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Microbial water quality in pangasius and tilapia aquaculture systems in five regions of Bangladesh

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ABSTRACT

Aims: To determine abundance of potential pathogenic microorganisms in pangasius and tilapia farms in five major fish-producing areas in Bangladesh by PCR approaches.
Methodology and results: Important microbial water quality indicators were studied in water of 38 fish farms producing pangasius (*Pangasianodon hypophthalmus*) and tilapia (*Oreochromis niloticus*) in five major fish-producing areas of Bangladesh. The parameters included physicochemical data and PCR detection of total coliforms and *E. coli*, species of potentially pathogenic *Vibrio*, and cyanobacterial genes encoding the toxins microcystin and saxitoxin. Quantitative PCR showed that coliform bacteria occurred in all fish farms with densities from one to 2.2×10^5 per mL, while *E. coli* ranged from none to 5.0×10^4 per mL. Numbers of total coliforms and *E. coli* were higher in pangasius farms than in tilapia farms, and when high abundances occurred, coliform bacteria and *E. coli* bacteria co-varied. Detection of *Vibrio*-specific genes indicated presence of *Vibrio* species in 76% of the farms and included *V. vulnificus* and *V. cholerae*. The human pathogen type of *V. cholerae* (carrying the *ctxA* gene) and the fish pathogen *V. parahaemolyticus* were not detected. The microcystin-encoding *mcyE* gene ranged from undetectable to 2.6×10^5 copies per mL and tended to be highest in pangasius farms. The saxitoxin-encoding gene *sxtA* was not found in any of the farms.
Conclusion, significance and impact of study: Based on the high abundance of especially coliform bacteria and *E. coli*, we recommend more efficient water quality monitoring systems to improve detection and control of fecal coliforms and to reduce presence of microcystin-producing cyanobacteria in aquaculture farms in Bangladesh.

Keywords: Bangladesh, total coliforms, *E. coli*, *Vibrio*, microcystin

INTRODUCTION

Bangladesh is the world's fifth-largest producer of fish from aquaculture (FAO, 2020). Next to carps which constitute 35% of the total fish production, the two most important species cultured in ponds are pangasius (*Pangasianodon hypophthalmus*) and tilapia (*Oreochromis niloticus*), making up 11% and 9% of the total fish production in Bangladesh, respectively (DoF, 2019). These two fish species have become popular due to high growth rates, disease resistance, survival capacity at adverse environmental conditions and economic viability of farming (Belton and Azad, 2012). The intensified production of the two species has opened a door for export (DoF, 2019), but concern has been raised with respect to the water quality due to a high stocking density, increased feeding rates and volumes of water needed for the production (Belton *et al.*, 2011). Farming of the two species is carried out in earthen ponds that depend on rainwater or groundwater in the dry season, but the ponds typically lack water treatment, biosecurity systems and monitoring facilities (Knappett *et al.*, 2011; Jahan *et al.*, 2017). Heavy rainfalls cause run-off into the ponds in the monsoon season, resulting in contamination with cattle dung and human waste (Belton *et al.*, 2011).

Monitoring of fecal contamination in Bangladesh is conducted according to international recommendations by quantification of total coliform bacteria or only on *Escherichia coli* (Geldreich, 1996) but as in many other developing countries, most analyses are based on

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traditional and labor-consuming plate culture-dependent methods that may not allow an accurate quantification or may lead to miscalculation of the bacterial numbers. For example, the presence of *Salmonella* and *E. coli* was recently detected, but could not be quantified, in fish farm water and fish products by agar plate cultivation (Ava *et al.*, 2020). Real *et al.* (2017) used selective media and found unrealistically high numbers of up to 14×10^{23} coliforms, 6×10^{13} *E. coli* and 10×10^{14} *Vibrio cholerae* per mL river water used for fish production. For comparison, typical bacterial densities in fish intestine digesta are 10^5 to 10^9 cells/g (Zarkasi *et al.*, 2016).

In addition to fecal coliforms, pathogenic *Vibrio* species also cause a major water quality concern in Bangladesh, especially in coastal fish farms due to the risk of spreading diseases in fish and shrimp, e.g. due to the pathogens *V. parahaemolyticus* and *V. vulnificus* (Alam *et al.*, 2006; Righetto *et al.*, 2015; Hasan *et al.*, 2019). For humans, dispersal of *V. cholerae* by water and fish can lead to severe epidemic infections if water quality in fish farms is compromised (Islam *et al.*, 1993).

Another risk in dense fish populations with high feeding rates is eutrophication and a stimulated growth of cyanobacteria (blue-green algae) that potentially can affect fish and fish farm performance (Rojas-Tirado *et al.*, 2018). Cyanobacterial blooms are common in fish farms in Bangladesh (Hossain *et al.*, 2012; Affan *et al.*, 2016). Although cyanobacteria at an optimum density may positively influence water quality, fish health and serve as fish feed (Liang *et al.*, 2015; Torres *et al.*, 2016), cyanobacteria can have profound negative effects due to production of hepatotoxins, e.g. microcystin and neurotoxins, e.g. saxitoxin (Jang *et al.*, 2003; Ibelings and Chorus, 2007; Galvão *et al.*, 2009; Valério *et al.*, 2010). The toxins not only cause damage to fish but may also be transferred in the food web and pose a risk to consumers of freshwater fish (Pereira *et al.*, 2004; Humpage, 2008). For example, accumulation of microcystin is reported in fish tissue during blooms (Tencalla *et al.*, 1994; Magalhães *et al.*, 2001; Soares *et al.*, 2004) and saxitoxin has been measured in two species of freshwater pufferfish, *Tetraodon cutcutia* and *Chelonodon patoca* in Bangladesh (Zaman *et al.*, 1998), causing multiple outbreaks of pufferfish-induced intoxication, including 14 deaths (Homaira *et al.*, 2010).

Awareness of microbial water quality in pangasius and tilapia farms is only progressing slowly due to the application of conventional methods, lack of laboratory facilities, and reliable measurements and monitoring systems. If microbial water quality has to be improved in production of farmed fish in Bangladesh, accurate and versatile techniques must be implemented. Traditional cultivation-based methods should be replaced by sensitive and accurate molecular techniques, particularly PCR methods, that can improve monitoring of microbial quality of water and fish. PCR methods have recently shown successful for quantification of bacterial contaminants in the popular Hilsa fish (*Tenualosa ilisha*) in Bangladesh (Foysal *et al.*, 2019).

In this study, we applied various PCR-based approaches to detect microorganisms that are known to negatively affect aquaculture production or serve as indicator organisms. Water samples were collected from 38 pangasius and tilapia farms in five major fish-producing areas of Bangladesh during a 3-years period to survey abundances of total coliforms, *E. coli*, various *Vibrio* species and cyanobacterial genes encoding production of the toxins microcystin and saxitoxin. The obtained data were related to important physicochemical parameters to find and explain variations in microbial status in terms of pathogenic and toxic characteristics in farms producing these two commercially important species.

MATERIALS AND METHODS

Sampling sites and general farming practices

Five commercially important aquaculture regions in Bangladesh were selected based on data by the Department of Fisheries (DoF) (DoF, 2016): Mymensingh, Bogura, Cumilla, Jashore and Khulna (Figure 2). Pangasius is the dominant species in Mymensingh, Bogura and Cumilla, while tilapia is dominant in Jashore and Khulna. The regions are geographically representative of fish production in Bangladesh and possess favorable biophysical resources and climatic conditions for fish breeding (DoF, 2014; Ahmed and Toufique, 2015; Jahan *et al.*, 2015). Moreover, in these regions there is availability of hatchery-produced fry, fish feed industries and access to marketing facilities (Ahmed *et al.*, 2010). Except for the Khulna region, the farms were established after conversion of rice fields and they depend on well or surface water during the dry season, while rain is the main water source in the wet season (Table 1). The Khulna region is located in the coastal zone of Bangladesh, and the studied farms are located close to the estuarine rivers and mainly receive water from the brackish Shoilmari River.

Pangasius and tilapia monoculture is practiced in Mymensingh and Jashore, while polyculture (pangasius-tilapia-carps or pangasius-tilapia) is practiced in other regions (Table 1). In Mymensingh, Bogura and Cumilla, relatively deeper ponds and multiple production cycles are common. The investigated fish farms in Mymensingh, Bogura and Cumilla were established earlier (7 to 22 years ago) than the farms in Jashore and Khulna (less than 10 years ago) (Table 1). Farms in Mymensingh and Bogura have a higher stocking density (50,000-75,000 fish/ha) and mainly use farm-made feeds and have some application of chemicals (Jahan *et al.*, 2010; Anka *et al.*, 2014).

Water sampling and physicochemical analyses

Collection of water samples and water quality measurements were performed between 9 and 11 am on the sampling days to ensure similarity in water quality data. Water was collected during 38 visits to fish farms

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Table 1: Characteristics of the sampled farms.

Regions	Farms	Culture species	Pond size (ha)	Pond age	Pond depth (m)	Annual production cycles (no.)	Stocking density (individual/ ha)	Feed type	Water sources and water exchange	Pesticides/ antibiotic/ fertilizer application
Mymensingh	Pangasius (n=9)	Pangasius 100%	0.41	8-20 years	2.04	2	60000-75000/ha	Commercial feed/Farm made feed	Underground/Surface water, less or no water exchange	High
	Tilapia (n=7)	Tilapia 100%	0.49	8-20 years	2	2	60000-75000/ha	Commercial feed/Farm made feed	Underground/Surface water, less or no water exchange	High
Bogura	Pangasius (n=4)	Pangasius 80% Tilapia 10% Carps 10%	0.71	7-22 years	2	2	50000-60000/ha	Farm made feed	Underground/Surface water, less or no water exchange	High
	Tilapia (n=2)	Tilapia 80% Pangasius 20%	0.61	8-17 years	1.9	2	50000/ha	Farm made feed	Underground/Surface water, less or no water exchange	High
Cumilla	Pangasius (n=4)	Tilapia 80% Pangasius 20%	0.41	8-17 years	2.1	2	40000-45000/ha	Commercial feed	Underground/Surface water, less or no water exchange	High
	Tilapia (n=4)	Tilapia 80% Pangasius 20%	0.41	10 years	1.7	2	40000-45000/ha	Commercial feed	Underground/Surface water, less or no water exchange	High
Jashore	Pangasius (n=2)	Pangasius 100%	0.41	5-10 years	1.2	1	40000-50000/ha	Commercial feed	Underground/Surface water, less or no water exchange	Moderate
	Tilapia (n=2)	Tilapia 100%	0.41	5-10 years	1.2	1	40000-50000/ha	Commercial feed	Underground/Surface water, less or no water exchange	Moderate
Khulna	Pangasius (n=2)	Pangasius 80% Tilapia 20%	0.41	5 years	1.5	1	40000-45000/ha	Commercial feed	River, Frequent water exchange	Low
	Tilapia (n=2)	Tilapia 90% Pangasius 10%	0.71	5 years	1.5	1	40000-45000/ha	Commercial feed	River, Frequent water exchange	Low

Data obtained from DoF (2019). Information on application of pesticides, antibiotics and fertilizers published by Ali *et al.* (2013) and Anka *et al.* (2014).

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(21 pangasius farms and 17 tilapia farms). Six farms were visited in October 2016, 4 farms were visited in October 2017 and 28 farms were visited in October 2018. Additionally, reference samples from underground well water in Mymensingh and Bogura, and water from the Shoilmari river in Khulna were also collected. Water samples were stored in sterile black bottles to protect light-sensitive microbes and carried to the laboratory from the sampling sites in coolers with ice.

Temperature, dissolved oxygen (DO) and pH of the water samples were measured by EXTECH (USA) portable devices (ExStik® II Temperature/DO/pH/Conductivity Kit) at the sampling sites. Water transparency was measured with a Secchi disk. Concentrations of nitrate, orthophosphate (ortho-P) and ammonia were measured immediately after arrival at laboratory by specific reagent kits by Lovibond (www.lovibond.com) by spectrophotometry (SpectroDirect, Tintometer Ltd., UK). All analyses were performed in triplicate.

DNA extraction

For genomic DNA extraction, 100-500 mL water were filtered through 0.2 µm pore-size mixed cellulose-ester membrane filters (Advantec MFS Inc., Japan). DNA was extracted from the filters using the PowerWater® DNA isolation Kit (MO BIO Laboratories Inc., USA) according to the manufacturer's protocol and stored at –70 °C. DNA samples were carried in frozen condition from the Bangladesh Agricultural University, Mymensingh, to the University of Copenhagen, Denmark for further use. The concentrations of the extracted DNA were quantified using a NanoDrop (ND-2000) spectrophotometer (USA), and quality of the DNA was assessed by gel electrophoresis.

Quantification of coliforms and *E. coli* by quantitative PCR (qPCR)

Numbers of total coliform bacteria were determined by quantitative PCR (qPCR) targeting the *lacZ* gene (encodes β-galactosidase) in the genera *Escherichia*, *Klebsiella*, *Citrobacter* and *Enterobacter* (Table 2). The qPCR was carried out according to Hu *et al.* (2016) with slight modification. Briefly, 20 µL PCR mixture containing 2 µL template (DNA extracted from the samples), 10 µL Premix Taq (Ex Taq Version 2.0) (Takara Bio Group, Japan), 1 µL 10 µM primer, 0.5 µL 10 µM probe and 4.5 µL water. The PCR conditions were: heat activation at 95 °C for 3 min; 40 cycles at 95 °C for 5 sec (denaturation) and annealing at 60 °C for 40 sec (annealing). Fluorescence (HEX) was measured at 60 °C. For calculation of cell numbers for coliform, one copy of the *lacZ* gene per cell was assumed (Hu *et al.*, 2016).

For quantification of *E. coli*, the TaqMan® qPCR assay adopted from Chern *et al.* (2009) and Petersen *et al.* (2019) was applied. The assay targets the *E. coli* specific *uidA* gene encoding the β-D-glucuronidase enzyme (Table 2). Briefly, 20 µL PCR mixture containing 2 µL template, 10 µL Premix Taq (TaqMan), 2 µL 10 µM primers, 0.16 µL 10 µM probe with fluorophore FAM, 0.2 µL bovine serum albumin (BSA), and 3.64 µL water. The PCR cycling conditions were: initial heat activation at 95 °C for 10 min, 45 cycles at 95 °C for 15 sec (denaturation) and 60 °C for 1 min (annealing). Calibration curves for both the total coliform and the *E. coli* assays were prepared by DNA extracted from a wildtype of *E. coli* at densities from 10^1 to 10^6 cells per µL. For calculation of cell numbers for *E. coli*, one copy of *uidA* gene per cell was assumed (Chern *et al.*, 2009).

Table 2: Primers and probes (Pb) used for PCR and qPCR assays in this study.

Target organisms	Primer	Target gene	Sequence (5' to 3')	Amplified product (bp)	Reference
Total coliforms	1130a-F	*lacZ*	TCCTGCTGATGAAGCAGAACAA	137	(Hu *et al.*, 2016)
	1130b-F		ATATCGAACTCATGAAGCAGCATAA		
	1266-R		CACCATGCCGTGGGTTTC		
	1156A- Pb		AACGCCGTGCGCTGYTCGCA		
E. coli	uidA-F	*uidA*	CAACGAACTGAACTGGCAGA	130	(Chern *et al.*, 2009)
	uidA-R		CATTACGCTGCGATGGAT		
	uidA-Pb		CCCGCCGGGAATGGTGATTAC		
Vibrio spp.	567-F	16S rRNA	GGCGTAAAGCGCATGC AGGT	114	(Thompson *et al.*, 2004)
	680-R		GAAATTCTACCCCCCTCTACAG		
Virulent *V. cholerae*	94-F	*ctxA*	CGGGCAGATTCTAGACCTCCTG	564	(Rivera *et al.*, 2001; Dobbs *et al.*, 2013)
	614-R		CGATGATCTTGGAGCATTCCCAC		
Saxitoxin-producing cyanobacteria	sxtA-RF1	*sxtA*	ACAAACCGGCGACATAGATG	196	(Podduturi *et al.*, 2021)
	sxtA-RR1		TTTCCCGATCTGCCAGCTTA		

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Presence of *Vibrio* spp. and specific *Vibrio* species

Presence of *Vibrio* spp. was determined by PCR using primers targeting the 16S rRNA region specific for the *Vibrio* genus by Thompson *et al.* (2004) (Table 2) and with modifications by Petersen *et al.* (2019). In brief, 20 µL PCR mixture containing 2 µL template, 0.2 µL of 10 µM of each primer, 0.4 µL of 10 mM dNTP's, 2 µL 10× buffer, 0.2 µL Taq polymerase (Takara Bio Group, Japan) and 15 µL water. The PCR reaction was initiated by heat activation at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 sec (denaturation) and at 65 °C for 30 sec (annealing) and at 72 °C for 30 sec (extension). The amplification products were separated by agarose gel electrophoresis and visualized by GelRed® nucleic acid stain and UV exposure.

The virulent *V. cholerae* was detected by PCR targeting the virulence gene *ctxA* according to methods by Dobbs *et al.* (2013) and Rivera *et al.* (2001) (Table 2) and with the slight modifications by Petersen *et al.* (2019). Briefly, the 20 µL PCR mixture consisted of 2 µL template, 0.2 µL (10 µM concentration) of each primer, 0.4 µL of 10 mM dNTP's, 2 µL 10× buffer ($MgCl_2$ plus), 0.25 µL rTaq polymerase (Takara) and 14.95 µL water. The positive control RC4 (serotype O139) was obtained from Professor F. Dobbs, Old Dominion University, VA, USA. The PCR reaction was initiated by heat activation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 65 °C for 30 sec and extension at 72 °C for 30 sec.

Vibrio parahaemolyticus and *V. vulnificus* were detected using commercial PCR kits (Onecup *Vibrio* Kits from Eurofins Q-Bioanalytic GmbH, Germany). Setup of the PCR reactions and thermal cycling conditions followed recommendations by the manufacturer.

Quantification of microcystin and toxin-encoding genes in cyanobacteria

The content of various microcystin toxins in particulate matter was determined in water from farms in Khulna and Bogura (one pangasius and one tilapia pond in each region). Around 250 to 500 mL of water samples were filtered through Whatman GF/F filters and immediately frozen and until analysis according to the following procedure by Schlüter *et al.* (2018). Initially, the filters were thawed and frozen 3 times and extracted in 1.0 mL 75% methanol (MeOH). Next, the filters were sonicated on ice with a Sonics VCX 750 W sonicator in pulse mode at 40% duty cycle for 1 min. This sonication step was repeated 3 times. After each step, the extracts were centrifuged at 10,000× *g* for 10 min, the supernatants were withdrawn and 0.6 mL 75% MeOH was added. The combined supernatants (1.8 mL in total) were evaporated to dryness at 60 °C in a stream of N_2, dissolved in 0.4 mL 75% MeOH, and finally refiltered through a 0.2 µm filter. The HPLC system consisted of a Shimadzu LC-10ADVP unit with 2 pumps (LC-10AS), autosampler and a column oven (set at 40 °C). The column was a 150 × 4.6 mm Synergy 4 µm Fusion-RP with a pre-column (both from Phenomenex). The mobile phases were 0.05% aqueous trifluoroacetic acid (TFA) (solvent A) and 0.05% TFA in acetonitrile (solvent B). The gradient program (23 min run-time) was as follows: 0 min, 25% B; 5 min, 60% B; 10.5 min, 100% B; 15 min, 100% B; 16 min, 25% B. The flow rate was 1.3 mL/min and the injection volume was 50 µL. The different microcystins were detected by retention times and absorption spectra by diode array detection operated between 200 and 300 nm and were quantified at 238 nm.

Copy numbers of the microcystin-encoding gene *mcyE* (one of the *mcy* genes involved in microcystin production in some cyanobacteria) were quantified by PCR, using a commercial qPCR kit (Phytoxigene CyanoDTec™ Toxin Genes Test; Diagnostic Technology, Sydney, Australia). The setup of the PCR reaction and thermal cycling conditions followed recommendations by the manufacturer.

For the detection of the saxitoxin-encoding gene *sxtA* (common to all saxitoxin-producing cyanobacteria), PCR method by Podduturi *et al.* (2021) was applied (Table 2). Briefly, 20 µL PCR reaction mixture was prepared with the final concentration of 1× Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent), 400 nM of each primer, 0.5 mg of BSA and 1 µL DNA template. The PCR cycling conditions were as follows: initial heat activation at 95 °C for 3 min, 40 cycles at 95 °C for 30 sec (denaturation), 66 °C for 30 sec (annealing) and 72 °C for 30 sec (extension). To determine numbers of the *sxtA* gene per volume water, a calibration curve was created by dilution series of DNA from the saxitoxin-producing cyanobacterium *Raphidiopsis raciborskii* T3. Cell numbers of *R. raciborskii* T3 were quantified by microscopy and *sxtA* numbers are expressed in units of cell numbers, since copy number of *sxtA* per cell is unknown.

Data analysis

All values are expressed as means and standard deviation (SD) by descriptive statistics. Significant differences for different microbial water quality parameters among the regions were tested applying the one-way analysis of variance (ANOVA) with 95% confidence. Tukey's HSD test was applied for post hoc detection of significant pair-wise comparisons between the regions. A Pearson correlation test was done to determine the association between different water quality and microbial parameters at 99% (p<0.01) and 95% (p<0.05) level of significance. All the statistical tests were carried out using the statistical software SPSS (version 26.0, SPSS, Chicago, IL, USA).

RESULTS

Physical and chemical attributes of farms

Analysis of physical and chemical attributes in water of the fish farms showed significant differences (ANOVA; p<0.05) among the regions and between the species.

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When comparing the tilapia and pangasius farms, more dissolved oxygen and less nitrate occurred in water in the tilapia farms and these farms had higher water transparency than the pangasius farms (Table 3). Regional differences showed that (a) the pond depth was higher in Mymensingh and Bogura than other regions (Table 1); (b) the oxygen level was highest in Jashore and the lowest in Bogura; (c) higher ammonia levels occurred in the Mymensingh and Bogura regions than at Khulna and Jashore (tilapia farms only); (d) the water transparency was higher in Jashore and Khulna, as compared to Mymensingh and Bogura. The mean water temperature varied from 30.1 °C in tilapia farms in Cumilla to 31.8 °C in pangasius farm in Mymensingh (Table 3).

Total coliforms and *E. coli*

Quantitative PCR analysis showed that coliform bacteria were common in all fish ponds. In the tilapia farms, numbers of coliforms ranged from none to 9.1×10^2 copies/mL, with mean numbers from 3.5×10^1 copies/mL (Khulna farm) to 5.9×10^2 copies/mL (Bogura farm) (Table 4). Higher numbers were found in the pangasius farms, ranging from 9 to 2.2×10^5 copies/mL and with mean values of 5.9×10^2 copies/mL (Mymensingh farm) to 5.5×10^4 copies/mL (Bogura farm).

For *E. coli*, the numbers ranged from 0 to 5×10^4 copies/mL in the farms and mean numbers varied from 8 copies/mL in tilapia farms in Mymensingh (no *E. coli* were found in four tilapia farms in this region) to 2.5×10^4 copies/mL in pangasius farms in Jashore. High abundances of coliforms co-varied with high numbers of *E. coli* bacteria, and total coliforms and *E. coli* were both more abundant in pangasius farms than in tilapia farms (although not statistically significant).

When comparing geographic regions, densities of coliforms varied significantly in the tilapia ponds ($p<0.05$) (due to a large pond-to-pond variation) (Figure 1). The number of coliforms also had a high variation among pangasius ponds. The highest numbers occurred in farms in the Bogura and Jashore regions, but differences between the five regions were not statistically different (Figure 1). The highest numbers of *E. coli* were found in pangasius farms in the Jashore region, while tilapia farms had highest abundance in the Bogura and Jashore regions ($p<0.05$).

In the underground well water and in the Shoilmari river at Khulna, few coliform bacteria (1-4 copies/mL) were found and *E. coli* was only detected

Table 3: Physicochemical parameters of water in the 38 fish farms.

Region	Species	Water temperature (°C)	Dissolved O_2 (mg/L)	pH	Nitrate (mg/L)	Orthophosphate (mg/L)	Ammonia (mg/L)	Transparency (cm)
Mymensingh	Pangasius	31.8 ± 1.3	$4.8 \pm 0.5^{a,b}$	7.0 ± 0.6^{a}	$17.5 \pm 2.6^{b,c}$	$0.90 \pm 0.41^{b,c}$	0.21 ± 0.08^{d}	$15.7 \pm 2.0^{a,b}$
	Tilapia	31.5 ± 1.6	5.5 ± 0.7^{x}	7.3 ± 0.5	17.1 ± 2.2^{z}	$0.62 \pm 0.45^{y,z}$	$0.08 \pm 0.13^{x,y}$	19.2 ± 4.9^{x}
Bogura	Pangasius	30.7 ± 1.9	4.5 ± 0.5^{a}	6.6 ± 0.4^{a}	17.9 ± 2.5^{c}	1.00 ± 0.40^{c}	$0.14 \pm 0.06^{c,d}$	14.3 ± 1.3^{a}
	Tilapia	31.3 ± 3.4	5.6 ± 0.4^{x}	7.5 ± 0.4	$15.6 \pm 1.1^{y,z}$	1.07 ± 0.38^{z}	0.16 ± 0.12^{y}	16.4 ± 2.2^{x}
Cumilla	Pangasius	30.8 ± 0.7	$5.7 \pm 1.2^{b,c}$	8.1 ± 0.5^{b}	17.9 ± 2.5^{c}	$0.64 \pm 0.50^{b,c}$	$0.09 \pm 0.01^{b,c}$	17.6 ± 0.8^{b}
	Tilapia	30.1 ± 0.4	6.7 ± 0.5^{y}	7.4 ± 0.4	13.3 ± 2.1^{y}	$0.31 \pm 0.17^{x,y}$	$0.05 \pm 0.04^{x,y}$	20.6 ± 0.6^{x}
Jashore	Pangasius	30.4 ± 0.5	6.5 ± 2.1^{c}	8.5 ± 0.4^{b}	14.6 ± 1.9^{b}	$0.42 \pm 0.15^{a,b}$	$0.04 \pm 0.05^{a,b}$	23.6 ± 1.8^{c}
	Tilapia	31.3 ± 0.3	7.6 ± 1.3^{y}	7.8 ± 0.5	13.9 ± 1.5^{y}	$0.26 \pm 0.06^{x,y}$	0.00 ± 0.00^{x}	27.1 ± 2.2^{y}
Khulna	Pangasius	31.7 ± 1.8	6.1 ± 0.6^{c}	8.4 ± 0.6^{b}	8.0 ± 0.8^{a}	0.11 ± 0.04^{a}	0.00 ± 0.01^{a}	33.2 ± 4.3^{d}
	Tilapia	31.8 ± 2.6	7.1 ± 0.6^{y}	8.0 ± 0.4	6.2 ± 1.1^{x}	0.12 ± 0.09^{x}	0.00 ± 0.00^{x}	36.8 ± 0.9^{z}

The values are reported as means ± SD of samples collected in 2016, 2017 and 2018. At each sampling time, the individual analyses were based on triplicates, but when the same farms were visited in all three years, the shown data are means of nine replicates ± SD. Superscripts indicate statistically significant differences ($p<0.05$) between pangasius farms (a,b,c) and between tilapia farms (x,y,z).

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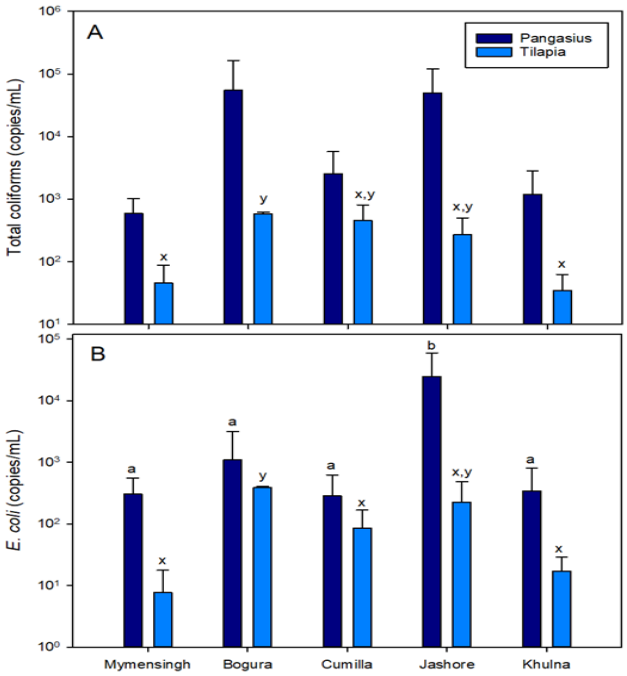

Figure 1: Total coliforms and *E. coli* in pangasius and tilapia fish farms in different regions. Same letters above bars indicate the data are not significant different (p>0.05). The data are reported as means ± SD (n=3 for each of the 2 to 9 samples locations in each region).

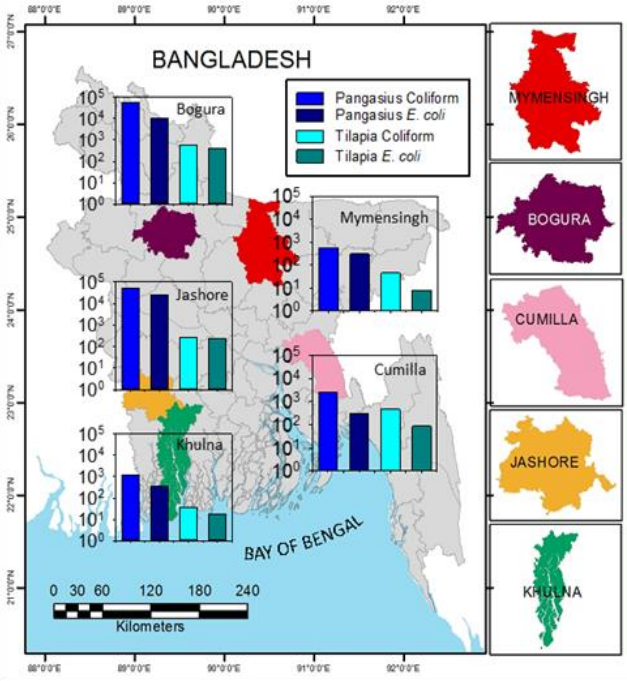

Figure 2: Map of Bangladesh showing the five regions studied. Mean densities of coliforms and *E. coli* in pangasius and tilapia farms in each of the regions are shown. Y axes (log scale) show numbers of cells per mL.

in the Mymensingh well water (0.6 copies/mL) (Table 4). Mean densities of coliforms and *E. coli* bacteria in the different regions are summarized in Figure 2.

Abundance of *Vibrio* spp.

Species of *Vibrio* bacteria were present in 76% of the farm samples and included *V. vulnificus* and *V. cholera*, while the severe fish and prawn pathogen *V. parahaemolyticus* was not detected in any of the samples (Table 4). The human pathogen type of *V. cholerae* (carrying the *ctxA* gene) was not detected in the farm samples, while *V. vulnificus* only occurred in tilapia farms in Khulna. *V. cholerae* occurred in 26% of the farms in all the regions. No *Vibrio* spp. were detected in the underground well water or in the Shoilmari river samples. Gel images of PCR analyses for detection of *Vibrio* strains are shown in Supplementary Figure S1.

Microcystin and saxitoxin

Copy numbers of the microcystin-encoding gene *mcyE* varied from none in 14 samples to a peak of 2.6×10^6 per mL in one tilapia farm in Jashore (Table 4). Except for the single peak in the Jashore region, when *mcyE* was detected, more *mcyE* copies were measured in the pangasius farms (mean of 7.2×10^2 copies/mL) than in tilapia farms (mean of 2.4×10^2 copies/mL) in Mymensingh, Bogura and Cumilla. No copies of *mcyE* were found in pangasius farms in Jashore or in pangasius and tilapia farms in Khulna, or in the well water or the Shoilmari river.

Concentrations of microcystins in the phytoplankton (measured in one pangasius and one tilapia pond from each of the Bogura and Khulna regions in selected months in 2016-2017 only) were dominated by the microcystin LR variant. Lowest concentrations of microcystin LR occurred in December (0.2 to 0.8 µg/L), while maximum concentrations were found in April in Bogura at 4.3 µg/L in pangasius farms and at 5.5 µg/L in tilapia farms (Figure 3).

The saxitoxin-encoding gene *sxtA* in cyanobacteria was not detected by the applied qPCR method in water from any of the 38 farms.

Correlating physicochemical properties with microbes

A correlation matrix of the observed parameters was determined by Pearson's correlation coefficients (Table 5). The correlations showed that numbers of total coliforms tended to decrease when temperature, pH, ammonia and transparency increased, but co-varied with dissolved oxygen, nitrate and ortho-P. Besides, the abundance of *E. coli* correlated positively with dissolved oxygen and total coliforms, and none of the water quality parameters appeared related to copy numbers of the *mcyE* gene. For nutrients, concentrations of ammonia, nitrate and ortho-P are covaried in the ponds.

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Table 4: Abundance of coliform bacteria and *E. coli*, presence of various *Vibrio* strains (+ and - indicate number of positive or negative ponds among the studied ponds, wells and the Khulna river) and copy number of the *mcyE* gene (encodes production of microcystin in cyanobacteria). For coliforms, *E. coli* and *mcyE* copy numbers, range (minimum to maximum of samples collected in 2016, 2017 and 2018) and means of triplicate samples are shown. NT: Not tested.

Region	Species and number of ponds studied	Indicator species		*Vibrio*										Microcystin
		Total coliforms (qPCR) Copies/mL	*E. coli* (qPCR) Copies/mL	*V.* spp. (PCR)		*V. cholerae* (*ctxA*) (PCR)		*V. parahaemolyticus* (PCR)		*V. vulnificus* (PCR)		*V. cholerae* (PCR)		*mcyE* (qPCR) Copies/mL
		Mean (min, max)	Mean (min, max)	+	-	+	-	+	-	+	-	+	-	Mean (min, max)
Mymensingh	Pangasius (n=9)	5.9×10^2 (8.6×10^1, 1.3×10^3)	3.1×10^2 (7, 6.8×10^2)	6	3	0	9	0	9	0	9	1	8	7.7×10^2 (0, 2.3×10^3)
	Tilapia (n=7)	4.6×10^1 (0, 1.2×10^2)	8.0×10^0 (0, 2.4×10^1)	5	2	0	7	0	7	0	7	1	6	6.6×10^2 (0, 3.6×10^3)
	Well	3.4	0.6		1	NT		NT			1		1	Not detected
Bogura	Pangasius (n=4)	5.5×10^4 (1.9×10^2, 2.2×10^5)	1.1×10^3 (2.6×10^1, 4.2×10^3)	3	1	0	4	0	4	0	4	1	3	8.5×10^2 (0, 3.3×10^3)
	Tilapia (n=2)	5.9×10^2 (5.7×10^2, 6.1×10^2)	3.9×10^2 (3.8×10^2, 4.1×10^2)	1	1	0	2	0	2	0	2	1	1	5.8×10^1 (2.3×10^1, 9.3×10^1)
	Well	1.9	0		1	NT		NT			1		1	Not detected
Cumilla	Pangasius (n=4)	2.6×10^3 (2.8×10^2, 7.2×10^3)	2.9×10^2 (1.0×10^1, 7.3×10^2)	4	0	0	4	0	4	0	4	0	4	5.3×10^2 (0, 1.4×10^3)
	Tilapia (n=4)	4.5×10^2 (4.3×10^1, 9.1×10^2)	8.5×10^1 (1.8×10^1, 2.0×10^2)	3	1	0	4	0	4	0	4	1	3	1.3×10^2 (0, 4.8×10^2)
Jashore	Pangasius (n=2)	5.0×10^4 (9, 1.0×10^5)	2.5×10^4 (0, 5.0×10^4)	1	1	0	2	0	2	0	2	1	1	Not detected
	Tilapia (n=2)	2.7×10^2 (1.1×10^2, 4.4×10^2)	2.3×10^2 (4.4×10^1, 4.1×10^2)	2	0	0	2	0	2	0	2	2	0	1.3×10^6 (1.2×10^2, 2.6×10^6)

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(Continued)

Khulna	Pangasius (n=2)	1.2×10^3 (2.8×10^1, 2.4×10^3)	3.4×10^2 (1.3×10^1, 6.7×10^2)	2	0	0	2	0	2	0	2	1	1	Not detected
	Tilapia (n=2)	3.5×10^1 (1.5×10^1, 5.4×10^1)	1.7×10^1 (9, 2.6×10^1)	2	0	0	2	0	2	2	0	1	1	Not detected
	River	1.5	0		1	NT		NT			1		1	Not detected

Table 5: Correlation matrix among physicochemical parameters and number of microbes (total coliforms and *E. coli*) and presence of the microcystin *mcyE* gene in water of the farms.

Parameters	Water temp.	DO	pH	Nitrate	Ortho-P	Ammonia	Transparency	Total coliform	*E. coli*	Microcystin
Water temp.	1									
DO	-0.055	1								
pH	-0.023	0.291**	1							
Nitrate	-0.070	-0.437**	-0.402**	1						
Ortho-P	0.230*	-0.314**	-0.213*	0.366**	1					
Ammonia	0.270**	-0.500**	-0.372**	0.371**	0.368**	1				
Transparency	0.067	0.462**	0.417**	-0.688**	-0.586**	-0.365**	1			
Total coliforms	-0.036	0.019	-0.171	0.268	0.053	-0.093	-0.071	1		
E. coli	-0.039	0.386*	0.084	0.075	-0.016	-0.150	0.047	0.468**	1	
Microcystin	0.027	0.181	0.183	-0.117	-0.125	-0.153	0.231	-0.039	-0.032	1

*Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).
Water temp.= water temperature; DO = dissolved oxygen

DISCUSSION

Physicochemical conditions of the fish farms

The coincidence of higher nutrient loads (nitrate, ortho-P and ammonia) and lower dissolved oxygen in ponds in the Mymensingh and Bogura regions, as compared to the other three regions, was probably related to higher fish densities, excess feeding and fertilization (for stimulation of algal growth as feed for the fish), all together causing a higher organic and inorganic load. The deeper ponds in these two regions (about 2 m depth), relative to <1.5 m in Jashore and Khulna, may also have impacted the water quality. In earthen ponds, shallow water depths tend to worsen the water quality (Nzeagwu *et al.*, 2017), but deeper ponds may speculatively also lower the water quality due to sedimentation of organic matter and reduced water circulation. In contrast to the farms in Mymensingh and Bogura, fish farms in the Jashore and Khulna regions usually apply less feed and fertilizer and may, therefore, maintain a better water quality.

In all regions, pH values were acceptable for fish farming (pH of 6.5-9.5) (Abedin *et al.*, 2017), but a negative relationship between pH and total coliforms was found. Hence, if alkaline pH can be maintained in farms by application of lime, presence of coliform bacteria in pangasius and tilapia farms may be reduced. Application of lime is common in aquaculture farms in Bangladesh and has been shown to improve ecosystem health of the water (Ali *et al.*, 2013).

The tendency of lower water transparency in the pangasius farms than in tilapia farms was probably caused by higher densities of cyanobacteria

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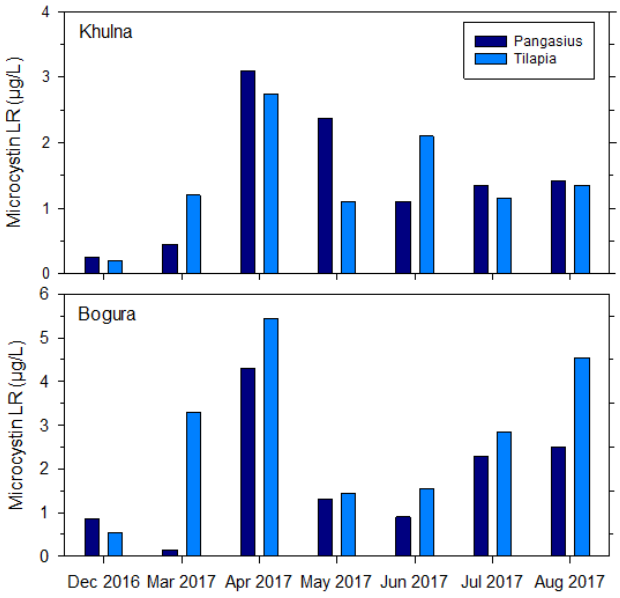

Figure 3: Concentrations of microcystin LR in water from individual pangasius and tilapia farms in Khulna (ponds in Jalma village in Batiaghata Upazila) and Bogura (ponds in Kalaikuri village in Adamdighi Upazila) in 2016 and 2017. Single concentrations are shown. Microcystin LR refers to content of the amino acids leucine (L) and argenine (R) in this microcystin congener.

and other phytoplankton in the pangasius farms. Tilapia is capable of filter-feeding on phytoplankton and has been shown to improve water transparency by ingestion of algae (Menezes *et al.*, 2010). Similar positive effects on transparency have been observed for omnivorous filter-feeders, such as silver carp (*Hypophthalmichthys molitrix*) and catla (*Catla catla*) (Turker *et al.*, 2003; Torres *et al.*, 2016). The higher water transparency observed in Khulna was caused by a low organic load in the water and frequent water exchange by river water.

Total coliforms and *E. coli*

Coliform bacteria and *E. coli* were present in water of the fish farms and occurred occasionally at densities up to 2.2 × 10^5 and 5.0 × 10^4 cells per mL, respectively. There was a tendency to co-occurrence of coliform bacteria and *E. coli* in the farms, irrespective of the fish species being cultivated (Table 5). The presence of *E. coli* and coliform bacteria in the fish farm water does most likely indicate fecal contamination (Godfree *et al.*, 1997). The source of these bacteria is assumed to be feces from humans and warm-blooded animals, e.g. cattle and birds (Doyle and Erickson, 2006; Roche *et al.*, 2013). Wash-out of nutrients from farm fields by rainwater, as well as unhealthy conditions in the fish farms, may also stimulate the growth of coliforms (Khanom *et al.*, 2014; Jahan *et al.*, 2017). In addition, well water is often used in fish production and may be another source of fecal pollution due to discharge of bacteria from nearby latrines, septic tanks and seepage from contaminated surface areas (Knappett *et al.*, 2011; Rahman *et al.*, 2019). Occasionally, extreme densities of fecal bacteria have been recorded in surface water polluted by dumping of waste and cattle wash (Jahan *et al.*, 2017).

High densities of 10^4 to 10^5 coliforms per mL have previously been found by cultivation assays in ponds and lakes in populated areas and in rivers receiving urban waste in the Dhaka region in Bangladesh (Shiekh *et al.*, 2006; Hasan *et al.*, 2019). Representative numbers of coliforms in water in tilapia or pangasius farms in Bangladesh are scarce, but Mandal *et al.* (2009) reported 200 coliforms and 3 *E. coli* bacteria (as CFU) per mL by plate counting in tilapia ponds in central Bangladesh. According to World Health Organization, water is suitable for aquaculture production when the coliform density is below 10 cells per mL (WHO, 1989). Based upon our data from 38 farms, fish farms in Bangladesh appear to have an unacceptable or alarmingly high density of fecal bacteria. This was especially true for farms in the Bogura and Jashore regions, while farms in the Mymensingh and Khulna regions appear to have a better microbial water quality (below 100 coliforms and 10 *E. coli* per mL in some farms) but in general, the water quality is unacceptable according to WHO recommendations. Farms in the riverine Khulna area usually have less contamination from nearby external sources, and this may explain the low number of *E. coli* detected in the water. However, river water in the Khulna region might be an important source of fecal contamination due to open latrines along the riverside and to local streams that enter the river (Knappett *et al.*, 2011; Jahan *et al.*, 2017; Real *et al.*, 2017). In Mymensingh, the absence of *E. coli* in four samples and the overall low *E. coli* numbers in tilapia farms probably reflect well-managed fish farms that are located at a distance from external sources, minimizing the risk of contamination by fecal coliforms.

Once coliform bacteria are present in the water, dense fish stocks, richness of nutrients in the water and high temperatures all together may stimulate the growth of bacteria (Petersen and Hubbart, 2020). After slaughter, the coliforms often remain on the fish, as shown for Hilsa fish (*Tenualosa ilisha*) (Foysal *et al.*, 2019). In marketed Hilsa fish, the presence of several coliform bacteria on the skin surface and in the gut suggests that careful hygiene must be practiced when preparing the fish for consumption.

***Vibrio* spp., microcystin and saxitoxin**

Several *Vibrio* species were present in the farms, including *V. cholerae* (without the toxic *ctxA* gene) and the fish- and human-infectious *V. vulnificus*. *Vibro cholerae* was found in farms in all regions, except in pangasius farms in Cumilla and ranged from few positive farms (Mymensingh, both species) to all-positive farms (tilapia, Jashore), while *V. vulnificus* only occurred in tilapia farms in Khulna. Presence of *V. cholerae* in water of some farms suggests that fish could serve as a potential vehicle for toxic and non-toxic *V. cholerae*

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transmission, when there is a high prevalence of *V. cholerae* in the water (Hossain *et al.*, 2018). The severe fish pathogen *V. parahaemolyticus* was not detected in any farms. Most *Vibrio* bacteria are marine species (Thompson *et al.*, 2004) and the appearance of *V. vulnificus* in tilapia farms in Khulna does most likely reflect intrusion of seawater through the Shoilmari River in this region. The incidence of *V. vulnificus* in only coastal tilapia farms along the Bay of Bengal in Bangladesh agrees with observations from a previous study (Mahmud *et al.*, 2010).

The presence of the *mcyE* gene in four of the five regions with densities of up to 2.6×10^6 copies/mL clearly indicates that microcystin production may occur in the fish farms, especially in some of the pangasius farms, where higher copy numbers tended to occur. Concentrations of up to 5.5 µg/L of microcystin LR in plankton in Khulna and Bogura in 2016 and 2017 support production of microcystin in the ponds. Surprisingly, despite microcystin LR was present in all water samples from Khulna and Bogura, the *mcyE* gene was only detected in Bogura (up to 3.3×10^3 copies/mL) and not in Khulna. Possibly, the density of microcystin-producing cyanobacteria in the Khulna farms was low at the sampling time. The measured concentrations of microcystin LR align concentrations observed in plankton in tilapia farms in Brazil (Schlüter *et al.*, 2018; Falcone-Dias *et al.*, 2020), but were lower than the concentration of 33 µg/L measured during a *Microcystis* bloom in a fish pond in central Bangladesh (Ahmed *et al.*, 2008).

A positive correlation between *mcyE* copy numbers and concentrations of microcystin in plankton cells was observed by Falcone-Dias *et al.* (2020) in Brazilian fish farms, as well as in a shallow temperate lake by Panksep *et al.* (2020) (observed for microcystin RR only) and in lakes in Iowa, USA, by Lee *et al.* (2020) (only the *mcyA* gene was studied). Thus, although microcystin only was measured in selected ponds in this study, the wide occurrence of the *mcyE* gene suggests that microcystin was present in phytoplankton cells and might be released to the water. Free dissolved microcystin may remain in the water but may also be removed by microcystin-degrading bacteria that appear common in freshwaters (Krishnan *et al.*, 2020).

Elevated nutrient levels might have triggered blooms of microcystin-producing cyanobacteria in the farms, since microcystin production by cyanobacteria has been shown to increase proportionally to the level of nutrients, especially of total phosphorus (Wang *et al.*, 2002). Supporting a relation between cyanobacterial blooms and microcystin production, farms in the Jashore region were characterized by heavy blooms with scum formation, and these farms had the highest *mcyE* copy numbers. As mentioned above, tilapia is a potential filter-feeding fish and may reduce the abundance of cyanobacteria (removal of up to 60% of the cyanobacterial biomass by tilapia has been observed) by Turker *et al.* (2003) and Torres *et al.* (2016). However, ingestion of microcystin-producing cells can lead to toxin accumulation in organs and muscle tissue in fish and threaten the health of both fish and consumers eating the fish (Singh and Asthana, 2014).

In contrast to the presence of the *mcyE* gene, no occurrence of the *stxA* gene (encodes production of saxitoxin) was detected in any of the five regions. Common saxitoxin producers are marine dinoflagellates but recently, cyanobacteria producing saxitoxin have also been found in freshwater environments (Carmichael *et al.*, 1997; Lagos *et al.*, 1999; Galvão *et al.*, 2009; Al-Tebrineh *et al.*, 2010). Galvão and co-authors observed accumulation of saxitoxin in tilapia, cultivated in freshwater, and this indicates an overlooked risk in tropical freshwater aquaculture, specifically during blooms of toxic cyanobacteria. In Bangladesh, content of saxitoxin was reported in two freshwater species of freshwater pufferfish, *Tetraodon cutcutia* and *Chelonodon patoca* (Zaman *et al.*, 1998). This suggests that further research on saxitoxin in Bangladeshi freshwaters is needed.

Consequences of a poor microbial water quality

In Bangladesh, villagers typically live close to their livestock, and the livestock is frequently left to graze near ponds, where open latrines are common and often located near wells. These conditions introduce a high risk of fecal contamination of both pond water and well water (Knappett *et al.*, 2011). Species within coliform genera (*Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella* and *Escherichia*) do not only include several human pathogens (Cabral, 2010). Certain coliforms are also fish pathogens, e.g. *Citrobacter* that causes mortality in tilapia (Bandeira Junior *et al.*, 2018) and *Enterobacter* that may lead to enteritis in catfish (Cao *et al.*, 2017). This means that a poor water quality, caused by fecal contamination, may threaten health of both local inhabitants and fish stocks. Therefore, understanding the relative contributions of fecal sources could lead to better public health interventions.

Cyanobacterial toxins may also negatively affect the commonly farmed species pangasius and tilapia. Pangasius can ingest surface scum with a high content of cyanobacteria while feeding on floating food pellets (Zimba *et al.*, 2001). Being planktivorous, tilapia intentionally ingests cyanobacteria and this introduces risk of accumulation of toxins in their liver/hepatopancreas in intensive aquaculture facilities (Beveridge *et al.*, 1993; Keshavanath *et al.*, 1994). Among cyanobacterial toxins, microcystins make up the most toxic group and are also the most commonly encountered toxins in eutrophic aquatic environments (Hoeger *et al.*, 2005). Microcystins accumulate in fish and remain stable and resistant to degradation, even during cooking (Harada *et al.*, 1996). The risk of human exposure to microcystins via fish consumption depends on concentration of microcystins in the fish (Chen *et al.*, 2009). Smith *et al.* (2008) reported that in about half of the cases where microcystins were found in edible tissues of aquatic animals, the concentration exceeded the tolerable daily intake (TDI) of 0.04 mg/kg body weight/day (Kuiper-Goodman *et al.*,

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1999), suggesting that human intoxication is possible when eating farmed fish. This causes great concern for public health, because chronic ingestion of microcystin in food and drinking water has a considerable potential to promote cancer (de Figueiredo *et al.*, 2004) and tumor growth (Dawson, 1998; Moreno *et al.*, 2004; Codd *et al.*, 2005). Tilapia is the second most important farmed and widely consumed fish species in Bangladesh, and its omnivorous and planktivorous feeding habits introduce a risk of ingestion of microcystin-producing phytoplankton, which might lead to the accumulation of microcystins. Therefore, it is important to assess the toxicity of microcystins in farmed fish, as well as the toxic effects on human health.

CONCLUSION

This study revealed that water quality in pangasius and tilapia farms in different regions of Bangladesh is challenged due to fecal contamination (high abundance of the indicator organism *E. coli* and total coliforms), microcystin-producing cyanobacteria, and pathogenic *Vibrio* spp. Sites with a high fish production (Mymensingh, Bogura and Cumilla) differed from sites with a relatively lower production (Jashore and Khulna) in terms of physicochemical properties and populations of selected microorganisms. An elevated level of fecal coliforms and microcystin producers in many of the fish farms illustrates the barriers that aquaculture in Bangladesh is facing. Likewise, the distribution of *V. vulnificus* in coastal farms and farm water sources needs further attention.

Our results show that there is a need for improved treatment and handling of water from private households and public facilities to reduce fecal contamination, as well for better control of cyanobacterial blooms to improve and safeguard an acceptable future water quality in fish farms and in natural freshwater ponds. Molecular methods have recently successfully been introduced in monitoring of drinking water quality in Bangladesh (Mina *et al.*, 2018) and we hope that the present results may serve as incentive for also using such methods in the aquaculture industry, e.g. by aquaculture associations. Implementation of accurate and reliable procedures for microbial water quality analyses can help to improve the aquaculture production for the benefit of both fish farmers, local consumers and international export markets.

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SUPPLEMENTARY INFORMATION

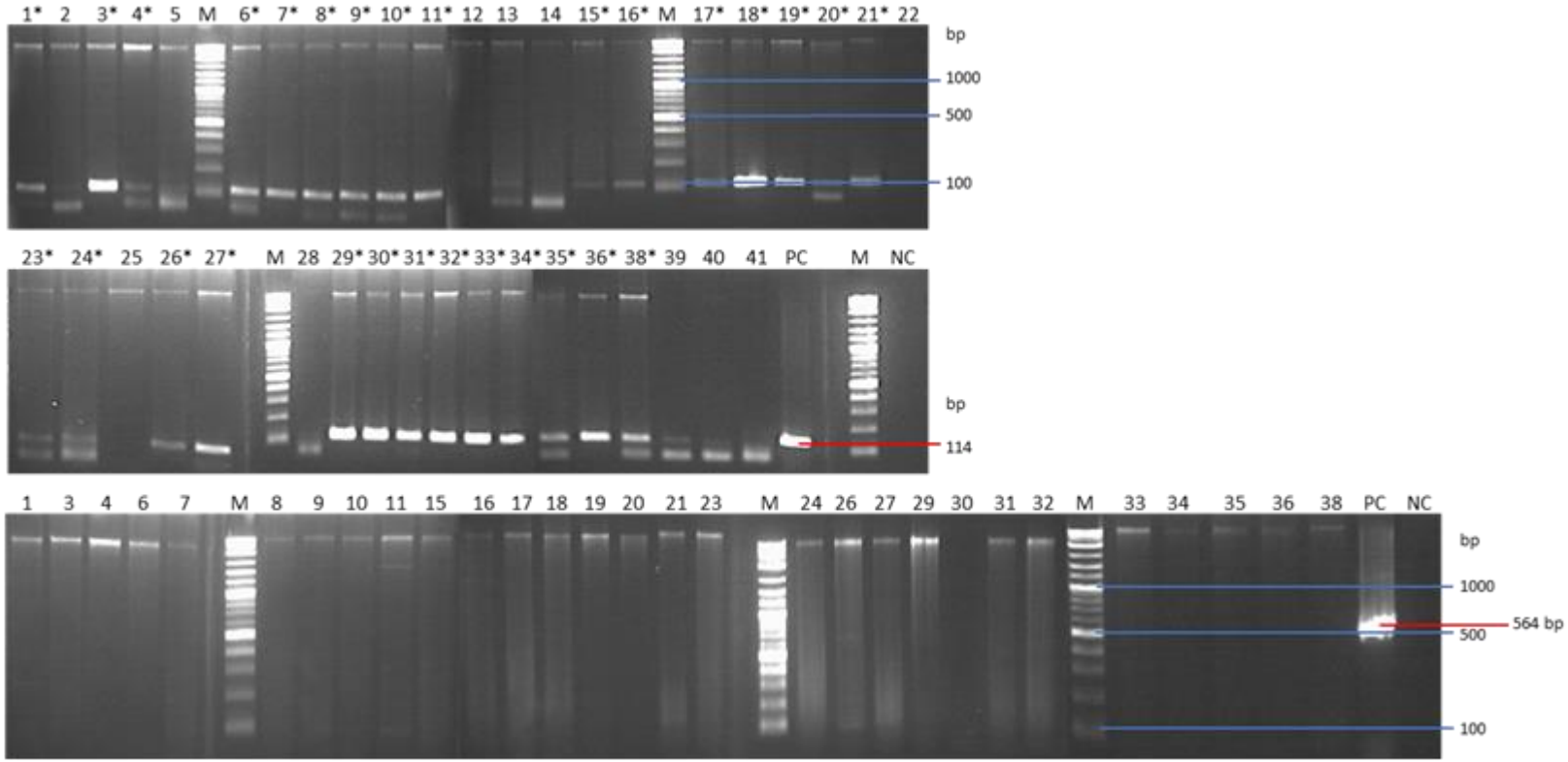

Figure S1: PCR products of the 16S rRNA gene of *Vibrio* spp. on 2% agarose gel. Upper and middle panel: Lane M: DNA marker, Lane 1: MP (Mymensingh pangasius), Lane 2: BP (Bogura pangasius), Lane 3: KP (Khulna pangasius), Lane 4: MT (Mymensingh tilapia), Lane 5: BT (Bogura tilapia), Lane 6: KT (Khulna tilapia), Lane 7: BP, Lane 8: KP, Lane 9: BT, Lane 10: KT, Lanes 11-18: MP, Lanes 19-20: BP, Lanes 21-26: MT, Lanes 27-28: JP (Jashore pangasius), Lanes 29-30: JT (Jashore tilapia), Lanes 31-34: CP (Cumilla pangasius), Lanes 35, 36, 38: CT (Cumilla tilapia), Lane 39: Background Mymensingh, Lane 40: Background Bogura, Lane 41: Background Khulna, Lane PC: Positive control, Lane NC: Negative control. * indicates positive amplification. Lower panel: PCR products of the *ctxA* gene of *V. cholerae* on 1% agarose gel. Lane M: DNA marker, Lanes 1 to 38 shows samples that were positive for *Vibrio* spp.

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