreasing of K values obtained from both experimental results (experimental K) and kinetic models (plotting method K) were similar and both values are close to each other. The values of counted algae from the experiments were compared with the number of algae obtained from kinetic models. The results show that the correlation coefficient between these values is higher than 0.8, which indicates that the models have sufficient validity.

3.2 The effect of HA on *Chlorella* algae growth

As shown in Fig. 3, the concentrations of 4 and 8 mg/l of HA, during 48 h, increased the relative growth rate of algae ($p > 0.05$). However, after 72 h, the relative growth rate was reduced ($p < 0.05$). Plotting the relative growth rate of *Chlorella* algae after 96 h of exposure against different concentrations of HA showed that the IC_{50} of HA is 25 mg/l.

As shown in Table 3, the growth constant coefficient of algae in all treatments after 24, 48, 72 and 96 h decreased compared to the control treatment, but the rate of decreasing the growth rate is very slower than the decreasing rate in the presence of CuONP.

The number of algae from the experiments and the number of algae obtained from the kinetic models were comprised using a regression model. The regression coefficients between these values were higher than 0.9.

3.3 Influence of dissolved HA on the Nano toxicity

In order to study the interaction between HA and cooper oxide nanoparticles in algal growth, the concentration ranges of independent variables were entered to the Design Expert software. The software proposed 36 experiment variables treatments with different values of CuONP and HA concentrations and contact time. The experiments were carried out with adding CuONP in mixture with HA and after counting the cells, the relative growth rate of each treatment was calculated. The results were entered to the software to statistical analysis. The results of analysis of variance are presented in Table 4.

The F value in the analysis of variance method shows the effect intensity of the variables. As shown in the Table 4, the F value (208.99) for the nanoparticle (A) is greater than the F value of two other variables of HA (B) and contact time (C), which shows that the nanoparticle plays the main role in algal growth over two other factors of time and humic acid. Meanwhile, the effect of time is significant and its effect intencity ($F = 33$) is higher than HA.

The results of ANOVA analysis show that the interaction effect of CuONP and HA is notable with the F value of 144.

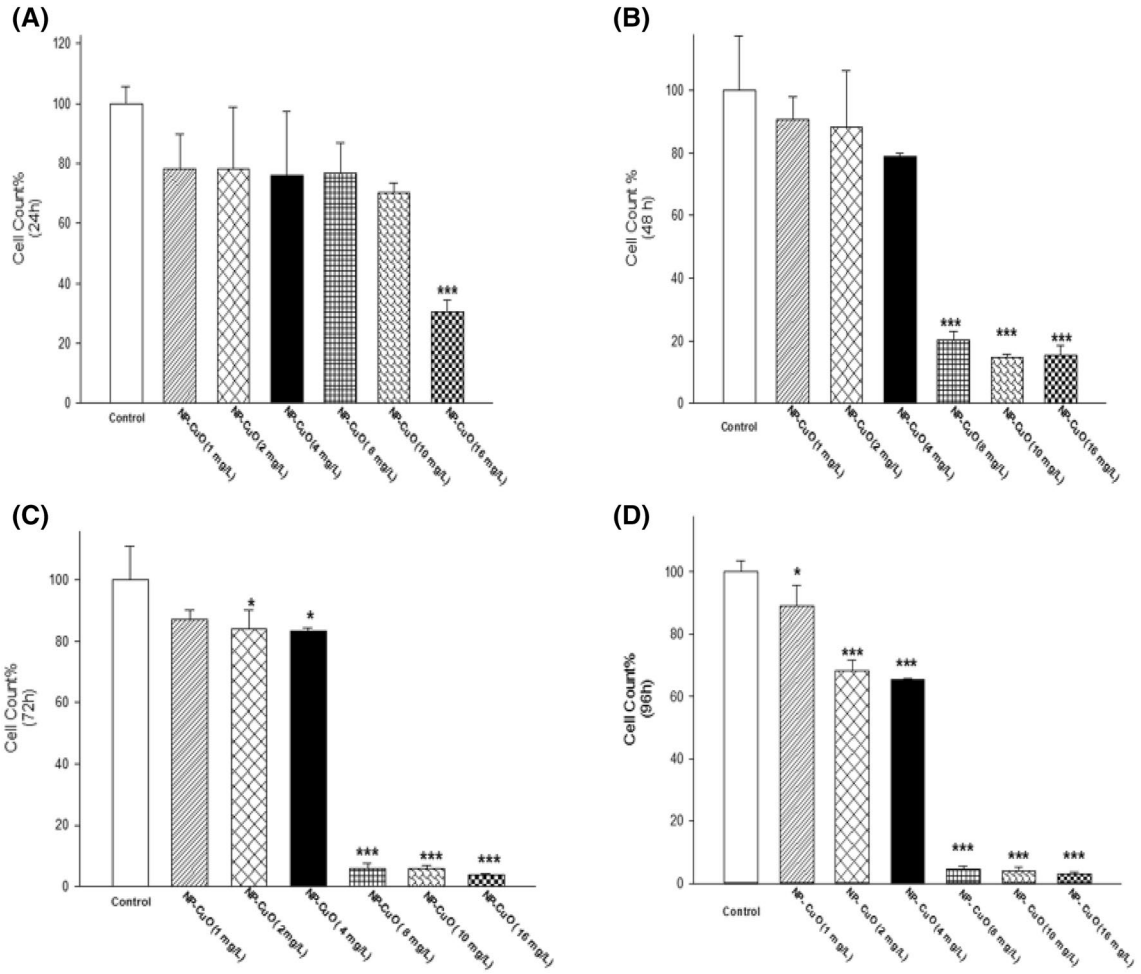

Fig. 2 The effect of copper oxide nanoparticles on the cell growth in time intervals. **a** 24 h, **b** 48 h, **c** 72 h, **d** 96 h. Results are reported as Mean ± SD. *$p < 0.05$; *$p < 0.01$; ***$p < 0.001$ indicates a significant difference with the control group

Table 2 *Chlorella* algal growth indexes in exposure to various concentrations of CuONP

CuONP (mg/l	Experimental K	Models obtained from the plotting method (kinetic model)	Plotting method K	Linearity of the models (R^2)	Correlation coefficient between numbers of algae from the experiments and kinetic models
0	0.73	$y = 0.66x + 0.19$	0.66	0.9674	0.9807
1	0.69	$y = 0.65x + 0.1067$	0.65	0.9694	0.9635
2	0.65	$y = 0.53x + 0.35$	0.53	0.8856	0.8678
4	0.63	$y = 0.57x + 0.1667$	0.57	0.871	0.8128
8	−0.11	$y = -0.16x + 0.1867$	−0.16	0.8848	0.9905
10	−0.16	$y = -0.11x + 0.13$	−0.11	0.9758	0.9712
16	−0.23	$y = -0.295x + 0.145$	−0.29	0.9595	0.9826

According to the analysis of variance results, the lack of fit of the model with the data was not significant ($p > 0.05$), it indicates that there is a good fitness between the growth rates predicted by the model and the data obtained from the experiments. The following equation is presented for 96 h contact time and has shown that the concentration of nanoparticles (A) has more effect on reducing the growth rate than HA (B).

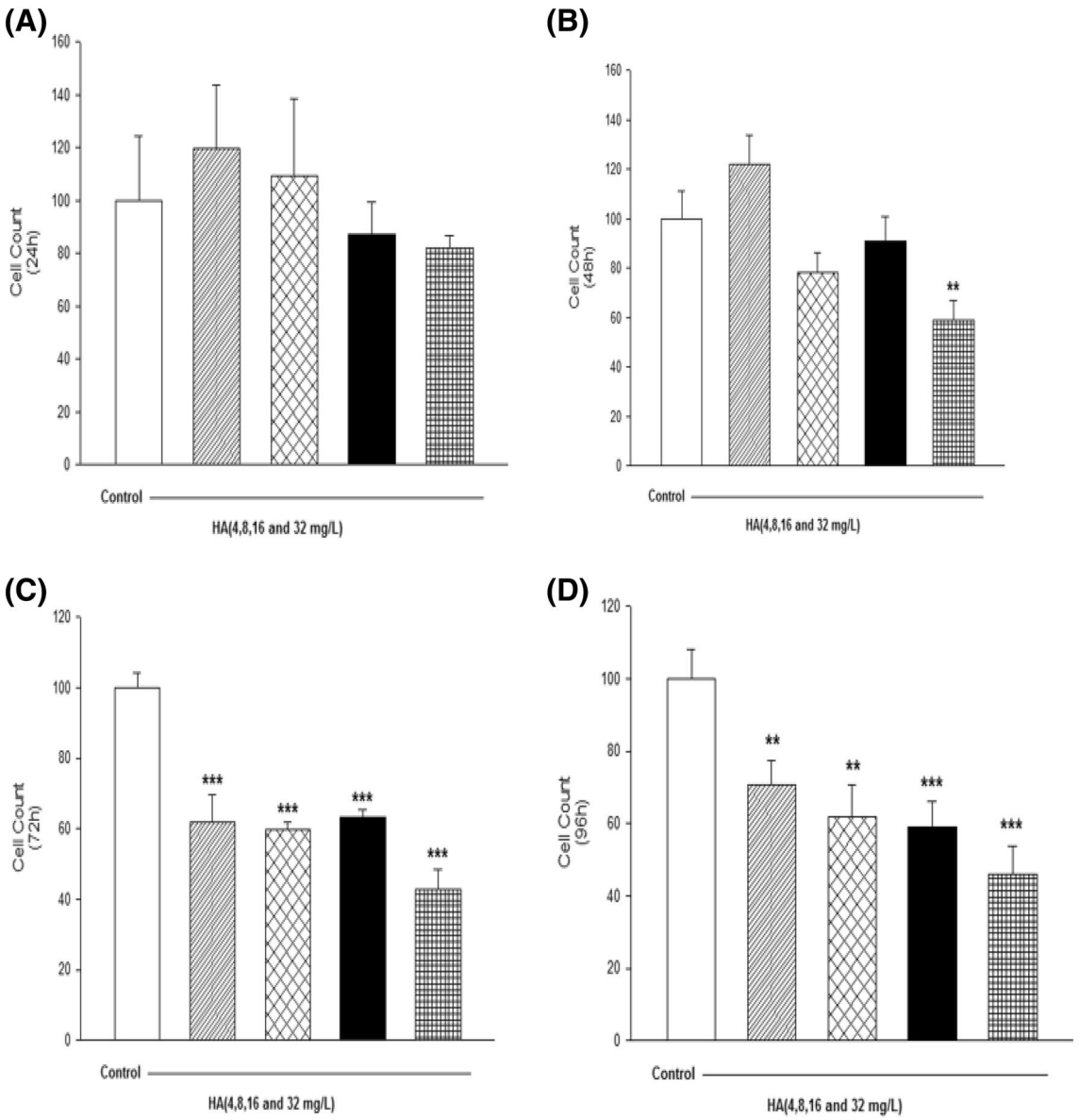

Fig. 3 The effect of HA on the cell growth in time intervals. **a** 24 h, **b** 48 h, **c** 72 h, **d** 96 h. Results are reported as Mean ± SD. *$p < 0.05$; *$p < 0.01$; ***$p < 0.001$ indicates a significant difference with the control group

Table 3 *Chlorella* algal growth indexes in exposure to various concentrations of HA

HA (mg/l)	Experimental K	Models obtained from the plotting method (kinetic model)	Plotting method K	Linearity of the models (R^2)	Correlation coefficient between numbers of algae from the experiments and kinetic models
0	0.74	$y = 0.8136x + 0.188$	0.81	0.973	0.999
4	0.7	$y = 0.5405x + 0.4263$	0.54	0.9741	0.992
8	0.61	$y = 0.6952x + 0.2471$	690.69	0.9951	0.995
16	0.63	$y = 0.5985x + 0.0961$	0.6	0.994	0.995
32	0.49	$y = 0.6594x + 0.464$	0.66	0.9967	0.997

Table 4 The summarized results of analysis of variance and modeling of relative growth rates of experiments

Parameter	Sum of squares	*df*	Mean square	F value	*p* value	Significances
Model	47,523.12	27	1760.12	22.12	< 0.0001	Significant
A	16,259.19	1	16,259.19	208.99	< 0.0001	Significant
B	72.52	1	72.52	0.93	< 0.0001	Significant
C	7780.18	3	2593.39	33.33	< 0.0001	Significant
AB	11,249.28	1	11,249.28	144.6	< 0.0001	Significant
AC	1642.61	3	547.54	7.04	0.0124	Significant
BC	1083.5	3	361.17	4.64	0.0367	Significant
Lack of fit	143.11	4	35.78	0.3	0.8657	Not significant
Residue	622.39	8	77.80			

A, CuONP; B, HA; C, contact time

$$\text{Grow percentage} = 106.6 - 10.6A - 7.4B - 0.78A^2 + 0.39B^2 + 0.51AB + 0.06A^3 - 0.0071B^3 - 0.017A^2B - 0.0034AB^2$$

To validate the model, the measured values were compared with the predicted values by the model, and the correlation coefficient between the results of the actual values of the analysis and the predicted values obtained by the model (R^2) was 0.91.

Figure 4 shows the graphical results of RSM method. The rate of algal growth in the experiments with higher concentrations of HA and CuONP was markedly lower than the other experiments. It means that the HA has a synergetic effect on the toxicity of CuONP.

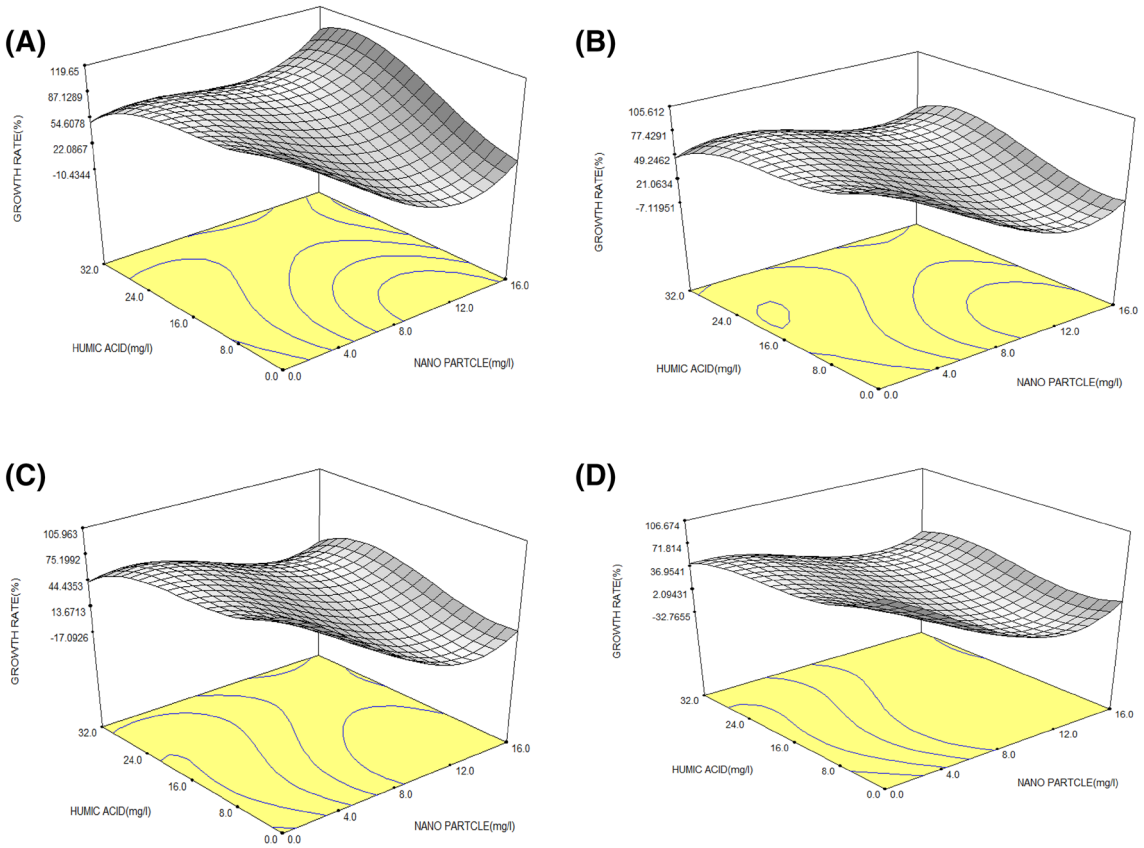

Fig. 4 Interaction of time, HA and of copper oxide nanoparticles on the relative growth percentage of *Chlorella* algae. **a** 24 h, **b** 48 h, **c** 72 h, **d** 96 h

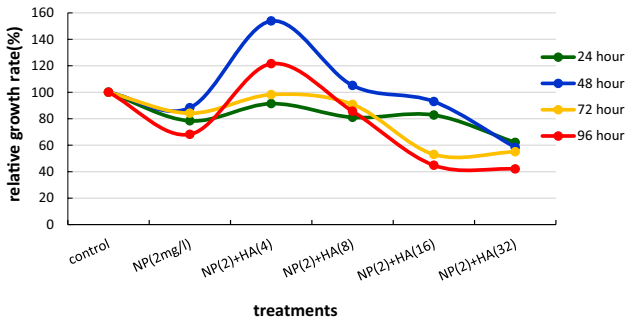

Fig. 5 The relative growth rates of algae in the experiments with 2 mg/l CuONP and different concentrations of HA. NP, copper oxide nanoparticle; HA, humic acid (mg/l)

In order to clear the effect of increasing of HA concentration on the growth rate, experiments were conducted with 2 mg/l of CuONP and 0, 4, 8, 16, and, 32 mg/l of dissolved HA. Increasing the concentrations of dissolved HA from 0 to 32 mg/l, decreased the relative growth rates from 68 to 42%. The results are presented in Fig. 5.

3.4 Measurement of reactive oxygen species (ROS)

Another tested parameter for determination of the toxicity effects of copper oxide nanoparticles and HA on the *Chlorella* was ROS. In some of the treatments the ROS values were measured and the results were compared with relative growth rates (Table 5).

The results showed that in treatments which were polluted with CuONP or HA (individually), the relative growth rate decreased and the ROS increased. The relative growth rates in the treatments with 8 and 16 mg/l of CuONP were 5% and 3% respectively and ROS increased from 77 to 124%. These increase in ROS and decrease in relative growth rate were also observed in the cultures polluted with HA (without CuONP).

In the flasks with high concentrations of CuONP (16 mg/l), the synergetic effects of HA on the toxicity of CuONP were clear. In these treatments, the ROS values reached to 315% and 340% in the last treatments (16 mg/l CuONP + 16 mg/l CuONP and 16 mg/l CuONP + 32 mg/l HA) compared to the blank controls.

3.5 Toxicity of CuONP–HA complex

Two milligram per liter of different prepared pre-loaded nanoparticles (CuONP–HA complexes) were added to the algae culture medium in different flaxes, after finishing the time, the growth constant coefficient and relative growth rates were calculated in each treatment.

In this step, the concentration of CuONP–HA complex was constant (2 mg/l), because the aim of the tests was to find out the effect of increasing the HA loading (bounding) on the nanoparticles. Therefore, our experiments were performed with different complexes which were loaded by different concentrations of HA. The adsorption tests which were conducted by spectrophotometric method showed that more than 80% of the dissolved HA was adsorbed on the nanoparticles. The measurement of remained HA in the supernatant of the preparation process of complex confirmed the adsorption of HA on the copper oxide nanoparticles.

The results showed that in the flasks containing CuONP–HA complexes with increasing loaded of HA (increasing HA content of the complexes), a significant increasing in the relative growth rates was observed after 96 h (Fig. 6).

4 Discussion

4.1 Effect of dissolved CuONP

The toxic effects of nanoparticles in research studies have been related to the small size and surface properties (large surface area per mass unit) of nanoparticles, the release of metal ions [4, 16–19] or the adhesion of nanoparticles to the cell wall [20, 21] and ROS production [17, 22, 23]. Oxidative stress plays the main role in

Table 5 The results of ROS test in different treatments in comparison with growth indexes

Treatments	ROS (%)				Relative growth rate (%)				K
	24 (h)	48 (h)	72 (h)	96 (h)	24 (h)	48 (h)	72 (h)	96 (h)	
CuONP: 8 mg/l	11	25	42	77	77	20	6	5	−0.16
CuONP: 16 mg/l	26	29	50	124	30	15	4	3	−0.29
HA: 16 mg/l	14	19	37	110	109	78	60	62	−0.6
HA: 32 mg/l	17	43	39	119	82	59	43	46	−0.66
CuONP: 8 mg/l + HA: 16 mg/l	40	43	103	153	72	41	25	10	−0.62
CuONP: 8 mg/l + HA: 32 mg/l	48	54	159	194	64	36	28	8	−0.67
CuONP: 16 mg/l HA: 16 mg/l	37	138	147	315	77	34	15	5	−0.87
CuONP: 16 mg/l HA: 32 mg/l	49	141	229	340	91	40	13	4	−1.06

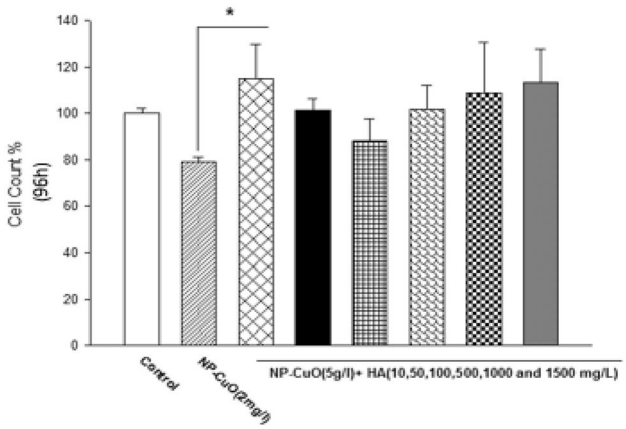

Fig. 6 The relative growth rates of algae in different treatments polluted with CuONP–HA complexes

the toxicity of various nanoparticles [24, 25]. The toxicity of copper oxide nanoparticles in various organisms is due to the high absorption rate of copper oxide nanoparticles and intracellular interactions in various organisms [26, 27]. Dissolution of toxic copper ions (Cu^{2+}) from nanoparticles with the induction of cytotoxicity, shading effect and reducing of light, adhesion, oxidative stress and other physical impairments are the other reported mechanisms in the literatures [27–30].

Decreasing of algae growth rate in the present study shows that algae growth rate are affected by one or more of the above factors.

Entering the CuONP into the cells is well explained by researchers. Ion channels and transporter proteins are the main ways of entering CuONP into the cells. Transport and ion/voltage-gated channels permit released Cu^{2+} from CuONP to enter into the cells. The main mechanism of toxicity of CuONP in the cells is induction of ROS by interaction with oxidative organelles such as mitochondria. One of the key factors in nanotoxicity is dissolution of metal ions from particles. ROS can be induced by Cu^{2+} and chemical reactions [2, 31].

The findings of this study are comparable to the results of some studies on the toxicity of copper oxide nanoparticles in different organisms [4, 9]. Increasing of ROS in the flasks contained 8 and 16 mg/l of CuONP from 77 to 124 shows that the oxidative stress is the main mechanism of nanotoxicity and releasing of Cu^{2+} is occurred and entered into the cell.

4.2 Effects of dissolved HA

Dissolved organic matter such as HA can absorb on algal cells and affects the permeability of the membrane or serve as a food source [32, 33]. The results of this study showed that HA alone in a 48-h period and in concentrations lower than 4 mg/l in algae culture medium acts as a nutrient and increases the growth of algae. It has been reported that there is a dose–response phenomenon characterized by low dose simulation and high dose inhibition [12]. HA can be a carbon source at low concentrations and has a xenobiotic-like effect on organisms at high concentrations. At long time, the HA is accumulate and shows the toxicity effect on the organisms. The intracellular ROS of algae was measured and compared with the control group. The results showed that HA at higher concentrations of 32 mg/l and more, after 48 h, increased the production of ROS significantly. Our findings were comparable to the results of the study of Lin et al. [12] in which the HA with concentrations of less than 5 mg/l act as a carbon source and improve the growth of microorganisms.

4.3 Effect of dissolved CuONP at the presence of dissolved HA

The previous studies have shown that the amount of releasing ions in the solution, and the most important factor of NOMs such as HA, have direct effect on the nanotoxicity.

The results of various studies suggest different mechanisms for the effect of HA on the nanoparticles and their toxicity such as: the change in the physical properties of nanoparticles such as nanoparticle deposition [12], blockage of the contact of the nanoparticle and the cell [10, 34–36], increasing the accumulation and deposition of nanoparticle [2], preventing the dissolution of the nanoparticle [37], or reducing the accumulation of nanoparticles, increasing the penetration of nanoparticles into the cell, forming of CuONP–HA complex and sulfidation [11, 38–40]. In this study at the variable and constant concentrations of nanoparticles and adding different concentrations of HA, the HA could increase the toxicity of nanoparticles. Agglomeration of CuONP by HA is one of the probable effective factors, however the results show that the CuONP had toxicity effect on the growth at low concentrations. It shows that CuONP was quiet widely dispersed in the solution and probability agglomeration is weak.

Peng et al. [31] have published a report in which, they explained the influence of pH, electrolytes and NOM on the CuO nanoparticles in the aquatic environment. They exactly have shown that NOM has the following clear cut effects on the CuO nanoparticles. (1) The presence of humic acid enhances the dispersion and stabilization of CuO nanoparticles suspension and alleviates the agglomeration of CuONP. (2) Humic acid improves the dissolution of CuONP. In their study the soluble Cu released from CuONP was 19% at PH 7, but the amount of released Cu^{2+} was markedly increased

by the presence of the humic acid. (3) The presence of humic acid significantly decreased sedimentation rate of CuONP.

The most important reason can be the releasing of Cu ions at the presence of HA and the role of this ion in induction of ROS. Similarly, in present study the releasing of the Cu^{2+} and its nanotoxicity increased with increasing of the HA, therefore, it can be concluded that the oxidative stress can be the main reason of toxic effect of CuONP. Dispersion of the CuONP by HA can be another reason of enhanced toxic effect. The F value of 144 in the ANOVA analysis shows the synergetic effect of HA.

4.4 Influence of CuONP–HA complex on the nanotaxicity

Figure 6 shows the effect of surface-bound HA on the growth rate of algae. The relative growth rate increased with increasing of HA content of the complexes. The results showed that the surface bound humic acid alleviated the nanotoxicity, and oppositely, dissolved HA enhanced the toxicity of CuONP. The main reason is that in the process of preparation of CuONP–HA complex, the HA (during 3 days) is gradually adsorbed on the surface of CuONP. During this time, Cu released from the CuONP, but we separated the complex by centrifuging and Cu^{2+} ions remain in the solution.

In the experiments with dissolved HA, HA increases the releasing of Cu^{2+} and these ions could have harmful damages on the cells, but in the experiments with CuONP–HA complex, the cells were protected because the CuONP was coated by HA.

Intracellular reactions such as ROS production and cellular uptake need close contact of nanoparticles and the cells, and ions of Cu^{2+} in the solution. Adsorption of HA on the nanoparticles can limit this contact and adhesion and result in the ROS production reduction.

Increasing of surface negativity of coated CuONP and also increasing of repulsion forces between CuONO and negatively charged algal cells is another effective mechanism of decreasing nanotoxicity effect of CuONP–HA complex.

5 Conclusion

This study showed that the copper oxide nanoparticle and HA have a toxic effect on *Chlorella* algae individually, and the effect of toxicity scaled up with increasing their concentrations and increasing the exposure time. The toxic effect of nanoparticles is practically significant by decreasing the growth rate as well as by increasing the production of intracellular algae ROS. The toxic effect of CuONP in the presence of dissolved HA in the culture medium was intensified and synergetic effect was observed. Surface-bound HA had a different effect on the nanotoxicity of CuONP and growth rate of *Chlorella* algae increased with increasing of HA contents of CuONP–HA complex.

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Author contributions MM is the main investigator and was involved in the study design, data collection, analysis and drafting of the manuscript. PF, MJ, GS, and ZF were involved in the data analysis. MM contributed to the theory and design of the manuscript, and critically revised the final article. All authors read and approved the final manuscript.

Availability of data and materials The datasets generated and analyzed during the current study are not publicly available in order to protect the anonymity, but they are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication Not applicable.

Ethics approval and consent to participate The study procedure was approved by the Medical Ethics Committee of Zanjan University of Medical Sciences.

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