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# Some nanotoxicity effects of copper (60-80 nm) and copper oxide (40 nm) nanoparticles on *Artemia salina*

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**Abstract.** In this study, nanotoxicity tests were made by exposure of *Artemia salina* to copper (Cu 60-80 nm) and copper oxide (CuO 40 nm) nanoparticles (NPs) at different concentrations (0.2, 1, 5, 10, 25, and 50 mg/L). The $LC_{50}$ value of Cu (60-80 nm) NPs on the *A. salina* individuals at the beginning (0), 24th, 48th and 72nd hours and elimination period was 52.37 mg/L while the $LC_{50}$ value of CuO (40 nm) NPs was 55.39 mg/L. The results of UV-Vis absorbance values showed that all statistical data revealed that maximum effect was observed between 24-30 hours and 25 ppm absorbance concentration was more effective. The multiple R, correlation coefficient (R2) and adjusted R2 values of Cu NP for the suitable Quadratic model were, respectively; 92.96 %, 86.42 % and 76.71 % while they are 98.31 %, 96.64 % and 94.25 % for CuO NP. Also, the data, was indicated effect size significantly changed based on the type and size of NP. Considering the microscope results, it was clearly noticed that *A. salina* organisms took the NPs in to their body. The accumulation in the gut of *A. salina* was observed and the images were taken with phase contrast microscope for both of NPs. The highest decrease for survival rates of *A. salina* individuals exposed to Cu NP was observed in the 10 ppm concentration (43.47 %) and in the 5 ppm concentration (46.20 %) for CuO NP. The results revealed that Cu and CuO NPS showed different toxic effects and that Cu NPs were more toxic than CuO.

**Keywords:** elimination; nanoparticles; $LC_{50}$; survival rates; zooplankton

## 1. Introduction

The nanotechnology is a new area of science and technology but also it shows a rising tendency in the worldwide markets (Ma *et al.* 2013, Vijayakumar *et al.* 2019, Mazzaglia *et al.* 2018, Vajargah *et al.* 2019) The usage of metal-based nanomaterials (NMs) in commercial products and applications is increasing rapidly, because of the current advances in nanotechnology (Khan *et al.* 2019, Naeemi *et al.* 2020, Wu *et al.* 2020). Although, there are discussions about the safety of nanomaterials, the production and application of nanomaterials has been continued to increase significantly (Zhu *et al.* 2019. The negative effects of metal and oxide nanoparticles (NPs) on aquatic organisms and ecosystems have been taken into consideration recently. It is known that metallic NPs has toxic effects on varied aquatic organisms, but the toxic functioning working of metallic NPs have not been clearly understood (Yang and Wang 2019). Therefore, researches are necessary to examine for the effects of the pollutants emerging like nanoparticles (Vajargah *et al.* 2019). The LC50 reference is an important parameter for different aquatic organisms to evaluate polluters toxicity and it was been used in many studies (Marwood *et al.* 2011, Lindh *et al.* 2019, Noureen *et al.* 2019, Ates *et al.* 2020, Dobretsov *et al.* 2020). Copper (Cu) and copper oxide (CuO) NPs are used in over a wide area in various industries, and this trouble raises worries for aquatic organisms. Also, usage areas of Cu based nanomaterials were been increasing especially in direct applications, therefore it is necessary to understand possible human and ecological hazards (Keller *et al.* 2017).

Taking into account the antifungal and antimicrobial properties of $Cu^{+2}$, the use of Cu NPs in agriculture and food preservation practices are being improved (Dugal and Mascarenhas 2015, Kalatehjari *et al.* 2015, Maniprasad *et al.* 2015, Park *et al.* 2016, Ray *et al.* 2015, Majumder and Neogi 2016, Montes *et al.* 2016, Ponmurugan *et al.* 2016, Villanueva *et al.* 2016). Moreover, Cu NPs utilization fields are been increasing (optical and electrical properties, cosmetic products, printers and electronic devices) and Cu NPs is of acquire an increasing attention (Song *et al.* 2015, Hou *et al.* 2017). CuO NPs are widely used in various applications (such as antimicrobial agent, photocatalyst and gas sensors) (Hou *et al.* 2017) and they attract attention due to their superior physicochemical properties (Dogan Calhan and Gundogan 2020). There are also many commercially different industrial fields for CuO NPs, multiple applications including wood preservation (Park *et al.* 2016), antimicrobial textiles, agricultural biocides and antifouling agents in antifouling paints (Batley *et al.* 2013, Kim *et al.* 2012, Llorens *et al.* 2012) and also, cancer treatments (Gnanavel *et al.* 2017, Dogan Calhan and Gundogan, 2020).

*Artemia* is has a non-selective filter-feeding system, it

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located in a high degree salinity waters throughout the world. It makes an ideal organism for toxicological experiments for their distribution over a wide area, strong structure and advantageous culture conditions. It is reported that *Artemia* sp. is used for the nanomaterial toxicity studies by some authors (Arulvasu *et al*. 2014, Ates *et al*. 2013, Rajabi *et al*. 2015, Ates *et al*. 2020, Cimen *et al*. 2020, Danabas *et al*. 2020). In this research, it was aimed to determine the some nanotoxic effects of Cu (60-80 nm) and CuO (40 nm) NPs at different concentrations on *Artemia salina*.

## 2. Material and methods

### *2.1 Nanoparticles and bioassay organism A. salina*

Metal based Cu (60-80 nm) and CuO (40 nm) NPs (powder forms) were got from SkySpring Nanomaterials, Inc. (Houston TX, USA). *A. salina*, was utilized obtained from a commercial company in Turkey. The incubation method of *A. salina* egg, has been made according to describe by Ates *et al*. (2013). Nearly 1 g of *A. salina* eggs were incubated in 1 L of sea water at 30±1 °C in a conical plastic container. *A. salina* eggs were set in the pH level was above 7.6, the ambient illumination was provided by fluorescent lamp with 1500 lx daylight and during the incubation period the continuously airing was been done with an aquarium air pump. 7 experimental groups (Control, 0.2, 1.0, 5.0, 10.0, 25.0 and 50.0 ppm) NPs were applied to *A. salina* with 3 repetitions.

### *2.2 Determination of $LC_{50}$ values of nanoparticles*

The sample (1.0 mL) was transferred to 10 mL and was diluted to 10 mL with seawater of the organism environment exposed to the NPs at the beginning (0), 24th, 48th and 72nd hours and elimination period to determine the $LC_{50}$ values of Cu and CuO NP used in this study. Then, 0.1 mL of diluted solution was sampled while whole of it was mixing and the numbers of the organisms were visually determined under the light in this mass. For every repetition, they were counted as to be three parallels and the arithmetic mean of counted values was calculated. This process was carried out for every NP and experimental concentration and the number and percentage of death individuals for every concentration were determined and converted to probit values using a computer program at the end of the 72-hour experiment to determine the fatal concentration.

### *2.3 Characterization analysis*

For the characterization analysis, the ultraviolet and visual light (UV-Vis) absorbance and the phase contrast microscopy analysis were made.

*Statistical Determination of UV-Vis Absorbance Values,*

UV-Vis spectroscopy is a method used to measure the light absorbed and emitted by a sample (the amount which is known as fading and defined as the sum of light absorbed and emitted). The absorption values of every NP at 300-800 nm wavelength were collected using a UV-Vis spectroscopy device (Optima Brand, SP-3000 Nano Model) in this spectroscopy method which has an important place on characterization analysis.

Table 1 Levels and ranges of independent variables used in measuring absorbance

| Variables | The variable codes | Levels | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| Concentration of NPs (mg/L) | X1 | 1 | 10 | 50 |
| Interaction time (hours) | X2 | 24 | 36 | 48 |

Table 2 Experimental design created according to BBD (Box Behnken Design) model with two independent variables

| Number of experiments | X1: Concentration (ppm) | X2: Time (hour) | Cu NPs (60-80 nm) | CuO NPs (40 nm) |
|---|---|---|---|---|
| 1 | | 48.00 | 0.169 | 0.168 |
| 2 | 10.00 | 24.00 | 0.424 | 0.117 |
| 3 | 10.00 | 48.00 | 0.171 | 0.166 |
| 4 | 50.00 | 48.00 | 0.138 | 0.343 |
| 5 | 1.00 | 48.00 | 0 | 0 |
| 6 | 10.00 | 48.00 | 0.174 | 0.166 |
| 7 | 10.00 | 72.00 | 0.206 | 0.085 |
| 8 | 50.00 | 72.00 | 0.111 | 0.147 |
| 9 | 10.00 | 48.00 | 0.175 | 0.165 |
| 10 | 1.00 | 24.00 | 0 | 0.047 |
| 11 | 50.00 | 24.00 | 0.25 | 0.383 |
| 12 | 10.00 | 48.00 | 0.17225 | 0.16625 |
| 13 | 1.00 | 72.00 | 0 | 0 |

Statistical experimental design results showing UV-Vis absorbance values of Cu and CuO NPs were examined using the polynomial equation methods. The regression process with the least-squares method was carried out on the data obtained as a result of the experiment design made in line with Tables 1 and 2. According to the polynomial equation obtained as a result of this design with three variables and three levels, the following Eq. (1) and (2) enabled the collection of response surface charts.

$$y_i = \beta_0 + \Sigma\ \beta_i X_i + \Sigma\ \beta_{ii} X_{ii}^2 + \Sigma \beta_{ij} X_i X_j \tag{1}$$

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \tag{2}$$

The statistical analysis of NP concentration (ppm) and activation time was carried out and the ANOVA analysis of the implemented model at 95% confidence interval was examined. The compatibility of the model with the experimental findings was reviewed to determine the difference between absorbance values based on the second-degree polynomial equation.

*Phase Contrast Microscopy Analysis,*

Images were taken using a phase contrast microscope (Olympus Brand, BX53+SC50 Model) to view the results of NP application such as deformation and NP accumulation

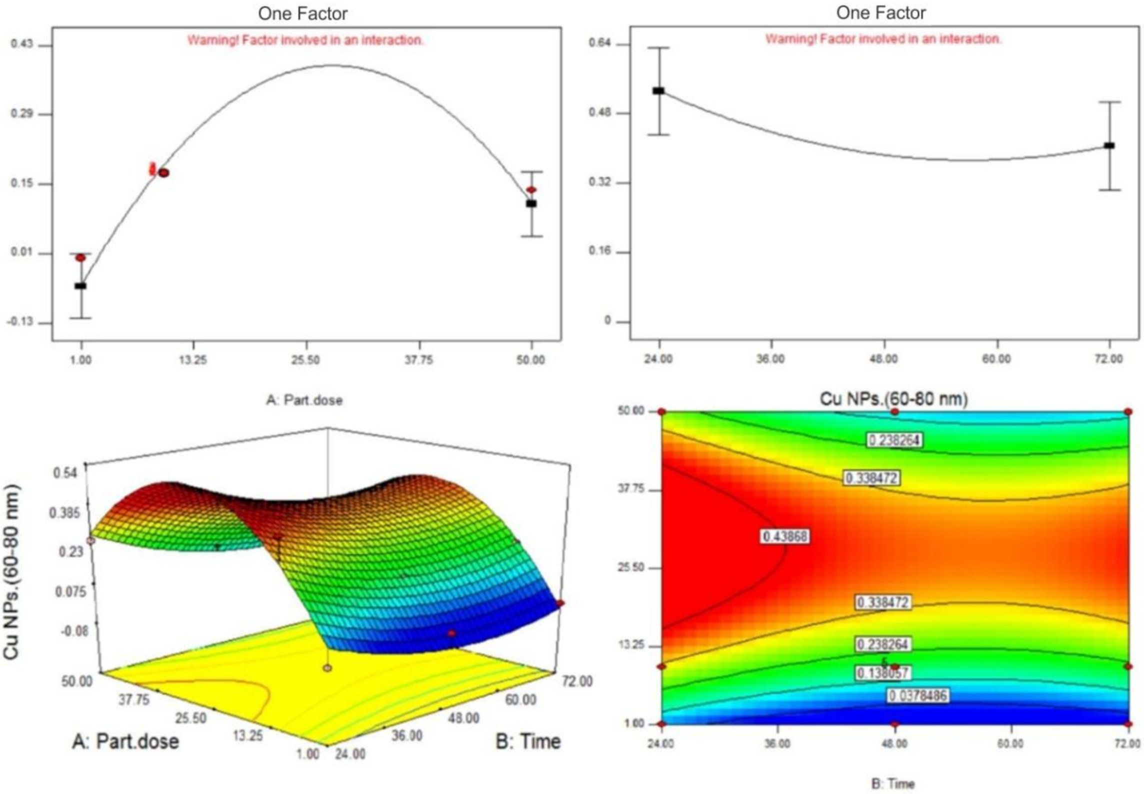

Fig. 1 UV-Vis absorbance values graphs of statistical data for Cu NPs (60-80 nm)

on organisms based on the duration and concentration of NP application.

### 2.4 Statistical analyses

Probit analysis used which was a computer program at the end of the 72-hour experiment to determine the $LC_{50}$. The UV-Vis absorbance values using Design Expert v.10., a statistical experiment design program was used to determine the mean effect of NPs at 60-80 nm in Cu and at 40 nm in CuO. Phase contrast images were obtained from live organisms inside a special slide through a Micron Imaging software.

## 3. Results and discussion

Our another manuscript, Cicek Cimen *et al*. (2020) can be examined for the results of the other characterization analyses (Transmission Electron Microscope (TEM), Dynamic Light Scattering (DLS), Zeta Potential, X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM), Fourier Transformation Infrared (FT-IR)) of Cu and CuO NPs, that cannot be included in this article containing the results of a comprehensive project.

### 3.1 Experimental design results of UV-Vis absorbance values

In the statistical analysis, the fitness of the selected model was shown in tables by naming every NP. Accpordingly Accordingly, the quadric model was used for all these experiments and the obtained results of Anova were shown on tables.

According to the statistical results of Cu (60-80 nm) NPs on the tables, the correlation coefficient ($R^2$) values for the suitable Quadratic model were, respectively, multiple R=92.96 %, $R^2$=86.42 % and adjusted $R^2$=76.71 %. Determining ($R^2$) value as 86.42 % showed that observed values were compatible with the estimated values and the implemented model was in the confidence interval. Determining multiple R value as 92.96 % showed that regression was statistically significant and only 7.04 % of total variables cannot be explained with this model. The “significance F” value in the ANOVA test was considered to determine the statistical value of the model. Obtaining the significance F value as lower than 0.05 (0.0061) and model F value as 8.91 showed that the model was statistically significant at the 95% confidence interval (Fig. 1). In mathematical modelling,

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \qquad (3)$$

$$\text{Abs.} = 0.38+0.083\,X_1-0.063\,X_2-0.017\,X_{12}\;\; 0.36\,X_1^2+0.084\,X_2^2 \qquad (4)$$

It was found that while the absorbance concentration among the parameters selected for Cu (60-80 nm) increased absorbance value, the duration decreased the absorbance value. Considering the absolute values of the coefficients, the order of action was concentration and duration. According to the statistical results of CuO (40 nm) NPs on the tables, the correlation coefficient ($R^2$) values for the Quadratic model suitable for CuO NP were, respectively, multiple R=98.31 %, $R^2$=96.64 % and adjusted $R^2$=94.25

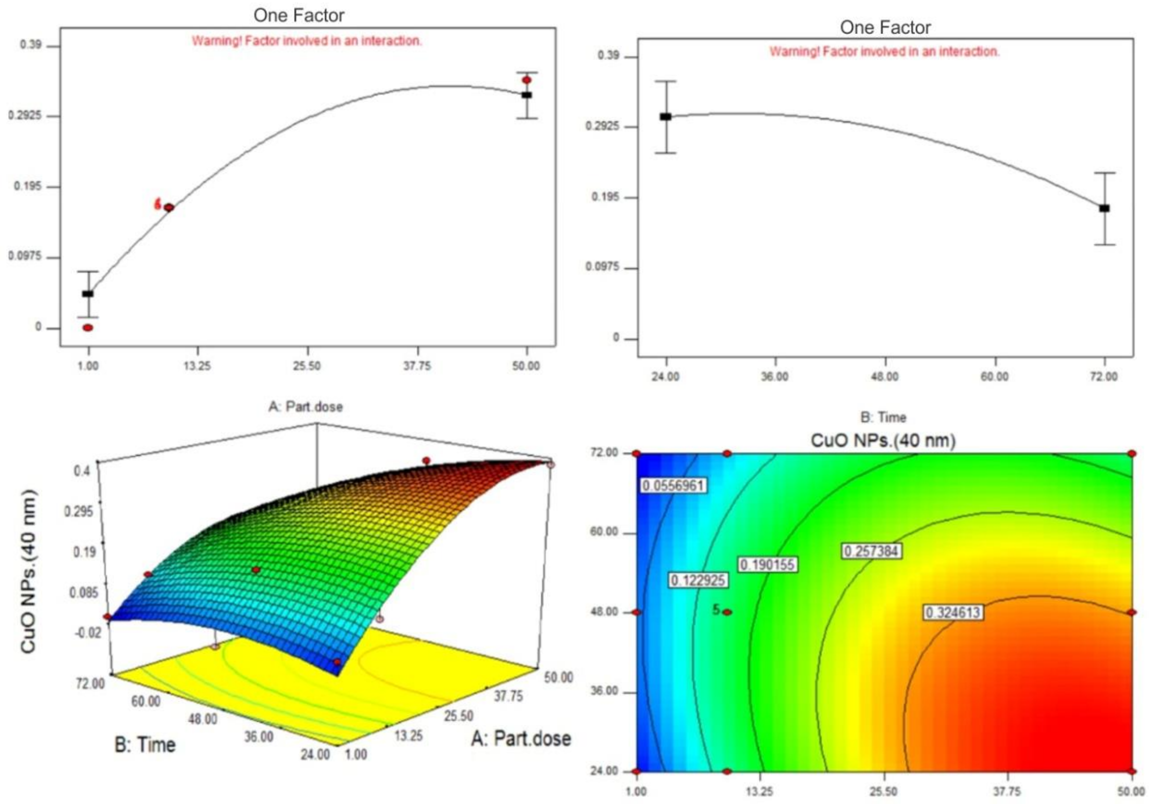

Fig. 2 UV-Vis absorbance values graphs of statistical data for CuO NPs (40 nm)

%. Determining ($R^2$) value as 96.64 % showed that observed values were highly compatible with the estimated values and the implemented model was in the confidence interval. Determining multiple R value as 98.31 % showed that regression was statistically significant and only 1.69 % of total variables cannot be explained with this model. The “significance F” value in the ANOVA test was considered to determine the statistical value of the model. Obtaining the significance F value as lower than 0.05 (<0.0001) and model F value as 40.31 showed that the model was statistically significant at the 95% confidence interval (Fig. 2). In mathematical modelling,

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \qquad (5)$$

$$\text{Abs.} = 0.15+0.11\ X_1+0.023\ X_2+0.012\ X_{12} +0.0094\ X_1^2+0.00607\ X_2 \qquad (6)$$

It was found that the absorbance concentration and duration among the parameters selected for CuO (40 nm) increased absorbance value. Considering the absolute values of the coefficients, the order of action is concentration and duration. All statistical data revealed that maximum effect was observed between 24-30 hours and 25 ppm absorbance concentration was more effective. Similar results were found in the studies on UV-Vis absorbance values of Alpha and Gamma $Fe_2O_3$ Nanoparticles and Zn, ZnO NPs (Ates *et al*. 2019a, b) Absorbance values of all NPs decreased during periods under 24 hours and over 36 hours. Similarly, effect size decreased in the cases of low and very high absorbance concentrations. Considering all these data, effect size significantly changed based on the type and size of NP.

### *3.2 Phase contrast microscopy analysis results*

The phase contrast images of the control group Fig. 3a which was not exposed to an application and *A. salina* where Cu and CuO NPs were administered were obtained separately. Generally, similar images were obtained on the phase contrast images of *A. salina* which was exposed to different concentrations of NPs in the bioexperiment. Therefore, Fig. 3b presents the phase contrast image of one group (50 µg/mL, 72 hours) and the images of the control group. Considering the phase contrast microscopy results, it was clearly observed that *A. salina* organisms took the NPs into their body in the environment parallel with increasing of the application concentrations. Similar images were obtained in the phase contrast images of the control group which was not exposed to an application and the *A. salina* which was exposed to CuO NPs. Therefore, Fig. 3c presents the phase contrast image of one group (50 µg/mL, 72 hours) and the images of the control group. It was also stated that he accumulation of Cu nanoparticles into the gut of *Artemia salina* was the major reason for toxicity (Madhav *et al*. 2017).

### *3.3 Results of toxicity and survival rates*

$LC_{50}$ value was calculated to determine the toxicity rate of Cu and CuO NPs on *A. salina* individuals. The probit value and estimated regression curves of the analysed NPs on *A. salina* were prepared on the computer using multimedia. Considering the figures showing the line of concentration effect values prepared for every NP and tables

Table 3 Mean lethal concentration (LC50) values of Cu and CuO NPs

| Nanoparticles | Concentration(mg/L) | Min. (mg/L)* | Maks. (mg/L)* |
|---|---|---|---|
| Cu (60-80 nm) | 52.370 | 32.807 | 104.471 |
| CuO (40 nm) | 55.393 | 32.584 | 121.777 |

* Values of $72^{nd}$ hours toxicity and 95 % confidence interval of NPs on organisms (min and max values)

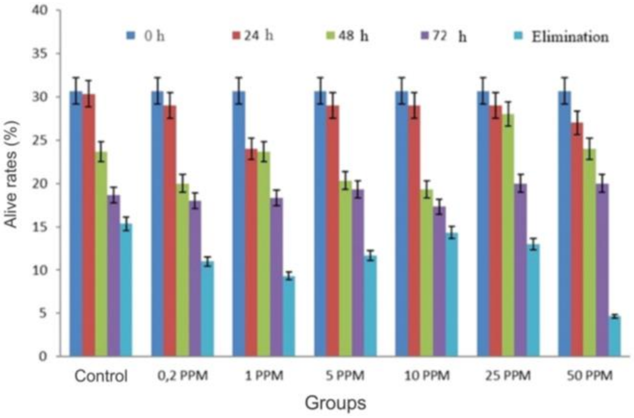

Fig. 4 Survival rates of *A. salina* exposed to Cu (60-80 nm) NPs

showing toxicity confidence interval and $LC_{50}$ value, the lethal concentration was different for every NP. The $LC_{50}$ value of Cu (60-80 nm) NPs on the *A. salina* individuals was 52.37 mg/L while the $LC_{50}$ value of CuO (40 nm) NPs was 55.39 mg/L (Table 3.) The results revealed that Cu and CuO NPS showed different toxic effects and that Cu NPs were more toxic than CuO. The median lethal concentration ($LC_{50}$) values obtained from the acute toxicity studies on different life stages of *Artemia salina* was found to be 61.4, 35, 12.2 and 175.2 mg/L for 1d, 2d, 7d old and adult, respectively (Madhav *et al*. 2017).

The survival rates of *A. salina* exposed to Cu and CuO NPs based on the application concentration were presented in Figs. 4 and 5. Considering the $72^{nd}$ hour survival rates of *A. salina* individuals exposed to Cu NPs, a decrease (43.47 %) in 10 ppm concentration was found and this decrease was the highest decrease percentage at this time compared to the other groups (Fig. 4). When the application groups were examined based on time, all groups tended to decrease in survival percentages in parallel with the passing time. Also, in a research about the impacts of Zn and ZnO NPs on the survival rates of *A. salina*, they found that, the survival rates decrease in parallel with the increase in the concentrations (Danabas *et al*. 2019). The highest decrease percentage in the survival rate during the elimination period was 84.78 % in 50 ppm group. Considering the $72^{nd}$ hour survival rates of *A. salina* individuals exposed to CuO NPs, a decrease (46.20 %) in 5 ppm concentration was found and this decrease was the highest decrease percentage compared to the other experimental groups (Fig. 5). When the time-dependent change of each application group was separately examined, all groups tended to decrease in survival percentages in parallel with the passing time. The differences between the groups in the $72^{nd}$ hour were statistically significant. The highest decrease percentage in the survival rate during the elimination period was 74.91 % in 10 ppm group (Fig. 5). The study also found a decrease (73.92 %) in the survival rate in 1 ppm group and a decrease of 70.29 % in the survival rates of 5 and 25 ppm groups.

Since this type of organisms generally filters feed, they absorb all the micro or macro-sized particles in the aqueous medium through food (Gophen and Geller 1984). Even now for the Cu NPs, there has argumentation as to whether the toxicity of NPs depends on the nanoparticle and ions or a combination of both (Yang and Wang 2019). It has been reported in many studies that metal ion released from NPs is an important driving force for toxicity (Zhao and Wang 2012, Adam *et al*. 2015, Bao *et al*. 2015, Song *et al*. 2015, Jiang *et al*. 2017, Zhu *et al*. 2017). However, other studies have shown that the toxicity of NPs is mainly due to the NPs themselves, given the low release ions in the exposure medium (Hua *et al*. 2014, Wang *et al*. 2016). These NPs could be passing through the cells or pass into different organs and tissues that may show toxicity (Takenaka *et al*. 2001, Samet *et al*. 2004). The images obtained as a result of the phase contrast microscopy analysis conducted to see the interactions of living organisms used in the experiment with NPs revealed that *A. salina* organisms got the NPs into their bodies in the environment and that the organs in the body were filled with particles. Ozkan *et al*. (2016) found that $AgTiO_2$ and $TiO_2$ NPs caused some anomalies (enlarged intestine, shrinkage, changes in the shape of the eye socket and deformations in the outer shell) in their studies in *A. salina* naupli. Huang *et al*. (2022) reported that the toxicity of Cu2+, nano-Cu, and nano-CuO to marine phytoplankton decreased in order.

However, how much of these particles absorbed in the body is NP and whether NPs accumulate in the organism can only be determined with the results of ICP-MS analyses. The results of the analysis that we made with ICP-MS to determine the accumulation and elimination rates, revealed that they increased in parallel with the increase in NP concentrations (Cimen *et al*. 2020). The accumulation rate increased as the NP concentration increased. Additionally, Danabas *et al*. (2020), the effects of Zn (40-60 nm) (80-100 nm) and ZnO (10-30 nm) were studied in *A. salina* and *D. magna* and the results of microscopy images, they found that the organisms were absorbed all the NPs in the medium levels. Another study for the survival rates of *A. salina* with the Zn and ZnO NPs, the results showed that, the highest survival rates were in the beginning and a decrease trend in parallel with the increase in the concentrations (Danabas *et al*. 2019). Similarly, Bhuvaneshwari *et al*. (2017), reported the ZnO and $Ti0_2$ NPs accumulate in the body of A. *salina* and some morphological changes in the organisms. Wu *et al*. (2020) hypothesized that Cu and CuO NPs can elicit differential toxicity to the organisms due to alterations in particle dissolution and variations in organismal uptake. They stated that actual concentrations of dissolved Cu released from the NPs were compared to ionic copper controls ($CuCl_2$) at the same concentrations to determine the relative contribution of particulate and dissolved Cu on organism uptake and toxicity and that both NPs had higher

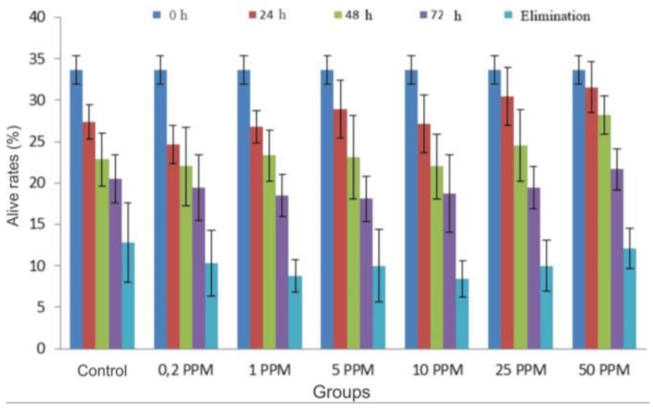

Fig. 5 Survival rates of *A. salina* exposed to CuO (40 nm) NPs

uptake in *D. magna* and zebrafish than equivalent ionic exposures, suggesting that both Cu-based NPs are taken up by organisms.

## 4. Conclusions

In conclusion, considering the accumulation and elimination results of Cu in *A. salina* individuals exposed to Cu (60-80 nm), accumulation and elimination occurred in parallel with the increase in concentration at each application hour and elimination. The accumulation amounts increased exponentially as the concentration rate increased. The results of this study showed that the presence of the NPs in the aquatic environment carries a potential risk for aquatic organisms.

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