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Original Article

Bioaccumulation of manufactured titanium dioxide (TiO_2), copper oxide (CuO) and zinc oxides (ZnO) nanoparticles in the soft tissues of tilapia (*Oreochromis mossambicus)*

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Authors' Contribution
KS: perform experimental work, **MNK, FJ:** supervise research, **NK:** contributed towards sample preparation and ICP-MS usage. **MS:** contributed in writing the manuscript, **MKAK:** helped out in data analysis, **MA:** contributed in experimental trial

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Abstract
The use of various nanoparticles in industry and domestic practices enviably end into the aquatic environment. Nanoparticles have the potential accumulative and toxic influence in the water environments. There is a need to exploremore likely accumulation of nanoparticles in the aquatic organisms. This study was designed to investigate the possible bioaccumulation of TiO_2, CuO and ZnO nanoparticles to tilapia (*Oreochromis mossambicus*). Fish were exposed to three doses of nanoparticles suspensions T0 (no dose administration), T1 (0.5 mg/L), T2 (1.0 mg/L) and T3 (1.5 mg/L) for 14 days. After 14 days, animals were slaughtered and organs (gills, liver and muscles) were freeze dried. Freeze dried tissue samples were digested with the help of nitric acid (HNO_3) and perchloric acid ($HClO_4$). Acid digested samples were analyzed by Inductively Coupled Plasma Mass Spectrophotometry (ICP-MS) foraccumulation of Ti, Cu and Zn nanoparticles in various tissues (gills, liver and muscles). Ti and Cu accumulation in gills was recorded to be 5.7033 and 3.7133 µg/Kg respectively,whereas, maximum Zn accumulation (2375.3 µg/Kg) was observed in liver. It was concluded from the present study that the strategy applied to assess the bioaccumulation of nanoparticles in the soft tissues of fish is very useful tracking method to determine the accretion in aquatic organisms.

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INTRODUCTION

Commodities with one dimension and size between 1 to 100 nm are regarded as nanomaterials (Masciangioli and Zhang, 2003). Nanoparticles are useful for mankind found as volcanic dust, soil sediments and colloids in water since millions of years on earth(Handy *et al.*, 2008b). In natural environment, nanoparticles are found in the form of metal oxides, clay, organic matter and other minerals (Klaine *et al.,* 2008). Now a days nanoparticles are being synthesized artificially because of different chemical and physical properties relative to naturally occurring nanoparticles bearing potential environmental hazards (Crane *et al.,* 2008). Anthropogenic activities are producing a wide range of nanoscale pollutants from ordinary materials such as airborne particles emitted from automobiles and frictional erosion over the road surfaces (Handy and Shaw, 2007).

Nanoparticles have a wide range of domestic and industrial applications. Titanium dioxide (TiO_2) are used in paints, sunscreen, cosmetics, medicine and sporting equipment and dumped into natural aquatic environment

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inducing a number of biological and physiological responses in fish (Federici *et al.,* 2007). Wide range of CuO-NPs applications made them suitable for making electronics, heat sensing devices and medicine (Bondarenko *et al.,* 2013). Zinc oxide (ZnO) nanoparticles are potentially used in sunscreen, cosmetics, deodorant and car polish similarly to titanium dioxide nanoparticles having potential environmental danger (Shaw and Handy, 2011). All activities leading to the dumping of these nanomaterials alongwith otherwastes into the natural aquatic systems. Fishes are larger than invertebrates and important group of organisms which become crucial victim to these nanomaterials (Buffle, 2006). Nanoparticles have the ability to accumulate in the cells as macrophages and hepatocytes, therefore, taken up by the aquatic organisms like, fish, mollusks, crustaceans and artemia into their bodies (Ward and Kach, (2009). The route of exposure to these nanoparticles in fish mostly done through gills to blood, skin mucosa and from gastrointestinal absorption by blood leading to the other parts of body such as liver, kidney, brain and muscles (Handy *et al.,* 2008).Studies have been conducted on the uptake of these materials. Ates *et al.* (2013) investigated the accumulation of titanium dioxide nanoparticles in intestine, gills, muscles and brain of gold fish (*Carracius auratus*).Similar study regarding the accumulation of heavy metals in the soft tissues of fresh water mussels from Chashma Lake was done by Sohail *et al.* (2016).Shaw *et al.,* (2012) observed copper accumulation in the gills of rainbow trout (*Oncorhyncus mykiss*) from waterborne copper nanoparticles and its bulk salt exposure. Zhao *et al.* (2011) also studied the distribution of CuO-NPs in juvenile carp (*Cyprinus carpio*) and their potential toxicity.

A comparative study on the bioaccumulation and sub-acute toxicity of zinc oxide nanoparticles in juvenile carp (*Cyprinus carpio*) to its bulk counterparts showed that ZnO-NPs have more potential to accumulate in the tissues and induce oxidative cellular responses Hao *et al.* (2013). Kaya *et al.* (2015) observed accumulation and oxidative stress in different organs of Nile tilapia (*Oreochromis niloticus*) induced by ZnO-NPS.

The aims and objectives of present study was to determine and assess the bioaccumulation of metals released from their nanoscale entities in the soft tissues of tilapia(*Oreochromis mossambicus*).

MATERIAL AND METHODS

Animal collection and placement

Fish were collected from aquaculture ponds at Pattoki District Kasur, Pakistan. Animals were sorted out with average weight of 22.980±0.368g and size 9.378±0.186cm. Animals were placed into plastic bags having freshwater and oxygen was diffused into water using oxygen cylinder pipe with no mortality during transportation. Animals were placed in rectangular water glass tanks fitted with aerators and aquarium heaters to maintain oxygen and temperature level. Fish were acclimatized for seven days in the water glass tanks before the start of experiment.

Experiment Design

Animals were graded into ten experimental water glass tanks (12 fish/tank) with triplicate having dimensions 45.72 x 60.96 x 45.72 cm for 14 days after acclimatization in a semi-static system. Commercial feed containing 35% crude protein, 4% crude fats, 5% crude fibre and 12% moisture in granular form was given to fish twice a day. Stock solutions of TiO_2, CuO and ZnO nanoparticles were prepared in Milli Q water by means of sonication. TiO_2-NPs were sonicated for 30 minutes at 35 KHz whereas CuO-NPs and ZnO-NPs were sonicated for 30 min. at 40 KHz frequency respectively by means of a sonicator (WUC-A06H). Three treatments identified as T1 (0.5 mg/L), T2 (1.0 mg/L) andT3 (1.5 mg/L) of were applied to separate tanks and one control having no nanoparticles with 3 tanks as replicates per treatment. While exposing to these nanoparticles the fish were not fed to reduce the adherent of nanoparticles to food. Water was changed each day before the treatment. About 80% of the water along with animals waste were taken out of each tank with the help of a suction pump. Fresh water was then added to the water glass tanks. Before dose administration to animals in glass tanks nanoparticles were sonicated. The volume of water in each glass tank was 40 liters.

At the end of 14^{th} day animals were taken out one by one into smaller water container. To anesthetize 3 to 4 drops of clove oil were added. Animals were slaughtered humanely. Gills, liver and muscles were excised with the help of scissors. Excised organs were placed in plastic bottles at -20ºC to assess the bioaccumulation.

Physicochemical Parameters

Water quality/ physicochemical parameters such as temperature and dissolved oxygen (DO) were measured with the help of YSI pro 20 DO meter, pHwas measured with the help of pH meter Hoelzle and Chelius 1687, Conductivity and TDS were measured by JENCO conductivity meter. Titration based standard APHA (2005) protocols were followed for the measurement of p-Alkalinity, total alkalinity, Ca-hardness, total hardness and Chlorides.

Sample preparation for Inductively Coupled Plasma Mass Spectrometry(ICP-MS)

One gram of freeze dried sample was taken in a digestion flask to which about 10 ml concentrated nitric acid (HNO_3) and 2ml perchloric acid ($HClO_4$) were added. The contents were then heated on hot plate in a fume hood at 100^oC until the yellow acid digested color was disappeared and two drops of hydrogen peroxide were added. Each digested sample was evaporated to 2 ml, cooled and diluted with distilled water to 50 ml and filtered with Whattman filter paper. These samples were analysed by using inductively coupled plasma mass spectrometry (ICP-MS) (APHA, 2005).

Statistical Analysis

Data for bioaccumulation from ICP-MS was analyzed using Minitab Version 17. The effects of glass tanks were not observed during the whole treatments in any experiment. ANOVA was applied using Tukey's test at 95% level of significance to compare means.

RESULTS

Physicochemical parameters

The mean values of temperature, pH, dissolved oxygen (DO), conductivity, total dissolved solids (TDS), carbon dioxide (CO_2), p-alkalinity, total alkalinity, Ca-hardness, total hardness and chloride were shown in Table I.

Table I: Physicochemical parameters of water.

Physicochemical parameters	Results
Temperature	27.997±0.0606˚C
pH	7.7500±0.0306
Dissolved Oxygen (DO)	7.00±0.153 mg/L
Conductivity	395.67±1.86 µS/m
Total Dissolved solids (TDS)	333.47±1.68 mg/L
Carbon Dioxide (CO_2)	0.00±0.00 mg/L
p-Alkalinity	8.677±0.145 mg/L
Total Alkalinity	202.67±1.20 mg/L
Ca-Hardness	35.0±0.577 mg/L
Total Hardness	51.667±0.882 mg/L
Chloride	25.00±0.577 mg/L

All values are mean ± S.D

Table II: Bioaccumulation of titanium from titanium dioxide (TiO_2) nanoparticles in µg/Kg

Tissues	Treatments			
	T0 (0 mg/L)	T1 (0.5 mg/L)	T2 (1.0 mg/L)	T3 (1.5 mg/L)
Gills	0.7033 ± 0.0116^{h}	1.4533 ± 0.0208^{g}	4.0533 ± 0.0473^{b}	5.7033 ± 0.0208^{a}
Liver	1.8933 ± 0.0513^{f}	2.3933 ± 0.0153^{e}	3.6167 ± 0.0306^{c}	4.1067 ± 0.0208^{b}
Muscles	0.4267 ± 0.0208^{i}	0.6734 ± 0.0153^{h}	0.7833 ± 0.0208^{f}	2.9400 ± 0.0200^{d}

All values are mean ± S.D at 95% level of significance
Superscript showing the mean values are significantly different from each other at 14th day

Bioaccumulation of titanium dioxide (TiO_2)

The accumulation of titanium (Ti) from titanium dioxide nanoparticles (TiO_2-NPs) in the gills, liver and muscles of tilapia (*Oreochromis mossambicus*) are shown in the Table II. It has been observed that with the increasing dose concentration the amount of Ti started to accumulate in the tissues. Maximum titanium was accumulated in the gills as 5.7033±0.0208 µg/Kg compared to liver and muscles to T3

(1.5mg/L) when compared to T0 (0 mg/Kg) as 0.7033±0.0116 µg/Kg. Significant difference of Ti accumulation was observed between gills, liver and muscles. The mean Ti concentration in liver was 4.1067±0.0208 µg/Kg whereas in muscles was 2.9400±0.0200µg/Kg. These results showed that Tihas the ability to accumulate more in the gills as compared to liver and muscles. The order of Ti accumulation in the fish tissues is gills>liver >muscles.

Bioaccumulation of copper oxide (CuO) nanoparticles

The accumulation of copper (Cu) from copper oxide nanoparticles (CuO-NPs) in the gills, liver and muscles of tilapia (*Oreochromis mossambicus*) are shown in the Table III. It has been observed that with the increasingdose concentration, the amount of Cu started to accumulate in the tissues. Maximum accumulation was observed in the gills as compared to liver and muscles at high dose concentration (1.5 mg/L) (*p<0.05*) which was 3.7133±0.0208 µg/Kg as compared to the control as 0.4933±0.028 µg/Kg. Significant difference of Cu accumulation was observed between gills, liver and muscles. The mean Cu concentration at high dose concentration in liver was 2.66±0.0300 µg/Kg whereas in muscles was 0.8867±0.0153 µg/Kg. Minimum Cu accumulation was observed in the liver. Cu has permeability to accumulate more in the gills as compared to liver and muscles. The order of Cu accumulation in the fish tissues is gills>liver >muscles.

Bioaccumulation of zinc oxide (ZnO) nanoparticles

The accumulation of Zinc (Zn) from zinc oxide nanoparticles (ZnO-NPs) in the gills, liver and muscles of tilapia *Oreochromis mossambicus* are shown in the Table IV.

Table III: Bioaccumulation of copper (Cu)from copper oxide (CuO) nanoparticles in µg/Kg

Tissues	Treatments			
	T0 (0 mg/L)	T1 (0.5 mg/L)	T2 (1.0 mg/L)	T3 (1.5 mg/L)
Gills	0.4933 ± 0.028^{J}	0.9367 ± 0.0321^{f}	1.8433 ± 0.0208^{c}	3.7133 ± 0.0208^{a}
Liver	0.6034 ± 0.0153^{i}	1.2233 ± 0.0208^{e}	1.7533 ± 1.0153^{d}	2.66 ± 0.0300^{b}
Muscles	0.7335 ± 0.0153^{gh}	0.7133 ± 0.0231^{h}	0.7833 ± 0.0058^{g}	0.8867 ± 0.0153^{f}

All values are mean ± S.D at 95% level of significance
Superscript showing the mean values are significantly different from each other at 14th day

Table IV: Bioaccumulation of zinc (Zn) from zinc oxide (ZnO) nanoparticles in µg/Kg

Tissues	Treatments			
	T0 (0 mg/L)	T1 (0.5 mg/L)	T2 (1.0 mg/L)	T3 (1.5 mg/L)
Gills	1181 ± 2.00^{K}	1295 ± 2.00^{J}	1338.3 ± 1.15^{h}	1431.7 ± 1.53^{g}
Liver	1136.3 ± 3.08^{l}	1548.7 ± 1.53^{e}	1244.7 ± 2.08^{b}	2375.3 ± 1.53^{a}
Muscles	1316 ± 2.65^{l}	1451.3 ± 1.53^{f}	1716.7 ± 2.08^{d}	2017 ± 2.00^{c}

All values are mean ± S.D at 95% level of significance
Superscript showing the mean values are significantly different from each other at 14th day

It has been observed that with the increasing dose concentration the amount of Zn started to accumulate in the tissues. Maximum accumulation was observed in the liver as compared to gills and muscles at high dose concentration (1.5 mg/L) (*p<0.05*) which was 2375.3±1.53 µg/Kg as compared to the control as 1136.3±3.08 µg/Kg. Significant difference of Zn accumulation was investigated between gills, liver and muscles. The mean Zn concentration at high dose concentration in gills was 1431.7±1.53 µg/Kg whereas in muscles was 2017±2.00 µg/Kg. Minimum Zn accumulation was also observed in the liver. Zn has permeability to accumulate more in the liver as compared to gills and muscles. The order of Zn accumulation in the fish tissues is liver> muscles>gills.

DISCUSSION

Nanoparticles are the most important commodities in the domestic and industrial fields. Anthropogenic activities are continuously

dumping the nanoscale wastes into the natural aquatic ecosystems. These wastes are going to be deposited in the watery environments forming sediments and suspensions in the water columns. Aquatic organisms feed on the sedimentation and living in water columns are in continuous contact with these entities. Nanoparticles are being absorbed by these organisms. They also have the ability to enter into the bodies of these organisms and accumulate in the tissues and other body organs. Fish is the most important bio indicator to the toxic materials present in their environment. Nanoparticles by means of skin and gills entered into the blood stream and distributed throughout the fish bodies like brain, liver, kidneys and muscles (Handy *et al*., 2008).

Sohail *et al.* (2016) studied the heavy metals accumulation in the soft tissues of freshwater mussels support our results. The results from the study conducted by Ates *et al.* (2013) on the accumulation of titanium (Ti) from titanium dioxide nanoparticles (TiO_2-NPs) in the gills of gold fish (*Carassius auratus*), where Ti accumulated more in the gills with the rising dose concentration. This study backings our present result where more titanium accumulated in the gills of tilapia (*Oreochromis mossambicus*) at high dose concentration as compared to the liver and muscles. The mean Ti accumulated in the gills was 5.7033±0.0208 µg/Kg as compared to control shown in Table II.

The accumulation of copper (Cu) from copper oxide nanoparticles (CuO-NPs) was observed more in the gills as mean value 3.7133±0.0208 µg/Kg at high dose concentration as compared to the liver and muscles. The increasing trend of copper accumulation in the tissues of tilapia (*Oreochromis mossambicus*) with rising dose concentration. The minimum mean value of Cu was observed in muscles as 0.8867±0.0153 µg/Kg. Liver had intermediate accumulation of Cu. Shaw and Handy (2006) came out with the result while exposing Nile tilapia (*Oreochromis niloticus*) with dietary copper that the copper accumulation was more in liver as compared to the gills and intestine. Another study conducted by Mansouri *et al.* (2016) on co-exposing common carp (*Cyprinus carpio*) with titanium and copper nanoparticles resulted an increased liver accumulation of copper than gills. In juvenile carp (*Cyrinus carpio*) Cu was more accumulated in the intestine as compared to the gills, muscles, skin, scales, liver and brain. (Zhao *et al.,* 2011). The results from the previous studies investigated that the copper had the ability to accumulate in liver more as compared to gills and muscles. These results are contrary to the present where copper is more accumulated in the gills Table III. This is because the gills are in continuous contact with water due to agglutinate ability more copper instead of penetrating into the blood stream via blood stick to the gill filaments and results an increase in copper accumulation in the gills as compared to liver and muscles. The study elaborated by Sohail *et al.* (2016) supported the present study, therefore, observed more Cu accumulation in the gills of freshwater of muscles as compared to other soft tissues.In the present study Zn was more accumulated in liver than muscles and gills Table IV. Hao *et al*. (2013) suggested that Zn from zinc oxide nanoparticles found to be more accumulated in the gills and gut of juvenile carp (*Cyprinus carpio*) when compared to its bulk counterparts. Similar study was conducted by Kaya *et al.* (2015) resulted the same trend of Zn accumulation in the liver of juvenile carp (*Cyprinus carpio*) in relation to gills, kidney and muscles. Sohail *et al.* (2016) also investigated the increasing metals deposition in the soft tissues of freshwater mussels. All these previous studies contributed towards the same trend of Zn accumulation in the liver with the increasing dose concentration of ZnO-NPs as compared to the other tissues of fish which have been found in the current data.

CONCLUSION

From the present study we can conclude that the rising concerns in using nanomaterials and its products are environmentally deposited into the aquatic ecosystems. Their deposition and suspensions in water column are being ingested by the aquatic organisms. Fish being a pivotal model and important bio indicator in aquatic environment are frequently come in contact with these materials and entered into the body systems of these organism.As they are toxic to the fish and change the biological processes. It is essential to prevent the entry of these nanoparticles to the aquatic environment to save the natural fauna.

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