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Effects of Zerovalent Iron and Iron Oxide Nanoparticles on White Leg Shrimp, *Litopenaeus vannamei* (Boone): A Comparative Study of Toxicity, Bioaccumulation and Oxidative Stress.

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Abstract

The present study was performed to compare the effect of green synthesized zerovalent iron (nZVI) and iron oxide (nIO) nanoparticles on mortality rate, bioaccumulation and oxidative stress biomarkers in *Litopenaeus vannamei*, *in vitro*. The investigation on toxicity assessment revealed that the nZVI cause high mortality even at low concentration compared to nIO. The results on oxidative stress biomarker enzymes and bioaccumulation demonstrated statistically significant variations, between the nanoparticles, with the increase of concentration and exposure time.

Keywords

Zerovalent iron oxide, iron oxide, toxicity, *L. vannamei*.

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INTRODUCTION

Due to some unique features like very small size, high surface area, good mobility, penetration ability and high reactivity [1,2], the metal nanoparticles have of special concern in various industrial applications. The production and utilization of nanomaterials for a wide variety of uses have resulted in their potential environmental impact, hence becoming an important topic of academic and industrial research. The numerous applications of nanomaterials resulted in an increase in their discharge into aquatic environment [3] and may interfere with physiological

processes, posing a potential risk to aquatic organisms. Hence, it is important to regulate the safe limits of nanomaterials for both environmental and occupational safety. Furthermore, the development of sensitive and effective analytical approaches for monitoring of nanomaterials discharged to in the environment becomes necessary [4].

Nanoparticles are used in different fields, such as, tissue engineering, cancer therapy, manipulation of cell and biomolecules, protein detection [5] pigment, dyes, UV protection, heterogeneous catalysis, antimicrobial additives, organic and inorganic sub micrometer UV fibers, organic color filters for LCD technology, inorganic solar cell constituents, membranes and filters [6]. Zero-valent iron nanoparticles (nZVI) are becoming an increasingly popular choice for treatment of toxic wastes and for remediation of contaminated sites [7]. There are report available on the toxicological effect of nZVI on bacteria, virus, fungi, phytoplankton's [8,9,10,11]. However, knowledge on the toxicity of nZVI on higher organisms have been scarcely explored.

The aquaculture industry continues to expand as a crucial segment of the global seafood market. The white leg shrimp, *Litopenaeus vannamei* is one of the most prominent marine species for aqua farming [12,13]. But, the development of the commercial culture of shrimp has generally been accompanied by increasing problems with diseases, which are mostly caused by opportunistic pathogens, such as viruses, bacteria and fungi [14]. Vibriosis, a disease associated to shrimp, caused by 14 strain of bacteria of the genus *Vibrio,* such as *Vibrio harveyi, V. parahaemolyticus, V. anguillarum, V. vulnificus*, *V. fischeri, V. pelagicus, V. logei* etc [15]. They are the natural inhabitants of estuarine and marine environments, well known for causing vibriosis in fish worldwide and the prevention has become a major challenge in aqua farming [16,17].

To study the toxicity of nZVI and nIO, post larval stage 5 (PL_5) of *L. vannamei* was chosen as model organisms, since it is commercially important species. In the present study, efforts were made to compare the toxic effect of biologically synthesized nZVI and iron oxide nanoparticles (nIO), on *L. vannamei* since it is expected that biologically synthesized nanomaterials would exhibit lesser toxicity then biologically synthesized nanoparticles [18,19].

MATERIALS AND METHODS

Experimental design

Green synthesised zerovalent iron nanoparticles (nZVI) [20] (average size 30nm) and Iron oxide nanoparticles (nIO) [21] (average size 48nm) were used for the present study. Suspensions of nZVI and nIO were dissolved in double distilled water and dispersed with water bath sonicator for 20 min (GT Sonic professional Ultrasonic cleaner, model no. GT1738QTB, Frequency 40 KHz). The stock solution was diluted to desired concentrations before treatment. The concentrations used for the study were 1, 10 and 100mg/L and these test concentrations were selected based on LC_{50} value.

Post larval stage 5 (PL_5) of white leg shrimp, *L. vannamei* (about 500 individuals) mean length of 1.5 cm, and mean weight of 6mg, were obtained from Gayathri shrimp hatchery, Bapatla, Andhra Pradesh, India in August, 2018. Shrimps were transported to the laboratory in oxygenated hatchery water. Upon reaching the laboratory, the shrimps were transferred to the rearing tanks (20 liters of water) filled with artificial seawater (3ppt). The average water quality parameters such as temperature, dissolved oxygen and pH were maintained as 26.8 ± 0.28°C, 5.05 ± 0.35mg/L and 7.6 ± 0.19 respectively. Suffocation was avoided by ensuring a water depth of 7–10 cm. Shrimps were allowed to acclimatize to the indoor laboratory condition for 7 days. During acclimatization, the shrimps were fed *ad libitum* with commercial shrimp feed. Artificial seawater was prepared by dissolving 3g of sea salt in 1L of water.

To study the effect of nanoparticles, shrimps were randomly divided into seven groups (G_1, G_2, G_3, G_4, G_5, G_6 & C). Each group contain ten healthy PL_5 shrimps and were exposed to different concentration nanoparticles such as 1, 10 and 100mg/L. Groups G_1, G_2 and G_3 were exposed to 1, 10, & 100mg/L of nZVI and the groups G_4, G_5 and G_6 were exposed to 1, 10, & 100mg/L of nIO respectively. The group without nanoparticles was considered as control (C). Sampling was done once in 24h interval for 5 days.

Acute Toxicity (LC_{50})

In median lethal toxicity study, lethality was the endpoints. Test concentrations of both the nanoparticles (nZVI and nIO) for lethality were 1, 6.25, 12.50, 25, 50 & 100mg/L. Test suspensions were prepared and dispersed using bath sonicator for 20min immediately prior to use without the addition of any stabilizing agents. Ten PL_5 were randomly exposed to each concentration for 5days in 2L container with 1.5L of the test solution. To ensure a constant concentration, all the test solutions were changed every 24 h (semi-static method). The control group was provided with distilled water without any nanoparticles. Each treatment was run in triplicate and placed under the same environmental conditions. In order to maintain water quality,

shrimps were not fed on the day before or during the experimental period to minimize the absorption of the nanoparticles in food and the production of faeces. The 120 hrs LC_{50} were calculated by probit analysis.

Bioaccumulation

Accumulated nZVI and nIO in the shrimp of all studied groups was determined using a flame atomic absorption spectrophotometer (Shimadzu, AA-6880) according to Ravikumar et al.[22]. Shrimps, exposed to nanoparticles, were randomly selected at 24h intervals for 5 days. The collected shrimps were washed with distilled water and digested with 70% HNO_3 at 90^0C and then diluted with deionized water to known volume. In order to correct the background absorption, the procedural blanks were aspirated throughout the measurement process. Samples with known concentration of standard solution were measured during the analysis procedure to check the measurement accuracy. The accumulated nZVI and nIO were expressed as percentage (%) dry weight.

Oxidative stress

At the conclusion of the toxicity tests, superoxide dismutase (SOD) [23] and catalase (CAT) [20] activities of the shrimps were determined to assess oxidative stress and damage as per the standard methods. Shrimp were randomly collected, rinsed with cold saline and homogenized in 1mL of phosphate buffer solution. This was then centrifuged at 4°C at 10,000 ×g for 10 min. Samples of the supernatant were used to determine SOD, CAT.

For SOD, 150 µL of chloroform was added to 100µL of supernatant and mixed well and centrifuged at 1000g for 15 minutes at 4^0C. The supernatant was collected and 2 mL of Tris-EDTA buffer (pH -7.4) and 0.5 mL pyrogallol (2mM) and 1 ml of double distilled water was added. Absorbance was read at 470nm using UV-vis spectrophotometer (Shimadzu, UV-1800). Enzyme activity was represented in terms of percentage (%) increased or decreased with respect to control.

For CAT, 100µL of the above supernatant was added to the reaction mixture containing 1.6mL of phosphate buffer (pH 7.3), 100 µL of EDTA and 200µl of 0.3% H_2O_2. Absorbance was read at 240nm by using UV-Vis spectrophotometer (Shimadzu, UV-1800). The catalase activity of nanoparticles was represented in terms of percentage (%) increased or decreased with respect to control.

Statistical analysis

Statistical analysis was carried out using SPSS software version 21(SPSS, Richmond, VA, USA) as described by Dytham [24]. One-way ANOVA was used to compare the effect of nZVI and nIO concentrations. Two-way ANOVA was used to examine the effect of different concentrations of both the nanoparticles and different exposure duration. Significant differences among treatments at $P < 0.05$ were performed using Duncan test as a post-hoc test.

RESULTS AND DISCUSSION

Due to the rapid discharge of nano-sized materials in to aquatic ecosystem, due to their huge industrial applications, a great deal of attention has recently been directed to their consequent impact to aquatic organisms. Zerovalent Iron and Iron oxide nanoparticles are produced in large quantities and used widely owing to their high stability, photocatalytic properties and anticorrosion attributes. Post larval stage 5 (PL_5) of white leg shrimp, *L. vannamei* was used in the present study as a model organism to determine the possible toxicity of nZVI and nIO in estuarine ecosystems.

Toxicity assessment

Ten post larval stages of shrimps (PL_5) were exposed to different concentrations, such as 1, 6.25, 12.50, 25, 50 & 100mg/L of both the nanoparticles (nZVI and nIO) separately for five days. Once in 24 h intervals, the mortality percentage was calculated and it was found to be increasing with the increasing concentration and duration of both the nanoparticles with respect to control (100% viability) (Fig. 1 & 2). However, 50% of mortality was observed at low concentration of nZVI (47.86ppm) when compared to nIO (63.09ppm). According to Ates et al., [25]. The size of the nanoparticle plays important role in causing more toxicity to the brine shrimp *Artemia salina.* Same result was observed in our results also, the small size nZVI (30nm) is more toxic to the *Litopenaeus vannamei*, compared to nIO.

Bioaccumulation

To quantify the amount of iron (Fe) accumulated in the PL_5 shrimps body were evaluated. The amount of Fe content in the control was considered as 100% and accordingly elevated level of Fe was considered as accumulated in the body. The results showed that the accumulation of Fe was increasing with increasing concentration and increasing duration of exposure and it was comparably similar in both the cases, nZVI and nIO exposed shrimps. Maximum accumulation of Fe (86.46±3.1%) was recorded at 120 h of exposures at the concentration of 100mg/L for nIO exposed shrimps, and it was 72±2.2% for exposed nZVI (Fig. 3 & 4). Same kind of result was obtained with juvenile carp (*Cyprinus carpio*) when they exposed to zinc oxide nanoparticles [26]. Similarly, Mehmet Ates et al. [27] also observed

increased accumulation of titanium oxide nanoparticles in Goldfish (*Carassius auratus*) with the increase in concentration and exposure time. The vital pathway of uptake of nanoparticles to fish body is through gut from diet [28], the vital pathway of uptake is through the gut from diet. Kumar et al. [20] and Kadar et al. [11] compared the impact of chemically and biologically synthesized nZVI on the brine shrimp, *Artemia salina* and *Daphnia magna* found chemically synthesized nZVI has more effect compared to biologically synthesized nZVI, whereas *vice versa* was observed on freshwater phytoplankton *P. subcapitata*. Recent study by Abd El-Atti et al. [29] revealed that the accumulation of Ti nanoparticles in different organs of red swamp crayfish showed a dose-dependent pattern. However, statistical analysis revealed that the difference between the concentration and duration of exposure was significant (P<0.001), whereas insignificant differences was observed between the different kinds of nanoparticles (nZVI or nIO) treated samples compared at a specific concentration and a given time (P>0.001).

Antioxidant enzymes.

Assaying antioxidant enzymes can be used as a potential biomarker for contaminant-mediated oxidative stress [30]. The catalase provides the first line of defense against reactive oxygen species and is used as a biomarker of oxidative stress [31], because it plays a vital role in the protection against oxidative damage by breaking down hydrogen peroxide to oxygen and water [32]. In the present study, catalase activity was found to be increasing with respect to increasing concentration of both nZVI and nIO nanoparticles and increased exposure duration. Highest catalase activity was recorded with higher concentration (100ppm) for all the duration of exposure of both the nanoparticles. Maximum activity of catalase 63.17±1.1% and 91.91±1.1% was recorded at 100ppm of 120hrs of exposure for nZVI and nIO respectively (Fig. 5 & 6). Statistical analysis revealed that the difference between the concentrations and duration of exposure were significant (P<0.001) and also differences between the catalase activity of nZVI and nIO were also significant. Increases in catalase activities may be explained as a response to the increased H_2O_2 levels and superoxide anions [33]. These results correlating the findings of Abd El-Atti et al. [30] with red swamp crayfish, Chiara Gambardella et al., [34] when *A. salina* larvae exposed Fe_3O_4 nanoparticles and Kumar et al. [20] with *Artemia salina* exposed to Chemically and biologically synthesized zerovalent Iron nanoparticles. However, these results differ from Hao et.al., [35] who reported catalase level decreased in the liver of common carp, *C. carpio* after exposure to TiO_2 nanoparticles.

SOD activity

Superoxide dismutase (SOD) activity was found decreasing with increasing concentration and duration of exposure, when compared to control (considered as 100% activity). Maximum SOD activity of 90.04±1.3% was observed in the case of 1ppm at 24hrs and minimum SOD activity of 55.5±1.3% was recorded in 100ppm at 120h of exposure in nZVI interacted samples and it was 93.95±1.7% and 42.35±1.1% respectively for nIO. In case of nIO treated samples, it was seen that the SOD activity was higher as compared with nZVI interacted samples (Fig. 7 & 8). Statistical analysis revealed that the difference between the concentrations and duration of exposure were significant (P<0.001) and also differences between the SOD activity of nZVI and nIO were also significant. Same trend of SOD was reported by Hongchengli et al. [36] with medaka (*Oryziaslatipes)* when exposed to iron nanoparticles toxicity and Sesha Srinivas Vutukuru et.al., [37] when *Esomus danricus*a fresh water teleost exposed to Cu nanoparticles.

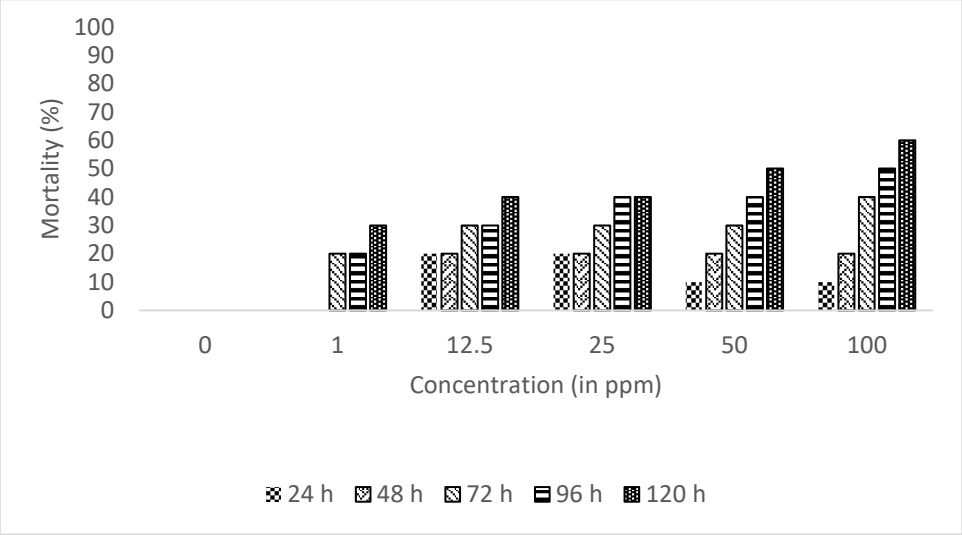

Figure 1: Mortality rate of PL_5 when exposed to nZVI.

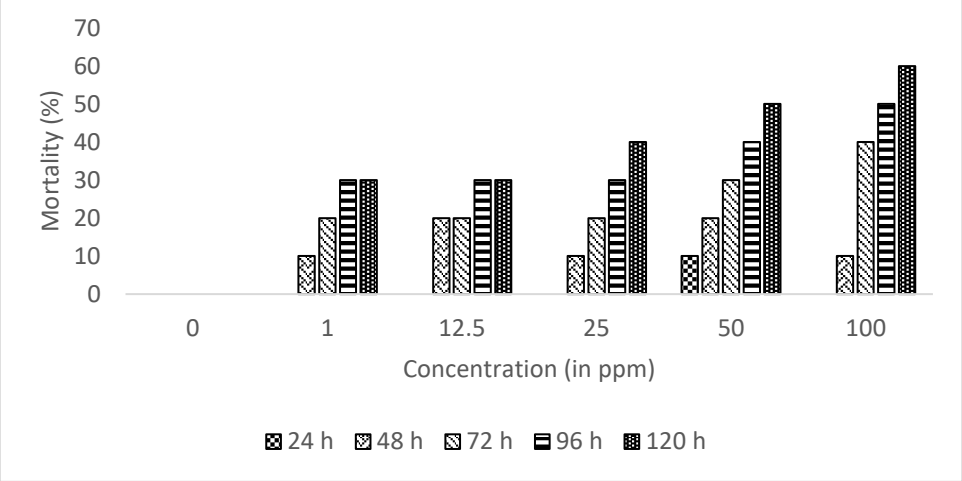

Figure 2: Mortality rate of PL_5 when exposed to nIO.

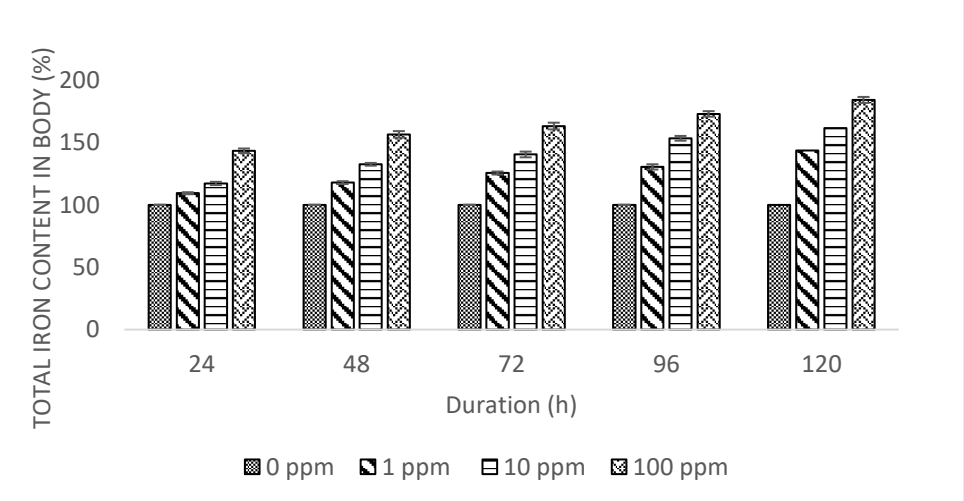

Figure 3: Total Iron content in the body of PL_5 when exposed to nZVI.

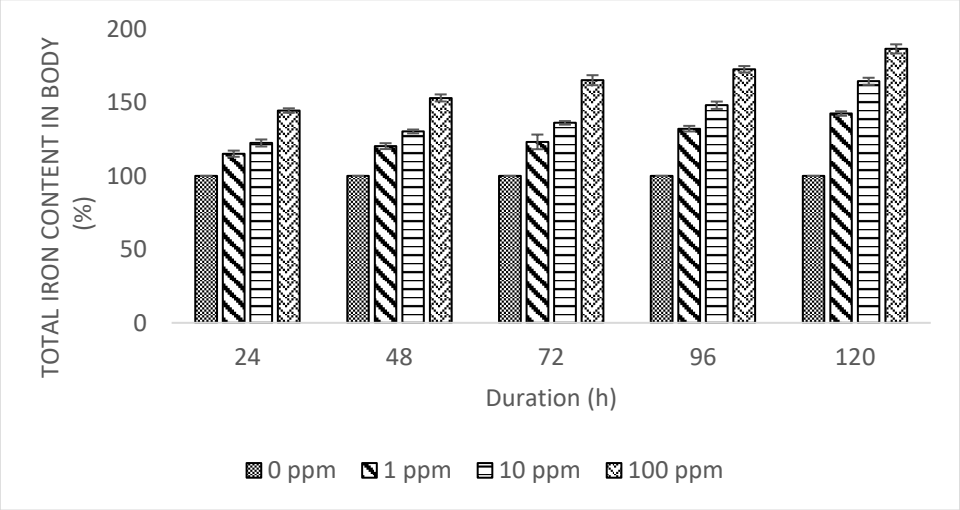

Figure 4: Total Iron content in the body of PL_5 when exposed to nIO.

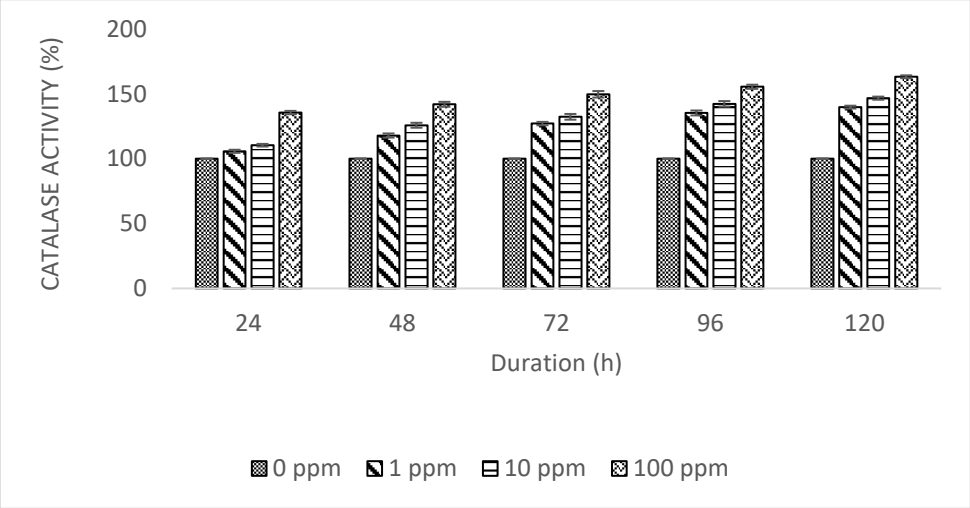

Figure 5: Catalase activity in PL_5 when exposed to nZVI.

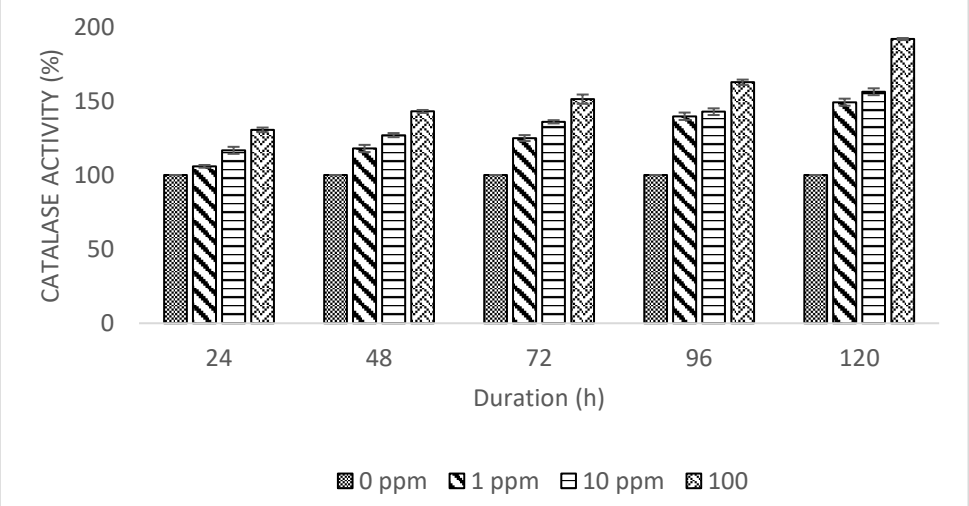

Figure 6: Catalase activity in PL_5 when exposed to nIO

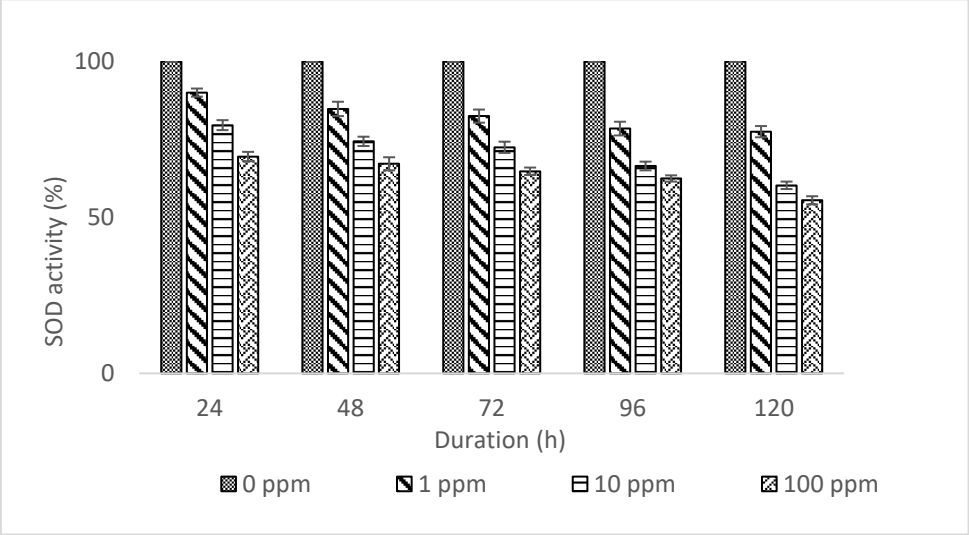

Figure 7: SOD activity in PL_5 when exposed to nZVI.

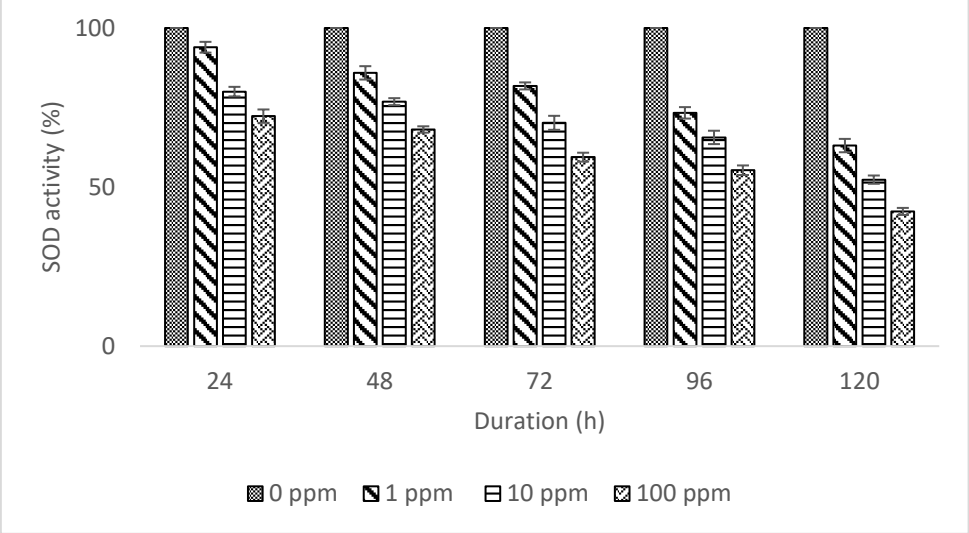

Figure 8: SOD activity in PL_5 when exposed to nIO.

CONCLUSION:

Post larval stage 5 of *Litopenaeus vannamei* was chosen to study the toxic effects of zerovalent iron oxide and iron oxide nanoparticles. Bio-accumulation, activity of antioxidant enzymes like Super-oxide dismutase and catalase were estimated to judge the toxicity levels. Results showed that both the nanoparticles were found to be toxic to selected organism. However, nZVI showed more toxic effect (in low concentration) compared to nIO (required more concentration. This may be due to variations in size of the nanoparticles. Thus, the present study suggests that the nZVI and nIO contaminated water should be treated properly before discharged into the aquatic environment for the safety of aquatic organisms.

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