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Study of Sanitary Conditions and Quantitative Estimation of Bacterial Flora in Tank Goby (*Glossogobius giuris*) Fish and Pond

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Abstract
A study was conducted to monitor the quantitative bacterial flora in the *Glossogobius giuris*, water and sediment of the pond. Mean water temperature (°C), dissolve oxygen (mg/l), and pH were 19±3, 30±4, 5.6±0.5, 5.1±0.2, 6.8±0.4 and 7.1±0.3 in winter and summer respectively. In winter total viable bacterial count in water ranged from 9.15×10^3 to 6.20×10^4 CFU/ml and in sediment ranged from 7.72×10^7 to 3.80×10^8 CFU/g. In summer, it ranged from 7.74×10^4 to 4.67×10^5 CFU/ml in water and 4.84×10^8 to 2.77×10^9 CFU/g in sediment. On the other hand, in fish, total viable plate count (TVC) ranged from 8.17×10^5 to 1.12×10^6 CFU/g in winter and 4.60×10^6 to 3.06×10^7 CFU/g in summer from the pond. Gram-negative rods (77%) and motile bacteria (78%) were dominated. Present study on the bacteriology of the pond was very useful in gathering a lot of valuable information regarding the physico-chemical parameters of water and the bacterial load of water, sediment and fish samples.

Keywords: dissolved oxygen, bele, gram positive, gram negative, enteric bacteria

1. Introduction
Bangladesh is very rich in inland freshwater resources. Bangladesh is a country with hundreds of rivers and ponds and is notable for being a fish-loving nation, acquiring the name "Machh-e Bhat-e Bangali" which means, "Bengali by fish and rice". The inland water bodies include ponds, rivers, canals, reservoirs, lakes, Oxbow lakes, floodplains and natural depressions. The closed inland water resources are estimated 7.74 million hectares of which 3.71 million-hectare ponds, 0.055 million-hectare oxbow lakes, 2.75-million-hectare shrimp farm, 1.22-million-hectare seasonal water bodies;
27.11 million-hectare floodplains [1]. Microorganisms are widely distributed in nature and are found mostly in natural water. In urban and densely populated rural areas, the microbiological quality of fresh water is frequently threatened by contamination with untreated domestic wastewater. Biological pollutants (human and livestock) as well as physical and chemical pollutants have negative influences on the microbial community in the water and sediments [2]. As an important part of the aquatic environment, the sediment has attracted great interest and emphases by many researchers. The bacteria living in the sediment have fundamental roles in the biogeochemical cycles of elements [3, 4] and can cause perceptible changes in the surrounding environment by their uptake and release of chemicals [5, 6]. An attempt was made in this report to investigate the occurrence of bacterial populations quantitatively at seasonal level by studying the micro flora of (Glossogobius giuris) for the development of preventive measures to safeguard against infectious agents that could cause disease and financial losses. By monitoring the bacteria contents of the fish, the quality of fish can be measured since these will affect the storage life and quality of the fishery products7. The fish Glossogobius giuris which are nutritionally valued is commonly known as Tank Goby and locally known as Bele, Bailla. This species is found in various types of river, pond and lakes in Bangladesh. It belongs to a family Gobidae of order Perciformes. For the assessment of microbial load, Tank goby fish species were selected because these species have high demand in market due to its scarcity in the water body in our country. Microbiological quality assessment of water and fresh fish is very important as it is related to Public health. Microbiological quality determination is very useful for both export and country consumption.

Materials and Methods
Sample Collection
The samples were collected in a sterile aseptic container. In the present study, microbiological parameters for examination of sample fishes were considered including-Standard Plate Counts (SPC), E. coli counts, Salmonella and Shigella counts, Gram-staining, motility test, stock maintenance, etc. Water samples were collected in sterile plastic bottles (200 ml) from three corners of the pond at every sampling. Bacteriological analysis was performed with the water samples separately and averaged. Sediment samples were collected, with sterile glass bottles submerged to the bottom, from three different locations in pond. Five gram of wet sediment sample was weighted and suspended in 200 ml physiological saline to make stock solution. One milliliter of the sample was serially diluted (10-1 to 10-9) and treated in the same way as the water samples. The collected samples were transported to the Fisheries Technology laboratory, department of Fisheries

Technology, Bangladesh Agricultural University, Mymensingh, Bangladesh within 30 minutes of collection. The sampling was done during winter and summer season respectively. Duration of the study was from December, 2015 to April, 2016.

Methods of Microbial Analysis

Bacterial load was assessed from water, sediment and fish samples of selected pond. Bacteriological parameters for examination of fish and water samples were- SPC, total E. coli and quantitative analysis of Salmonella spp. and Shigella sp.

Determination of Standard Plate Count (SPC)

Media were sterilized before using them in order to kill any bacterial and fungal cells or spores present in the media or in the glass wares containing them. After the completion of sterilization process it was allowed to cool down at around 50°C temperature and poured into previously sterilized petri-dish. After solidification of the media, the plates were inverted and incubated at 30˚C for 48 hours. For preparation of media, 23.5 g of plate count agar media was weighed and suspended into 1.0 liter of distilled water in a conical flask. Then the mixture was boiled on an electric heater to dissolve completely and sterilized. Eosin Methylene Blue (EMB) agar and Salmonella-Shigella (S-S) agar were used as a selective media to detect the presence of enteric bacteria.

Sample preparation and culture

Standard plate count expressed as colony forming units per gram (CFU/g) were determined by using consecutive decimal dilution technique using spread plate method. Six to seven grams of sampled fish were taken aseptically, mixed, and homogenized in a mortar. Five g of homogenate was suspended in a bottle containing 200 ml of sterile (121ºC, 15 min) physiological saline separately. One milliliter sample (fish stock solution) was transferred with a micropipette to test tube containing 9 ml of physiological saline. The test tube was shaken thoroughly on a vortex mixture in order to get 10-1 dilution of original sample solution. Using the similar process several dilutions of 10-2, 10-3, 10-4, 10-5 and10-6 were made for fish respectively. Volumes (0.1 ml) of the dilutions were spread onto previously prepared agar media plate in triplicate. In case of water samples 250 ml of water collected from three different corners in pond at every sampling. Three samples were combined to make a composite sample for bacteriological analysis. After thorough mixing one milliliter sample (water stock solution) was transferred with a micropipette to test tube containing 9 ml of physiological saline. The test tube was shaken thoroughly on a vortex mixture in order to get 10-1 dilution of original sample solution. Using the similar process several dilutions of 10-2, 10-3 and 10-4 were made for water respectively.

Aerobic plate count (SPC)

Plates containing 30-300 colonies were used to calculate bacterial load results, recorded as CFU per unit of sample by using following formula:

$$\text{CFU/g} = \frac{\text{No.of colonies on petridish} \times 10 \times \text{dilution factor} \times \text{volume of total stock solution}}{\text{Wt.of sediment (g)}}$$

Presence of enteric bacteria was determined by using selective media EMB- agar and S-S agar. From each stock solution of fish, 0.1 ml samples were inoculated into the selective media. Growth of bacterial colony in EMB agar and S-S agar media indicate the presence of enteric bacteria. For confirmation, the suspected colonies from each selective agar plate were selected and streaked onto the surface of nutrient agar plates to obtain pure culture. Pure culture obtained from nutrient agar plates were used for Gram staining, motility test, agar slant preparation, etc.

Gram's staining

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Alternatively, Gram negative bacteria stain red, which is attributed to a thinner peptidoglycan wall, which does not retain the crystal violet during the discoloring process. From the incubated culture plate after 24 hours incubation at 37ºC, a portion of the culture was taken with sterile loop and suspended in a drop of saline over a clean glass slide and mixed.

Motility Test

Motility is an inheritable phenotype and is a useful criterion for identification and classification of bacteria. Microscopic examination of live bacteria in wet mounts reveals whether the bacteria are motile or non-motile. The motility of bacterial colonies was performed by hanging drop method. Motility is interpreted using high dry magnification to locate the bacteria within the drop of water. If they swim randomly and "against the current" of water streaming across the slide surface, they are positive for motility. If they seem to be buffeted around, all moving the same direction and at the same speed, there is no motility.

Results

Physico-chemical parameters

Water temperature (°C), pH, and dissolved oxygen (mg/l) were recorded. Water temperature in the pond during winter ranged from 16 to 22°C. The pH of water ranged from 6.4 to 7.2. The dissolved oxygen (DO) of water ranged from 5.1 to 6.1 mg/l.

During the period of study, bacterial loads in pond were 9.15×103 to 6.2×104 CFU/ml in water; 7.72×107 to 3.8×108 CFU/g in sediment and 4.48×105 to 1.12×106 CFU/g in Bele when analysis was done by using fresh samples in the pond (Table 2).

Quantitative Analysis of Bacteria in Water, Sediment and Fish (Bele) in winter season

During the period of study, bacterial loads in pond were 9.15×103 to 6.2×104 CFU/ml in water; 7.72×107 to 3.8×108 CFU/g in sediment and 4.48×105 to 1.12×106 CFU/g in Bele when analysis was done by using fresh samples in the pond

Table 2: Bacterial load in water, sediment and fish (Bele) in winter season

Category	Sample	Bacterial load (Sampling)			Mean (±SD)
		1st	2nd	3rd	
Water	01	1.16×10^4	9.80×10^3	5.20×10^4	$3.63 \pm 2.64\times10^4$
	02	6.40×10^4	8.50×10^3	7.20×10^4	
	Average	3.78×10^4	9.15×10^3	6.20×10^4	
Sediment	01	4.08×10^8	9.76×10^7	4.32×10^8	$2.33 \pm 1.51\times10^8$
	02	7.6×10^7	5.68×10^7	3.28×10^8	
	Average	2.42×10^8	7.72×10^7	3.80×10^8	
Bele	01	1.26×10^6	5.76×10^5	3.84×10^5	$7.95 \pm 3.36\times10^5$
	02	3.74×10^5	3.2×10^5	1.86×10^6	
	Average	8.17×10^5	4.48×10^5	1.12×10^6	

Table 3: Presence of enteric bacteria in water, sediment and fish sample in winter season

Types	In water samples CFU / ml	In sediment samples CFU / g	In fish samples CFU / g
EMB	26.64±24.38	95.36±63.73	191.36±153.18
SS	57.00±8	138.36±88.54	106.36±88.12

**EMB=Eosine methylene blue, SS=*Salmonella- Shigella*

Quantitative Analysis of Bacteria in Water, Sediment and Fish (Bele) in Summer Season

During the period of study, bacterial loads in pond were 7.74×10^4 to 4.67×10^5 CFU/ml in water; 4.84×10^8 to 2.77×10^9 CFU/g in sediment and 4.60×10^6 to 3.06×10^7 CFU/g in Bele when analysis was done by using fresh samples of the pond.

Table 4: Number of enteric bacteria in water, sediment, and fish sample in the pond

Types	In water samples CFU / ml	In sediment samples CFU / g	In fish Samples CFU / g
EMB	103.36±104.16	175.64±155.11	259.64±195.30
SS	133±71.76	102.64±87.76	143.36±197.10

**EMB=Eosine methylene blue, SS=*Salmonella- Shigella*

During the period of study, bacterial loads in pond were 7.74×10^4 to 4.67×10^5 CFU/ml in water; 4.84×10^8 to 2.77×10^9 CFU/g in sediment and 4.60×10^6 to 3.06×10^7 CFU/g in Bele when analysis was done by using fresh samples of the pond (Table 5).

Table 5: Bacterial load in water, sediment and fish (Bele) in summer season

Category	Sample	Bacterial load (Sampling)			Mean (±SD)
		1st	2nd	3rd	
Water	01	2.68×10^4	2.28×10^5	1.55×10^4	$2.23 \pm 2.12\times10^5$
	02	1.28×10^5	2.08×10^4	9.20×10^5	
	Average	7.74×10^4	1.24×10^5	4.67×10^5	
Sediment	01	9.92×10^8	8.64×10^8	1.92×10^8	$1.49 \pm 1.16\times10^9$
	02	4.56×10^9	1.05×10^8	5.44×10^8	
	Average	2.77×10^9	4.84×10^8	1.23×10^9	
Bele	01	2.64×10^6	4.08×10^6	5.68×10^7	$1.75 \pm 1.30\times10^7$
	02	3.24×10^7	5.12×10^6	4.48×10^6	
	Average	1.75×10^7	4.60×10^6	3.06×10^7	

In different samplings the numbers of bacterial isolates were different and, in the pond, (December-January) the percentage of Gram (+ve) was 25% and Gram (–ve) was 75% where motile and non-motile bacteria was 77and 23%, respectively. In different samplings the numbers of bacterial isolates were different and in summer, the percentage of Gram (+ve) was 22% and Gram (–ve) was 78% where motile and non-motile bacteria was 79 and 21% respectively. The bacterial load in water, sediment and Bele in the pond during winter and summer were 4.93×10^4 to 8.5×10^4 CFU/ml and 4.02×10^8 to 8.17×10^8 CFU/g respectively in case of pond analysis (Table 6).

Table 6: Comparative study of bacterial load in water, sediment and fish between two different sampling period

Category	Sample	Bacterial load (Sample)		
Winter				
Water	Average	3.78×10^4	9.15×10^3	6.20×10^4
Sediment	Average	2.42×10^8	7.72×10^7	3.80×10^8
Bele	Average	8.17×10^5	4.48×10^5	1.12×10^6
Summer				
Water	Average	7.74×10^4	1.24×10^5	4.67×10^5
Sediment	Average	2.77×10^9	4.84×10^8	1.23×10^9
Bele	Average	1.75×10^7	4.60×10^6	3.06×10^7

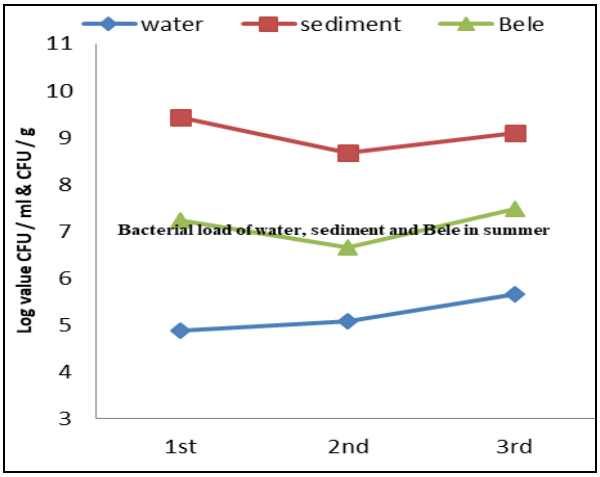

Fig 1: Changes in bacterial load in winter

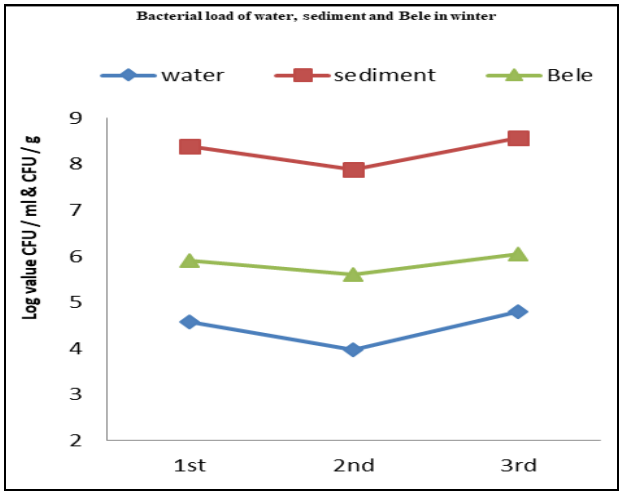

Fig 2: Changes in bacterial load in summer

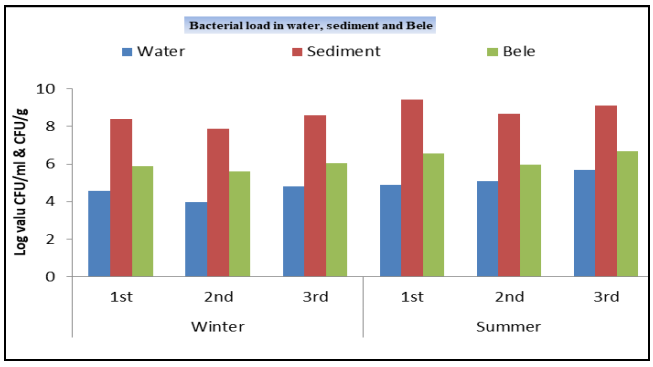

Fig 3: Changes in bacterial load of water, sediment and Bele in winter and summer season

Discussion

The suitable pH range for fish culture was between 6.7 and 9.5 and ideal pH level was between 7.5 and 8.5 described by Santhosh and Singh [8]. Ideally, an aquaculture pond should have a pH between 6.5 and 9 examined by Bhatnagar [9]. All of the above studies agreed with the present study pH 7.0 or nearly neutral which was suitable for bacterial growth.

The present study results of bacterial load in pond ranged from 9.15×10^3to6.20×10^4 CFU/ml and 7.74×10^4 to 4.67×10^5 CFU/ml in water; 7.72×10^7 to 3.80×10^8 CFU/g and 4.84×10^8 to 2.77×10^9 CFU/g in sediments and 4.48×10^5 to 1.12×10^6 CFU/g and 4.60×10^6 to 3.06×10^7 CFU/gin fish during winter and summer when analysis was done by using samples in the pond. Bacterial loads of water and sediment samples at two different times were different because the sampling seasons were not similar. Al-Harbi and Uddin [10] found that the total viable counts in water and sediments of catfish pond were $7.9\pm4.4\times10^3$ to $4.3\pm5.7\times10^4$ CFU/ml and $1.3\pm2.7\times10^8$ to $7.4\pm4.6\times10^9$ CFU/g respectively. Total viable counts of bacteria (measured as colony-forming units, CFU) were in the range of $1.2\pm2.9\times10^4$ to $2.5\pm3.5\times10^5$ CFU/ml in pond water; $9.3\pm2.1\times10^7$ to $2.7\pm3.5\times10^9$ CFU/g in sediment of common carp pond observed by Al-Harbi and Uddin[11]. Bacterial density was observed to be ten times higher at the pond sediment than in the water medium. This is expected because organic matter content is greater at the pond as the total solids of manure are suspended. Similar observations were recorded by Al-Harbi and Uddin[12]. On the other hand, as Bele are bottom dwelling animals, the livelihood of their becoming contaminated with bacteria from the muddy substrate have always possibility. The results showed that bacterial load in Bele varied during winter and summer season. The seasonal change in intestinal bacterial load of fresh water tilapia in Saudi Arabia as 8.9×10^5 CFU/g to 1.3×10^9 CFU/g [13] which is more or less similar with the present study.

Present study showed the predominant bacterial flora consisted of Gram-negative rods. The composition of the bacterial micro flora found in fish farms is typical of freshwater environments and as generally recognized, is dominated by Gram-negative bacteria [14] which is more or less similar with the present study. *Escherichia coli* was found round the year in freshwater, whereas *Salmonella* was found in summer only [15] and observed significant coliform bacteria, including *E. coli* in fish culture ponds at 25-29°C temperature. The recommended limits for *Salmonella* spp. by ICMSF [16] is 0 (zero). The presence of pathogenic bacteria in the pond indicated unhygienic environment and the sources of contamination of the pond. In the present study, *Salmonella* spp. Fecal coliforms, and *Vibrio cholerae* were not detected in any of the samples. *Salmonella* spp. in aquaculture shrimp products mainly originates from the environment rather than from poor standards of hygiene and sanitation. But sometimes, incidence of this bacterium in fish, shrimp or similar foods of aquatic habitats may be happened due to external contamination [17]. On the other hand, in the present study, raw freshwater fish contained more enteric bacteria counts because not eliminated head and intestine contain maximum microbiological load of total body. The overall results indicated that the bacterial loads in ponds were somewhat different in their bacteriological condition during the study period in summer and winter. High temperature may be the major factor in increasing bacterial loads in the pond water, sediment and Bele during the summer period.

Conclusion

Bacteriological condition of the pond water, sediment and fish (Bele) was good as they showed bacterial counts in an acceptable limit in all the bacterial parameters. So, the level of contamination was within the allowed range for fish. From the result it might be pointed out that the processing, handling and culturing condition of the selected sample is good and the quality of fish is better for consumption. It might also indicate the better-quality preservation, handling, hygiene and sanitary maintenance during collection, transportation and culture system. Therefore, taking into

consideration the recommendations of ICMSF, it could be concluded that microbiological load of water was found to be higher than the approved safety standard which was inacceptable. The highest contamination level with *E. coli* was observed in water, sediment and fish samples. The share of *Salmonella* spp. from the total microbial load was considerable. Bacteriological condition of the water, sediment and Tank Goby fish (Bele) were safe for consumer's health. To avoid public health risks, it is necessary to follow the Good Hygienic Practices (GHPs) concerning handling of the catch, post-harvesting procedure and storage. Keeping up good sanitary conditions in the farms might improve water quality of the pond which might inhibit unwanted bacterial populations.

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