Ultra structure alteration of sublethal concentrations of zinc oxide nanoparticals on Nil Tilapia (*Oreochromis niloticus*) and the protective effects of vitamins C and E

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Abstract: In the present study, the ultra structure toxic effects of ZONPs on *Oreochromis niloticus* (*O. niloticus*) and the protective role of vitamin C and E were evaluated. Two hundred male *O. niloticus* were exposed to sub-lethal concentrations (1 and 2 mgL^{-1}) of characterized ZONPs nanoparticles either with or without vitamin C and E mixture for a period of 15 days. Livers and gills ultra-structure were investigated using transmition electron microscope. ZONPs leads to a severe vacuolation of gill cells and necrosis of pavement and epithelial cells with dilated mucous cells. These cells were separated from secondary lamella by edema containing fibrin and inflammatory cells. While the hepatocyte subcellular had a sever alteration manifested by rarefaction, complete disappearance of mitochondria, aggregation of rough endoplasmic reticulums, indentation of nucleus, Partial lysis of the outer and intactness of the inner nuclear membrane, appearance of irregular shaped nucleus (polypoid) and fragmentation of the nuclear chromatin. Vitamin E and C combination some what modified and overcume the gills and hepatocytes suborganills deformity that induced by ZONPs. In conclusion, ZONPs are toxic in a concentration of 1 and 2 mgL^{-1} for Nil Tilapia (*Oreochromis niloticus*), that manifested by ultra-structure alteration in gills and liver sub cellular organilles as nucleus, golgy apparatus, endoplasmic reteculam and cell membrans. The addition of vitamin E combination with vitamin C in the diet appeared to modulate the ultra-structure alteration induced with ZONPS with the time.

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1. Introduction

Nanoparticles (NPs) are a diverse class of small scale (usually <100 nm) substances formed by

molecular-level engineering to achieve unique mechanical, optical, electrical and magnetic properties for a large variety of applications. Due to their unique physical and chemical properties, nanoparticles are increasingly being produced and applied **(Ramesh et al., 2013).** Nanomaterials are used in many industrial areas (e.g. material science, personal care products and electronics) and will provide a promising technology in many other areas (e.g. medicine) **(Ferre et al. 2009)**. Thus far, more than 1,000 products with nanomaterials are already on the market **(Nanovip 2010)**. Revenues from nanotechnology-based products are expected to grow worldwide to US $3.1 trillion by 2015 **(Jonathan 2009)**. Due to the great increase in the production volume and widespread use, they can pose a potential threat to the environment and human health. Since nanomaterials differ in origin, size and material, they are expected to exhibit different biological effects. However, concerns over the health risks to humans and biota have recently drawn global attention.

Among the various types of nanoparticles, Zinc Oxide nanoparticles (ZONPs) are widely produced and applied in many products including sunscreen (**Maier and Korting, 2005**), waste water treatment (**Chen et al., 2004**), and environmental remediation (**Aitken et al., 2006**). Direct and indirect release of these nanoparticles (NPs) into aquatic environments via bathing, sewage effluent (**Daughton and Ternes, 1999; Handy and Shaw, 2007**) and other engineering applications **(Chen et al., 2004; Nagaveni et al., 2004)** have increased the exposure chances of humans and ecosystems to NPs **(Nowack and Bucheli, 2007).** Recent studies have indicted the risks of ZONPs to aquatic organisms **(Reddy et al., 2007; Handy et al., 2008 ; Wang et al., 2009; Farre´ et al., 2009; Xiong et al., 2011; Linhua and Lei, 2012)**. ZnONPs toxicity has been reported in human and mammalian models, but there are very limited data on the toxicological effects of ZnONPs nanoparticles in aquatic organisms specially animal

model of nil tilapia (*Oreochromis niloticus*). The present study was carried out to estimate the potential effects of ZnONPs nanoparticles on nil tilapia based on determination of the ultra- structure alteration in the liver and gills tissues.

2. Materials and Methods

Experimental Fish

Two hundred male *O. niloticus* (Nile tilapia, Perciformes, Cichlidae; weight 90 ±5 g, length 15 ±3cm) were obtained from Abrahem El-Solimani farms for fish, Kholes, Saudi Arabia Kingdom. Fish were transferred to the Biotechnology Lab, Faculty of Science, King Abdul Aziz university north campus, where they were held in twenty glass aquaria (n=10 individuals/aquarium), with 100 L of healthy water (pH 7.6 ± 0.3, conductivity of 287mS/cm, 0.60 m MCa 2 þ, and 0.3 m MMg2) that was changed daily, a continuous system of water aeration (Eheim Liberty 150 Bio-Espumador cartridges), and a 12:12-h light:dark photoperiod. Temperature was maintained at 28±2C and dissolved oxygen, at 7.0±0.5 mgL^{-1}. Fish were fed with commercial fish food, containing 6% lipids, 31% proteins, 37% carbohydrates, 2.5% fiber, 1.5% total phosphorus, 12% ash, 200 mg α-tocopherol/kg, 1,700 IU vitamin D_3/kg feed, and 10,000 IU vitamin A/kg feed. The amount of feed (on dry matter basis) given daily to fish was 10% of body weight and the fish were fed 3 times daily. Fish were acclimatized for 15 days before the beginning of the experiments. All experimental procedures were approved by the Ethical Committee of the University of King Abdul-Aziz.

Chemicals

ZnONPs suspension (30-nm sized particle, a surface area of 50 m^2/g, with a purity of 99.90%), vitamin C and E were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Riedstrasse 2, D-89555 Steinheim, Germany). Product Number: 721077, A5960 and T3251, respectively. The particle size was nearly spherical and very fit with the nano-scale, and the measured particle size was close to the manufacturer information (≈30).

Experimental Protocol

Fish were randomly divided into 5 groups (40 fish for each), the first group was kept as control, the 2nd and 3rd, groups were exposed to ZnONPs of 1 and 2 mg/L, respectively, the 4th and 5th group were exposed to ZnONPs of 1 and 2 mg/L and treated with a mixture of vitamin C and E in a dose of 500 mg/kg diet (250 mg of each vitamin). After 15 days of exposure twenty fish of each group were first anaesthetized with 100 mg/L of Ms-222. Fish were immersed in the anesthetic solution until they reached a stage of complete immobility, sacrificed in ice-water, dried with filter paper and finally necropsied for the collection of target organs including liver and gills. These organs were carefully removed, weighed, washed with ice-cold saline and preserved in 2.5% Glutraldehyde until Transmition electron microscope examination.

Transmition electron microscope examination

Specimens from the liver and gills were fixed in 6.25 % cacodylate buffer gluteraldehyd followed by 1% osmium tetraoxide. After dehydration, the specimens were embedded in poly-ethylene capsules containing the embedding mixture (Epon mixture and hardener).Ultra-thin sections were prepared and stained by Uranyl acetate and lead citrate **(Weakly 1981).**

3. Results

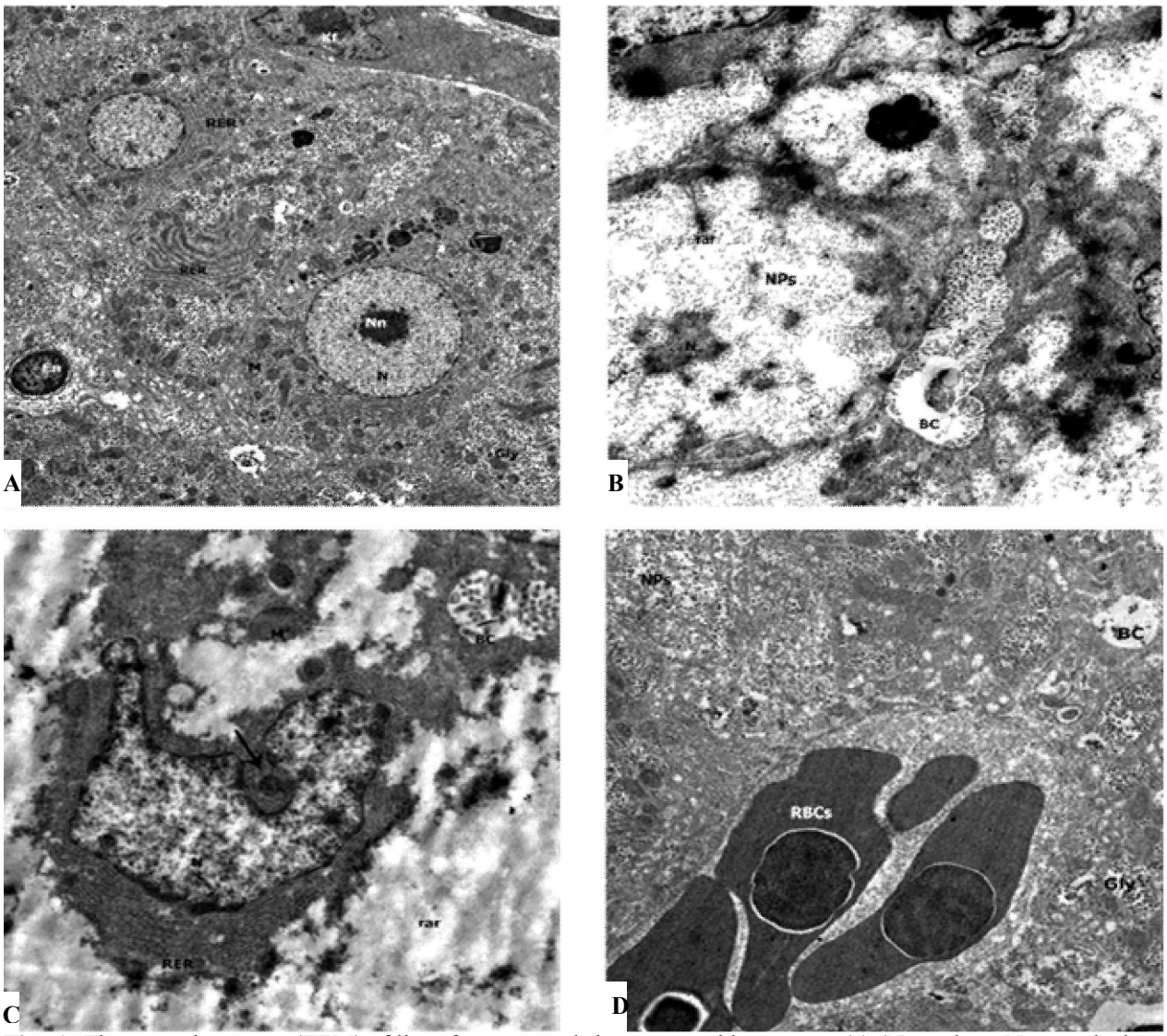
TEM examination of gills

The gills of control were normal with gill filaments, lamellae and surface epithelium (Fig 1 a). Meanwhile the gills received ZnONPs showed severe vacuolation and necrosis pavement and epithelial cells with dilated mucous cells. These cells were separated from secondary lamella by edema containing fibrin and inflammatory cells (Fig 1 b). The previous findings were lowered or absent with restored the lining epithelium when treated with vitamin C and E. Mild activation of the mucous and chloride cells were detected (Fig 1 c).

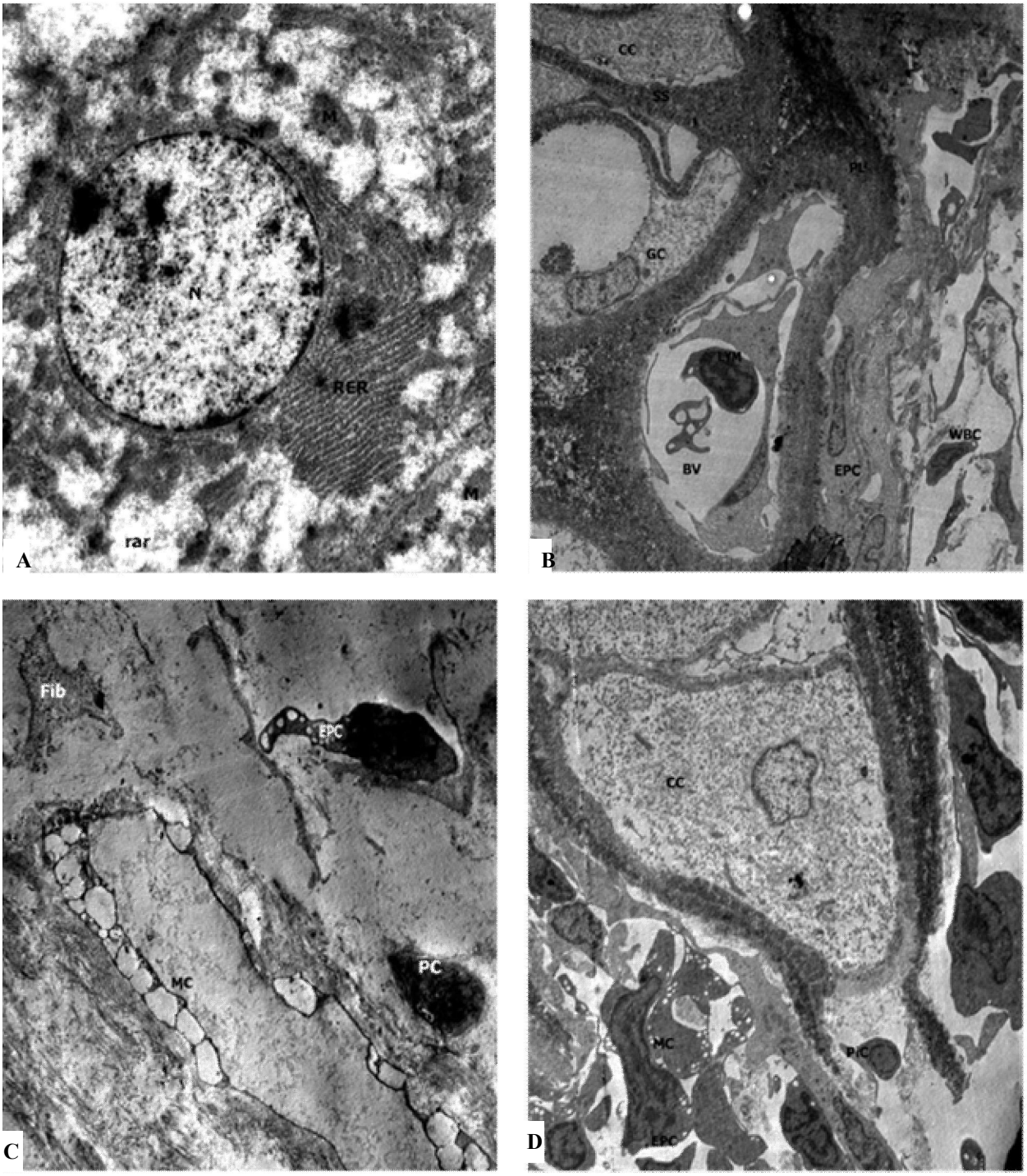
TEM examination of Liver

The liver cells of control were identical with that generally known and previously described. The cytoplasm showed numerous mitochondria, rough endoplasmic reticulum, Golgi apparatus, glycogen granules and few fat lipid globules. Meanwhile, the nucleus was normal round with centrally located nucleolus and dispersed granular chromatin (Fig 2a). The hepatocytes received ZnONPs showed severe vacuolation and lysis of the cytoplasm (rarefaction) with aggregation of ZnONPs, complete disappearance of the mitochondria, Golgi apparatus and glycogen. Narrowing or complete obliteration of the spaces among the hepatocytes were visualized with proliferation of bile canaliculi (Fig 2b). Few rough endoplasmic reticulums were aggregated and condensed around the nuclei with partial lysis of its membranes and detached ribosomes. The nucleus showed marked crenation (indentation) and vacuolar detaching of nuclear membranes, dislocation of nucleolus, clumping and condensation of chromatin in the shape of an irregular homogenous ring of a snowflake form with peripheral migration into the cytoplasm (Fig 2c). Partial lysis of the outer and intactness of the inner nuclear membrane was also observed with appearance of irregular shaped nucleus (polypoid). Fragmentation of the nuclear chromatin was detected and dispersed in the cytoplasm with

absence of the nuclei inside the hepatocytes. The hepatic sinusoids were congested (Fig 2d) and stuffed with numerous inflammatory cells of macrophages and neutrophils containing numerous lysosomes were seen among the degenerated or necrotic hepatocytes. The hepatocytes of treated groups with vitamin C and E showed mild to moderate vacuolations of the cytoplasm with few mitochondria and dilated regular shaped RER. Depletion or complete absence of glycogen was detected. Activated cytosomes (peroxisome) were also detected. The nucleus was nearly normal with mild clumping of chromatin. The inflammatory cells of predominate neutrophils were noticed (Fig 2e).



Figs 1: Electron microscopy (TEM) of liver from: control shows normal hepatocyte (a) (N=nucleus, Nn= nucleolus, KF=kupffer cell, En= endothelium, M=mitochondria, RER=rough endoplasmic reticulum, FC=Golgi apparatus, Gly=glycogen, F=lipid globule, Lys=lysosome). ZnONPs received group shows hepatocyte with clumping and condensation of chromatin in the shape of polypoid mass, rarefaction of the cytoplasm and aggregation of ZnONPs (b). Marked crenation (indentation) and vacuolar detaching of nuclear membranes (c). Congestion of the hepatic sinusoid (d). TEM x 5850.



Figs 2: Electron microscopy (TEM) from: hepatocyte of treated with vitamin C and E shows mild vacuolation of the cytoplasm (rar) with few mitochondria, RER and peroxisome (a). Gills shows normal primary (PL) and secondary (SS) lamellae, epithelial lining (EPC), chloride (CC), pavement (PC), goblet (GC) and pillar (PiC) cells besides normal blood vessels (BV) (b). Gill of ZnONPs received group shows Moderate vacuolations of the cytoplasm (rar) with edema (E) containing fibrin (Fib), dilated mucous cells (MC) and necrotic pavement (PC) and epithelium (EPC) (c). Gill of vitamin C and E treated group shows slight activation of mucous (MC) and chloride (CC) cells with normal other lining epithelia (d). TEM x 5850.

4. Discussion

Electron microscopy analyzes have been extensively used tool in monitoring of fish exposed to contaminants and showing the initial signs of lesions or alterations not easily identifiable during the morphological or macroscopic examination. And, in this study, the changes of ultrastructure of gill and liver tissues could be regarded as the effective indicators of water contamination and the degrees of toxicity to fish induced by ZnONPs.

Liver is the main organ of body's metabolism. Previous reports have shown that nano-TiO2 exposure aggravated liver's load and resulted in liver injuries, which might lead to lipidosis of carp (**Hao et al., 2009)** or rainbow trout **(Handy et al., 2008). Linhua et al (2013)** reported the highest Zn content was detected in the liver of carp, indicating that ZnONPs would simultaneously reach the liver through multiple pathways via gill circulation and intestinal adsorption. Additionally, another reason of high Zn content in liver was likely due to the formation of many metal sulfur proteins in liver tissue induce by NPs **(Wang et al., 2011). Smith et al. (2007)** reported that the livers o f some fish exposed to nano-TiO2 and single walled carbon nanotubes (SWCNTs) showed condensed nuclear bodies (probably apoptotic bodies) and minor fatty change. In the present study ZONPs leads to ultrastracture changes in the liver tissue as severe vacuolation and lysis of the cytoplasm (rarefaction) with aggregation of ZnONPs, complete disappearance of the mitochondria, Golgi apparatus and glycogen. Narrowing or complete obliteration of the spaces among the hepatocytes were visualized with proliferation of bile canaliculi (fig. 1b). and alteration in hepatocyt nucleus, endoplasmic reticulum as well the hepatocyt membrane (fig 1 c and d) **Linhua et al. (2013)** was found that exposure to ZnONPs caused the cellular pathological changes in liver tissue. However, only according to TEM images, it could not be definitive as to whether the detected particles were o n t he surf ac e o f t he cells or incorporated into them, but their presence and uptake were supported by the results from t he above IC P- OES analysis. Additionally, TEM images demonstrated that ZnONPs caused more severe histopathology than its bulk particles, which was in accordance with the induction of higher levels of intracellular oxidative stress occurred with ZnONPs from the above biomarkers analysis. Vitamin E and C accompanied with improvement of all pathological and ultrastracture lesions (fig. 2a)

It is well known that gill is the important respiratory tissue/ organ participating in many physiological activities such as metabolites excretion, body fluid permeability balance and acidbase regulation balance. And gill is also the first tissue/organ that contacts with ambient water. Uptake of nano-TiO2, nano-Ag and nano-CuO into fish has been demonstrated both by ingestion and across the gills through water **(Zhao et al., 2011). Linhua et al (2013)** reported the exposed gill showed a significant Zn accumulation, which might be a combination of NPs adsorption directly on the gill surface with static negative charges and the subsequent penetration across gill membrane. Therefore, it was concluded that liver and gill might be the target tissues exposed to nano-ZnO.

Our results indicated that, the gills of fish received ZnONPs had a severe vacuolation and necrosis pavement and epithelial cells with dilated mucous cells. These cells were separated from secondary lamella by edema containing fibrin and inflammatory cells(figure 2 c). This results were consistent with the reports from other researchers **(Linhua et al., 2013).** The swollen and disrupted gill cells exposed to nano-ZnO might reduce the contact surface and affect the exchanges of air and ion. And, some black blocks accumulated on the mucus of chloride cell, which was suspected as nano-ZnO aggregates, showing that nano-ZnO might directly enter into the fish body through the injured epithelial cell membrane and induce the undesirable toxic effects **(Linhua et al., 2013).** Gill is the major target organ for chemical pollutants to elicit toxic effects. Previously, some researchers defined two types of gill injuries: the first type of injury results from defense response, including hyperplasia of the gill filaments epithelium, oedema of gill lamellae; the second type is the direct injury, including necrosis and shedding of gill epithelium **(Jinyuan et al., 2011).**In the present study,Vitamin E and C had the ability to reduced the pathological lesions induced by ZnONPs in gills tissues (Figure2 d).

5. Conclusion

ZONPs are toxic in a concentration of 1 and 2 mgL^{-1} for Nil Tilapia (*Oreochromis niloticus*), that manifested by ultra-structure alteration in gills and liver sub cellular organilles as nucleus, golgy apparatus, endoplasmic reteculam and cell membrans. The addition of vitamin E combination with vitamin C in the diet appeared to modulate the ultra-structure alteration induced with ZONPS with the time.

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Conflicts of Interest

The authors declare no conflict of interest.

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