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Effect of Dietary Supplementation of Spirulina on Growth and Phosphatase Activity in Copper-Exposed Carp (*Labeo rohita*)

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Key words: spirulina, *Labeo rohita*, copper elimination, growth, phosphatase activity

Abstract

The impact of spirulina supplementation at 0, 2, 4, 6, or 10% on the alleviation of copper toxicity was studied in the freshwater cultivable carp, *Labeo rohita*. Evaluation was based on selected food utilization parameters and phosphatase activities. Copper concentrations in the aquatic medium, selected body tissues, and fecal matter were analyzed to determine the mechanism of toxicity reduction. Dietary supplementation of spirulina significantly improved the tested physiological and biochemical parameters and reduced the metal burden in tissues. Reduction of metal toxicity seems to be achieved via elimination of metal through feces. A significant positive correlation ($r = 0.714$; $p < 0.01$; $n = 18$) was obtained between supplementation of dietary spirulina and copper defecation. The addition of 4% spirulina was optimum since this dose produced the maximum elimination of copper from the body and better physiological and biochemical parameters. The treatment period (21 days) was not sufficient for complete removal of the copper. Therefore, a longer period of supplementation is recommended.

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Introduction

Metal contamination in freshwater bodies is a matter of serious concern from the human health point of view since many aquatic organisms, particularly fish, form an integral part of the human diet. *Labeo rohita* is one of the important cultured carp species in Asia. Among the freshwater fishes, carps are most affected to environmental contamination (Stouthart et al., 1996; Toth et al., 1996). The reduction of toxic elements in aquatic systems and organisms by acceptable methods is a need of the hour (Boyd, 1990; James et al., 2004).

Spirulina is one of the most concentrated natural sources of nutrients for all animals. Spirulina contains protein (60-70%), essential amino acids and fatty acids, phycocyanin (14%), chlorophyll (1%) and carotenoid pigments (0.37%), vitamin B-12, and minerals that play important roles in animals in various ways (Venkataraman, 1993). Spirulina improves the intestinal flora in fish by breaking down indigestible feed components (Ramakrishnan et al., 2008). It stimulates the production of enzymes that transport fats in fish for growth instead of storage (Henrikson, 1994). β -carotene in spirulina firmly maintains the mucous membrane and thereby prevents the entry of toxic elements into the body (Henrikson, 1994). Chlorophyll in spirulina acts as a cleansing and detoxifying factor against toxic substances (Henrikson, 1994). Researchers have reported the therapeutic effects of spirulina as a growth promoter, probiotic, and booster of the immune system in animals including fishes (Venkataraman, 1993; James et al., 2006).

So far, spirulina is known for its nutritive value only; its role in alleviating metal toxicity in fishes and other cultivable organisms remains unexplored. Phosphatase activities are involved in active transport, glycogen metabolism, protein synthesis, secretory activities, and synthesis of certain enzymes in fishes (Garg et al., 1987; Palanivelu et al., 2005). Any change in phosphatase activity may affect the biochemical and physiological activity in animals in several ways (Palanivelu et al., 2005). Copper is a common metal used in industries in India for electroplating and the most common contaminant of freshwater systems (James et al., 2008). In the present study, experiments were designed to investigate the impact of dietary spirulina supplementation on the growth, phosphatase activity, and alleviation of copper toxicity in carp, *Labeo rohita*.

Materials and Methods

Fish and maintenance. Experimental fish (*Labeo rohita*) were collected from Manimuthar Dam in Tirunelveli, Tamil Nadu, and acclimated for 30 days to laboratory conditions in water with the following characteristics: DO 4.08 ± 0.6 ml/l, $29.1 \pm 0.6^\circ\text{C}$, pH 7.7 ± 0.06 , salinity 0.16 ± 0.003 ppt, and hardness (CaCO_3) 83 ± 2.3 ppm. During acclimation, water was changed daily and fish were fed a pelletized diet containing 35% protein *ad libitum*.

Copper toxicity. Acclimated fish (1.27 ± 0.10 g) were exposed to different toxic concentrations of copper (0, 0.10, 0.15, 0.20, 0.30, 0.40, or 0.50 ppm) and mortality was observed for 96 h. A control group was maintained in metal-free water. A stock solution of copper was prepared by dissolving 3.93 g analar grade $\text{CuSO}_4 \cdot 7 \text{H}_2\text{O}$ (Merck) in one liter of distilled water. The desired concentration was obtained by dilution with fresh water. A static renewable bioassay method was used to determine the 96-h median lethal concentration (Sprague, 1973). Probit analysis was used to calculate 96-h LC_{50} (Litchfield and Wilcoxon, 1949). The 96-h LC_{50} value of copper for *L. rohita* was 0.23 ppm and its 95% confidence limits were -1.10 (lower limit) and 1.82 (upper limit).

Feed. Fish were fed a 35% protein diet containing dried fishmeal, ground nut oil cake, cod liver oil, egg yolk, tapioca flour, and vitamin and mineral mixtures in proportions determined by the Square method (Hardy, 1980). In addition to the control diet, five test diets containing 0, 2, 4, 6, or 10% spirulina) were prepared (Table 1). The appropriate level of spirulina was added to the ingredients and an aliquot of boiled water. The mixture was well mixed and steam cooked for 15-20 min. After moderate cooling, pellets (2 mm) were prepared with a hand-operated pelletizer and dried in sunlight. After drying, diets were separately stored in a refrigerator. The protein and lipid contents of the diets were determined in a spectrophotometer following Lowry et al. (1951) and Bragdon (1951), respectively. The moisture content was analyzed by drying in an electric hot air oven at 100°C and the remaining minerals (ash) were estimated following the method of Paine (1964). Nitrogen-free extract was calculated by subtracting the protein, lipid, and mineral contents from the dry weight of the feed samples.

Table 1. Formulation and composition of the experimental diets and their proximate composition (% dry matter basis).

Ingredient (g)	Diet (spirulina content)					
	Control	0	2%	4%	6%	10%
Fishmeal	398	398	376	362	348	320
Ground nut oil cake	368	368	351	337	323	297
Tapioca flour	145	145	150	154	162	173
Wheat flour	87	87	101	105	105	108
Cod liver oil (ml)	1	1	1	1	1	1
Mineral mix	1	1	1	1	1	1
Spirulina	0	0	20	40	60	100
<i>Proximate composition (%)</i>						
Crude protein	34.80 ± 1.56	34.80 ± 1.56	35.09 ± 1.03	35.75 ± 1.67	37.60 ± 2.15	39.25 ± 2.01
Crude fat	7.16 ± 0.05	7.16 ± 0.05	6.70 ± 0.03	6.10 ± 0.03	5.50 ± 0.04	4.96 ± 0.03
Ash	15.67 ± 1.15	15.67 ± 1.15	16.58 ± 1.23	16.87 ± 1.13	17.37 ± 1.30	18.25 ± 1.18
Nitrogen free extract	42.37 ± 3.20	42.37 ± 3.20	41.63 ± 2.33	41.28 ± 2.65	39.53 ± 2.11	37.54 ± 1.89

Experiment 1: growth and efficiency. Active healthy juveniles (1.43 ± 0.27 g) were chosen from the acclimation tank and starved for 24 h prior to the commencement of the experiment. The fish were divided into 18 groups of ten individuals, each, three replicates of each of the six treatments. The control group was reared in copper-free fresh water and fed a spirulina-free diet. Animals in the remaining five groups were exposed to 0.12 ppm copper (50% of the LC_{50} level). The experiment was conducted in epoxy-coated cement tanks (0.6 x 0.6 m; 110 l) containing 100 l of water. Water was not changed during the experiment but was aerated 24 h to avoid oxygen depletion. Hydrobiological parameters were measured once a week and averaged: DO 4.28 ± 0.25 ml O_2/l , $28.5 \pm 0.3^\circ C$, pH 7.8 ± 0.1 , salinity 0.32 ± 0.2 ppt, and hardness 77 ± 3.30 mg $CaCO_3/l$.

Fish were fed weighed quantities of the experimental diet twice a day at 07:00 and 18:00. Unconsumed feed was removed 1 h after feeding and dried in a hot air oven at $80^\circ C$ for two days. Feed intake was estimated by subtracting the amount of unconsumed dry feed from the total dry weight of the offered feed. The feeding rate (mg/g live fish/day) was computed as the amount of feed consumed/(initial wet wt of the fish x no. days). Feed samples and unconsumed feed were weighed in an electric monopan balance to 1 mg accuracy. The duration of the experiment was 21 days.

At the beginning the experiment, the total wet weight of the fish in each group was weighed in an electric monopan balance. Five fish from the stock were sacrificed to estimate water content (Maynard and Loosli, 1962) and determine the initial dry weight of the fish. All fish in each group were weighed at the end of the experiment and dry weight was calculated using the percent water content of the fish sacrificed at the beginning of the experiment. Weight gain (growth) was calculated as the difference between initial and final dry fish weight. Growth rate (mg/g live fish/day) was calculated as growth/(initial wet wt of fish x no. days). Gross conversion efficiency (%) was calculated as growth/feed intake x 100. Feed conversion ratio (FCR) was computed as the relation between feed intake and growth.

Experiment 2: phosphatase activity and copper content. A parallel experiment was conducted simultaneously for 21 days to study the impact of dietary spirulina on phosphatase (acid and alkaline) activity and metal accumulation. Fish were fed experimental diets *ad libitum* twice a day at 07:00 and 18:00 h for 1 h, each feeding. Fecal matter was randomly collected before feedings using enamel trays and dried in a hot air oven at $60^\circ C$ to estimate the copper content. At the end of the experiment, test animals were starved for 24 h.

The acid and alkaline phosphatases were estimated according to the method of Bergmayer (1963) using p-nitrophenyl phosphate as a substrate. Three fish were removed from each experimental group on day 23. They were dissected and gill, liver, and muscle tissues were subjected to phosphatase analysis. The activity of each enzyme and the copper content in the fecal matter were estimated three times and the data were subjected to Student's *t* test, correlation, and regression analysis (Zar, 1984).

Copper content in the liver, muscle, gill, feces, and water was estimated on day 23. The remaining seven fishes of each group were dissected and their tissues were pooled. Three samples from each tissue from each group were digested in a water bath at 100°C with a mixture of concentrated nitric acid and perchloric acid at a ratio of 1:2 until the formation of a white residue. The cooled residue was completely dissolved by adding 1 N HCl and made up to 25 ml with distilled water (FAO, 1975). The copper concentration in the water was estimated following the method of APHA (1993). The solution was filtered through cotton wool and the filtrate was subjected to metal analysis in atomic absorption spectrophotometry (GBC Avantha model). The instrument was calibrated using standards prepared from copper sulfate. The bioconcentration factor (BF) was determined as $(tc_{21} - tc_0)/\text{copper concentration in water}$, where tc_{21} = tissue concentration on day 21 and tc_0 = tissue concentration of day 0.

Results

Dietary spirulina supplementation significantly ($p < 0.05$) improved feeding and growth parameters in copper-exposed fish (Table 2). The FCR of fish fed 4% spirulina was lower (4.70) than the other test groups and similar to that of the control fish (4.22). Similar trends were obtained in the acid and alkaline phosphatase activity experiment; increased supplementation of spirulina diets enhanced both phosphatase activities up to a midpoint (4% spirulina) after which it reached a plateau; Student's t test revealed that 6% and 10% levels of spirulina did not significantly ($p > 0.05$) increase phosphatase activity beyond that of 4% spirulina (Fig. 1). Among the tested tissues, the liver registered the highest activity for both phosphatases, followed by gill and muscle.

The highest copper accumulation was found in the gill tissue, followed by the liver and muscle (Table 3). Copper accumulation was significantly ($p < 0.05$) highest in the 0 spirulina group and gradually decreased as the spirulina level increased. The maximum reduction of copper accumulation in tissues occurred with the 10% spirulina diet, followed by 6%, 4%, and 2% diets, but there were no significant ($p > 0.05$) differences between the 4%, 6%, and 10% diets. The elimination of accumulated copper through feces increased as the spirulina level increased. A statistically significant ($r = 0.714$; $p < 0.01$; $n = 18$) positive correlation coefficient was obtained for the relationship between the level of dietary spirulina and the elimination of copper through feces (Fig. 2). The bioconcentration factor was highest in copper-exposed fish fed the spirulina-free diet and drastically dropped in fish fed diets containing spirulina.

Table 2. Effect of dietary supplementation of spirulina levels on selected food utilization parameters in copper exposed *Labeo rohita*. Each value is the mean±SD of three observations.

Parameter	Diet (spirulina content)					
	Control	0	2%	4%	6%	10%
Feed intake (g dry matter)	32.66±2.10	16.04±1.26	22.73±1.80	27.73±2.30	28.32±2.45	27.45±2.64
Consumption rate (mg/g live fish/day)	51.85±4.60	26.06±2.17	33.08±3.01	46.11±4.53	42.54±3.96	42.29±4.28
Weight gain (g wet wt)	7.73±0.48	1.13±0.02	2.55±0.14	5.90±0.43	5.58±0.51	5.10±0.43
Weight gain (%)	25.75±0.64	3.42±0.65	7.79±0.62	20.60±0.55	17.56±0.68	16.49±0.62
Growth rate (mg/g live fish/day)	12.27±1.13	1.63±0.02	3.73±0.03	9.81±0.61	8.36±0.78	7.86±0.55
Gross conversion efficiency (%)	23.66±2.06	7.05±0.38	11.22±1.08	21.28±2.11	19.66±1.67	18.56±1.39
Feed conversion ratio	4.22±0.35	14.19±1.21	8.91±0.74	4.70±0.33	5.09±0.48	5.38±0.53

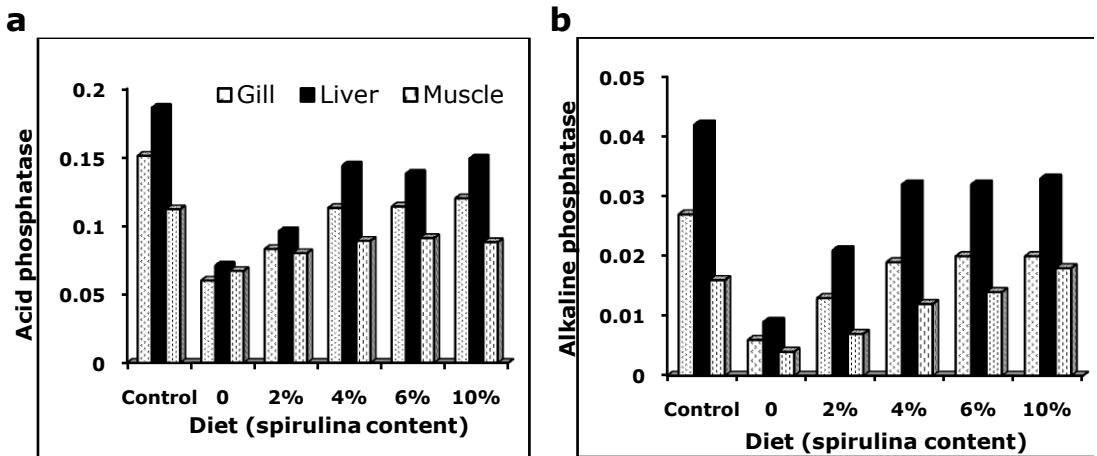


Fig. 1. Effect of feeding different levels of dietary spirulina on (a) acid and (b) alkaline phosphatase activity (mg p-nitrophenyl phosphate/mg protein/h) in copper-exposed *Labeo rohita*.

Table 3. Effect of dietary spirulina level on metal distribution in tissues ($\mu\text{g Cu/g}$ wet tissue), feces ($\mu\text{g Cu/g}$ dry matter), and water ($\mu\text{g Cu/l}$) in *Labeo rohita* exposed to a sublethal level of copper for 21 days (means±SD; n = 3).

Component	Diet (spirulina content)					
	Control	0	2%	4%	6%	10%
Gill	ND	0.445±0.030	0.380±0.027	0.268±0.018	0.244±0.024	0.235±0.017
Liver	ND	0.338±0.025	0.289±0.021	0.202±0.011	0.195±0.018	0.189±0.020
Muscle	ND	0.113±0.010	0.092±0.007	0.062±0.005	0.058±0.006	0.056±0.004
Feces	ND	0.025±0.001	0.061±0.004	0.980±0.012	1.080±0.015	1.050±0.006
Water	ND	0.107±0.002	0.094±0.005	0.088±0.006	0.086±0.009	0.088±0.009
BF	ND	26.84±2.020	9.24±0.025	0.40±0.030	0.33±0.040	0.33±0.020

BF = bioconcentration factor, ND = not detected.

Students *t* test comparing 0 spirulina diet vs 2%: for gill, $t = 2.040$, $p < 0.05$; for liver, $t = 5.097$, $p < 0.01$; for muscle, $t = 2.040$, $p < 0.05$; for feces, $t = 34.100$, $p < 0.01$

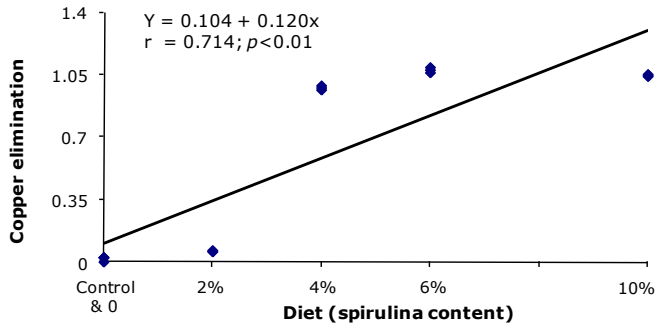


Fig. 2. Effect of dietary supplementation of *Spirulina* levels on elimination of copper ($\mu\text{g Cu/g}$ dry matter) through feces in copper-exposed *Labeo rohita*.

Discussion

The study showed that feeding and growth parameters improved in copper-exposed fish fed spirulina-supplemented diets. Spirulina reduced copper accumulation in tissues and increased copper elimination through feces, lessening the metal burden and its toxicity to fish. Likewise, addition of an ion-exchanging agent, zeolite, to cadmium-contaminated media significantly reduced the cadmium (Cd) level in water and fish (elimination of metals through feces), reducing Cd toxicity and improving biochemical and growth parameters in *Heteropneustes fossilis* (James and Sampath, 1999).

The reduced growth rate in fish given a sublethal level of copper was probably due to the tissue burden of copper which, in turn, could have caused a reduction in feed intake, an increase in metabolic cost, or poor food conversion efficiency. Growth reduction in copper-exposed *Salmo gairdneri* was partly due to increased metabolic costs and reduced food consumption (Lett et al., 1976). The RNA:DNA ratio is a sensitive measure of growth rate in fish (Love, 1980). A reduction in the RNA:DNA ratio in copper-exposed *Oreochromis mossambicus* was due to the low production of RNA for protein synthesis due to the copper burden in the tissues (James et al., 2004).

Acid and alkaline phosphatase enzyme activity improved in copper-exposed *L. rohita* fed spirulina-supplemented diets. This agrees with previous studies of Garg et al. (1987) and Palanivelu et al. (2005). Copper-exposed *L. rohita* fed spirulina-supplemented diets might have eliminated the copper from the body tissues through feces, enhancing phosphatase activities. Spirulina reduced genotoxicity and oxidative stress of several antibiotics in mice (Premkumar, 2004) and lead (Pb) toxicity in rats (Upasani, 2003).

The present study concludes that dietary supplementation of spirulina significantly improves food utilization and phosphatase activity in metal-contaminated *L. rohita* even within a short period of 21 days. However, the feeding duration (21 days) was not sufficient for complete removal of the

metal from the fish body. Four percent spirulina is considered the optimum level of supplementation for *L. rohita* since lower and higher levels did not elicit better performance. Spirulina can be used not only as a feed additive to enhance growth in fishes but also as a metal-alleviating substance in a contaminated medium.

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