RESEARCH ARTICLE

IMPACT OF DIFFERENT CONCENTRATION OF COPPER NANOPARTICLES AND ORGANOMETALLIC COMPOUNDS ON BIOMARKERS OF TILAPIA FISH.

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Manuscript Info	Abstract
	Copper-oxide and dibutyltin are used as antifouling underwater hall paints. Copper-oxide nanoparticles (CuO-NPs) are serious water pollutants but their impact in fish's performance remains poorly understood. In the present study we have investigated the effects of different concentration of copper oxide nanoparticles (CuO-NPs) and Dibutyltin on Tilapia fish and their bioaccumulation in the gills, liver and brain and also to check minimum lethal dose. We have exposed Mozambique Tilapia (*Tilapia mossambica*), a freshwater edible fish to different doses of copper nanoparticles (15mg/L, 10mg/L, 5mg/L, 2mg/L) and Dibutyltin (0.08mg/L, 0.04mg/L, 0.12mg/L) for 96 hours. The results indicated that the activity of oxidative stress enzymes GSH, AChE and glutathione -S-transferase were significantly decreased. The results showed more serious deleterious impart in the tissues in case of CuO-NPs than dibutyltin which may affect fish growth and development, protein content and causes death. The GST level in liver was maximum affected, when fish treated with CuO-NPs at 10mg/L and dibutyltin at 0.04mg/L. In case of gills GST, CuO-NPs at a concentration of 15mg/L was more deleterious. The level of AChE was more affected in gills and brain when treated with CuO-NPs at 15mg/L. In CuO treated with 10mg/L, the GSH level was maximum affected in liver and gills. In CuO treated with 10mg/L, the protein content was maximum reduced in liver and brain but in gills at 15mg/L. In case of dibutyltin treated with 0.12mg/L, liver, gills, brain most affected. The present study investigated that CuO NPs are more toxic than dibutyltin.

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Introduction:-

Organotin compounds are ubiquitous contaminants in the environment. The high toxicity of organotin derivatives towards aquatic organisms has resulted in deleterious impacts on aquatic ecosystems. Although regulations were effective in some respects, dibutyltin and copper concentrations remain high enough to cause toxicity to aquatic and benthic organisms. In this communication, the ecotoxicology of organotins is critically reviewed with emphasis on fish as key organisms in aquatic ecosystems.

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Ecotoxicology investigates the effects of environmental chemicals (xenobiotic) in ecosystems. Fish are key species in aquatic systems and their protectiomportant for both ecological and economic reasons, as they represent a major protein source for the nutrition of mankind.

There is a growing body of evidence that toxic organotins are making their way into terrestrial and aquatic mammals including humans. In the United States, one possible route of environmental exposure to organotin (specifically dibutyltin and triphenyltin) is via fresh surface waters and fish taken from those waters.

Antifouling paints containing Tributyltin (TBT) began in the late 1980s. Although it still may be available in some parts of the world, antifouling paints containing TBT were ultimately banned in 2008. Copper began to become a concern in California in the 1990s. Copper has been used in antifouling paints for centuries because it is effective, available and relatively inexpensive compared to other biocides. The devastating effects of heavy metals are mainly due to the dispersal performance and bio-magnification of metals into aquatic food chains in addition to their toxicity and accumulative behavior in the biological tissues (Matta et al., 1999; Islam and Tanaka, 2004; Yi et al., 2011).

Oxidative stress is a convenient parameter to measure toxicity and ecotoxicity. Also oxidative stress has been proposed as a common mechanism of cell damage induced by many types of NPs (Stone et al., 2007). Cu-NPs are also used as one of the main constituents of fungicides, algaecide and herbicides however they can cause genotoxicity and oxidative DNA damage at cellular level (Song, Li, Kasai, & Kawai, 2012). Cu-NPs have the ability to cross the plasma membrane, cause oxidative stress via interacting with subcellular organelles (Fahmy & Cormier, 2009; Melegari, Perreault, Costa, Popovic, & Matias, 2013; Wang, Li, Zhao, & Xing, 2011; G_omez, Martínez-A, Gonz_alez, & Rebollo, 1998) and can accumulate in the tissues such as liver and gills of fish (Wang et al., 2013; Griffitt et al., 2007). In liver, Cu-NPs were shown to induce necrosis and to alter sinusoidal spaces in the gills (Al-Bairuty, Shaw, Handy, & Henry, 2013; Griffitt et al., 2007). Cu-NPs disrupt normal bronchial ionoregulatory homeostasis causing efflux of electrolytes from the blood by the gill epithelium and can lead to death due a subsequent cardiovascular collapse (Handy, 2003). In this respect, it is important to study the effect of Cu-NPs using an edible fish as the animal model. Unfortunately, lots of studies have shown that DBTs are able to accumulate in and even contaminate the aquatic environment. In our previous study we investigated the effect of copper-oxide nanoparticles and Dibutyltin of Tilapia fishes. Therefore, to continue our study (Saif Al Ghais, 2019) we purposed to further investigate the effects of different concentration of copper oxide nanoparticles (CuO-NPs) and Dibutyltin on Tilapia fish and their bioaccumulation in the gills, liver and brain and also to check minimum lethal dose.

Methods:-
Experimental fish maintenance and Treatment
Experimental fish in the present study were Tilapia. They were taken from unpolluted private fish farm located in Ras Al Khaimah, UAE. The initial body length and weight of fish were (9-14.5 cm) and (13.2-64 g), respectively. All Tilapia were transported in plastic containers with continuous aeration to the lab. All fishes (4fish/aquarium) were maintained for one week in glass aquaria with 50 L aerated, dechlorinated tap water. Water temperature was maintained at 25 °C, while salinity and pH were 1.12–1.002, and 8.74–7.61, respectively. Photoperiod was 12 h light: 12 h dark. During the acclimatization period, fish were fed once daily with commercial pellet food (20% crude protein, 4% crude fat, 5% crude fiber, 12% crude ash and 10% crude moisture). Dead fish as well as any fish showing any unusual performances were excluded.

Treatment with CuO and Dibutyltin
After acclimatization period (1 week), each four fishes were transferred to small glass aquaria for lethal concentration determination. Concentrations used for CuO were 15mg/L, 10mg/L, 5mg/L, 2mg/L and for dibutyltin were 0.08mg/L, 0.04mg/L, 0.12mg/L. The exposure period was 96 h; with the same temperature, dissolved oxygen and pH as in the acclimatization period. The dead fish was recorded in each concentration. A control was handled identically but without exposure to CuO and dibutyltin. The conditions of the experiments were as those of acclimatization period and water was constantly (every day) checked for pH, temperature, salinity and dissolved oxygen. Fish were fed everyday one time.

Sample collection
At the end of the treatments, the weights of whole fish as well as of the brain, liver and gill were measured. Tissues were dissected out and used for analysis. Samples were obtained from all fishes (control and treated) in replicates

similar procedure was followed. Tissues were used for biochemical and protein analysis. Tissues from fishes were pooled to obtain biological samples and a total sample were used for all experimentations.

Measurement of Biomarkers
For evaluation of oxidative damage, liver, brain and gills were homogenized in cold buffer (pH 7.4) per gram tissue using a homogenizer. Then the homogenates were centrifuged at 4000 rpm for 15 min and the supernatants were stored in refrigerator until used. Oxidative stress was detected in supernatant of the tissue homogenate (GST, GSH, AChE, Protein).

Glutathione-S-transferase (GST) assay
Jakob et al. (1980) protocol was followed for measuring GST activity. GST catalyzes the conjugation of GSH to CDNB through the thiol group of the glutathione and making CDNB-GSH adduct and this CDNB-GSH adduct was used to measure GST activity.

Glutathione reduced (GSH)
According to Saif et al. (2019) this reaction mechanism involves oxidation of GSH (Glutathione, CAS No:1.04090.005, Merck) by 0.01 Mole DTNB (5,5 dithio bis-2-Dinitrobenzoic acid, CAS No:422592J, VWR UK) to form glutathione disulfide and yellow derivative of 5, thio 2-nitrobenzoic acid and its measured at 412 nm by spectrophotometer.

Acetylcholine esterase (AChE)
According to method described in our previous paper (Saif Al Ghais et al ,2019) for estimation of AChE. There is rate of production of thiocholine (Acetocholine iodide, CAS No:1866-15-5, VWR UK) and this is measured by continuous reaction of hydrolysis of thiocholine with DTNB (0.01 M) (5,5 dithio bis-2-Dinitrobenzoic acid, CAS No:422592J, VWR UK) to produce yellow colour compound 5-thio-2-nitro benzene ion. the rate of colour production of the reaction is measured at 412 nm by spectrophotometer.

Protein Estimation
Protein was estimated by the method of Lowry et al., 1951. The liver, brain and gills samples of fish muscle was taken out, washed with ice-cold normal saline, dried and weighed (Bhardwaj et al. 2014: Saif Al Ghais et al 2018).

Statistical analysis
Data are expressed as mean. Pair wise comparisons were performed. Experimental error was determined for triplicate assays and expressed as standard deviation (SD).

Results
### Effect of Cu-NPs and Dibutyltin on fish and tissue weight
At the end of experiment, weight of the whole fish and tissues (liver, gills, brain) were noted for each group and changes in relation to control were found. However, more pronounced effect and a significant increase of tissue (liver, gills, brain) and body weight were observed in treated group as compared to the control.

Effect of concentration of CuO-NPs and Dibutyltin on enzymes (GSH, AChE, GST) and protein
The levels of various enzymes were analyzed in liver, brain and gills of control and treated groups and exposure to CuO –NPs was found to modify the enzyme performance more.

Glutathione reduced (GSH)
For GSH, CuO-NPs effected more gills than in Dibutyltin as compared to control. When treated with CuO at a concentration of 10mg/L then the level of toxicity was more in case of gills and liver as compared to other concentrations (Fig 1). But in case of dibutyltin liver was more affected at concentration of 0.08mg/L and gills were at 0.04mg/L. When compared with control, the level of GSH in treated fishes reduced to more than half the original concentration.
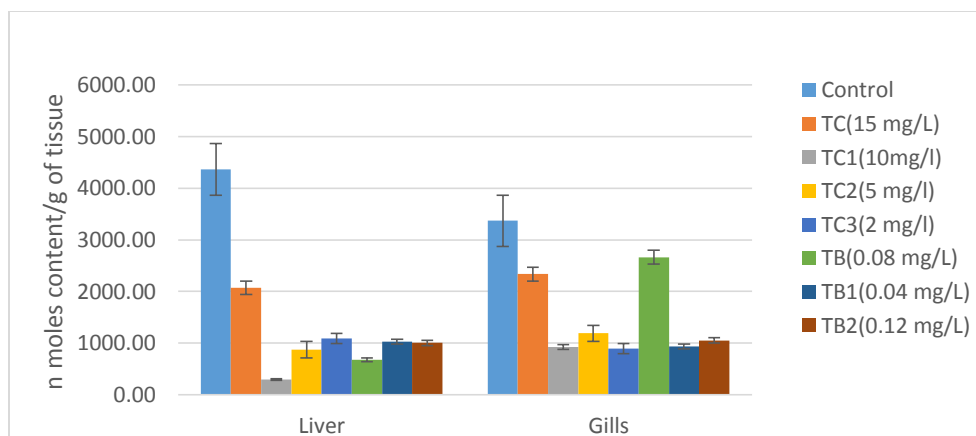
Figure1:-Average GSH content in Tilapia in liver and gills in Control, Cu Treated and dibutyltin treated. Data were expressed as mean and with standard deviation error.

Acetylcholine esterase (AChE)
For AChE, the brain and gills were most effected in case of Cu treated at 15mg/L and Dibutyltin at 0.08mg/L as compared to control. But the liver was most affected when treated with CuO concentration 2mg/L. It was observed that the toxicity was almost 80% in liver, 50% in gills and 90% in brain when treated with CuO as compared with control (Fig 2).
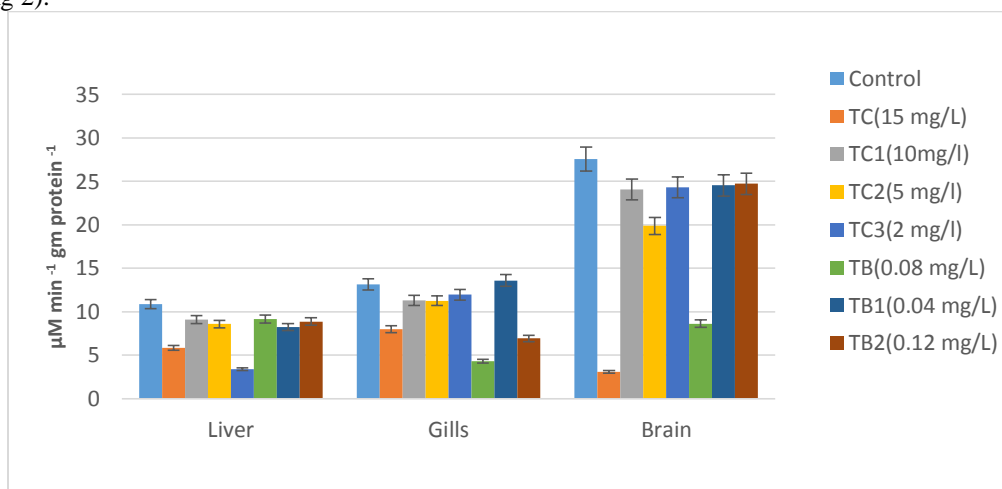
Figure 2:-Average AChE content in Tilapia in liver and gills in Control, Cu Treated and dibutyltin treated. Data were expressed as mean and error bars indicate SD

**Glutathione-S-transferase (GST) assay**
For GST, the toxicity with CuO-NPs was more in case of liver (10mg/L) and gills (15mg/L) than in dibutyltin (Fig 3). There was 80% effect of CuO in liver as compared to control but in case of gills was 50%. In case of dibutyltin the effect was 50% in liver and gills.
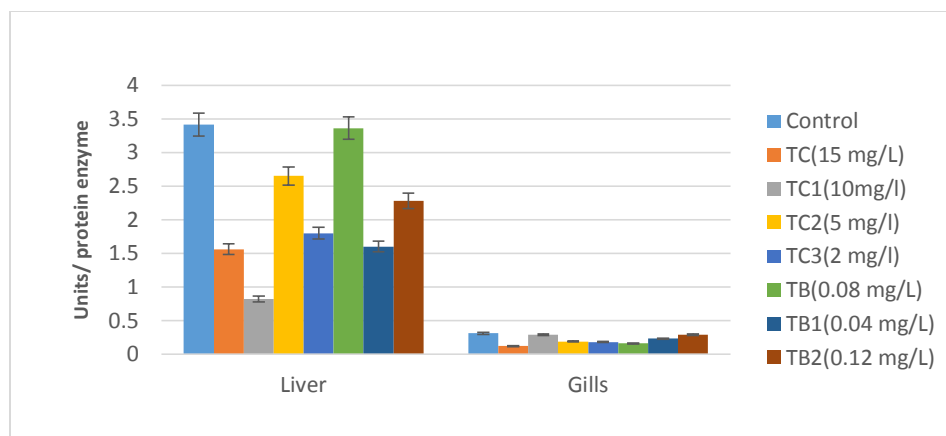
Figure 3:-Average GST content in Tilapia in liver and gills in Control, Cu Treated and dibutyltin treated. Data were expressed as mean and error bars indicate SD

Protein

The amount of protein was reduced to 89% in case of gills treated with CuO-NPs. But in case of brain 70% and liver 75%, fish treated with Cu-NPs and dibutyltin showed 95% effect in gills, 65% in case of brain and 65% in liver as compared to control (Fig 4).
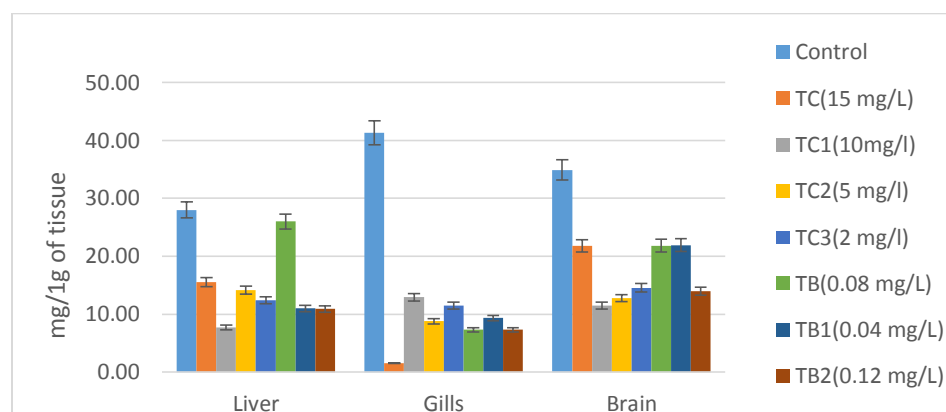
Figure 4:-Average protein content in Tilapia in liver and gills in Control, Cu Treated and dibutyltin treated. Data were expressed as mean and error bars indicate SD

Discussion:-

In this study, the activity of oxidative stress enzyme AChE, GSH, and GST indicate the alteration of normal homeostasis. Cu-NPs are causative molecules for generating oxidative stress and responsible for cell death (Fahmy & Cormier, 2009). Further, Cu has redox property and is involved in several enzymatic reactions such as cytochrome-c oxidase, SOD, quercetin 2, 3-dioxygenase, indole 2, 3-dioxygenase. Cu is a well-known inhibitor of gill respiration and ionoregulation. The present study also analyzed the liver as a central compartment for Cu metabolism (Saif Al Ghais et al 2019). Previous reports documented that fish exposed to Cu-NPs displayed blood accumulation and increase in sinusoid space, which is an indication of liver damage (Arellano, Storch, & Sarasquete, 1999; Shaw & Handy, 2011). In present study, exposure to Cu-NPs even to lower doses showed a pronounced increase in the number of pyknotic nucleus indicating dead nuclei that may progress to tissue necrosis, displayed accumulation of lipid droplet in the hepatocytes or forming vacuole and cellular swelling with a clear cytoplasm due to the presence of small vacuoles, with indistinct shape.

GSH plays an important role in non-enzymatic antioxidant system, since it acts as a reductant in conjugation with xenobiotics (Kanak et al., 2014). GSH depletion could probably be caused also by a significant dissolution of metal oxide NPs that released metal ions in the media (Jozefczak et al., 2012).

Results indicated that, CuO (NPs) have more toxic effect than dibutyltin in liver and gill tissues in most oxidative stress parameters. Therefore, CuO potential toxicity should not be ignored.

Conclusion:-
It has been concluded on basis of our analysis, that short-term exposure of Cu-NPs and dibutyltin even at a low dose can cause oxidative stress and this may lead to growth disarray in the Tilapia and also increased the activity of oxidative stress enzymes that might lead to disturbance of internal homeostasis indicating that this compound has a profound adverse effect on fish health and protein. CuO- NPs could cause more toxic effects than dibutyltin as antifouling agent. In this study we investigated the effects of different concentration of copper oxide nanoparticles (CuO-NPs) and Dibutyltin on Tilapia fish and their bioaccumulation in the gills, liver and brain. Also their toxicity to aquatic organisms but those in laboratory, so further studies are required to assess the current environmental burden of NPs in aquatic ecosystems.

Abbreviations
CuO-NPs: Copper-oxide nanoparticles GSH: reduced glutathione AChE: acetylcholinesterase GST: glutathione -S-transferase NPs: Nanoparticles TBT: Tributyltin Cu: Copper DBT: Dibutyltin CDN: 1-Chloro 2,4 Dinitrobenzene CF1: Control Fish TC1: Treated Copper1 TB1: Treated Dibutyltin1.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
The relevant data and materials are available in the present study.

Competing interests
The authors declare that they have no competing interests. All procedures followed were in accordance with the ethical standards (institutional and national). All institutional and national guidelines for the care and use of laboratory animals were followed.

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Authors' contributions
SAG supervised the entire project. VB and PK performed all the experiments. The supervision of the laboratory work was performed by VB. PK and OAS helped in fish dissection. VB analysed the data and wrote the manuscript.

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