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Study on bacteriological and physicochemical conditions of fish hatcheries water along with the antimicrobial traits

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ABSTRACT

Fisheries sectores have been playing significant role in our economy. Therefore, proper maintenances of fish hatcheries is so important however fish hatcheries are now in tremendous threat due to the undesired changes in the physical, chemical and biological characteristics of water. The motive of the current study was to determine the physicochemical and microbiological quality of fish hatcheries water along with their antibacterial effect on different environmental bacteria through conventional culture methods as well as agar well diffusion method. The physical conditions of the water (temperature, pH, electric conductivity, dissolved oxygen, total dissolved solids, alkalinity, total hardness, turbidity, biological oxygen demand, ammonia, chlorine,) were in marginal value. The total viable count was estimated within the range of $1.8x10^5$ to $5.6x10^5$cfu/ml. The presence of specific pathogens (*E.coli*, *Klebsiella* spp., *Vibrio* spp., *Staphylococcus* spp. *and Pseudomonas* spp.) were confirmed through series of biochemical tests. Among the nine antibiotics, six were very effective against the isolated bacteria while the other isolates were found to be highly resistant against three (vancomycin, polymyxin, nalidixic acid) antibiotics. Interestingly, hatcheries water unveiled their antibacterial activity by producing clear zone of inhibition against the bacteria, which might be one of the issue to think about the pre-treatment procedure of the hatchery water. Proper monitoring and hygienic practice is obligatory for maintaining the quality of fish.

Keywords: Water quality, Physicochemical properties, Fish borne pathogen, Antimicrobial activity

INTRODUCTION

Fish hatchery is one of the most important non-natural habitat of fish for their artificial breeding, hatching as well as rearing in the very early stage of their life. In Bangladesh most of the fish biologist and fish farmers are trying to excel the overall quality of the hatchery based fish farming due to

the maximum production and sustainable benefit (Bhatnagar and Devi, 2013). From the very ancient time, fish and fish products are the most significant items in our daily diet especially for the Asian and European people because it contains protein substances and sufficient amount of micro-nutrients (Acharjee et al., 2019; Nur et al., 2020a). Beside the nutritive values, export quality fishes have been paying huge role since very early period to gross the foreign currency every year in Bangladesh (Belton et al., 2011). As reported by Food and Agriculture Organization (FAO, 2018), Bangladesh became third fish producing country from inland water-bodies just next to the China and India (Dof, 2017). Therefore, scientist and fish biologists are now very careful for successful and sustainable fish cultivation (Acharjee et al., 2019). Although, for a successful fish farming some important factors have to be considered such as physical, chemical and biological parameters of fish habitat water along with the sufficient amount of nutrient (Sapktoa et al., 2008). Several studies showed the huge microbial contamination in fish hatcheries water especially *Salmonella* spp., *Shigella* spp., *Vibrio* spp., and coliform bacteria as well as the imbalance physical condition of water such as DO, salinity, temperature, pH, TDS, turbidity, arsenic. (Sapktoa et al., 2008, Gorlach-Lira et al, 2011; Majumder et al., 2018). Aquatic life becomes stressful when the physicochemical and biological parameters have been altered and then adversely affect fish growth and reproduction by causing infections (Iwama et al., 2000; Kiran et al., 2010, Tiwari and Chauhan, 2006; Bureau & Cho, 1999, Carr & Goulder, 1990). Poor management of water quality and heavy metal accumulation may be the vital reason of fish dead and illness, slow growth of fishes and most significantly the fish borne diseases take place (Barker et al., 2009; Shil et al., 2017). So, good water quality is very essential for the survival and growth of fish (Majumder et al., 2018, Moriarty, 1997).

Recently, fish farmers are very frequently applying antibiotics and other antimicrobial agents to prevent the fish diseases but they don't have enough knowledge about the appropriate dose of such synthetic drugs as a result the drug resistant bacteria is increasing in the fish bodies and transmitted into the consumers (Agoba et al, 2017;Angulo 1999; Chenia and Vietze, 2012; Cabello et al.,2013; Dang et al., 2011; Rodgers and Furones 2009; Sapkota et al., 2008; Stachowiak et al, 2010). Meanwhile, some of the effective antibiotics for human like oxytetracycline, sulfamerazine, and trimethoprim are being using now for the treatment of bacterial infections in salmon, catfish, trout and other commercially-raised fish (Chenia and Vietze, 2012; Cabello et al., 2013).

The economy of our country largely depends on aquaculture throughout the different regions of Bangladesh. Thus, contaminated water can hamper the production of fish which might be an extreme intimidation towards our whole economy. Therefore, the present study was designed to determine the physicochemical parameter, microbiological quality and antimicrobial activity of aquaculture water collected from the Khulna division in Bangladesh. The deduction of these parameters will provide useful information to the fish farmer and they will be concern about the quality of water and the negative impact of misuses of antibiotics.

MATERIALS AND METHODS

Study area and sampling

Five hatcheries water samples were collected from different districts of Khulna division from February to march in 2019. Samples were collected aseptically in sterile screw-capped bottles kept in a thermal stabilizing box maintained at 25 °C, transported to the laboratory and immediately subjected to microbiological analysis (Acharjee et al., 2014).

Physico-Chemical status of the water samples

The Physico-chemical properties of all the samples such as dissolved oxygen, temperature, pH, Electrical conductivity (EC), Salinity, Total dissolved solid (TDS) and Turbidity were measured following the standard protocols and methods of American Public Health Organization (APHA) (APHA 1995) and American Society for Testing and Materials (ASTM) using different calibrated standard instruments (DeZuane et al., 1997). The pH of the water samples was measured by using a pH meter (model HI 98130 HANNA, Mauritius, IramacSdn. Bhd.). The pH meter was calibrated, with three standard solutions (pH 4.0, 7.0, and 10.0), before taking the measurements. The conductivity of the samples was measured using a conductivity meter (model HI 98130 HANNA, Mauritius, IramacSdn. Bhd.). The turbidity of the samples was measured by using a turbidity meter (model 2100P Turbidimeter HACH, Colombia, USA,

Arachem (M) Sdn. Bhd.). After achieving the reading stability, the value was recorded. TDS in water samples were determined according to the standard methods of APHA (APHA 1995) and Sawyer et al. 1994 by the filtration process. The BOD was calculated by: BOD (ppm) = (DO_0-DO_5), where, DO_0= Initial DO in the sample, andDO_5= DO after 5 days (Mou et al., 2018).The temperature of the water samples were measured in the lab to avoid a change of temperature with time using a thermometer. Ammonia was examined by the method of Harwood and Kuhn, 1970.

Isolation and enumeration of Total viable bacteria (TVC) and Total fecal coliform (TFC)

0.1 ml of each sample was spread on to Nutrient agar and Membrane fecal coliform (MFC) agar respectively. Afterward, for total bacterial count plates were incubated at 37 °C for 24 hours and for obtaining fecal coliform count plates were incubated at 44.5 °C for 24 hours (Habiba et al., 2019; Nur et al.,2020).

Identification of *E. coli, Klebsiella spp., Staphylococcus spp.* and *Pseudomonas spp.*

0.1 ml of each sample was spread on to MacConkey agar, Mannitol Salt Agar (MSA), and cetrimide agar for the isolation of *E. coli, Klebsiella* spp*., S. aureus, and Pseudomonas* spp., respectively. Subsequently, the plates were incubated at 37 °C for 24 hours. Eosin Methylene Blue (EMB) agar media were further used for the observation of the production of green metallic sheen (if any) as the specific characteristic of *E. coli* strains (Nur et al.,2020b; Islam et al., 2020).

Isolation *of Salmonella spp.,Shigellaspp.* and *Vibrio spp.*

For the assessment of *Salmonella* spp., *Shigella* spp. and *Vibrio* spp., 0.1 ml of each sample was spread on to Xylose lysine deoxycholate (XLD) agar and Thiosulfate Citrate Bile Salt (TCBS) Sucrose agar respectively. For the final identification of all isolates, several biochemical tests were performed including the triple sugar iron test, motility, urease test, methyl red test, VogesProskauer test, indole utilization test, and the oxidase test (Acharjee et al., 2014; Nur et al., 2020a).

Determination of antimicrobial susceptibility of Isolates

All the isolates were prepared to determine their antibiotic susceptibility pattern against nine antibacterial drugs (including first, second and third-generation drugs) by disc diffusion assay on Mueller-Hinton Agar (Difco, Detroit, MI). A single colony of each isolate was inoculated into 9 ml of Mueller-Hinton broth and incubated at 37 °C for 4 hours. The culture turbidity was then adjusted to a 0.5 McFarland standard. Sterile cotton swabs were dipped into the suspensions and spread evenly over the entire surface of Muller-Hinton agar. Antibiotic discs of appropriate concentrations (amoxicillin 10µg, ciprofloxacin 5 µg, ceftazidime 30µg, nalidixic acid 30 µg, imipenem 10 µg, tetracycline 30 µg, gentamycin 10µg, vancomycin 30µg) were placed aseptically over the surface at appropriate spatial distance of 5 mm. Plates were then inverted and incubated at 37 °C. After 24 hours, plates were examined and the diameters of the zones of inhibition were measured and interpreted as susceptible, intermediate and resistant (Ferraro et al., 2001; Acharjee et al., 2014; Nur et al., 2020b).

Antimicrobial activity of aquaculture water

As antibiotics were used some hatcheries for the treatment of fishes so this water could have antimicrobial activity. To examine the antibacterial activity of water laboratory isolates were used. Study was introduced agar well diffusion methods on Muller Hinton Agar (Sharmin et al., 2015). According to the suggested method by Clinical and Standard Laboratory Institute; a loop full culture of the tested bacteria (*Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Staphylococcus* spp.) was inoculated into the appropriately labeled sterile tubes containing Mueller Hinton (MH) broth (Oxoid Ltd, England) and the bacterial lawn was prepared onto the surface of the MHA media. Then wells (8 mm) were made on the inoculated MHA media and 100µL water was added into the wells along with a positive control antibiotic disc (Gentamicin, 10µg) and negative control (normal saline). After incubation at 37 °C for 24 hours, the presence of a clear zone around the sample solution (if any) was analysis for the existence of the antibacterial activity of the samples tested.

RESULTS

Physico-chemical status of the water samples

Total 14 parameters such as DO, Temperature, pH, EC, Salinity, TDS, Turbidity, Chlorine, Iron, Arsenic of the water samples were monitored. The dissolve oxygen, temperature, pH, EC, Salinity, TDS and Turbidity were found within the Marginal limit for all samples but the concentration of chlorine was overloaded in case of sample 5. (table 1 & 6).

Table 1: Physicochemical properties of the water samples

Sample Number	Hardness (ppm)	DO (mg/l)	Temperature (º C)	BOD (mg/l)	Ammonia (mg/l)	pH	EC (µs/cm)	Salinity (ppt)	Alkalinity (mg/l)	TDS (ppm)	Chlorine (mg/l)	Turbidity (NTU)	Iron (ppm)	Arsenic (ppm)
S-1	23	7.4	27	0.3	0.05	9.8	298	0.14	20	132	2.5	0.72	0.5	Nil
S-2	32	7.2	26	0.4	0.06	9.6	466	0.22	53	201	0.5	0.53	0.5	Nil
S-3	25	5.9	26	0.1	0.04	9.6	540	0.27	45	251	2.0	2.09	0.2	Nil
S-4	22	4.9	26	0.3	0.055	10.0	392	0.16	60	174	0.5	0.72	0.3	Nil
S-5	30	4.8	26	0.8	0.07	10.4	372	0.22	50	189	5	0.75	0.5	Nil

All the experiments have been done three times and the results were reproducible.One representative data have been shown.

Table 2: Microbiological quality of fish hatcheries water samples.

	Sample Number	TVB (cfu/ml)	TFC (cfu/ml)	*E. coli* (cfu/ml)	*Klebsiella* spp. (cfu/ml)	*Salmonella* spp. (cfu/ml)	*Shigella* spp. (cfu/ml)	*Vibrio* spp. (cfu/ml)	*Staphylococcus spp.* spp (cfu/ml)	*Pseudomonas* spp. (cfu/ml)
Water Sample	S-01	1.8×10^5	0	0	2×10^2	0	0	7.2×10^3	2.3×10^3	6.2×10^3
	S-02	2.8×10^5	0	2×10^2	0	5.9×10^3	0	4.1×10^3	1.9×10^3	1.3×10^3
	S-03	3.5×10^5	4×10^2	0	2.7×10^3	0	0	1.5×10^3	1.8×10^3	6.6×10^3
	S-04	3.7×10^5	0	1.5×10^3	1.4×10^3	3.3×10^3	0	0	3.3×10^3	2.7×10^3
	S-05	5.6×10^5	0	2.9×10^3	0	0	0	0	7.2×10^3	1.4×10^3

All the experiments have been done three times and the results were reproducible. One representative data have been shown.

Table 3: Biochemical tests of isolated bacteria

Assumed Pathogenic microorganisms	TSI				Motility	Indole Producti	MR	VP	Citrate utilizatio	Catalase	Oxidase
	Slant	Butt	Gas	H_2S							
E.coli	Y	Y	+	-	+	+	+	-	-	+	-
Salmonella spp.	R	Y	-	+	+	-	+	-	-	+	-
Klebsiella spp	Y	Y	+	-	+	-	-	-	+	+	-
Vibrio spp.	R	Y	-	-	+	-	+	-	-	+	+
Staphylococcus spp.	Y	Y	-	-	+	-	+	-	-	+	-
Pseudomonas spp.	R	Y	-	-	+	-	+	-	-	+	+

All the experiments have been done three times and the results were reproducible. One representative data have been shown.

TSI : Triple Sugar Iron Test , **Y**: Yellow (Acid), **R:** Red (Alkaline), **MR :** Methyl red, **VP:** Voges-Proskaur

Prevalence of pathogenic bacteria in hatcheries water

Pathogenic bacteria was observed in aquaculture water which was further biochemically identified (Tables 2 & 3). Total viable bacteria was estimatedin all tested samples up to 10^5cfu/ml in all tested samples. Fecal coliforms (4.0×10^2cfu/ml) was found in sample 3, *Klebsiella* spp. (2×10^2 - 2.7×10^3cfu/ml) was found in sample 1, 3 and 4, and *E. coli* (2×10^2 - 2.9×10^3cfu/ml) were found in samples 2, 4 and 5. *Salmonella* spp. were present within the range of (10^2 – 10^3cfu/ml) in sample 2 & 4 but the growth of *Shigella* spp.was absent in all samples and*Vibrio* spp. was observed in samples 1,2 and 3. The growth of *Pseudomonas* spp. was noticed up to 10^3 cfu/ml and present in all tested samples likewise the presence of

Staphylococcus spp. was found up to 10^3cfu/ml in all samples (table 2).

A Total eight types of the biochemical tests showed their result for the confirmation of all isolated bacteria (Table 3). The growth of *E. coli* and *Klebsiella* spp. on MacConkey agar plates were further transferred on to the EMB media and confirmed 3 were *E. coli* and 3 were *Klebsiella* spp. (Table 3)

Prevalence of drug-resistant pathogenic bacteria in the hatcheries water

For most of the pathogenic isolates, higher rates of resistance were found against Vancomycin (30 µg), Polymyxin B (300 units), Nalidixic acid (30 µg) (Table 4). On the other hand, most of the isolates were found to retain higher sensitivity against Imipenem (10 µg), Ceftadizime (10 µg), Gentamycin (10µg), Amoxicillin (30 µg).

Table 4: Antibiotics susceptibility pattern of different bacteria isolated from fish hatcheries water

Sample	Organism	CAZ (10 µg)	VAN (30 µg)	TET (30 µg)	GEN (10 µg)	CIP (5µg)	PB (300 units)	AMX (30 µg)	IPM (10 µg)	NA (30 µg)
S1	*Vibrio* spp.	32 mm	12 mm	10 mm	15 mm	18 mm	7 mm	25 mm	25 mm	20 mm
	*Pseudomonas*spp.	30 mm	25 mm	28 mm	25 mm	20 mm	22 mm	22 mm	30 mm	18 mm
	Staphylococcus spp	28 mm	16 mm	24 mm	28 mm	17 mm	ND	23 mm	24 mm	ND
	*Klebsiella*spp.	30 mm	21mm	16 mm	27 mm	19 mm	21 mm	18 mm	14 mm	30 mm
S2	*Vibrio* spp.	30 mm	10 mm	20 mm	20 mm	20 mm	12 mm	25 mm	40 mm	13 mm
	Staphylococcus spp.	25 mm	18 mm	20 mm	22 mm	11 mm	ND	25 mm	22 mm	ND
	Salmonella spp.	34 mm	25 mm	15 mm	30 mm	23mm	15 mm	20 mm	30 mm	25 mm
	E. coli	30 mm	5 mm	28 mm	25 mm	30 mm	17 mm	15 mm	20 mm	10 mm
	Pseudomonas spp.	28 mm	23 mm	27 mm	26 mm	21 mm	20 mm	25 mm	32 mm	20 mm
S3	*Staphylococcus* spp.	30 mm	28 mm	30 mm	25 mm	20 mm	ND	30 mm	25 mm	ND
	*Klebsiella*spp.	28 mm	20 mm	15 mm	25 mm	18 mm	20 mm	18 mm	15 mm	25 mm
	*Pseudomonas*spp	30 mm	18 mm	20 mm	20 mm	22 mm	10 mm	20 mm	30 mm	15 mm
	Vibrio spp.	32 mm	8 mm	22 mm	25 mm	10 mm	12 mm	26 mm	38 mm	15 mm
S4	*Pseudomonas*spp.	35 mm	25 mm	10 mm	30 mm	20 mm	18 mm	25 mm	42 mm	20 mm
	*Klebsiella*spp.	30 mm	22 mm	20 mm	28 mm	21 mm	23 mm	16 mm	15 mm	27 mm
	Salmonella spp.	36 mm	0 mm	15 mm	20 mm	20 mm	15 mm	25 mm	30 mm	20 mm
	Vibrio spp.	32 mm	10 mm	20 mm	26 mm	28 mm	10 mm	25 mm	36 mm	15 mm
	Staphylococcus spp.	30 mm	15 mm	22 mm	22 mm	25 mm	ND	25 mm	30 mm	ND
	E.coli	29 mm	0 mm	25 mm	30 mm	10 mm	18 mm	12 mm	22 mm	13 mm
S5	*Pseudomonas* spp.	32 mm	12 mm	7 mm	20 mm	18 mm	20 mm	20 mm	35 mm	15 mm
	E. coli	30 mm	10 mm	18 mm	15 mm	10 mm	12 mm	15 mm	15 mm	10 mm
	Klebsiella spp.	28mm	23 mm	20 mm	26 mm	24 mm	18 mm	19 mm	20 mm	25 mm
	Staphylococcus spp.	32 mm	27 mm	23 mm	19 mm	15 mm	24 mm	ND	26 mm	ND

All the experiments have been done three times and the results were reproducible. One representative data have been shown.

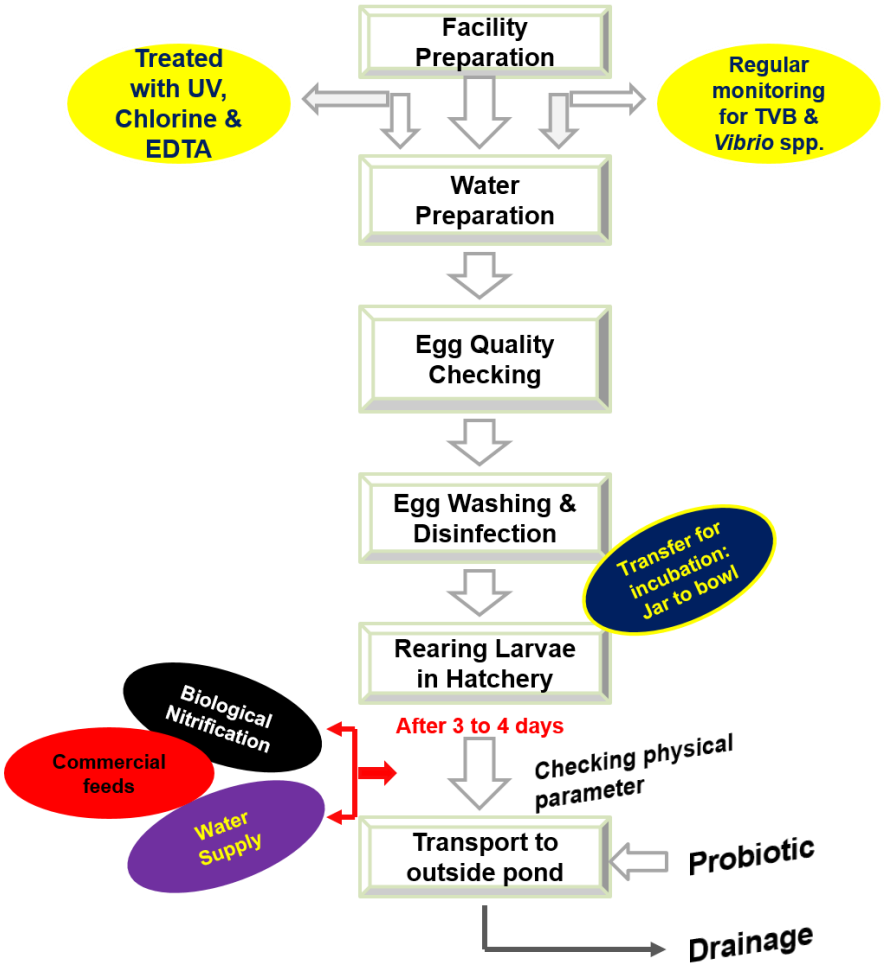

Figure 1: Incubation and hatching layout for a commercial fish farm (Nur et al., 2020)

In general, microbiological safety procedure and precautions should taken from facility preparation to drainage. Hatcheries water should be treated with UV radiations, chlorine and other recommended cleaning agents. Monitoring of Total viable bacteria and pathogenic *Vibrio* spp. is mandatory. Quality check of eggs and disinfection of eggs are preliminary step before embryos development and rearing larvae in hatchery. After 3 to 4 days newly hatched larvae transferred into outside pond where they provided by commercial feeds and probiotics. Therefore, great care must be taken to provide them with the proper incubating and hatching environment. Regular draining should practices to reduce the contamination and to maintain the ecosystem of hatcheries.

Table 5: Antimicrobial activity of hatchery water against laboratory microbial isolates

	Zone diameter (mm) against bacteria				
	E. coli	***Klebsiella* spp.**	***Salmonella* spp.**	***Pseudomonas* spp.**	***Staphylococcus* spp.**
Aquaculture water					
1	10mm	12 mm	0mm	8mm	0 mm
2	8 mm	10 mm	0mm	0 mm	12 mm
3	12mm	0 mm	0mm	11 mm	0 mm
4	12mm	0 mm	0mm	0 mm	0 mm
5	8 mm	8 mm	0mm	10 mm	10 mm

The experiments have been done three times and the results were reproducible. One representative data have been shown

Table 6: Ideal range for water quality parameters in maturation/hatchery facilities According to (FEPA 1991)

Parameter	Ideal range
pH	5.5-10
Alkalinity	50-300 mg/l
TDS	≥ 500 ppm
Chloride	10-25 mg/l
Ammonia	0.01-0.09 mg/l
BOD	3-6 mg/l
DO (Dissolve oxygen)	> 5 mg/l
Turbidity	20-30 NTU
Salinity	29–34 ppt
Temperature	28-30^{o}C
Iron	1ppm
Arsenic	0.05 mg/l
Hardness	20-60mg/l

Antimicrobial activity of water sample

Surprisingly antibacterial activity of hatcheries water was recorded against some bacteria. Sample 1 exhibited the antibacterial activity against *E. coli* (10mm) and *Klebsiella* spp. (12mm) while the sample 2 produced the 8mm, 10mm and 12mm against *E. coli*, *Klebsiella* spp and *Staphylococcus* spp. consecutively. Sample 3 showed the activity against *E. coli* (12mm) and *Pseudomonas* spp. (11mm). 12mm zone diameter produced by sample 4 against *E. coli*. Sample 5 showed antibacterial activity against almost all the bacteria except *Salmonella* spp. (Table 5)

DISCUSSIONS

Microbiological examination of fish hatchery water is crucial to detect the prevalence of pathogenic microorganisms that might hazardous for fish and water bodies.Water quality significantly influences the bacterial load of an aquaculture pond and the microbial load of an aquatic organism (Roy et al., 2011). Presence of pathogenic *Vibrio* spp. in an aquatic system indicate the poor microbiological quality of water (Colwell and Kaper,1977). Thus *Vibrio cholera* and *V. parahaemolyticus* were the major pathogens which causing diseases to the aquatic organism by putrefying the water quality (Ruangpan and Kitao,1991). Occurrence of *E. coli* indicates fecal contamination of warm-blooded animals (Mandal et al., 2009) and it also poses public health hazards (Rao and Gupta 1978). In an aquaculture system, the source of *E. coli* is mainly through terrestrial water bodies in which contamination has been taken place *S. aureus* is a pathogenic bacterium which causes food poisoning that leads to public health problem associated with fish and fishery products (Henson and Humphrey, 2009). Humans are the main source of *S. aureus* and get contaminated through the environment due to improper hygienic and sanitary conditions. *Salmonella* is very harmful to humans, and its occurrence in a raw or cooked product is considered to be an adulterant (Lunestad et al. 2007) suggested that homemade feed is one of the major sources for the contamination of Salmonella spp. which can be transmitted to cultured species and finally to consumers. In an aquaculture system, *Salmonella* tends to form biofilms on both inert and organic surfaces, which provide better shelter against environmental stresses (Donlan and Costerton, 2002). The Presence of *Salmonella* should be absent for a good aquaculture system. The bacteriological quality of water plays a vital role in the diseases spreading in farmed fish, the fact that must be well known by the fish farmers who should understand the importance of maintaining proper bacteriological water quality of the pond. In aquaculture, bacterial pathogens represent an important cause of fish infections and mortalities (Wamala et al., 2018; Arifin et al., 2013). Moreover, the fish handlers and other aquatic organisms were affected by the fish bacterial flora. On the other hand, normal flora of water bodies emulate for the formation

of humus and maintain a balance in the ecosystem. These may show positive or negative correlations in the management of the commercial systems. That is why there is a correlation between the bacterial number and the water ecosystem (Behera et al., 2012). Moreover, some other factors greatly influence the microbial contamination in water bodies. For instance location of toilet is very close to pond and discharges from latrine introduces fecal coliform and other pathogenic microorganisms into the fish pond water (Ogedengbe and Aina, 1980; Robert, 2012).

CONCLUSION

Fish hatcheries play a key role in our economy by producing huge amount of export quality fish which have substantial amount of protein. To ensure the fish quality and consumers health safety, using of antibiotics and other chemicals in fish habitat should be in marginal amount to avoid the side effect of synthetic drugs in fish as well as to shield the possibility of drug resistance. However, during hatchery fish farming several factors must have to consider for the safety of aquatic fish such as the integrity of the environment, the safety of target animals, and the safety of the persons administering the compounds. To prevent the detrimental effect of harmful bacteria, ministry of fisheries, fisheries scientist and microbiologist should have to work combindly regarding the proper training system for the fish farmer on the aseptic management of fish and the sustainable maintenance of hatchery water. Finally, our findings will help to understand the actual scenario of the most popular hatchery of Khulna division in Bangladesh, which would help the fisheries scientist to provide the long-term controlling measures of such harmful bacteria.

Conflict of interest

The authors have declared no conflict of interest

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