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Bioaccumulation of copper nanoparticle in gill, liver, intestine and muscle of Siberian sturgeon (*Acipenser baerii*) juvenile

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ABSTRACT

Copper (Cu) is an essential element required by all living organisms, since at least 30 enzymes are known to use Cu as a cofactor. Cu is also toxic in excess and liver and gills are known to be target organs for it. In the present study, 240 Siberian sturgeon juvenile (with initial weight 29.2 ± 3.1 g and initial length 21.8 ± 1.4 cm) were randomly distributed in 12 fiberglass tanks at 4 different copper nanoparticle (Cu-NPs) treatments with 3 replicates. Treatments included control (T_0 = no added Cu-NPs), 50 (T_{50}), 100 (T_{100}), 200 (T_{200}) $\mu g.l^{-1}$ Cu-NPs (mean primary particle size of 2 - 6 nm) in a semi-static waterborne exposure regime. Water exchanged were 20% daily with re-dosing after each change. The experimental period lasted 28 days, 14 days exposure to Cu-NPs and 14 days as recovery time. Fish liver, gill, intestine and muscle were sampled at days 0, 7, 14, 21 and 28. Samples were weighed, dried (100 °C for 48 h) then digested in concentrated nitric acid in a water bath, cooled, and analyzed for Cu concentration in the tissues with graphite furnace atomic absorption spectroscope. Most of the Cu-NPs were accumulated in the intestine, gill, liver and muscle. The accumulation of NPs in tissues was increased in all treatments from day 7 through 14. In the recovery period, Cu-NPs in tissues decreased but it was still higher than the control treatment. The current findings indicate that preventing the entry of Cu-NPs into the aquatic environment would seem to be essential.

Key words: Fish, Nanoparticles, Copper accumulation, Atomic absorption.

INTRODUCTION

Nanotechnology is a major innovative scientific and economic growth area, which may present a variety of hazards for environmental and human health (Moore 2006). NPs are set of several atoms from a specific material. Important features of NPs are small size, wider surface, and specific optical features and their particular surface coverage which increases their activity when they enter to the body and poisoning caused by such particles lasts longer (Kagan *et al.* 2005; Kipen & Laskin, 2005).

Fish are excellent sentinels of environmental health because of their position in the aquatic food chain. Since fish is an important part of human's food resources, presence of heavy metals in water and their accumulation in fish has been well studied (Dural *et al.* 2006; Storelli *et al.* 2006; Alibabić *et al.* 2007; Erdoğrul and Erbilir 2007; Keskin *et al.* 2007). So far, most attentions have been attracted to the accumulation within the muscle because muscle is the main part that is consumed by human (Storelli *et al.* 2006; Keskin *et al.* 2007). Due to metal absorption, regulation, storage and excretion mechanisms, role of different tissues in these processes and bioaccumulation rates in tissues are different (Storelli *et al.* 2006).

In most cases, muscle is not a good indicator of the whole body fish contamination, thus analyzing other tissues such as liver and gill is important (Has-Schön *et al.* 2006). Accurate pattern of accumulation depends on the type of metal and the animal species, thus special attentions have been paid to these studies (Olojo *et al.* 2005; Storelli *et al.* 2006). Some tissues like gill, intestine, liver and brain are the targets of toxicity of NPs (Handy *et al.* 2011).

Understanding the effects of NPs on fish is therefore an important aspect when considering the effects of NPs on the aquatic environment as a whole (Ates *et al.* 2013). Potential routes of uptake for NPs in fish include absorption via the gill and intestine epithelium as a result of dietary exposure and drinking, or via the skin (Handy *et al.* 2008 b). Bioaccumulation of heavy metals can reflects the amount of toxin ingested by organism, pattern in which the metals are distributed through different organs and its extent to which the metals remained in organs (Senthil *et al.* 2008). As the presence of NPs in commercial products increases, worries about poisoning potential and its risks in environment increases (Colvin, 2003). Despite the studies about poisoning of nanomaterial (Moore, 2006; Handy *et al.* 2008 b ; Klaine *et al.*, 2008; 2010; Handy *et al.* 2011), little information about bioavailability of metal NPs, their accumulation and effects on internal organs of fishes is available.

Cu is an essential element required by all living organisms, since around 30 enzymes are known to use Cu as a cofactor (Sarkka *et al.* 1978; Grosell *et al.* 1998). Recently, a new type of Cu is designed which is a type of nanomaterial containing Cu-NPs (Shaw & Handy 2011). Cu-NPs in the sub-50-nm range exhibit increased efficiency in inhibiting a wide range of bacteria and fungi. Although Cu-NPs are already widely found in multiple products, an actual assessment of their environmental implications is lacking. There is evidence indicating that Cu-NPs have entered to the fish organs and fish are at risk of high concentration of NPs through water (Griffitt *et al.* 2007). Since Cu-NPs are designed and produced recently, few reports have been issued about the effects of this NPs. Bagherzadeh Lakani *et al.* (2014 a, b, 2015) have investigated the effect of Cu-NPs on gill, liver and intestine of rainbow trout, *Oncorhynchus mykiss*. In another study, effects Cu-NPs and Cu sulfate on rainbow trout organs have been investigated (Al-Bairuty *et al.* 2013). Accumulation of CuO-NPs in different tissues of common carp, *Cyprinus carpio* has been studied and maximum accumulation of Cu is reported in intestine, gills, muscle, skin and scale, liver and brain (Zhao *et al.* 2011). Effect of metal NPs including Cu on tissues of zebrafish, *Danio rerio* has also been studied (Griffitt *et al.* 2007, 2009). Effect of Cu on gill of rainbow trout (Wilson and Taylor, 1993) and $CuSo_4$ on Nile tilapia, *Oreochromis niloticus* has been reported (Figueiredo-Fernandes *et al.* 2007).

Siberian sturgeon, *Acipenser baerii* is one of the most important sturgeon species that is used for caviar and meat production in sturgeon aquaculture (Bronzi *et al.* 2011; Wei *et al.* 2011). According to the International Union for Conservation of Nature (IUCN) Red List classification (IUCN, 2011), Siberian sturgeon is a threatened species. This species is greatly invaluable for research on sturgeon physiology (Eslamloo & Falahatkar 2014). To date, there is no report about effects of Cu-NPs on Siberian sturgeon. A few studies have focused on the reversibility of damages caused by Cu exposure in fish (Cerqueira *et al.* 2002). In general little is known about the potential toxicity of metals such as Cu to Siberian sturgeon. Therefore, the aim of the present study was to determine the bioaccumulation of Cu-NPs in the gill, liver, intestine and muscle of Siberian sturgeon juvenile and elimination of Cu-NPs from these tissues.

MATERIALS AND METHODS

Juvenile Siberian sturgeon (n = 300) were obtained from International Sturgeon Research Institute, Rasht, Iran, and held for two weeks in a fiberglass tank with flowing, aerated water. Fish were fed 3% body weight by commercial

sturgeon food (Skretting, Classic L.F. 1P, Italy). Two hundred and forty Siberian sturgeon juveniles (with initial weight 29.2 ± 3.1 g and initial length 21.8 ± 1.4 cm) were randomly distributed into 12 fiberglass tanks (with 200 L water volume and 20 fish/tank) and acclimated for 7 days prior to experiments. Three tanks per treatment were randomly allocated. Treatments included control (T_0) (no added Cu-NPs), 50 (T_{50}), 100 (T_{100}) and 200 (T_{200}) $\mu g.L^{-1}$ Cu-NPs (mean primary particle size of 2 - 6 nm). A semi-static exposure regime (20% water change every day with re-dosing after each change) was employed. Photoperiod was 12 h light: 12 h dark. The experimental period lasted 28 days, 14 days exposure to Cu-NPs and 14 days as recovery time. Fish were fed 1% body weight (30 min after water change) in order to minimize the risk of the Cu-NPs absorbing to food or fecal material, and to help maintaining water quality. Cu-NPs were added 30 min after feed were given to the fish. Highly stable dispersed Cu-NPs were prepared by means of the chemical reduction of metal salt precursor ($CuCl_2$) in water with L-ascorbic acid as reducing and stabilizing agents (Xiong *et al.* 2011). The nominal versus actual Cu concentrations in the test tanks containing the Cu-NPs are shown in Table 1. Subsequent image analysis by scanning electron microscopy (SEM) and particle size distribution revealed a mean primary particle of 2-6 nm (Figs. 1 - 2). The actual concentration of Cu in the treatment was measured every day by sampling 50 mL of water from the central part of the water column. The Cu concentrations were then analyzed using graphite furnace atomic absorption spectroscope (Analytik Jena, novAA 400, Germany). Water samples were taken before and after each water change in exposure period to Cu-NPs and once a day in recovery period for pH, temperature, dissolved oxygen. There were no significant differences (ANOVA, $P < 0.05$) between any tanks in water quality or Cu concentrations within treatments, so water quality data were pooled per treatment including mean ± SD, n = 504; pH 7.24 ± 0.08; temperature 18.0 ± 0.05 °C and dissolved oxygen 7.82 ± 0.07 $mg.L^{-1}$.

On days 0, 7, 14, 21 and 28, six fish (two fish per tank) were randomly sampled from each treatment. The fish were individually anesthetized with 150 $mg.L^{-1}$ of clove powder (Falahatkar *et al.* 2011) and then weighed. The liver, gill, intestine and muscle were dissected out and placed in a freezer (-20 °C). Tissues Cu analyses were done according to Shaw *et al.* (2012). Samples were weighed, dried (100 °C for 48 h) and moisture content was calculated from wet and dry tissue weights. Samples (typically 0.1 - 0.5 g dried tissue) were then digested in concentrated nitric acid (69% analytical grade) for 2h at 70°C in a water bath, cooled, and then diluted to 16 mL using ultrapure deionized water. For small tissue samples (less than 0.1 g dry weight), the volumes of reagents were reduced pro rata (1 ml of nitric acid, diluted to a final volume of 4 ml). Samples were then analyzed for Cu concentration in the tissues with graphite furnace atomic absorption spectroscope (Analytik Jena, novAA 400, Germany). This study was implemented as split plot in time design and in a completely randomized design with three replications. The statistical analysis performed using SPSS software (Version 16). No tank effects were observed throughout the experiment, so data were pooled by treatment for statistical analysis. Data were tested for treatment, time and treatment × time interaction effects by multifactor ANOVA followed by Tukey's post-hoc test. When a statistically significant effect was indicated by this model, Two-way ANOVA was employed to assess treatment and time. Differences among groups were considered significant at $P < 0.05$.

Table 1. Nominal versus actual Cu concentrations during toxicity experiments (mean ± SD).

Treatments	Nominal concentrations	Actual concentrations
Control	0	0
50 µg.L^{-1}	50	51.25 ± 0.7
100 µg.L^{-1}	100	100.9 ± 0.1
200 µg.L^{-1}	200	203.8 ± 0.2

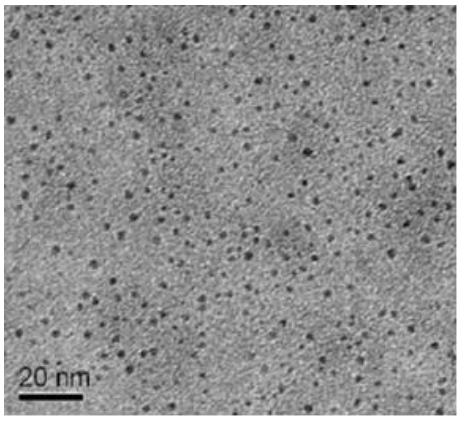

Fig. 1. TEM micrographs of Cu-NPs.

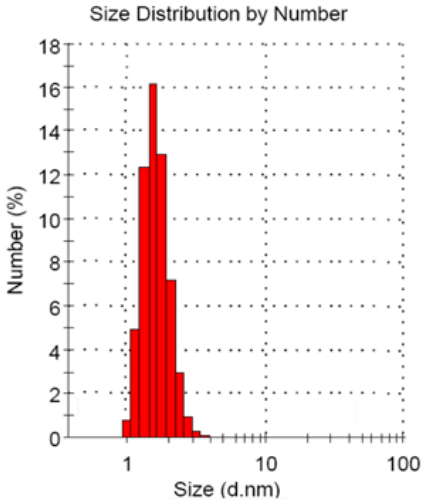

Fig. 2. Cu-NPs size distribution.

RESULTS

During the experiment, no mortality was observed in the control and the treatments. Fig. 3 shows accumulation of Cu-NPs in the tissues of Siberian sturgeon in different sampling times. During the experiment, Cu concentration in different tissues followed the order: intestine > gill > liver > muscle. In all tissues, there were interactions between time and Cu-NP levels, and also some significant differences were observed in Cu levels throughout the experiment ($P < 0.05$). Cu accumulation displayed a general dose-dependent pattern in all tissues. From the beginning till day 7 of exposure, amount of Cu in all tissues showed an increasing trend and this increase continued till day 14 of exposure. Maximum amount of Cu was found in T_{200} (200 µg.L^{-1} Cu-NP) and on day 14 which has a significant difference with other treatments ($P < 0.05$). In recovery period, from day 14 through 21, concentration of Cu in tissues was reduced and this trend continued up to day 28.

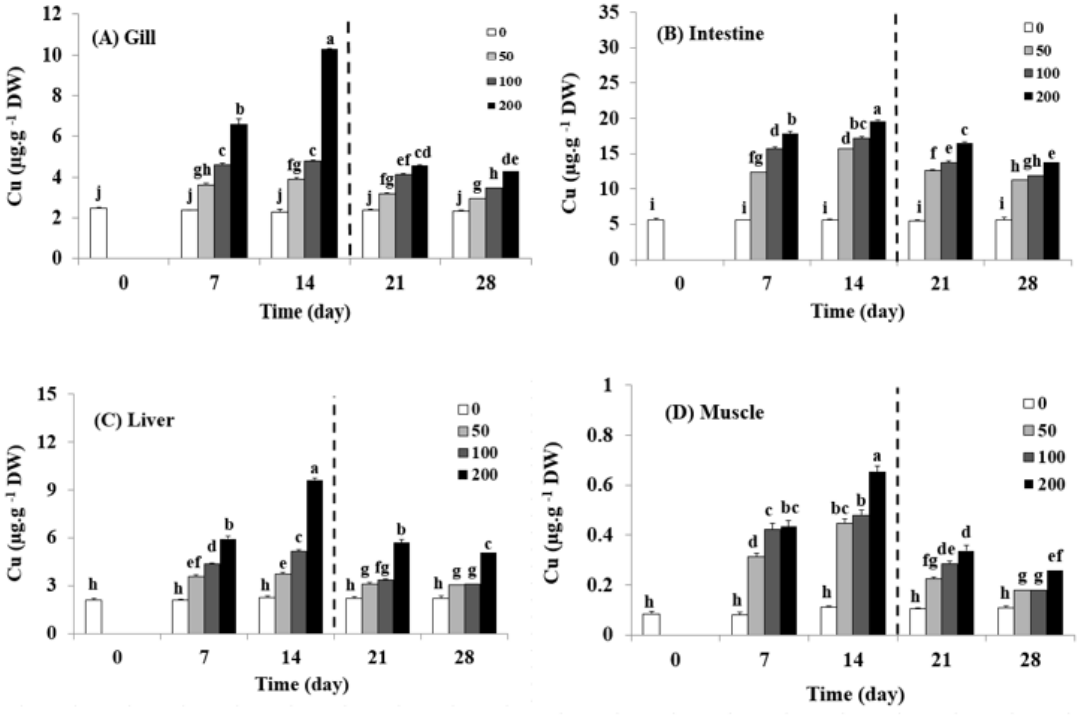

Fig. 3. Accumulation of Cu (µg.g^{-1} DW) in the gill (A), intestine (B) liver (C) and muscle (D) of Siberian sturgeon (*Acipenser baerii*) juvenile exposed to Cu-NPs. The experimental period were 28 days, 14 days exposure to Cu-NPs and 14 days as recovery time (n = 6). Values are mean ± SD of three replicate tanks and values with different letters are significantly different ($P < 0.05$). The dashed line indicates the end of exposure and the return of all fish to normal food (recovery period).

DISCUSSION

In this study fish were exposed to sub-lethal concentration of Cu-NP for 14 days followed by a recovery period up to day 28. Fish were examined for alteration in tissue burden in gill, liver, intestine and muscle. The pattern of Cu tissue accumulation showed some evidences of a dose-dependent increase in accumulation. A significant increase in tissue Cu concentration over the time was also observed exhibiting the effect of time on accumulation of this metal in tissues. In other study, effect of time on bioaccumulation of zinc oxide nanoparticles (ZnO-NPs) was reported in juvenile carp, *Cyprinus carpio* (Hao *et al.* 2013).

In the present study, maximum Cu accumulation was observed in the intestine, gill, liver and muscle, respectively. Cu concentration in intestine was 5.7 µg.g^{-1} DW on day 0. On day 7, Cu concentration in T_{50}, T_{100} and T_{200} increased to 12.32, 15.63 and 17.78 µg.g^{-1} DW, respectively. On day 14, Cu concentration increased to 15.64, 17.18 and 19.61 µg.g^{-1} DW, respectively. These values are higher than those measured in gill, liver and muscle. Similarly, after exposure to titanium dioxide nanoparticles (TiO_2-NPs) maximum amount of accumulation of Ti in common carp, *C. carpio* was reported in intestine, stomach, gill and muscle, respectively (Sun *et al.* 2007). In rainbow trout after exposure to the powdered silver nanoparticles (Ag-NPs), maximum amount of Ag accumulation was reported in intestine, liver, gill and muscle, respectively (Johari *et al.* 2015). After exposure to CuO-NP, maximum amount of Cu accumulation in common carp was reported in intestine, gill, muscle, skin, scale, liver and brain (Zhao *et al.* 2011). Contaminants can increase the drinking of water by freshwater fish (Best *et al.* 2003),

thereby increasing the potential exposure of their digestive system to pollutants (Johari *et al.* 2015). Thus, the ingestion of water containing NPs and the direct effects of NPs on the tissue structure of the digestive system can affect the absorption of these materials by the internal organs through endocytosis (Handy *et al.* 2008 a). So, presence of Cu in intestine in the present study might be due to drinking water containing Cu-NPs as a result of stress. Shaw & Handy (2011) mentioned that NPs are too large for ion transport mechanisms into tissue, so the most likely route for the uptake and accumulation of NPs in intestinal tissue is endocytosis. Drinking water containing NPs due to stress has been previously reported in rainbow trout after exposure to TiO_2-NP (Federici *et al.* 2007) and Cu-NP (Shaw *et al.* 2012).

In the present study, accumulation of Cu in liver and gill in T_{200} was higher than T_{50} and T_{100} such that in this treatment the amount of Cu in the gill on day 7 was 6.59 $\mu g.g^{-1}$ DW and reached 10.29 $\mu g.g^{-1}$ on day 14. On day 14, Cu concentration in T_{50} reached to 3.88 $\mu g.g^{-1}$ DW and in T_{100} to 4.8 $\mu g.g^{-1}$ which is lower than T_{200}. In another study exposure to ZnO-NPs increased Zn concentration in gill, liver and brain of common carp, but there was no accumulation in muscle (Hao *et al.* 2013). In rainbow trout, after exposure to 5000 $\mu g.L^{-1}$ TiO_2-NP, Ti was accumulated in gill (Moger *et al.* 2008). Increase in metal accumulation of the gill could be associated with the formation of metal complex with the mucus on the gill, because of its direct contact with the surrounding water. Gill is the main route for the entrance of Cu into the aquatic organisms due to its direct exposure to toxicant in water and acts as a reservoir of metal accumulation. When metals concentration is high in aquatic ecosystems, gill is the first tissue that is affected by contaminants (Oronsaye & Brafield 1984).

In general, gill is mostly exposed to contaminants in water (Oliveira-Filho *et al.* 2010), thus metals concentration in gill indicates their concentration in the water column (Ikem *et al.* 2003).

In liver, accumulation of Cu in T_{200} was 5.92 $\mu g.g^{-1}$ DW on day 7 and reached to 9.61 $\mu g.g^{-1}$ on day 14. In T_{50}, Cu accumulation increased from 4.35$\mu g.g^{-1}$ DW to 5.61 $\mu g.g^{-1}$, while in T_{100}, it increased from 5.92 $\mu g.g^{-1}$ DW to 9.61 $\mu g.g^{-1}$ Cu metabolism is mostly controlled by the liver. The liver not only tends to accumulate Cu, but also plays an important role in Cu homeostasis (Grosell *et al.* 1997). So, liver is an active organ in fish and high amount of Cu accumulation can possibly be attributed to the involvement of liver in detoxification and removal of toxic substances circulating in the blood stream (Asagba *et al.* 2008). In other studies, it has been shown that liver plays an important role in contaminant storage, redistribution, detoxification and transformation of contaminants (Licata *et al.* 2005; Norouzi *et al.* 2012; Baramaki *et al.* 2012). Liver can also be studied for the pathologic effects induced by metal contamination (Tekin-Ozan & Kir 2008). Vinodhini & Narayanan (2008) reported that the maximum amount of bioaccumulation of lead occurs in the liver of common carp resulting in different biochemical parameters and damage the liver. In rainbow trout after exposure to colloidal Ag-NP, the maximum amount of accumulation was reported in liver, gill and intestine, respectively (Johari *et al.* 2015).

In the present study, accumulation of Cu in muscle was 0.08 $\mu g.g^{-1}$ DW on day 0, increasing to 0.31, 0.42 and 0.43 $\mu g.g^{-1}$ for T_{50}, T_{100} and T_{200} on day 7 followed by 0.45, 0.48 and 0.65 $\mu g.g^{-1}$ on day 14 respectively which is much lower than in the other tissues. Muscle has the lowest amount of Cu accumulation because it is not an active organ in accumulating metals (Alam *et al.* 2002) and usually has the lowest essential and nonessential metal concentrations (Wen *et al.* 2003). Similarly, bioaccumulation of nickel and cobalt in muscle was lower than those in liver and gill of black fish (*Capoeta fusca*) (Mansouri *et al.* 2011, 2012). On the other hand, in other studies minimum amounts of TiO_2-NP were reported in common carp (Sun *et al.* 2007) and goldfish (*Carassius auratus*) (Ates *et al.* 2013), ZnO-NP in common carp (Hao *et al.* 2013) and

also colloidal and powdered Ag-NP in rainbow trout (Johari *et al.* 2015).

This may be related to the fact that the flesh and bone are not involved in detoxification, hence the transportation of Cu from other tissues to flesh and bone may not arise (Das & Gupta 2013).

In the present study, accumulation of Cu in the tissues decreased in the recovery period. At the end of this period, Cu level in intestine in T_0 was 5.63 µg.g^{-1} DW, while in T_{50}, T_{100} and T_{200} reached to 11.31, 11.83 and 13.79 µg.g^{-1} respectively, which was higher than the control. The Cu level in gill was 2.63 µg.g^{-1} DW in T_0, while reached to 2.95, 3.49 and 4.29 µg.g^{-1} in T_{50}, T_{100} and T_{200} respectively.

Amount of Cu in gill and liver decreased from day 14 to day 21 in T_{200} and this decrease continued to day 28. Reduction of Cu in T_{100} was lower than T_{200}.

Amount of Cu in muscle decreased in the recovery period and at the end of the experiment in T_{50}, T_{100} and T_{200} reached to 0.18, 0.18 and 0.26 µg.g^{-1} DW respectively.

At the end of the recovery period amount of Cu in muscle in T_{50} and T_{100} was the same but in T_{200} was higher than the former treatments. In all treatments amount of Cu in muscle decreased from day 14 to 21 faster while from day 21 to day 28 it reduced slower.

Results of the present study indicate that even at the end of recovery period, there was Cu in tissues and was not eliminated completely. Therefore, this recovery time is not sufficient and should be increased to study the recovery trend.

The present findings indicate that preventing the entry of Cu-NPs into the aquatic environment would seem to be essential.

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تجمع زیستی نانوذره مس در بافت‌های آبشش، کبد، روده و عضله بچه تاسماهی سیبری (*Acipenser baeri*)

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چکیده

مس یک عنصر ضروری مورد نیاز تمام ارگانیسم های زنده بوده و حدود ۳۰ آنزیم از مس به عنوان کوفاکتور استفاده می کنند. مس در مقدار زیاد سمی بوده و کبد و آبشش به عنوان اندام هدف آن شناخته شده اند. در مطالعه حاضر مجموعاً ۲۴۰ بچه تاسماهی سیبری (با میانگین وزنی ۳/۱ ± ۲۹/۲ گرم و میانگین طول ۱/۴ ± ۲۱/۸ سانتیمتر) (میانگین ± SD) به صورت تصادفی در ۱۲ تانک فایبرگلاس در چهار تیمار مختلف نانوذره مس با سه تکرار توزیع شدند. تیمارها شامل شاهد (بدون اضافه کردن نانوذره مس)، ۵۰، ۱۰۰ و ۲۰۰ میکروگرم در لیتر نانوذره مس (با میانگین اندازه ۶-۲ نانومتر) در شرایط نیمه ساکن بودند. کل دوره ۲۸ روز، شامل ۱۴ روز قرارگرفتن در معرض نانوذره مس و ۱۴ روز دوره بهبود بود. روزانه ۲۰٪ آب تعویض شده و مجددا نانوذره مس به آب اضافه شد. از بافت های کبد، آبشش، روده و عضله ماهی در روزهای صفر، ۷، ۱۴، ۲۱ و ۲۸ نمونه برداری شد. نمونه ها وزن و خشک شده (به مدت ۴۸ ساعت در دمای $100^{\circ}C$) سپس در اسید نیتریک در بن ماری هضم شده و پس از خنک شدن با آب مقطر به حجم رسیدند. میزان مس در بافت ها توسط دستگاه جذب اتمی مجهز به سیستم کوره گرافیتی اندازه گیری شد. بیشترین میزان تجمع نانوذره مس در بافت ها به ترتیب در بافت های روده، آبشش، کبد و عضله بود. در تمام تیمارها از روز ۷ تا ۱۴ روند افزایشی در تجمع نانوذره در بافت ها مشاده شد. در دوره بهبود میزان نانوذره مس در بافت ها کاهش یافت، اما همچنان نسبت به تیمار شاهد بالاتر بود. نتایج مطالعه حاضر نشان می‌دهد که جلوگیری از ورود نانوذرات مس به محیط‌های آبی ضروری به نظر می‌رسد.

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