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Research Article

Nanoparticles (Al_2O_3, CuO, TiO_2) Decrease ATPase Activity in the Osmoregulatory Organs of Freshwater Fish (*Oreochromis niloticus*); Histopathological Investigations of Tissues by Transmission Electron Microscope

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Abstract

The use of nanoparticles (NPs) in diverse field of nanotechnology has been increased in the last decades and especially the aquatic environments have been contaminated by waste products. Because the potential toxic effects of NPs have been documented in different groups of animals, the present study was carried out to help understanding the toxic effects of three NPs in freshwater fish. In this work, *Oreochromis niloticus* were exposed to aluminum oxide (Al_2O_3), copper oxide (CuO) and titanium dioxide (TiO_2) NPs in differing concentrations (0, 1, 5, 25 mg/L) for 14 d. Following the exposures, the activities of total ATPase, Mg-ATPase and Na, K-ATPase in the osmoregulatory organs (gill, kidney) of fish were measured. Additionally, the aggregates of NPs in the gill and intestine of fish which are the possible entrance to the blood stream were demonstrated by a transmission electron microscope (TEM) to demonstrate the histopathological effects of NPs. Thus, the aim of this study was to evaluate the relationship between the histological view of tissues and changes in ATPase activity. Data showed that all NPs decreased significantly ($P < 0.05$) ATPase activities in most cases. In general, Al-NPs and Cu-NPs decreased significantly ($P < 0.05$) all ATPase activities in the gill and kidney at the highest and medium exposure concentrations. Similarly, Ti-NPs also decreased significantly all ATPase activities in the kidney at the highest and medium exposure concentration, though none of Ti-NP concentrations caused significant change ($P > 0.05$) in ATPase activity in the gill. Overall, data demonstrated that the least affected enzyme was Mg-ATPase and the least toxic NP was Ti-NP. TEM images demonstrated that all NPs accumulated in the tissues of fish in 14 days of exposure period, but not eliminated completely after 14 days of depuration period. There were considerable changes in histological images between control fish tissues and NP exposed fish tissues. NP aggregates in the intercellular space and in the cells can be seen from the TEM images, indicating the decreases in ATPase activities occurred due to histopathological changes. This study demonstrated that NPs are able to cause changes in tissue structures and ATPases were very sensitive to NP exposures. Data suggested that there should be more studies to understand better the toxic effects of NPs in fish.

Keywords: *Metal; Nanoparticle; ATPase; Biomarker; Toxicity; Histopathology; Fish*

Introduction

Although metals have been being used for a long time, a considerable contribution of metals to the environment began after industrialization which may mean that the history of metal pollution begins mostly early in the 20th century. It is well known that "contamination" of an aquatic environment is accepted as the presence of elevated concentrations of metal presents in water (above the natural background

level for the area), though "pollution" means the introduction of metals by man directly or indirectly to the aquatic environment resulting in deleterious effects to living resources [1]. In other words, contamination may provide a warning signal, but it does not constitute pollution unless, first, it is caused by human activities and second, it has some damaging effects. The fate of the aquatic environments, especially the freshwater ones (due to smaller volume compared to a sea), is not good because nearly all xenobiotic find their destination in the aquatic systems as a result of washing up by rain water.

In recent years, metals have entered human life in a different form called metal-oxide nanoparticles. Materials ranging between 1 - 100 nm are called nanoparticle and metal-oxide NPs are named after metals they contain. Most important characteristics of NPs are their reactivity, surface structure, high surface to volume ratio and unique electronic properties [2,3]. Some NPs contain extremely toxic metals such as silver and some contain metals known for lower toxicities such as iron. Nevertheless, ionic form and NP form of a metal may exhibit different toxic effects [4-6], therefore nanotoxicology has emerged as new area for toxicology. Because many products that human use contain NPs such as suntan cream, toothpaste, medicals, textile, electronic, toys, moisturizer and some are used in food, nanotoxicology studies should be carried out in different classes of animals in the aquatic environments and also in terrestrial animals [7-10]. This warning was possibly done due to the capacity of NPs to pass through cell membranes and accumulates in tissues and consequently cause hazardous effects on organs, tissues, cells and molecules. Studies have also indicated that while studying toxic effects of NPs, size, shape, coating, dose, duration and metals in NPs should be taken into account to understand better their effects in different groups of animals [3,9,11-15].

ATPases are membrane-bound enzymes responsible for the transport of ions through cell membranes. ATPases found in the gill and kidney of fish are responsible for the transport of ions such as Na and K and through the cell membranes. Therefore, ATPases play important roles in the regulation of osmotic pressure, cell volume and membrane permeability. Na, K-ATPase transports 2 K ions into the cell and 3 Na ions out of the cell to maintain the required ionic balance. Mg-ATPase is required in the oxidative phosphorylation in the mitochondria and also Mg ion transport for regulation of Mg ions in the cell. Actively maintaining of osmotic concentrations in extracellular fluids, in spite of the osmolarity of the surrounding environment is called osmoregulation that is a fundamental physiological adaptation of animals living in the aquatic systems [16,17]. Studies have shown that ATPases, primary osmoregulation enzymes, were found to be sensitive to metals due to their binding ability onto the active sites of enzymes that cause inhibition or stimulation of ATPases [18-21].

Because the use of biomarkers in evaluating the physiological condition of fish against the toxic effects of metals were found to be beneficial, they might also be useful for determining the effects of nanoparticles. A key mechanism of toxicity of metals is osmoregulatory impairment associated with ATPase inhibition in the osmoregulatory tissues such as gill and kidney and determination of the response of biomarkers may be vital as an early warning signal for nanoparticle toxicity. Thus, the present study was undertaken to investigate the effects of 3 nanoparticles on the histological views of gill and intestine and their effects in ATPase activity osmoregulatory system of fish. For this aim, freshwater fish, *O. niloticus* were exposed to Al_2O_3, CuO and TiO_2 NPs in differing concentrations (0, 1, 5, 25 mg/L) for 14 days and subsequently, the activities of total ATPase, Mg-ATPase and Na, K-ATPase in the gill and kidney were measured. To associate the occurred changes in the activity, NP aggregates and histopathological observations were also investigated by means of Transmission Electron Microscopy techniques.

Materials and Methods

Freshwater fish (*Oreochromis niloticus*) were transferred from the raring pools of Faculty of Fisheries and Aquatic Sciences in Cukurova University (Adana, Turkey) where they have been cultured for more than 30 years and were acclimatized to laboratory conditions for one month before beginning to the experiment (12h dark; 12h light; 21 ± 1°C) in glass aquaria sized 40 x 40 x 100 cm. The mean size and associated standard deviation of fish used in the experiments were 18.6 ± 1.50 cm. There was no significant ($P > 0.05$) difference among different exposure regimes and controls. All the chemicals were supplied from Sigma-Aldrich unless otherwise stated.

Fish were individually exposed to Al_2O_3, CuO and TiO_2 NPs using exposure concentrations of 0 (control), 1, 5 and 25 mg NP/L for 14 days, aerating continuously with an air compressor to supply oxygen (5.7. ± 0.87 mg O_2/L). During the experiments, the total hardness, pH and conductivity of water were measured and the mean values and associated standard deviations of these were 315.8 ± 19.9 mg $CaCO_3$/L, 7.99 ± 0.16 and 569 ± 10.1 µS/cm respectively. Each NP concentration and control contained 6 fish. Every 2 days, the exposure media were renewed just after fish feeding to prevent the contamination of the exposure media with food remains and also to renew NP concentrations. Fish were fed with commercial fish food (Pinar Sazan, Izmir, Turkey) using the ratio of their weight (2%). The aquaria were aerated from the bottom to minimize rapid aggregation of NPs. Uptake and depuration experiments were run at the same time, using 60 fish for each protocol (a total of 120 fish). At the end of 14 days of uptake period, 60 fish (6 for each exposure and control) were killed by transaction of the spinal cord according to the decision of an Ethic Committee of Cukurova University and some parts of gill and kidney tissue samples were dissected out and frozen at -85°C (Esco UUS-480A) for the measurement of ATPase activity. Remaining fish were moved to depuration aquaria which contained only decholorinated tap water. Depuration experiments were carried out for 14 days, using the same experimental conditions, except NP presence and fish was dissected out as above. Unfrozen samples of gill tissues from the uptake and depuration experiments were immediately processed to use in TEM analysis.

ATPase activity assay

Activities of total-ATPase, Na,K-ATPase and Mg-ATPase were carried out using following final assay concentrations; 40 mM Tris-HCl, 120 mM for NaCl, 20 mM for KCl, 3 mM for $MgCl_2$, 7.7 for pH, and 1 mM for ouabain. The measurement of ATPase activity was started adding 50 µl of enzyme suspension (~100 µg protein) to 850 µl of incubation media and preincubated for 5 min at 37°C in a disposable test tube. After preincubation, the reaction was started after addition of 100 µl ATP (3 mM) and incubated in a water bath with shaker (Wise Bath WSB-30). After 30 minutes, the reaction was stopped by adding 500 µl of ice-cold distilled water. ATPase activity in the samples was measured using the method of Atkinson., *et al* [22]. This method was used to measure inorganic phosphate produced from the degradation of ATP, using appropriate blanks. A series of KH_2PO_4 (25 - 250 µM) was used as an inorganic phosphate standard. All readings were done in a spectrophotometer (Schimadzu UV-1800) at 390 nm. Specific Na,K-ATPase activity in the gill and kidney was calculated the differences between the presence (Mg-ATPase activity) and absence (total-ATPase activity) of the ouabain. All assays were carried out in triplicate and mean values were used as individual data. Total protein levels in the gill and kidney were measured using the method of Lowry., *et al.* [23] and bovine serum albumin used as a standard. ATPase activities were expressed as µmol Pi/mg prot./h. Our group has started measuring ATPase activities in 1996 [24] and since then we have published many papers concerning ATPase activities in lobster, fish and rat.

Characterization of nanoparticles

Metal-oxide nanoparticles (Al_2O_3, CuO, TiO_2) were purchased from Sigma-Aldrich Germany) or Nanografi (Turkey). Company information of nanoparticles was as follows; Al_2O_3 (~40 nm, > 99% purity, > 30 m^2/g surface area, 2.70 g/cm^3 density), CuO (~40 nm, > 99% purity, > 20 m^2/g surface area, 6.50 g/cm^3 density) and TiO_2 (~21 nm, > 99% purity, > 30 m^2/g surface area, 4.26 g/cm^3 density). Further characterizations were done on these NPs in our labs. XRD (X-Ray Diffraction) analysis were done on NPs using a Rigaku RadB SmartLab diffractometer system (CuKα1, λ=1.5405 *Å*, 30 kV, 15 mA, 2θ = 10° - 90°, scanning rate 2°/min). These analyses showed that gamma Al_2O_3 NP had a cubic phase and polycrystal structure, CuO NP had monoclinic phase and polycrystal structure and anatase TiO_2 NP had a tetragonal phase and polycrystal structure. EDX (Energy-Dispersive X-ray) data were obtained using a Field Emission Scanning Electron Microscope (Zeiss/Supra 55 VP). EDX data showed that percent ratios of aluminium, copper and titanium atoms in Al_2O_3, CuO and TiO_2 NP powders were 38.26, 48.74 and 33.83, respectively. Percent weight ratios of aluminium, copper and titanium atoms in Al_2O_3, CuO and TiO_2 NP powders were also calculated. These were 51.10, 79.06 and 60.49 percent for aluminium, copper and titanium, respectively and remaining percentages contained only oxygen atoms. TEM images of Al_2O_3, CuO and TiO_2 NPs in stock solutions (Figure 1) and TEM images of the nanoparticle accumulation in gill and intestine tissue samples (Figure 2-8) were obtained using a Jeol JEM-1010 TEM (80kW) connected to a GATAN 782 ES500W Erlangshen camera.

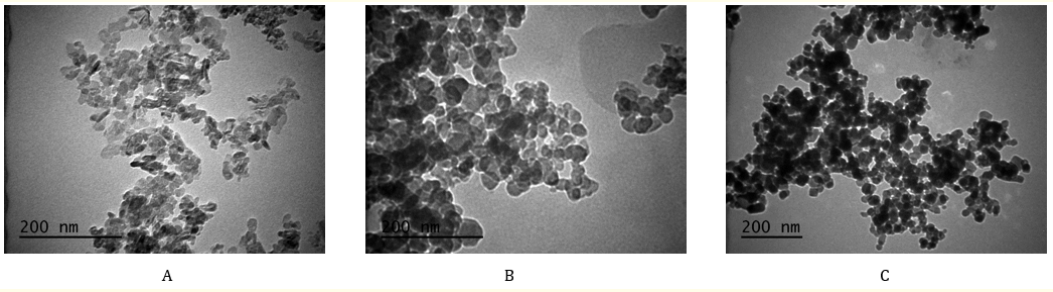

Figure 1: *TEM images of Al_2O_3 (A), CuO (B) and TiO_2 (C) nanoparticles in stock solutions.*

Statistical analysis

A statistical package program (SPSS 15, Chicago, IL, USA) was used for the statistical analysis of data. Homogeneity of variance of the data was first checked before the analysis. Data was first analysed using One-way ANOVA. Significant ($P < 0.05$) results were re-analysed by post-hoc tests to find out which individual group caused the variation over controls. Mean enzyme activity and associated standard errors were presented in figures (Figure 5-7) indicating significant variations between control and individual exposures. Percent variation of ATPase activity over control values were also calculated and given in a table (Table 1).

Results and Discussion

None of NP concentrations killed the fish during the uptake and depuration experiments and also there were no apparent health problems such as swimming performance, eating habit and color change which are readily apparent in fish under physiological stress [16]. Figure 2 shows the TEM images of gill and intestine tissue samples of control fish. As the images demonstrate there are no NP aggregates in control tissues. However, all NPs at all concentrations accumulated in the gill and intestine of fish after 14 days of the exposure period. However, accumulated NPs were not depurated completely after 14 days of elimination period (Figure 3-8). Accumulation of NPs in tissues may mean that metal-oxide NPs, regardless of their size, are able to pass through the membranes (either in the gut or gill) and enter into the blood stream and finally aggregate in the tissues, causing histopathological effects. This also suggests that NP aggregates could also cause histological changes either blocking capillaries or changing membrane permeability. Potential toxic and pathological effects of NP aggregates were also remarkable as some NP aggregates were still remained in the tissues after 14 days of depuration period. However, histopathological effects of NPs still remain to be investigated. Especially, surface structures of NPs containing different metals may affect occurred histological changes. Because NPs are rather large matters for the intercellular space, it is likely that they would intervene communications among tissues and cells.

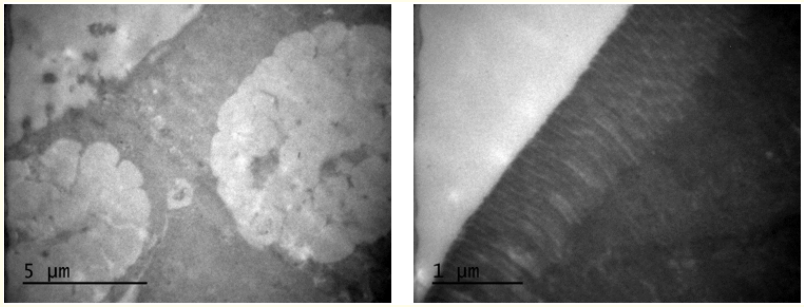

Figure 2: *TEM images of gill and intestine tissue samples of control fish (O. niloticus).*

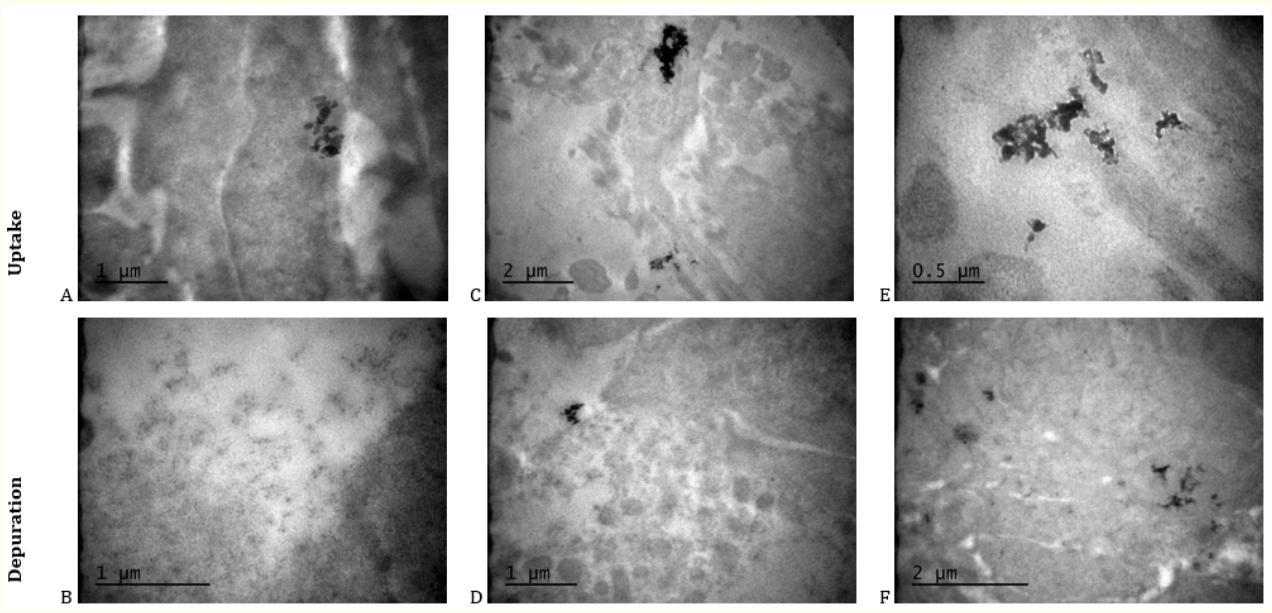

Figure 3: *TEM images of gill tissue sample of fish (O. niloticus) exposed to 1 (A and B), 5 (C and D) and 25 (E and F) mg/L of Al_2O_3 NPs for 14 days of uptake and 14 days of depuration periods, respectively.*

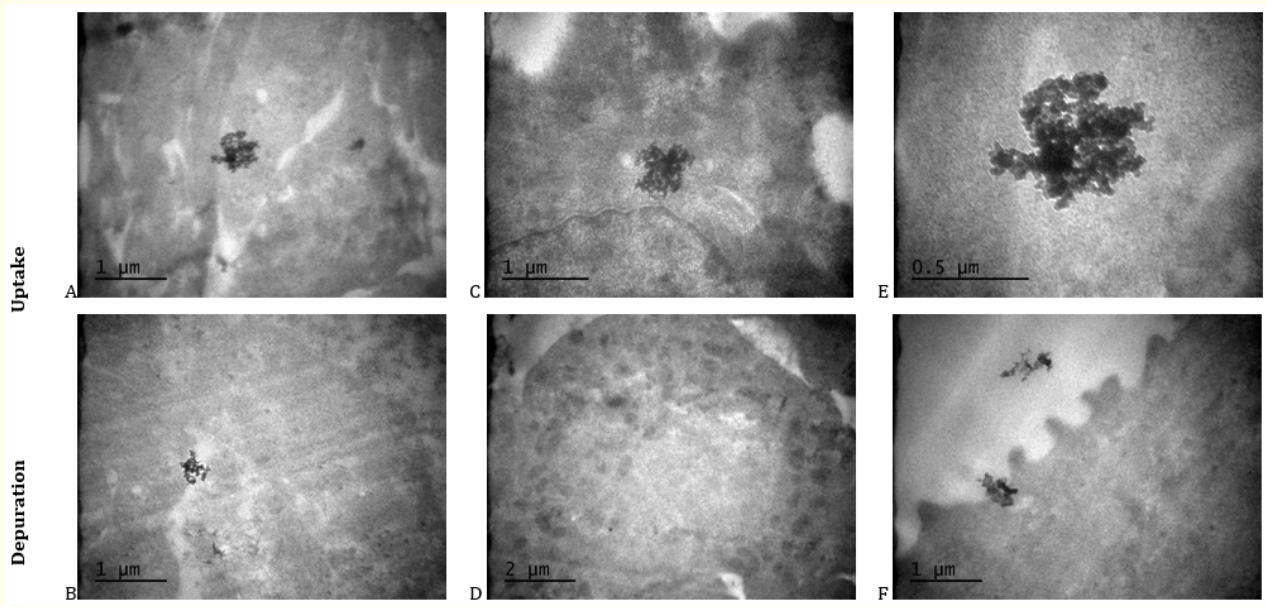

Figure 4: *TEM images of gill tissue sample of fish (O. niloticus) exposed to 1 (A and B), 5 (C and D) and 25 (E and F) mg/L of CuO NPs for 14 days of uptake and 14 days of depuration periods, respectively.*

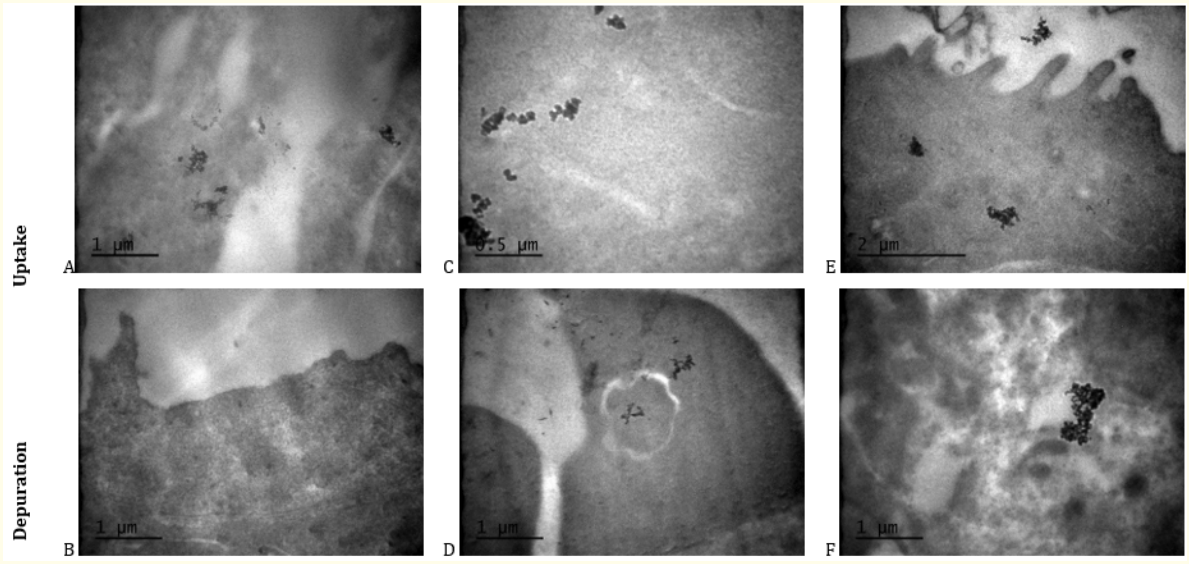

Figure 5: *TEM images of gill tissue sample of fish (O. niloticus) exposed to 1 (A and B), 5 (C and D) and 25 (E and F) mg/L of TiO_2 NPs for 14 days of uptake and 14 days of depuration periods, respectively.*

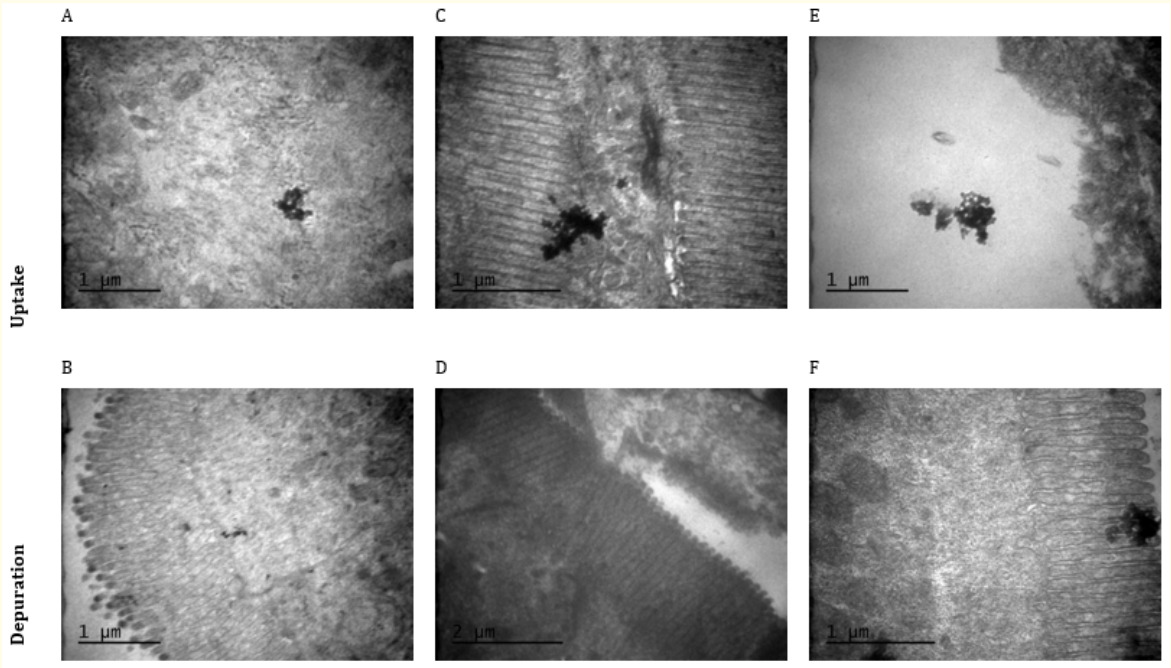

Figure 6: *TEM images of intestine tissue sample of fish (O. niloticus) exposed to 1 (A and B), 5 (C and D) and 25 (E and F) mg/L of Al_2O_3 NPs for 14 days of uptake and 14 days of depuration periods, respectively.*

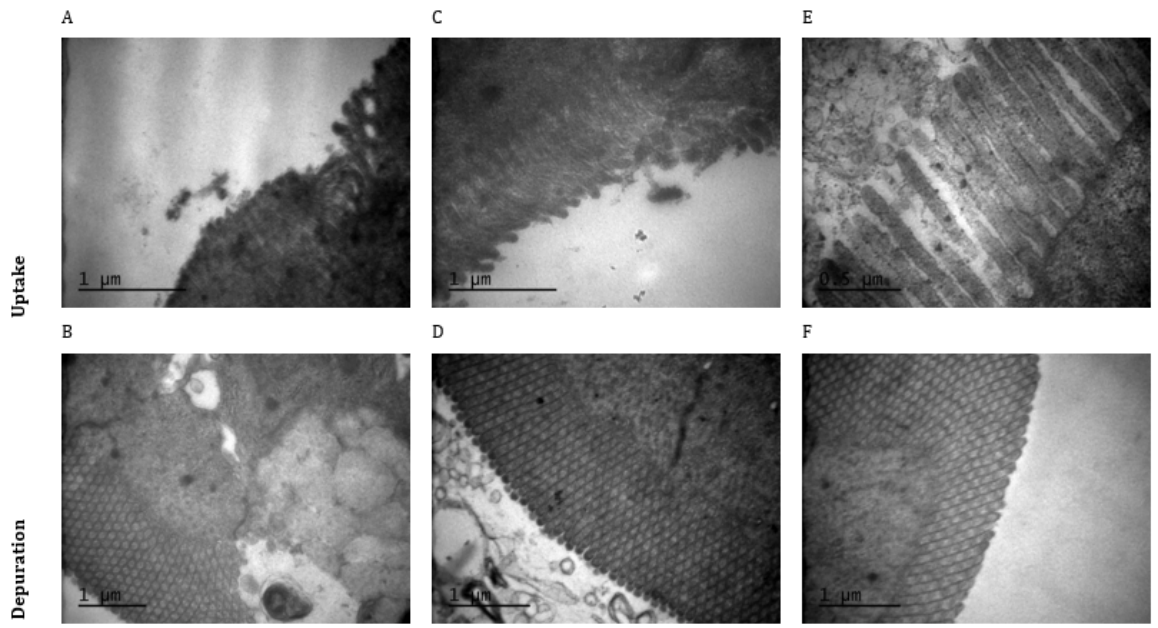

Figure 7: *TEM images of intestine tissue sample of fish (O. niloticus) exposed to 1 (A and B), 5 (C and D) and 25 (E and F) mg/L of CuO NPs for 14 days of uptake and 14 days of depuration periods, respectively.*

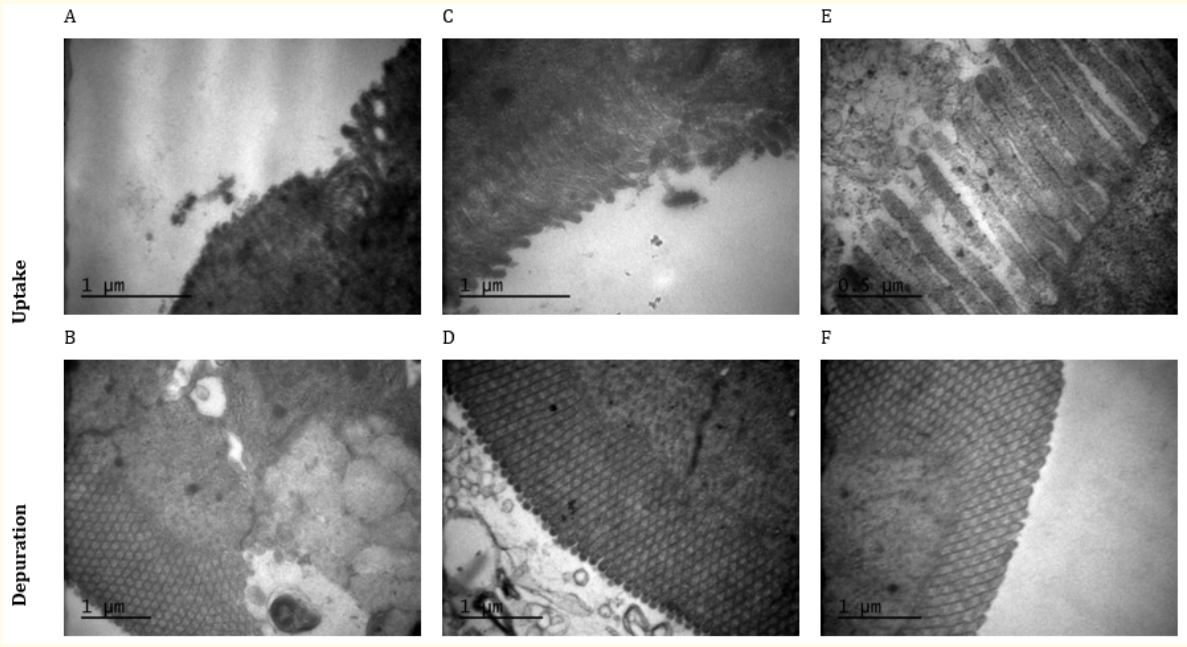

Figure 8: *TEM images of intestine tissue sample of fish (O. niloticus) exposed to 1 (A and B), 5 (C and D) and 25 (E and F) mg/L of TiO_2 NPs for 14 days of uptake and 14 days of depuration periods, respectively.*

Studies have also shown that different metal-oxide NPs accumulated in the tissues of animals, regardless of uptake route, indicating their potential toxic effects [25-27]. Although there is limited data on the depuration of NPs from fish tissues, it may be accepted that their depuration is much slower than uptake. This is also a remarkable point of environmental toxicology point of view. Another remarkable point is the accumulation of Al_2O_3, CuO and TiO_2 NPs in the muscle of fish and low depuration (data in preparation) which may be accepted as a warning signal for the human consumption point of view. Zhang., *et al.* [28] also demonstrated the differences in accumulation and elimination rates of two different iron NPs (Fe_2O_3 and Fe_3O_4) in fish (*Danio rerio*). Similarly, TiO_2 and CuO NPs accumulated in the tissues of carp (*C. carpio*) and eliminated thereafter following a depuration period, though there were differences in combination exposure experiments [29]. However, the TEM images in the present study demonstrated that some of accumulated NPs were not eliminated from the gill after 14 days of depuration period. Osborne., *et al.* [13] also showed that different Ag-NP types accumulated in the tissues of zebra fish and some Ag-NPs were cleared in the gill but retained in the intestines after depuration period. Similarly, Jang., *et al.* [30] measured silver accumulation in the carp (*C. carpio*) following exposure to Ag-NP for 7 days followed by a 2-week depuration period. They found that most accumulated silver in some tissues returned to the control levels after 14 days of depuration period, except liver, gastrointestinal tract and gills, according to the present study.

In the present study, data showed that the activity of ATPases in the gill and kidney of fish mostly decreased following exposure to NP. Table 1 shows the percent decreases in the activities. The mean ATPase activities and their associated standard errors in the gill and kidney of fish exposed to Al_2O_3 NP were shown in figure 9a and 9b, respectively. All ATPase activities in the gill significantly decreased following Al_2O_3 NP exposures at the medium and highest exposure concentrations. Similarly, total-ATPase and Na, K-ATPase activities in the kidney also decreased significantly at the highest exposure concentration. Significant decreases in the ATPase activities ranged between 25 - 44% in the gill and 36 - 46% in the kidney. The mean ATPase activities and their associated standard errors in the gill and kidney of fish exposed to CuO NP were shown in figure 10a and 10b, respectively. Effects of Cu-NPs were similar to Al-NPs. Cu NPs also caused significant decreases in all ATPase activities the gill and kidney (except Mg-ATPase activity) at the medium and highest exposure concentrations. Significant decreases in the ATPase activities ranged between 30 - 35% in the gill and 38 - 62% in the kidney. The mean ATPase activities and their associated standard errors in the gill and kidney of fish exposed to TiO_2 NP were shown in figure 11a and 11b, respectively. Ti-NP exposures did not cause any significant change in the gill, but it decreased significantly all ATPase activities in the kidney at the medium and highest exposure concentrations. Significant decreases in the ATPase activities ranged between 27 - 69%. As explained above, the decrease in ATPase activities seem to be the results of histological changes of tissue structures and especially, the interaction between the surface of NPs and cell membranes might played significant in occurred toxicity.

According to the literature data titanium nanoparticles have been studied widely perhaps due to their wide usage areas. Additionally, some very toxic NPs such as Ag-NPs have also been investigated widely due to their highly potential toxic effects and their usage for antibacterial activity. Although lethal values of different NPs may differ on the mortality point of view, all NPs may become toxic after a certain threshold [2]. As occurred in the present study, chronic uptake of NPs can cause toxic effects in different biomarkers belonging to different metabolic system, despite their low mortality values [31] In the literature, data show great variation in the effects of NPs in freshwater fish, depending on experimental protocols, metal contents, surface characteristics and the size of NPs [32,33]. ATPases in fish are responsible for ion transporting across the membranes and in the cell have been shown to be very sensitive to environmental contaminants. The present study also supported this, as ATPase activities in the gill and kidney of fish showed the significant decreases following NP exposures. However, the response of ATPases may vary greatly following exposures to metal containing products and also exposure protocols such as *in vitro* and *in vivo* [34]. Additionally, compensatory mechanisms play significant roles in measured inhibition and activity lost due to inhibited enzymes may be compensated by the other enzymes with their increases in turnover rates [24]. However, it seems that the recovery of ATPases in the present study did not mostly occur, as ATPase activity in general decreased significantly.

Tissue	ATPase	Al_2O_3 NP (mg/L)			CuO NP (mg/L)			TiO_2 NP (mg/L)		
		1	5	25	1	5	25	1	5	25
Gill	Total ATPase		↓30	↓39		↓30	↓32			
	Na_2K-ATPase		↓40	↓30			↓30			
	Mg-ATPase		↓25	↓44		↓35	↓34			
Kidney	Total ATPase			↓36			↓41			↓27
	Na_2K-ATPase		↓41	↓46	↓46	↓38	↓62		↓52	↓69
	Mg-ATPase								↓53	↓65

Table 1: *Percent alterations in the activities of ATPases in the gill and kidney of fish following exposure to Al_2O_3, CuO and TiO_2 nano particles for 14 days. The differences in mean values between control group and NP exposed groups were used in % calculations given in the table. Downward arrows indicate significant decreases in ATPase activity. Only significant differences ($P < 0.05$) were given in this table.*

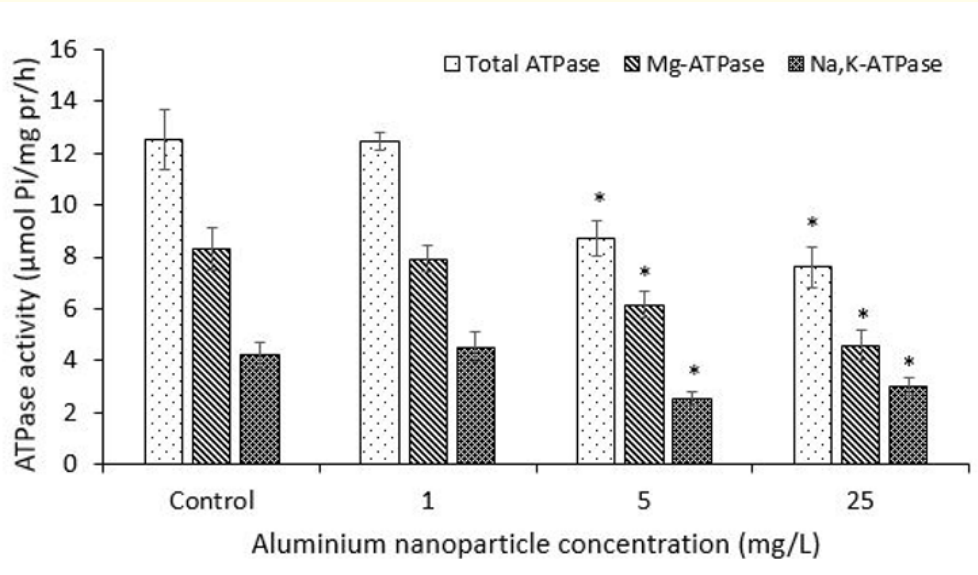

Figure 9a: *The mean ATPase activity and associated standard errors in the gill of O. niloticus exposed to different concentrations (0, 1, 5, 25 mg/L) of Al_2O_3 NP for 14 d (N = 6). See figure 1. * indicate significant ($P < 0.05$) differences resulted from the Duncan tests between control and NP-exposed fish.*

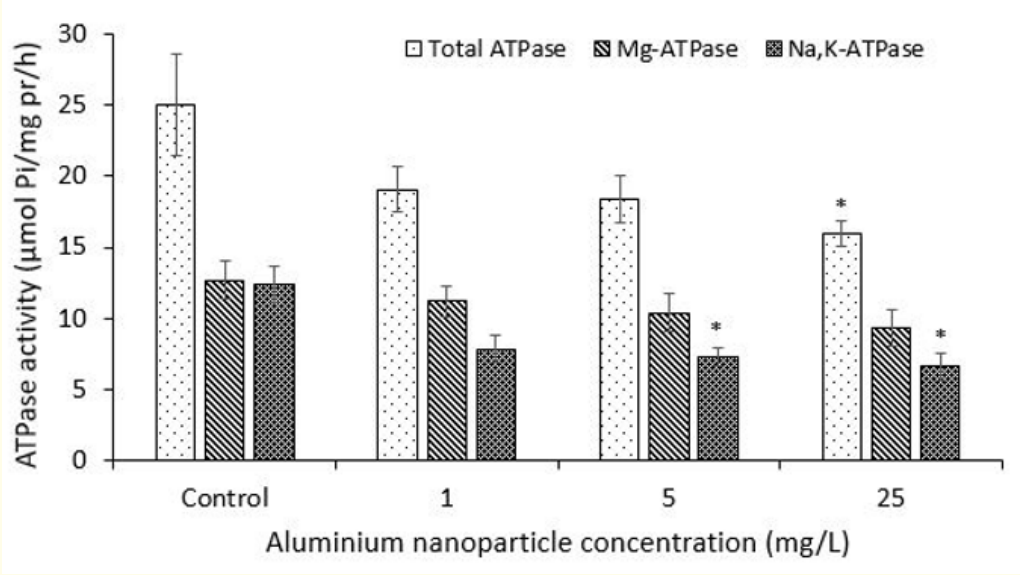

Figure 9b: *The mean ATPase activity and associated standard errors in the kidney of O. niloticus exposed to different concentrations (0, 1, 5, 25 mg/L) of Al_2O_3 NP for 14 d (N = 6). See figure 9a for detail.*

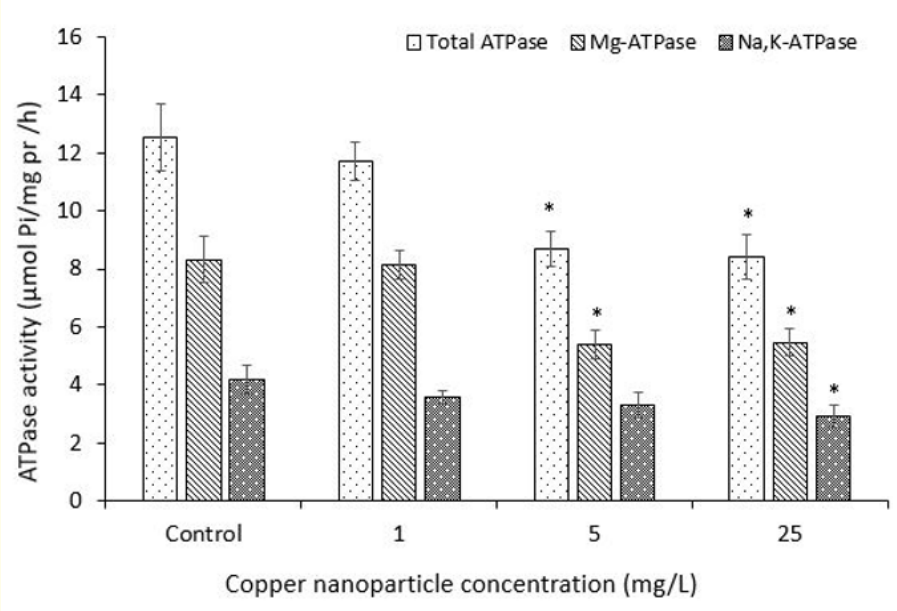

Figure 10a: *The mean ATPase activity and associated standard errors in the gill of O. niloticus exposed to different concentrations (0, 1, 5, 25 mg/L) of CuO NP for 14 d (N = 6). See figure 9a for detail.*

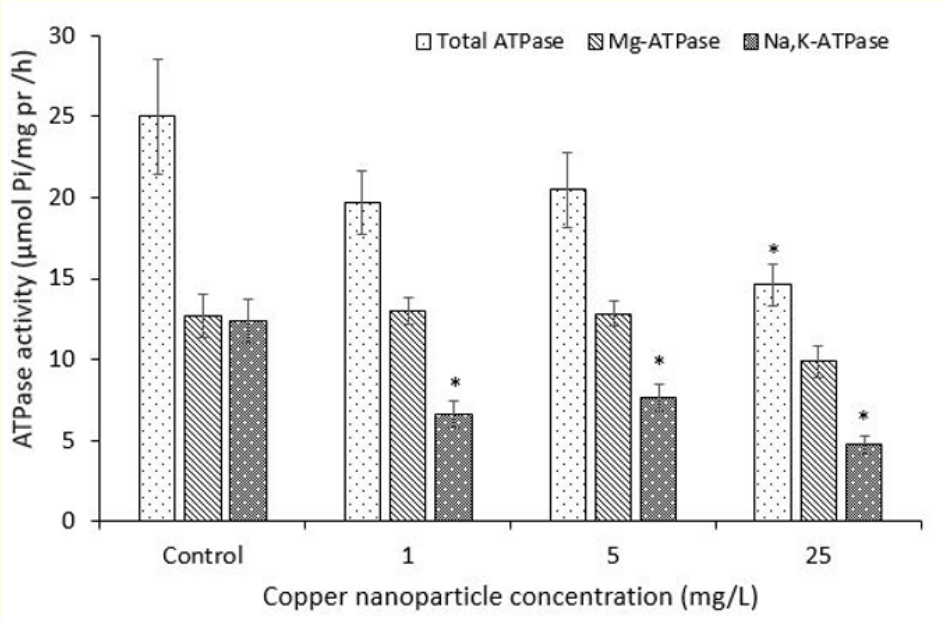

Figure 10b: *The mean ATPase activity and associated standard errors in the kidney of O. niloticus exposed to different concentrations (0, 1, 5, 25 mg/L) of CuO NP for 14 d (N = 6). See figure 9a for detail.*

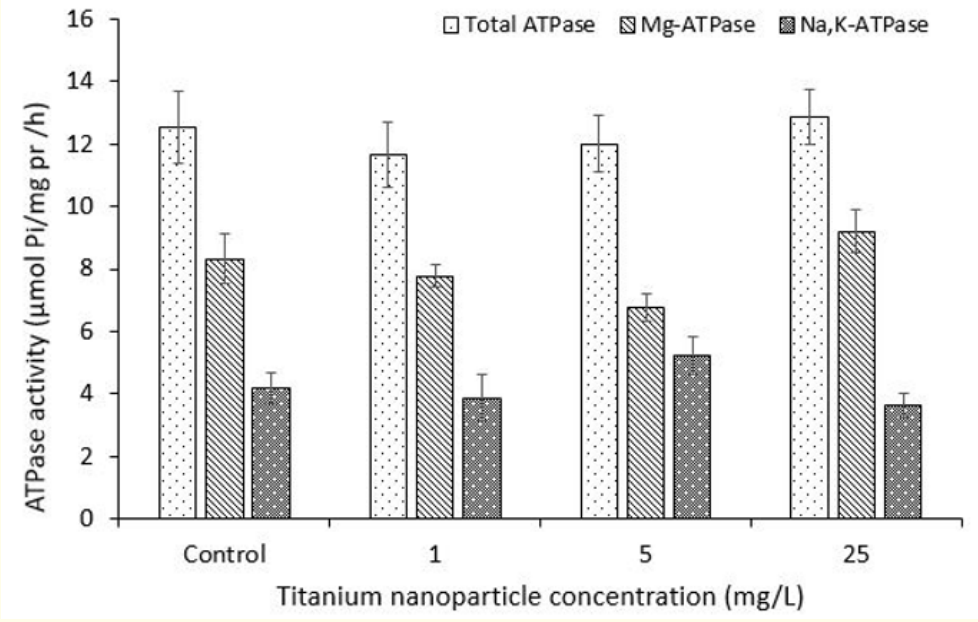

Figure 11a: *The mean ATPase activity and associated standard errors in the gill of O. niloticus exposed to different concentrations (0, 1, 5, 25 mg/L) of TiO_2 NP for 14 d (N = 6). See figure 9a for detail.*

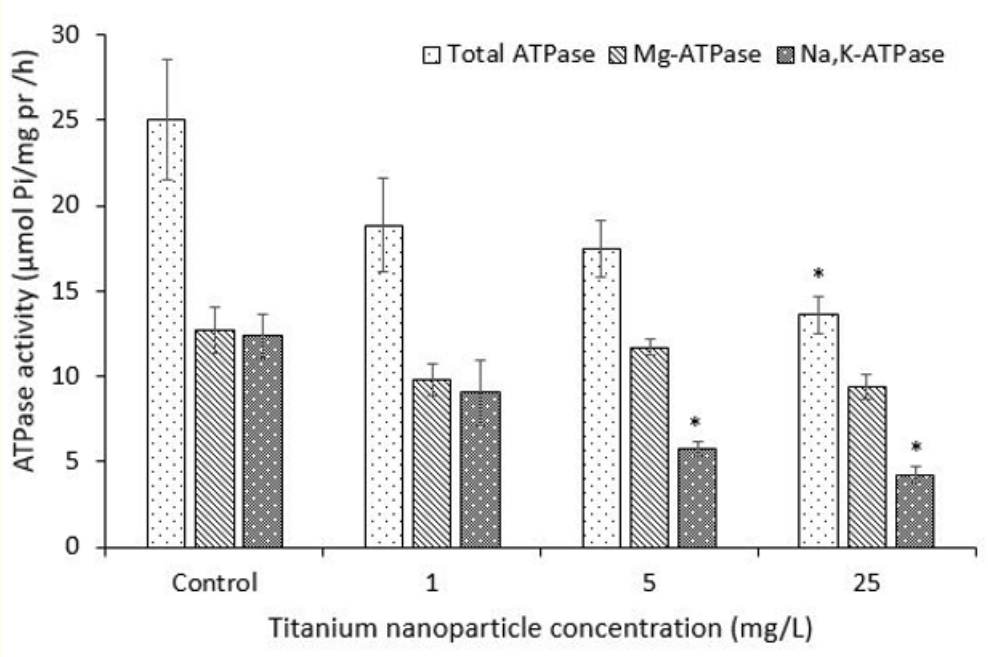

Figure 11b: *The mean ATPase activity and associated standard errors in the kidney of O. niloticus exposed to different concentrations (0, 1, 5, 25 mg/L) of TiO_2 NP for 14 d (N = 6). See figure 9a for detail.*

Literature data mostly showed the inhibitory effects of different NPs on ATPases in different fish species, though there are conflicting results from different experiments. As occurred in the ionic metal effects, the effects of NPs on fish ATPases differ depending on fish species, uptake routes, exposure concentrations and periods. Shaw and Handy [2] indicated that the chemistry and behavior of NPs involve dynamic aspects of aggregation theory, rather than the equilibrium models traditionally used for free metal ions. Osborne., *et al.* [13] studied different sizes of Ag-NPs in zebrafish following exposure for 4h, 4 days, or 4 days plus a 7-day depuration period. They found different toxicokinetic profiles for different the sizes of Ag-NPs. Their data demonstrated that the gill had higher Ag content for the 20 nm NPs than the 110 nm NPs. Their data also showed that the gill and intestine confirmed prominent Ag deposition in the basolateral membranes for the smaller Ag-NP, but not for the larger Ag-NP. Their study suggests the possibility of the site of tissue deposition to disruption of the Na/K ion channel, which is also localized to the basolateral membrane and this was confirmed by a reduction in ATPase activity and immunohistochemical detection of the R subunit of this channel in both target organs, with the smaller NPs causing significantly higher inhibition and disruption than the larger size particles or $AgNO_3$. Federici., *et al*. [35] demonstrated the toxic effects of Ti-NPs on gill injury, oxidative stress and ATPase activity in rainbow trout (*Oncorhynchus mykiss*), confirming the present data. Although their study showed that there were no alterations in the levels major ions, exposure to Ti-NPs decreased in Na, K-ATPase activity in the gill and intestine, possibly occurred due to the pathologies in the gill including oedema and thickening of the lamellae. Griffitt., *et al.* [4] exposed zebrafish to either Al-NPs or aluminium chloride for up to 48 hours in moderately hard fresh water. Exposure to both aluminium chloride and Al-NP resulted in a concentration dependent decrease in Na, K-ATPase activity, although Al-NP exposure did not alter gill morphology as measured by filament widths. They emphasized that decreases in ATPase activity coincided with decreases in filamental ATPase staining and mucous cell counts. Carmo., *et al.* [26] exposed the Neotropical fish (*Prochilodus lineatus*) to Ti-NP (0, 1, 5, 10 and 50 mg/L) and evaluated the osmo- and ionic balance, Na, K-ATPase activity in the gills and kidney up to 4 days. Acute exposure to TiO_2-NP decreased plasma os-

molality and Na, K-ATPase in the gills, but not in the kidney. After subchronic exposure, there was no change in plasma osmolality, ionic balance and enzyme activities, suggesting a cellular, physiological and morphological response to restore and maintain osmotic and ionic homeostasis after subchronic exposure.

Metals known for their low toxicities such as iron may also become toxic when given in NP form. For example, Remya., *et al.* [36] studied the chronic toxicity of iron oxide NPs on gill Na, K-ATPase activity of an Indian major carp (*Labeo rohita*) for a period of 25 days. Fe NP exposure of fish caused alterations in ion levels and activity of gill Na, K-ATPase, emphasizing adverse physiological effects on fish of Fe-NPs. Similarly, essential metal zinc could also cause toxic effects in NP form [37]. Their work showed that zinc-oxide NPs caused oxidative and cellular stress in freshwater fish known as the white sucker (*Catostomus commersonii*), though interestingly it caused increases in gill Na, K-ATPase activity, probably due to increased epithelial permeability. They also noted that the fish exhibited a 35% decrease in heart rate during ZnO NP exposures. Although copper is an essential metal, it can be very toxic to fish in ionic form and evidence suggest that Cu-NPs may also exhibit high toxicity. Wang., *et al.* [6] found an increase in ATPase activity in juvenile *Epinephelus coioides* exposed to Cu-NPs and soluble Cu (0, 20 or 100 µg Cu/L), despite time-dependent Cu accumulation in all tissues increased with increases in Cu dose. Oppositely, the inhibitory effects of Cu NPs were demonstrated by Shaw., *et al.* [25] in juvenile rainbow trout. They exposed fish to either copper as $CuSO_4$ or Cu-NPs (20 or 100 g/L) and found that most important alterations in Na, K-ATPase activity occurred in all Cu treatments, suggesting Cu-NPs are an ionoregulatory toxicant to rainbow trout. Similarly, Kaya., *et al.* [14] found that tilapia (*Oreochromis niloticus*) exposed to different sizes (10 - 100 nm) Zn-NPs in 1 and 10 mg/L concentrations showed that ZnO NPs inhibited the Na, K-ATPase activity at all concentrations and increased serum Ca and Cl levels especially in gill, emphasizing Zn-NPs were osmoregulatory and toxicant for tilapia fish. They concluded that both sizes of the particles have led to organ damage, osmoregulatory changes and immune disorder in tilapia fish. In this respect, this study and the present study in accord in terms of NP toxicity in tilapia. Griffitt., *et al.* [18] indicated the toxic potential of Cu-NPs, emphasizing Cu-NP was very toxic to zebra fish even though most Cu-NPs in water aggregated rapidly. Similarly, the toxic effects of Al-NP were shown in zebra fish, despite rapid aggregation of Al-NP [4], suggesting the toxic effects of NPs could increase when they suspended in water.

Dietary uptake of NPs was also shown in both fish and mammals. Ramsden., *et al.* [38] demonstrated the dietary (10 and 100 mg/kg) uptake of Ti-NPs by juvenile rainbow trout (*Oncorhynchus mykiss*), emphasizing its important for the fish physiology in chronic exposures. They found that the accumulation occurred in the tissues of fish and Na, K-ATPase activity in the brain showed a 50% decrease, though there was a significant change in the gills and intestine. The toxic effects of NPs were also demonstrated in terrestrial vertebrates such as mammals. In a previous paper, we also demonstrated that there were significant inhibitions of Na,K-ATPase activity following oral administration of Al_2O_3, CuO and TiO_2 NPs in the erythrocytes of female Wistar rats [15]. Similarly, different studies showed the toxic effects of different NPs on ATPases from different tissues of mammals [19,39,40]. It seems that the most likely route for the absorption of NPs from either the gut and gills mostly occur via the endocytosis and measuring NPs in tissue samples may help understanding the body distribution, metabolism, and excretion of NPs [2]. TEM images in the present study clearly support this assumption, as all tissues accumulated NPs.

Conclusion

Data from the literature and the present work demonstrated that different metal NPs had different toxic effects depending on species and exposure protocols. Metal NPs mostly affected ATPases in the present study, general trend being a decrease in the activity. Overall, data demonstrated that Al-NPs and Cu-NPs behaved similarly as they both decreased ATPase activities in the gill and kidney. However, Ti-NPs behaved somewhat differently, as they only caused significant decreases in the kidney. This may be attributed to the differences in the physio-chemical behavior of different nanoparticles and specific interaction with the tissues. The present study also showed that NPs accumulated by the gill and intestine within 14 days did not completely removed from the gill following 14 days of depuration per-

iod. This suggests that NP aggregates are able to cause histological changes either blocking capillaries or membrane permeability. The decreases in ATPase activities could be due to the potential toxic and pathological effects of NP aggregates in the tissues. NP aggregates in the intercellular space and in the cells can be seen from the TEM images, indicating the decreases in ATPase activities occurred due to histopathological changes. There were considerable changes in histopathological changes of the tissues and the present data suggest further investigations in any possible correlation between ATPase activity and histopathological effects. The present study and most literature data emphasized that metal-oxide NPs were not innocent compounds as they are able to alter many metabolic parameters in fish, suggesting there must be some criteria and limits for their usage and discharge to the environment.

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