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Reecha Sharma
Department of Zoology, CCS Haryana Agricultural University, Hisar, Haryana, India

Rachna Gulati
Department of Aquaculture, CCS Haryana Agricultural University, Hisar, Haryana, India

Paramveer Singh
Department of Aquaculture, CCS Haryana Agricultural University, Hisar, Haryana, India

Corresponding Author:
Reecha Sharma
Department of Zoology, CCS Haryana Agricultural University, Hisar, Haryana, India

Bioefficacy of *Azolla indicus* in sewage treated water and its effect on *Pangasius hypophthalmus* growth

Reecha Sharma, Rachna Gulati and Paramveer Singh

Abstract
The experiment was conducted to evaluate the bioefficacy of *Azolla indicus* in sewage treated water and its effect on *Pangasius hypophthalmus* growth in *vitro* condition. The physico-chemical parameters of water were recorded on 0, 7, 14 and 21 days to see the role of *Azolla* as bio-filter. The bio-filtered water from *Azolla* culture was used to observe its effect on *P. hypophthalmus* growth parameters and compared with fresh and sewage treated water with feed. The results on the growth performance of fishes in fresh, sewage treated and biofiltered *Azolla* water showed that the maximum growth was observed in sewage treated water followed by biofiltered *azolla* water and least in freshwater culture. *A. indicus* was successfully cultured and maintained in fresh and sewage treated water. Bio-filtrate from both the cultures was estimated for water quality parameters. Additionally, effect of *A. indicus* culture on heavy metal concentration was also evaluated.

Keywords: *Azolla indicus*, bio-filtered *azolla* water, *P. hypophthalmus*, sewage treated water

Introduction

Sewage-fed aquaculture is a unique system which acts as a major source of nutrients for crop farming and aquaculture, economical for sustainable production and helps to combat environmental pollution. The use of municipal wastewater fed to fertilize ponds began in Calcutta in the 1930s; the city now has perhaps the largest wastewater-fed aquaculture system in the world (Jana 1998) [6]. A large number of people derive their livelihood from the sewage-fed aquaculture in many states. A sewage fed pond does not require fertilizers and supplemented food. This reduces the cost of culture and at the same time the growth rate of fish in such ponds is also faster (Drechsel *et al*. 2009) [3]. Water quality is an important factor which directly affects the fish's growth rate, health, survival and economics of the system. The Biological Oxygen Demand (BOD), Dissolved Organic Matter (DOM), dissolved oxygen and total alkalinity were higher in pond with raw water than pond with treated water (Osman and El-Khateeb, 2016) [8]. The presence of high organic matter in water also increases population of microorganisms. The higher concentrations of pH, BOD, COD and ammonia were found to be positively correlated with the bacterial count (Dosoky *et al*. 2015) [2]. Presence of heavy metals in sewage treated water also affect the quality of the aquatic environment, with its long-term impact on living organisms especially, some metals which are not biodegradable and accumulated in different organs of human and animals as well as plant (Saad *et al*. 2012) [9].
Azolla (water fern) is a unique freshwater small-leaf floating fern, being one of the fastest growing plants on the planet due to its symbiotic relation with a cyanobacterium called *Anabaena*. It was found that *Azolla* has the potential to remove the heavy metals (Cohen-Shoel *et al*. 2002) [1] and caused reduction in physicochemical parameters (Noorjahan and Jamuna 2015) [7] from polluted water. *Azolla* were also shown to have a high potential to remove COD, phosphorus, and nitrogen by 44 to 98.8 percent in 28 days (Golzary *et al*. 2018) [5]. It was considered one of the most promising agents to remove COD and treat nitrogen-free and phosphorus-rich wastewaters. Keeping these facts in mind, present study was planned to evaluate the bioefficacy of *Azolla indicus* in sewage treated water and its effect on *Pangasius hypophthalmus* growth.

Materials and Methods

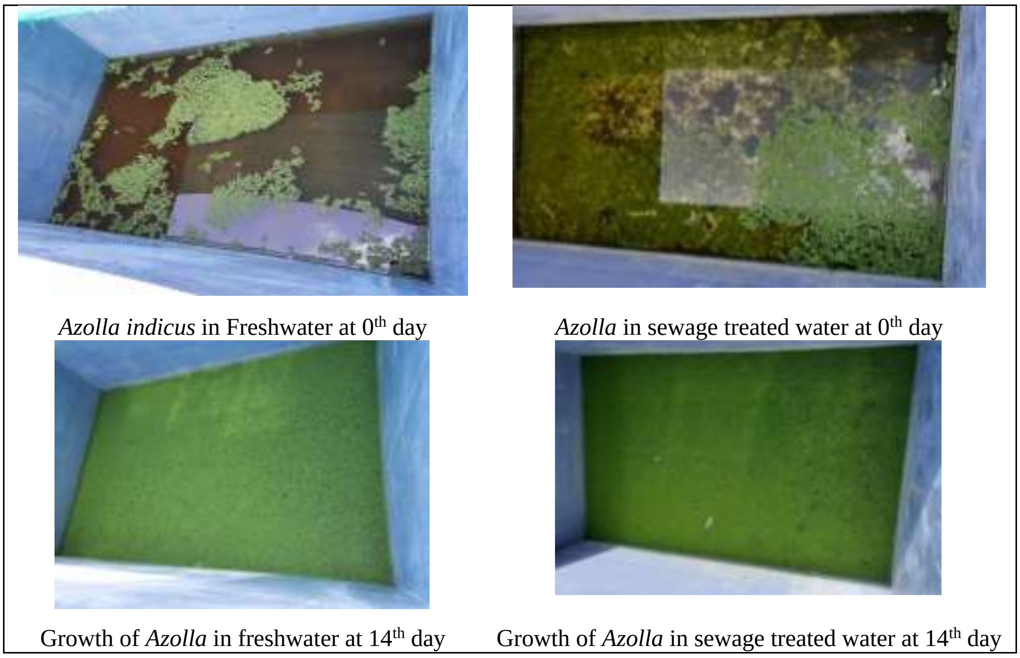
For present study, sewage treated water was collected from Sewage treated water ponds located near Vegetable Research Farm, CCSHAU, Hisar and brought to the water quality laboratory of the College of Fisheries Science, CCSHAU, Hisar. Small inoculums of *Azolla indicus* was-procured from fish farmer to raise its culture in fresh water and sewage treated water.

The culture so reared were used in experimentation.

Cultivation of *Azolla*: The *Azolla* was cultivated in cemented water tanks. The tank was filled with freshwater/ sewage treated water up to a certain depth. The cow dung (1Kg): super phosphate (100 g) slurry was prepared and poured into the tanks (Fig 1). Fresh and pure culture of *A. indicus* was inoculated (500 g) into tanks containing fresh and sewage treated water and tanks were covered with a net to provide partial shade and to prevent the falling of leaves and other debris. After 15 days, the water tanks were fully covered with *Azolla* (Fig 2). Periodic application of slurry was carried out to ensure the proper growth of the *Azolla*. Biomass was removed on every fourth day to avoid overcrowding

Water Analysis: Physico-chemical parameters of fresh water and sewage treated water viz., temperature, conductivity, total dissolved solids, salinity, pH, ammonia, nitrite, hardness, alkalinity and heavy metals were estimated following standard methods before and after the experiment.

Fig 1: Preparation of *Azolla* culture



Azolla indicus in Freshwater at 0th day

Azolla in sewage treated water at 0th day

Growth of *Azolla* in freshwater at 14th day

Growth of *Azolla* in sewage treated water at 14th day

Fig 2: Growth of *Azolla* in freshwater and sewage treated water

Effect on *Pangasius hypophthalmus* growth

The experiment was conducted in aquariums of 200 liter capacity under three treatments: sewage treated water, biofiltered water from *A. indicus* and fresh water under triplicate conditions. After acclimatization, five *P. hypophthalmus* fish of known weight and length were released in each aquarium with two aerators. The fishes were fed with commercially available pelleted feed at the rate of 8 percent of the body weight. Observations on weight, length, weight gain, percent increment in weight, percent increment in length, average daily weight gain, specific growth rate, survivability and Feed conversion ratio for *P. hypophthalmus* and water parameters were recorded at 0, 7, 14 and 21 days following standard methodology.

Statistical Design: Water quality parameters of *A. indicus* cultured fresh water and sewage treated water were analysed statistically by calculating Critical Difference (CD) through ANOVA. Two factorial ANOVA was applied on comparative evaluation of fresh, sewage treated and biofiltered A. indicus water on weight and length of *P. hypophthalmus*.

Results

Water quality parameters of fresh water cultured *Azolla indicus*

The change in physico-chemical parameters (temperature, conductivity, total dissolved solids, salinity, pH, ammonia concentration, nitrite concentration, hardness and alkalinity) of fresh water was estimated after 0, 7, 14 and 21 days of growing *A. indicus* (Table 1). There was no significant difference in the conductivity, total dissolved solids, salinity, nitrite and hardness of water due to *Azolla* culture during 21 days of study period. The water pH, ammonia and alkalinity declined significantly in *Azolla* cultured fresh water. It was 7.6, 0.40 mg/l and 213 mg/l at 0 day which significantly decreased to 7.15, 0 and 103.5 mg/l at 21st day.

Table 1: Water quality parameters of *Azolla indicus* cultured fresh water

Days	Temperature (°C)	Conductivity (ms/cm)	Total Dissolved Solids (ppm)	Salinity (ppt)	pH	Ammonia (mg/l)	Nitrite (mg/l)	Hardness (mg/l)	Alkalinity (mg/l)
0	28.95	1.00	248.25	0.56	7.60	0.40	0.002	181.00	213.00
7	30.15	0.85	207.50	0.46	7.40	0.20	0.000	185.50	122.50
14	33.00	0.64	196.75	0.42	7.20	0.10	0.000	178.00	106.00
21	33.50	0.53	132.25	0.30	7.15	0.00	0.000	159.00	103.50
CD (p=0.05)	1.84	N/A	N/A	N/A	0.22	0.10	0.000	N/A	81.13
SE(m)	0.56	0.19	48.46	0.11	0.07	0.03	0.000	17.09	24.50

Water quality parameters of sewage treated water cultured *Azolla indicus*

A significant change in physico-chemical parameters of sewage treated water was recorded in which *Azolla* was cultured (Table 2). There was significant decrease in the conductivity (0.79 ms/cm; CD =0.52, p=0.05), total dissolved solids (196.17 ppm; CD =8.78, p=0.05), salinity (0.44 ppt; CD =0.05, p=0.05), ammonia (0.00 mg/l; CD =0.23, p=0.05), hardness (196 mg/l; CD =12.32, p=0.05) and alkalinity (112 mg/l; CD =8.60, p=0.05) of sewage treated water due to *Azolla* culture after 21 days as compared to 2.69 ms/cm, 588.67 ppm, 1.32 ppt, 0.50 mg/l, 310 mg/l and 276 mg/l, respectively at 0 day. The water pH and nitrite concentration did not show any significant difference.

Table 2: Water quality parameters of *Azolla* cultured sewage treated water

Days	Temperature (°C)	Conductivity (ms/cm)	Total Dissolved Solids (ppm)	Salinity (ppt)	pH	Ammonia (mg/l)	Nitrite (mg/l)	Hardness (mg/l)	Alkalinity (mg/l)
0	29.80	2.69	588.67	1.32	8.20	0.50	1.002	310.00	276.00
7	33.83	2.04	510.00	1.14	7.93	0.40	1.000	280.00	190.00
14	32.60	0.80	203.00	0.58	7.60	0.20	1.000	210.00	128.00
21	35.70	0.79	196.17	0.44	7.40	0.00	1.000	196.00	112.00
CD (p=0.05)	0.49	0.52	8.78	0.05	N/A	0.23	0.000	12.32	8.60
SE(m)	0.15	0.16	2.65	0.02	0.19	0.07	0.000	3.72	2.60

Effect of *Azolla indicus* culture on heavy metals in sewage treated water

Heavy metal concentration in sewage treated water were compared before and after *Azolla* treatment (Table 3). The results showed that *Azolla* culture significantly reduced the heavy metal concentration (0.035 mg/l) in sewage treated water after 21 days from 0.107 mg/l at 0 day (CD=0.025; p=0.05). Among the heavy metals, iron concentration was significantly higher (0.140 mg/l) than zinc (0.113 mg/l), copper (0.027 mg/l) and manganese (0.005 mg/l) (CD=0.039; p=0.05) although all were well below the permissible limits. The interaction between heavy metal concentration and duration was significant (CD=0.055; p=0.05). Iron zinc copper and manganese concentrations were significantly lower after 21 days of *Azolla* culture as compared to initial concentrations.

Table 3: Heavy metal concentration in sewage treated water before and after *Azolla* treatment

Heavy metals	Heavy metal concentration in sewage treated water (mg/l)		Mean
	Before *Azolla* treatment (0th day)	After *Azolla* treatment (21st day)	
Cu	0.034	0.020	0.027
Mn	0.010	0.000	0.005
Fe	0.183	0.097	0.140
Zn	0.201	0.024	0.113
Mean	0.107	0.035	

CD (p=0.05) for heavy metals =0.039; SE(m) = 0.013
CD (p=0.05) for duration=0.025; SE(m) =0.008
CD (p=0.05) for heavy metals × duration =0.055; SE(m) = 0.019

Effect on *Pangasius hypophthalmus* weight

The effect of fresh, sewage treated and biofiltered *Azolla* water on the weight of *P. hypophthalmus* is presented in Table 4. Weekly analysis showed a significant effect of observation days on *P. hypophthalmus* weight (CD=0.968; p=0.05). It was 8.25 g at 0 day which significantly increased to 10.16, 12.41 and 15.19 g at 7, 14 and 21 days. All the treatments; fresh, sewage treated and biofiltered *Azolla* water were statistically comparable with each other in terms of change in *P. hypophthalmus* weight. Interaction between treatments and observation days was non significant meaning that the weight of fish in these treatments did not differ significantly with each other at 0, 7, 14 and 21 days.

Table 4: Effect of fresh, sewage treated and biofiltered *Azolla* water on *Pangasius hypophthalmus* weight

Treatments	Weight (g) of *Pangasius hypophthalmus* at				Mean
	0 day	7th day	14th day	21st day	
Fresh water	8.30	9.92	11.82	14.22	11.06
Sewage treated water	8.11	10.32	13.07	16.20	11.92
Biofiltered *Azolla indicus* water	8.36	10.25	12.34	15.15	11.52
Mean	8.25	10.16	12.41	15.19	

CD (p=0.05) for Treatments =N/A; SE(m) = 0.30
CD (p=0.05) for Days =0.96; SE(m) =0.34
CD (p=0.05) for Treatments × Days =N/A; SE(m) = 0.60

Effect on *Pangasius hypophthalmus* length

Concurrent with the above trend, the length of fish reared in fresh (11.69 cm), sewage treated (12.14 cm) and biofiltered *Azolla* (12.18 cm) water was statistically at par with each other. Observation days exhibited significant difference on the length of *P. hypophthalmus* (Table 5). At the time of initiation of experiment, it was 10.35 cm which significantly increased to 11.59, 12.43 and 13.66 cm at 7, 14 and 21 days (CD=0.55; p=0.05). Interaction between treatments and observation days showed higher length of fish in sewage treated water followed by biofiltered *Azolla* and fresh water at all observation days (CD=0.96; p=0.05).

Effect of these treatments on other growth parameters of *P. hypophthalmus* are represented in graphical form. The weight gain, percent increment in weight and length was comparatively higher in sewage treated water (121.43 g, 99.9%, 48.7%) followed by fishes cultured in biofiltered *Azolla* water (101.81 g, 81.2%, 26.8%) (Fig 6). Least weight gain (88.86 g), percent increment in weight (71.7%) and length (21.9%) was recorded in fresh water cultured fishes. In all the three treatments, 100 percent fishes survived, no mortality was recorded (Fig 6). Likewise, average daily weight gain and specific growth rate was maximum in sewage treated water (0.38 g/day, 1.43%) followed by biofiltered *Azolla* water (0.32 g/day, 1.23%) and fresh water (0.28 g/ day, 1.11%) (Fig 7). Feed conversion ratio was calculated as 1.74, 1.59 and 1.36 in fresh, biofiltered *Azolla* and sewage treated water, respectively.

Table 5: Effect of fresh, sewage treated and biofiltered *Azolla* water on *Pangasius hypophthalmus* length

Treatments	Length (cm) of *Pangasius hypophthalmus* at				Mean
	0 day	7th day	14th day	21st day	
Fresh water	10.56	11.38	11.97	12.87	11.69
Sewage treated water	9.71	11.60	12.81	14.44	12.14
Biofiltered *Azolla indicus* water	10.79	11.8	12.50	13.66	12.18
Mean	10.35	11.59	12.43	13.66	

CD (p=0.05) for Treatments =N/A; SE(m) = 0.19
CD (p=0.05) for Days =0.55; SE(m) =0.19
CD (p=0.05) for Treatments × Days =0.96; SE(m) = 0.34

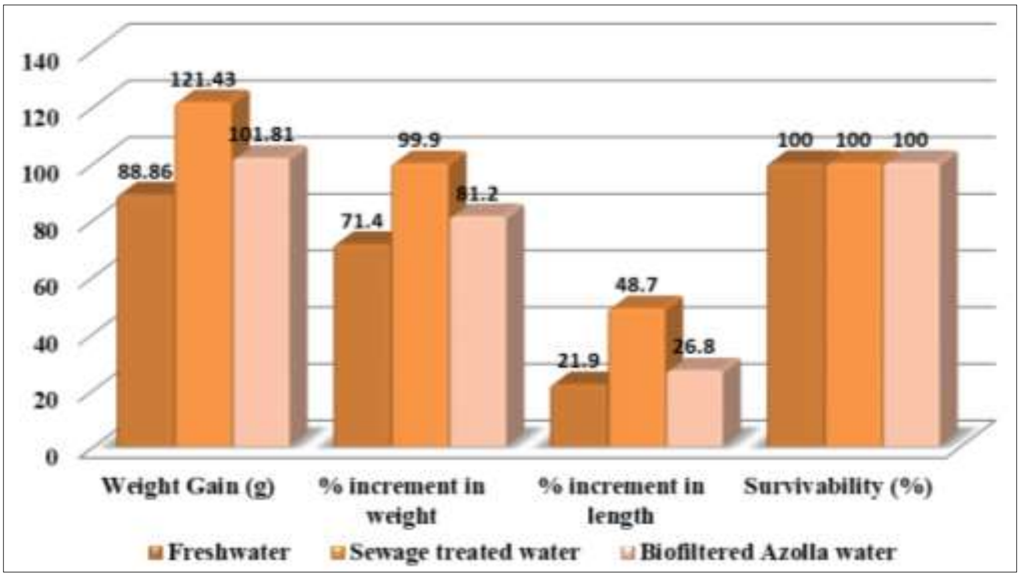


Fig 6: Growth parameters of *Pangasius hypophthalmus* in fresh, sewage treated and bio-filtered *Azolla* water

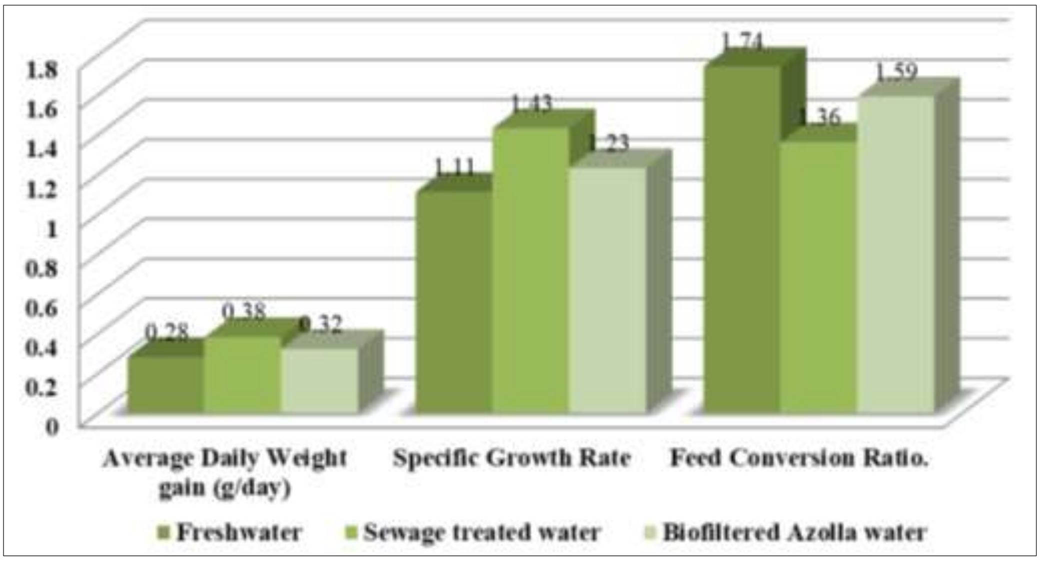


Fig 7: Daily weight gain, specific growth rate and feed conversion ratio in *Pangasius hypophthalmus*

Discussion

Effect of *Azolla* on sewage treated water quality

The effect of *A. indicus* on water quality parameters showed that there was significant decrease in the water parameters such as conductivity (2.69 to 0.79 mS/cm), total dissolved solids (588.67 to 196.17 ppm), salinity (1.32 to 0.44 ppt), pH (8.2 to 7.4), ammonia (0.50 to 0.00 mg/l), nitrite (1.002 to 1.0 mg/l), hardness (310 to 196 ppm) and alkalinity (276 to 112 mg/l) of sewage treated water. Similar results were observed for *Lemna* and *Azolla macrophytes* cultured in a fish pond

(Ferdoushi *et al*. 2008). A significant difference was observed for pH (7.53, 7.61, 8.09), alkalinity (133.87, 144.93, 224.41 mg/L), Nitrogen content (1.29, 1.33, 1.87 mg/l), Phosphorus content (1.04, 1.04, 1.64 mg/l) in *Lemna* biofiltered pond, *Azolla* biofiltered pond and control pond, respectively. Nitrogen and Phosphorus removal at 100 ppm of each component in separate medium turned out to be 36 and 44 percent, respectively represented the similar results. (Golzary *et al*. 2018) [5].

During present investigation, the concentration of various heavy metals decreased significantly. It was as follows: Cu (0.034 to 0.020 mg/l), Mn (0.010 to 0.00 mg/l), Fe (0.183 to 0.097 mg/l) and Zn (0.201 to 0.024 mg/l) in a time duration of 21 days. *Azolla's* potential as an efficient and simple tool for the removal of polluting heavy metals from water was also demonstrated (Cohen-Shoel *et al*. 2002) [1]. *A. microphyta* has the highest ammonia bio filtration efficiency which can remove ammonia up to 32.2±3.0 percent of the total NH_3-N and NH_4+-N (TAN) signified the similar results (Sumoharjo *et al*. 2018).

Effect of *Azolla* filtered water on fish growth

The effect of *A. indicus* biofiltered water on fish growth was comparable to the growth performance of fish in sewage treated water. The weight and length of *P. hypophthalmus* reared in sewage treated water (11.92g and 12.14 cm) was statistically comparable with the *P. hypophthalmus* reared in *A. indicus* biofiltered water (11.52 g and 12.18 cm) at the end of the present study. The introduction of aquatic macrophytes (*Lemna* and *Azolla*) in ponds enhance the water quality in a sustainable way, especially reducing the nutrient loading to the aquatic environment for sustainable aquaculture (Ferdoushi *et al*. 2008) [10].

Conclusion

Azolla culture significantly improved the water quality parameters and reduced the heavy metal concentration in sewage treated water. All the treatments; fresh, sewage treated and biofiltered *Azolla* water were statistically comparable with each other in terms of change in *P. hypophthalmus* weight and length.

Acknowledgement

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Ethics approval: The present study was approved by the Advisory committee appointed by Dean PGS of Chaudhary Charan Singh Haryana Agricultural University, Hisar and by Dean PGS himself. Moreover, no anesthesia was given or the animal was sacrificed during the study. All methods involved in the study was carried out in accordance with relevant guidelines and regulations.

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