Water quality and fish production

Scientific report from the BangFish project Work Package 1

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Preface

This report on *Water quality and fish production* is part of the BangFish project *Upgrading pang as and tilapia value chains in Bangladesh,* funded by the Danish International Development Agency (DANIDA project number F38A26778).

Research in this work package has focused on microbial and chemical water quality in fish farms producing pangasius and tilapia. In addition, factors affecting flesh colour of pangasius, and flavour of fish flesh in pangasius and tilapia were examined. The research data were obtained by field work in fish farms in different regions of Bangladesh and by experimental studies at Patuakhali Science and Technology University (PSTU) and Bangladesh Agricultural University (BAU). Analysis of collected samples were performed at either PSTU, BAU or University of Copenhagen (UCPH).

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1. INTRODUCTION TO WORK PACKAGE 1

The most important factor for successful and profitable aquaculture production is clean water. Low water quality in terms of pollution, lack of oxygen, toxic algae and transfer of diseases, etc. can have numerous negative effects on production. The negative effects can be identified by higher mortality, non-optimal growth rates, diseases and a low quality of the fish produced. Low quality can affect human welfare if the fish contain human pathogens, pollutants such as arsenic, lead, and toxins. Furthermore, low quality can affect farm income through the price received, both locally and for export of fish.

Typical sites for aquaculture in Bangladesh are rural ponds and lakes. In most cases, water in the ponds originates from rain and flooding of rivers and adjacent fields, especially during the monsoon period, but larger pond systems have controlled water passage (inlet-outlet). Pond systems that receive run-off from agricultural and cattle fields benefit from the washout of nutrients that promote algal growth. Algae and phytoplankton serve as supplementary feeds to pangasius and tilapia in the ponds. However, there is a potential risk that chemicals (insecticides and fungicides) from crop treatment may enter the ponds and accumulate in the fish. Several pesticide residues have recently been detected in surface waters in Bangladesh (Chowdhury et al. 2012), and in an earlier study, organochlorine pesticides were detected in organs of the Ganges perch, although at levels below FAO recommendations (Jabber et al. 2001). In some regions of Bangladesh, bottom sediment in the ponds has a high content of the metals lead and arsenic (mainly from weathering of Himalayan mountainous), and these metals have been found in several freshwater fish from ponds in Bangladesh (Rahman et al. 2012). In addition to these external and potential sources of contaminants, water in most ponds is used for general household purposes (dishwashing, personal hygiene, animal watering, etc.).

The origin of water in fishponds has high seasonal variability due to precipitation and flooding of fields, as well as the human activity, which makes it difficult to maintain optimum water quality for fish breeding. The input of nutrients stimulates growth of beneficial algae (phytoplankton) that may benefit the fish, however, the algal growth also promotes dense populations of different bacteria, as observed in a Danida-sponsored study in the Patuakhali region in Bangladesh (Mahmud et al. 2013; 2016). A high abundance of bacteria may lower the water quality and negatively affect the health of fish, and most fish farmers in Bangladesh encounter fish diseases caused by bacteria (Faruk et al. 2004).

In this Work Package 1, the focuses have been the most important issues that threaten Bangladeshi production of fish in village ponds, as well as in large-scale fish farms. These issue are (1) microbial and chemical water quality and (2) quality of the farmed fish with respect to flesh colour and taste.

1.1 Microbial water quality

Aquaculture production is prone to stimulate growth of microorganisms in water in fish farms. Dense fish stocks, regular feeding, production of fecal matter, debris from fish, excretion of organic and inorganic phosphorus and nitrogen compounds stimulate bacterial growth. The dissolved nutrients can cause eutrophication, blooms of algae, including also toxic species, and reduction of oxygen. Organic substances reduce the oxygen concentration in the water column and in sediments, and may modify pelagic and benthic food chains (Islam and Yasmin 2017).

In a study of fishponds in Bangladesh, Khanom et al. (2014) observed that conditions for fish production were not recommended in some ponds due to a high concentration of nutrients, low oxygen content and presence of coliform bacteria in the water. In addition, the authors found a relatively high abundance of coliform bacteria and presence of *Salmonella* bacteria. The fecal bacteria may be of human or animal origin since many fishponds are multi-purpose ponds that are used for several household purposes, as well as water source for animals, but these bacteria may also originate from run-off from farm fields. Coliform and pathogenic bacteria may spread via skin and organs in fish and introduce risk to consumers. Supporting dispersal of pathogenic bacteria by fish in Bangladesh, Foysal et al. (2019) used molecular methods to detect bacteria on Hilsa fish and found a high abundance of *Vibrio cholerae*, *Aeromonas* spp., *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and other potential human pathogens on skin and organs of the fish. Yet, little published information on pathogens in water and fish in Bangladesh is available, but the study on Hilsa fish emphasizes that attention must be paid to risks of spreading diseases to consumers when eating freshwater fish.

Other microorganisms that threaten aquaculture production is phytoplankton in the water. A high abundance of algal cells is commonly observed during spring and summer in fishponds due to increasing temperature and precipitation (Affan et al. 2005). Adverse effects from the algae include risk of anoxic conditions (when the algae die and are degraded), irritation and clogging of fish gills, and growth of toxic and fish-killing cyanobacteria (blue-green algae) (Smith et al. 2008). In Bangladesh, toxic cyanobacteria are reported to commonly occur in ponds (Abu et al. 2016), but specific knowledge on the biology of these organisms and mechanisms that control the toxin production await to be examined.

Over the past decade, the globally increasing use of antibiotics, particularly in Asian countries, has been questioned. This also applies to aquaculture production in Bangladesh (Ali et al. 2016). Several studies have pointed out that most aquaculture farmers lack proper training and institutional support on how to use antibiotics in aquaculture (Ali et al. 2016). Insufficient knowledge on disease diagnosis, mode of action, proper doses, and withdrawal period of antibiotic compounds is of major concern. The consequences of this lack of knowledge are overuse or misuse of antibiotics in many fish farms and accumulation in finfish and shellfish tissues, resulting in a potential health hazard for humans and animals, as well as a barrier for export of aquaculture products (Sapkota et al. 2008). The use of antimicrobial agents in fish farms presents a risk to public health because of the development of acquired antimicrobial resistance in fish pathogens and other aquatic bacteria, since many people are exposed to aquaculture systems in different ways. Such drug-resistant bacteria can act as reservoirs of resistance genes, from which genes can disseminate to human pathogens (Heuer et al., 2009). Additional adverse effects of fish farming can be the application of various chemicals used for control of bacterial diseases for optimization of the fish production (Cabello 2006).

1.2 Chemical water quality

Metals are naturally present in all environments, but anthropogenic sources of metals such as mining, industry, traffic, domestic sewage, and atmospheric deposition vastly elevate the metal concentration in the environment, including also the aquatic environments (Saha et al. 2016) Metals are non-biodegradable and persistent and may result in bioaccumulation in organisms (Zhang et al. 2016). For food safety reasons, metal accumulation in fish has been studied for many years (Hsi et al. 2016). Routes for metal exposure in fish include direct uptake through gills and other body surfaces, and digestion of food and sediment material in the digestive tract, causing metals to accumulate in the body of fish. Rajkowska and Protasowucki (2013) showed that metal distribution among varies tissues in fish mainly depended on the metal content in water and food, and therefore it can serve as a pollution indicator of the environments. If fish are farmed in metal-contaminated environments, fish could accumulate metals through water, sediment, and feeds; consequently, the human who has high consumption rate of fish could be exposed to the higher health risk. Under certain conditions, humans accumulate metals through the consumption of aquaculture organisms and could have adverse effects on the body (Saha et al. 2016).

1.3 Quality of the farmed fish with respect to flesh colour

In the current international export market, the main criteria applied for fish quality are colour, size, disease and antibiotic residues. The first two criteria, colour and size, are important for the price of fish and success in export markets. Consumers in USA and the EU prefer white and pink fillets and are willing to pay higher prices for these, while yellow fish fillets are sold at lower prices (with a lower quality standard) to markets in Eastern Europe, such as Russia and Ukraine, and some southeast Asian countries, such as Singapore and South Korea (VASEP 2008).

Vietnam is the World´s largest producer of pangasius with export to many well-established markets due to acceptability and affordability. Europe is the largest market for pangasius and is a potential target for exports from Bangladesh (Belton, et al., 2011). While pangasius fillets from Vietnam are white, fillets produced in Bangladesh are often discoloured yellow. Since European consumers prefer white fillets, the discoloured yellow fillets are considered of lower quality (Little et al. 2012; Kulawik et al. 2015). Consequently, European importers are not interested in Bangladeshi pangasius fillets (Belton et al., 2011). A large and growing pangasius industry exists in Bangladesh, but the produced fillets typically have a yellow colour, which limits the export of pangasius to Europe since the discolouration is a critical issue (SEAFISH, 2015). The existing literature indicates that a white pangasius fillet reflects (a) good farming practices at a low stocking density; (b) frequent water exchange and usage of water with high oxygen content (Haque et al. 2016; Belton et al. 2011); (c) low levels of carotenoids in feed (Amaya & Nickell 2015); (d) low levels of chromatophore cells (Qiufen et al. 2012); and (e) good handling procedures after harvest, including draining the blood from the fillet to prolong its shelf life (Love 2001). Yet, most of the pangasius produced in Bangladesh still have yellow discoloured flesh, showing that more efforts are needed to farm fish with white flesh to develop an export-oriented pangasius industry.

1.4 Quality of the farmed fish with respect to taste and flavour

Fish from aquaculture farms are sometimes reported to be tainted by off-flavours, especially by an earthy and musty compound such as geosmin and 2-methylisoborneol (2-MIB) (Gutierrez et al. 2013). In Bangladesh, some consumers hesitate to buy pond-raised fish due to the flavour and taste of the fish. Even though the fish may not have an undesirable taste, consumers may still opt-out certain fish species on markets in Bangladesh.

The presence of off-flavours in fish from Bangladeshi ponds was examined in a pilot study of pangasius by Mahmud et al. (2013). The study confirmed an off-taste in some pangasius fish but the authors also observed that depuration of the fish in clean water for about one day significantly improved the taste of the fish. A local consumer panel confirmed the improved taste.

The off-flavours are produced by certain microorganisms and algae in the water (Lylloff et al. 2012). Fish are assumed to be tainted by off-flavours when digesting microorganisms and algae, or by uptake from the water via the gill or body surface. Despite the off-flavour problem is growing internationally due to increased production of fish in farms, there is limited knowledge on the biology of the off-flavour producing organisms and on mechanisms controlling the production of these compounds.

In a recent study, Podduturi and co-authors (2017) carried out chemical analysis of volatile compounds in tilapia and pangasius, in the fish feeds and in phytoplankton in ponds in Bangladesh to determine the source of the off-flavours. The authors identified geosmin and 2-MIB and several other off-flavours in the samples, including many terpenes that are known from different biological sources. The analyses showed that fish flesh may contain a number of naturally occurring organic compounds (several compounds also contribute pleasant taste to fish) and that the fish feed, rather than algae or organic matter in the water, was the most abundant source of the taste compounds.

Despite this study only included fish from a few farms, the observation suggests that the feed industry should be careful when selecting ingredients for farmed fish to minimize potential off-flavour problems related to feeding of the fish.

1.5 Short review on aquaculture systems and fish production in Bangladesh

The freshwater aquaculture systems used in Bangladesh are pond aquaculture, rice-fish culture (either rice and fish together or rice followed by fish) on seasonal farmland, cage culture in rivers and lakes, pen culture in closed and open water bodies, and fish culture in such commonly held perennial water bodies as oxbow lakes. Aquaculture systems generally use free water sources (precipitation), but some receive commercial irrigation or hire irrigation equipment. Summary of cultivation methods is given below (from review by Dey et al. 2008).

Pond fish culture. Today, almost every household in lowland floodplains has at least one pond excavated initially to acquire soil to raise the homestead floor above the high water mark during the rainy season. Those without a river nearby also need to have a pond to supply water for household use. Most of the ponds connect with other water bodies during the rainy season, receiving runoff and becoming home to indigenous fish, which people catch during and after the rainy season. Many small ponds in northern and central Bangladesh dry up completely during the dry season. Fish farmers have adopted a variety of culture systems to suit the diversity of ponds.

Rice-fish culture. Rice-fish culture started in the 1990s through pioneering adaptive research done by the WorldFish Center (Gupta et al. 1999) in open water bodies, particularly with carp polyculture and tilapia mixed culture in the greater Mymensingh region. A large number of poor people are gradually adopting to this production type. The ponds of small farmers are usually dominated by tilapia, sarpunti and catfish (pangas), with lesser numbers of carp. The major difficulties of this culture system are poor water quality and vulnerability to seasonal flooding, which allows fish to swim away, leaving farmers with serious economic losses. Farmers see this culture system as second only to rice in terms of importance to their food, nutrition and income security. In supplement to the permanent rice-fish cultures, local entrepreneurs and farm communities increasingly practice fish culture in seasonally flooded paddies.

Cage cultures. This technique involves net boxes in open water bodies and is a new concept to Bangladeshi fish farmers and a highly commercial enterprise. It first started in 1997 in an oxbow lake in Jessore (Chowdhury and Yakupitiyage 2000). The Bangladesh Fisheries Research Institute (BFRI) encourages private entrepreneurs to expand this aquaculture system in other perennial water bodies, especially in freshwater rivers.

Pen cultures. Application of pens for fish production consist of netting in semi-closed water bodies, e.g. irrigation and drainage canals, but sometimes also in large, open water bodies. Traditionally, in pen cultures fish feed mainly on naturally occurring plankton, though farmers sometimes apply low-protein natural materials as supplemental feed. Carp, tilapia and pangasius are suitable for this type of aquaculture.

Hatcheries and nursery cultures. Hatcheries are both privately and publicly owned, with a number of specialized hatcheries for specific species. In freshwater environments, hatcheries choose carp and catfish. In the coastal saline environments of the southeast and southwest, most hatcheries choose shrimp. Nursery cultures are found in almost every district in Bangladesh, most of which produce carp for freshwater aquaculture. During the past decade, private nurseries have expanded in parts of the country.

Aquaculture production in Bangladesh. Bangladesh is one of the World's leading fish producing countries with a total production of 4,277,000 MT in fiscal year (FY) 2017-18, where aquaculture production contributes about 56% to the total fish production in the country (DoF, 2018). Globally, Bangladesh is ranked as the 3rd most important producer of inland open-water fish capture and 5th most important in aquaculture production (FAO, 2018). Currently, Bangladesh ranks as the 4th dominant tilapia producer in the world and the 3rd most important in Asia (FAO, 2018). Besides, this sector contributes 3.6% to the national GDP and more than one-fourth (25.3%) to the agricultural GDP. More than 11% of the total population of Bangladesh is engaged in fish production at a full-time or part-time basis for their livelihoods. The sector is contributing significantly to food and nutrition security through consistently providing safer and good quality animal protein, almost 60% of total animal protein supply (DoF, 2018).

Production of pangasius and tilapia. Pangasius has become an economically very important species to the south-east Asian aquaculture industry (FAO, 2019a), and tilapia is one of the most cultured freshwater fish in the World (FAO, 2019b). The production of pangasius and tilapia has increased from 260,990 and 136,541 MT in 2011-2012, respectively, to 453,383 and 384,737 MT in 2017-2018 (FRSS 2013, DoF, 2018). In Bangladesh, the largest pangasius producing district is Mymensingh (189,388 MT), followed by Comilla, Bogra, Jessore and Barishal. The highest tilapia producing district is Comilla with 36,064 MT, followed by Mymensingh, Jessore, Chittagong and Barishal (DoF, 2018).

1.6 Purpose and hypotheses of Work Package 1

The purpose of this work package has been to provide a knowledge base on the impact of water quality on the production of fish. Important research questions have been: (a) to which extent do bacteria negatively affect fish production, (b) does the natural presence of arsenic and other metals in water and soils negatively influence fish production, (c) what are the environmental implications of application of antibiotics and other additives in fish farms; (d) can water quality initiatives benefit the farmers in the aquaculture industry?

We hypothesize that improved water quality, with respect to biological and chemical contaminants, will improve growth, quality, and marketability of the produced fish. To confirm these hypotheses, we conducted fieldwork, pilot studies, and laboratory experiments and applied state-of-the-art methods within water chemistry (nutrients and metals), microbial ecology (obnoxious and beneficial microorganisms), DNA technology (detection of specific genes) and environmental monitoring (turbidity, temperature, oxygen and water level).

1.7. Study sites and methods

1.7.1. Microbial water quality in pangasius and tilapia aquaculture systems in Bangladesh

Research approach and study area: This investigation was carried out to evaluate the microbial status of the pond water in five commercially important fish producing areas of Bangladesh, including Mymensingh, Bogra, Comilla, Jessore, and Khulna. Among these regions, Khulna differs from others as it is situated at the coastal belt of Bangladesh. The ponds we investigated in Khulna receive water from the adjacent river Shoilmari. Water in the river is a mixture of freshwater and seawater due to the adjacent estuary. In other regions, mostly underground water is used. Water from different farms in the five regions was sampled in pangasius and tilapia ponds in 2016, 2017 and 2018.

Methods: Environmental parameters such as temperature, dissolved oxygen, and pH were measured by EXTECH (USA) portable devices and Secchi disc at the sampling sites. Ammonia, nitrate, and ortho-phosphate were measured immediately in the laboratory by Lovibond SpectroDirect Spectrophotometry (Tintometer GmbH, Germany). Genomic DNA was extracted using

PowerWater® DNA isolation Kit (Mobio Laboratories Inc., USA). Total coliforms, *E. coli*, *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholera*, as well as gene copy numbers of genes encoding the toxins microcystin and saxitoxin were determined by quantitative PCR (qPCR) using an Agilent AriaMx Real-Time PCR system. Standard PCR methods were used for the detection of general *Vibrio* sp. and genes encoding toxic *Vibrio* spp. (*ctxA*), geosmin synthase and 2-MIB.

1.7.2. Off-flavour in cultured pangasius and tilapia with respect to regional differences and environmental influences

Research approach and location: A field study was conducted in four fishponds in two geographically different regions (Bogra and Khulna) in Bangladesh. At each region, pangasius and tilapia fish ponds were selected. The purpose of the study was to test if flesh from pangasius and tilapia contains off-flavour compounds. In addition, commercial feeds, feed ingredients and plankton in the water (supplementary fish feed source) were examined for the content of off-flavours.

Khulna differs from Bogra as it is situated at the coastal belt of Bangladesh and water source in most fish farms is the adjacent Shoilmari river. Water in the river is typically tidal water from the nearby estuary. Exchange of pond water with river water is supposed to help to reduce waste from the ponds and lower the growth of planktonic organisms that potentially may add off-flavours to the fish. Pangasius and tilapia are the major culture species in the ponds. In Bogra, the ponds receive rainwater and are fed by underground water when needed.

Fish were sampled in July one month before harvest and at harvest in August in 2017. Previously, fish had been sampled in December to compare differences with respect to seasonal variation. Each sampling was done by randomly sampling five fish. The fish samples were subsequently prepared for sensory evaluation and odorous compounds detection.

Methods: Water quality and phytoplankton community were investigated in December 2016 (as winter sample) and each month from March to August in 2017 to cover a full culture cycles. Water quality parameters such as temperature, pH, dissolved oxygen, transparency, salinity, nitrate, phosphate, and ammonia were measured.

For extraction of volatile, off-flavour compounds form the fish, a dynamic headspace sampling method was employed according to Petersen et al. (2011). An optimized method based on Höckelmann et al. (2009) was used to extract volatiles from filters with collected phytoplankton biomass. Volatile compounds in the water were extracted on Tenax TA traps by transferring water samples to gas washing flasks, followed by purging with N_2. Commercial feeds, major feed ingredients and phytoplankton from the ponds (collected on filters) were also tested for the content of volatiles.

The trapped volatiles were desorbed in an automatic PerkinElmer thermal desorption unit (PerkinElmer, Norwalk, USA) before transfer to an Agilent 7890A GC-MS with an Agilent 5975C with a Triple-Axis detector (Agilent Technologies, Palo Alto, California). Separation of the volatiles was carried out on a DB-Wax capillary column using H_2 as the carrier gas. The mass spectrometer was subjected to electron ionization mode at 70 eV and mass-to-charge (m/z) ratio between 15 and 300 were scanned. The GC-MS data were processed using MSD Chemstation software (Agilent Technologies). Volatile compounds were identified by the probability-based matching of their mass spectra with those from the commercial database (Wiley275.L). Retention indices (RI) were calculated for all detected compounds by running an alkane standard mixture. The identity of 10 compounds in fish, 7 in phytoplankton, and 4 in fish feed were confirmed by running authentic standards, and the remaining compounds were tentatively identified using MS library suggestions from Wiley and confirmed with the RI values from the literature.

1.7.3. Effects of feed and water exchange on the off-flavour in cultured pangasius

Research approach and location: An additional study on the possible content of off-flavours in farmed fish was conducted in seven uniformly-sized soil fish ponds to examine the effects of feed and water exchange on off-flavour of Pangasius for 9 months between May 2018 and January 2019. The study was conducted at the Faculty of Fisheries at Bangladesh Agricultural University (BAU) at Mymensingh, Bangladesh. The ponds were arranged as follows. Pond 1 was a control pond with commercial feed (FT-1) and no water exchange. Pond 2, 3 and 4 received own formulated feed type 2 (FT-2) and had no, fortnightly or weekly water exchange, respectively. Pond 5, 6 and 7 received own formulated feed type 3 (FT-3) and had no, fortnightly or weekly water exchange, respectively.

The same ponds and fish were also used to examine the abundance of the yellow colour of the fish flesh and reasons for the colour (section 1.7.4 below).

Methods: Water quality parameters were investigated for consecutive six months from May to October in 2018, and the latest in January 2019. Water quality parameters such as temperature, pH, dissolved oxygen, transparency, nitrate, phosphate, and ammonia were measured. Fish were sampled at three-time points (September, October, and January) using nets. Off-flavours were identified and quantified as described above using GC-MS technology at the University of Copenhagen.

1.7.4. Yellow tainting of flesh in pangasius: Source of the colour and procedures for removal

Field experiment setup: The controlled field study (including seven ponds with pangasius) described in section 1.7.4 above, was also used to study the prevalence of yellow colour in the fish flesh. Based on the hypothesis that certain ingredients in standard pangasius feed cause the yellow colouration of fish flesh, two experimental feeds were formulated and fed the fish, while a commercial pangasius feed type (Pangasius floating feed; http://www.megafeedbd.com) served as control. The two experimental feed types were formulated without maize and mustard oil cake, and each feed had a content of crude protein of 30%. Feed type 2 (FT-2) contained fish meal (25%), soybean meal (25%), rice bran (24%), wheat flour (24%), salt (1%), and vitamin-mineral premix (1%). Feed type 3 (FT-3) was made from meat and bone meal as the main animal protein source (30%), while soybean meal (30%) was the main plant-based protein source. The remaining ingredients were rice bran (19%), wheat flour (19%), salt (1%), and vitamin-mineral premix (1%). These ingredients were finely ground, mixed-together with clean water and made into pressed pellets by a fish feed pelleting machine. The fish were fed the three feed types as 3.0-4.0 mm pellets at a rate of 4-5% of their average body weight twice a day and adjusted bi-weekly with the body weight.

Possible effects of water exchange on the colour of the flesh were tested as mentioned above in section 1.7.3. For the commercial feed FT-1, no water exchange was applied to resemble real farming practices in which fish farmers usually do not exchange the water.

Methods: Water quality parameters were investigated for consecutive six months from May to October in 2018, and the latest in January 2019. Colour of fish fillets was measured using the Commission Internationale de I'Eclairage (CIE) colour system by measurement of *L*, a*, b**- values which are widely used to determine the colour intensity of fish (Wathne et al., 1998 and Gouveia et al., 2003).

The procedure for extraction of carotenoids was modified from the technique given by Liu et al. (2012). Extracted pigments were analyzed by HPLC according to Schlüter et al. (2016). The HPLC-system was calibrated using pigment standards from DHI Lab Products.

1.7.5 Detection of metals in fish farms and prevalence of antibiotic resistance genes

Pangasius and Tilapia are cultured more or less all over Bangladesh, but the intensity of culture of these two species is very high in some particular regions of Bangladesh. The major regions are Mymensingh, Comilla, Bogra and Jessore. Besides these areas, pangasius and tilapia are also being cultured in some other regions of Bangladesh on a medium scale. In this study, samples were collected from all of these regions. A prescribed questionnaire was used to collect information on general pond characteristics and culture practices from the selected farmers. Water was collected in 100 ml bottles and kept in icebox with coolants to maintain a water temperature around 10-12˚C during transportation and to prevent direct exposure to light. After arrival in the laboratory, samples were stored in deep freezer at -20˚C.

Methods Analysis of general water quality parameters were conducted during the experimental period. General water quality parameters like temperature, pH, dissolved oxygen of the selected ponds were recorded by using portable measuring devices during the time of sampling on the spot. Chemical parameters (ammonia, nitrate, and phosphate) were measured by using spectrophotometry analysis according to Alica et al. (2012) in the Water Quality Laboratory of Dept. of Aquaculture, Patuakhali Science and Technology University.

Bioavailable arsenic was detected using bioluminescent biosensor bacteria (*E. coli* K12 pJAMA arsR). The natural resistance mechanism of bacteria against arsenic (As) was used for the determination of the bioavailable fraction of As in the collected samples by employing a whole-cell bacterial biosensor strain that responds to bioavailable As by increased *luxAB* gene expression resulting in increased bioluminescense. The biosensor strain used in this protocol (*E.coli* pJAMA-arsR) was constructed and described by Stocker et al. (2003) and biosensor analysis was carried out as described previously for soil extracts (Frick et al., 2019).

Bioavailable tetracyclines were detected using biosensor bacteria (*E. coli* K12 pTetLux1). The whole-cell bacterial biosensor strain responds to bioavailable tetracyclines (e.g. tetracycline, oxytetracycline and chlorotetracycline) by increased *luxAB* gene expression resulting in increased bioluminescense (Korpela M.T. et al.,1998). Biosensor analysis was carried out as described previously for soil extracts (Song et al., 2017).

High-quality DNA was extracted from water samples using the MoBio PowerWater® DNA Isolation Kit according to the instruction manual. The concentration and quality of DNA were checked by fluorometer (Qubit 2.0, Invitrogen Life Technologies) and agarose gel electrophoresis. The extracted DNA was stored at −80 °C until freeze-drying and molecular analyses.

For high throughput qPCR analysis of antibiotic resistance genes, a total of 296 primer sets were used to quantify antibiotic resistance determinants in water samples collected from different aquaculture ponds. A total of 285 primer sets targeted antibiotic resistance genes (ARGs) and 10 primer sets targeted mobile genetic elements (MGEs) known to harbor ARGs. The 16S rRNA gene was quantified as a reference gene allowing for normalization of average gene abundance per cell (Zhao et al., 2018; Zhu et al., 2017). The HT-qPCR was performed with an HT-qPCR based ARG chip using the WaferGen SmartChip Real-time PCR system. Negative controls were included. All HT-qPCR were performed in technical triplicates.

Network analysis and visualization were applied using the CoNet Cytoscape plug-in method to explore the co-occurrence patterns among the detected ARGs, MGEs in all the samples as previously described (Soffer et al. 2015; Hu et al. 2017). The pairwise interaction scores were calculated with Pearson and Spearman correlation methods and Kullback-Leibler and Bray-Curtis dissimilarity methods. The significant pairwise correlations (with a correlation coefficient above 0.6 and a significance level below 0.05) were utilized to construct the co-occurrence network and visualized using the yFiles Layout.

Analysis of metals in water samples (filtered through a 0.45 μm membrane filters) were analyzed directly by ICP-MS according to Öztürk et al. (2009). Sediment sample preparation procedure, digestion and analysis were performed according to ICP-MS method described by Gilbert and Oldewage (2014).

2. SCIENTIFIC RESULTS

2.1 STUDIES ON GROWTH, TASTE AND COLOUR OF FISH

2.1.1. Effects of water treatment on fish growth

S. Mahmud, Md. L. Ali, Md. A. Alam, Md. M. Rahman, N.O.G. Jørgensen (2016). Effect of probiotic and sand filtration treatments on water quality and growth of tilapia (*Oreochromis niloticus*) and pangasius (*Pangasianodon hypophthalmus*) in earthen ponds of southern Bangladesh. Journal of Applied Aquaculture, 28:199-212

Abstract Effects of water treatment by two probiotic products (PondPlus® and AquaPhoto®) and sand filtration were studied on the growth performance of tilapia (*Oreochromis niloticus*) and pangasius (*Pangasianodon hypophthalmus*) stocked at tilapia: pangasius ratio of 5:3 in traditional earthen ponds in Bangladesh. The fish were stocked at a density of 20,000 fish ha^{-1} and reared for 7 months. Compared to untreated ponds, treatments of probiotic products or sand filtration in earthen ponds resulted in higher O_2 content, higher water transparency, less ammonium, and fewer cyanobacteria. Weight gain for individual tilapia was lowest in the AquaPhoto-treated ponds (177 g), while similar gains (188–194 g) occurred in the other ponds. For pangasius, the lowest weight gain (627 g) was obtained in the sand filter-treated ponds, as compared to 690–797 g in the other ponds. Thus, a general positive effect from the treatments on fish growth was not observed, possibly due to a reduced content of detritus (supplementary feed for both fish species) in the treated ponds.

2.1.2. Flavour compounds in pangasius and tilapia from experimental ponds

R. Podduturi, M. A. Petersen, S. Mahmud, Md Rahman, N. O. G Jørgensen (2017). Potential contribution of fish feed and phytoplankton to the content of volatile terpenes in cultured pangasius (*Pangasianodon hypophthalmus*) and tilapia (*Oreochromis niloticus*). Journal of Agricultural and Food Chemistry 65: 3730-3736.

Abstract Geosmin and 2-methylisoborneol are the most recognized off-flavours in freshwater fish, but terpenes may also contribute off-flavour in fish. We identified six monoterpenes, 11 sesquiterpenes, and three terpene-related compounds in pangasius and tilapia from aquaculture farms in Bangladesh. The concentrations of most of the volatiles were below published odor thresholds, except for α-pinene, limonene, β-caryophyllene, α-humulene, and β-ionone in tilapia, and limonene and β-ionone in pangasius. To identify sources of the terpenes, terpene profiles of fish feed and phytoplankton in the ponds were analyzed. In feed and mustard cake (feed ingredient), five monoterpenes and two sesquiterpenes were identified, and five of these compounds were also detected in the fish. In phytoplankton, 11 monoterpenes were found and three also occurred in the fish. The higher number of terpenes common to both fish and feed, than to fish and phytoplankton, suggests that feed was a more abundant source of odor-active terpenes in the fish than phytoplankton.

2.1.3. Environmental effects on off-flavour compounds in pangasius and tilapia

S. R. Islam, Md. M. Haque, M. E. Ahsan, R. Podduturi, M. A. Razzak, Niels O. G. Jørgensen (2019). Off-flavour in cultured Pangasius (*Pangasianodon hypophthalmus*) and tilapia (*Oreochromis* *niloticus*) in terms of regional differences and environmental influences. The final manuscript will be ready mid-December.

Abstract Off-flavours represent one of the most economically significant problems encountered in pangasius and tilapia aquaculture. Here, we carried out a seasonal study covering a full growth cycle of pangasius and tilapia in two commercially important, but environmentally different areas of Bangladesh, Bogra, and Khulna. The analyses included estimation of water quality and composition of the phytoplankton community in culture farms and quantification of odoriferous volatile compounds in fish. Samples were analyzed for physico-chemical parameters of water, quantitative and qualitative analysis of plankton and off-flavour analysis of fish and water. Concentrations of dissolved off-flavour compounds (2-methylisoborneol and geosmin) were assessed by gas chromatography mass spectroscopy of water column samples. Cell count data showed differences in dominant species, bio-volume, and diagnostic pigment signatures among ponds. Pigment and microscopic analyses of algal cells were well-correlated. Preliminary analyses show that off-flavour producers mainly were found in summer and in ponds that were densely colonized by cyano-bacteria. This suggests that corrective measures need to be taken to maintain acceptable water chemistry in the ponds to control plankton communities and suppress of off-flavour producers.

2.1.4. Effects of feed and water exchange on the off-flavour in pangasius

S. R. Islam, M. M. Haque, M. E. Ahsan, R. Podduturi, M. A. Razzak, Niels O. G. Jørgensen (2019). Effects of feed and water exchange on the off-flavour in cultured pangasius (*Pangasianodon hypophthalmus*). Manuscript in preparation.

Abstract Over the last 20 years, the production of pangasius (*Pangasianodon hypophthalmus*) in aquaculture farms in Bangladesh has significantly expanded and now contributes about 16% of the total aquaculture production of 2.3 million MT. However, pangasius cultured in Bangladesh has received less international attention than pangasius from Vietnamese fish farms. Among reasons for the limited export of Bangladeshi pangasius, tainting by off-flavours is an important quality issue. The objectives of this study were to characterize off-flavour production in the water of the farms and the extent of off-flavours in the fish flesh. A range of studies in-field ponds and experimental ponds were conducted over the last two years to assess the water quality of pangasius farmers' ponds, and to identify the reasons behind off-flavour in pangasius flesh by application of different water exchange frequencies and testing of various feed types in experimental ponds. The experimental work included analysis of major parameters controlling the water quality, analysis of off-flavours in pangasius flesh by GC-MS and feeds. Off-flavours profiles of the fish are still being studied, but preliminary studies suggest that certain phytoplankton organisms, especially cyanobacteria, maybe mainly responsible for the tainting, but more analyses are needed before a general conclusion can be given. Conclusions on the extent of off-flavouring of the fish await analysis at the University of Copenhagen.

2.1.5. Colour of fish flesh

S. R. Islam, Md. M. Haque, L. Schlüter, M.A. Razzak, M. E. Ahsan, R. Podduturi, Niels O. G. Jørgensen (2019). Yellow tainting of flesh in pangasius (*Pangasianodon hypophthalmus*): origin of the colour and procedures for removal. Submitted to Journal of Agriculture and Food Chemistry.

Abstract Pangasius (*Pangasianodon hypophthalmus*) is a commonly farmed fish in ponds in Bangladesh but a yellow flesh colour creates a barrier for export. Here, we investigated if feed ingredients and environmental parameters might impact yellow tainting of the flesh. In new feeds, maize (typical ingredient in commercial feeds) was replaced by fish meal or meat and bone meal, and

regular exchange of water was introduced. Chemical analyses showed that commercial feed was high in carotenoids (zeaxanthin and lutein; 420 ng/g) as compared to ≤6 ng/g in the experimental feeds. After feeding fish different diets for 9 months, total carotenoids in the flesh was reduced by 36-48% and visual yellowness by 47% (one experimental feed only). Weekly or biweekly water exchange reduced the yellow colouration to 22% relative to fish given commercial feed and without water exchange. Our study demonstrates that pangasius with white flesh can be produced in Bangladesh by combining feeds low in pigments with frequent water exchange.

2.2 STUDIES ON MICROBIAL WATER QUALITY

2.2.1. Microbial water quality in tilapia and pangasius production

S. R. Islam, Md. M. Haque, M. E. Ahsan, R. Podduturi, M. A. Razzak, Niels O. G. Jørgensen (2019). Microbial water quality in pangasius and tilapia aquaculture systems in Bangladesh. Manuscript in preparation

Abstract In this study, physicochemical parameters, water quality indicator organisms (total coliforms and *E. coli*), toxic microorganisms (microcystin, saxitoxin, toxic *Vibrio* sp.), and off-flavour producers (geosmin and 2-MIB) were evaluated in pangasius and tilapia aquaculture systems in Bangladesh. To maintain good water quality and to produce quality and healthy fish, the systems depend on a diverse microbial community involved in different processes. PCR techniques were applied to identify and quantify the presence of potentially pathogenic microorganisms. The analyses showed that species of *Vibrio* bacteria were present in 80% of the pond samples and included *V. parahaemolyticus, V. vulnificus and V. cholerae.* The human pathogen type of *V. cholerae* (carrying the *ctxA* gene) was not found. The severe fish and prawn pathogen species *V. parahaemolyticus* was not detected in the pond samples, while *V. vulnificus* and *V. cholerae* occurred in ponds in Khulna, Mymensingh and Bogra region. Coliform bacteria were found in all fish ponds and varied from few (60 bacteria per ml) to more than 160,000 bacteria per ml, while *E. coli* ranged from none to 25,000 bacteria per ml. The highest numbers were found in ponds in the Mymensingh and Bogra region, while the lowest numbers occurred in the Jessore region. No differences between ponds with pangasius and tilapia farms were obvious, but high abundances of coliform bacteria co-varied with high numbers of *E. coli* bacteria. The results showed that microbes producing the off-flavour geosmin were common in pond water in all regions, yet our studies of fish only revealed tainting of fish with geosmin at few sites. Genes encoding the toxin microcystin was dominant in pangasius aquaculture system, while saxitoxin-encoding genes were not detected in any of the farms. Based on our observations, we recommend the establishment of regular monitoring systems to control the occurrence of faecal coliforms and microcystin in the aquaculture in Bangladesh, since the extensive and semi-intensive aquaculture systems are very vulnerable to expose of pathogens and toxins.

2.3 STUDIES ON CHEMICAL WATER QUALITY

2.3.1. Abundance of different metals in fish farms

Water in a total of 212 pond located at Barura, Bakra, Chandina, Jhikorgacha, Kachua, Kotalipara, Sachar, Trishal and Tala were sampled during the period of January 2017 to May 2018. Most of the ponds are used for commercial fish culture and had an average age of approximately 10 years and were about 1.70 m deep. Surface water is the main source for cultivation but farmers also use shallow tube well's water during the drought.

Bioavailable As concentrations were detected from pangasius and tilapia aquaculture system from these selected areas of Bangladesh using biosensor bacteria (*E. coli* pJAMA ArsR).

Results Highest level of AsV and AsIII were found at Kotalipara in Gopalgonj and were 188 µg/l and 14 µg/l, while the lowest concentrations of 14 µg/l and 3 µg/l were found at Rajoir under Madaripur district, respectively. Within the total samples, only 6.13% (14 samples) were found to detectable by biosensor and above the safety limit of 50 µg/l for As-affected waters. The calculated average value of temperature, dissolved oxygen, pH, ammonia, nitrate and phosphate of all water samples were 30.7±0.77°C, 6.24±1.30 mg/l, 7.5±1.37, 0.29 mg/l, 2.45 mg/l, and 0.22 mg/l, respectively.

2.3.2. Assessment of heavy metal from pangasius and tilapia aquaculture in Bangladesh and Human consumption risk

Heavy metal levels in pangasius, tilapia, water, and sediments was studied in four commercial aquaculture regions of Bangladesh and related human consumptionrisk was estimated. Inductively coupled plasma mass spectrometry (ICPMS) was used to assess the heavy metal concentration from water, sediment and different tissues of fish. Bio-concentration factor (BCF) and biota-sediment accumulation factor (BSAF) were applied to assess the metal accumulation capacity of biotas, while metal pollution index (MPI) was calculated to compare the pollution level of total five metals (As, Pb, Cd, Cr and Cu). Cr and Cu were detected from pangasius from all sampling regions. Metal level in tissues revealed a tissue-specific bioaccumulation pattern. Except for Cu, the highest metal load was found in kidney, with significant differences than the other tissues of tilapia. Muscle accumulated the low concentrations of As (0.07 ± 0.05), Pb (0.57 ± 0.2), Cd (0.14 ± 0.02), Cr (1.50 ± 0.82), and Cu (1.48 ± 0.68) µg/g dry wt of tilapia and Cr (0.12 ± 0.05), and Cu (0.24 ± 0.08) µg/g dry wt of pangasius. Results permitted putting the mean MPI of tissues in order from the highest to lowest values, kidney (2.52) > liver (1.30) > muscle (0.14) and kidney (3.75) > liver (1.03) > muscle (0.35) for pangasius and tilapia, respectively. In addition to Cd, the estimate of BCF was greater that of BSAF for other metals, demonstrating that the bioaccumulation of Cd for tilapia was from the sediments, as well as the bioaccumulation of other metal was from water. Correlation analysis showed that Fulton's condition factor (K) of both pangasius and tilapia had the negative relationship with MPI for liver and kidney. Decreasing K of pangasius and tilapia were with the increasing estimates of MPI. Pangasius and tilapia cultured in these four regions of Bangladesh were found to be low-risk for consumption and do not surface any possible threat to the health of consumers. Results of this study can assist the government in determining the strategies of food safety and its implementation in Bangladesh.

2.4 STUDIES ON ANTIBIOTIC RESISTANCE IN FISH FARMS

2.4.1. Bioavailable tetracycline concentration in fish farms

In this study, bioavailable tetracycline (TC) concentrations were detected from pangasius and tilapia aquaculture systems in Bangladesh using biosensor bacteria (*E. coli* K12 pTetLux1) from the pond surface waters of selected pangasius and tilapia farms. In 62 of 325 samples, tetracycline was detected at relatively high concentrations from 2.6 to 24.9 µg/l. The relationships between different variables were studied. The fluctuation of tetracycline concentrations at different ranges of water temperature and age of the pond did not show any correlations.

2.4.2. Prevalence of antibiotic resistance genes in pangas and tilapia aquaculture production systems in Bangladesh

Antibiotic resistance genes (ARGs) were studied in 33 pangasius and tilapia farms in different regions of Bangladesh using high-throughput qPCR ARG chip. A total of 160 unique ARGs and 10 MGEs were detected across the pond water samples with an average relative abundance of 0.06 ARG gene copies per 16S rRNA gene. The multidrug resistance genes were the most frequently encountered ARGs, accounting for 27% of the total number of ARGs in the pond water. Other frequently detected ARGs included resistance genes to β-lactam (17%), aminoglycoside (15%), tetracycline (12%) and macrolidelincosamide-streptogramin B (MLSB) (10%). The relative abundance of mobile genetic elements (MGE) ranged from 0.009 to 0.07 copies per 16S rRNA gene with an average at 0.02. A significant correlation between the abundance of ARGs and MGEs was observed. Positive correlations were also found between the absolute abundance of ARGs and the MGEs Class 1 integrons (Pearson's r=0.97, P<0.01) and transposons (Pearson's r = 0.66, P <0.01), suggesting the importance of horizontal gene transfer for ARG dissemination. Network analysis revealed significant co-occurrence patterns between specific MGEs (Integrase and transposase) and associated ARGs. The abundance of ARGs also significantly correlated with several environmental variables, including the age of the pond, nitrate content, and ammonia content. The ARG compositions showed a biogeographical pattern, which could be related to feed-type used (commercial and farm made). Our results indicate that ARGs are well-spread in pangasius and tilapia aquaculture systems in Bangladesh and indicate a risk for environmental ARG dissemination via horizontal gene transfer.

3. DISCUSSION

3.1. Microbial water quality

Analysis of the pond water showed presence of coliform bacteria in all fish ponds (occasionally more than 100,000 bacteria per ml). Similarly, *E. coli* was also abundant in largely all ponds with densities in some cases exceeding 25,000 cells per ml. There was a tendency to co-occurrence of coliform bacteria and *E. coli* in some ponds, irrespective of the fish species being cultivated. It is uncertain whether the bacteria originated from external sources or the fish.

Most coliform bacteria and *E. coli* in natural waters are assumed to originate from faeces from humans and warm-blooded animals, e.g. cattle and birds. In most ponds in Bangladesh, densities of faecal coliforms range from about 100 to more than 5,000 cells per ml (Hasan et al. 2019). In pond water used for fish production, there is 60% risk that coliform bacteria will be present (Hasan et al. 2019). Water in wells (often used in fish production) is another source of faecal pollution due to the discharge of bacteria from nearby latrines, septic tanks and seepage from contaminated surface areas (Rahman et al. 2019).

According to WHO, water is suitable for aquaculture production when the number of coliform bacteria is <10 bacteria per ml (WHO, 1989). This means that largely all aquaculture ponds in Bangladesh have an unacceptable – or alarmingly – high density of faecal bacteria. This is especially true for farms in the Mymensingh and Bogra regions, while farms in the western Jessore region appear to have a better, but still unacceptable water quality according to WHO recommendations.

The difference of coliform bacteria between the northern (Mymensingh and Bogra) and southern region (Jessore) of Bangaldesh is possibly due to relatively higher salinity in the ponds of Jessore district. According to Rozen and Belkin (2001), salinity introduces osmotic shock in many enteric bacteria. Thus, the increased salinity in the Jessore region most likely introduces decreasing survival of coliform bacteria. This suggests that the use of saltwater in the ponds might help reduce the abundance of obnoxious bacteria. Recently, it was observed that many farmers actually apply salt in the freshwater pangasius and tilapia ponds to improve water quality and reduce the disease occurrence. This suggests that the treatment of pond water with salt can improve the overall environmental health and can ensure safe fish production for consumption.

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

Analysis of bacterial DNA from the pond water showed the presence of several *Vibrio* species in the ponds, including also *V. cholerae*, but not the human-virulent type. *V. vulnificus* and *V. cholerae* were found in ponds in the Khulna, Mymensingh and Bogra regions. *V. vulnificus* is a severe human pathogen that may cause fatal infections, but it is also a fish pathogen. Most *Vibrio* bacteria are marine species and the appearance of *V. vulnificus* in tilapia ponds in Khulna does most likely reflect intrusion of seawater through the Shoilmari River in this region.

Our results indicate that improved treatment and handling of water from private households and public facilities are required to improve and safeguard an acceptable future water quality in fish farms and natural freshwater ponds. Consumers eating fish from freshwater ponds must be careful when preparing the meals with fish, e.g. by heating to temperatures that kill potential human pathogens.

3.2. Off-flavours in fish

In the BangFish project, the potential contribution of fish feed and phytoplankton in the water to flavour compounds in pangasius and tilapia, providing both attractive and non-attractive flavours and taste, were examined (Podduturi et al. 2017). The study was conducted since fish from ponds in Bangladesh sometimes are reported to have an unattractive taste, but most information appears to be anecdotal. Also, scientific evidence for off-flavour compounds in the fish in Bangladesh is missing.

Our results showed that the common off-flavour compound in fish, geosmin, only was detected at low amounts in the fish, while several other aroma compounds (various terpenes, some with fruity or woody flavours) occurred in the fish, but were also found in feeds and feed ingredients. Phytoplankton in the water (ingested by some fish species) had a low contribution to the fish flavour, indicating that feeds probably are the most abundant flavour source in the fish. Chemicals that accidentally are discharged into the pond water may also contribute taste to the fish, but more studies must be done to substantiate this.

In pilot studies at Patuakhali Science and Technology University on procedures to treat water for improvement of the water quality and improve fish taste, Petersen et al. (2014) found that sand filtration and addition of certain probiotic bacteria could improve the water quality. Local consumer panels conducted a sensory tasting of the fish and found that depuration in clean well water for about one day significantly improved the taste and flavour of the fish. In the same study, Mahmud et al (2016) showed that the treatments for improvement of the water quality were not always reflected in higher fish growth, probably because the treatment procedures removed organic matter that serves as supplementary feed to the fish.

The above-mentioned results on fish flavour from the experimental ponds were related to the occurrence of known off-flavour producing microorganisms in several commercial fishponds in the Mymensingh, Bogra, Comilla, Jessore, Khulna regions. Analysis water samples collected in 2018 is still ongoing, but preliminary results show that microorganisms producing the earthy compound geosmin were found in more than 80% of the water samples, whereas organisms producing the musty compound 2-MIB were present in about 20% of the analyzed samples.

3.3. Yellow colour of pangasius flesh

Pigments in the feed are reported to be the main source for colouring of flesh in catfish (Qiufenet al. 2012). This was confirmed in our study of pangasius flesh colour. When a commercial feed containing maize and mustard oil cake was replaced by experimental feeds without maize and with a low content of zeaxanthin and lutein, the total carotenoid content in the feeds was reduced from 420 ng/g to 6 ng/l or less. The reduced concentration of carotenoids in the feed caused a lower pigment content in the flesh, which led to a reduced yellow colour of the flesh.

Maize is often used in commercial fish feeds to reduce costs, but maize is known to introduce the risk of yellow tainting of the flesh (Amaya et al. 2015). Supporting this, the yellow colouring of pangasius in Bangladeshi farms appears also to reflect the common use of maize as protein and carbohydrate source. Maize is mixed with mustard oil cake, fish meal, meat and bone meal as the main ingredients in many Bangladeshi fish feeds (Ali et al., 2013). In contrast to the Bangladeshi farms, maize is not used in pangasius production in Vietnam and here farm-produced pangasius is known for its white flesh colour (Phan et al. 2009. The absence of maize in Vietnamese feed and the white flesh colour strongly suggests that maize is the main source of yellow tainting of pangasius flesh in Bangladesh due to carotenoids (Uddin et al. 2019). In general, it is not recommended to use feeds rich in carotenoids for catfish species, e.g., channel catfish, pangasius and African catfish, because consumers expect these fish to have white flesh (Amaya et al. 2015).

Carotenoid pigments in fish can be removed, if the fish feeds are replaced with feeds without carotenoids, as observed for channel catfish (*Ictalurus punctatus*) (Li et al. 2011). We observed that a frequent water exchange in the ponds also reduced the pigment concentrations. This may have been caused by a higher oxygen content, relative to no water exchange, causing stimulation of the metabolic activity and release of lipid-bound carotenoids.

As an alternative approach to replacement of feeds and water exchange, the yellow colouring of catfish flesh can also be reduced after harvest by chemical treatment, typically by dipping in sulfite solutions, e.g. sodium bisulfite, as shown for channel catfish by Li et al. (2017). However, from a consumer viewpoint, low-carotenoid feeds appear a more attractive approach than post-harvest treatment with chemicals for the production of fish with a low yellow colour.

The analysis of fish colour demonstrates that pangasius with low yellow colour can be produced by culture in Bangladesh if feeds without or with low carotenoid content and regular exchange of the pond water are practiced. Replacement of maize as a common feed component to other ingredients, e.g. soybean meal and varying percentage of fishmeal, or meat and bone meal, may raise the production costs of the fish, but it may open for export to new markets for the benefit of Bangladeshi aquaculture.

3.4 Chemical water quality

Arsenic concentrations are generally higher in groundwater than surface water. But, As concentration of surface water like freshwater ponds is a matter of concern nowadays. Food grown in As contaminated water is also detrimental and attributed the threat of arsenicosis. Arsenical toxicity depends on the amount of intake of arsenic laden groundwater and arsenic concentration in the water. Of the 212 collected water samples, only 6.13% (14 samples) were found to detectable by *E. coli* pJAMA ArsR where concentrations of As found highest at Kotalipra pond water and lowest at Rajoir pond water. The highest concentration was observed 14.19 µg/l among the detectable samples and it was the only sample scored above 10 µg/l while the standard arsenic limit in case of Bangladesh is 50 µg/l (UNICEF, 2008). From the study result, it can be said that the highest value of pond's water arsenic is inconsiderable for Bangladesh standard but an apple of discord in case of international standard since WHO and USEPA recently reduced the safety limit of arsenic permissible in public water supplies from 50 µg/L to 10 µg/L. In conclusion, As did not appear to constitute an important problem for water quality although it should be mentioned that surface water shortage in some regions may occasionally force aquaculture farmers to rely on ground water resources prone to contain higher As concentrations than surface waters.

Use of antibiotics and other chemicals can contribute to the increased productivity and growth of the aquaculture sector, but has also faced criticism due to possible negative consequences for human and environmental health (Ashbolt et al. 2013; Brandt et al. 2015). Antibiotic residues may accumulate in the treated animals, resulting in a potential hazard for consumers and for the marketing and export of aquaculture products (Heuer et al. 2009; Sapkota et al. 2008). Bioavailable tetracyclines were detected in almost 20% (62 out of 317) of all analyzed samples at concentrations of 2.57 to 24.85 µg/l. Considering the rather high detection limit of the used tetracycline biosensor assay, this suggest widespread use of tetracyclines in pangasius and tilapia aquaculture systems in Bangladesh. The extensive use of antibiotics in aquaculture can contribute to the development of antimicrobial-resistant pathogenic bacteria both inside and outside the aquaculture facilities (Le et al. 2005).

3.5 Heavy metal contamination in fish and human consumption risk

Fish can be exposed to metals in many ways, including direct uptake through gill and biological membranes (waterborne) and digestion of food and sediment matters in the digestive tract (dietary), and metals end up in the body of fish. Rajkowska and Protasowucki (2013) indicated that metal distribution among various tissues of fish mainly depends on the metal content in water and feed supplied.

In this study, we assessed heavy metals in water, sediments and different tissues of pangasius and tilapia. The concentration of Cu and Cr in water and sediments were higher than other metal in both pangasius and tilapia farms samples in all sampling regions. The relatively high content of Cr and Cu may be due to use of $CuSO_4$, $K_2Cr_2O_7$ especially that was not properly regulated. Higher content of Cr in the present study might be due to the fish feed, as poultry and/tannery wastes are thought to add in the fish feed where $K_2Cr_2O_7$ play a key role in cleaning (Sarker et al. 2016). Another source of heavy metal is the pellet feed that was not eaten by fish and settled in the sediment (Tao L et al. 2012).

The highest metal load was found in the kidney, except for Cu, showing the significant differences in metal concentration of kidney with the other tissues of tilapia and pangasius. Muscle accumulated the low concentrations of As (0.07 ± 0.05), Pb (0.57±0.22), Cd (0.14 ± 0.02), Cr (1.50±0.82), and Cu (1.48 ±0.68) µg/g dry wt.). Farkas et al. (2003) suggested that low concentration in muscle might be attributed to the growth dilution of fish if growth is faster than metal accumulation. Moreover, it is mostly considered that muscle is not the targeted tissue for metal biotransformation and accumulation (El-Moselhy et al., 2014).

For all tissues of tilapia, the Bioconcentration factor (BCF) of Cr and Cu were higher than 1.0, showing the tendency to accumulate Cr and Cu from water. We also found that the Biota-sediment accumulation factor (BSAF) of Cd in all tissues of tilapia were greater than 1.0, indicating that tilapia was readily subjected to the bioaccumulation of Cd from sediments. Moreover, the BSAFs of Cd in kidney and liver and Cu in the liver were higher than 1. As tilapia has the habit to clean the pond bottom of detritus and decaying algae (Zhou and Wong, 2000), sediments may be a potential media for tilapia exposed to metal in aquaculture.

Results of health risk assessment showed a negligible harmful effect (Hazard quotient value less than 1) on adult population consuming pangasius and tilapia cultured in the regions studied. These studied regions play an important role in pangasius and tilapia aquaculture industry in Bangladesh. In future, to decrease the human health risk caused by consuming cultured fish, monitoring must be executed to control the contents of heavy metal in the fish farms.

3.6 Prevalence of antibiotic resistance genes (ARGs) in fish farms

Antibiotics are applied to increase animal growth and reduce infectious diseases (Holmström *et al.*, 2003; Cabello *et al.*, 2013). Consequently, aquaculture is now considered a hot spot for the dissemination of antibiotic resistance to the environment and into the food chain, causing great concern for public health (Miller and Harbottle, 2017; Shen *et al.*, 2018). In this study, we used high-throughput qPCR to investigate the diversity and abundance of ARGs and mobile genetics elements (MGEs) in pangasius and tilapia aquaculture production systems in different regions of Bangladesh. We observed high diversity of ARGs and MGEs in all investigated ponds, with a total of 160 ARGs and 10 MGEs detected across all ponds. Of all genes, 76 were shared between all sampling regions.

The absolute abundance (copies/L) of ARGs in the ponds ranged from 1.34×10^8 (BAU pond) to $7.75 \text{x} 10^8$ (Cumilla pond), with a mean of $3.55 \times 10^8 \pm 1.43 \times 10^8$ in all analyzed pond samples. The absolute abundance of ARGs was lower in the BAU ponds than the other ponds and significantly lower than the Mymensingh ($p=0.01$), and Khulna ($p=0.04$) ponds. The overall lower abundance of ARGs in the BAU ponds could likely be a result of proper management (proper feed management, regular exchange of water, use of probiotic, avoid unnecessary use of chemical etc) as the ponds are administered by the Faculty of Fisheries at Bangladesh Agricultural University. The other ponds were, on the contrary, maintained by commercial farmers, and previous reports have suggested that aquaculture farmers, in general, lack the proper training on how to administer antibiotics (Cabello, 2006; Ali et al., 2016). The lower abundance of ARGs in the BAU ponds thus indicates that the management practices of BAU could be considered as guidelines for aquaculture management to reduce the overall abundance of ARGs, and thereby reduce the antibiotic resistance burden in aquaculture.

Furthermore, we discovered a high abundance of MGEs, which significantly correlated with the abundance of ARGs, indicating a potential for horizontal gene transfer (HGT) of the ARGs. The ARG compositions of the ponds displayed a biogeographical pattern based on the sampling region. However, the composition also appeared to be affected by feed-type used, as most northern ponds were given farm-made feed, while southern ponds were given commercial feed. Overall, our results indicate that ARGs are well distributed in pangas and tilapia commercial aquaculture systems in Bangladesh and suggest a risk for environmental ARG dissemination via HGT.

4 Conclusions

Research in WP1 can be summarized in the following conclusions:

- Presence of high densities of *E. coli* and coliform bacteria in many fish farms indicate that fecal pollution should be better monitored to avoid spreading of diseases with fresh fish products.
- An increased salt content, either by application of salt or by intrusion of seawater, has been shown to reduce abundance of obnoxious bacteria and improve the water quality in fish farms.
- Off-flavours in fish flesh that sometimes are reported in Bangladeshi fish were occasionally observed and might be caused by feed ingredients or plankton in the water.
- Presence of yellowish colour of pangasius flesh appeared to be related to maize as a feed ingredient but plankton in the water may also have contributed pigments to the flesh.

- Metal concentrations in both pangasius and tilapia are most strongly accumulated in kidney, except for copper that accumulated in the liver. Muscle tissue contains low concentration of heavy metal as it is considered as non-targeted tissue for metal biotransformation and accumulation.
- None of the estimated hazard quotients (HQ) are greater than 1, indicating that there is no adverse health effects for adult males or females by consumption of pangasius and tilapia.
- Various environmental factors (pond age, water temperature, oxygen content, nitrate, ammonia and phosphate) and feed type influenced the abundance and composition of antibiotic resistance genes in ponds, suggesting that proper water quality management might reduce the risk for spreading of these genes.
- The strong correlations between the mobile genetic elements (e.g. “jumping” genes, plasmids and spreading of genes by virus) and antibiotic resistance genes indicate that the antibiotic resistance genes are found on and may spread with these mobile genes.
- The obtained data on antibiotic resistance genes are valuable in health risk assessment of aquatic environment with production of pangasius and tilapia.
- Our study suggests that it is imperious to give due attention to monitor the antibiotic contamination in the fast-growing aquaculture sector of Bangladesh to reduce the potential risks of antibiotics on aquatic organisms as well as human health.

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