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Sub lethal effect of antifouling paints on marine organism

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Abstract
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. However, excessive concentrations of copper in aquatic ecosystems can exert detrimental effect on marine life such as fish.

In the present study, we have exposed marine fishes, to two different doses (15 mg/L) of Cu-NPs and (2mg/L) dibutyltin for six days. The doses selected were eco-relevant considering the contamination levels of certain water resources. The results indicated that the activity oxidative stress enzymes reduced glutathione (GSH), acetylcholinesterase (AChE) and glutathione -S-transferase (GST) were significantly decreased in the liver, brain and gills of the treated groups when compared to control. Taken together, the results of suggest that short-term exposure of marine fish to Cu-NPs and dibutyltin causes oxidative stress and impart serious deleterious effects in the tissues which may affect fish growth and development and causes death. In the case of gills, liver and brain when exposed to both concentrations of CuO and dibutyltin (NPs), although activity of these enzymes showed an inhibition in the liver when exposed to both concentrations of NPs. The present study investigated that CuO NPs are more toxic than dibutyltin.

It has been suggested that the antifouling agents can cause aquatic pollution and subsequent accumulation in fish constitute a substantial risk to human health and to the environment. Therefore, our study purposed to further investigate the effects of copper-oxide nanoparticles (CuO-NPs) and dibutyltin on marine fish to compare CuO-NPs and dibutyltin bioaccumulation in the gills, liver, and brain. The present study aims to provide a comparative study between CuO and dibutyltin (NPs) at a concentration to declare their deleterious effects on oxidative biomarkers of marine fish and the measurement of oxidative stress.

Keywords: Copper nanoparticles, dibutyltin, reduced glutathione, acetylcholinesterase, glutathione -s-transferase, antifouling

1. Introduction
Marine fouling is an unwanted growth of biological organisms on a surface immersed in water. Deleterious effects of biofouling cause increase in fuel consumption, reduction in vessels speed, increase in greenhouse gases, accelerated corrosion and propagation of invasive species. These biological organisms introduced accidently or intentionally and they spread rampantly due to the lack of natural predators on new locations in which they become leading threats to biodiversity. They impose enormous cost to fisheries, agriculture, forestry and human health. To prevent the attachment of fouling organisms, antifouling paints have been developed and used [1]. They contain chemical compounds (biocides), which are released from the paint matrix, to provide a constant threshold concentration of the biocide in water, therefore inhibiting the development of fouling communities [2, 3]. Unfortunately, Tributyltin (TBT) adversely affect the environment [4]. Due to its high toxicity to molluscs, fish reproduction and fish behavior at very low concentrations [5-10], the use of tributyltin (TBT) has been restricted since the early 1990s and marine paint companies have been developed alternatives to antifouling to substitute TBT [11]. Currently, new alternatives to antifouling paints are based on copper compounds such as cuprous oxide (Cu2O) and copper thiocyanate (CuSCN), with supplementation of booster biocides to control Cu resistant fouling organisms [12,13]

Copper nanoparticles (CuNPs) are among the most used nanomaterials; they are used in textiles, food storage containers, home appliances, paints, and dietary supplements, among

others. Because of their exclusive specifications, such as small size, proportion of the surface to high volume ratio, and more activities, much attention has been paid to them [14]. The production and use of CuNPs make their release into wastewaters and industrial effluents easy; they eventually spread to the aquatic environment, even though they are hazardous to the aquatic organisms [15, 16]. The aquatic organisms receive nanoparticles through the gills, digestive tract, olfactory organ, and the skin [17]. At the cellular level, nanoparticles enter the cell via endocytosis and then induce toxic effects on the living organisms [18]. Furthermore, CuNPs could be accumulated in the organism and transferred to higher trophic levels. Hence, there is a growing concern about the adverse effects of CuNPs on the body system of fish [19].
Therefore, our study purposed to further investigate the effects of copper-oxide nanoparticles (CuO-NPs) and dibutyltin on marine fish to compare CuO-NPs and dibutyltin bioaccumulation in the gills, liver, and brain. In our previous study we investigated the effect and minimal lethal concentration of copper-oxide nanoparticles and Dibutyltin on Tilapia fishes [20]. Therefore, to continue our study we purposed to further investigate the comparative study between CuO and dibutyltin (NPs) at a concentration to declare their deleterious effects on oxidative biomarkers of marine fish and the measurement of oxidative stress.

2. Materials and methods

2.1 Experimental fish maintenance and treatment

Experimental fish in the present study were marine fishes (*Pomacanthus-maculosus)*. They were taken from sea area located in Ras Al Khaimah, UAE. The initial body length and weight of fish were (11-25.2 cm) and (12.3-289 g), respectively. All marine fishes were transported in plastic containers with continuous aeration to the lab. All fishes (4 fish/aquarium) were maintained for one week in glass aquaria with 50 L aerated, sea water. Water temperature was maintained at 25 °C, while salinity and pH were 39.20–39.70, and 8.74–7.98, respectively. Photoperiod was 12 hrs. light: 12 hrs. 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.

2.2 Treatment with CuO and dibutyltin

After acclimatization period (1 week), each four fishes were transferred to small glass aquaria for lethal concentration determination. Nominal concentrations used for CuO was 15mg/L and for dibutyltin was 2 mg/L. The exposure period was 96 hrs.; 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 particles. The conditions of the experiments were as those of acclimatization period. A semi-static exposure regime (20% water change every day with re-dosing after each change) was employed. Fish were fed, 30 min. after water change in order to minimize the risk of the NPs absorbing to food or fecal material, and to help maintaining water quality. NPs were added 30 min. after feed were given to the fish. Water was constantly (every day) checked for pH, temperature, salinity and dissolved oxygen. Fish were fed everyday one time. A control was handled identically but without exposure to CuO and dibutyltin.

2.3 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 total samples were used for all experimentations.

2.4 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).

2.5 Glutathione-S-transferase (GST) assay

[21] 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 [22, 23].

2.6 Glutathione reduced (GSH)

According to [22, 24] 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.

2.7 Acetylcholine esterase (AChE)

According to method described in our previous paper [22] 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.

2.8 Protein estimation

Protein was estimated by the method of Lowry [25]. The liver, brain and gills samples of fish muscle was taken out, washed with ice-cold normal saline, dried and weighed [26, 27]

2.9 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).

3. Results

3.1 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.

3.2 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.

3.3 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 15mg/L then the level of toxicity was more in case of gills as compared liver (Fig. 1). Same results were observed in case of dibutyltin, that both liver and gills were effected but gills effected more than liver. When compared with control, the level of GSH in treated fishes reduced to more than half the original concentration in case of CuO treated gills. But in DBT treated liver the GSH level reduced to 30%. Similar results were observed when fish livers treated with 0.5 mg/L of CuNPs demonstrated the vacuolization of hepatocytes and necrosis. The necrosis is one of the most severe cases of tissue damage and final stage of cells' lives. In other hand, most of the cells die because of damage. As a result, tissues cannot perform their functions. Similar observations were reported by other authors [28, 29].

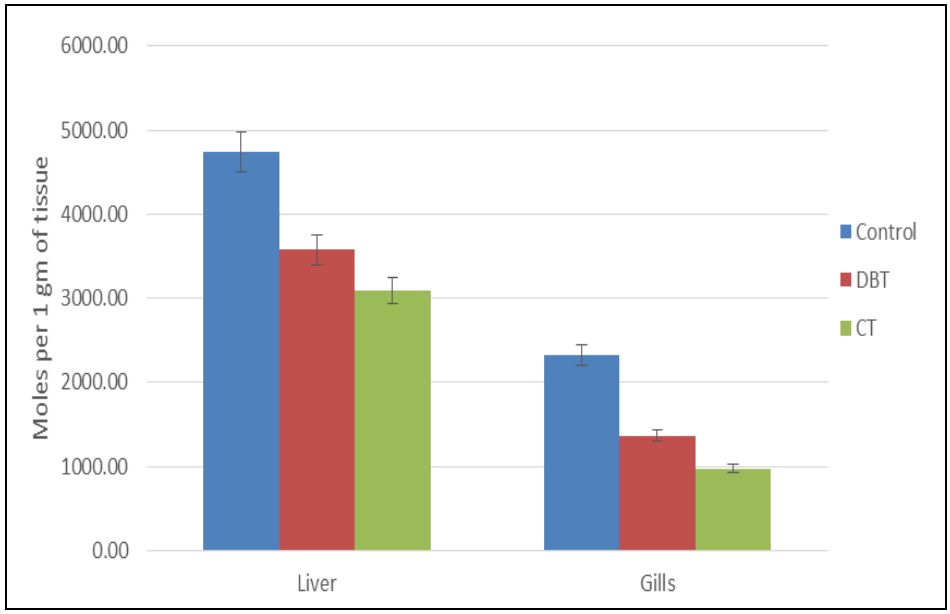

Fig 1: GSH content in Marine fish in liver and gills in Control, Cu Treated and dibutyltin treated. Data were expressed as mean and with standard deviation error.

3.4 Acetylcholine esterase (AChE)

For AChE, the gills were most affected in case of CuO treated at 15mg/L as compared to control. But the liver was 50% affected when treated with CuO. It was observed that the toxicity was almost 50% in liver, 90% in gills and 90% when treated with CuO as compared with control (Fig. 2). In case of DBT treated, liver is most affected as compared to gills and brain.

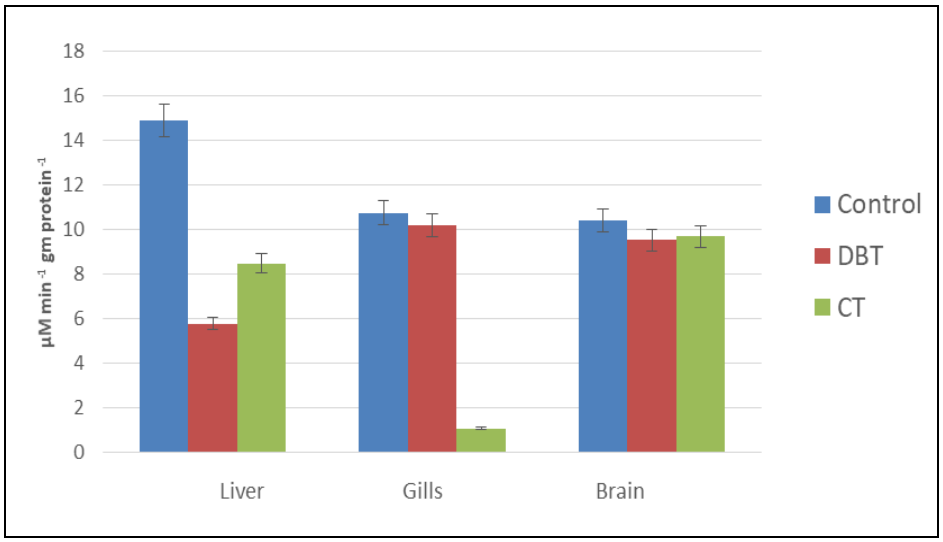

Fig 2: AChE content in Marine fish in liver and gills in Control, Cu Treated and dibutyltin treated. Data were expressed as mean and error bars indicate SD

3.5 Glutathione-S-transferase (GST) assay

For GST, the toxicity with CuO-NPs and DBT was more in case of gills as compared to control (Fig. 3). There was 90% effect of CuO in gills as compared to control in treated fishes but in case of liver was 50%. In case of dibutyltin the effect was 90% in gills.

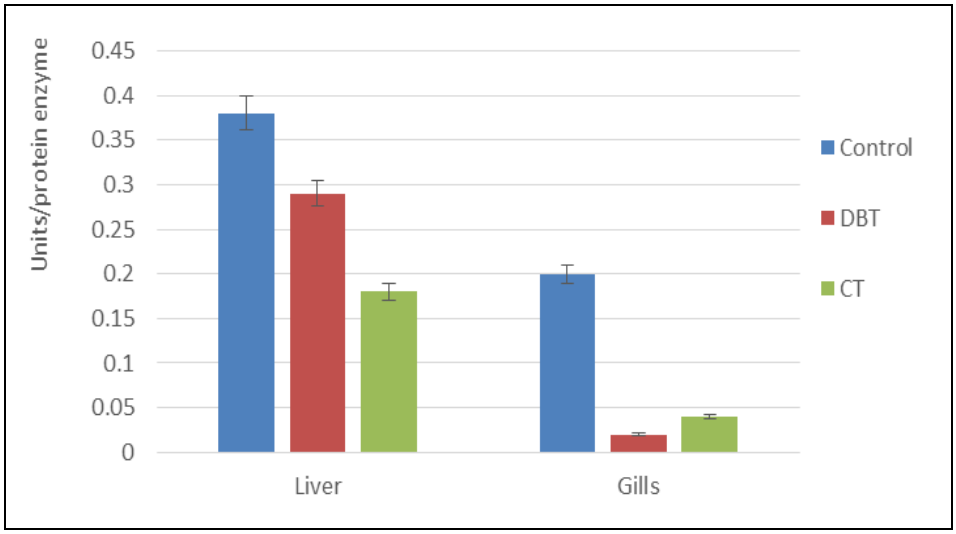

Fig 3: GST content in Marine fish in liver and gills in Control, Cu Treated and dibutyltin treated. Data were expressed as mean and error bars indicate SD

3.6 Protein

The amount of protein was reduced to 50% in case of liver treated with CuO-NPs. But in case of brain 30%, when fish treated with Cu-NPs and dibutyltin showed 30% effect in case of brain as compared to control (Fig. 4).

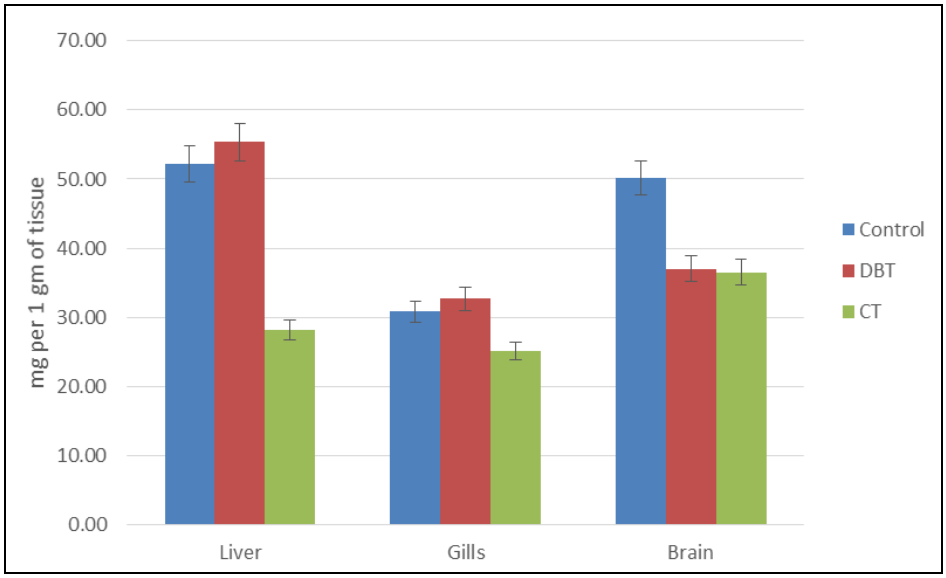

Fig 4: Protein content in Marine fish in liver and gills in Control, Cu Treated and dibutyltin treated. Data were expressed as mean and error bars indicate SD

4. Discussion

In this study, the activity of oxidative stress enzyme AChE, GSH, and GST indicate the alteration of normal homeostasis. The present study also analyzed the liver as a central compartment for Cu metabolism [22]. Previous reports documented that fish exposed to Cu-NPs displayed blood accumulation and increase in sinusoid space, which is an indication of liver damage [30, 31]. 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. GSH depletion could probably be caused also by a significant dissolution of metal oxide NPs that released metal ions in the media [32]. 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.

5. Conclusion

This study concluded that the toxicity evaluation of organs was indispensable for recognition of any damage to tissues by nanoparticles. 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 marine organisms 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. Among the tissues, the gill, liver and brain might be the most sensitive to CuNPs. According to the findings, CuNPs can induce severe necrosis and other tissue injuries to the gill, liver, and brain of the marine fish. The extent of tissue damage depends on the

degree of the CuNPs exposure. This study could help researchers to determine water quality criteria, and it is important to make regulations that forbid companies to spread nanoparticles into aquatic environments and conserve valuable marine environment.

6. 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 **CT:** Copper Treated

7. Declarations

7.1 Ethics approval and consent to participate

Not applicable.

7.2 Consent for publication

Not applicable.

7.3 Availability of data and materials

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

7.4 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.

7.5 Funding

Not applicable.

7.6 Authors' contributions

SAG supervised the entire project. Supervision of the laboratory work was performed by VB. VB analysed the data and wrote the manuscript. PK did experimental work.

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