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EFFECT OF COPPER OXIDE NANOPARTICLES TO SHEEPSHEAD MINNOW (*CYPRINODON VARIEGATUS*) AT DIFFERENT SALINITIES

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Nanotechnologies research has become a significant priority worldwide. Many engineered nano-sized materials have been increasingly used in consumer products. But the adverse effects of these nanoparticles on the environment and organisms have recently drawn much attention. The present study investigated the effects of different concentrations of copper oxide nanoparticles (CuO NPs) on the sheepshead minnow (*Cyprinodon variegatus*) at different salinity regimes, since it is able to withstand a wide range of salinities. The results indicated that CuO NPs could cause behavioral changes in the fish, such as increased mucus secretion, less general activity and loss of equilibrium. No mortality was observed at the presence of CuO NPs during the experiments. But higher oxidative stress was determined at half strength seawater than seawater exposure medium, which can be associated with the decreasing toxicity of CuO NPs as salinity increases. In addition, Cu contents in the tissues of the fish were significantly higher ($p<0.05$) in the low salinity. The order of Cu accumulation in the fish's organs was intestine > gills > liver.

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1. Introduction

Nano-sized copper oxide (CuO) particles are commonly used as bactericides and have the potential to replace noble metal catalysts for carbon monoxide oxidation [1]. CuO NPs suspensions have excellent thermal conductivity and are used as a heat transfer fluid in machine tools [2]. The increased use of engineered CuO NPs is likely to result in the release of these particles into the environment with increased exposure to both aquatic and terrestrial ecosystems and species.

Aquatic species are at risk of NP exposure, and a body of literature is emerging concerning the chemical behavior of NPs in aquatic systems, including their accumulation and toxicity in aquatic species. NPs have been shown to accumulate in cells such as macrophages and hepatocytes [3, 4]. Moreover, they could be sorbed, causing toxic effects on aquatic organisms such as phytoplankton, artemia, mollusks, crustaceans and fish in freshwater and seawater [5-9]. Therefore, understanding the effects of NPs on fish is an important aspect when considering the effects of NPs on the aquatic environment as a whole. Potential routes of uptake for NPs in fish include absorption *via* the gills, *via* the intestine epithelia as a result of dietary exposure and drinking and *via* the skin [10]. *In vitro* results demonstrated that CuO NP is genotoxic, increases haemolysis as a result of membrane damage and accumulates in higher concentrations in the gills for trout erythrocytes [11]. CuO NPs significantly inhibited growth of carp during 30 day sub-acute toxicity tests, intestine and gill of the carp were the cumulative organs and CuO NPs had

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higher toxicity than bulk CuO [12]. Studies with Cu NPs have demonstrated quite lower acute toxicity than the fish exposed to dissolved Cu [13, 14].

The sheepshead minnows (*Cyprinodon variegates)* are euryhaline, possess the ability to adjust quickly and effectively to fluctuations in environmental salinity [15, 16]. The sheepshead minnows live in coastal waters often encounter both salinity changes and elevated contaminant levels. Their salinity acclimation involves physiological and biochemical changes, which may influence how organisms respond to a stressor. They are generally tolerant to contaminants such as Cu and efficient at regulating Cu at sublethal levels [17]. Because Cu possibly accumulates in different tissues of the fish which form a link in a coastal food chain, Cu can be transported to higher tropic levels.

In this study, the exposure of different concentrations of CuO NPs to the sheepshead minnow at seawater and half-strength seawater medium was evaluated. Particle stability in exposure medium, survival of the fish, the accumulation of CuO NPs in fish tissues and lipid peroxidation levels in the liver, gills and muscles were investigated.

2. Materials and Methods

2.1. Test organism

A group of healthy sheepshead minnows was collected from the Mexican Gulf Coast (USA). The initial body weight and length of the fish (head to the fin) were 3.2±0.2 g and 4.1±0.4 cm, respectively. The seawater was prepared by dissolving appropriate amount of Instant Ocean® salt in deionized water, stirred for 24 h under aeration and then filtered through 30-μm Millipore cellulose filters. All fish were maintained in a 30-L glass aquarium. They were acclimated for a period of 7 days and fed with commercial fish food (Tetramin flake food, Germany) during the acclimation and experimental period. The sheepshead minnows were anesthetized using clove oil at a lethal dose for dissection. All animal protocols in this study were conducted under the supervision and approval of the Jackson State University Institutional Animal Care and Use Committee.

2.2. Reagents and chemicals

Copper oxide nanoparticles (CuO 40 nm NPs, 99.5% pure) were purchased, as uncoated nanoparticles, from Skyspring Nanomaterials, Inc. (Houston, TX). The CuO NPs were stored at room temperature in the laboratory until the implementation of the experimental studies. Deionized water produced by a Barnstead E-pure system with 18.0 MΩ-cm resistivity was used to prepare the exposure medium and experimental solutions. Trace metal grade nitric acid (HNO_3, Fisher Scientific) was used for the dissolution of the organs of sheepshead minnow collected after exposure to determine the total uptake levels. Stock Cu standard solution (1000 μg mL^{-1}) was purchased from SCP Science (Champlain, NY). Calibration standards for ICP-MS determinations were prepared within a range from 0 to 500 μg L^{-1} from the stock Cu solution in 5% HNO_3. Carbon-coated Cu TEM grids (300 mesh) were purchased from Electron Microscopy Sciences (Hatfield, PA).

2.3. Preparation of nanoparticles suspension

For preparation of exposure medium, appropriate amounts of CuO NPs (10% w/v) were weighed into polypropylene tubes and dispersed in deionized water. To achieve maximum dispersion, the suspension was homogenized by vortex (20 s at 2000 rpm), exposed to ultrasound sonication bath for 10 minutes and immediately transferred into the exposure glass tanks. To determine 1) total Cu concentration in the exposure medium and 2) total CuO NP level below 0.2 μm (e.g., 200 nm) and Cu ions in the exposure medium after filtration of the aggregates, 5 mL of each medium was filtered through a 0.2-μm PTFE membrane disk filter and analyzed by ICP-MS. Additionally, the colloidal solution in 10 μL of the water sample was dropped onto 50 Å thick carbon-coated copper grids and allowed to dry overnight at room temperature, and transmission electron microscopy (TEM) images of CuO NPs in the stock solution and exposure medium were taken. To estimate the mean particle size, approximately 100 NPs were measured in random fields

of view of three images. The images were recorded by a JEOL-1011 TEM instrument, providing a resolution of JEM-1011 with 0.2 nm lattice and magnification of 50 to $1x10^6$ under the accelerating voltage of 40 to 100 kV.

2.4. Experiment design

Nanoparticle exposure experiments for sheepshead minnows were conducted with different doses of the CuO NPs (5 and 50 mgL^{-1}) at two different salinities (about half and full strength seawater, 1.5 and 3.0 % respectively) for seven-days according to OECD 203 testing guidelines [18]. Two control groups with 1.5 and 3.0 % salinities were also setup without the test compound. Studies were carried out in an aquarium (30 L inner volume). The volume for the 20 L level was marked and filled with seawater or half strength seawater, followed by the addition of the NPs' suspensions prepared. Slight aeration was provided from bottom of the aquarium. The fish's individual length and weight were measured at the beginning and end of the experiment. Water quality parameters were measured with a model HI 9828 HANNA multiparameter from HANNA Instruments (Michigan, USA). Details of the experimental conditions were summarized in Table 1. The water in each tank was manually changed every day, leading to an exchange rate of 10 L/day. Contaminated water was collected and treated with activated charcoal before being discharged in a sewer. The turbidity increased after NPs were added to the tanks as a result of the formation of visible CuO NP aggregates. No mortality of fish was observed in any groups during the experiment.

Table 1. Expanded design summary of sheepshead minnow (Cyprinodon variegatus)

Parameters	1.5 % Salinity			3.0 % Salinity		
	Control 1	Group A	Group B	Control 2	Group C	Group D
Volume of water in aquarium (L)	20	20	20	20	20	20
NPs concentrations (mgL^{-1})	0	5	50	0	5	50
Duration of exposure (Day)	7	7	7	7	7	7
Temperature (ºC)	20 ± 2	19 ± 1	20 ± 1	20 ± 2	19 ± 2	20 ± 2
Oxygen (ppm)	6.75 ± 1.1	6.01 ± 1.6	6.82 ±1.9	6.95 ±0.2	6.70 ±1.3	6.29 ±1.3
pH	7.05	7.57	7.61	7.95	8.03	7.82
Number of fish	10	10	10	10	10	10
Replicate	2	2	2	2	2	2

2.5. Instrumental analysis

Both unfiltered and filtered suspensions (section 2.3) and fish tissues dissected [19] after exposure experiment were digested in Teflon vessels in 2 mL of concentrated HNO_3 for 2 hours by using a DigiPrep MS digestion block from SCP Science at 160 ^oC according to protocols described elsewhere [20]. At the end, the contents were visually inspected for the complete dissolution of CuO NPs (e.g., clear solution without any turbidity) and were diluted to 10 mL with water. The sample solutions were analyzed by inductively-coupled plasma mass spectrometry (ICP-MS) using a Varian 820MS ICP-MS instrument (Varian, Australia). The solutions' copper content was measured to determine the accumulation pattern of the NPs across the dose of exposure. Total Cu concentration detected was then translated into the corresponding CuO NP concentration.

2.6. Oxidative stress parameter analysis

The experiment was designed to allow for sub-lethal physiological effects (some physiological or biochemical responses) to the exposure over the exposure period of seven days [21]. The exposure time of seven days was chosen to enable some physiological or biochemical responses to the exposure. Five fish per treatment were collected from each tank at the end of each experiment day for biochemical analysis. The extent of lipid peroxidation in the tissues was determined by monitoring the formation of malondialdehyde (MDA) [22]. The quantification of MDA was done following the methods described by Esterbauer and Cheeseman [23]. Briefly, 10 mL of butylated hydroxytoluene (BHT), 0.25 mL of sample supernatant, 0.25 mL of phosphoric

acid (1.0 M), and 0.25 mL of TBA were added to a vial. A set of MDA standards were freshly prepared from tetramethoxypropane in a concentration range of 0 to 10 mM. All samples and standards were incubated at 90 ºC for one hour and centrifuged at 12,000 rpm for 15 minutes to separate the suspending tissue. The method is based on the formation of pink MDA-Thiobarbituric acid (TBA) adduct, which has an absorption maximum in acidic solution at 532 nm. The concentration of the MDA formed was calculated based on a standard curve for the MDA (Sigma Chemical Co.) complex with TBA. The extent of lipid peroxidation was expressed in nanomoles (or micromoles) of MDA.

2.7. Statistical analysis

All experiments were repeated twice independently, and data were recorded as the mean with standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons was used to detect significant differences among groups. Student's t-test was used for paired comparisons of two groups. In all data analyses, a p-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Characterization of nanoparticles

The stability and aggregation behavior of NPs within aquatic media are determined by both the physicochemical properties of the media and the charge on the surface of the NPs. The degree of aggregation of NPs has been shown in some cases to affect uptake *in vitro*. The aggregation of NPs when suspended in water is a known issue for some metal-based NPs [24-26]. In this study, the CuO NPs were exposed to ultrasound for 10 minutes to improve dispersion in water. The uniform continuous aeration allowed stable NP suspensions to be formed in the exposure medium, even for the 50 mgL^{-1} CuO NPs medium. However, the water's visibility decreased substantially with increasing concentration of CuO NPs, such as at the 50 mgL^{-1} level the solution was completely grey cloudy. CuO NPs did aggregate substantially in solution as reported previously by other groups [24-26]. TEM images for CuO NPs from the stock and seawater (3% salinity) exposure medium were displayed in Fig. 1. Particle size of CuO NP aqueous dispersion ranged from 30 to 75 nm for stock solution (Fig. 1a). Mean particle size was highly variable in exposure medium; from about 100 nm to about 2 μm of large aggregates containing strips of NPs (Fig. 1b). The electrophoretic mobility of the various particles was measured using a Malvern Instrments Ltd (Holtsville, NY). The electrophoretic mobilities of the CuO NPs were measured at a concentration of 10 mg L^{-1} to minimize aggregation. As indicated in Fig. 2, the average size distribution of CuO NPs from aqueous suspensions was 530 nm. Surface charge is critical in maintaining the stability of NPs in solution through electrostatic repulsions. Our results showed that CuO NPs possessed negatively-charged surfaces (Zeta Potential: -15.1 mV).

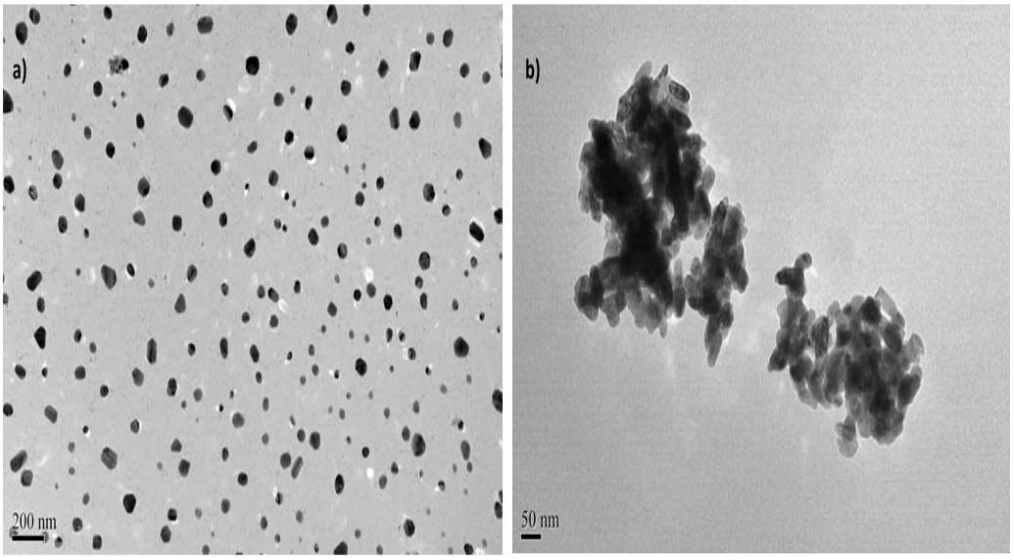

Fig 1. TEM images for CuO NPs from the stock (a) and seawater (3% salinity) exposure medium (b)

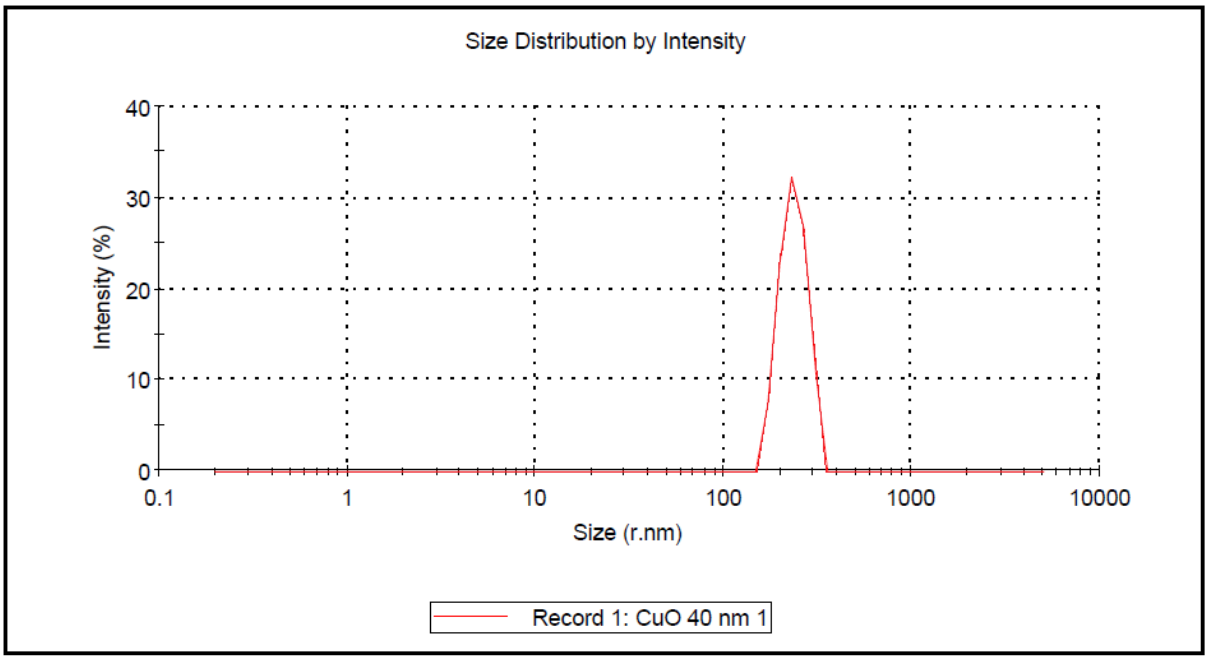

Fig 2. Size distribution of CuO NPs suspensions by dynamic light scattering

3.2. Accumulation of nanoparticles in different fish tissues

Potential routes of uptake for NPs in fish include absorption *via* gill epithelia, intestine epithelia as a result of dietary exposure and drinking, and the skin [27]. Fish gills serve as an organ for gas exchange, osmoregulation, acid-base regulation, nitrogenous waste excretion and endocrine regulation [28]. Gills are one of the main target organs for NPs exposure in fish [29], having a large surface area and a thin epithelial barrier. The gills are uptake routes for environmental toxicants and could act as such for NPs and pesticides. Primary cultures of fish gill cells have been used as model systems for toxicological measurements [30]. The same three general mechanisms as for gills (metal-specific carriers, substitution on nutrient ion transporters, and simple diffusion) apply to metal or NPs uptake *via* the gastrointestinal tract [31]. NPs behavior, and hence their bioavailability, uptake, accumulation, and the consequent toxicity of nanoparticles in aquatic organisms depend on particle properties and environmental conditions. This includes the particle's surface properties and the physico-chemical properties as particle size and shape, surface charge

and structure, particle chemistry and solubility, aggregation state and ionic strength, pH and the presence of organic matter [32-36].

In this study, the fish tissues (gill, intestine, liver, brain, heart and muscle) were sampled by dissection and analyzed for total copper content using ICP-MS. At seawater and half strength seawater, uptake in intestine significantly increased ($p<0.05$) to 9 and 14 ppm, respectively, with increasing doses to 50 $mg.L^{-1}$ of CuO NPs. Higher salinity also increased CuO NPs uptake in the intestine. In contrast, a higher accumulation of CuO NPs in the liver and gills were determined at half strength seawater. In both salinities, the order of the Cu concentrations in the tissues of fish is intestine > liver > gills (Fig. 3). The ICP-MS analysis showed that Cu in the muscle, brain, and heart after seven days of short-term exposure (data not shown) for both exposure mediums were not significantly different ($p\geq0.05$) than control groups.

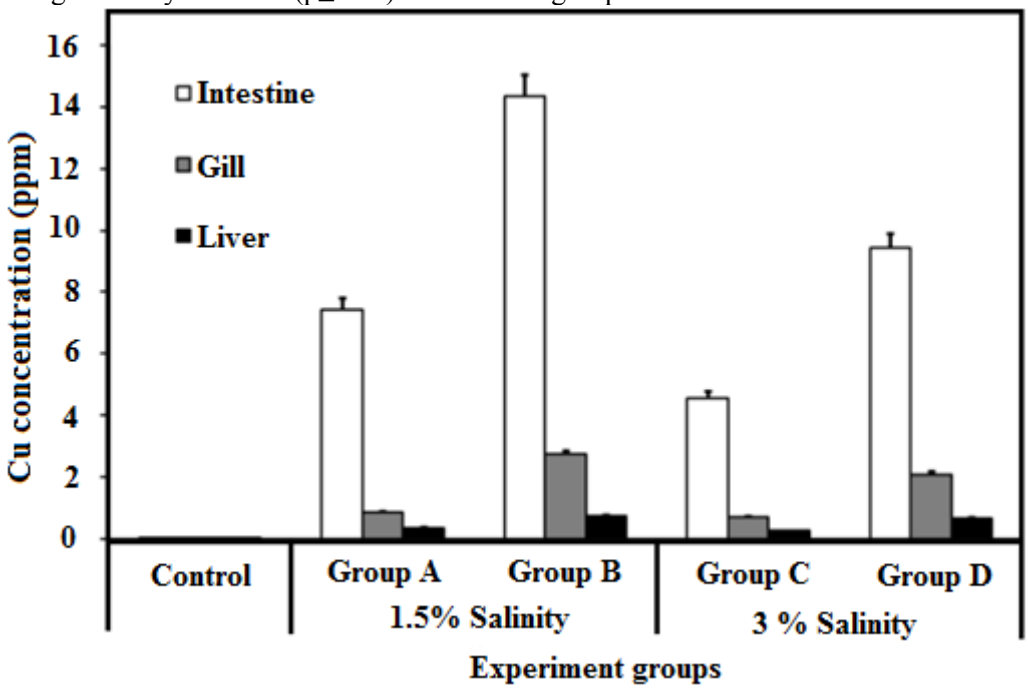

Fig 3. Copper (Cu) concentration in the tissues of sheepshead minnow. (Since the results of Control 1 and Control 2 are not statistically different ($p\geq0.05$), just one control value was used in the figure for better presentation)

Some previous studies also reported that water salinity influences the biodistribution and bioaccumulation of copper and can affect its toxicity [37-41]. The biodistribution of copper throughout the gills, intestine and liver of the common killifish, *Fundulus heteroclitus*, is salinity-dependent [37]. According to these authors, the gills and the liver are important target organs for copper accumulation at low salinities, whereas the intestine is a target organ at high salinities. Water salinity may be more important to species that actively regulate internal osmotic pressure. The results from these recent studies also support the bioaccumulation patterns of this study.

3.3. Dissolution of CuO NPs in the exposure medium

Copper is a trace element that is needed for the proper functioning of many enzymes in biological systems. However, it is one of the most toxic metals to aquatic organisms and ecosystems. Copper is moderately soluble in water and binds easily to sediments and organic matter [42]. CuO dissociates in water, and the most prevalent copper oxidation state is Cu^{2+} (cupric ion) [43]. Cu^{2+} is also a form primarily responsible for coppers' biocidal effects.

Copper is generally more toxic to organisms in freshwater than in saltwater. One of the reasons for this difference is that freshwater lacks cations, which compete with Cu^{2+} at the biological action sites, thus reducing copper toxicity [44]. Consequently, pH and water hardness play more important roles in freshwater than in saltwater environments. Increased pH accentuates copper toxicity because of the reduced competition between copper and hydrogen ions at the cell

surface [45]. Cations that are involved in water hardness (i.e., Ca^{2+} and Mg^{2+}) also compete with Cu^{2+} for biological binding sites [46]. Therefore, Cu^{2+} is less bioavailable in hard water than in soft water. The mechanism by which electrolytes, metal ions and organic molecules are taken up across epithelial cell layers is well described [47]. The toxic effect of copper salts is more strongly manifested in soft water; in hard water, part of the copper becomes bonded, forming copper carbonate or hydrocarbonate. Initial prepared synthetic seawater also contained copper ions. The concentration of dissolved CuO in the solution was increased with increasing initial concentration of CuO NPs and salinity (see Fig. 4). However, a large fraction of the NPs was still intact (e.g., undissolved) in the medium.

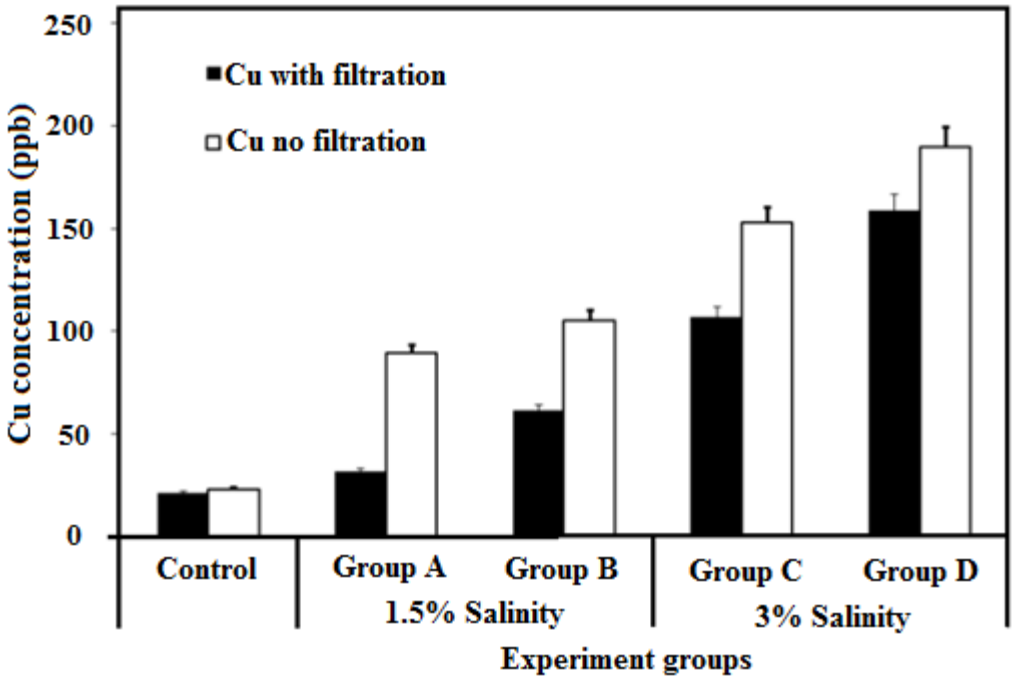

Fig 4. Copper (Cu) concentrations in medium with filtration (0.2-µm PTFE membrane disk filter) and without filtration (Since the results of Control 1 and Control 2 are not statistically different (p≥0.05), just one control value was used in the figure for better presentation).

3.4. Oxidative stress

The oxidative deterioration of cell membrane lipids has been used extensively as a marker of oxidative stress in the lipid peroxidation detection method. Lipid peroxidation generates a group of products, among which are reactive electrophiles such as epoxides and aldehydes [23, 48-49]. MDA is a major product of lipid peroxidation in aqueous solution. Metals are well-known inducers of oxidative stress. Metal contamination of the aquatic environment can be reflected in the assessment of oxidative damage and antioxidant defenses in fish [50]. In this study, the analysis of MDA showed that lipid peroxidation in the liver and gills from the CuO NPs treatment was significantly higher ($p<0.05$) than that in the controls (Table 2.). But the results of lipid peroxidation in the muscles were not significantly different ($p\geq0.05$) than the control (data were not shown). During the experiment, cases of mortality were not observed. But some behavioral changes in the fish, such as increased mucus secretion, less general activity and loss of equilibrium were observed in all exposure groups. Higher oxidative stress was determined at the low level of salinity, which may be associated with the decreasing toxicity of NPs as salinity increases. As displayed in Table 2, higher lipid peroxidations were observed in the liver than in the gills at both salinity levels. The bioaccumulation results of this study also supported these oxidative stress levels.

Table 2. Malondialdehyde levels (nmolg^{-1}) measured from sheepshead minnow (Cyprinodon variegatus) at different salinities

Tissues	1.5 % Salinity			3.0 % Salinity		
	Control 1	Group A	Group B	Control 2	Group C	Group D
Liver	5.01±0.4	7.47±1.4	9.79±0.9	5.07±0.7	6.17±1.5	7.03±1.3
Gills	4.07±0.5	4.81±1.2	4.40±1.1	4.01±0.2	4.60±1.4	5.01±0.7

4. Conclusion

We conclude that a short period of exposure (seven days) to CuO NPs is not lethal to the sheepshead minnow at the concentrations of 5 and 50 ppm at different salinities. But abnormal physiological and behavioral changes occurred at the higher NP concentrations during the experimental period. The result of this study showed that half strength seawater acclimated sheepshead minnows exhibited greater sensitivity to subsequent CuO NP exposure. In addition, higher oxidative stress was determined at the low level of salinity. Therefore, release of CuO NPs into the aqueous environment may potentially pose more risks to freshwater aquatic organisms. Since the knowledge of the fate, behavior and bioavailability of these types of particles in natural systems is limited, a need for longer-term and more environmentally realistic NP exposure regimes should be studied to fully determine the transport capabilities of NPs in the aquatic environment.

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