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ORIGINAL RESEARCH ARTICLE

# Effects of dietary Cu nanoparticles on growth performance, physiology and bioaccumulation in Asian walking catfish (*Clarias batrachus*)

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**ABSTRACT**

The present investigation was conducted to determine the optimal dietary Cu-NPs requirement of Asian walking catfish, *Clarias batrachus* (7.46 ± 0.15 cm; 5.28 ± 0.10 g) by feeding with diets supplemented with different concentrations of Cu-NPs (10, 20, 30, 40 and 50 mg/kg) and control group. Each experimental diet was hand-fed to triplicate groups of fish for 60 days in glass aquarium. Results showed that fish group fed with 20 mg/kg Cu-NPs in feed exhibited highest ($P < 0.05$) growth performance and feed utilization compared to the control group. However, increased level of Cu-NPs from 30 to 50 mg/kg in feed significantly reduced the growth performance. Significantly higher protein and lipid were also recorded at 20 mg/kg Cu-NPs supplemented group. Haematological parameters, serum lipid and enzymatic profile were found to influence significantly with the addition of Cu-NPs in feed compared to the control group. Based on the polynomial regression analysis between FW, WG and $SGR_W$ against dietary Cu-NPs levels, the optimal dietary supplementation of Cu-NPs for *C. batrachus* were estimated to be ranged between 19.98 to 20.05 mg/kg per diet, respectively. Bioaccumulation of Cu was the highest in liver compared to muscle and serum, whereas the highest Cu accumulation was observed at 50 mg/kg Cu-NPs supplemented group. The findings of the present study will be helpful for formulating nutrient rich low cost catfish feed.

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## INTRODUCTION

Copper (Cu) is a necessary trace element in the diet for all living organism. Fish obtained Cu from the environment through diet. However, the amount of copper in water is insufficient to satisfy the needs of aquatic organisms, and dietary sources are essential for Cu acquisition. Waterborne Cu only represents about 10% of whole-body Cu content, while a diet containing 0.8 mg/kg Cu may increases the Cu content up to 60% in body constituent (Kamunde *et al*., 2002). Typical feedstuffs used in fish feed formulation, such as fishmeal and plant protein sources contain insufficient Cu to cover the fish's needs (Mondal *et al*., 2007). Thus, it becomes necessary to supplement Cu to formulated fish feed to adjust the Cu content to the proper level required by fish. Cu deficiency disrupts the functionality of multiple enzymes involved in antioxidant defense mechanisms and adenosine triphosphate (ATP) synthesis. These effects may compromise the cellular immune function, impacting the ability to kill bacteria and rendering the animals more vulnerable to infection (Cerone *et al*., 2000). The insufficiency of dietary Cu leads to stunted growth, heightened oxidative stress, diminished appetite, anemia, and decreased copper levels in the tissues of juvenile fish (Abdel-Hameid *et al*., 2017; Moazenzadeh *et al*., 2018). Contrarily, an overabundance of dietary Cu can result in

toxicity, which can cause changes in various physiological processes, damage to the liver, and cholestasis (Domínguez *et al*., 2019). Furthermore, high levels of dietary Cu in fish causes a toxic syndrome, which includes growth depression, increased mortality, oxidative stress and reduced immune response (Berntssen *et al*., 2000; Mohseni *et al*., 2014). High bioavailability of Cu in fish diet can decrease the need for Cu supplements and the excreted Cu in wastes, which accumulate, pollute the rearing water, and harm the fish's health. Many studies have investigated the effective role of dietary Cu concentrations and types on various fish species including *Sparus aurata* (Tseng *et al*., 2023), *Heteropneustes fossilis* (Zafar and Khan, 2019), *Channa punctatus* (Abdel-Hameid *et al*., 2016), *Larimichthys croceus* (Li *et al*., 2016) and *Ctenopharyngodon idella* (Tang *et al*., 2013).

Bioavailability of Cu is depending on its available forms, concentration, water quality interactions with other elements, age, size and fish species (Eisler, 2000). Inorganic copper salts, including copper sulfate, copper oxide, and copper chloride, are frequently employed as feed additives to enhance growth promotion in various applications. However, the bioavailability of Cu was found to be higher in fish species, including Nile tilapia rainbow trout, grouper, and channel catfish, when administered in the nano or chelated form as opposed to the inorganic form (EL-Erian *et al*., 2023; Apines-Amar *et al*., 2004). Therefore, in recent years, copper nanoparticles (Cu-NPs) are becoming a popular dietary supplement in the aquatic nutrition industry (El Basuini *et al*., 2016; Wang *et al*., 2015). The enhanced absorption of Nano Cu can be attributed to its small size, which facilitates its uptake through endocytosis and cell bypass mechanisms (Bellmann *et al*., 2015). There is limited data regarding the utilization of nano Cu as a dietary supplement. However, previous research has demonstrated that Cu-NPs exhibit enhanced biological traits and improved bioavailability compared to inorganic Cu salts, particularly in red sea bream (El Basuini *et al*., 2016). Muralisankar *et al*. (2016) reported enhanced growth, survival, feed intake and boosted digestive enzyme activity in *Macrobrachium rosenbergii* fed with the feed supplemented with 20 mg/kg Cu-NPs.

In the field of aquaculture, the production of fish is significantly influenced by two crucial factors: feed and water quality. Feed contributes significantly to commercial fish production and is the determining factor between profitable and unprofitable aquaculture business (Jewel *et al*., 2018; Bolivar *et al*., 2006). Hossain *et al*. (2022) stated that the feed accounted for 56.45–58.49% of the production costs in aquaculture, which may be up to 70% in some cases (FAO, 2009). Gandotra *et al*. (2015) and Obirikorang *et al*. (2015) also reported that feed constitute 40-70% of the variable cost in aquaculture production. Therefore, quality feed production is a major challenge for small farm-made feed producers and commercial feed millers (Hossain *et al*., 2021; Bhuyain *et al*., 2019). In this context, the utilisation of nanoparticles containing trace elements holds potential for promoting the growth and development of fish. This can be achieved through their incorporation into fish feed as a means of appropriate supplementation (Jewel *et al*., 2023).

Therefore, the present study investigated the efficacy of Cu-NPs supplementation in feed on growth performance, proximate composition, hematological parameters, serum lipid and enzyme profile and bioaccumulation in Asian walking catfish *Clarias batrachus*.

## MATERIALS AND METHODS

### Synthesizing and characterization of Cu-NPs

The synthesis of copper nanoparticles (Cu-NPs) was conducted following the procedure described by Alam *et al*. (2015), with some modifications. The aquostic method was utilized to carry out the Liquid Phase Synthesis. In this procedure, Cupric acetate monohydrate, $(CH_3COO)_2Cu.H_2O$, ethylene glycol (EG, 322 99.8 %), and Polyvinylpyrrolidone (PVP, M. Wt. 130, 000) were combined in a 25 ml water solution and subjected to heating at a temperature of 70 °C for a duration of 45 minutes using an oil bath heater. Nanoparticles are prepared through a process of heating. Nanostructures with specific shapes and sizes were synthesized in large quantities by manipulating different experimental factors, including the concentrations of reagent surfactants (such as PVP), the type and amount of solvents (such as EG or other polyols), gas bubbling, as well as temperature and heating rate adjustments (Alam *et al*., 2015).

The synthesized nanoparticles underwent purification using the precipitation method. The crystal structures and growth mechanisms of nanoparticles were observed using an Atomic Force Microscope (AFM-Park Systems, XE-70, South Korea), while their morphologies were also characterized. The product solutions underwent centrifugation at a speed of 6,000 revolutions per minute (rpm) on three separate occasions, with each centrifugation lasting for duration of 30 minutes. This process was carried out to guarantee the thorough retrieval of all products during each centrifugation cycle. The solid particles are gathered and subsequently dispersed again in ethanol. Samples for AFM measurements were prepared by dropping a droplet of the colloidal solutions on the glass slides.

### Experimental diet preparation

The basal diet was prepared by combining various ingredients including fish meal, mustard oil cake, soybean meal, maize bran, wheat bran, rice bran, soybean oil and Cu free minerals and vitamin pre-mixture. The feed ingredients underwent a series of technical processes including grinding, sieving, weighing, and thorough mixing in order to produce the final diets. The basal feed that was prepared in advance was supplemented with synthesized copper nanoparticles (Cu-NPs) at five different concentrations (10, 20, 30, 40, and 50 mg/kg of dry feed). The mixture of basal diet and Cu-NPs were blended together for approximately five minutes using the appropriate amount of water until the mixture achieved a dough-like texture. The dough underwent the process of pelletization using a manual pelletizer equipped with 3 mm diameter dies. The resulting pellets were then gathered and placed in an aluminum tray and oven dried with a thermostatic hot air oven (Microsil, India) until

**Table 1.** Formulation and proximate composition of basal diet*.

| Ingredients | g/kg | Proximate composition | (%)† |
|---|---|---|---|
| Fish meal[a] | 275 | Protein | 31.86±0.10 |
| Mustard oil cake[a] | 200 | Lipid | 8.25±0.16 |
| Soybean meal[a] | 125 | Moisture | 8.10±0.12 |
| Maize bran[a] | 125 | Ash | 11.23±0.30 |
| Wheat bran[a] | 90 | | |
| Rice bran[a] | 90 | | |
| Soybean oil[a] | 60 | | |
| Choline chlorid[a] | 2.5 | | |
| Cu-free premix[b] | 32.5 | | |

*Cu-NPs were added to the basal diet at 10, 20, 30, 40 and 50 mg/kg diet.
†Values are presented as mean ± SEM, n = 3.
[a]Ingredients purchased from local market of Rajshahi, Bangladesh.
[b]Cu-free premix (mg/kg of premix): vitamin A-156000 IU, vitamin D3-31200 IU, vitamin E-299, vitamin K3-26, vitamin B1-32.5, vitamin B2-65, vitamin B6-520, vitamin B12-0.16, Nicotinic Acid-520, Folic Acid-10.4, Copper-130, Iodine-5.2, Manganese-780 and Selenium-1.95. Premix was supplied by Reneta Animal Health Pharma Co. Ltd. Bangladesh.

the moisture content reached a level below 10%.

Following the completion of the drying process, the formulated diets were stored at a temperature of 20°C until they were utilized for the experiment. Table 1 displays the inclusion rate of feed ingredients and the proximate composition of the prepared diet.

## Experimental design and animal care

The experiment was carried out in accordance with the research protocols established by the University for conducting experiments involving animals. A total of 216 juvenile *C. batrachus* specimens, with an average body weight of 5.28±0.10 g (mean ± SEM), were procured from a government fish seed hatchery. The specimens were then transported in a plastic bag with aeration to the laboratory of the Department of Fisheries, University of Rajshahi, Bangladesh. The fish were kept in a circular cemented tank (1 $m^3$) with a continuous water flow for two weeks. In the tank, the fish were provided with a control diet that consisted of 31.86% crude protein at a rate of 5% of the total biomass three times a day. After the acclimatization period, the fish were dispensed in 18 glass aquaria (12 fish/ aquarium) including three tanks as control. Each aquarium had dimensions of 1.5 m × 0.8 m × 0.8 m with 500 L volume of water holding capacity. The fish were provided with experimental diets three times a day, manually, at a rate of 3% of their body weight, for duration of 60 days. The fish were subjected to fortnightly weighing, and the daily ration was adjusted based on their weight gain. In each aquarium, a routine water exchange was conducted on a daily basis, involving the replacement of roughly 50% of the total water volume with pre-dosed water. Regular syphoning was conducted to remove the remaining food particles from each aquarium after the allocated feeding period. The amount of residual feed were dried in oven and weighted. The amount of feed consumed (FC) was determined by subtracting the amount of feed given and the total dry weight of residual feed. Routine monitoring of physicochemical parameters (Temperature, °C; DO, mg/L; pH and ammonia, mg/L) were done in every 15 days interval using a Celsius thermometer for temperature, HACH kit (FF-2, USA) for dissolved oxygen and ammonia, and pH meter (Jenwary 3020) for pH. The ranges of water temperature, DO, pH and ammonia were 27.88–28.70°C, 5.14–5.50 mg/L, 7.00–7.21 and 0.001–0.003 mg/L, respectively.

## Estimation of fish growth indices and feed utilization

The initial body weight (IBW), initial body length (IBL) and final body weight (FBW), final body length (FBL) of each fish in each aquarium was measured at the beginning and end of the experiment. The number of fish in each aquarium was also quantified and documented during each sampling event. Growth indices and feed utilization of the experimental fish were measured following Ibrahim *et al.* (2021) as follows:

Weight gain (WG) = FBW (g) − IBW (g)

Length gain (LG) = FBL (g) − IBL (g)

Percentage weight gain (%WG) = ((FBW − IBW)/ IBW) ×100

Percentage length gain (%WG) = ((FBL − IBL)/IBL) ×100

Average daily gain of weight ($ADG_W$, g) = (FBW− IBW)/Culture duration

Average daily gain of length ($ADG_L$, cm) = (FBL − IBL)/Culture duration

Specific growth rate of weight ($SGR_W$, %/day) = ((lnFBW − lnIBW)/Culture duration) × 100

Specific growth rate of length ($SGR_L$, %/day) = ((ln FBL − ln IBL)/ Culture duration) × 100

Condition factor (CF) = (Weight/length$^3$) × 100

Survival rate (SR, %) = (Number of fish stocked/Number of fish harvested) × 100

Feed conversion ratio (FCR) = Feed fed (dry weight, g)/live weight gain (g)

Feed conversion efficiency (FCE) = Live weight gain (g)/feed fed (dry weight, g)

Protein efficiency ratio (PER) = Total weight gain (g)/total protein intake (g)

Protein growth rate (PGR, %) = ((lnFP – lnIP)/Culture duration) × 100

Apparent net protein utilization (ANPU, %) = (Net increase in carcass protein/protein consumed) × 100

Where, ln = natural log, IBW = initial body weight, FBW = fianl body weight, IBL = initial body length, FBL = fianl body length, IP = initial protein, FP = final protein

### Proximate composition analysis

The proximate composition of fish muscle was assessed both at the beginning and end of the experiment. At the end of the experiment, 10 fishes from each feeding group were randomly selected for proximate composition (Protein, lipid, ash and moisture) analysis according to AOAC (2016). Crude protein was determined by the Kjeldahl method and crude lipid by petroleum ether extraction using the Soxhlet method. Ash content was determined by muffle furnace at 550 °C for 5 h. The moisture levels were determined by oven drying to a constant weight at 70 and 105°C, respectively.

### Blood assay

Ten fish were chosen at random from each treatment group and were anesthetized using clove oil (Merck, Germany) at a concentration of 50 µl per liter of water in order to collect blood samples. Blood samples (500 µm) were collected from the caudal vein of three fish per replicate using a 10% ethylenediaminetetraacetate (EDTA) solution. The samples were subsequently divided into two distinct groups. The initial blood group was isolated for the purpose of evaluating hematological parameters. The second aliquot of blood was subjected to centrifugation at 3000g for 10 minutes to separate the blood plasma. The collected plasma samples were stored at a temperature of -20 °C for subsequent analysis. The blood samples were diluted with diluting medium (Turk's solution and Toisson's solution) before the estimation of total erythrocyte (RBC) and leucocytes (WBC) and the cells were counted by a neubauer haemocytometer (Behera *et al*., 2014). The cell counts were determined according to Parida *et al.* (2011). Total RBCs per $mm^3$ = 20050N 10,000N (N = number of RBCs counted, dilution factor = 200) and total WBCs per $mm^3$ = (201L) (0.4) cells 50L (L = number of WBCs counted, dilution factor = 50). The hemoglobin concentration in the blood was determined using the cyanomethemoglobin method with the aid of Drabkin's solution (Drabkin 1950). The standard micro-haematocrit method was employed to measure the haematocrit (Bull *et al*., 2000). Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) of erythrocytes were estimated following the formulae given by Dacie and Lewis (1999) by using microhematocrit tubes. The spectrophotometric method was used to evaluate the levels of total protein and albumin, while the globulin levels were determined by subtracting albumin from total protein.

### Lipid profile and enzymatic analysis

Total cholesterol, triglycerides, 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®. The amylase, lipase and protease activity of the serum was estimated by a colorimetric enzymatic method.

### Bioaccumulation of Cu-NPs

The quantification of Cu uptake from Cu-NPs) in the liver, muscle and serum was assessed using inductively coupled plasma mass spectrometry (ICP-MS) analysis following the method described by Shahzad *et al.* (2017). A solution comprising of 10 ml of concentrated nitric acid and 2 ml of perchloric acid was added to a 1 g portion of freeze-dried samples. The digestion process was performed by applying thermal energy to the mixture using a hot plate set at a temperature of 100°C. The samples underwent a process where two drops of hydrogen peroxide were added, followed by dilution with distilled water and subsequent filtration using a Whatman filter paper. The concentration of copper (Cu) was measured and recorded in micrograms per gramme (µg/g).

### Statistical analysis

All data were provided as Mean ± SEM. The Shapiro-Wilk test established the normality of the data, and all variables except percentage and ratio were studied untransformed to allow treatment comparison. Percentage and ratio data were arc-sign-transformed. A one-way analysis of variance (ANOVA) using SPSS 25 ver. was used to examine how the different Cu-NPs levels affected growth, feed utilization, hematological, serum lipid, and enzymatic parameters. Duncan's multiple range test (DMRT) was used to compare means with significant F values at 5% level of significance. Polynomial regression analysis determined the linear effects of Cu-NPs supplementation's on final weight, weight gain, and specific growth rate (Yossa and Verdegem, 2015).

## RESULTS AND DISCUSSION

### Water quality parameters

The water quality parameters of all the feeding groups did not exhibit significant variation throughout the duration of the study period (Table 2) and all the parameters remained within the ideal range for cultivating tropical fish (Hossain *et al*., 2022; Jewel *et al*., 2020; Akter *et al*., 2018). This indicates that the rearing conditions were favorable throughout the duration of the experiment. The water temperature, dissolved oxygen (DO), pH, and ammonia levels in the experimental groups ranged from 27.88±0.19 to 28.70±0.42 °C, 5.14±0.23 to 5.50±0.26 mg/L, 7.00±0.20 to 7.21±0.19, and 0.001±0.000 to 0.003±0.001 mg/L, respectively.

**Table 2.** Water quality parameters.

| Parameters | Dietary Cu-NPs supplementation level in feed | | | | | | F-value | P-value |
|---|---|---|---|---|---|---|---|---|
| | Control | 10 | 20 | 30 | 40 | 50 | | |
| Temperature (°C) | 28.50±0.50$^{a}$ | 28.20±0.35$^{a}$ | 28.16±0.19$^{a}$ | 28.70±0.42$^{a}$ | 27.90±0.23$^{a}$ | 27.88±0.19$^{a}$ | 0.55 | 0.85 |
| DO (mg/L) | 5.25±0.20$^{a}$ | 5.30±0.22$^{a}$ | 5.17±0.15$^{a}$ | 5.50±0.26$^{a}$ | 5.14±0.23$^{a}$ | 5.20±0.10$^{a}$ | 1.56 | 0.36 |
| pH | 7.00±0.20$^{a}$ | 7.16±0.23$^{a}$ | 7.20±0.14$^{a}$ | 7.10±0.13$^{a}$ | 7.09±0.25$^{a}$ | 7.21±0.19$^{a}$ | 12.85 | 0.16 |
| Ammonia (mg/L) | 0.001±0.000$^{a}$ | 0.003±0.001$^{a}$ | 0.002±0.001$^{a}$ | 0.001±0.001$^{a}$ | 0.001±0.001$^{a}$ | 0.003±0.001$^{a}$ | 0.85 | 0.36 |

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

**Table 3.** Growth performance of *Clarias batrachus* fed with different levels of Cu-NPs.

| Parameters | Dietary Cu-NPs supplementation level in feed | | | | | | F-value | P-value |
|---|---|---|---|---|---|---|---|---|
| | Control | 10 | 20 | 30 | 40 | 50 | | |
| IW (g) | 5.25±0.13$^{a}$ | 5.32±0.07$^{a}$ | 5.28±0.09$^{a}$ | 5.30±0.15$^{a}$ | 5.24±0.11$^{a}$ | 5.30±0.09$^{a}$ | 0.27 | 0.92 |
| IL (cm) | 7.58±0.15$^{a}$ | 7.51±0.04$^{a}$ | 7.43±0.19$^{a}$ | 7.44±0.22$^{a}$ | 7.40±0.20$^{a}$ | 7.42±0.08$^{a}$ | 0.57 | 0.72 |
| FW (g) | 14.44±1.02$^{bc}$ | 16.83±0.60$^{b}$ | 24.27±0.97$^{a}$ | 15.53±1.91$^{bc}$ | 13.84±1.49$^{c}$ | 10.65±1.39$^{d}$ | 37.31 | 0.00 |
| FL (cm) | 8.48±0.16d$^{e}$ | 8.92±0.19$^{c}$ | 9.76±0.22$^{a}$ | 9.30±0.18$^{b}$ | 8.59±0.22$^{d}$ | 8.20±0.11$^{e}$ | 29.98 | 0.00 |
| WG (g) | 9.19±1.02$^{bc}$ | 11.51±0.55$^{b}$ | 18.99±1.06$^{a}$ | 10.23±1.98$^{bc}$ | 8.61±1.41$^{c}$ | 5.35±1.31$^{d}$ | 37.31 | 0.00 |
| LG (cm) | 0.89±0.17$^{d}$ | 1.41±0.15$^{c}$ | 2.33±0.34$^{a}$ | 1.86±0.06$^{b}$ | 1.19±0.40$^{cd}$ | 0.78±0.19$^{d}$ | 17.51 | 0.00 |
| %WG | 174.67±20.21$^{bc}$ | 216.33±8.50$^{b}$ | 360.00±26.06$^{a}$ | 193.33±40.05$^{bc}$ | 164.00±25.00$^{c}$ | 100.67±23.46$^{d}$ | 34.46 | 0.00 |
| %LG | 11.80±2.31$^{d}$ | 18.77±1.91$^{bc}$ | 31.50±5.36$^{a}$ | 25.08±1.39$^{ab}$ | 16.13±5.79$^{cd}$ | 10.53±2.72$^{d}$ | 14.54 | 0.00 |
| $ADG_W$ (g) | 0.15±0.02$^{c}$ | 0.19±0.01$^{b}$ | 0.32±0.02$^{a}$ | 0.17±0.03$^{bc}$ | 0.14±0.03$^{c}$ | 0.09±0.02$^{d}$ | 43.31 | 0.00 |
| $ADG_L$ (cm) | 0.02±0.01$^{c}$ | 0.02±0.01$^{bc}$ | 0.04±0.01$^{a}$ | 0.03±0.00$^{b}$ | 0.02±0.01$^{bc}$ | 0.01±0.01$^{c}$ | 9.14 | 0.00 |
| $SGR_W$ (%/day) | 1.68±0.12$^{bc}$ | 1.92±0.04$^{b}$ | 2.54±0.10$^{a}$ | 1.78±0.23$^{bc}$ | 1.61±0.16$^{c}$ | 1.15±0.19$^{d}$ | 26.57 | 0.00 |
| $SGR_L$ (%/day) | 0.19±0.04$^{d}$ | 0.29±0.03$^{bc}$ | 0.45±0.07$^{a}$ | 0.37±0.02$^{ab}$ | 0.25±0.08$^{cd}$ | 0.17±0.04$^{d}$ | 15.11 | 0.00 |
| CF | 2.38±0.30$^{ab}$ | 2.37±0.08$^{ab}$ | 2.61±0.07$^{a}$ | 1.94±0.28$^{b}$ | 2.20±0.39$^{ab}$ | 1.94±0.32$^{b}$ | 2.99 | 0.06 |
| SR (%) | 100.00±0.00 | 100.00±0.00 | 100.00±0.00 | 100.00±0.00 | 100.00±0.00 | 100.00±0.00 | .- | .- |

Values with different superscript letter in the same column indicate significant difference at P < 0.05.
IW = initial weight, IL = initial length, FW = final weight, WG = weight gain, %WG = percentage weight gain, LG = Length gain, %LG = percent length gain, $ADG_W$ = average daily gain of weight, $ADG_L$ = average daily gain of length, $SGR_W$ = specific growth rate of weight, $SGR_L$ = specific growth rate of length, CF = condition factor, SR = Survival rate

## Growth performance

The results indicate that there was no significant difference (P>0.05) in the initial weight and length of *C. batrachus* among the feeding group. The average initial weight and length of the fish under study were 5.28±0.10 g and 7.46±0.15 cm, respectively (Table 3). At the end of the feeding trial, a statistically significant difference (P<0.05) was detected in all growth indices when compared to the control group. Fish fed with a diet containing 20 mg/kg of Cu-NPs exhibited a notable increase in their final weight (24.27±0.97 g) and length (9.76±0.22 cm). In addition, a notable increase in weight gain (WG), length gain (LG), percentage of weight gain (%WG), percentage of length gain (%LG), average daily weight gain ($ADG_W$), average daily length gain ($ADG_L$), specific growth rate in weight ($SGR_W$), specific growth rate in length ($SGR_L$), and condition factor (CF) was observed in fish group fed with 20 mg/kg Cu-NPs in feed. Based on the polynomial regression analysis between FW, WG and $SGR_W$ against dietary Cu-NPs levels, the optimal dietary supplementation of Cu-NPs for *C. batrachus* were estimated to be ranged between 19.98 to 20.05 mg/kg per diet respectively (Figure 1). According to Wang *et al.* (2018), the utilization of Cu has been found to be more effective in promoting growth in Russian Sturgeon (*Acipenser guldenstaedtii*) compared to inorganic Cu ($CuSO_4$). El Basuini *et al.* (2016) also confirmed the growth-promoting effect of Nano-Cu particles in red sea bream. The enhanced intestinal absorption, bioavailability, synthesis of growth hormone, and catalytic activity are just some of the ways in which nanoparticles have been shown to benefit fish growth, development, and physiology (Alishahi *et al.*, 2011; Eide, 2006; Albrecht *et al.*, 2006; Dube *et al.*, 2010; Maret and Kr'el, 2007).

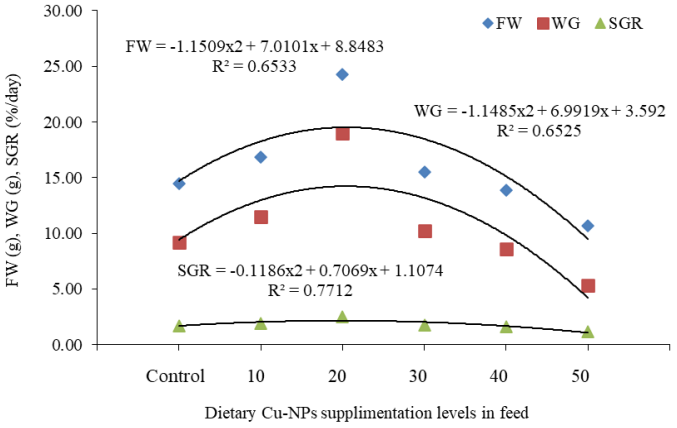


**Figure 1.** *The relationship between final weight (g), weight gain (g) and specific growth rate (SGR; %g/day), of C. batrachus fed diets supplemented with varying levels of Cu-NPs for 60 days.*

Nevertheless, in the particular concentration of Cu-NPs in feed reduced the growth characteristics of *C. batrachus*. The fish group fed with 50 mg/kg Cu-NPs in feed exhibited the lowest growth performance, even lower than the control group. It is possible that this is owing to the toxicity of copper in higher concentrations, and it suggests that *C. batrachus* may be able to make effective use of dietary copper nanoparticles up to a certain threshold. Evidence of depressed growth due to higher Cu doses was also observed by Murai *et al.* (1981) and Tan *et al.* (2011) in channel catfish and juvenile yellow catfish. In contrast, the survival rate for all feeding groups was 100%, suggesting that the presence of Cu-NPs did not have any adverse impact on *C. bactrachus*, which is known to be potentially lethal to the fish.

**Table 4.** Growth performance of *C. batrachus* fed with different levels of Cu-NPs.

| Parameters | Dietary Cu-NPs supplementation level in feed | | | | | | F-value | P-value |
|---|---|---|---|---|---|---|---|---|
| | Control | 10 | 20 | 30 | 40 | 50 | | |
| FC (g) | 25.20±0.63$^{a}$ | 25.53±0.31$^{a}$ | 25.36±0.45$^{a}$ | 24.46±0.72$^{a}$ | 24.13±0.53$^{a}$ | 24.46±0.41$^{a}$ | 0.27 | 0.92 |
| FCR | 2.77±0.33$^{b}$ | 2.22±0.08$^{c}$ | 1.34±0.10$^{c}$ | 2.56±0.57$^{b}$ | 2.97±0.45$^{b}$ | 4.93±1.04$^{a}$ | 14.66 | 0.00 |
| FCE | 0.36±0.04$^{bc}$ | 0.45±0.02$^{b}$ | 0.75±0.06$^{a}$ | 0.40±0.09$^{bc}$ | 0.34±0.05$^{c}$ | 0.21±0.05$^{d}$ | 34.25 | 0.00 |
| PER | 1.11±0.13$^{bc}$ | 1.37±0.05$^{b}$ | 2.27±0.16$^{a}$ | 1.22±0.25$^{bc}$ | 1.04±0.16$^{c}$ | 0.64±0.15$^{d}$ | 35.22 | 0.00 |
| PGR (%) | 1.13±0.04$^{d}$ | 1.20±0.03$^{c}$ | 1.38±0.01$^{a}$ | 1.28±0.05$^{b}$ | 1.20±0.01$^{c}$ | 1.06±0.05$^{e}$ | 29.55 | 0.00 |
| ANPU (%) | 15.24±1.31$^{c}$ | 18.87±0.18$^{b}$ | 32.37±1.98$^{a}$ | 18.08±2.79$^{b}$ | 15.06±1.82$^{c}$ | 9.53±1.30$^{d}$ | 57.57 | 0.00 |

Values with different superscript letter in the same column indicate significant difference at P < 0.05.
FC = feed consumed, FCR = feed conversion ratio, FCE = feed conversion efficiency, PER = protein efficiency ratio, PGR = protein growth rate, ANPU = annual net protein utilization.

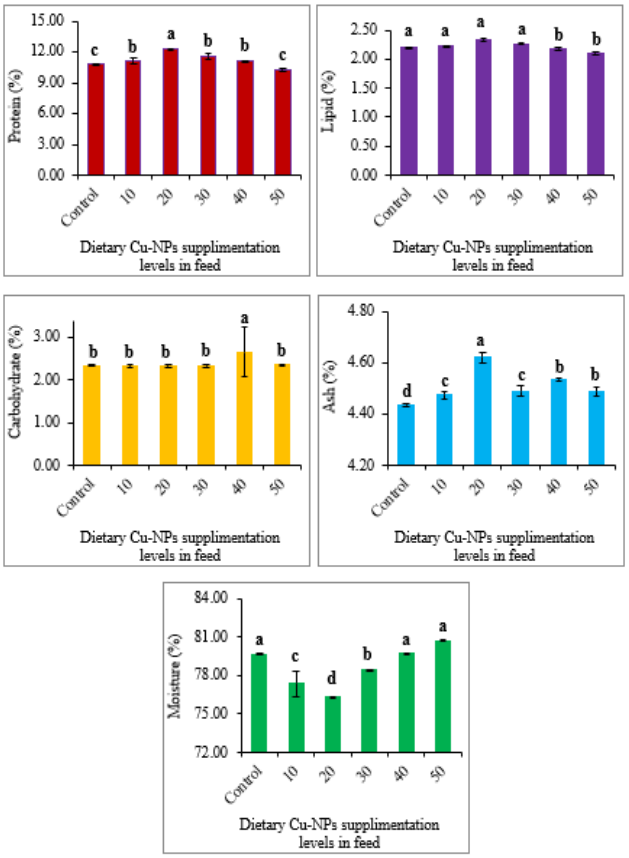


**Figure 2.** *Proximate composition of Clarias batrachus fed on different levels of Cu-NPs enriched diets.*

## Feed utilization parameters

Different concentrations of Cu-NPs did not have any significant effect on feed consumption of *C. batrachus* (Table 4). However, feed performance was improved significantly (P < 0.05) in the Cu -NPs supplemented diet compared to the control group in a dose -dependent manner. Feed conversion ratio was ranged between 1.34±0.10 (20 mg/kg Cu-NPs supplemented group) to 4.93±1.04 (50 mg/kg Cu-NPs supplemented group). Feed efficiency ratio is converse parameters of FCR which showed significantly higher value at 20 mg/kg Cu-NPs supplemented group. Improved feed utilization in Nile tilapia was also reported by EL-Erian *et al.* (2023) fed with diet supplemented with 3 and 6 Cu-NPs mg/kg. Furthermore, several studies also have documented improved feed consumption by fish fed with Cu supplemented diet (Faramarzi, 2012; Mohseni *et al.*, 2014; Sabatini *et al.*, 2009; Tang *et al.*, 2013; Wang *et al.*, 2009). At higher dose of 50 mg/kg Cu-NPs supplemented group, significant reduction was observed in the protein efficiency ratio (PER), protein growth rate (PGR) and apparent net protein utilization (ANPU). Previous studies conducted by Tan *et al.* (2011) and Liang *et al.* (2012) have documented the occurrence of reduced feed utilization in channel catfish, juvenile yellow catfish, *Cyprinus carpio*, and *Ctenopharyngodon idella* when exposed to higher concentrations of copper (Cu).

## Proximate composition

The muscle proximate composition of *C. batrachus* is depicted in Figure 2. A statistically significant difference was observed in all the parameters between the group fed with Cu-NPs and the control group. The group that received a supplement of 20 mg/kg Cu-NPs exhibited a notable increase in protein and lipid levels compared to the control group. Experimental fish groups supplemented with 40 mg/kg Cu-NPs in feed, exhibited higher carbohydrate content in muscle. The ash content was observed to be significantly elevated at a concentration of 20 mg/kg of Cu -NPs supplemented group, while the control group exhibited the lowest ash content. However, a notable decrease in moisture content was observed at 20 mg/kg of Cu-NPs, while supplementation of 50 mg/kg of Cu-NPs exhibited higher moisture content. The dose dependent variation in protein and lipid content were 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. Protein and lipid content of muscle are associated with factors such as feed intake, metabolic use and intestinal absorption of feed and all of these factors are influenced by elevated dietary Cu concentrations (Chatzifotis *et al.*, 2010) which might be the reason behind the lower protein and lipid content of muscle at higher doses of Cu-NPs in the present study.

## Hematological parameters

Dose-dependent significant (P<0.05) responses of Cu-NPs supplemented feeding groups were observed during the study period (Table 5). At the higher doses of 50 mg/kg, Cu-NPs showed toxic effect on the hematological parameters of *C. batrachus*. Nevertheless, the inclusion of Cu-NPs at a concentration of 20 mg/kg in the feed exhibited noteworthy enhancements in RBC count, haemoglobin levels, hematocrit levels and blood indices (MCV, MCH and MCHC) when compared to the control group. The addition of 20 mg/kg of Cu-NPs to the diet resulted in a 6.73% increase in RBC count, while at 50 mg/kg Cu-NPs

**Table 5.** Hematological parameters of *C. batrachus* fed on different levels of Cu-NPs.

| Parameters | Dietary Cu-NPs supplementation level in feed | | | | | | F-value | P-value |
|---|---|---|---|---|---|---|---|---|
| | Control | 10 | 20 | 30 | 40 | 50 | | |
| RBC ($10^6$/mm$^3$) | 2.91±0.07$^{c}$ | 3.12±0.03$^{b}$ | 3.42±0.15$^{a}$ | 2.63±0.08$^{d}$ | 2.44±0.10$^{d}$ | 1.94±0.18$^{e}$ | 65.08 | 0.00 |
| WBC ($10^6$/mm$^3$) | 5.38±0.11$^{d}$ | 6.36±0.21$^{d}$ | 6.49±0.16$^{e}$ | 7.23±0.02$^{c}$ | 7.53±0.07$^{b}$ | 8.22±0.02$^{a}$ | 202.85 | 0.00 |
| Hemoglobin (g/dl) | 8.70±0.13$^{bc}$ | 8.87±0.01$^{ab}$ | 9.15±0.09$^{a}$ | 8.45±0.11$^{c}$ | 7.66±0.20$^{d}$ | 7.17±0.30$^{e}$ | 63.23 | 0.00 |
| Hematocrit (%) | 22.16±1.18$^{ab}$ | 23.51±0.91$^{b}$ | 23.60±0.49$^{a}$ | 21.06±2.15$^{bc}$ | 19.40±0.81$^{c}$ | 16.03±0.72$^{d}$ | 18.18 | 0.00 |
| MCV (fL) | 69.15±2.42$^{c}$ | 71.04±3.79$^{bc}$ | 80.86±1.24$^{ab}$ | 79.98±7.66$^{abc}$ | 79.80±6.58$^{abc}$ | 83.24±8.90$^{a}$ | 2.96 | 0.04 |
| MCH (pg/cells) | 26.81±1.19$^{d}$ | 28.43±0.26$^{cd}$ | 29.96±1.16$^{bc}$ | 32.12±0.74$^{b}$ | 31.46±0.47$^{b}$ | 37.15±2.54$^{a}$ | 23.11 | 0.00 |
| MCHC (%) | 3.88±0.05$^{b}$ | 4.01±0.22$^{ab}$ | 3.71±0.20$^{b}$ | 4.04±0.35$^{ab}$ | 3.96±0.27$^{b}$ | 4.48±0.37$^{a}$ | 2.88 | 0.03 |
| Total protein (g/dl) | 3.21±0.28$^{d}$ | 4.29±0.04$^{c}$ | 4.58±0.07$^{ab}$ | 4.46±0.05$^{bc}$ | 4.75±0.09$^{a}$ | 4.77±0.08$^{a}$ | 61.51 | 0.00 |
| Albumin (g/dl) | 2.28±0.12$^{c}$ | 2.51±0.27$^{c}$ | 3.01±0.19$^{b}$ | 3.25±0.17$^{ab}$ | 3.41±0.13$^{a}$ | 3.50±0.07$^{a}$ | 25.41 | 0.00 |
| Globulin (g/dl) | 2.23±0.03$^{d}$ | 2.69±0.22$^{c}$ | 2.73±0.17$^{c}$ | 3.28±0.22$^{b}$ | 3.25±0.01$^{b}$ | 3.76±0.09$^{a}$ | 40.64 | 0.00 |

Values with different superscript letter in the same column indicate significant difference at P < 0.05.
RBC = red blood cells, WBC = white blood cells, MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean corpuscular haemoglobin concentration.

**Table 6.** Serum lipids and enzyme profile of *C. batrachus* fed on different levels of Cu-NPs.

| Parameters | Dietary Cu-NPs supplementation level in feed | | | | | | F-value | P-value |
|---|---|---|---|---|---|---|---|---|
| | Control | 10 | 20 | 30 | 40 | 50 | | |
| Total cholesterol (mg/dl) | 129.02±6.55$^{c}$ | 126.22±16.55$^{c}$ | 130.01±15.80$^{c}$ | 168.99±15.93$^{ab}$ | 190.92±6.71$^{a}$ | 183.01±7.10$^{ab}$ | 20.68 | 0.00 |
| HDL (mg/dl) | 34.48±5.03$^{c}$ | 38.23±2.01$^{c}$ | 33.97±1.35$^{c}$ | 50.57±0.41$^{b}$ | 52.95±0.73$^{ab}$ | 55.31±0.41$^{a}$ | 39.67 | 0.00 |
| LDL (mg/dl) | 132.59±2.04$^{c}$ | 137.03±1.72$^{c}$ | 130.11±3.36$^{c}$ | 144.48±2.10$^{b}$ | 149.29±1.32$^{a}$ | 150.22±2.75$^{a}$ | 27.22 | 0.00 |
| Triglycerides (mg/dl) | 132.41±2.00$^{d}$ | 137.79±2.34$^{c}$ | 133.21±2.63$^{d}$ | 150.77±4.02$^{b}$ | 154.51±2.23$^{ab}$ | 158.07±1.93$^{a}$ | 43.76 | 0.00 |
| ALP (mg/dl) | 10.21±0.40$^{c}$ | 10.55±0.56$^{c}$ | 11.22±1.00$^{b}$ | 12.68±2.15$^{b}$ | 14.21±0.34$^{b}$ | 16.14±0.59$^{a}$ | 13.79 | 0.00 |
| ALT (U/L) | 25.11±0.51$^{d}$ | 26.62±0.52$^{d}$ | 26.82±1.13$^{d}$ | 33.43±0.89$^{c}$ | 36.61±0.77$^{b}$ | 39.96±1.39$^{a}$ | 113.90 | 0.00 |
| AST (U/L) | 31.96±1.32$^{c}$ | 31.81±1.56$^{c}$ | 30.05±1.45$^{c}$ | 41.18±0.95$^{b}$ | 43.22±1.06$^{ab}$ | 45.30±1.05$^{a}$ | 66.90 | 0.00 |
| Amylase (U/L) | 0.33±0.03$^{e}$ | 0.35±0.06$^{d}$ | 0.36±0.03$^{d}$ | 1.08±0.06$^{c}$ | 1.23±0.03$^{b}$ | 1.35±0.11$^{a}$ | 138.80 | 0.00 |
| Lipase (U/L) | 0.27±0.04$^{e}$ | 0.30±0.04$^{d}$ | 0.29±0.03$^{d}$ | 0.83±0.07$^{c}$ | 1.08±0.05$^{b}$ | 1.23±0.03$^{a}$ | 246.23 | 0.00 |
| Protease (U/L) | 0.93±0.03$^{e}$ | 1.02±0.03$^{d}$ | 1.04±0.04$^{d}$ | 1.42±0.03$^{c}$ | 1.53±0.02$^{b}$ | 2.09±0.07$^{a}$ | 321.98 | 0.00 |

Values with different superscript letter in the same column indicate significant difference at P < 0.05.
HDL = high density lipoprotein, LDL = low density lipoprotein, ALP = alkaline phosphatase, AST = aspartate aminotransferase, ALT = alanine aminotransferase.

supplementation to the diet caused a 33.33% reduction compared to the control group. Supplementation of 50 mg/kg Cu-NPs in feed also caused 34.55% increase in WBC count compared to the control group. The blood indices, specifically MCV, MCH, and MCHC exhibited fluctuations throughout the study, while the group that received a supplementation of 50 mg/kg of Cu-NPs demonstrated higher values for these indices. In addition, the levels of total protein, albumin, and globulin exhibited a statistically significant increase of 32.70, 34.86, and 40.69%, respectively, in the group supplemented with 50 mg/kg of Cu-NPs compared to the control group. 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). Similar to the present findings, elevation in the hematological parameters was reported by EL-Erian *et al*. (2023) when comparing the effect of two different doses of Cu-NPs (3 and 6 mg/kg feed) on Nile tilapia and recorded the higher values at higher doses. Research also showed that excess nanoparticles reduces the number of red blood cells and thus result in anemia by diminishing their life span or suppressing the activity of bone marrow stem cells (Faiz *et al*., 2015).

### Serum lipid and enzyme profile

The addition of Cu-NPs to the feed resulted in a statistically significant increase (P < 0.05) in the values of all serum lipid and enzyme profile parameters when compared to the control group. Supplementation of Cu-NPs at a concentration of 10 and 20 mg/kg feed did not yield any statistically significant differences when compared to the control group. This suggests that these two feeding groups did not have any harmful effects on *C. batrachus*. However, a subsequent elevation in the concentration of Cu-NPs in the feed led to a progressive rise in both the lipid and enzyme profile, indicating their potential toxicity at higher doses. Increment of serum lipids and enzymes were 29.50, 37.66, 11.74, 16.23, 36.74, 37.16, 29.45, 75.56, 78.05 and 55.50% higher at 50 mg/kg Cu-NPs supplemented group compared to the control group. The 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 findings of the present study agreed with Zaghloul *et al*. (2006) who studied the effect of Cu- NPs 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 is maybe due to hepatic cell injury or increased synthesis of the enzymes by the liver.

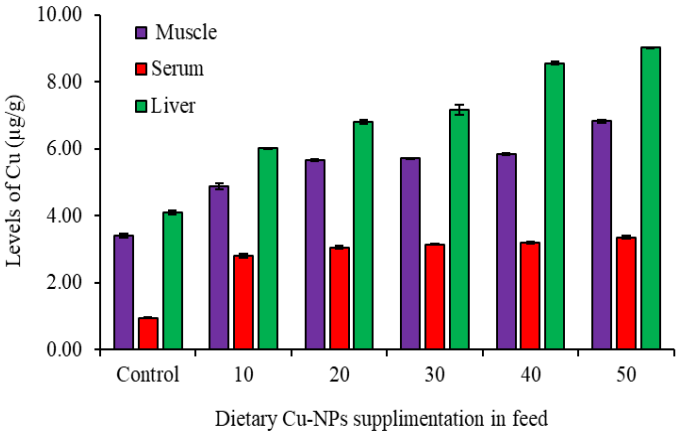


**Figure 3.** *Concentrations of Cu in muscle, serum and liver of Clarias batrachus fed on different levels of Cu-NPs enriched diets.*

### Bioaccumulation of Cu in muscle, serum and liver

The concentration of Cu in the liver was found to be the highest, followed by the muscle and serum, in the order of liver > muscle > serum (Figure 3).The average Cu accumulation in liver was 22.33 and 60.37% higher compared to muscle and serum, respectively. Additionally, the fish group fed with 50 mg/kg Cu-NPs in feed showed higher accumulation of Cu compared to the control group. Specifically, the Cu accumulation was 50% higher in the muscle, 71.73% higher in the liver, and 54.71% higher in the serum. The differences in the accumulation of metals among different tissues are often related to the metabolic activity of those tissues (Cicik, 2003; Tuncsoy *et al*., 2017). Therefore, higher metabolic activity of liver might be the reason for higher accumulation of Cu in this organ compared to muscle and serum.

## Conclusion

In conclusion, the results of the present experiments indicated that excessive (30 to 50 mg/kg feed) reduced growth performance of Bagridae catfish, *C. batrachus*, while a concentration of 20 mg/kg resulted better growth performance, feed utilization and improved physiological status. The study also revealed that 20 mg/kg Cu-NPs in feed increased the protein and lipid of fish muscle compared to the control group. Furthermore, higher accumulation of Cu was observed in liver and the lower in serum. The findings of the present study might be helpful in improving the quality of formulated feed for *C. batrachus*.

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