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Competitive Research Grant

Sub-Project Completion Report

on

Development of Nanomaterial Mediated Feed for Improving Growth and Immunity of Fish

Project Duration

July 2017 to September 2018

Department of Agronomy & Agricultural Extension
Department of fisheries
University of Rajshahi
Rajshahi-6205

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
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Acronyms

NPs: Nanoparticles
Fe-NPs: Iron Nanoparticles
Cu-NPs: Copper Nanoparticles
Zn-NPs: Zinc Nanoparticles
B. gonionotus: Barbodes gonionotus
L. rohita: Labeo rohita
cm: Centimeter
l: litre
^{0}C: Degree Celsius
mg: Milligram
gm: Gram
nm:Nanometer
mM.: Mille Molar
mg/l: Milligram per liter
g/dl: Grams per deciliter
Kg: Kilogram
mg/Kg: Milligram per kilogram
FW: Final Weight
DO: Dissolve oxygen
RBC: Red blood cell
WBC: White blood cell
SGR: Specific growth rate
ADG: Average daily gain
FCR: Feed conversion ratio
FCE: Feed conversion efficiency
PER: Protein efficiency ratio
PPV: Protein productive value
PGR: Protein growth rate
LDL: Low-density lipoprotein
HDL: High-density lipoprotein
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
ALP: Alkaline phosphatase
SEM: Scanning electron microscope

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Executive Summary

Nanotechnology has become the forefront of research and has the tremendous potential to revolutionize the aquaculture sector. A discussion regarding the advantages, approaches and limitations on the use of metallic NPs as feed additives in aquaculture is unrevealed. The understanding of challenges in terms of the potential toxic effects of NPs, the possible mechanisms and cellular consequences as a result of NPs interactions with host cells is necessary to provide better understanding of NPs use. This research work deals with the potential applications of NPs in aquaculture as feed additives. The present study concluded that dietary supplementations of Fe-NPs, Cu-NPs and Zn-NPs at the level 30, 20 and 40 mg/kg in diet improved the growth efficiency (final weight, weight gain, % weight gain, average daily gain and specific growth rate), feed utilization (FCR, FCE, PER, PPV and PGR), muscle composition (protein, lipid, carbohydrate, ash and moisture), hematological parameters (RBCs, WBCs, hemoglobin, total protein, albumin and globulin) and blood lipid profile (total cholesterol, HDL, LDL and triglyceride) of *B. gonionotus* in experiment-1. However, comparison of the aforementioned three NPs showed comparatively better performance by Zn-NPs followed Fe-NPs and Cu-NPs. In experiment-2, *L. rohita* fed diets enriched with Fe-NPs, Cu-NPs and Zn-NPs also showed better growth and physiological performance at the doses of 30, 20 and 40 mg/kg feed of NPs, respectively. Here also NPs based comparison showed better performance by Zn-NPs followed by Fe-NPs and Cu-NPs. In experiment-3, prepared alloy with the combination of Zn-NPs and Fe-NPs depicted a dose dependent effect on the growth and physiology of experimental fishes. Alloy enriched diets at a dose of 30 mg/kg feed showed better growth and physiological performance for both *B. gonionotus* and *L. rohita*. However, comparing the three experiment, alloy used in the third experiment at the dose of 30 mg/kg feed showed better growth and physiological performance followed by Zn-NPs, Fe-NPs and Cu-NPs. Species wise comparison also showed better growth and physiology by *B. gonionotus* compared to *L. rohita* feed NPs supplemented diets. The use of serum enzymatic (AST, ALT, amylase, lipase, protease and ALP) responses in the present study signifies the physiological responses of *B. gonionotus* and *L. rohita*, which favours dietary supplementations of Fe-NPs, Cu-NPs, Zn-NPs and alloy at the level 30, 20, 40 and 30 mg/kg of feed. However, the increased levels of AST, ALT and ALP and decrease level of amylase, lipase and protease in serum at the highest dose may be a sign of toxic effect that ultimately reduced the growth efficiency and feed utilization of experimental fishes. The field experiment was conducted in 2 earthen ponds with 1 replication each (17 bigha) for a period of 180 days (March'2018 - Auguest'2018) in selected farmer's ponds in Mohonpur, Rajshahi district. Pond 1 (Nano feed) and Pond 2 (commercial Pellet feed). The Growth and production performance of fishes of field experiment, There was no significant difference ($P<0.05$) in the FCR between the two experimental ponds. However, comparatively low FCR was observed at Pond-1 (2.12±1.51) than Pond-2 (2.80±1.80). Significantly higher ($P<0.05$) total production was recorded at Pond-1 (3521.97±392.76 kg/ha/180 days) than Pond-2 (2843.96±208.66 kg/ha/180 days) during the study period. Moreover, results of present study indicate the scope of nanotechnology for the enhancement of fish production. This result will enable the scientific community to formulate a suitable diet for fish that will be helpful to reduce feed cost and enhance aquaculture production.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. Title of the CRG sub-project: Development of Nanomaterial Mediated Feed for Improving Growth and Immunity of Fish
2. Implementing organization: Department of Agronomy & Agricultural Extension & Dept. of Fisheries, Rajshahi University, Rajshahi- 6205.
3. Name and full address with phone, cell and E-mail of PI (s): Dr. Md. Jahangir Alam, Professor, Department of Agronomy & Agricultural Extension, Rajshahi University, Rajshahi-6205, Cell no - 01716587448.
   Name and full address with phone, cell and E-mail of Co-PI: Dr. Md. Abu Sayed Jewel, Professor, Department of Fisheries, Rajshahi University, Cell no. 01727144520.
4. Sub-project budget (Tk):
   a. Total: 46,42,267.00/=
   b. Revised (if any):
5. Duration of the sub-project:
   5.1 Start date (based on LoA signed) : July 2017
   5.2 End date : 30 September 2018
6. Justification of undertaking the sub-project:

The aquaculture industries can be revolutionized by using nanotechnology with new tools to enhance the ability of cultivable organisms to uptake drugs like hormones, vaccines and nutrients (Rather et al. 2011). The metal nanoparticles (NPs) such as Se, Al, Fe, FeO, and ZnO play a crucial role in aquaculture operations (Zhou et al. 2009). Nanotechnology holds promise for both medication and nutrition, because materials at the nanometer dimension exhibit novel properties different from those of isolated atom and bulk material (Albrecht et al. 2006; Wang et al. 2007). Moreover, food additives in the nano forms are being increasingly used including aquaculture, iron-fortified cereals and drinks for human consumption (Hilty et al. 2010).

Recently, nanotechnology has emerged as an excellent field of technology that shows its application in various sectors including agro-food system, aquaculture (Defra, 2009) and aqua-feed (Handy, 2012). Nanotechnology involves the synthesis of nanoscale particles that exhibit unique physiochemical properties like higher intestinal absorption, bioavailability and enhanced bactericidal and catalytic activities (Dube et al. 2010).

Over the years, technological applications in aquaculture have been associated with intensification of the applied systems for increased production with economic profitability. Besides high density culture systems, efforts are also being made to achieve high-growth performances and early weaning by shortening productive cycles. Nanoparticles received considerable attention in the recent years because of their ability to deliver a wide range of molecules to the body and for a sustained period of time.

Recently quality fish feed is a major challenge for aquaculture industry of Bangladesh. Fish farmers are frequently reported that the growth responses of culture fishes are not satisfactory by feeding commercial fish feeds. Commercial fish feed cost is increasing day by day. So, there is an urgent need to develop a quality fish feed (nanomaterial mediated feed) for better growth responses of culture fishes that will help to feed industry for improving their feed quality. Finally fish farmers will be economically benefited from better growth performance of healthy fish.

In Bangladesh, population density is increasing day by day and demand of animal protein is also increasing. To meet this growing demand, need to proper utilization our limited resources. The introduction of nanoparticles as feed additives will enhance feed quality and that will be ensured better fish growth, production and health condition. If applying this nanotechnology in fish feed industry then the fish feed industry will be enriched and fish farmer will be economically benefited through higher fish production. It is the first attempt to incorporate nanoparticles as feed additives serve as micronutrient to observe growth performance and physiological status of culturable fishes in Bangladesh.

After completion of this project, it can be developed nanoparticle enriched quality fish feed that will ensured nutritional quality of fish and produce healthy fish for human consumption. The commercial feed industries will be benefited by adopting this project finding technology and can improve their fish feed quality.

7. Sub-project goal: Improving nutritional quality of fish feed by applying nanoparticles that would ensure better growth and health of fish for human consumption.
8. Sub-project objective (s):
    a) To prepare shape and size controlled nanoparticles of different metals under oilbath heating.
    b) To synthesize nanomaterials (micronutrients) mediated feed for disease free fish growth.
    c) To observe growth performance, meat quality (proximate compositions), haematological parameters and immune responses of fish by adding different doses of nanoparticles in experimental diet.
9. Implementing location (s): Dept. of Agronomy & Agricultural Extension, and Dept. of Fisheries, Rajshahi University, Rajshahi-6205.
10. Methodology in brief: Nanoparticles are fundamental to modern science and technology. Nanoparticulate material delivery to fish technology also holds the promise of controlled release smart fish feed formulation and site targeted delivery of various macromolecules needed for improved fish disease resistance, efficient nutrient utilization and enhanced fish growth.

10.1. Formulation of diet

10.1.1 Raw materials: (a) Metallic salts (Fe, Cu and Zn) (b) water, (c) Polyvinylpyrrolidone (PVP), (d) Basal diets prepared with locally available feed ingredients (Balance diet).

10.1.2 Synthesis of Nanoparticles

The aquatic method is a typical technique to prepare metallic nanoparticles in water by reducing their ionic salts. In general, a mixture of reagent and Polymer surfactant in water was heated in an oil-bath heater for several minutes, as a result of heating nanoparticles were prepared. Iron, Copper and Zinc nanoparticles were prepared under oilbath heating. An aqueous solution of 1.11 gm PVP as a polymer surfactant mixed with precursor salt of NPS {$FeCl_3$. H_2O for Fe, $(CH_3\text{-}COO)_2$ Cu. H_2O for Cu and $(CH_3\text{-}COO)_2$ Zn. $2H_2O$ for Zn NPS)} separately in a three neck round bottom flask. The mixture of aqueous solution was heated for 60 min under oilbath heater with reflask for the preparation of each nanoparticle. The final concentrations of Fe, Cu and Zn nanoparticles were 80 mM. The overall technique is depicted in Figure 1.

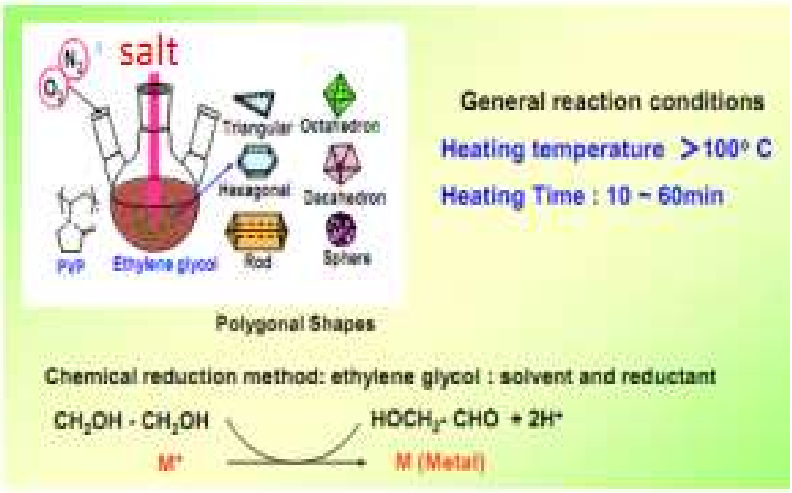


Figure 1. Synthesis of NPs under oil bath heating

***10.1.3 Characterization of Nanoparticles*:**

Morphologies of the Fe, Cu and Zn nanoparticles were characterized using a Scanning electron microscope (SEM; EVO 18, Courl Zesis, Germany at 200 kV). Product solutions were centrifuged at 12000 rpm three times for 30 min to ensure complete collection of the products each time. The precipitates were collected then re-dispersed in distilled water. Samples for SEM measurements were prepared by dropping a droplet of the colloidal solutions on the slide class. Ultraviolet–visible (UV–vis) extinction spectra were obtained (UV-1280; Shimadzu Corp.) using a quartz cell. The sample solution was diluted with water.

10.1.4 Preparation of diet:

Ingredients and proximate composition of prepared control diet were shown in Table 1. All feed ingredients were purchased from local market and they were grinded in the laboratory to acquire fine powder. The powdered and sieved feed ingredients were weighed out and mixed thoroughly in 6 different ratios for preparing six different diets, one control and five different diets containing Fe-NPs, Cu-NPs, Zn-NPs and alloy (Fe-NPs and Zn-NPs) at various doses such as 0 (control-free from NPs), 10, 20, 30, 40 and 50 mg/kg dry feed weight. Then distilled water was added and blended well (5 min) until the mixture achieves a dough consistency. The dough was pelletized in a manual pelletizes fixed with 3 mm diameter and the pellets were collected in aluminum trays. A thermostatic hot air oven (Microsil INDIA, Universal Lab Product Co., Chennai, India) was used to dry the diets until the moisture content was reduced below 10%. After drying diets were kept at 20 ^{o}C until used. Ingredients and proximate composition of control diet were same due to prepare a balance fish diet, only differ in dose of NPs for evaluating the effective performance of all types of fishes.

Table 1. Ingredients and proximate composition of control diet mixed with Fe-NPs (0-50 mg).

Ingredients	gm/kg	Proximate composition	(%)†
Fish meal[a]	275	Protein	33.11±0.14
Mustard oil cake[a]	200	Lipid	9.38±0.03
Soybean meal[a]	125	Carbohydrate	36.45±0.44
Maize bran[a]	125	Moisture	7.07±0.01
Wheat bran[a]	90	Ash	11.23±0.60
Rice bran[a]	90		
Soybean oil[a]	60		
Choline chlorid[a]	2.5		
Fe-free premix[*b]	32.5		

†Values are presented as mean ± SD, n= 3

*Fe-free premix (mg/kg of premix): vitamin A-156000 IU, vitamin D_3-31200 IU, vitamin E-299, vitamin K_3-26, vitamin B_1-32.5, vitamin B_2-65, vitamin B_6-520, vitamin B_{12}-0.16, Nicotinic Acid-520, Folic Acid-10.4, Copper-130, Iodine-5.2, Manganese-780, Zinc-650 and Selenium-1.95.

[a] Ingredients were collected from chemical store of Rajshahi city, Bangladesh.

[b] Supplied by Reneta Animal Health Pharma Co. Ltd. Bangladesh.

Table 2. Ingredients and proximate composition of control diet mixed with Cu-NPs (0-50 mg).

Ingredients	g/kg	Proximate composition	(%)†
Fish meal[a]	275	Protein	33.11±0.14
Mustard oil cake[a]	200	Lipid	9.38±0.03
Soybean meal[a]	125	Carbohydrate	36.45±0.44
Maize bran[a]	125	Moisture	7.07±0.01
Wheat bran[a]	90	Ash	11.23±0.60
Rice bran[a]	90		
Soybean oil[a]	60		
Choline chlorid[a]	2.5		
Cu-free premix[*b]	32.5		

†Values are presented as mean ± SD, n= 3

*Cu-free premix (mg/kg of premix): vitamin A-156000 IU, vitamin D_3-31200 IU, vitamin E-299, vitamin K_3-26, vitamin B_1-32.5, vitamin B_2-65, vitamin B_6-520, vitamin B_{12}-0.16, Nicotinic Acid-520, Folic Acid-10.4, Iodine-5.2, Manganese-780, Zinc-650 and Selenium-1.95.

[a] Ingredients were collected from chemical store of Rajshahi city, Bangladesh.

[b] Supplied by Reneta Animal Health Pharma Co. Ltd. Bangladesh.

Table 3. Ingredients and proximate composition of control diet mixed with Zn-NPs (0-50 mg)

Ingredients	g/kg	Proximate composition	(%)†
Fish meal[a]	275	Protein	33.11±0.14
Mustard oil cake[a]	200	Lipid	9.38±0.03
Soybean meal[a]	125	Carbohydrate	36.45±0.44
Maize bran[a]	125	Moisture	7.07±0.01
Wheat bran[a]	90	Ash	11.23±0.60
Rice bran[a]	90		
Soybean oil[a]	60		
Choline chlorid[a]	2.5		
Zn-free premix[*b]	32.5		

†Values are presented as mean ± SD, n= 3

*Zn-free premix (mg/kg of premix): vitamin A-156000 IU, vitamin D_3-31200 IU, vitamin E-299, vitamin K_3-26, vitamin B_1-32.5, vitamin B_2-65, vitamin B_6-520, vitamin B_{12}-0.16, Nicotinic Acid-520, Folic Acid-10.4, Copper-130, Iodine-5.2, Manganese-780, and Selenium-1.95.

[a] Ingredients were collected from chemical store of Rajshahi city, Bangladesh.

[b] Supplied by Reneta Animal Health Pharma Co. Ltd. Bangladesh.

Table 4. Ingredients and proximate composition of control diet mixed with alloy (combination of Fe-NPs and Zn-NPs, 0-50 mg)

Ingredients	g/kg	Proximate composition	(%)†
Fish meal[a]	275	Protein	33.11±0.14
Mustard oil cake[a]	200	Lipid	9.38±0.03
Soybean meal[a]	125	Carbohydrate	36.45±0.44
Maize bran[a]	125	Moisture	7.07±0.01
Wheat bran[a]	90	Ash	11.23±0.60
Rice bran[a]	90		
Soybean oil[a]	60		
Choline chlorid[a]	2.5		
alloy-free premix[*b]	32.5		

†Values are presented as mean ± SD, n= 3

*Fe, Zn-free premix (mg/kg of premix): vitamin A-156000 IU, vitamin D_3-31200 IU, vitamin E-299, vitamin K_3-26, vitamin B_1-32.5, vitamin B_2-65, vitamin B_6-520, vitamin B_{12}-0.16, Nicotinic Acid-520, Folic Acid-10.4, Copper-130, Iodine-5.2, Manganese-780 and Selenium-1.95.

[a] Ingredients were collected from chemical store of Rajshahi city, Bangladesh.

[b] Supplied by Reneta Animal Health Pharma Co. Ltd. Bangladesh.

10.2 Collection and maintenance of experimental fishes

Juveniles of *B. gonionotus* and *L. rohita* having an average weight of 33.45±0.23 and 33.53±0.20 gm, respectively were purchased from Fish Seed Hatchery, Rajshahi and transported live in aerated plastic bags to the laboratory of Department of Fisheries, University of Rajshahi. Fishes were kept in a circular cemented tank having flow through system and were acclimatized for a period of two weeks. During the acclimatization period water temperature was maintained at optimum range as 27-30 ^{o}C, with a photoperiod of 12 hrs light and 12 hrs darkness.

10.3 Experimental design

Three experiments were conducted during the study period. In the first experiment Effect of different nanoparticle on growth and physiology of *B. gonionotus* In experiment-2 Effect of different NPS on growth and physiology of L*. rohita*. In the third experiment, Effect of alloy on growth and physiology of B. *gonionotus* and L*. rohita*. Two best NPs among the three were selected, based on their growth and physiological performance on experimental fishes and mixed together to form alloy. The alloy was then used to evaluate the growth and physiological performance of experimental fishes in a dose dependent manner. Finally, statistical analysis was done to select best NPs and their corresponding fish species based on growth and physiological parameters.

Experiment 1: Effect of different NPS on growth and physiology of B. *gonionotus*.

Nanoparticles (NPS)	Dose of NPS (mg/kg feed)						Feeding rate	No of fish/Aquarium	Nos/Aqu-arium	Days of culture	Replication
Fe-NPS	0	10	20	30	40	50	3% body weight	10	18	60	3
Cu-NPS	0	10	20	30	40	50					
Zn-NPS	0	10	20	30	40	50					

Experiment 2: Effect of different NPS on growth and physiology of L*. rohita*.

Nanoparticles (NPS)	Dose of NPS (mg/kg feed)						Feeding rate	No of fish/Aquarium	Nos/Aqu-arium	Days of culture	Replication
Fe-NPS	0	10	20	30	40	50	3% body weight	10	18	60	3
Cu-NPS	0	10	20	30	40	50					
Zn-NPS	0	10	20	30	40	50					

Experiment 3: Effect of alloy (Fe NPS and Zn NPS) on growth and physiology of B. *gonionotus* and L*. rohita*.

Nanoparticles (NPS)	Dose of NPS (mg/kg feed)						Feeding rate	No of fish/Aquarium	Nos/Aqu-arium	Days of culture	Replication
Alloy (Fe-Zn) NPS	0	10	20	30	40	50	3% body weight	10	18	60	3

Figure 2. (A) nanoparticle (B) supplemented diets (C) *B. gonionotus* and *L. rohita* in aqurium (D and E) measurment (F) biochemical analysis.

For each experiment, after an acclimatization period, healthy and uniform sized fishes were selected, individually weighed by using electronic top-loading balance and evenly distributed in eighteen fiber glass aquaria at 10 fish per aquarium with similar initial weight. The experiment was conducted as a Completely Randomized Design (CRD) with six treatments as control, 10, 20, 30, 40 and 50 mg/kg NPs or alloys each with three replications. Fishes were fed daily (twice in a day) with a feeding rate of 3% body weight. After feeding period, the diet remaining in each tank was collected by siphoning before the second day's feeding. A routine work of exchanging 50% water from each aquarium was done daily. Figure 2 showing experimental setup and in each experiment fishes were fed for a period of 60 days and after that period growth performance, feed utilization and physiological parameters of experimental fishes were measured.

10.4 Water quality analysis

10.4.1 Water temperature

Water temperature was recorded with the help of a Celsius thermometer. The temperature was expressed as °C.

10.4.2 Dissolved Oxygen (DO)

The dissolved oxygen concentration of water was determined by the aid of a water quality test kit (HACH kit FF-2, USA). Alkaline Iodide-Azide powder pillows, Manganous sulfate powder pillows, Sodium thiosulfate titration cartridge (0.2000 N), Starch indicator solution and Sulfuric acid powder

pillows were used for determination of dissolved oxygen. The concentration of dissolved oxygen thus estimated was expressed in milligram per litter (mg/l) of water.

10.4.3 Hydrogen Ion concentration (pH)

Water pH of cage water was measured by using a pH meter (Jenwary 3020).

10.4.4 Ammonia (NH_3)

Ammonia-nitrogen was measured by using a HACH Kit (FF-2, USA). Rochelle salt solution and Nessler reagent were used to measure the NH_3. A color comparator (value ranging from 0 to 3.0 mg/l) was also used for the same. The concentration of ammonia-nitrogen thus estimated was expressed in milligram per litter (mg/l) of water.

10.5 Growth and feed utilization parameters

All fish in different experimental groups were weighed at the end of 60 days feeding trial for the estimation of growth parameters. Growth parameters were calculated according to the following formulae:

Weight gain (gm) = Final weight (gm) – Initial weight (gm)

Percent weight gain (%) = $\frac{\text{Final weight (gm)} - \text{Initial weight (gm)}}{\text{Initial weight (gm)}} \times 100$

Specific growth rate (% bwd^{-1}) = $\frac{ln\ \text{Final weight (gm)} - ln\ \text{Initial weight (gm)}}{\text{Study period}} \times 100$

Survival rate (%) = $\frac{\text{Final fish number}}{\text{Initial fish number}} \times 100$

Food conversion ratio (FCR) = $\frac{\text{Feed given (dry weight)}}{\text{Total wet weight gain (g)}}$

Food conversion efficiency (FCE) = $\frac{\text{Total wet weight gain (gm)}}{\text{Feed given (dry weight) (gm)}}$

Protein efficiency ratio (PER) = $\frac{\text{Total weight gain (gm)}}{\text{Protein intake (gm)}}$

Protein productive value (PPV %) = $\frac{\text{PT} - \text{P}}{\text{Protein intake (gm)}} \times 100$

Where, PT = Protein content in fish carcass at the end, PI = Protein content in fish carcass at the start.

10.6 Proximate composition of diets and fish carcass

Different chemical compositions of feeds and fish carcass such as moisture, lipid, ash, crude protein and carbohydrate were measured according to Association of Official Analytical Chemists (AOAC, 2000).

10.7 Determination of crude protein content

About 2 gm of sample was taken to the digestion tube. For each sample two digestion tubes was taken and one digestion tube was used as blank. Then 1.1 gm digestion mixture was taken to each tube by weighting in electric balance and 10 ml conc. H_2SO_4 was added to each sample. All the digestion tubes were transferred to the digestion unit and the exhaust system was placed on the top of the tube with fume extraction system turned on. The sample was digested for 45 minutes at 420 °C and the colour become light green after that the tube was removed from digestion unit. After cooling for sometimes 5 ml $Na_2S_2O_3$ (33%) were added to each tube and mixed with the vortex mixture. Other side, 25 ml 4% Boric acid was added to a conical flask with distilled water and transferred to the distillation unit. Before transferring to distillation unit two drops mixed indicator

was added to each of the flask which appears violet colour. The extraction found in conical flask during distillation was titrated with 0.2 N HCl by using magnetic stirrer for well mixing. When the pink colour of the solution was found then the titration was completed. For each titration the necessary data was recorded. Then protein content was calculated by the following formula.

$$\% \text{ Nitrogen } (N_2) = \frac{\text{ml of titration} \times \text{strength of HCl (0.2N)} \times \text{mili equivalent of N}}{\text{Weight of the sample}} \times 100$$

Here, mili equivalent of Nitrogen (N_2) = 0.014

% Crude protein = % N_2 × 6.25 (animal source)

= % N_2 × 5.85 (plant source)

10.8 Determination of lipid content

At first, a small amount of sample (about 2-3 kg) was taken in a previously marked thimble paper with the help of spatula. Then the thimble paper was placed in a Soxhlet apparatus with the help of tong (specialized forcep). Two-third of the round bottom ground joint flask was filled with acetone (180 ml) and attached to the Soxhlet apparatus. The Soxhlet apparatus was left on an electric heater for being heated at 70°C for 3 hrs. Thus acetone was evaporated. The evaporated acetone was condensed in the condenser and dropped slowly on the sample inside the paper thimble. The acetone was gradually accumulated in the hollow space of the main body and drained out to the round bottom flask with lipid through siphoning process. The acetone containing lipid was allowed to be evaporated by keeping the beaker in a hot air oven at 105 °C for 30 minutes. After this the beaker was transferred to the desiccators for few minutes to be cooled. Then the beaker containing lipid was weighted by electric balance. Then lipid content was calculated by the following formula.

$$\% \text{ Lipid content} = \frac{\text{Weight of the lipid}}{\text{Weight of the sample}} \times 100$$

10.9 Determination of carbohydrate content

Extraction of sugar: 4-6 gm of sample were plunged into boiling ethyl alcohol and allowed to boil for 5-10 minutes (5 to 10 ml alcohol was used for each g of sample). Then the extract was filtered through two layers of muslin cloth and re-extracted the ground fish for three minutes in hot 80% alcohol, using 2 to 3 ml of alcohol for each gm of fish sample. The second extract ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whitman no-41 filter paper. The volume of the extract was evaporated to about ¼ of the volume over a steam bath and cooled. This reduced volume of the extract was then transferred to a 100 ml volumetric flask and made up to the mark with distilled water. Then 1 ml of diluted solution was taken into another 100 ml volumetric flask and made up to the mark with distilled water (Working standard).

Procedure: A liquor of 1 ml of the fish extract from each part was pipette into different test tubes and 4 ml of the anthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on the top of each to prevent loss of water bath then cooled. A reagent blank was prepared by taking 1ml of water and 4ml of anthrone reagent in a tube and treated similarly. The absorbance of the blue-green solution was measured at 680 nm in a colorimeter. A standard curve of glucose was prepared by taking 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1ml of standard glucose solution in different test tubes containing 0.0, 0.01mg, 0.02 mg, 0.03 mg, 0.04 mg, 0.05, 0.06, 0.08 and 0.1 mg of glucose respectively and made the volume up to 1 ml with distilled water. Then 4 ml of anthrone reagent was added to each test tube and mixed well. All these solutions were treated similarly as described above. The absorbance was measured at 680 nm using the blank containing 1

ml of water 4 ml of anthrone reagent. The amount of free sugar was calculated from the standard curve of glucose. The carbohydrate content was calculated by the following formula.

$$\% \text{ of carbohydrate} = \frac{\text{Amount of Carbohydrates}}{\text{Weight of the feed}} \times 100$$

10.10 Determination of ash content

At first the marked empty crucible was taken and weighted by using by electric balance. Then 2-3 gm of sample was taken into the crucible and weighted. Then the crucible with sample was kept in a muffle furnace at 550 °C for 6 hrs. Then the muffle furnace was stopped and allowed to cool but it was not open because of its high temperature. After a certain period, when the muffle furnace was fully cooled, the sample was taken out by using spatula. Then the sample was weighted by using same electric balance. Then ash content was calculated by the following formula.

$$\% \text{ Ash content} = \frac{\text{Weight of ash}}{\text{Weight of the sample}} \times 100$$

10.11 Determination of moisture content

Marking the empty crucible according to the sample used. Their weight was taken by using an electric balance and recorded. Then about 2-3 gm of each of the sample was weighted out into the clean weighted crucible by using the sample balance. Then the crucible with samples was placed in a hot air oven at 105 °C for 24 hrs. Then the sample was carefully taken out from oven by using a specialized forceps and kept in desiccators for cooling. Finally the weight was taken again. The difference in weights represents the moisture content of the sample. Then moisture content was calculated by the following formula.

$$\% \text{ of the moisture} = \frac{B - D}{C} \times 100\ \%$$

Where, B = Weight of crucible + Sample (gm)

D = Weight of crucible + Dry sample (gm)

C = Weight of sample (gm)

10.12 Serum biochemical profile

At the end of feeding trial, two fish from each treated group was randomly selected for measurement of serum biochemical profile. The blood was drawn from caudal vein of individual fish and were transferred into sterile tubes without any addition of anticoagulant and kept for 3 hours in slanting position. Samples were centrifuged at 5000 rpm for 10 minutes at 4 ^{o}C. Sera were collected by one ml auto-pipette. The collected sera samples were stored in deep freeze at -20 °C for serum biochemical studies. Red blood cells (RBCs) and white blood cells (WBCs) diluting fluids were used for determining total erythrocyte and leucocyte counts. It was done by mixing 20 µl of blood with 3,980 µl of the corresponding diluting fluid in a clean test tube. The hemoglobin level of blood was analyzed following the cyanomethemoglobin method using Drabkins Fluid (Qualigens Chemicals) (Darbkin, 1945). The absorbance was measured using a spectrophotometer at 540 nm and the final concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens Chemicals). The hemoglobin concentration was then calculated using the following formula: hemoglobin (g/dl) = [OD (T)/OD (S)] × [251/ 1,000] × 60 where OD (T) is the absorbance of the test and OD (S) the absorbance of the standard. Total protein, cholesterol, triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were estimated by atomic absorption spectrophotometry using the kits prepared by Crest Biosystems®. Serum iron content was estimated by Biuret and bromocresol green (BCG) dye binding method (Dumas et al., 1971).

10.13 Statistical analysis

In the experiments, the data were analyzed by one-way analysis of variance to select suitable dose of each nanoparticles. The percentage and ratio data that didn't show normal distribution by Kolmogorov-Smirnov test ($P > 0.05$) were analyzed after normalization using arcsine transformed data. All analyses were performed using SPSS (Statistical Package for Social Science) version 20.0 (IBM Corporation, Armonk, NY, USA). Data were expressed as mean ± SD.

10.14 Materials and methods (Field experiment)

10.14.1 Experimental design

The experiment was conducted in 2 earthen ponds with 1 replication each (17 bigha) for a period of 180 days (March'2018-Auguest'2018) in selected farmer's ponds in Mohonpur, Rajshahi district. Pond 1 (Nano feed) and Pond 2 (commercial Pellet feed).

10.14.2 Preparation and stocking of experimental ponds

The ponds used for this experiment were rectangular in shape and were fully exposed to prevailing sunlight. The main sources of water of the ponds were rainfall and deep tube well. Before starting the experiment the aquatic weeds of the ponds were removed completely by manual effort. All unwanted fishes and other larger aquatic organisms were eradicated by application of rotenone at the rate of 2.5 gm m^{-3} followed by repeated netting. After one week of rotenone application, the ponds were limed at the rate of 247 kg/ha. One week after liming, the ponds were filled with water from adjacent deep tube-well. Then the ponds were fertilized with urea and TSP at the rate of 38 and 20 kg/ha, respectively. After the preparation of the ponds, fishes such as *L.rohita, Cirhinus cirrhosis, Catla catla, Ctenopharyngodon idellus, Mylopharyngodon piceus, Hypophthalmichthys molitrix* and *L.calbasu* were stocked.

10.14.3 Water quality monitoring

Water samples were collected fortnightly (twice in a month) between 10:00 and 11:00 hours for the analysis of various physico-chemical parameters using dark bottles. Water temperature and transparency were measured using a Celsius Thermometer and a black and white standard colour coded Secchi disc of 30 cm diameter. Water pH was measured using an electronic pH meter (Jenwary, 3020) and dissolved oxygen (DO) was measured directly with a DO meter (Lutron, DO-5509). Total alkalinity was measured using a HACH water analysis kit (Model FF-2, USA).

10.14.4 Formulation of diet

Ingredients and proximate composition of prepared control diet were shown in Table 1-5. All feed ingredients were purchased from local market and in the laboratory they were grinded to acquire fine powder. The powdered and sieved feed ingredients were weighed out and mixed thoroughly for preparing diets. Then distilled water was added and blending well (10 min) until the mixture achieves a dough consistency. The dough was pelletized in a manual pelletizer fixed with 3 mm diameter and the pellets were collected in aluminum trays. A thermostatic hot air oven (Microsil INDIA, Universal Lab Product Co., Chennai, India) was used to dry the diets until the moisture content was reduced below 10%. After drying diets were kept at 20 ^{o}C until used. The cost of the formulated diet was 30 BDT/kg. A commercial diet was collected from the market to compare with experimental diet. The cost of commercial diet was 28 BDT/kg.

Table 5. Ingredients and proximate composition of control diet mixed with Zn-NPs

Ingredients	g/kg	Proximate composition	(%)†
Fish meal[a]	275	Protein	33.11±0.14
Mustard oil cake[a]	200	Lipid	9.38±0.03
Soybean meal[a]	125	Carbohydrate	36.45±0.44
Maize bran[a]	125	Moisture	7.07±0.01
Wheat bran[a]	90	Ash	11.23±0.60
Rice bran[a]	90		
Soybean oil[a]	60		
Choline chlorid[a]	2.5		
Zn-free premix[*b]	32.5		
Zn-NPs	0.04		

†Values are presented as mean ± SD, n= 3

*Zn-free premix (mg/kg of premix): vitamin A-156000 IU, vitamin D_3-31200 IU, vitamin E-299, vitamin K_3-26, vitamin B_1-32.5, vitamin B_2-65, vitamin B_6-520, vitamin B_{12}-0.16, Nicotinic Acid-520, Folic Acid-10.4, Copper-130, Iodine-5.2, Manganese-780, and Selenium-1.95.

[a] Ingredients purched from local market of Rajshahi, Bangladadesh.

[b] Supplied by Reneta Animal Health Pharma Co. Ltd. Bangladesh.

10.14.5 Fish sampling, growth parameters and yield analysis

Fish sampling was carried out in the morning between 7:00 and 9:00 am using a scoop net. Around 10% of fish in each treatment were sampled monthly in order to determine weight of fishes. At the final harvest, all fish were weighed, measured and the survival rate and mean weight were determined. To determine the growth response of fish, the following parameters were calculated by following formulas:

$$\text{Percent weight gain (gm)} = \frac{\text{Mean Final weight} - \text{Mea Initial weight}}{\text{Mean initial weight}} \times 100$$

$$\text{SGR (\% } bwd^{-1}\text{)} = \frac{\ln[\text{Final weight}] - \ln[\text{Initial weight}]}{\text{Culture period}} \times 100$$

$$\text{Survival rate (\%)} = \frac{\text{No. of fish harvested}}{\text{No. of fish stock}} \times 100$$

$$\text{Food conversion ratio} = \frac{\text{Weight of feed fed}}{\text{Fish weight gain}}$$

Fish yield (kg/ha/180 days) = Fish biomass at harvest — fish biomass at stock

10.14.6 Economic analysis

At the end of the experiment, an economic analysis was performed to estimate the net return and benefit–cost of the experimental diets in ponds. The following simple equation was used according to Asaduzzaman *et al.* (2010):

$$R = I - (FC+VC+Ii)$$

Where, R= net return, I= income from *L.rohita* sale, FC= fixed/common costs, VC= variable costs and Ii=interest on inputs

The benefit-cost ratio was determined as:

Benefit cost ratio (BCR) = Total net return/Total input cost

10.14.7 Statistical analysis

Water quality, fish growth and yield parameters and economic performance were analyzed by independent sample t-test and significance level was evaluated at 5%. The percentages and ratio data were analyzed using arcsine transformed data. All the analyses were performed using SPSS (Statistical Package for Social Science) version 20.0 (IBM Corporation, Armonk, NY, USA).

11. Results and discussion

11.1 SEM observation:

Fe, Cu and Zn Nanoparticles were Prepared under oil bath heating. Figure (3-A - 3-C) show typical SEM images of Fe, Cu and Zn nanocrystals obtained from (Fe/Cu/Zn) salts/PVP/H_2O at 80 °C for 60 min, respectively. Yields and average sizes of each Fe Cu and Zn nanostructure at 60 min were obtained using more than three SEM images. The yields were determined by counting the total numbers of each product and evaluating its fraction in all products. Sizes of spherical particles stand for their average diameters and the dentition of sizes of other anisotropic products is shown in Figure 3 (A-C). Dominant products (total 99%) at 60 min were nearly spherical based on SEM in Figure 3.

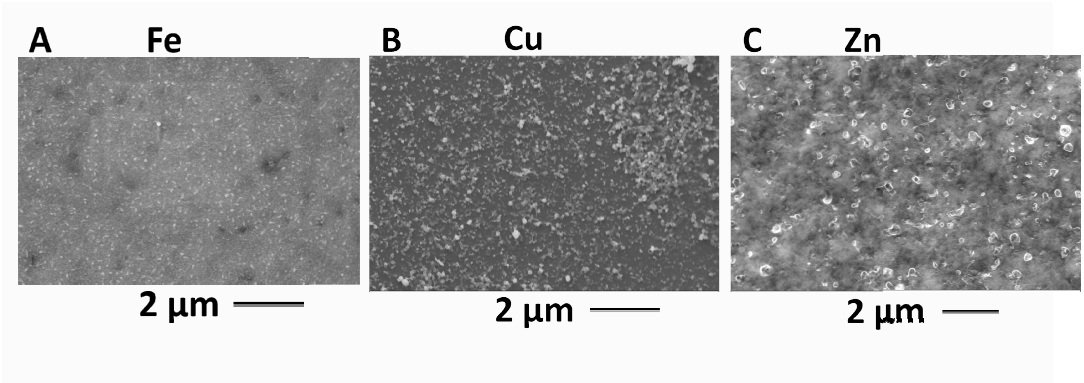


Figure 3. SEM images of nanoparticles obtained at 80 °C in an oil-bath heating under (1-A) for Fe nanoparticles, (1-B) for Cu nanoparticles and (1-C) for Zn nanoparticles dispersed on slide glass.

11.2 UV-Vis extinction spectra of nanoparticles:

UV-vis extinction spectra of Fe, Cu and Zn solutions under oil bath heating were measured to obtain information on changes in products with heating time in Figures 4 (A1 – A3). Figures 4 (A1-A3) are UV- vis spectra of product obtained from 80 mM concentration of Fe, Cu and Zn salts and A1 is sharp spectra of Fe nanoparticles at 450 nm after that it was gone downward with the increase of particles sizes under heating up to 60 min.

Similarly A2 is sharp spectra of Cu nanoparticles arise at 350 nm and the peak of a surface plasmon resonance (SPR) band is decreased with the increase of heating time from 30 min to 60 min that depicted that the particles sizes are bigger with the increase of heating time by means of Ostwald ripening process. On the other hand, A3 is sharp spectra of Zn nanoparticles arise at 460 nm and the peak of a surface plasmon resonance (SPR) band is decreased with the increase of heating time from 30 min to 60 min that predicted that the particles sizes are bigger with the increase of heating time by means of .Ostwald ripening and melting process.

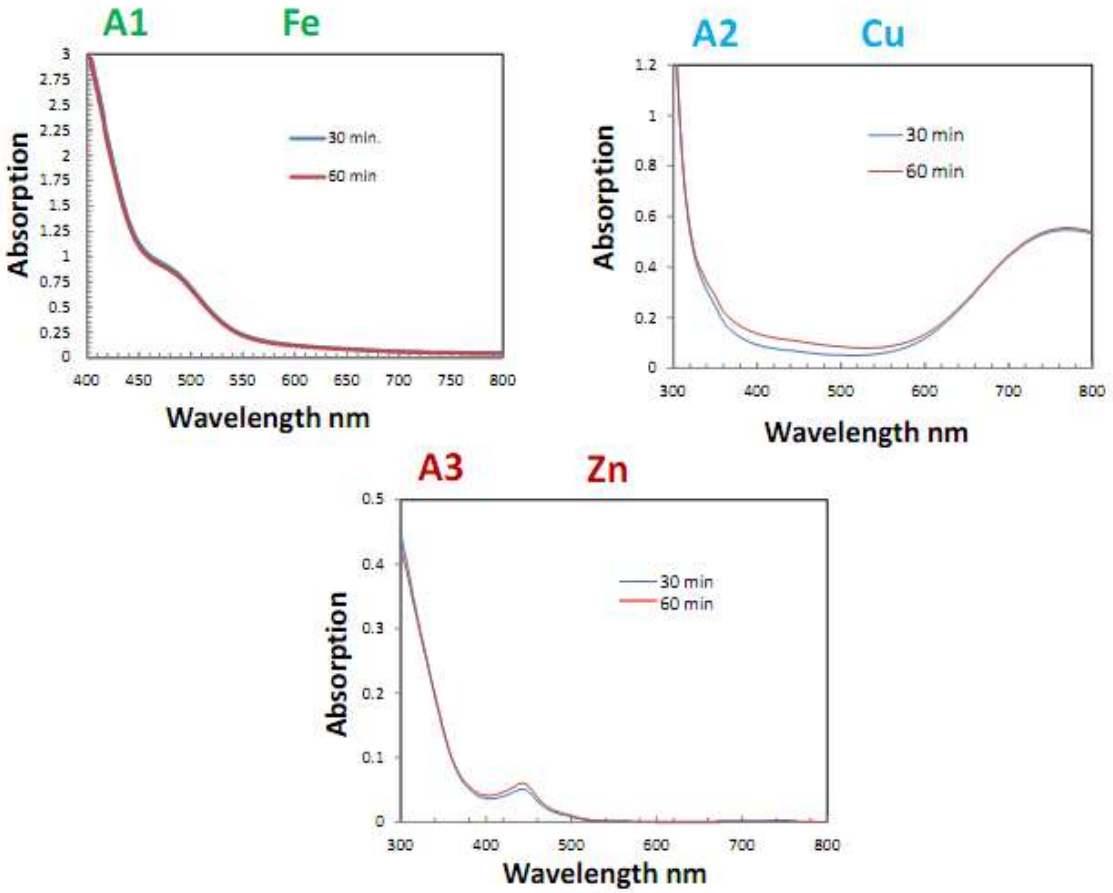


Figure 4. UV-vis extinction spectra of different nanoparticles A1 for Fe, A2 for Cu and A3 for Zn.

Similarly A2 is sharp spectgra of Cu manuparticles arise at 350 nm and the peak of a surface plasmon resonance (SPR) band is decrerased with the increase of heating time from 30 min to 60 min that depicted that the particles sizes are bigger with the increase of heating time by means of Ostwald ripening process. On the other hand A3 is sharp spectra of Zn nanoparticles arise at 460 nm and the peak of a surface plasmon resonance (SPR) band is decreased with the increase of heating time from 30 min to 60 min that predicted that the particles sizes are bigger with the increase of heating time by means of Ostwald ripening and melting process.

11.3 (Experiment-1): Effect of different nanoparticle on growth and physiology of *B. gonionotus*

11.3.1 Water quality

Water quality parameters were maintained as temperature 27.73°C to 28.23°C, dissolved oxygen (DO) 5.75 mg/l to 6.23 mg/l, pH 6.96 to 7.29 and ammonia 0.001 mg/l to 0.002 mg/l throughout the study period. There were no significant differences ($P > 0.05$) in water quality parameters among the different doses of nanoparticles (NPs) during the study period (Table 6).

Table 6. Water quality parameters

Parameters	NPs	Doses of NPs (mg/l)					
		Control	10	20	30	40	50
Temperature (°C)	Fe-NPs	27.93±0.53[a]	27.90±0.35[a]	28.03±0.57[a]	27.76±0.68[a]	27.73±0.33[a]	27.80±0.29[a]
	Cu-NPs	27.99±0.43[a]	27.89±0.34[a]	28.03±0.61[a]	27.83±0.63[a]	27.74±0.45[a]	28.09±0.57[a]
	Zn-NPs	27.74±0.33[a]	27.84±0.28[a]	27.94±0.49[a]	27.83±0.27[a]	28.19±0.21[a]	28.23±0.23[a]
DO (mg/l)	Fe-NPs	5.87±0.26[a]	5.97±0.12[a]	6.07±0.14[a]	5.90±0.25[a]	6.04±0.13[a]	6.00±0.14[a]
	Cu-NPs	6.14±0.17[a]	5.75±0.16[a]	6.19±0.29[a]	6.03±0.19[a]	6.07±0.29[a]	6.20±0.08[a]
	Zn-NPs	6.01±0.08[a]	5.90±0.29[a]	6.11±0.14[a]	6.01±0.19[a]	6.02±0.07[a]	6.23±0.03[a]
pH	Fe-NPs	6.99±0.11[a]	7.06±0.13[a]	7.16±0.04[a]	6.97±0.03[a]	7.09±0.15[a]	7.09±0.09[a]
	Cu-NPs	7.10±0.11[a]	7.29±0.30[a]	6.96±0.03[a]	7.16±0.08[a]	7.06±0.18[a]	7.01±0.08[a]
	Zn-NPs	7.14±0.10[a]	7.05±0.14[a]	7.16±0.08[a]	7.00±0.05[a]	7.18±0.25[a]	7.05±0.17[a]
Ammonia (mg/l)	Fe-NPs	0.001±0.000[a]	0.002±0.001[a]	0.002±0.001[a]	0.002±0.001[a]	0.001±0.001[a]	0.001±0.001[a]
	Cu-NPs	0.002±0.001[a]	0.001±0.001[a]	0.002±0.001[a]	0.001±0.001[a]	0.001±0.001[a]	0.002±0.001[a]
	Zn-NPs	0.001±0.001[a]	0.001±0.001[a]	0.002±0.001[a]	0.001±0.001[a]	0.001±0.001[a]	0.001±0.001[a]

Values in the same row having same superscript letter indicates no significant difference ($P > 0.05$). DO = Dissolved oxygen, Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.3.2 Growth performance and survival

At the beginning, no significant difference ($P > 0.05$) was observed in the initial weight between NPs enriched feed fed fish groups and control group but at the end of the study period *B. gonionotus* fed feed supplemented with 30 mg/kg Fe-NPs showed significantly ($P < 0.05$) enhanced growth performance in the forms of final weight, weight gain, %weight gain, average daily gain (ADG) and specific growth rate (SGR) ($P < 0.05$) (Table 7). A comparison with control feed showed that the final weight of *B. gonionotus* increased with an increment rate of 8.63%, 22.39%, 32.31%, 15.21% and 4.40% for the doses of 10, 20, 30, 40 and 50 mg/kg feed of Fe-NPs over control feed. On the contrary, although a doses of 20 mg/kg feed of Cu-NPs gave better growth performance of *B. gonionotus* than the fish groups fed the feeds containing 10 mg/kg, 30 mg/kg, 40 mg/kg feed of Cu-

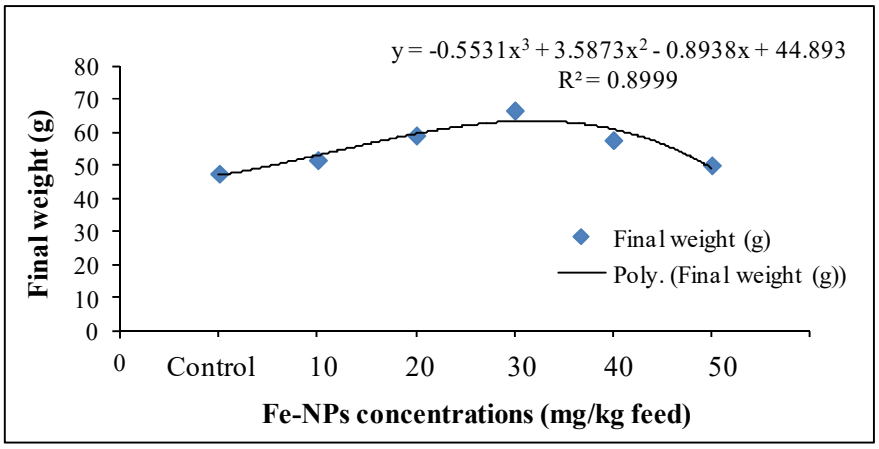
NPs and control feed, a severe toxic effect of Cu-NPs was observed for the fish group fed the feed containing 50 mg/kg feed of Cu-NPs. Even the final weight (48.14±0.68 gm) and weight gain (14.67±0.64 gm) were also found to reduce than that of control group (48.18±0.52 and 14.70±0.40 gm), a decrement of -0.08% was recorded in final weight over the control feed. SGR (%bwd^{-1}) was also found to remain as the same as its control group. The fish groups fed with 40 mg/kg feed of Zn-NPs showed significantly ($P < 0.05$) better growth performance compared to other fish groups. Zn-NPs enriched feeds gave an increment rate of 14.37%, 19.52%, 29.12%, 35.84% and 11.67% over the control feed for the doses of 10, 20, 30, 40 and 50 mg/kg feed of Zn-NPs.

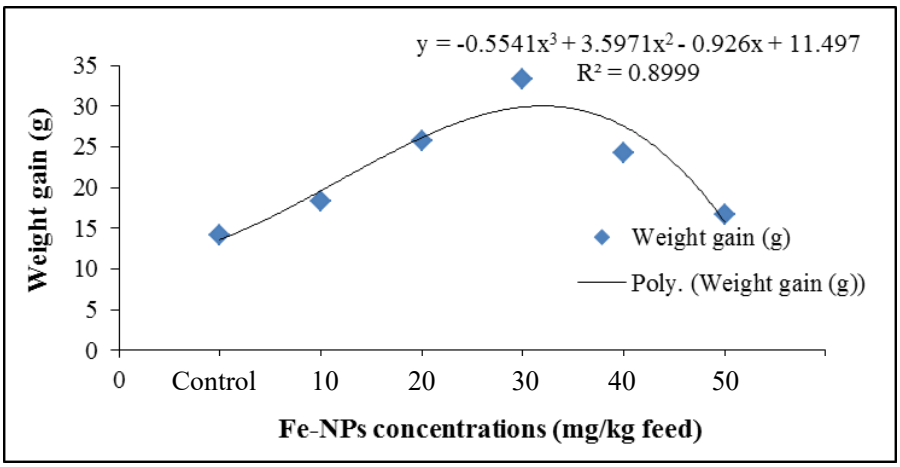
During the study period, different doses of Fe-NPs showed a negative correlation between the doses of NPs and final weight (gm), weight gain (gm) and SGR (%bwd^{-1}) with R^2 values of 0.899, 0.899 and 0.923. However, after a certain dose of the 30 mg/kg feed of Fe-NPs growth of *B. gonionotus* began to decrease with increasing the doses of NPs (Figures 5 & 8). Positive correlation with R^2 values of 0.941, 0.941 and 0.942 were observed between different doses of Cu-NPs in feed and final weight (gm), weight gain (gm) and SGR (%bwd^{-1}). Cu-NPs at the doses of 20 mg/kg feed gave the better growth performance and after that a gradual decrease in growth performance was observed with increasing the doses (Figures 6 & 8). Zn-NPs showed negative correlations between different doses in feed and growth parameters (final weight, $R^2 = 0.922$; weight gain, $R^2 = 0.923$ and SGR, $R^2 = 0.934$). At the doses of 40 mg/kg feed of Zn-NPs the fishes showed best growth performance and a gradual decrease in final weight, weight gain and SGR was observed at higher doses (Figures 7 & 8). There was no significant difference in survival rate was observed among the experimental groups during the study period (Table 7).

Table 7. Growth parameters of *B. gonionotus* fed different doses of dietary nanoparticles.

NPs	Growth parameters	Doses of NPs (mg/kg feed)					
		Control	10	20	30	40	50
Fe-NPs	Initial weight (gm)	33.42±0.39^{a}	33.44±0.19^{a}	33.43±0.44^{a}	33.43±0.42^{a}	33.43±0.05^{a}	33.44±0.35^{a}
	Final weight (gm)	47.60±0.18^{f}	51.71±0.39^{d}	59.18±0.48^{b}	66.72±0.36^{a}	57.75±0.31^{c}	50.14±0.45^{e}
	Weight gain (gm)	14.18±0.48^{f}	18.28±0.57^{d}	25.75±0.80^{b}	33.29±0.08^{a}	24.32±0.29^{c}	16.70±0.35^{e}
	% weight gain	42.45±1.88^{f}	54.67±2.01^{d}	77.05±3.28^{b}	99.58±1.41^{a}	72.74±0.85^{c}	49.94±1.26^{e}
	ADG (gm)	0.24±0.01^{f}	0.31±0.01^{d}	0.43±0.01^{b}	0.55±0.01^{a}	0.40±0.01^{c}	0.28±0.01^{e}
	SGR (% bwd^{-1})	0.59±0.03^{f}	0.73±0.02^{d}	0.95±0.03^{b}	1.15±0.01^{a}	0.91±0.01^{c}	0.68±0.01^{e}
	Survival (%)	100	100	100	100	100	100
Cu-NPs	Initial weight (gm)	33.48±0.19^{a}	33.47±0.23^{a}	33.46±0.10^{a}	33.48±0.08^{a}	33.47±0.19^{a}	33.48±0.24^{a}
	Final weight (gm)	48.18±0.52^{d}	54.08±0.41^{b}	58.49±0.47^{a}	54.03±0.67^{b}	51.16±1.50^{c}	48.14±0.68^{d}
	Weight gain (gm)	14.70±0.40^{d}	20.61±0.61^{b}	25.02±0.48^{a}	20.55±0.60^{b}	17.68±1.55^{c}	14.67±0.64^{d}
	% weight gain	43.91±1.11^{d}	61.57±2.21^{b}	74.78±1.52^{a}	61.37±1.69^{b}	52.83±4.76^{c}	43.81±1.94^{d}
	ADG (gm)	0.24±0.01^{d}	0.34±0.01^{b}	0.42±0.01^{a}	0.34±0.01^{b}	0.30±0.03^{c}	0.25±0.01^{d}
	SGR (% bwd^{-1})	0.61±0.02^{d}	0.80±0.02^{b}	0.93±0.01^{a}	0.80±0.02^{b}	0.71±0.06^{c}	0.61±0.02^{d}
	Survival (%)	100	100	100	100	100	100
Zn-NPs	Initial weight (gm)	33.43±0.36^{a}	33.44±0.12^{a}	33.43±0.44^{a}	33.42±0.34^{a}	33.43±0.25^{a}	33.44±0.37^{a}
	Final weight (gm)	47.52±0.77^{f}	54.35±0.79^{e}	58.13±0.24^{c}	64.45±0.08^{b}	70.62±0.48^{a}	55.76±0.52^{d}
	Weight gain (gm)	14.09±0.43^{f}	20.91±0.67^{e}	24.70±0.46^{c}	31.02±0.28^{b}	37.19±0.55^{a}	22.32±0.73^{d}
	% weight gain	42.13±0.89^{f}	62.54±1.79^{e}	73.92±2.24^{c}	92.83±1.77^{b}	111.27±2.14^{a}	66.74±2.76^{d}
	ADG (gm)	0.24±0.01^{f}	0.35±0.01^{e}	0.41±0.01^{c}	0.52±0.01^{b}	0.62±0.01^{a}	0.37±0.01^{d}
	SGR (% bwd^{-1})	0.58±0.01^{f}	0.81±0.02^{e}	0.92±0.02^{c}	1.10±0.02^{b}	1.25±0.02^{a}	0.85±0.03^{d}
	Survival (%)	100	100	100	100	100	100

ADG = Average daily gain, SGR = Specific growth rate, NPs = NanoparticlesValues with different superscripts in the same row for each feedary nanoparticle indicate significant differences ($P < 0.05$). Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.





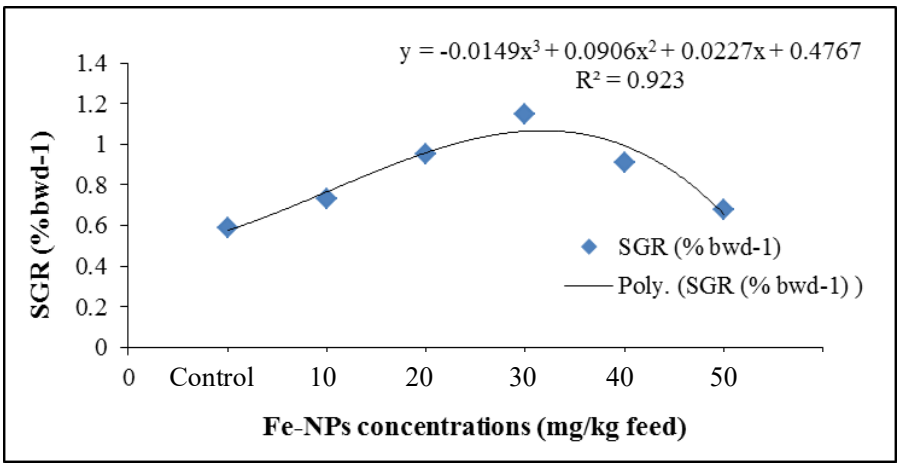


Figure 5. Relationship between different concentrations of Fe-NPs in feed with growth performance (final weight, weight gain and SGR) of *B. gonionotus*

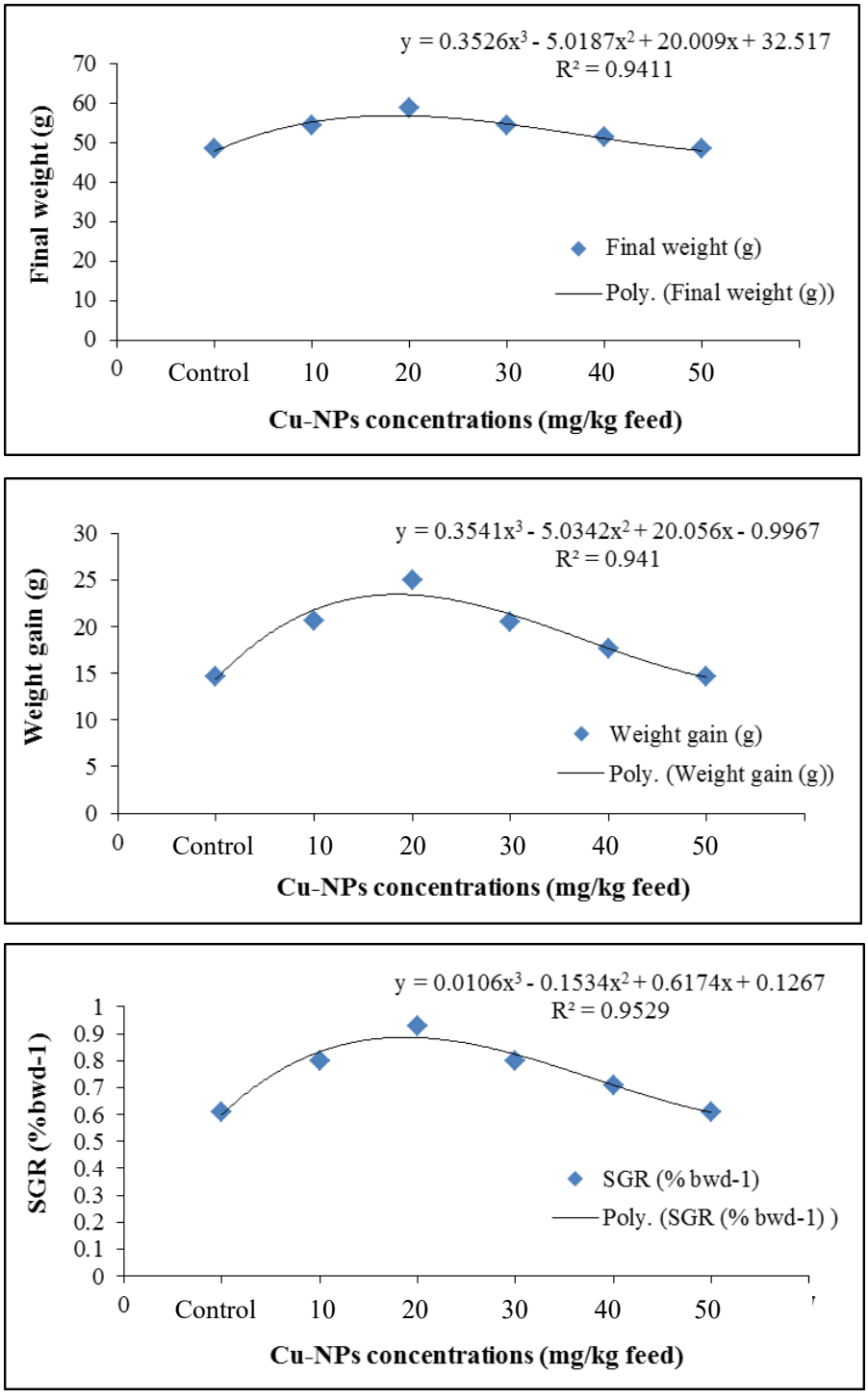


Figure 6. Relationship between different concentrations of Cu-NPs in feed with growth performance (final weight, weight gain and SGR) of *Barbonymus gonionotus*

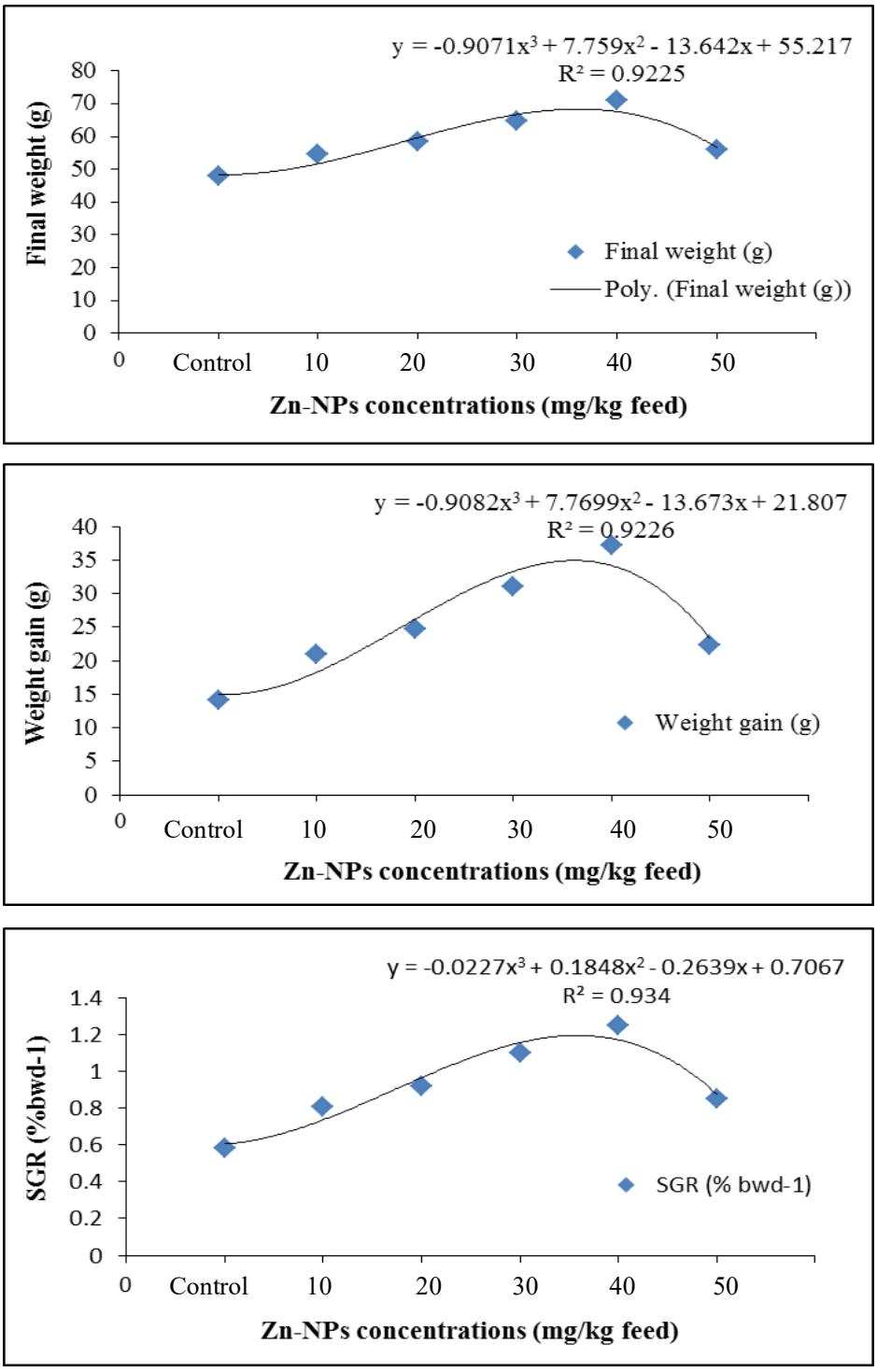


Figure 7. Relationship between different concentrations of Zn-NPs in feed with growth performance (final weight, weight gain and SGR) of *Barbonymus gonionotus*

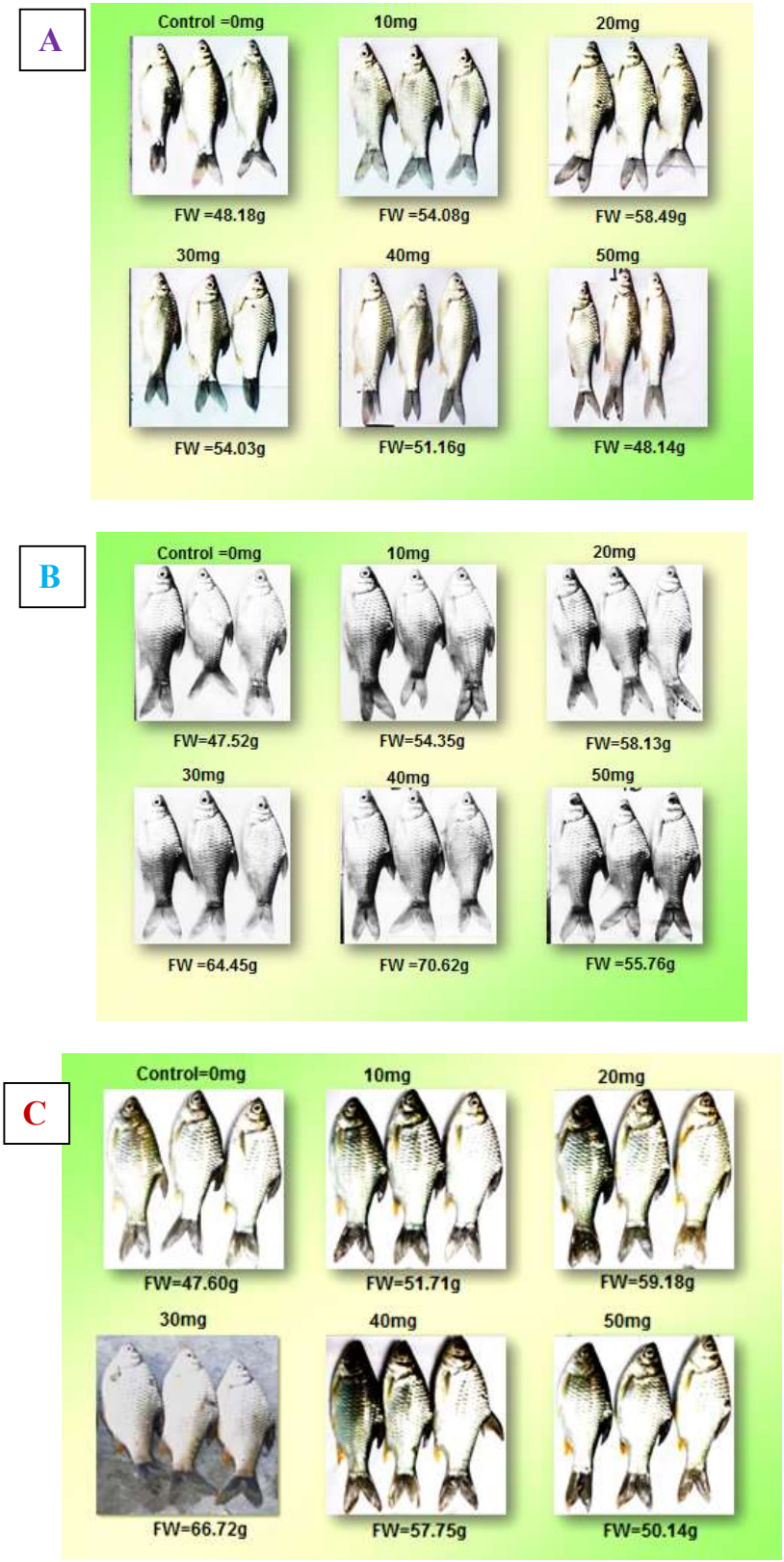


Figure 8. Typical images of *B. gonionotus* groups fed (A) Fe- NPs, (B) Cu-NPs and (C) Zn-NPs supplemented feeds.

40 and 50 mg/kg feed of Fe-NPs doses, respectively. However, in case of Cu-NPs mediated feeds, the FCR and PPV% of feed containing 50 mg/kg feed of Cu-NPs showed reduced performance even than control group. Along with this no improvement in FCE and PER was noted at this feed compared to the control feed. However, some shorts of improvement that observed in PGR% was negligible to be realized. Better performance in feed utilization parameters for fishes fed with Cu-NPs enriched feeds was found at the doses of 20 mg/kg feed of Cu-NPs. In case of Zn-NPs the best performance of the feed utilization parameters were observed in fish group fed with 40 mg/kg feed of Zn-NPs mixed feed. FCR found to be 4.10±0.11 for the control group and 2.92±0.11, 2.41±0.05, 2.94±0.08, 3.42±0.31 and 4.11±0.18 for group of fishes fed feed containing 10, 20, 30, 40 and 50 mg/kg feed of Zn-NPs, respectively. Whereas, PER for different feed groups was found as 0.71±0.02 for the control group and 1.05±0.03, 1.24±0.04, 1.56±0.03, 1.87±0.04 and 1.12±0.05 for 10, 20, 30, 40 and50 mg/kg feed Zn-NPs, respectively.

Table 8. Feed utilization parameters of *B. gonionotus* fed different NPs enriched feeds.

NPs	Parameters	Doses of NPs (mg/kg feed)					
		Control	10	20	30	40	50
Fe-NPs	FCR	4.25±0.19^{a}	3.30±0.12^{c}	2.34±0.10^{d}	1.81±0.03^{e}	2.47±0.03^{d}	3.60±0.09^{b}
	FCE	0.23±0.01^{f}	0.30±0.02^{d}	0.43±0.02^{b}	0.55±0.01^{a}	0.40±0.01^{c}	0.28±0.01^{e}
	PER	0.71±0.03^{f}	0.92±0.03^{d}	1.30±0.06^{b}	1.67±0.03^{a}	1.22±0.02^{c}	0.84±0.02^{e}
	PPV (%)	11.23±0.44^{e}	14.03±0.42^{d}	18.63±0.61^{b}	31.82±0.46^{a}	17.35±0.21^{c}	13.37±0.09^{d}
	PGR (%)	1.28±0.06^{d}	1.55±0.05^{c}	1.86±0.05^{b}	2.47±0.05^{a}	1.79±0.04^{b}	1.51±0.02^{c}
Cu-NPs	FCR	4.10±0.11^{a}	2.92±0.11^{c}	2.41±0.05^{d}	2.94±0.08^{c}	3.42±0.31^{b}	4.11±0.18^{a}
	FCE	0.24±0.01^{d}	0.34±0.02^{b}	0.42±0.01^{a}	0.34±0.01^{b}	0.30±0.03^{c}	0.24±0.01^{d}
	PER	0.74±0.02^{d}	1.03±0.04^{b}	1.26±0.03^{a}	1.03±0.03^{b}	0.89±0.08^{c}	0.74±0.03^{d}
	PPV (%)	10.40±0.36^{d}	13.35±0.27^{c}	21.09±0.24^{a}	15.36±0.23^{b}	13.06±0.59^{c}	10.32±0.43^{d}
	PGR (%)	1.27±0.05^{d}	1.50±0.02^{c}	2.00±0.03^{a}	1.64±0.02^{b}	1.49±0.04^{c}	1.26±0.05^{d}
Zn-NPs	FCR	4.27±0.09^{a}	2.88±0.09^{b}	2.44±0.07^{d}	1.94±0.04^{e}	1.62±0.03^{f}	2.70±0.12^{c}
	FCE	0.24±0.01^{e}	0.35±0.01^{d}	0.41±0.01^{c}	0.51±0.01^{b}	0.62±0.02^{a}	0.37±0.02^{d}
	PER	0.71±0.02^{f}	1.05±0.03^{e}	1.24±0.04^{c}	1.56±0.03^{b}	1.87±0.04^{a}	1.12±0.05^{d}
	PPV (%)	10.43±0.33^{f}	14.81±0.43^{e}	17.44±0.27^{c}	22.53±0.44^{b}	36.03±0.36^{a}	14.08±0.34^{d}
	PGR (%)	1.27±0.05^{e}	1.61±0.04^{d}	1.75±0.02^{c}	2.07±0.04^{b}	2.67±0.04^{a}	1.55±0.03^{d}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$).
FCR = Feed conversion ratio, FCE = Feed conversion efficiency, PER = Protein efficiency ratio, PPV = Protein productive value, PGR = Protein growth rate, Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.3.4 Proximate composition of muscle

In *B. gonionotus* total protein content were recorded as 8.75±0.01% in control group and 8.93±0.02, 9.32±0.02, 12.31±0.02, 9.09±0.06 and 8.91±0.04% at 10, 20, 30, 40 and 50 mg/kg feed of Fe-NPs fed fish groups (Figure 9. a). There was a significant difference ($P < 0.05$) in protein content of different feed groups fed with feeds containing 10, 20, 30, 40 and 50 mg/kg feed of Fe-NPs. The fish group fed feed containing 30 mg/kg feed of Fe-NPs showed better performance compared to other fish groups and even from control group. In case of Cu-NPs enriched feeds, significantly ($P < 0.05$) higher protein content was obtained from fish group fed the feed containing 20 mg/kg feed of Cu-NPs. However, the fish group fed feed containing 50 mg/kg feed of Cu-NPs showed reduced protein content from control group (Figure 9. a). Significantly ($P < 0.05$) higher protein content in fish group fed feed containing 40 mg/kg feed of Zn-NPs was observed for Zn-NPs enriched feeds. Higher lipid content in

fish fed feed containing Fe-NPs, Cu-NPs and Zn-NPs were found as 3.95±0.02% for 30 mg/kg feed of Fe-NPs, 2.96±0.03% for 20 mg/kg feed of Cu-NPs and 3.67±0.04% for 40 mg/kg feed of Zn-NPs, respectively (Figure 9. b). There was significant ($P < 0.05$) differences found in carbohydrate contents in all the experimental and control feed for accumulation of carbohydrate (Figure 9. c). Significant ($P < 0.05$) difference in total ash contents was also observed in the fish group fed with experimental and control feed, whereas increase in NPs content in feed significantly increases the ash content of muscle (Figure 9. d). Significant ($P < 0.05$) difference was noted in moisture content in muscle of *B. gonionotus* in Fe-NPs, Cu-NPs and Zn-NPs enriched feeds. Maximum moisture content was found in 50 mg/kg feed of NPs fed fish groups for all the NPs types (Figure 9. e).

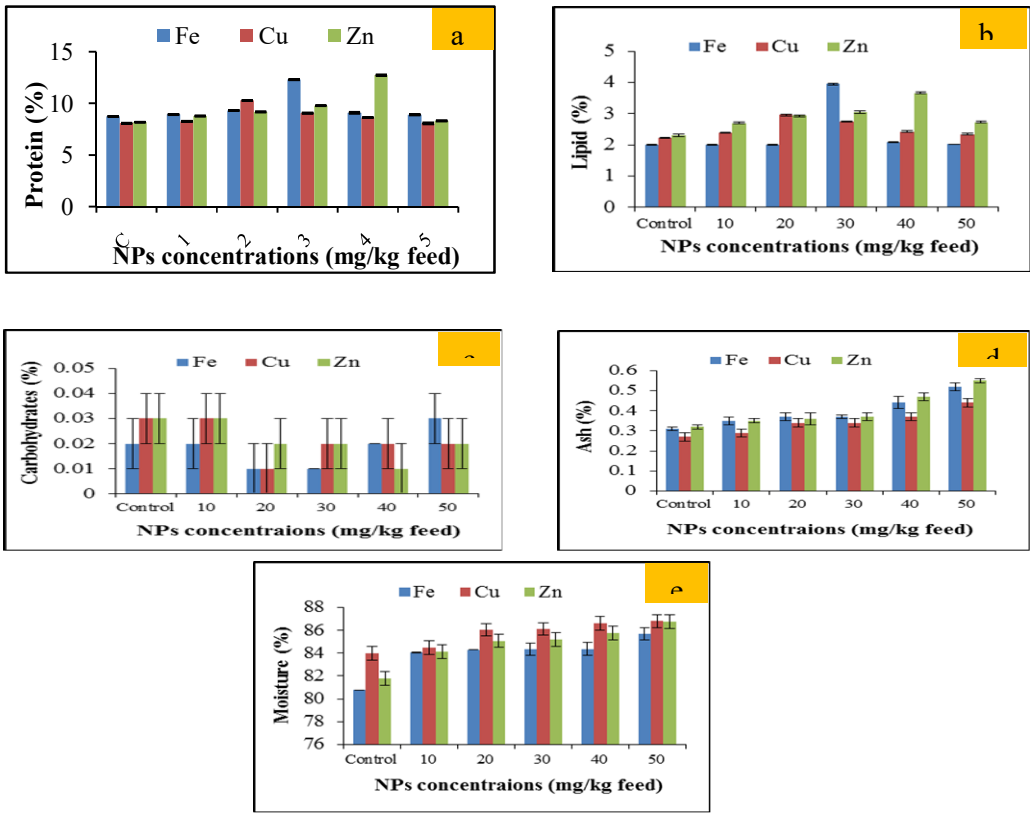


Fig. 9. Proximate composition of muscle of *B. gonionotus* fed diets with different concentrations of Fe-NPs, Cu-NPs and Zn-NPs (a, protein; b, lipid, c, carbohydrate; d, ash and e, moisture).

11.3.5 Hematological parameters

The hematological parameters of *B. gonionotus* fed NPs at different doses are shown in Table 9. The fishes fed feeds containing Fe-NPs showed increasing RBC content with increasing the doses and the highest value was obtained from the fish group fed 50 mg/kg feed of Fe-NPs containing feed (192.55±0.02 %). WBC (392.25±0.02 %) and hemoglobin (6.45±0.02 %) content was found to increase up to 30 mg/kg feed of Fe-NPs containing feed fed fish group and afterwards a decreasing trend was observed. However, total protein (15.75±0.03 g/dl) and globulin (3.59±0.02 g/dl) content were found

to show their maxima at the doses of 30 mg/kg feed of Fe-NPs. Overall significant differences ($P < 0.05$) was observed among the fish groups fed feeds containing different doses of Fe-NPs and control group (Table 9). Fish groups fed feeds containing different doses of Cu-NPs also varied significantly ($P < 0.05$) among them and from control group. In case of Cu-NPs mediated feeds, maximum value of the parameters *viz.* WBC (276.16±0.02%), hemoglobin (4.73±0.02%), total protein (14.67±0.02 gm/dl) and globulin (3.76±0.02 gm/dl) were observed at 20 mg/kg Cu-NPs enriched feed. Fish groups fed feeds containing 40 and 50 mg/kg feed of Cu-NPs showed a decrease in total protein content than control group. However, apart from the above parameters RBC (122.66±0.01%) showed its maxima with increased doses of NPs and albumin with decrease doses of NPs, whereas the highest value of albumin was found in control group (1.72±0.01 gm/dl) (Table 9). WBC (358.39±0.02%), hemoglobin (5.21±0.02%), total protein (15.87±0.02 gm/dl) and globulin (3.85±0.02 gm/dl) content of fish groups fed feeds containing 40 mg/kg feed of Zn-NPs showed maximum value followed by 30 mg/kg feed of Zn-NPs enriched feed (WBC, 306.86±0.01%; hemoglobin, 4.87±0.025; total protein, 14.93±0.02 gm/dl and globulin, 3.75±0.03). The highest value of blood albumin content of *B. gonionotus* was observed for control group of fishes indicating lack of influence of Zn-NPs in this parameter. However, all the blood parameters were found varied significantly ($P < 0.05$) among different doses of Zn-NPs and control group (Table 9).

Table 9. Hematological parameters of *B. gonionotus* fed different NPs enriched feeds.

NPs	Parameters	Doses of NPs (mg/kg feed)					
		Control	10	20	30	40	50
Fe-NPs	RBC (%)	23.87±0.03^{f}	83.64±0.03^{e}	94.49±0.02^{d}	105.71±0.02^{c}	113.17±0.02^{b}	192.55±0.02^{a}
	WBC (%)	121.36±0.02^{f}	249.85±0.02^{d}	327.97±0.02^{c}	392.25±0.02^{a}	362.44±0.03^{b}	220.45±0.03^{e}
	Hemoglobin (%)	3.75±0.03^{f}	4.79±0.02^{d}	4.97±0.02^{c}	6.45±0.02^{a}	5.26±0.02^{b}	4.43±0.02^{e}
	Total protein (gm/dl)	12.66±0.02^{f}	13.55±0.02^{d}	13.65±0.02^{c}	15.75±0.03^{a}	14.15±0.02^{b}	13.05±0.02^{e}
	Albumin (gm/dl)	1.83±0.02^{d}	1.82±0.02^{a}	1.67±0.01^{b}	1.64±0.02^{b}	1.53±0.02^{c}	1.43±0.02^{a}
	Globulin (gm/dl)	2.38±0.01^{d}	3.37±0.01^{b}	3.35±0.01^{b}	3.59±0.02^{a}	3.34±0.03^{b}	3.19±0.02^{c}
Cu-NPs	RBC (%)	21.83±0.02^{f}	82.83±0.02^{e}	84.76±0.02^{d}	109.23±0.02^{c}	111.71±0.02^{b}	122.66±0.01^{a}
	WBC (%)	113.17±0.02^{f}	207.85±0.02^{e}	276.16±0.02^{a}	251.47±0.02^{b}	215.35±0.03^{c}	209.36±0.01^{d}
	Hemoglobin (%)	3.29±0.02^{f}	4.38±0.02^{b}	4.73±0.02^{a}	4.13±0.02^{c}	3.44±0.01^{d}	3.35±0.01^{e}
	Total protein (gm/dl)	12.64±0.01^{d}	13.73±0.02^{c}	14.67±0.02^{a}	14.23±0.03^{b}	12.63±0.02^{d}	12.54±0.02^{e}
	Albumin (gm/dl)	1.72±0.01^{a}	1.53±0.02^{b}	1.47±0.02^{c}	1.53±0.02^{b}	1.39±0.02^{d}	1.29±0.02^{e}
	Globulin (gm/dl)	2.73±0.01^{f}	3.64±0.01^{b}	3.76±0.02^{a}	3.54±0.03^{c}	3.45±0.02^{d}	3.15±0.01^{e}
Zn-NPs	RBC (%)	25.17±0.02^{f}	95.65±0.02^{e}	103.24±0.03^{d}	109.27±0.02^{c}	112.57±0.02^{b}	115.27±0.02^{a}
	WBC (%)	122.63±0.02^{f}	269.74±0.01^{e}	287.87±0.02^{c}	306.86±0.01^{b}	358.39±0.02^{a}	271.86±0.01^{d}
	Hemoglobin (%)	3.43±0.02^{f}	3.87±0.02^{e}	4.63±0.01^{c}	4.87±0.02^{b}	5.21±0.02^{a}	3.74±0.01^{d}
	Total protein (gm/dl)	12.93±0.02^{e}	13.97±0.02^{d}	14.76±0.02^{c}	14.93±0.02^{b}	15.87±0.02^{a}	13.97±0.02^{d}
	Albumin (gm/dl)	1.73±0.02^{a}	1.69±0.02^{b}	1.58±0.02^{c}	1.55±0.01^{c}	1.43±0.02^{d}	1.29±0.02^{e}
	Globulin (gm/dl)	2.73±0.01^{f}	3.33±0.02^{d}	3.49±0.02^{c}	3.75±0.03^{b}	3.85±0.02^{a}	3.23±0.02^{e}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$). Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.3.6 Blood lipid profile

Lipid profile (total cholesterol, HDL, LDL and triglycerides) of blood of *B. gonionotus* is shown in Table 10. The fish groups fed Fe-NPs enriched feeds at different doses showed higher total cholesterol, HDL, LDL and triglyceride level compared to control feed fed fish group. However, HDL was found to increase and LDL was found to decrease with increase in Fe-NPs doses in feeds. Increasing trend in total cholesterol, HDL and triglyceride content was observed up to 30 mg/kg feed of Fe-NPs and there after it showed decreasing trend at 40 mg/kg and 50 mg/kg feed Fe-NPs mixed feed. However, significant differences ($P < 0.05$) were observed between the Fe-NPs enriched feeds and control group (Table 10). Similar results were also obtained from fish fed with Cu-NPs enriched feeds where control group showed minimum total cholesterol, HDL, LDL and triglyceride and 50 mg/kg feed of Cu-NPs group showed the highest LDL compared to other Cu-NPs mediated feeds. However, LDL was found to decrease with increasing Cu-NPs doses in feeds and it gave maximum value at control group. Significant differences ($P < 0.05$) in lipid profile of blood were also observed for Zn-NPs mediated feeds and control group. Similar to Fe-NPs and Cu-NPs enriched feeds, Zn-NPs enriched feed groups also showed higher total cholesterol and HDL for fish group fed 40 mg/kg feed of Zn-NPs and the lowest in control group. Significant ($P < 0.05$) decrease in LDL was observed with increasing the doses of Zn-NPs in feeds. However, triglycerides was found to increase up to 40 mg/kg feed of Zn-NPs enriched feed fed fish group and showed decreasing trend there after (Table 10).

Table 10. Blood Cholesterol, HDL, LDL and triglycerides of *B. gonionotus* fed different NPs enriched feeds.

NPs	Parameters	Doses of NPs (mg/kg feed)					
		Control	10	20	30	40	50
Fe-NPs	Total cholesterol (mg/dl)	206.53±0.02^{e}	213.17±0.02^{d}	213.15±0.02^{d}	221.35±0.03^{a}	217.25±0.02^{c}	219.75±0.02^{b}
	HDL (mg/dl)	49.43±0.02f	51.15±0.02^{e}	52.39±0.02^{d}	54.19±0.02^{a}	53.27±0.02^{b}	52.65±0.02c
	LDL (mg/dl)	140.35±0.02^{f}	140.54±0.03^{e}	141.95±0.03^{d}	143.15±0.03^{c}	143.93±0.02^{b}	147.89±0.02^{a}
	Triglycerides (mg/dl)	151.25±0.03^{f}	157.44±0.03^{e}	161.17±0.02^{d}	168.35±0.03^{a}	161.86±0.01^{c}	162.45±0.02^{b}
Cu-NPs	Total cholesterol (mg/dl)	213.33±0.02^{f}	214.15±0.02^{d}	214.93±0.02^{a}	214.74±0.02^{b}	214.63±0.02^{c}	213.76±0.02^{e}
	HDL (mg/dl)	50.84±0.02^{e}	50.25±0.02^{f}	55.83±0.02^{a}	55.66±0.01^{b}	55.35±0.02^{c}	50.94±0.03^{d}
	LDL (mg/dl)	154.30±0.02^{a}	153.39±0.02^{b}	153.29±0.02^{c}	153.12±0.01^{d}	152.87±0.02^{e}	142.43±0.02^{f}
	Triglycerides (mg/dl)	151.23±0.02^{e}	161.53±0.02^{b}	166.42±2.32^{a}	160.55±0.02^{b}	157.67±0.02^{c}	155.17±0.02^{d}
Zn-NPs	Total cholesterol (mg/dl)	212.25±0.02^{f}	212.37±0.02^{e}	213.17±0.02^{d}	213.27±0.02^{c}	213.63±0.02^{a}	213.34±0.02^{b}
	HDL (mg/dl)	53.93±0.02^{f}	54.04±0.03^{e}	54.67±0.02^{d}	55.93±0.02^{b}	56.73±0.02^{a}	55.87±0.02^{c}
	LDL (mg/dl)	154.60±0.02^{a}	154.35±0.03^{b}	152.86±0.01^{c}	152.73±0.02^{d}	152.67±0.02^{e}	152.41±0.02^{f}
	Triglycerides (mg/dl)	156.15±0.03^{e}	158.29±0.02^{d}	161.39±0.02^{b}	167.07±1.17^{a}	167.25±0.02^{a}	159.23±0.02^{c}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$). HDL = High density lipoprotein, LDL = Low density lipoprotein, Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.3.7 Blood enzymes profile

Enzymatic profile of *B. gonionotus* fed NPs (Fe-NPs, Cu-NPs and Zn-NPs) is shown in Table 11. Significant differences ($P < 0.05$) in enzymatic profile (alanine aminotransferase, ALT; aspartate aminotransferase, AST; amylase; lipase; protease and Alkaline phosphatase, ALP) were observed among the Fe-NPs enriched feed fed fish groups and control group. Increasing trend in AST, ALT and ALP was evident with increasing NPs doses in feed. However, amylase, lipase and protease were also showed increasing trend up to 30 mg/kg feed of Fe-NPs and after that the performance was reduced with increasing Fe-NPs doses in feeds. Protease was found to influence positively by the incorporation of Fe-NPs in feeds and showed better performance compared to their control groups. However, in case of amylase and lipase, increasing the doses of Fe-NPs in feed up to 50 mg/kg of feed reduced these enzymes activity than their control groups (Table 11). Significant differences ($P < 0.05$) were also observed in enzymatic profile of fish groups fed with feed enriched with Cu-NPs and their control groups. Here the increasing trend was evident up to a doses of 20 mg/kg feed of Cu-NPs in feeds and the values of amylase, lipase and protease were found to reduce with increasing the doses of Cu-NPs in feeds. Enzyme activity of the blood of fishes fed with Zn-NPs enriched feed showed their maximum value at 40 mg/kg feed of Zn-NPs and further increase in doses reduced these values at 50 mg/kg feed of Zn-NPs. Significant differences ($P< 0.05$) were also observed among doses of Zn-NPs enriched feeds and control group. The inclusion of Zn-NPs was found to enhance the performance of blood enzymes compared to their control groups in terms of amylase, lipase and protease content but up to a suitable dose of 40 mg/kg feed of Zn-NPs (Table 11).

Table 11. Blood enzymes of *B. gonionotus* fed different NPs enriched feeds.

NPs	Parameters	Doses of NPs (mg/kg feed)					
		Control	10	20	30	40	50
Fe-NPs	AST (U/L)	31.18±0.01^{e}	31.45±0.03^{d}	32.57±0.01^{c}	32.57±0.01^{c}	32.74±0.03^{b}	32.86±0.01^{a}
	ALT (U/L)	35.28±0.01^{f}	35.83±0.02^{e}	36.35±0.03^{d}	36.73±0.02^{c}	37.65±0.02^{b}	38.23±0.02^{a}
	Amylase (U/L)	0.44±0.03^{e}	0.58±0.02^{d}	0.94±0.02^{b}	1.63±0.02^{a}	0.75±0.02^{c}	0.27±0.02^{f}
	Lipase (U/L)	0.25±0.02^{d}	0.45±0.03^{c}	0.49±0.02^{b}	0.56±0.01^{a}	0.25±0.01^{d}	0.22±0.01^{d}
	Protease (U/L)	0.74±0.02^{f}	0.79±0.02^{e}	1.17±0.02^{b}	2.13±0.01^{a}	1.15±0.02^{c}	1.14±0.01^{d}
	ALP (mg/dl)	14.07±0.02^{c}	14.12±0.01^{b}	14.14±0.03^{b}	14.13±0.02^{b}	14.15±0.03^{b}	15.15±0.02^{a}
Cu-NPs	AST (U/L)	32.36±0.02^{f}	32.45±0.02^{e}	32.52±0.01^{d}	32.73±0.02^{b}	32.66±0.01^{c}	32.86±0.01^{a}
	ALT (U/L)	35.66±0.01^{f}	36.37±0.02^{e}	36.59±0.02^{d}	36.67±0.02^{c}	37.14±0.01^{b}	37.31±0.02^{a}
	Amylase (U/L)	0.45±0.02^{f}	1.07±0.01^{d}	1.85±0.02^{a}	1.34±0.02^{b}	1.16±0.01^{c}	0.66±0.01^{e}
	Lipase (U/L)	0.33±0.01^{f}	0.67±0.02^{c}	0.83±0.02^{a}	0.76±0.02^{b}	0.63±0.01^{d}	0.47±0.01^{e}
	Protease (U/L)	0.75±0.01^{e}	0.87±0.02^{d}	1.33±0.02^{a}	1.23±0.02^{b}	0.97±0.01^{c}	0.78±0.02^{e}
	ALP (mg/dl)	13.23±0.02^{e}	13.37±0.02^{d}	13.65±0.02^{b}	13.62±0.01^{c}	13.66±0.01^{b}	13.74±0.01^{a}
Zn-NPs	AST (U/L)	32.65±0.02^{e}	32.73±0.02^{d}	32.73±0.02^{d}	32.87±0.02^{c}	33.15±0.03^{b}	33.27±0.02^{a}
	ALT (U/L)	35.33±0.01^{e}	35.77±0.02^{d}	35.76±0.01^{d}	36.66±0.01^{c}	37.24±0.03^{b}	37.75±0.03^{a}
	Amylase (U/L)	0.52±0.01^{f}	0.66±0.01^{d}	1.14±0.01^{c}	1.35±0.02^{b}	1.73±0.02^{a}	0.59±0.02^{e}
	Lipase (U/L)	0.36±0.01^{e}	0.43±0.02^{d}	0.56±0.02^{c}	0.63±0.02^{b}	0.77±0.02^{a}	0.43±0.02^{d}
	Protease (U/L)	0.78±0.01^{e}	0.87±0.02^{d}	0.98±0.02^{c}	1.19±0.02^{b}	1.27±0.02^{a}	0.85±0.02^{d}
	ALP (mg/dl)	13.33±0.02^{f}	13.66±0.01^{e}	14.08±0.01^{d}	14.17±0.02^{c}	14.25±0.02^{b}	14.33±0.02^{a}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$). ALT = alanine aminotransferase, AST = aspartate aminotransferase, ALP = Alkaline phosphatase, Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.3.8 Bioaccumulation of NPs in muscle, liver and serum

During the experiment, significantly ($P < 0.05$) higher NPs (Fe-NPs, Cu-NPs and Zn-NPs) were found to accumulate in fishes fed feeds containing 50 mg/kg feed of Fe-NPs, Cu-NPs or Zn-NPs (Figure 10). However, doses of NPs were found to increase in muscle, liver and serum with increasing the doses of feedary NPs (Fe-NPs, Cu-NPs and Zn-NPs) in the feeds compared to control group. The accumulation of Fe-NPs and Zn-NPs showed the trend of liver > muscle > serum, whereas this trend was muscle > liver > serum in case of fishes fed the feeds enriched with Cu-NPs (Figure 10).

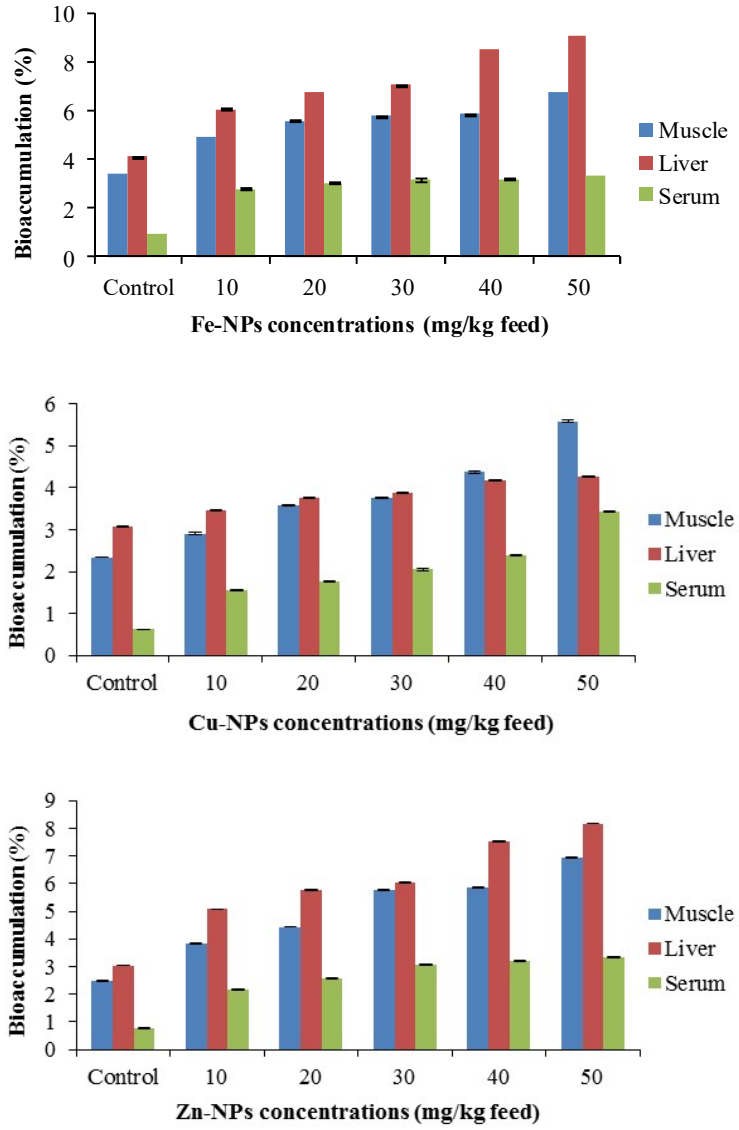


Figure 10. Concentrations of NPs (Fe-NPs, Cu-NPs and Zn-NPs) in muscle, liver and serum of *Barbonymus gonionotus* fed diets enriched with different NPs.

11.4 (Experiment-2): Effect of different nanoparticle on growth and physiology of *L. rohita*.

11.4.1 Water quality

Water quality parameters were maintained as temperature 27.73°C to 28.32°C, DO 5.83 mg/l to 6.19 mg/l, pH 6.96 to 7.14 and ammonia 0.001 mg/l to 0.002 mg/l throughout the study period. There were no significant differences ($P < 0.05$) in water quality parameters among the different doses of NPs during the study period (Table 12).

Table 12. Water quality parameters

Parameters	NPs	Doses of NPs (mg/l)					
		Control	10	20	30	40	50
Temperature (°C)	Fe-NPs	27.73±0.72[a]	28.09±0.19[a]	28.15±0.62[a]	28.17±0.32[a]	27.86±0.61[a]	28.03±0.16[a]
	Cu-NPs	28.04±0.13[a]	27.99±0.45[a]	28.32±0.33[a]	28.04±0.56[a]	28.00±0.51[a]	28.00±0.51[a]
	Zn-NPs	27.99±0.23[a]	27.90±0.39[a]	28.03±0.12[a]	27.82±0.11[a]	28.04±0.45[a]	27.89±0.38[a]
DO (mg/l)	Fe-NPs	5.87±0.21[a]	5.96±0.14[a]	6.19±0.04[a]	5.97±0.01[a]	6.06±0.17[a]	6.16±0.08[a]
	Cu-NPs	6.03±0.16[a]	6.10±0.14[a]	6.12±0.13[a]	6.10±0.22[a]	6.19±0.08[a]	5.96±0.01[a]
	Zn-NPs	5.83±0.52[a]	6.06±0.15[a]	6.15±0.08[a]	5.95±0.05[a]	6.18±0.07[a]	6.07±0.04[a]
pH	Fe-NPs	7.05±0.06[a]	7.01±0.05[a]	7.03±0.10[a]	7.03±0.04[a]	7.04±0.10[a]	7.06±0.11[a]
	Cu-NPs	7.10±0.10[a]	7.14±0.14[a]	7.02±0.04[a]	7.03±0.10[a]	7.07±0.10[a]	7.00±0.08[a]
	Zn-NPs	7.10±0.27[a]	7.11±0.16[a]	7.12±0.15[a]	7.02±0.08[a]	7.12±0.03[a]	6.96±0.02[a]
Ammonia (mg/l)	Fe-NPs	0.001±0.001[a]	0.001±0.001[a]	0.002±0.001[a]	0.002±0.001[a]	0.001±0.001[a]	0.002±0.001[a]
	Cu-NPs	0.001±0.001[a]	0.002±0.001[a]	0.001±0.001[a]	0.002±0.001[a]	0.001±0.001[a]	0.001±0.001[a]
	Zn-NPs	0.001±0.001[a]	0.002±0.001[a]	0.001±0.000[a]	0.001±0.001[a]	0.002±0.001[a]	0.001±0.001[a]

Values in the same row having same superscript letter indicates no significant difference ($P > 0.05$). DO = Dissolved oxygen, Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.4.2 Growth performance

Overall growth performance of *L. rohita* fed diets enriched with different types of NPs (Fe-NPs, Cu-NPs and Zn-NPs) for a period of 60 days are shown in Table 13. The fishes were all homogeneous in size during their releasing period and the homogeneity in fish size was also confirmed by ANOVA test as the differences in initial body weight was not significant ($P < 0.05$) at all. However, after the 60 days of experimental period the fishes showed significant differences ($P < 0.05$) in their body weight among different feed groups. In case of fish groups fed diets enriched with Fe-NPs, the highest final weight (61.67±0.47 g) was obtained from the group that fed diet containing 30 mg/kg feed of Fe-NPs. However, after this doses decrease in final weight was observed to the fish groups the feed diets containing 40 and 50 mg/kg feed of Fe-NPs. Final weight of fishes in control group compared to other groups of fishes fed with Fe-NPs enriched diet indicates the effects of Fe-NPs on growth of *L. rohita*. Similar trend was also observed in other growth parameters (weight gain, % weight gain, ADG and SGR) of *L. rohita* fed with Fe-NPs supplemented diets. The regression analysis revealed dose dependent negative correlation between doses and final weight, weight gain and SGR with R^2 values of 0.8981, 0.8987 and 0.9173, respectively of *L. rohita* fed diets containing Fe-NPs (Figure 11 and 14 A).

Fishes fed the diets supplemented with Cu-NPs also showed significant differences ($P < 0.05$) in growth parameters after the feeding period of 60 days (Table 13). The highest growth performance in terms of final weight, weight gain, % weight gain, ADG and SGR was observed in the fish group fed diets containing 20 mg/kg feed of Cu-NPs. Final weight differs significantly ($P < 0.05$) among the doses and control groups, while there were no significant difference ($P < 0.05$) in weight gain, % weight gain, ADG and SGR between the fish group fed diets supplemented with 10 and 30 mg/kg feed of Cu-NPs. However, increase in doses of Cu-NPs in diets up to 30, 40 and 50 mg/kg feed of Cu-NPs showed decreasing trend in fish growth performance. On the other hand, 50 mg/kg feed of Cu-NPs fed fish group showed a negative growth performance even from control group. The regression parameter R^2 was 0.8433, 0.8412 and 0.8584 for final weight, weight gain and SGR, respectively (Figure 12 and 14 B).

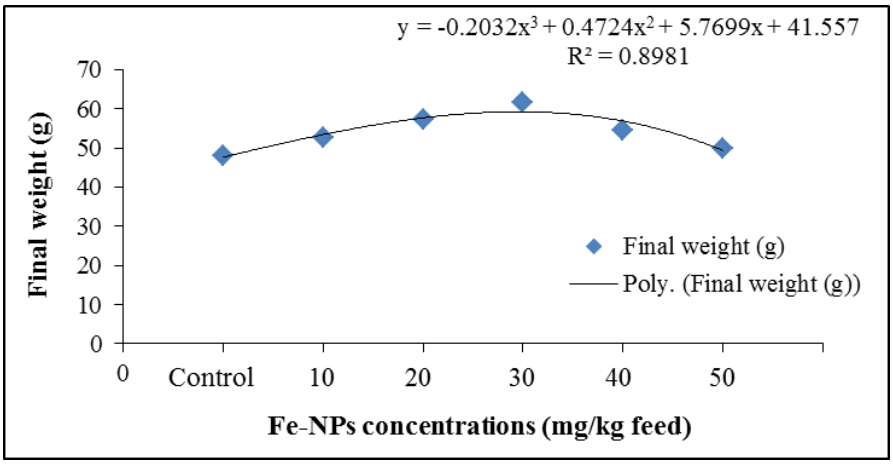
During the study period, fish fed diet supplemented with 40 mg/kg feed of Zn-NPs showed significantly higher final weight, weight gain, % weight gain, ADG and SGR compared to other diets groups and even from control group (Table 13). Afterword's increasing the dose of 50 mg/kg feed of Zn-NPs reduced the growth performance compared to 40 mg/kg feed of Zn-NPs fed fish group. Dose dependent regression analysis revealed negative correlations among the doses of Zn-NPs and growth performance with R^2 values of 0.876 (final weight), 0.8757 (weight gain) and 0.8958 (SGR) (Figure 13 and 14 C). During the feeding trial fish survival in the entire NPs group was 100%.

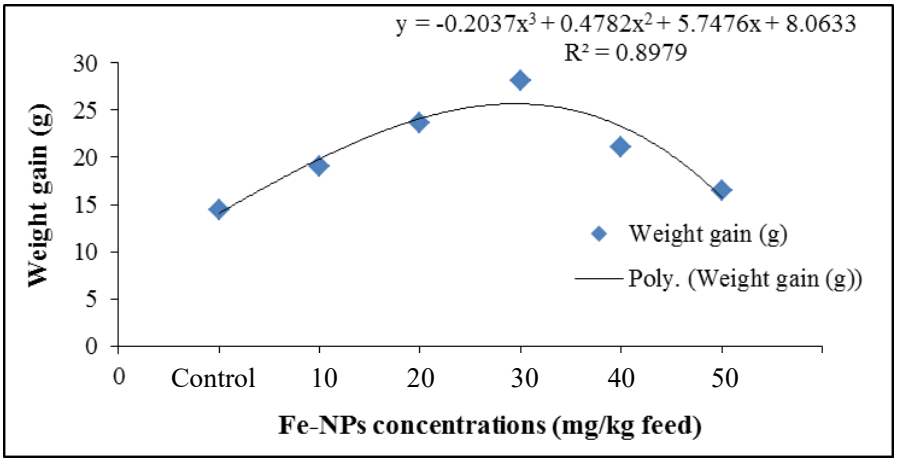
Table 13. Growth parameters of *L. rohita* fed different doses (mg/kg feed) of dietary nanoparticles.

NPs	Growth parameters	Doses of NPs (mg/kg feed)					
		Control	10	20	30	40	50
Fe-NPs	Initial weight (gm)	33.51±0.33^{a}	33.52±0.19^{a}	33.52±0.23^{a}	33.52±0.11^{a}	33.53±0.28^{a}	33.52±0.24^{a}
	Final weight (gm)	47.96±0.26^{f}	52.56±0.49^{d}	57.19±0.23^{b}	61.67±0.47^{a}	54.55±0.14^{c}	49.94±0.33^{e}
	Weight gain (gm)	14.45±0.29^{f}	19.04±0.59^{d}	23.67±0.23^{b}	28.15±0.50^{a}	21.03±0.18^{c}	16.42±0.15^{e}
	% weight gain	43.12±1.18^{f}	56.80±1.98^{d}	70.63±1.01^{b}	83.97±1.60^{a}	62.72±1.03^{c}	48.97±0.39^{e}
	ADG (gm)	0.24±0.01^{f}	0.32±0.01^{d}	0.39±0.01^{b}	0.47±0.01^{a}	0.35±0.00^{c}	0.27±0.01^{e}
	SGR (% bwd^{-1})	0.60±0.02^{f}	0.75±0.02^{d}	0.89±0.01^{b}	1.01±0.02^{a}	0.81±0.01^{c}	0.67±0.01^{e}
	Survival (%)	100	100	100	100	100	100
Cu-NPs	Initial weight (gm)	33.56±0.29^{a}	33.56±0.30^{a}	33.54±0.10^{a}	33.56±0.37^{a}	33.57±0.29^{a}	33.55±0.08^{a}
	Final weight (gm)	47.87±0.23^{d}	51.05±0.14^{c}	55.81±0.35^{a}	51.76±0.33^{b}	48.39±0.42^{d}	47.23±0.36^{e}
	Weight gain (gm)	14.32±0.32^{cd}	17.48±0.28^{b}	22.27±0.44^{a}	18.20±0.68^{b}	14.82±0.13^{c}	13.67±0.43^{d}
	% weight gain	42.67±1.27^{cd}	52.10±1.26^{b}	66.41±1.50^{a}	54.24±2.58^{b}	44.15±0.08^{c}	40.75±1.37^{d}
	ADG (gm)	0.24±0.00^{c}	0.29±0.01^{b}	0.37±0.01^{a}	0.30±0.01^{b}	0.25±0.01^{c}	0.23±0.01^{d}
	SGR (% bwd^{-1})	0.59±0.02^{cd}	0.70±0.02^{b}	0.85±0.02^{a}	0.72±0.03^{b}	0.61±0.00^{c}	0.57±0.02^{e}
	Survival (%)	100	100	100	100	100	100
Zn-NPs	Initial weight (gm)	33.51±0.25^{a}	33.51±0.20^{a}	33.52±0.27^{a}	33.51±0.28^{a}	33.51±0.25^{a}	33.53±0.19^{a}
	Final weight (gm)	48.14±0.20^{e}	54.95±0.34^{d}	57.63±0.65^{c}	59.53±0.32^{b}	65.66±0.29^{a}	57.06±1.57^{c}
	Weight gain (gm)	14.63±0.28^{e}	21.44±0.43^{d}	24.11±0.84^{c}	26.02±0.05^{b}	32.15±0.27^{a}	23.53±1.51^{c}
	% weight gain	43.65±1.10^{e}	63.98±1.54^{d}	71.95±2.99^{c}	77.64±0.58^{b}	95.93±1.26^{a}	70.17±4.41^{c}
	ADG (gm)	0.24±0.01^{e}	0.36±0.01^{d}	0.40±0.01^{c}	0.43±0.00^{b}	0.53±0.01^{a}	0.39±0.03^{c}
	SGR (% bwd^{-1})	0.60±0.02^{e}	0.82±0.02^{d}	0.90±0.03^{c}	0.96±0.01^{b}	1.12±0.01^{a}	0.88±0.04^{c}
	Survival (%)	100	100	100	100	100	100

*ADG = Average daily gain, SGR = Specific growth rate, NPs = Nanoparticles

*Values with different superscripts in the same row for each dietary nanoparticle indicate significant differences ($P < 0.05$). Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.





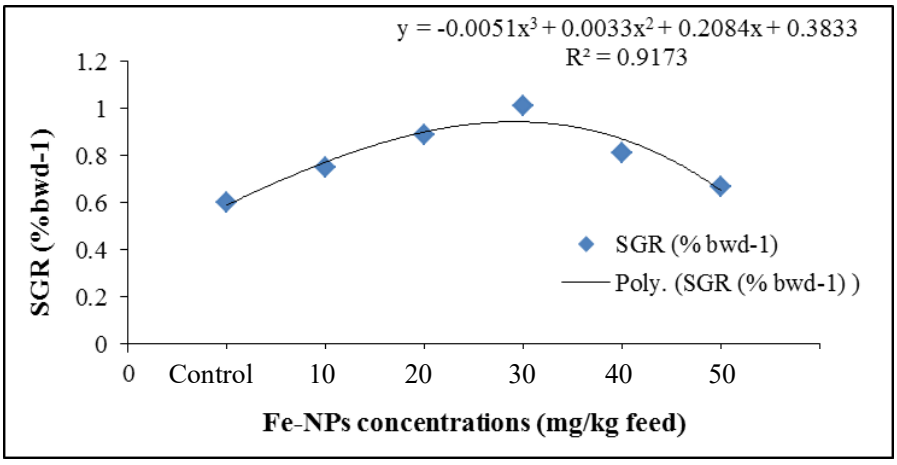


Figure 11. Relationship between different concentrations of Fe-NPs in feed with growth performance (final weight, weight gain and SGR) of *L. rohita.*

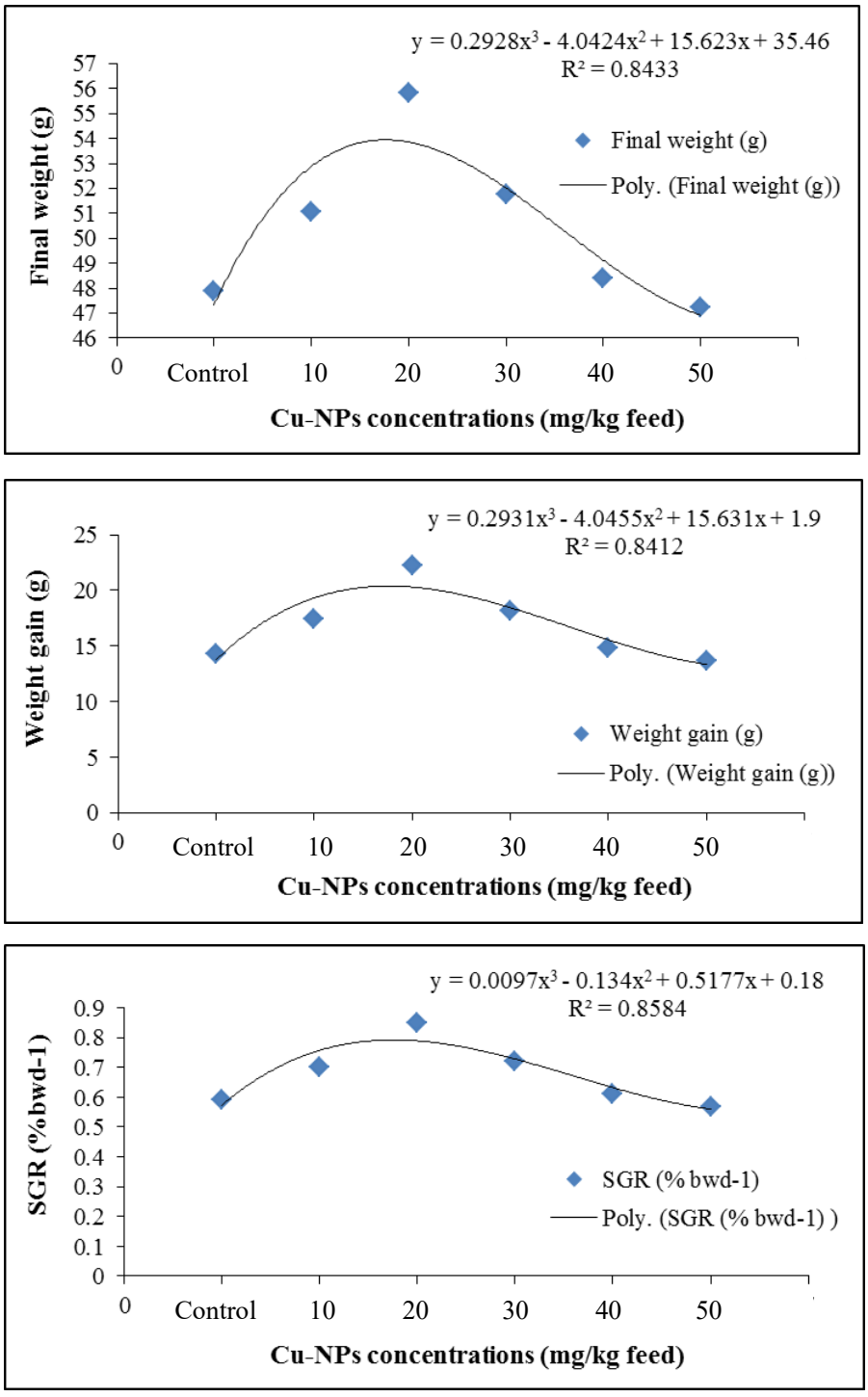


Figure 12. Relationship between different concentrations of Cu-NPs in feed with growth performance (final weight, weight gain and SGR) of *L. rohita*

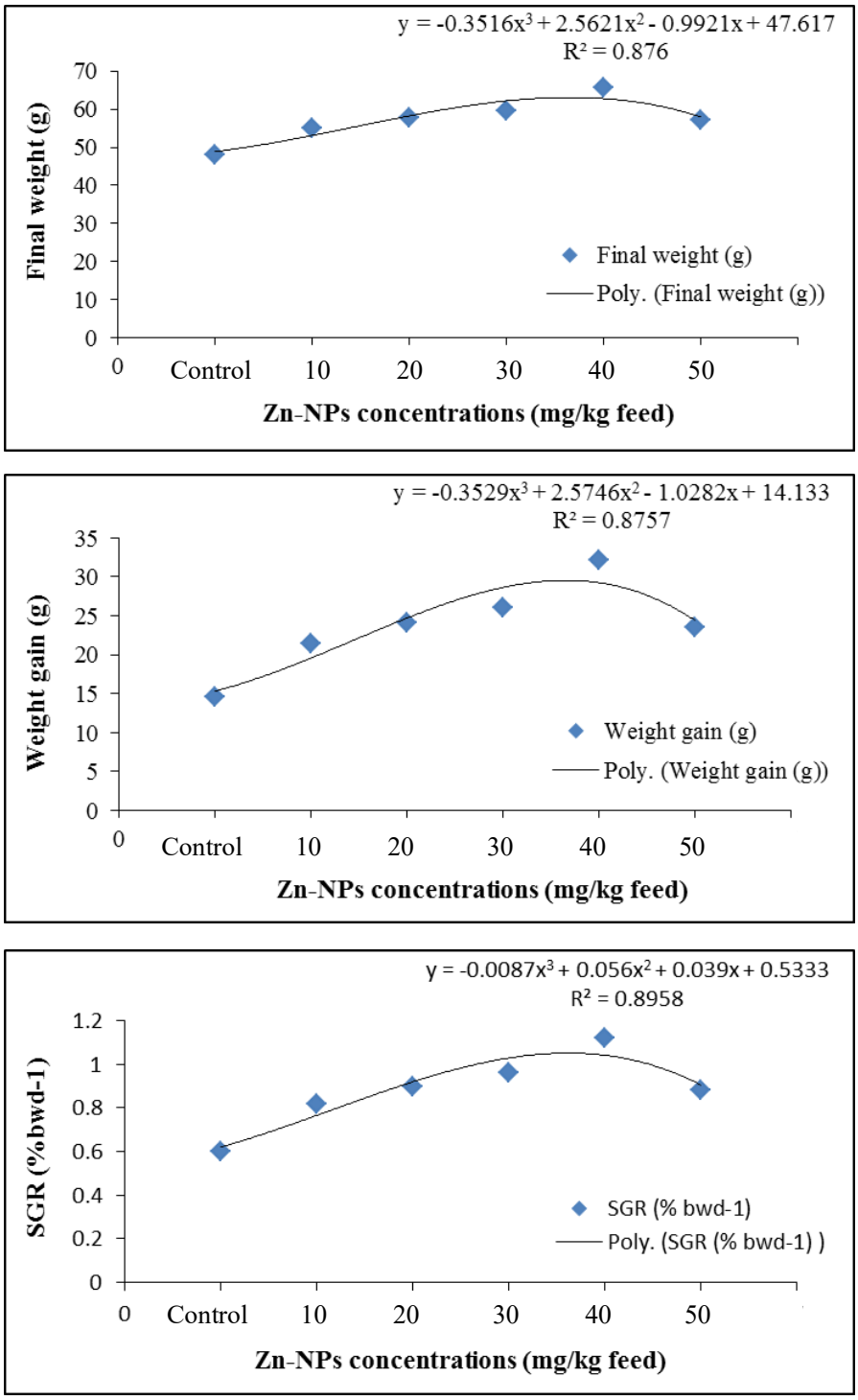


Figure 13. Relationship between different concentrations of Zn-NPs in feed with growth performance (final weight, weight gain and SGR) of *L. rohita*.

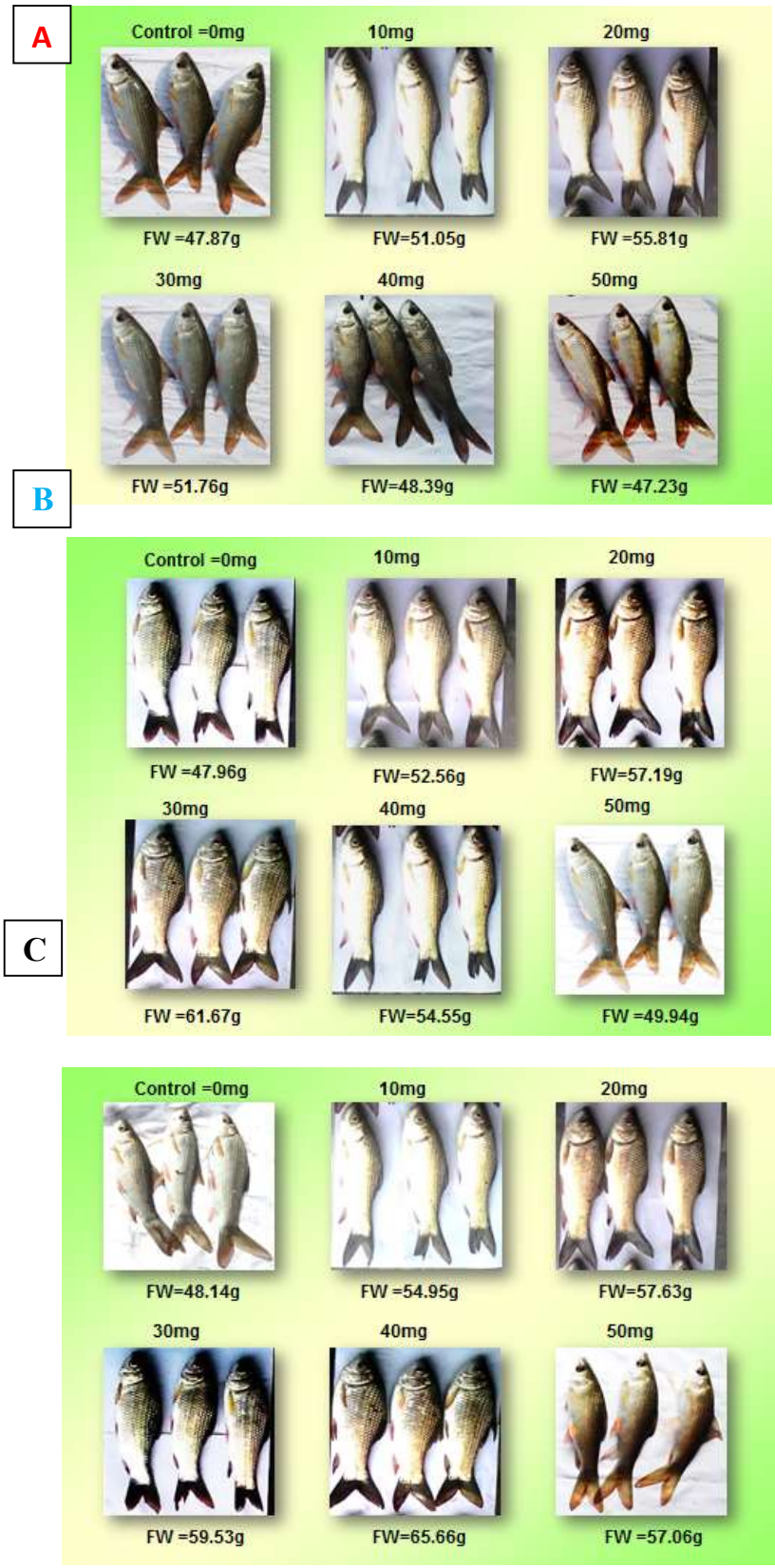


Figure 14. Graphical representation of *L. rohita* groups fed (A) Fe-NPs, (B) Cu-NPs and (C) Zn-NPs supplemented diets.

11.4.3 Feed utilization parameters

Feed utilization parameters of *L. rohita* fed diets containing different doses of NPs are shown in Table 14. In fish groups fed diets supplemented with Fe-NPs showed significant differences ($P < 0.05$) in FCR with better performance obtained from diet containing 30 mg/kg feed of Fe-NPs (2.14±0.04). Increase in doses of NPs up to certain level increase the value of FCR. However, the highest value of FCR was noted in control group. Other parameters such as PER, PPV and PGR also showed significant differences ($P < 0.05$) among the doses and control group, whereas the highest value of these parameters were obtained from the fish group fed 30 mg/kg feed of Fe-NPs (FCE 0.47±0.01; PER, 1.41±0.03; PPV, 27.37±0.25% and PGR, 2.55±0.02%).

Significant difference ($P < 0.05$) was also observed in FCR value of Cu-NPs supplemented diets; whereas the better performance was obtained in 20 mg/kg feed of Cu-NPs (2.71±0.06) fed fish group. FCE, PPR, PPV and PGR were also found significantly ($P < 0.05$) influence by the doses of Cu-NPs in feed. The highest values of these parameters were obtained at 20 mg/kg feed of Cu-NPs (FCE, 0.37±0.01; PER, 1.12±0.02; PPV, 19.31±0.55% and PGR, 2.16±0.09%). However, further increasing the doses of Cu-NPs in feed reduced the performance of feed utilization parameters. Increase in doses of Cu-NPs in feed up to 50 mg/kg Cu-NPs of feed reduced the performance of feed utilization parameters even from control group.

Supplementation of Zn-NPs significantly ($P < 0.05$) influenced the FCR of different fish groups. In case of Zn-NPs enriched diets, better FCR (1.88±0.02) was found in the fish group fed with 40 mg/kg Zn-NPs of feed. In contrast, other feed utilization parameters such as FCE (0.54±0.01), PER (1.61±0.02), PPV (32.38±0.54) and PGR (2.83±0.08%) were also found at their best for the fishes fed 40 mg/kg feed of Zn-NPs which differ significantly ($P < 0.05$) from other Zn-NPs enriched feed groups and even from control group.

Table 14. Feed utilization parameters of *L. rohita* fed different NPs enriched diets.

NPs	Parameters	Doses of NPs (mg/kg feed)					
		Control	10	20	30	40	50
Fe-NPs	FCR	4.18±0.12^{a}	3.17±0.11^{c}	2.55±0.04^{e}	2.14±0.04^{f}	2.87±0.05^{d}	3.68±0.03^{b}
	FCE	0.24±0.01^{f}	0.31±0.01^{d}	0.39±0.01^{b}	0.47±0.01^{a}	0.35±0.01^{c}	0.27±0.00^{e}
	PER	0.72±0.02^{f}	0.95±0.03^{d}	1.19±0.02^{b}	1.41±0.03^{a}	1.05±0.02^{c}	0.82±0.01^{e}
	PPV (%)	13.61±0.12^{f}	15.97±0.32^{d}	19.15±0.24^{b}	27.37±0.25^{a}	17.33±0.27^{c}	14.78±0.22^{e}
	PGR (%)	1.74±0.00^{f}	1.90±0.03^{d}	2.11±0.03^{b}	2.55±0.02^{a}	2.00±0.02^{c}	1.81±0.03^{e}
Cu-NPs	FCR	4.22±0.13^{b}	3.46±0.08^{c}	2.71±0.06^{d}	3.32±0.16^{c}	4.08±0.01^{b}	4.42±0.15^{a}
	FCE	0.24±0.01^{cd}	0.29±0.01^{b}	0.37±0.01^{a}	0.30±0.02^{b}	0.25±0.01^{c}	0.23±0.01^{e}
	PER	0.72±0.02^{cd}	0.87±0.02^{b}	1.12±0.02^{a}	0.91±0.04^{b}	0.74±0.00^{c}	0.68±0.02^{e}
	PPV (%)	2.65±0.16^{e}	9.03±0.28^{c}	19.31±0.55^{a}	9.95±0.20^{b}	6.43±0.06^{d}	2.49±0.09^{e}
	PGR (%)	0.51±0.03^{e}	1.33±0.06^{c}	2.16±0.09^{a}	1.43±0.03^{b}	1.04±0.01^{d}	0.48±0.02^{e}
Zn-NPs	FCR	4.13±0.10^{a}	2.81±0.07^{b}	2.51±0.11^{c}	2.32±0.02^{d}	1.88±0.02^{e}	2.57±0.16^{c}
	FCE	0.24±0.01^{e}	0.35±0.01^{d}	0.40±0.02^{c}	0.43±0.00^{b}	0.54±0.01^{a}	0.39±0.03^{c}
	PER	0.73±0.02^{e}	1.07±0.03^{d}	1.21±0.05^{c}	1.30±0.01^{b}	1.61±0.02^{a}	1.18±0.07^{c}
	PPV (%)	3.97±0.20^{e}	8.19±0.08^{d}	9.70±0.40^{c}	13.34±0.12^{b}	32.38±0.54^{a}	8.34±0.35^{d}
	PGR (%)	0.71±0.04^{e}	1.25±0.03^{d}	1.39±0.05^{c}	1.71±0.01^{b}	2.83±0.08^{a}	1.24±0.03^{d}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$).

FCR = Feed conversion ratio, FCE = Feed conversion efficiency, PER = Protein efficiency ratio, PPV = Protein productive value, PGR = Protein growth rate, Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.4.4 Proximate composition of muscle

Whole body proximate composition of muscle of *L. rohita* fed with Fe-NPs enriched diets showed significant difference ($P < 0.05$) among the different groups of fishes. Supplementation of Fe-NPs at doses of 10, 20 and 30 mg/kg feed of Fe-NPs significantly increased protein content of muscle compared to control group (Figure 15). Although decreasing trend in protein content was observed at 40 and 50 mg/kg Fe-NPs fed fish groups but these were not below the value obtained in control group of fish. Lipid content was also significantly ($P < 0.05$) influenced by addition of Fe-NPs in diets where the highest lipid content was noted for the fish group that fed diets containing 30 mg/kg feed of Fe-NPs. Addition of Fe-NPs also caused the ash and moisture content of muscle to be significantly ($P < 0.05$) increased from control group and the highest value was obtained from fishes fed diets containing 50 mg/kg feed of Fe-NPs.

Cu-NPs enriched fish diets caused a significantly ($P < 0.05$) increased protein (9.53±0.02%) and lipid (2.85±0.03%) content in fish muscle at a dose of 20 mg/kg feed of Cu-NPs and after that increase in doses of this NP caused a reduction in protein and lipid content. However, at the dose of 50 mg/kg feed of Cu-NPs showed lower value of protein and lipid compared to control group. Here also ash and moisture of fish muscle fed diets containing diets enriched with Cu-NPs showed significant differences ($P < 0.05$) from control group and increasing trend with increase in doses of Cu-NPs in feed.

Supplementation of Zn-NPs also influenced the proximate composition of fish muscle. In case of Zn-NPs mediated fish diets, significantly ($P < 0.05$) higher protein (12.06±0.02%) and lipid (3.19±0.02%) content were obtained from fish group fed 40 mg/kg feed of Zn-NPs enriched diets and the lower from control group (protein, 4.74±0.02; lipid, 2.18±0.01%). Higher dose treated fish (50 mg/kg feed of Cu-NPs) showed significantly ($P < 0.05$) higher ash and moisture level when compared to control group indicating absorption of more Zn-NPs with increasing doses. Although carbohydrate content showed significant difference ($P < 0.05$) among the NPs feed fish groups and control group, the values were not differ much.

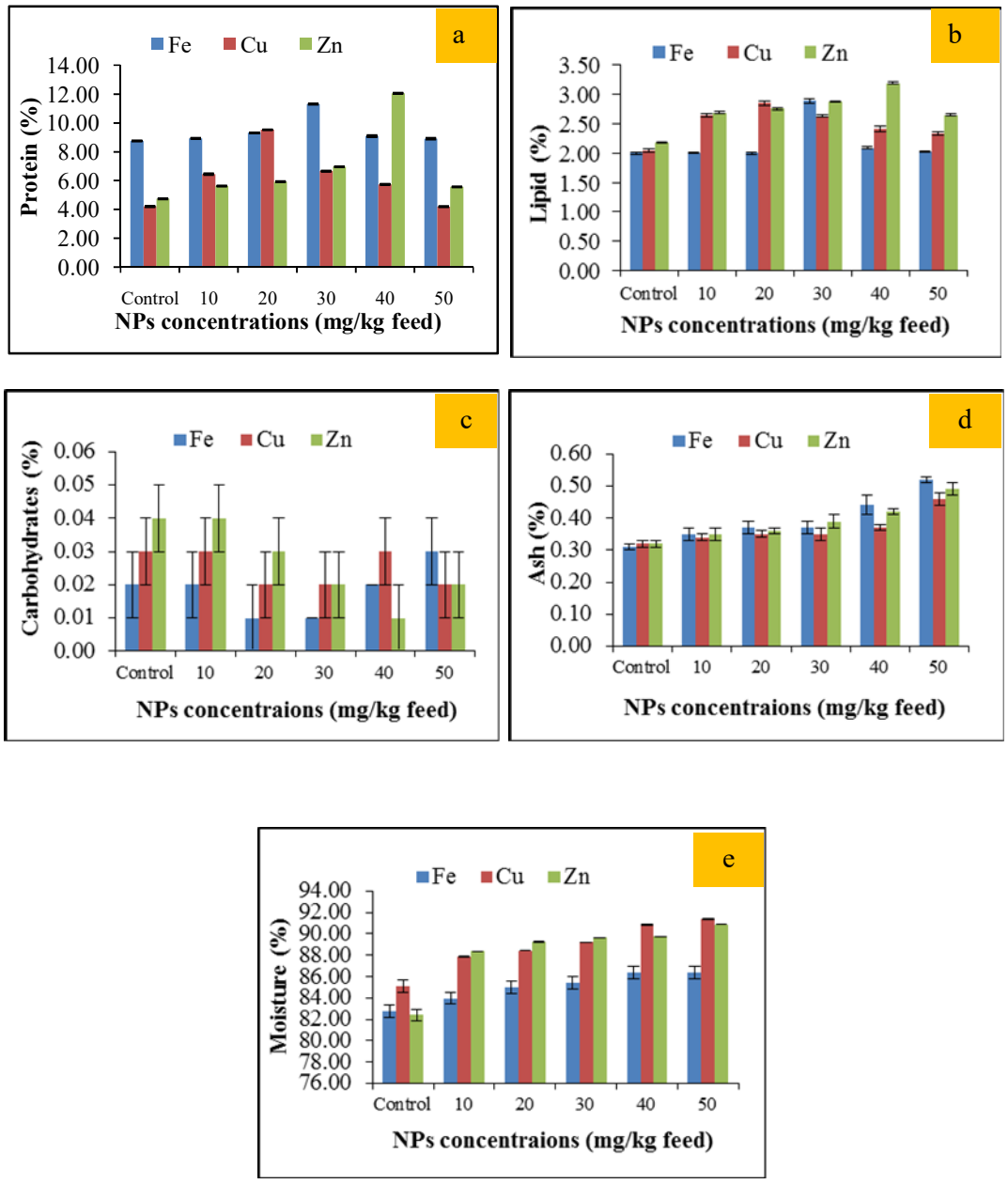


Figure 15. Proximate composition of muscle of L. *rohita* fed diets with different concentrations of Fe-NPs, Cu-NPs and Zn-NPs (a, protein; b, lipid; c, carbohydrate%; d, ash % and e, moisture %).

11.2.5 Hematological parameters

Blood parameters of *L. rohita* fed diets supplemented with different NPs at different doses are shown in Table 15. Significant ($P < 0.05$) increase in RBC level was observed with increasing the doses of Fe-NPs in diets compared to control group. WBC content also varied significantly ($P < 0.05$) among the feed groups and control group; whereas significantly ($P < 0.05$) higher WBC content was observed in fish group fed diets enriched with 30 mg/kg feed of Fe-NPs. However, further increase in doses of Fe-NPs reduced the immune system of fish which was evident by decreasing trend of WBC content. Similar to RBC, hemoglobin content was also found to increase with increasing Fe-NPs doses in diets. Significant ($P < 0.05$) increase in total protein content was observed in the Fe-NPs supplemented diet fed fish compared to control group and significantly higher total protein content was noted for fishes fed 30 mg/kg feed of Fe-NPs. Albumin and globulin content also influenced by addition of Fe-NPs in diets compared to control group whereas lowest albumin content were found to decrease with increase in doses of Fe-NPs in diets. However, the highest globulin (3.85±0.02 gm/dl) content was found in fish group fed 30 mg/kg feed of Fe-NPs and the lowest in control group (2.57±0.01 gm/dl).

Addition of Cu-NPs in diets significantly ($P < 0.05$) influenced the RBC, WBC, hemoglobin, albumin and globulin content of fish blood. A dose of 20 mg/kg feed Cu-NPs significantly increase the hematological parameters compared to other feed group of fishes and control group. However, Cu-NPs doses of 30, 40 and 50 mg/kg feed showed significantly ($P < 0.05$) reducing trend in RBC, WBC, hemoglobin, total protein and globulin content compared to the fish group fed 20 mg/kg feed of Cu-NPs.

In case of fish groups fed diets containing Zn-NPs showed significantly ($P < 0.05$) highest RBC (114.73±0.02%), WBC (347.55±0.02%), hemoglobin (5.15±0.02%), total protein (15.83±0.02 g/dl) and globulin (3.89±0.02 g/dl) content in 40 mg/kg feed of Zn-NPs enriched diet compared to other doses and control group (Table 15). However, increase in doses up to 50 mg/kg feed of Zn-NPs significantly ($P < 0.05$) reduced these aforementioned blood parameters. Albumin content was found to significantly ($P < 0.05$) reduced with increasing Zn-NPs content in diets compared to control group.

Table 15. Hematological parameters of *L. rohita* fed different NPs enriched diets.

NPs	Parameters	Doses of NPs (mg/kg feed)					
		Control	10	20	30	40	50
Fe-NPs	RBC (%)	22.56±0.01^{f}	72.77±0.02^{e}	97.67±0.02^{d}	112.27±0.02^{c}	113.76±0.02^{b}	117.25±0.02^{a}
	WBC (%)	132.58±0.03^{f}	265.77±0.02^{d}	312.85±0.02^{b}	372.85±0.01^{a}	285.76±0.02^{c}	234.76±0.01^{e}
	Hemoglobin (%)	3.15±0.03^{f}	3.56±0.02^{e}	4.25±0.02^{d}	4.96±0.02^{c}	5.23±0.02^{b}	5.76±0.02^{a}
	Total protein (gm/dl)	12.59±0.02^{f}	12.78±0.02^{e}	13.58±0.02^{c}	15.14±0.02^{a}	14.25±0.02^{b}	13.47±0.02^{d}
	Albumin (gm/dl)	1.88±0.01^{a}	1.84±0.01^{b}	1.76±0.02^{c}	1.56±0.02^{d}	1.54±0.01^{d}	1.35±0.01^{e}
	Globulin (gm/dl)	2.57±0.01^{e}	3.14±0.02^{d}	3.47±0.02^{b}	3.85±0.02^{a}	3.46±0.01^{b}	3.33±0.02^{c}
Cu-NPs	RBC (%)	22.85±0.03^{f}	108.54±0.02^{c}	112.39±0.02^{a}	110.44±0.01^{b}	107.72±0.02^{d}	101.53±0.02^{e}
	WBC (%)	131.53±0.02^{f}	203.47±0.02^{d}	264.53±0.02^{a}	225.25±0.02^{b}	206.84±0.01^{c}	201.74±0.01^{e}
	Hemoglobin (%)	3.26±0.01^{f}	4.26±0.01^{b}	4.76±0.01^{a}	4.19±0.02^{c}	3.59±0.01^{d}	3.34±0.02^{e}
	Total protein (gm/dl)	12.57±0.01^{f}	13.35±0.02^{d}	14.73±0.02^{a}	14.08±0.01^{b}	13.72±0.02^{c}	12.49±0.02^{e}
	Albumin (gm/dl)	1.76±0.01^{a}	1.63±0.02^{b}	1.58±0.01^{c}	1.53±0.01^{d}	1.44±0.01^{e}	1.32±0.01^{f}
	Globulin (gm/dl)	2.56±0.01^{e}	3.55±0.01^{c}	3.88±0.01^{a}	3.58±0.02^{b}	3.55±0.01^{c}	3.47±0.02^{d}
Zn-NPs	RBC (%)	25.57±0.01^{f}	75.73±0.02^{e}	87.26±0.02^{d}	92.56±0.01^{c}	114.73±0.02^{a}	102.23±0.02^{b}
	WBC (%)	132.56±0.01^{f}	258.27±0.02^{e}	285.85±0.02^{c}	291.38±0.01^{b}	347.55±0.02^{a}	261.27±0.02^{d}
	Hemoglobin (%)	3.36±0.01^{f}	3.65±0.22^{e}	4.59±0.02^{c}	4.82±0.01^{b}	5.15±0.02^{a}	4.08±0.01^{d}
	Total protein (gm/dl)	12.84±0.02^{f}	12.95±0.02^{e}	13.63±0.01^{c}	14.85±0.02^{b}	15.83±0.02^{a}	13.18±0.01^{d}
	Albumin (gm/dl)	1.74±0.01^{a}	1.64±0.02^{b}	1.58±0.01^{c}	1.64±0.01^{b}	1.47±0.02^{d}	1.26±0.01^{e}
	Globulin (gm/dl)	2.63±0.02^{f}	3.23±0.02^{e}	3.54±0.02^{c}	3.68±0.01^{b}	3.89±0.02^{a}	3.27±0.02^{d}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$). Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.4.6 Blood lipid profile

Blood lipid profile of *L. rohita* fed diets enriched with NPs is shown in Table 16. Blood total cholesterol was significantly ($P < 0.05$) influenced by addition of Fe-NPs in diets where diets containing 10, 20 and 30 mg/kg feed of Fe-NPs showed increasing trend in total cholesterol level compared to control group. However, diets containing Fe-NPs at doses of 40 and 50 mg/kg feed of Fe-NPs showed decreasing trend and even significantly ($P < 0.05$) differ from control group. HDL was significantly ($P < 0.05$) and positively influenced by the addition of Fe-NPs in diets compared to control group and showed an increasing trend of 10 > 20 > 30 and decreasing towards 40 < 50 mg/kg feed of Zn-NPs in diets. Decreasing trend in LDL was observed with increasing the doses of Fe-NPs and the higher value was obtained from control group. On the other hand, triglyceride content was found to increase up to 30 mg/kg feed of Fe-NPs and after that sudden decrease was noted due to toxic effect at higher doses.

In case of Cu-NPs supplemented diets, total cholesterol, LDL and triglyceride were found significantly ($P < 0.05$) influenced compared to control group and the highest value was obtained from fish group fed diets containing 20 mg/kg feed of Cu-NPs. However, increase in doses of Cu-NPs in diets significantly ($P < 0.05$) reduced the value of the aforementioned parameters and at the doses of 30, 40 and 50 mg/kg feed of Cu-NPs showed lower value even from control group. Similar to Fe-NPs mediated diets, addition of Cu-NPs in diets of *L. rohita* also increased the level of HDL up to 20 mg/kg feed of Cu-NPs and the lower value were obtained from control group of fishes.

Toxicity of Zn-NPs was evident at the dose of 50 mg/kg feed of Zn-NPs where the total cholesterol level was significantly ($P < 0.05$) reduce even from control group. Similar to Fe-NPs and Cu-NPs mediated diets, Zn-NPs enriched diets also showed significantly ($P < 0.05$) increasing trend in HDL and triglyceride level up to 40 mg/kg feed of Zn-NPs and decreasing trend in LDL compared to control group.

Table 16. Blood Cholesterol, HDL, LDL, triglycerides and alkaline phosphates of *L. rohita.*

NPs	Parameters	Doses of NPs (mg/kg feed)					
		Control	10	20	30	40	50
Fe-NPs	Total cholesterol (mg/dl)	213.43±0.02^{d}	215.75±0.04^{c}	217.33±0.02^{b}	219.15±0.02^{a}	209.67±0.02^{e}	205.12±0.56^{f}
	HDL (mg/dl)	51.36±0.02^{f}	52.46±0.01^{e}	53.97±0.02^{d}	55.14±0.02^{a}	54.37±0.02^{b}	54.17±0.02^{c}
	LDL (mg/dl)	147.59±0.02^{a}	143.31±0.02^{b}	142.17±0.02^{c}	141.39±0.02^{d}	140.55±0.02^{e}	140.28±0.03^{f}
	Triglycerides (mg/dl)	151.05±0.03^{f}	156.05±0.01^{e}	160.33±0.02^{d}	167.53±0.01^{a}	160.97±0.02^{c}	161.56±0.02^{b}
Cu-NPs	Total cholesterol (mg/dl)	216.17±0.02^{b}	216.24±0.03^{b}	216.59±0.02^{a}	216.08±0.01^{b}	214.70±0.23^{c}	214.25±0.02^{d}
	HDL (mg/dl)	54.13±0.02^{f}	55.46±0.01^{e}	56.47±0.02^{a}	55.84±0.01^{b}	55.75±0.03^{c}	55.57±0.01^{d}
	LDL (mg/dl)	154.15±0.02^{a}	153.63±0.02^{b}	153.26±0.01^{c}	153.16±0.01^{d}	152.74±0.01^{e}	152.33±0.02^{f}
	Triglycerides (mg/dl)	154.19±0.02^{f}	162.53±0.02^{b}	164.97±0.02^{a}	161.65±0.01^{c}	158.88±0.02^{d}	154.86±0.02^{e}
Zn-NPs	Total cholesterol (mg/dl)	214.65±0.02^{d}	215.02±0.01^{c}	215.14±0.02^{b}	215.15±0.02^{b}	215.26±0.01^{a}	214.17±0.02^{e}
	HDL (mg/dl)	53.83±0.02^{f}	54.19±0.02^{e}	54.73±0.02^{d}	55.29±0.02^{c}	56.34±0.02^{a}	55.39±0.02^{b}
	LDL (mg/dl)	154.75±0.01^{a}	154.15±0.02^{b}	153.75±0.01^{c}	153.38±0.01^{d}	152.47±0.01^{e}	152.26±0.02^{f}
	Triglycerides (mg/dl)	153.27±0.02^{f}	154.26±0.01^{e}	163.19±0.02^{c}	164.66±0.01^{b}	168.19±0.02^{a}	161.14±0.02^{d}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$). HDL = High density lipoprotein, LDL = Low density lipoprotein, Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.4.7 Serum enzyme profile

Data in Table 17 showed a significant ($P < 0.05$) increase in the serum level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in all treated groups of Fe-NPs, Cu-NPs and Zn-NPs enriched diets compared to control group of fishes. However, amylase, lipase and protease were found significantly ($P < 0.05$) increase in fish groups fed diets containing Fe-NPs supplemented diets up to 30 mg/kg feed of Fe-NPs compared to control group. After that, further increase in doses of Fe-NPs in feed significantly ($P < 0.05$) reduced the above mentioned enzymatic activities. Enzymatic activities in fish serum fed diets supplemented with Cu-NPs showed gradual increase up to 20 mg /kg feed of Cu-NPs. Further increase in dose gradually reduced the activity of amylase, lipase and protease in fish serum. However, fish fed diet containing 50 mg/kg feed of Cu-NPs did not showed significant ($P < 0.05$) difference from control group. In fishes fed the diets enriched with Zn-NPs showed the activity of amylase, lipase and protease in order of 40 > 30 > 20 > 50 > 10 mg/kg feed of Zn-NPs and these groups were significantly ($P < 0.05$) different from control group. Alkaline phosphatase (ALP) was found to increase with increasing the doses of NPs (Fe-NPs, Cu-NPs and Zn-NPs) in diets compared to control group. Although ALP showed significant ($P < 0.05$) difference among the control group and Cu-NPs enriched diets, no significant differences ($P < 0.05$) were observed among the diets containing different doses of Cu-NPs in feed.

Table 17. Serum enzymes profile of *L. rohita* fed different NPs enriched diets.

NPs	Parameters	Doses of NPs (mg/kg feed) Control	10	20	30	40	50
Fe-NPs	AST (U/L)	31.25±0.02^{f}	31.37±0.02^{e}	32.18±0.02^{d}	32.53±0.02^{c}	32.58±0.02^{b}	32.78±0.02^{a}
	ALT (U/L)	35.36±0.01^{f}	35.84±0.02^{e}	35.87±0.02^{d}	36.15±0.02^{c}	37.26±0.02^{b}	37.34±0.02^{a}
	Amylase (U/L)	0.27±0.02^{f}	0.76±0.02^{c}	0.83±0.02^{b}	1.26±0.01^{a}	0.67±0.02^{d}	0.64±0.01^{e}
	Lipase (U/L)	0.24±0.01^{d}	0.35±0.02^{c}	0.47±0.02^{b}	0.55±0.01^{a}	0.24±0.01^{d}	0.22±0.02^{d}
	Protease (U/L)	0.73±0.02^{d}	0.73±0.02^{d}	0.73±0.02^{d}	2.08±0.01^{a}	1.23±0.02^{c}	1.26±0.02^{b}
	ALP (mg/dl)	13.33±0.02^{f}	13.64±0.01^{e}	14.08±0.01^{d}	14.17±0.02^{c}	14.25±0.02^{b}	14.33±0.02^{a}
Cu-NPs	AST (U/L)	31.66±0.14^{d}	31.73±0.02^{d}	31.84±0.01^{c}	31.85±0.02^{c}	32.19±0.02^{b}	32.53±0.02^{a}
	ALT (U/L)	35.54±0.02^{f}	36.72±0.02^{e}	36.75±0.01^{d}	36.85±0.02^{c}	37.19±0.02^{b}	37.54±0.02^{a}
	Amylase (U/L)	0.34±0.02^{f}	0.97±0.02^{d}	1.73±0.02^{a}	1.14±0.01^{b}	1.07±0.02^{c}	0.74±0.01^{e}
	Lipase (U/L)	0.29±0.01^{e}	0.56±0.01^{cd}	0.74±0.02^{a}	0.64±0.01^{b}	0.57±0.02^{c}	0.54±0.01^{d}
	Protease (U/L)	0.73±0.02^{d}	0.74±0.01^{d}	1.23±0.02^{a}	1.15±0.01^{b}	1.06±0.01^{c}	0.73±0.02^{d}
	ALP (mg/dl)	14.12±0.01^{b}	14.16±0.01^{a}	14.16±0.01^{a}	14.16±0.01^{a}	14.16±0.02^{a}	14.17±0.02^{a}
Zn-NPs	AST (U/L)	32.53±0.02^{f}	32.67±0.02^{e}	32.69±0.02^{d}	32.84±0.01^{c}	32.94±0.01^{b}	33.21±0.02^{a}
	ALT (U/L)	35.27±0.01^{f}	35.68±0.01^{e}	35.74±0.01^{d}	36.84±0.02^{c}	37.14±0.01^{b}	37.43±0.02^{a}
	Amylase (U/L)	0.37±0.02^{e}	0.75±0.01^{d}	0.85±0.01^{c}	0.92±0.01^{b}	1.67±0.01^{a}	0.74±0.01^{d}
	Lipase (U/L)	0.33±0.02^{f}	0.43±0.02^{d}	0.48±0.01^{c}	0.54±0.01^{b}	0.73±0.02^{a}	0.36±0.01^{e}
	Protease (U/L)	0.73±0.01^{e}	0.85±0.02^{d}	0.91±0.01^{c}	0.97±0.02^{b}	1.23±0.02^{a}	0.86±0.01^{d}
	ALP (mg/dl)	14.08±0.02^{c}	14.12±0.01^{b}	14.13±0.02^{b}	14.13±0.02^{b}	14.14±0.02^{b}	15.15±0.02^{a}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$). ALT = alanine aminotransferase, AST = aspartate aminotransferase, ALP = Alkaline phosphatase, Fe-NPs = Iron nanoparticles, Cu-NPs = Cupper nanoparticles, Zn-NPs = Zinc nanoparticles.

11.5 (Experiment-3): Effect of alloy (Fe-NPs and Zn-NPs) on growth and physiology of *B. gonionotus* and *L .rohita*.

11.3.1 Water quality

Water quality parameters were maintained as temperature 27.73°C to 28.08°C, DO 5.92 mg/l to 6.16 mg/l, pH 6.98 to 7.13 and ammonia 0.001 mg/l to 0.002 mg/l throughout the study period. There were no significant differences ($P < 0.05$) in water quality parameters among the different doses of NPs during the study period (Table 18).

11.3.2 Growth parameters and survival

Growth parameters of *B. gonionotus* and *L .rohita* fed diets supplemented with alloys of Fe-NPs and Zn-NPs at different doses are shown in Table 19. At the start of the experiment, the initial weight of the experimental fishes was homogeneous and was not significantly different ($P < 0.05$) from each other. However, at the end of the experiment significant ($P < 0.05$) influence of the addition of alloys in fish diets were observed throwgh the increase in growth performance compared to control group for *B. gonionotus*. Final weight, weight gain, % weight gain, ADG and SGR were found to increase significantly ($P < 0.05$) up to a dose of 30 mg/kg feed of alloy, whereas further increase in doses significantly ($P < 0.05$) reduced these growth performance compared to the fish group fed diets containing 10, 20 and 30 mg/kg feed of alloy. Weight gain, % gain, ADG and SGR were not significantly ($P < 0.05$) different among the fish groups fed diets enriched with 40 and 50 mg/kg feed of alloy. In case of *L. rohita* dose of 30 mg/kg feed of alloy also gave significantly ($P < 0.05$) better growth performance in terms of final weight, weight gain, % weight gain, ADG and SGR compared to other alloy enriched feed fed groups and control group. Here also increase in doses up to 40 and 50 mg/kg feed of alloy significantly ($P < 0.05$) reduced the growth performance compared to the fish groups fed diets containing 10, 20 and 30 mg/kg feed of alloy. However, growth performance of fish group fed diet containing 50 mg/kg feed of alloy was not significantly ($P < 0.05$) different from control group (Table 19). The correlation of alloy doses and final weight, weight gain and SGR were positive with R^2 values of 0.675, 0.676 and 0.727 for *B. gonionotus* (Figure 16 and 18 A) and negative correlation for *L. rohita* with R^2 values of 0.775, 0.774 and 0.821 (Figure 17 and 18 B).

Table 18. Water quality parameters.

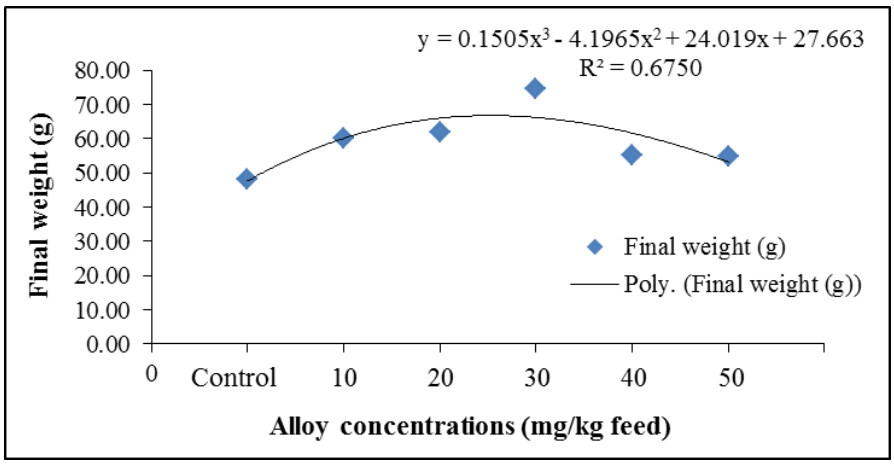
Parameters	Species	Doses of alloy (mg/l)					
		Control	10	20	30	40	50
Temperature (°C)	*B. gonionotus*	27.73±0.25[a]	28.08±0.18[a]	27.77±0.28[a]	27.94±0.14[a]	27.81±0.34[a]	27.99±0.13[a]
	L. rohita	27.99±0.11[a]	27.87±0.37[a]	27.95±0.14[a]	28.05±0.33[a]	27.76±0.27[a]	27.97±0.25[a]
DO (mg/l)	*B. gonionotus*	5.94±0.04[a]	6.02±0.20[a]	5.99±0.21[a]	5.92±0.05[a]	6.16±0.07[a]	6.04±0.19[a]
	L. rohita	6.06±0.12[a]	6.05±0.10[a]	6.10±0.12[a]	5.92±0.22[a]	6.14±0.08[a]	5.94±0.05[a]
pH	*B. gonionotus*	7.08±0.05[a]	6.98±0.01[a]	7.06±0.09[a]	7.04±0.09[a]	6.98±0.02[a]	7.11±0.05[a]
	L. rohita	7.02±0.11[a]	7.09±0.10[a]	7.05±0.10[a]	7.01±0.04[a]	7.07±0.10[a]	7.13±0.26[a]
Ammonia (mg/l)	*B. gonionotus*	0.001±0.001[a]	0.002±0.001[a]	0.001±0.001[a]	0.001±0.001[a]	0.002±0.001[a]	0.001±0.001[a]
	L. rohita	0.002±0.000[a]	0.001±0.001[a]	0.002±0.001[a]	0.001±0.000[a]	0.002±0.001[a]	0.001±0.001[a]

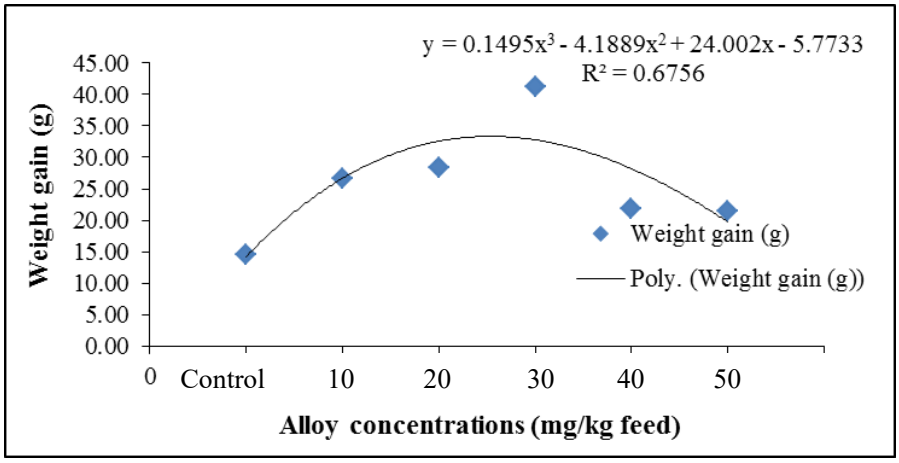
Values in the same row having same superscript letter indicates no significant difference (P > 0.05). DO = Dissolved oxygen

Table 19. Growth parameters of *B. gonionotus* and *L. rohita* fed diets enriched with alloy.

Species	Growth parameters	Doses of alloy (mg/kg feed)					
		Control	10	20	30	40	50
B. gonionotus	Initial weight (gm)	33.44±0.23[a]	33.44±0.27[a]	33.45±0.16[a]	33.44±0.17[a]	33.44±0.22a	33.47±0.20[a]
	Final weight (gm)	48.05±0.26[f]	60.14±0.27[c]	61.79±0.37[b]	74.63±0.22[a]	55.36±0.10[d]	54.88±0.30[e]
	Weight gain (gm)	14.60±0.40[e]	26.70±0.38[c]	28.34±0.51[b]	41.18±0.39[a]	21.92±0.31[d]	21.41±0.18[d]
	% weight gain	43.67±1.43[e]	79.86±1.57[c]	84.75±1.89[b]	123.15±1.78[a]	65.56±1.41[d]	63.98±0.58[d]
	ADG (gm)	0.24±0.01[e]	0.45±0.01[c]	0.47±0.01[b]	0.69±0.01[a]	0.37±0.01[d]	0.36±0.01[d]
	SGR (% bwd^{-1})	0.60±0.02[e]	0.98±0.01[c]	1.02±0.02[b]	1.34±0.02[a]	0.84±0.02[d]	0.82±0.01[d]
L. rohita	Initial weight (gm)	33.52±0.06[a]	33.53±0.32[a]	33.53±0.23[a]	33.51±0.20[a]	33.53±0.15[a]	33.52±0.24[a]
	Final weight (gm)	48.63±0.43[e]	56.73±0.36[c]	58.91±0.24[b]	68.58±0.22[a]	54.37±0.47[d]	49.23±0.79[e]
	Weight gain (gm)	15.11±0.41[e]	23.20±0.39[c]	25.38±0.15[b]	35.07±0.32[a]	20.84±0.44[d]	15.72±0.57[e]
	% weight gain	45.08±1.19[e]	69.21±1.30[c]	75.69±0.75[b]	104.66±1.49[a]	62.16±1.56[d]	46.89±1.41[e]
	ADG (gm)	0.25±0.01[e]	0.39±0.01[c]	0.42±0.01[b]	0.59±0.01[a]	0.35±0.01[d]	0.26±0.01[e]
	SGR (% bwd^{-1})	0.62±0.02[e]	0.88±0.02[c]	0.94±0.01[b]	1.19±0.01[a]	0.81±0.02[d]	0.64±0.02[e]

ADG = Average daily gain, SGR = Specific growth rate, NPs = Nanoparticles. Values with different superscripts in the same row for each fish species indicate significant differences (P < 0.05).





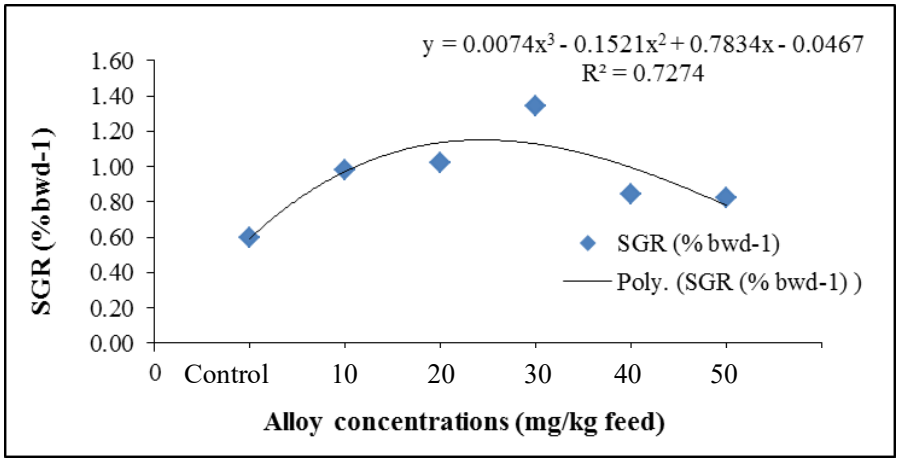
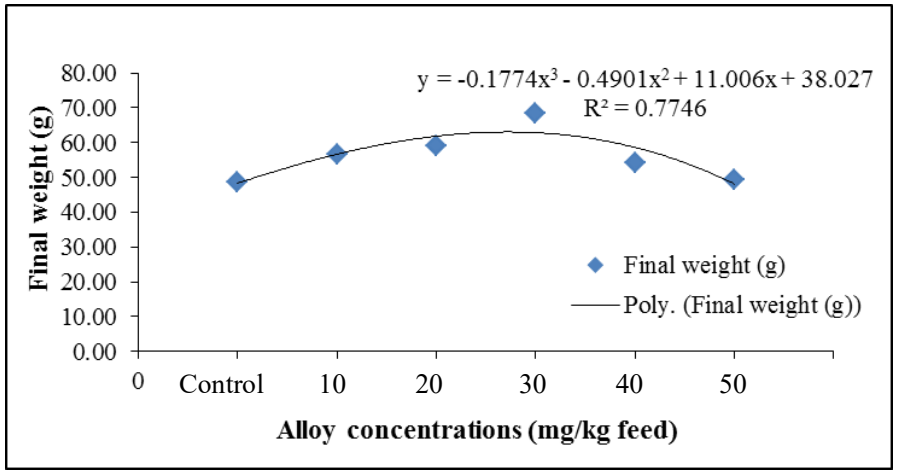
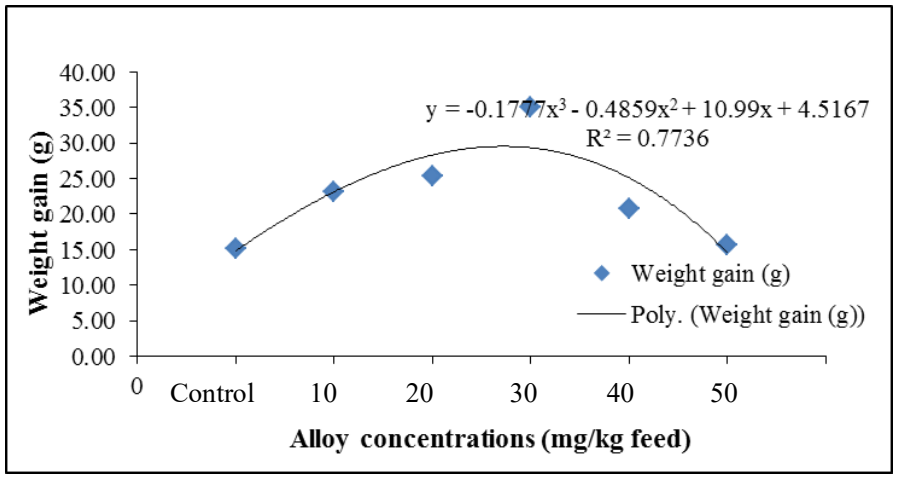


Figure 16. Relationship between different doses of alloy in feed with growth performance (final weight, weight gain and SGR) of *B. gonionotus.*





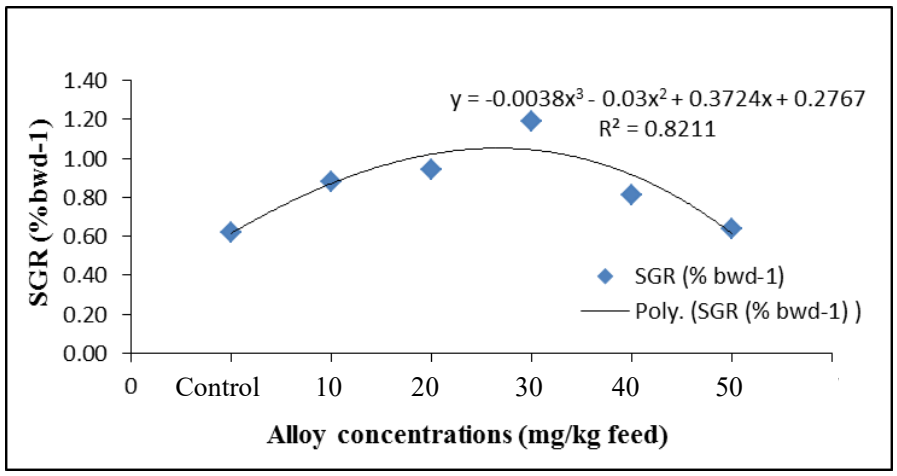


Figure 17. Relationship between different doses of alloy in feed with growth performance (final weight, weight gain and SGR) of *L. rohita.*

Figure 18. Photo graphical representation of (A) *B. gonionotus* (B) *L. rohita* groups fed alloy supplemented diets.

11.5.3 Feed utilization parameters

In the present experiment, supplementation of alloys in diets significantly enhanced the feed utilization parameters of *B. gonionotus* and *L. rohita* (Table 20). In case of *B. gonionotus*, better performance of FCR was observed in fish group fed diet containing 30 mg/kg feed of alloy (1.46±0.02) compared to control group (4.12±0.14). Similar to growth performance, further increase in dose of alloy in diets up to 40 and 50 mg/kg feed significantly increased the FCR compared to 10, 20 and 30 mg/kg feed alloy fed fish groups. However, fish fed diet containing 10 and 50 mg/kg feed of alloy was not significantly different ($P < 0.05$) from each other. Significantly ($P < 0.05$) higher PER and PGR were observed in fish group fed 30 mg/kg feed of alloy compared to other NPs fed and control group. However, PPV showed its highest value in fish group fed 40 mg/kg feed of alloy and lowest in control group. In case of *L. rohita*, better performance in FCR was noted for the fish group feed 30 mg/kg feed of alloy, where also FCE and PER were found to give significantly ($P < 0.05$) higher value compared to other groups. Addition of 50 mg/kg feed of alloy caused significantly ($P < 0.05$) impact on PPV and reduced the value even lower than control group. However, PGR was not significantly ($P < 0.05$) different among 10, 50 mg/kg feed of alloy fed fish group and control group. Significant difference ($P < 0.05$) was also not observed between 20 and 30 mg/kg feed of alloy fed fish groups. The highest value of PGR were recorded in fish group fed 40 mg/kg feed of alloy.

Table 20. Feed utilization parameters of *B. gonionotus* and *L. rohita* fed diets enriched with alloy.

Species	Parameters	Doses of alloy (mg/kg feed)					
		Control	10	20	30	40	50
B. gonionotus	FCR	4.12±0.14^{a}	2.75±0.06^{b}	2.12±0.05^{d}	1.46±0.02^{e}	2.26±0.04^{c}	2.82±0.03^{b}
	FCE	0.24±0.01^{e}	0.37±0.01^{d}	0.47±0.01^{b}	0.68±0.01^{a}	0.44±0.01^{c}	0.36±0.01^{d}
	PER	0.73±0.03^{e}	1.10±0.03^{d}	1.42±0.03^{b}	2.07±0.03^{a}	1.34±0.03^{c}	1.07±0.01^{d}
	PPV (%)	10.58±0.25^{f}	15.40±0.32^{d}	19.32±0.47^{c}	27.65±0.25^{b}	29.30±0.45^{a}	13.82±0.10^{e}
	PGR (%)	1.27±0.03^{f}	1.66±0.03^{c}	2.33±0.01^{a}	2.38±0.05^{a}	1.88±0.05^{b}	1.54±0.01^{d}
L. rohita	FCR	3.99±0.11^{a}	2.90±0.07^{c}	2.38±0.02^{e}	1.72±0.03^{f}	2.60±0.05^{d}	3.84±0.11^{b}
	FCE	0.25±0.01^{e}	0.35±0.01^{d}	0.42±0.01^{b}	0.58±0.01^{a}	0.38±0.01^{c}	0.26±0.01^{e}
	PER	0.76±0.02^{e}	1.05±0.03^{d}	1.27±0.01^{b}	1.76±0.03^{a}	1.16±0.02^{c}	0.79±0.03^{e}
	PPV (%)	6.66±4.01^{c}	8.06±0.15^{c}	14.21±6.97^{b}	16.62±0.16^{b}	26.98±0.28^{a}	6.40±0.16^{c}
	PGR (%)	1.03±0.44^{c}	1.24±0.02^{c}	1.75±0.51^{b}	1.97±0.02^{b}	2.57±0.02^{a}	1.04±0.03^{c}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$).

FCR = Feed conversion ratio, FCE = Feed conversion efficiency, PER = Protein efficiency ratio, PPV = Protein productive value, PGR = Protein growth rate

11.5.4 Proximate composition of muscle

Supplementation of alloy in diets of *B. gonionotus* significantly ($P < 0.05$) influence the protein content of muscle in a decreasing trend of 30 > 40> 20 > 10 > 50 mg/kg feed of alloy > control (Figure 19). Lipid content was also significantly ($P < 0.05$) varied among the fish groups fed diets containing NPs compared to control group, whereas the highest lipid content was noted in fish group fed diet enriched with 30 mg/kg feed of alloy. Ash and moisture content showed significantly ($P < 0.05$) increasing trend toward the increase in doses of alloy in diets. In case of *L. rohita* protein content was the highest in fish group fed diet enriched with 30 mg/kg feed of alloy, however, further increase in dose showed a decreasing trend in protein content compared to 30 mg/kg feed of alloy fed fish group. Similar observation was also found for lipid content of muscle whereas significantly ($P < 0.05$) highest value was recorded in fish group fed 30 mg/kg feed of alloy. Ash and moisture content here also showed significantly ($P < 0.05$) increasing trend with increasing the dose of alloy compared to control group. However, during the experiment, carbohydrate content of both *B. gonionotus* and *L. rohita* did not showed any significant difference ($P < 0.05$) among the alloy fed and control group.

11.5.5 Hematological parameters

Blood parameters of two experimental fishes fed different doses of alloys are shown in Table 21. Significant ($P < 0.05$) increase in RBC was observed both for *B. gonionotus* and *L. rohita* with increase in doses of alloy in diet compared to control group. However, WBC (*B. gonionotus*, 375.95±0.02%; *L. rohita*, 379.45±0.02%), hemoglobin (*B. gonionotus*, 4.88±0.01%; *L. rohita*, 5.74±0.01%), total protein (*B. gonionotus*, 15.24±0.03 gm/dl; *L. rohita*, 16.26±0.01 gm/dl) and globulin (*B. gonionotus*, 3.95±0.02; *L. rohita*, 3.86±0.01 gm/dl) showed an increasing trend up to the dose of 30 mg/kg alloy of diet and after that values of above parameters were found in decreasing trend with increasing level of alloy in diet. During the study period, another important parameter albumin was found in decreasing trend towards the increasing dose of alloy in diet for both the fish species.

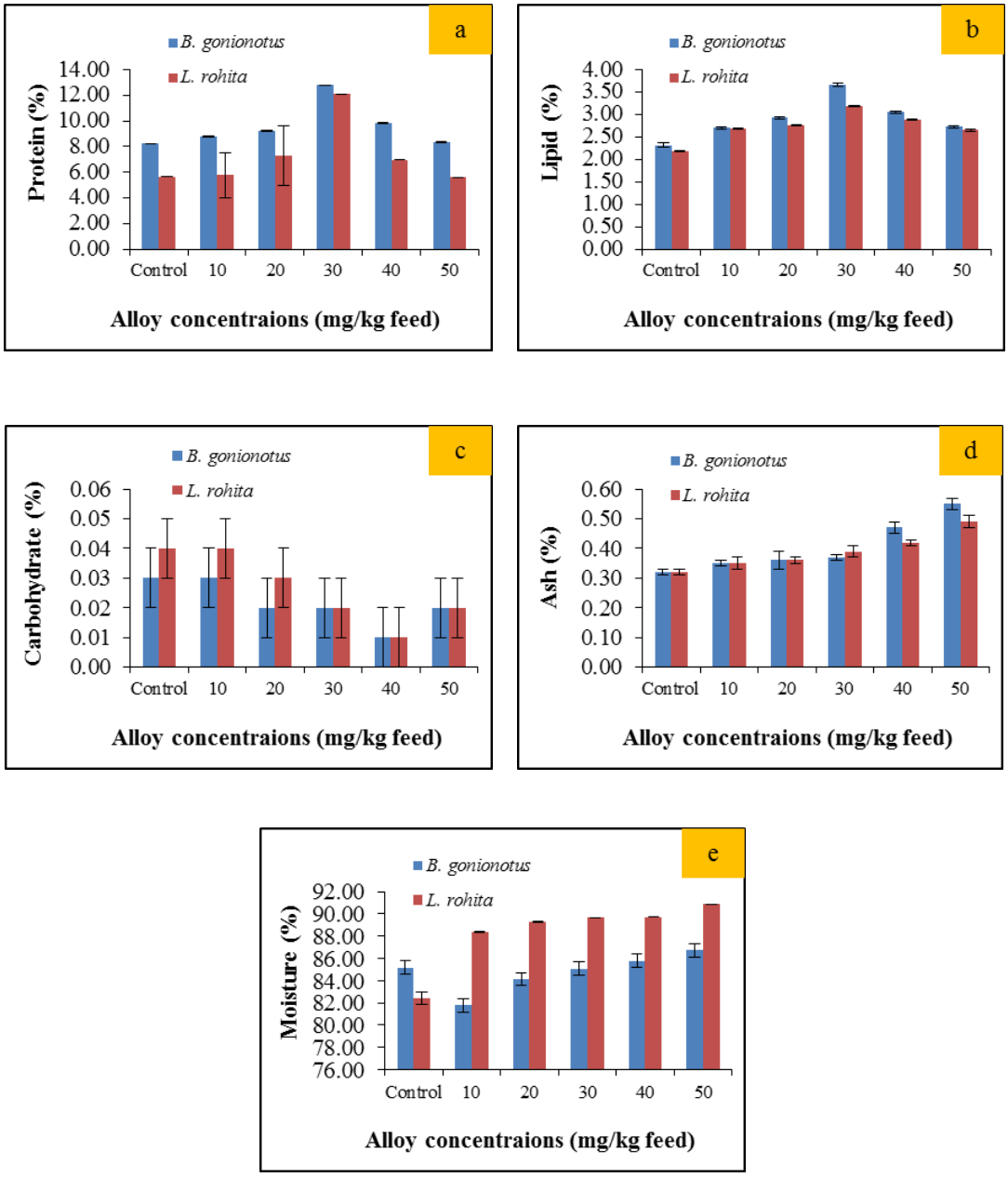


Figure 19. Proximate composition of muscle of *B. gonionotus* and *L. rohita* fed diets with different doses of alloy (a, protein; b, lipid; c, carbohydrate; d, ash and e, moisture).

Table 21. Hematological parameters of *B. gonionotus* and *L. rohita* fed diets enriched with alloy.

Species	Parameters	Doses of alloy (mg/kg feed)					
		Control	10	20	30	40	50
B. gonionotus	RBC (%)	22.75±0.03^{f}	71.58±0.03^{e}	98.77±0.02^{d}	113.55±0.03^{c}	115.55±0.03^{b}	117.44±0.03^{a}
	WBC (%)	133.65±0.03^{f}	268.87±0.02^{c}	310.39±0.02^{b}	375.95±0.02^{a}	252.50±0.02^{d}	243.59±0.02^{e}
	Hemoglobin (%)	3.24±0.03^{f}	3.69±0.02^{e}	4.35±0.02^{c}	4.88±0.01^{b}	4.19±0.02^{d}	5.15±0.03^{a}
	Total protein (gm/dl)	12.74±0.03^{f}	12.84±0.02^{e}	13.66±0.02^{d}	15.24±0.03^{a}	14.36±0.02^{b}	14.15±0.03^{c}
	Albumin (gm/dl)	1.88±0.01^{a}	1.87±0.02^{a}	1.69±0.02^{b}	1.67±0.02^{b}	1.45±0.02^{c}	1.37±0.02^{d}
	Globulin (gm/dl)	2.63±0.02^{e}	3.25±0.02^{d}	3.54±0.03^{c}	3.95±0.02^{a}	3.75±0.03^{b}	3.26±0.02^{d}
L. rohita	RBC (%)	23.67±0.02^{f}	78.59±0.02^{e}	105.43±0.02^{d}	112.73±0.02^{c}	116.56±0.01^{b}	127.54±0.01^{a}
	WBC (%)	135.63±0.02^{f}	257.84±0.01^{c}	321.35±0.02^{b}	379.45±0.02^{a}	256.47±0.02^{d}	232.55±0.02^{e}
	Hemoglobin (%)	3.42±0.01^{f}	3.66±0.01^{e}	4.32±0.02^{d}	5.74±0.01^{a}	5.64±0.02^{b}	5.38±0.01^{c}
	Total protein (gm/dl)	12.93±0.02^{f}	13.95±0.02^{d}	14.17±0.02^{c}	16.26±0.01^{a}	14.33±0.02^{b}	13.54±0.02^{e}
	Albumin (gm/dl)	1.87±0.01^{a}	1.84±0.01^{b}	1.76±0.01^{c}	1.55±0.02^{d}	1.56±0.01^{d}	1.35±0.01^{e}
	Globulin (gm/dl)	2.67±0.02^{f}	3.27±0.01^{d}	3.54±0.01^{c}	3.86±0.01^{a}	3.73±0.02^{b}	3.24±0.02^{e}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$).

11.5.6 Blood lipid profile

During the study period, total cholesterol and triglycerides of blood were found to increase with increasing the dose of alloy in diets and reached its highest value for fishes fed diets containing 30 mg/kg feed of alloy for both *B. gonionotus* and *L. rohita*. However, further increase in dose of alloy in diets significantly ($P < 0.05$) reduced the cholesterol level of blood for both the animals. HDL and LDL also showed significant ($P < 0.05$) variation among the alloy enriched diet groups and control group; whereas HDL showed an increasing trend with increasing the dose of alloy in diets up to 30 mg/kg feed of alloy and decreasing trend was noted for LDL content of blood for both *B. gonionotus* and *L. rohita* ($P < 0.05$) in Table 22.

11.5.7 Serum enzyme profile

During the study period, the stress indicator enzymes (alanine aminotransferase, ALT; aspartate aminotransferase, AST; amylase; lipase; protease and Alkaline phosphatase, ALP) of serum were found to increase with increasing alloy doses in diets and therefore, the alloy fed fish groups were significantly ($P < 0.05$) different from their control group for both *B. gonionotus* and *L. rohita* (Table 23). However, in case of *L. rohita*, ALP was found not significantly ($P < 0.05$) different in fish groups fed diets containing 10, 20, 30 and 40 mg/kg feed of alloy. On the other hand, enzymes such as amylase, lipase and protease were also found to vary significantly ($P < 0.05$) among the fish groups and significant ($P < 0.05$) increase of these parameters were observed up to a dose of 30 mg/kg feed of alloy fed fish group. However, further increase in dose of alloy in feed significantly reduced the activity of amylase, lipase and protease in blood of both *B. gonionotus* and *L. rohita*.

Table 22. Blood Cholesterol, HDL, LDL and triglycerides of *B. gonionotus* and *L. rohita* fed diets enriched with alloy.

Species	Parameters	Dose of alloy (mg/kg feed)					
		Control	10	20	30	40	50
B. gonionotus	Total cholesterol (mg/dl)	210.74±0.03^{e}	212.17±0.02^{d}	217.27±0.02^{b}	218.34±0.03^{a}	213.21±0.02^{c}	203.57±0.02^{f}
	HDL (mg/dl)	51.57±0.02^{d}	52.64±0.03^{c}	54.69±0.02^{a}	54.85±0.03^{a}	53.77±0.02^{b}	53.75±0.02^{b}
	LDL (mg/dl)	145.85±0.02^{a}	144.94±0.03^{b}	142.76±0.02^{c}	141.95±0.03^{d}	140.53±0.02^{e}	140.36±0.05^{f}
	Triglycerides (mg/dl)	151.24±0.03^{f}	155.45±0.02^{e}	161.17±0.02^{b}	168.39±0.02^{a}	160.85±0.03^{c}	159.47±0.02^{d}
L. rohita	Total cholesterol (mg/dl)	213.15±0.02^{d}	217.25±0.02^{c}	219.74±0.02^{b}	221.33±0.02^{a}	213.16±0.01^{d}	206.53±0.02^{e}
	HDL (mg/dl)	49.43±0.02^{f}	51.15±0.02^{e}	52.40±0.02^{d}	52.64±0.01^{c}	53.26±0.01^{b}	54.18±0.01^{a}
							)2^{f}
							)2^{b}

Table 23. Serum enzyme profile of *B. gonionotus* and *L. rohita* fed diets enriched with alloy.

Species	Parameters	Dose of alloy (mg/kg feed)					
		Basal	10	20	30	40	50
B. gonionotus	AST (U/L)	31.43±0.02^{f}	31.85±0.03^{e}	32.17±0.02^{d}	32.59±0.02^{c}	32.81±0.02^{b}	33.84±0.03^{a}
	ALT (U/L)	35.15±0.03^{f}	35.29±0.02^{e}	35.39±0.02^{d}	35.76±0.02^{c}	37.64±0.03^{b}	37.85±0.02^{a}
	Amylase (U/L)	0.31±0.03^{f}	0.57±0.02^{e}	0.97±0.02^{b}	1.40±0.02^{a}	0.78±0.03^{c}	0.65±0.02^{d}
	Lipase (U/L)	0.27±0.02^{d}	0.39±0.02^{c}	0.49±0.02^{b}	0.59±0.02^{a}	0.26±0.01^{d}	0.25±0.02^{d}
	Protease (U/L)	0.75±0.03^{d}	0.75±0.02^{d}	0.78±0.02^{d}	2.15±0.03^{a}	1.15±0.02^{b}	0.97±0.02^{c}
	ALP (mg/dl)	13.35±0.03^{f}	13.67±0.02^{e}	14.09±0.02^{d}	14.18±0.01^{c}	14.25±0.02^{b}	14.35±0.03^{a}
L. rohita	AST (U/L)	31.18±0.01^{e}	31.43±0.02^{d}	32.58±0.01^{c}	32.59±0.02^{c}	32.73±0.02^{b}	32.87±0.02^{a}
	ALT (U/L)	35.28±0.01^{f}	35.83±0.02^{e}	36.33±0.02^{d}	36.73±0.02^{c}	37.65±0.02^{b}	38.22±0.02^{a}
	Amylase (U/L)	0.27±0.01^{e}	0.58±0.02^{d}	0.92±0.01^{b}	1.62±0.02^{a}	0.74±0.01^{c}	0.25±0.01^{f}
	Lipase (U/L)	0.25±0.02^{d}	0.43±0.02^{c}	0.49±0.02^{b}	0.56±0.01^{a}	0.25±0.01^{d}	0.22±0.01^{e}
	Protease (U/L)	0.74±0.02^{e}	0.79±0.02^{d}	1.17±0.02^{b}	2.13±0.01^{a}	1.14±0.01^{c}	1.16±0.01^{bc}
	ALP (mg/dl)	14.09±0.02^{c}	14.13±0.02^{b}	14.12±0.01^{b}	14.12±0.02^{b}	14.13±0.01^{b}	15.14±0.02^{a}

Values in the same row with different superscript letter indicate significant differences ($P < 0.05$). AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = Alkaline phosphatase

11.5.8 Bioaccumulation of alloy in muscle, liver and serum

During the study period, liver was found to be the major organ to accumulate higher amount of NPs compared to muscle and serum, whereas accumulation of Zn-NPs were higher than Fe-NPs for both *B. gonionotus* (Figure 20 A) and *L. rohita* (Figure 20 B). However, the accumulation of NPs in all the organs (muscle, liver and serum) were found to be dependent on the dose of alloy in diets and the increase in dose of alloy in diets significantly ($P < 0.05$) increases the accumulations of these NPs in muscle, liver and serum of *B. gonionotus* and *L. rohita* (Table 23).

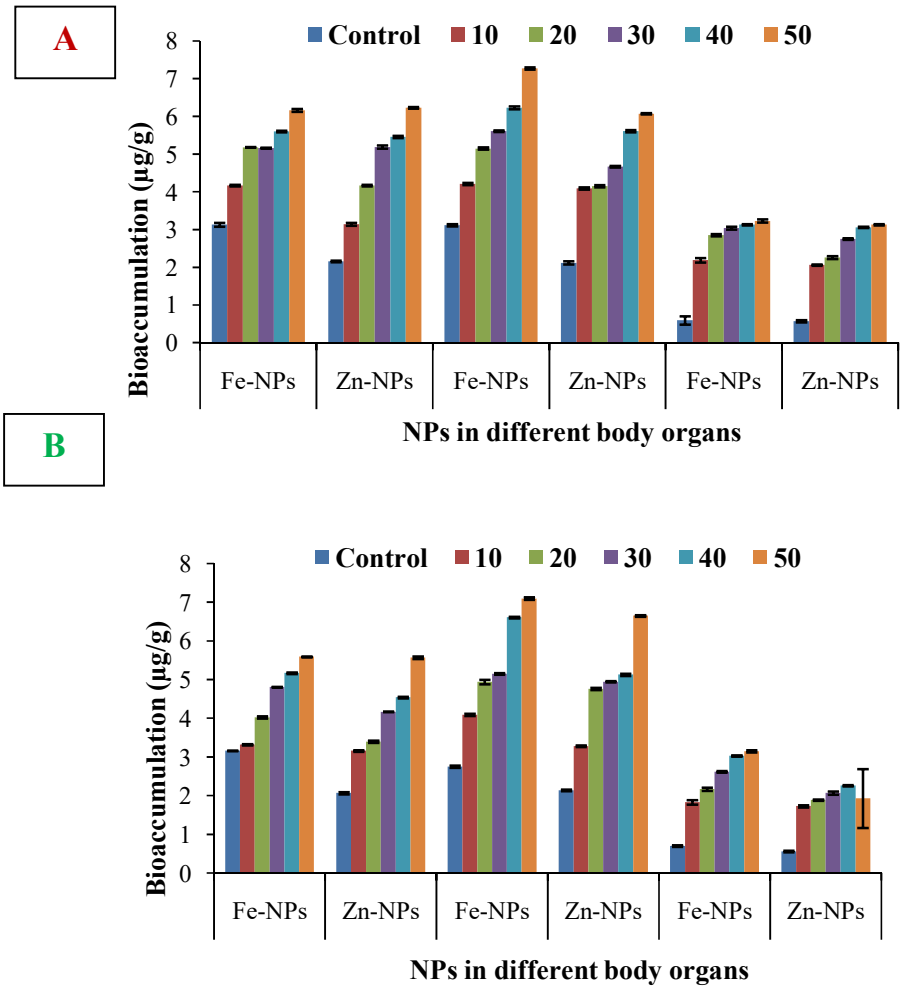


Figure 20. Muscle, liver and serum alloy concentrations of (A) *B. gonionotus* and (B) *L. rohita* fed diets enriched with alloy.

11.6 Result (Field Experiment)

11.6.1 Water quality parameters

Water quality parameters analysed during the study period are shown in Table 24. There was no significant difference (P>0.05) in the values of water quality parameters during the study period. However, comparatively higher NH_3 content was observed in the water of Pond-1 (0.014±0.014) that increased the plankton abundance and reduced transparency (26.71±5.05 cm) compared to Pond-2.

Table 24. Mean ± SD values of water quality parameters.

Parameters	Pond-1	Pond-2
Temperature (ºC)	27.67±2.47[a]	27.22±2.77[a]
Transparency (cm)	26.71±5.05[a]	27.26±3.57[a]
pH	6.91±0.23[a]	6.96±0.34[a]
DO (mg/l)	4.37±0.71[a]	4.44±0.76[a]
NH_3 (mg/l)	0.014±0.014[a]	0.012±0.009[a]
Total alkalinity (mg/l)	65.87±2.36[a]	66.03±1.72[a]

Mean values in each raw with different superscripts are significantly different (P<0.05)

11.6.2 Growth and production performance

Growth and production performance of fishes are shown in Table 25. There was no significant difference (P>0.05) in FW, %WG, SGR and survival of *L. rohita*. However, final production was varied between the studied ponds. Growth and production performance of *C. cirrhosis* were found significantly varied (P<0.05) between the two ponds, whereas only FW and production of C. c*Satla* were varied significantly (P<0.05) between two ponds during the study period. Significant (P<0.05) variation was also observed between the experimental pond in FW, %WG, SGR and production of *Ctenopharyngodon idellus.* Final production of *M. piceus* was found significantly (P<0.05) varied, whereas there was no significant difference observed in the growth and production performance of *H. molitrix* between the experimental ponds. FW, %WG and production of *L. calbasu* were also significantly varied (P<0.05) between the two ponds.

11.6.3 FCR and total production

There was no significant difference (P<0.05) in the FCR between the two experimental ponds (Figure 21). However, comparatively low FCR was observed at Pond-1 (2.12±1.51) than Pond-2 (2.80±1.80). Significantly higher (P<0.05) total production was recorded at Pond-1 (3521.97±392.76 kg/ha/180 days) than Pond-2 (2843.96±208.66 kg/ha/180 days) in (Figure 22) during the study period.

11.6.4 Economic analysis

Comparison of economic analysis between the two experimental ponds is shown in Table 26. There was no significant difference (P<0.05) in the fish fingerling cost between the two ponds, whereas feed cost was varied significantly with higher cost was observed at Pond-1 (192916.68±472.37 BDT). Although total cost was not varied significantly between the ponds, significantly higher (P<0.05) total return at fish sale was recorded at Pond-1 (814051.81±12599.35 BDT). Significantly (P<0.05) higher net return (343527.63±11024.64 BDT) was also enquired from Pond-1 which results in significantly higher BCR at Pond-1 (0.73±0.01) compared to Pond-2 (0.42±0.01).

Table 25. Growth and production performance of stocked fishes

Species	Ponds	Initial weight (kg)	Final weight (kg)	WG (%)	SGR (%/day)	Survival (%)	Production (kg/ha/180 days)
L. rohita	Pond-1	0.79±0.03[a]	1.68±0.24[a]	112.17±36.75[a]	0.41±0.10[a]	91.16±1.77[a]	1683.67±254.85[a]
	Pond-2	0.80±0.03[a]	1.45±0.11[a]	81.52±7.77[a]	0.33±0.03[a]	86.48±1.10[b]	1383.04±93.99[a]
C. cirrhosus	Pond-1	0.25±0.03[a]	1.22±0.10[a]	394.32±28.94[a]	0.89±0.04[a]	90.40±0.86[a]	678.28±62.87[a]
	Pond-2	0.25±0.02[a]	1.02±0.03[b]	306.31±43.62[b]	0.78±0.06[b]	85.14±2.13[b]	536.98±14.58[b]
C. catla	Pond-1	0.52±0.02[a]	2.04±0.17[a]	293.84±24.71[a]	0.76±0.04[a]	91.67±3.04[a]	410.72±33.03[a]
	Pond-2	0.52±0.02[a]	1.57±0.31[b]	199.74±58.88[b]	0.60±0.11[a]	86.53±3.01[a]	298.1±55.31[b]
C. idellus	Pond-1	1.02±0.02[a]	3.72±0.10[a]	265.54±6.40[a]	0.72±0.01[a]	90.11±1.50[a]	442.27±8.36[a]
	Pond-2	1.00±0.04[a]	3.27±0.19[b]	226.93±11.63[b]	0.66±0.02[b]	87.67±2.18[a]	378.96±30.46[b]
M. piceus	Pond-1	1.01±0.04[a]	4.75±0.48[a]	370.99±54.07[a]	0.86±0.07[a]	91.67±1.67[a]	115.02±11.62[a]
	Pond-2	1.01±0.02[a]	4.04±0.04[a]	301.76±10.00[a]	0.77±0.02[a]	86.67±3.34[a]	92.58±4.02[b]
H. molitrix	Pond-1	0.53±0.03[a]	3.47±0.62[a]	559.95±122.27[a]	1.04±0.11[a]	90.00±2.50[a]	109.71±17.6[a]
	Pond-2	0.52±0.02[a]	3.12±0.08[a]	503.44±16.73[a]	1.00±0.02[a]	88.33±0.72[a]	96.97±2.65[a]
L. calbasu	Pond-1	0.53±0.04[a]	2.10±0.10[a]	295.70±44.56[a]	0.76±0.06[a]	89.00±1.00[a]	82.3±4.43[a]
	Pond-2	0.53±0.04[a]	1.51±0.19[b]	186.17±52.01[b]	0.58±0.11[a]	86.00±2.65[a]	57.33±7.65[b]

Values in the same column for each species having different superscript letter differs significantly ($P<0.05$).

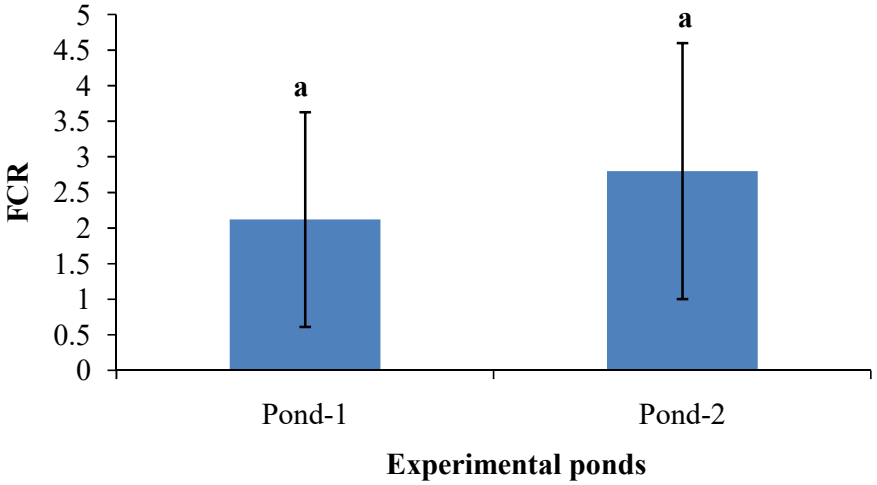


Figure 21. Feed conversion ratio (FCR) of the experimental diets.

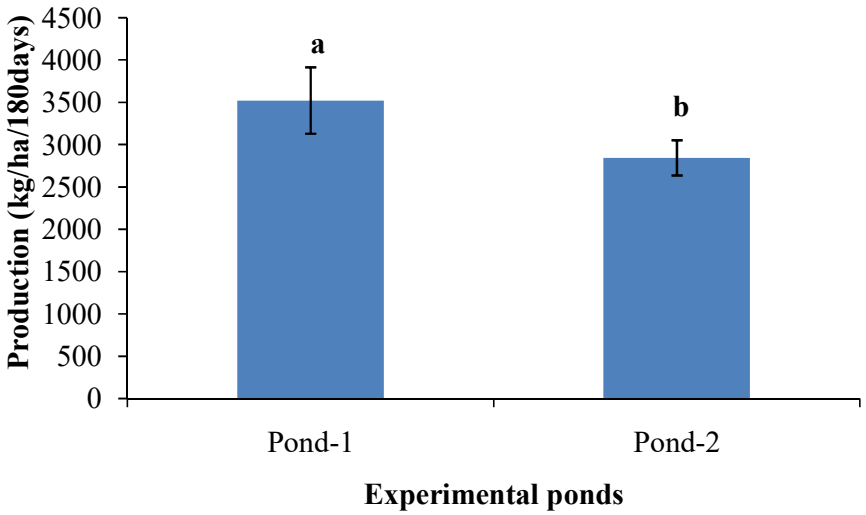


Figure 22. Total production (kg/ha/180 days) of experimental fishes in experimental ponds.

Table 26. Economic analyses among three treatments for 1 ha pond and 180 days of culture period

Variables	Treatments	
	Pond-1	Pond-2
Land used cost	15060.00	15060.00
Pond preparation and management cost (Rotenone, lime, Urea, TSP)	23668.00	23668.00
Fish fingerling	228746.50±2047.07[a]	225124.00±5467.34[a]
Feed cost	192916.68±472.37[a]	182495.28±1056.76[b]
Labour cost	4940.00	4940.00
Fish harvesting & marketing cost	5193.00	5193.33
Total cost	464504.86±6937.88[a]	462499.59±12923.18[a]
Fish sale as total return	814051.81±12599.35[a]	647089.30±2131.13[b]
Total net return	343527.63±11024.64[a]	190609.03±2279.45[b]
BCR	0.73±0.01[a]	0.42±0.01[b]

Values in the same raw having different superscripts are significantly different (P<0.05)

11.7 Discussion

11.7.1 Growth performance

According to the results of the present thesis, an improvement of the growth performance of the experimental fishes (*B. gonionotus* and *L. rohita*) was observed as a result of the supplementation of NPs (Fe-NPs, Cu-NPs, Zn-NPs) and alloy in feed in a dose-dependent manner. In case of *B. gonionotus* in experiment-1, a dose of 30 mg/kg feed of Fe-NPs, 20 mg/kg feed of Cu-NPs and 40 mg/kg feed of Zn-NPs showed better growth performance compared to other NPs treated fish and control fish groups. In experiment-3, *B. gonionotus* showed its higher growth performance at 30 mg/kg feed of alloy (combined Fe-NPs and Zn-NPs) group. However, On the basis of comparison, the growth parameters among the experiments using suitable doses for growth performance, significantly ($P < 0.05$) higher growth performance was obtained from the fish group fed 30 mg/kg feed of alloy enriched feed. In case of *L. rohita*, significantly ($P < 0.05$) higher growth performance was noted for the fish group fed 30 mg/kg feed of Fe-NPs, 20 mg/kg Cu-NPs and 40 mg/kg Zn-NPs. Alloy diet also showed significantly ($P < 0.05$) higher growth performance compared to Fe-NPs, Cu-NPs and Zn-NPs at the dose of 30 mg/kg feed of alloy. Supplementation of NPs in the feeds in the present study showed better growth performance compared to control fish group in a dose dependent manner. It is well know that the nano material have higher intestinal absorption, bioavailability and catalytic activities (Albrecht *et al*., 2006; Dube et al., 2010; Alishahi et al., 2011). Therefore, it might possible that conversion of metals in nano form increase the efficiency of metals by enhancing its absorption and bioavailability in the gastrointestinal tract. Dose dependent growth performance of NPs was also reported by many researchers. Behera et al. (2014) reported that in *L. rohita*, iron-supplementation in feed improved the growth performance of fish in a dose dependent manner. In the studies conducted by Gatlin and Wilson (1986); Lim et al. (1996) and Sealey et al. (1997) showed the total dietary Fe requirement for optimum growth, feed efficiency, hematological values and immune response of juvenile channel catfish was about 30 mg/kg of feed, which was similar to the dose used in the present study. However, intake of Fe-NPs with the doses more than 30 mg/kg of feed significantly ($P < 0.05$) reduced the growth performance. Similar observation was also made by Baker et al. (1997) who reported that ingestion of the dietary iron in high ratio resulted in decreased growth in the catfish by accumulating in the tissues. During the study period, toxic effect of Cu-NPs was observed at beyond the dose of 20 mg/kg feed of Cu-NPs indicating more toxic nature of Cu-NPs compared to Fe-NPs and Zn-NPs that caused the muscle tissue to disrupt. Evidence of depressed

growth due to higher Cu doses were also observed by Murai et al. (1981) and Tan et al. (2011) in channel catfish and juvenile yellow catfish. Chen et al. (2013) reported that the reduction in growth performance was most likely due to two reasons: first, Cu exposure caused increased metabolic expenditure for detoxification and maintenance of homeostasis; second, higher Cu exposure reduced feed intake, which would in turn lead to reduced growth. Comparatively higher growth performance showed by Zn-NPs might be due to the higher intestinal absorption, bioavailability and catalytic activities as reported by Alishahi et al. (2011). Many investigators have suggested the role of Zn in the growth, development and physiology of animals (Eide, 2006; Maret and Kre et al., 2007) and evaluated its role in the synthesis of growth hormone (Imamoglu et al., 2005). Therefore, positive effect of Zn nanoparticles on growth performance may be attributed to somatic growth by stimulation of DNA and RNA synthesis and cell division (Siklar et al., 2003). It was observed that both fishes (*B. gonionotus* and *L. rohita*) fed feed supplemented with Zn-NPs at the rate of 40 mg/kg feed showed significantly ($P < 0.05$) higher growth performance compared to other doses and even from control group. This level lies within the range reported by many investigators for different fish species such as for hybrid striped bass (Buentello et al., 2009), juvenile abalone (Tan and Mai, 2001) and Atlantic salmon (Maage et al., 2001) but somewhat higher than Faiz et al. (2015), Clearwater et al., (2002) and Apines et al., (2001). In the present study, supplementation of Zn-NPs at the dose of 50 mg/kg feed of Zn-NPs caused reduced growth performance compared to 40 mg/kg feed of Zn-NPs might be due to toxic effect at higher doses. It is well known that deficiency of Zn leads to growth retardation (Lim et al., 1996) and immunological impairment in fish (Kiron et al., 1993). Moreover higher intake of Zn cause deleterious effects on fish growth (Hayat et al., 2007). It has also been reported that dietary Zn and their nano sized forms beyond optimum concentration have also produced adverse effects on survival and growth of *M. rosenbergii* (Muralisankar et al., 2014; 2015). In an experiment with medaka fish (*Oryzias latipe*) and their embryos the effects of ZnO-NPs were examined. The finding of these studies revealed that both the exposed embryos and medaka adults showed dose dependent toxicity (Li et al., 2009), which is similar to the findings of the present study. However, the best growth performance of both *B. gonionotus* and *L. rohita* fed feeds containing 30 mg/kg feed of alloy over other NPs indicates superiority of alloy (combination of Fe-NPs and Zn-NPs) in animal production and the possible reason of this incidence was unknown because experiment regarding the use of alloy in animal feed is not present in literature and the present experiment is unique in its nature. Alloy also showed reduced growth performance of fishes at 40 and 50 mg/kg doses. In the present study, the decreased growth rate in fishes fed high levels of dietary NPs above the optimal dose was probably due to an increased expenditure of energy for sustaining normal metabolism, leaving less energy available for growth. Comparison of fish species regarding growth performance revealed that *B. gonionotus* gave comparatively better growth performance than *L. rohita* using both NPs and alloy.

11.7.2 Feed utilization parameters

Feed utilization parameters have close relationship with growth rate. Better feed utilization was observed at 30 mg/kg feed of Fe-NPs, 20 mg/kg feed of Cu-NPs and 40 mg/kg feed of Zn-NPs. In case of *B. gonionotus*, significantly ($P < 0.05$) higher feed utilization was observed for the fish group feed 30 mg/kg feed of Fe-NPs (experiment-1) alloy diet (experiment-3). Similar result was also observed for *L. rohita*. Comparison between two species has shown significantly ($P < 0.05$) better feed utilization by *B. gonionotus*. In the present study, deviation of feed utilization after a certain dietary NPs (40-50 mg/kg for Fe-NPs, 30-50 mg/kg for Cu-NPs, 50 mg/kg for Zn-NPs and 40-50 mg/kg for alloy) level was observed for both the fishes. In the present study, increased FCR and decreased PER were associated with diminished growth at upper optimal doses of NPs and alloy in feeds. Similarly, impaired FCR and PER in channel catfish and juvenile yellow catfish, *Cyprinus carpio* and *Ctenopharyngodon idella* at high levels of copper and zinc has been reported by Tan et al. (2011) and Liang et al. (2012). These studies suggested that feed intake and weight gain are influenced by levels of dietary NPs. However, comparatively poor performance of feed utilization parameters of Cu-NPs

supplemented feeds might be due to more toxic nature of these NPs compared to other NPs used in the present experiment. Although, several researchers (Faramarzi, 2012; Mohseni et al., 2014; Sabatini et al., 2009; Tang et al., 2013; Wang et al., 2009) reported that feed utilization can be enhanced by supplementation of Cu with vitamin C in feeds.

11.7.3 Proximate composition

During the study period, significant difference ($P < 0.05$) in proximate composition of both the *B. gonionotus* and *L. rohita* treated with NPs and alloy supplemented feeds were observed. In case of *B. gonionotus*, significantly higher protein and lipid content were recorded for the fish groups fed 30 mg/kg of alloy diet. Significantly ($P < 0.05$) higher protein and lipid content were also observed in the muscle of *L. rohita* fed feeds containing 30 mg/kg feed of alloy. However, higher protein content was found to be estimated in the muscle of *B. gonionotus* compared to *L. rohita*. Dose dependent reduction in total protein and lipid content of the muscle of *B. gonionotus* showed that Fe-NPs, Cu-NPs, Zn-NPs and alloy at the dose of 30, 20, 40 and 30 mg/kg feed, respectively were optimal for these species. Similar result was also noted for *L. rohita*. Dose dependent variation in protein and lipid content were also observed by Muralisankar et al. (2014, 2015) in case of *Macrobachium rosenbergii* PL. The decrease in the level of protein and lipid in muscle tissue may be due to overutilization of protein on stress. Therefore, the increase in energy demand, as well as the altered enzyme activities, will result in the decrease of protein content. It is likely that the protein and lipid in fish can be used as energy source for detoxification and the maintenance of homeostasis during metal exposure (Stefanni et al., 2014; Zheng et al., 2013). In the present study, crude proteins and crude lipids decreased with an increase in NPs and alloy doses indicating NPs and alloy up to the optimal level were harmful to energy stores (such as crude proteins and crude lipids) and weight gain of both *B. gonionotus* and *L. rohita*. Similar observation was also made by Abdel-Tawwab et al. (2008) who assumed that changes in body composition such as crude protein and crude lipid contents could be linked to changes in their synthesis, deposition rate in muscle, and differential growth rates. However, depending on toxicity of NPs and alloy can be categorized as Cu-NPs > Fe-NPs, alloy > Zn-NPs for both the experimental fish species. Protein and lipid content of muscle are associated with factors such as feed intake, metabolic use and intestinal absorption of feed (Chatzifotis et al., 2010) these factors can all be influenced by elevated dietary Cu concentrations (Berntssen et al., 1999). Tan et al. (2011) reported that decreased whole-body and muscle lipid content in juvenile yellow catfish when exposed to high dietary Cu. Berntssen et al. (1999) also observed a negative correlation between dietary Cu concentrations and energy stores in Atlantic salmon fed practical feeds. Carbohydrates are the primary as well as an immediate energy source (Umminger, 1977). A decline in the carbohydrate levels in muscle of both *B. gonionotus* and *L. rohita* fed the feeds treated with NPs and alloy was observed and the decrease in the level of carbohydrates may be due to more of utilization towards the energy requirement during stress condition at supra-optimal doses of these NPs and alloy. Similar result was also found by in Rajan et al. (2016) in *Oreochromis mossambicus* and by Obula (1994) in *Cyprinus carpio*. Manufactured NPs in the present study may conjugate with biological molecules and gain soluble properties which may affect the fishes through oxidative stress resulting damages in lipids, carbohydrates and proteins (Kohen and Nyska, 2002; Niazi and Gu, 2009).

11.7.4 Hematological parameters

The hematological parameters were determined as an index of fish health status were greatly used to evaluate the toxic stress of the fishes (Ranzani-Paiva and Silva-Souza, 2004, Saravanan et al., 2011; Romani et al., 2003; Barcellos et al., 2004; Kavitha et al., 2010). Blood parameters of both *B. gonionotus* and *L. rohita* were found influenced significantly ($P < 0.05$) by the incorporation of NPs and alloy in feeds compared to their control group. *B. gonionotus* showed higher level of RBC, WBC, hemoglobin, total protein and globulin content at the dose of 30 mg/kg of feed for Fe-NPs, 20 mg/kg

feed of Cu-NPs, 40 mg/kg feed of Zn-NPs and 30 mg/kg feed of alloy. However, beyond these doses the values of blood parameters were found to reduce. A similar result was also obtained for *L. rohita*. These results corroborated the study by Murai et al. (1981) that observed a decrease in the RBC number of catfish (*Ictalurus punctatus*) fed feeds formulated with levels above the requirement. In the blood of fish under stress, an increase in RBC counts and hemoglobin concentrations levels are frequently observed by Sevcikova et al., 2016). In this study, the increased RBC and hemoglobin values could be due to enhanced erythropoiesis as a result of chronic toxicity of high doses of NPs (Kondera and Witeska, 2013). Research showed that nanoparticle reduces the number of red blood cells and thus result in anemia by diminishing the life span of red blood cells or suppressing the activity of bone marrow stem cells (Faiz et al., 2015). The dose dependent reduction in RBCs of both the fish species fed NPs supplemented feed may be due to the swelling of the red cells that lead to hemolysis. Therefore, free radicals produced by nanoparticles can cause inflammation of red cells (Alkaladi et al., 2015). Hemolysis of erythrocytes has also been reported in *Heteroclarias* (Oti and Avoaja, 2005; Kori-Siakpere et al., 2008) and rainbow trout (Koyama et al., 1984) in response to Zn. Moreover, significantly higher RBCs value in response to Fe-NPs enriched feed as compared to other NPs types may be due to the causes that iron is important parameters of RBC and thus increase in Fe increased the RBC content of blood. In all vertebrates including fish, the WBCs count increase or decrease in response to various stressors like infections and chemical pollutant (Olurin et al., 2012; Moharram et al., 2011). Due to the role of WBC in non-specific or innate immunity (Kumar et al., 2007), increase in the WBC count and its functions is quite likely to result in an enhancement of the non-specific defense. The increasing trend in WBC count could be related to a stimulation of the immune system. These findings indicated that, the decrease in the WBC level observed in the fishes (*B. gonionotus* and *L. rohita*) fed feeds containing Fe-NPs 40-50 mg/kg of feed, Cu-NPs 30-50 mg/kg of feed, Zn-NPs 50 mg/kg of feed and alloy 40-50 mg/kg of feed may be associated with a decrease in nonspecific immunity of the fish due to exposure to toxicity. Thus doses of NPs below the above mentioned level were having beneficial effects on fish health and enhance their immune system. However, lower value of WBC in control group of each NPs and alloy indicates deficiency of minerals in fishes. Like these results, other scientists also reported the decrease in WBCs count in *Clarias* and "*Heteroclarias*" species (Oti and Avoaja, 2005; Kori-Siakpere et al., 2008) in response to Zn. The decreasing trend in the level of white blood cells in the present study or previous studies may either be the result of bioaccumulation of NPs and alloy in different tissues that cause toxicity and effect on cell production from spleen (Firat, 2007) or due to an increased level of corticosteroid hormones (Celik et al., 2013) because these hormones are important for prevention and healing of inflammation. In *B. gonionotus*, Fe-NPs mediated feeds at 30 mg/kg of feed were found to more active to develop nonspecific immunity as it processes significantly higher value of WBC compared to other doses of NPs and alloy. However, in *L. rohita*, alloy at the dose of 30 mg/kg of feed were found to give the same result. Decreased hemoglobin after exposure to the optimal doses of NPs have been observed in the present study indicated hemodilution in response to toxic effect of NPs. Similar observation was also made by Svobodova et al., (1994), where they showed that high concentrations of heavy metal or long term exposure of fish to sub lethal concentrations usually decrease the haematocrit, haemoglobin, and red blood cell. Again *B. gonionotus* gave significantly ($P < 0.05$) higher hemoglobin level at 30 mg/kg Fe-NPs feed fed group and *L. rohita* at 30 mg/kg feed of alloy fed group. However, Buentello et al., (2009) reported that Zn in nanoform was more efficiently absorbed, utilized and showed no negative impact on the absorption and bioavailability of other trace elements. Measurement of total protein, albumin and globulin, in serum is of considerable diagnostic value in fish, as it relates to general nutritional status (Schaperclaus et al., 1992). Albumin is the most abundant blood protein and is responsible for nutrient transportation and the maintenance of osmotic balance, and globulin is involved in the defense mechanism of animals. During the present study, total protein content of serum in all the three experiments was influenced in a dose dependent manner of NPs. Significant ($P < 0.05$) increase in serum total protein and globulin were observed up to 30 mg/kg feed of Fe-NPs, 20 mg/kg feed of Cu-NPs and 40 mg/kg feed

of Zn-NPs for both *B. gonionotus* and *L. rohita*. In case of alloy, significant increase in serum to total protein and globulin were observed up to 30 mg/kg feed of alloy for both the fish species compared to control group. However, inclusion of NPs in feeds up to the above mentioned doses significantly reduced the serum total protein and globulin in fishes. On the contrary, addition of NPs in feed significantly increases the albumin content of serum compared to control group of fishes. In this study, high levels of NPs significantly influenced serum total protein and globulin, with a decrease in albumin emphasizing the toxic effect of high levels of NPs in fishes. In this sense, it can be inferred that toxicity was so severe that it impaired the defense systems of the fish. The decrease in serum total protein and globulin levels along with decreasing albumin level may be valued for energy production during pollutant toxicity and/or due to other several pathological processes including renal damage and elimination in urine, decrease in liver protein synthesis, alteration in hepatic blood flow and plasma dissolution (Gluth and Hanke, 1985). The decrease in serum total protein may also be due to increased lipolysis (Ghosh and Chatterjee, 1989) and detoxification mechanism during stress (Neff, 1985). Haliwell (2007) and Wang et al. (2008) suggested that depletion in serum total protein after NPs exposure may be due to over production of reactive oxygen species (ROS) within the tissue, which can damage proteins. Also NPs are coated with proteins, resulting in an NP-protein corona (Nel et al., 2009) and this may be the cause of depletion in serum total protein levels. At the dose of 50 mg/kg feed of NPs for *B. gonionotus* and *L. rohita* at experiment-1 and 2 predicted that the total protein content of serum was lower for Cu-NPs mediated feed indication its more potential toxic nature compare to Fe-NPs and Zn-NPs. In experiment-3, alloy showed more toxicity for *L. rohita* compared to *B. gonionotus*.

11.7.5 Blood lipid profile

During the study period, total cholesterol, HDL and triglyceride content of serum were found to increase up to 30 mg/kg feed of Fe-NPs, 20 mg/kg feed of Cu-NPs and 40 mg/kg feed of Zn-NPs both for *B. gonionotus* and *L. rohita* at experiment-1 and 2, respectively. Alloy treated fishes (experiment-3) also depicted that significant increase in total cholesterol, HDL and triglyceride content of serum up to 30 mg/kg feed of alloy enriched feed. Further increase in doses significantly reduced the blood lipid profile of both the fishes. Similar observation was also made by Herzig et al. (2009) who also noticed significant decrease of plasma cholesterol when broilers were fed with high amounts of zinc in feed. The decreased level of serum HDL up to the certain doses in the present study was accompanied with increased concentrations of LDL. These results may indicate that experimental fishes were made stronger and protected when they were fed feeds containing optimal doses of NPs in feed. HDL-cholesterol and LDL-cholesterol are important indicators for lipid metabolism. Oberdörster, (2004) stated that exposure to nano-materials causes oxidative stress and severe lipid peroxidation in fish brain tissue and this lipid peroxidation can be repealed by increased HDL activity, which prevents LDL oxidation, eventually reducing serum lipids (Mackness et al., 1993; Cesar et al., 2010). Massarsky et al. (2014) showed that Ag-NPs induced lipid peroxidation by generating reactive oxygen species (ROS) extracellularly or within close proximity to the cell membrane in rainbow trout hepatocytes. This is an indication that dietary NPs have the ability to enhance HDL activity, which inhibit LDL activity, and subsequently reduce serum lipid at higher doses.

11.7.6 Serum enzyme profile

The serum activities of ALP, AST and ALT revealed a significant increase in all treated groups along the experimental periods. Serum enzymes such as AST, ALT and ALP could be used as sensitive biomarkers in ecotoxicology, because they provided an early warning of potentially hazardous alterations in contaminated aquatic organisms (Levesque et al., 2002; Kim and Kang, 2004; Nel et al., 2009). The results in the present study indicated a significant increase in serum enzyme (AST, ALT and ALP) activities, when the experimental fishes were exposed to NPs and alloy enriched feeds compared to control feed. These results were in agreement with Zaghloul et al. (2006) who studied

the effect of copper toxicity on three fish species: *Clarias gariepinus*, *Oreochromis niloticus* and *Tilapia zillii*. They showed a significant increase in serum enzyme (AST, ALT and ALP) activities in comparison to the control group. Wu et al. (2003) recorded an increase of AST and ALT activities in stressed juvenile areolate grouper (*Epinephelus areolatus*) and this may be due to hepatic cell injury or increased synthesis of the enzymes by the liver. Changes in the ALP activity also could be due to the result of physiological and functional alterations in metal exposed fish (Jiraungkoorskul et al., 2003). Increase in AST, ALT and ALP activities in the present investigation could be due to a variety of conditions, including muscle damage, intestinal and hepato-pancreatic injury, and toxic hepatitis especially at higher doses (Sevcikova et al., 2016; Farkas et al., 2004). Serum enzyme activity (e.g., protease, amylase, and lipase) can be used as an indicator of potential feed utilization and growth differences and to some extent may serve as an indicator of the digestive capacity in relation to the type of feed offered and the properties of aquaculture environments. In this study, the activities of protease, amylase, and lipase found in serum decreased with increasing NPs and alloy dose, suggesting that exposure of NPs up to the optima doses decreased digestive capability of *B. gonionotus* and *L. rohita*.

11.7.7 Bioaccumulation of NPs in muscle, liver and serum

Liver accumulation of Fe-NPs and Zn-NPs were high compared to the muscle and serum was found for *B. gonionotus* and *L. rohita* in the present study. Differences among various tissues in accumulating metals are generally attributed to their metabolic activities (Cicik, 2003; Tuncsoy and Erdem, 2014). However, Cu-NPs accumulation was found higher in muscle tissue compared to liver and serum during the present study. In experiment-3, liver accumulation of alloy was higher compared to muscle and serum.

12. Research highlight/findings (Bullet point – max 10 nos.):

a) Preparation of more active nanoparticles for different metals under oilbath heating.
b) Synthesis of nanomaterials (micronutrient) mediated feed for disease free fish growth and development.
c) The meat quality of fish was found improved.
d) The formulated fish feed was cost effective.
e) The feed would not leave any foot print of pollution
f) Building the evidence base by mapping good policy and practice models.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment					
(b) Lab &field equipment					
GD_4 -UV visible spectrosphotometer	As required	5,00,000.00	100%	4,99,000.00	
GD_5- High speed centrifugal machine	As required	4,25,000.00	100%	4,23,500.00	
GD_6 a) Oven dryer	As required	2,55,000.00	100%	2,54,500.00	
b) Combine fish feed machine	As required	2,35,000.00	100%	2,34,000.00	

(c) Other capital items					
GD_1 Chemicals	As required	290,000.00	100%	2,88,500.00	
GD_2 Chemicals	As required	3,00,000.00	100%	2,97,998.00	
GD_3 **Apparatus**					
a. Micro pipette	As required	30,000.00	100%	30,000.00	
b. Aquarium with aerators	As required	2,00,000.00	100%	1,99,500.00	
c. Digital dissolved oxygen meter	As required	70,000.00	100%	69,500.00	

2. Establishment/renovation facilities: N/A

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: NA

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	639882	600427	584427	55455	100	
B. Field research/lab expenses and supplies	2453460	2349559	2402400	51060	100	
C. Operating expenses	99660	98279	88048	11612	100	
D. Vehicle hire and fuel, oil & maintenance	0	0	0	0		
E. Training/workshop/seminar etc.	0	0	0	0		
F. Publications and printing	80000	68552	0	80000	100	
G. Miscellaneous	38265	27800	28500	9765	100	
H. Capital expenses	1411000	1380344	1404559	6441	100	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	**Major technical activities performed in respect of the set objectives**	**Output(i.e. product obtained, visible, measurable)**	**Outcome(short term effect of the research)**
Synthesis of nanoparticles	Desired shape and size of nanoparticles	Action performed in lab and field experiments	Diseases free growth of fish and

			development
Synthesis of nanomaterials mediated feed	Fish feed formulation	Laboratory investigation and validation of results	Safe adsorption of nanoparticles mediated fish feed

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.			
Journal publication	Under preparation		**Title , journal etc**
Information development			
Other publications, if any			

F. Technology/Knowledge generation/Policy Support (as applied):

i. **Generation of technology (Commodity & Non-commodity)**

Development of nanoparticles mediated feed for disease free growth and development of fish.

ii. **Generation of new knowledge that help in developing more technology in future**

Proper utilization and safe adsorption of nanomaterials in Agriculture sector.

iii. **Technology transferred that help increased agricultural productivity and farmers' income**

The nobel technology that helps increased agricultural productivity and farmers' income in short time.

iv. **Policy Support**

Nanomaterials in Agriculture supports the Agriculture policy of Bangladesh - 2018.

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring [description & output of consultation meeting, monitoring workshops/seminars etc.):

- CRG Sub- Project Implementation Progress Workshop, held in BARC, Farmgate Dhaka on 21 December 2017. Appreciated by NATP and other stakeholders.

- CRG Sub- Project Progress review Workshop held in BARC, Farmgate Dhaka on 10 April 2018. Found satisfactory

ii) Field Monitoring (time& No. of visit, Team visit and output):

No. of visit	Team members of BARC	Date	Visiting area	Output
01	02	07.03.2018	Lab and Field	Satisfactory

H. Lesson Learned/Challenges (if any):

I.

i) Nanoparticles are more active than bulk materials.

ii) It has peculiar behavior in aquaculture due to tiny amount of nanomaterials serve as micronutrient as well as enhance of growth and development of fish.

iii) Save and safe adsorption of nanomaterials facilitated disease free growth and development.

I. Challenges (if any):

I. Lack of pure raw materials for the preparation nanomaterial mediated fish feed in local market.
II. Scarcity of sophisticated instrument for characterization of nanoparticles and sample.
III. Discontinuous power supply in laboratory.
IV. Availability of room and field space for research work.
V. Delivery of pure chemicals takes much more time.

Signature of the Principal Investigator
Date …….
Seal

Counter signature of the Head of the organization/authorized representative
Date ……..
Seal

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