ASSESSMENT OF ANTIBIOTIC RESIDUES IN FARMED TILAPIA *(Oreochromis niloticus)* OF SOUTHWEST REGION OF BANGLADESH

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ID: MS-170631

SESSION: 2016-2017

Fisheries and Marine Resource Technology Discipline

Khulna University

Khulna

November, 2018

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A Thesis

By

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Session: 2016-2017

A thesis submitted to Fisheries and Marine Resource Technology Discipline in partial fulfillment of the requirements for the Degree

Of

Master of Science in Fish Genetics and Biotechnology

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November, 2018

DECLARATION

ASSESSMENT OF ANTIBIOTIC RESIDUE IN FARMED TILAPIA *(Oreochromis niloticus)* OF SOUTHWEST REGION OF BANGLADESH

The results presented in this thesis are entirely the candidate's own investigations and no part of the results has been accepted for any degree, not it is being concurrently submitted for any degree.

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DEDICATED TO

MY BELOVED PARENTS

Acknowledgement

The author expresses his gratitude and praises to the Almighty God who enabled the author to pursue higher studies as well as to submit the thesis for the degree of Master of Science in Fish Genetics and Biotechnology.

The author feels fortunate to get **Shimama Sultana**, Assistant Professor, Fisheries and Marine Resource Technology Discipline, Khulna University not only as his supervisor but also as a mentor and would like to express deep respect and gratitude for her scholastic supervision, pretty guidance, affectionate support, and immense help throughout the entire period of planning, conducting, and completing research work and preparation of the thesis.

With equal gratitude, the author would like to express heartfelt respect to **Md. Zillur Rahaman**, Fish Inspection and Quality Control Officer (Quality Manager), Quality Control Laboratory, Department of Fisheries, Boyra, khulna as a co-supervisor for his valuable suggestion, sympathetic cooperation, helpful advice, constructive criticism and affectionate feeling at all stage of this study period, research work and preparation of this thesis.

The author express his profound gratitude to Professor **Dr. Ghausiatur Reza Banu,** Head, Fisheries and Marine Resource Technology Discipline, Khulna University for her valuable suggestions and providence of all necessary facilities.

The author feels proud to express his sincere appreciation to S.M Shahin Hossain and Md. Ashikur Rahman for their immense help throughout the entire period of research work.

The author is also grateful to the owner of different fish farms of Khulna, Satkhira and Bagerhat district.

Finally, the author expresses his deepest respect to his beloved parents, brother and sister for their, inspirations, moral support, kindness and blessings and endless love not only during the study period but also for the entire period of life.

The Author

Abstract

The objectives of this investigation were to seek out the residue level of nitrofurans and chloramphenicol in cultured tilapia muscle in south west region of Bangladesh. Ninety samples were collected randomly from various fish farm of different Upazillas of Khulna, Satkhira and Bagerhat districts during August-December, 2017, where each district was contained 30 samples. Antibiotic residues level present in the animal muscles were detected using ELISA in the Quality Control Laboratory of the Department of Fisheries, Khulna. To collect information about the status of feed type questionnaire interviews were performed during sample collection from the respective farmers. The overall results revealed that nitrofuran metabolites and CAP residue existed in all the samples within the MRL (Maximum Residue Level) with the exception of 3.33%, 6.66% and 3.33% samples of Khulna, Bagerhat and Satkhira district respectively. Antibiotic residue data showed all nitrofuran metabolites like SEM, AOZ, AMOZ, AHD and CAP were present in all samples in which SEM was highest in Shatkhira district and lowest in Khulna district. In case of AHD, highest residue was found in Khulna and lowest was Satkhira district. Additionally, AOZ was highest in Khulna district and lowest in Bagerhat district and AMOZ was highest in Bagerhat district and lowest Satkhira district. CAP was found highest in Satkhira district while that was lowest in Bagerhat district. The results of the also revealed that contamination of nitrofuran metabolites and chloramphenicol were more in tilapia farms where home-made feed was used compared to commercial feed.

Table of Contents

Title	Page No.

List of Table

List of Figure

Chapter One

Introduction

1. Introduction

Aquacultural production in Bangladesh is increasing day by day through intensification (Al Mahmud *et al.*, 2012; Ahmed *et al.*, 2012). The role of fisheries sector to national economy has always been significant and it is considered as the main source of animal protein, employment opportunities, food security, foreign incomes and socio-economic improvement (Siddiq *et al.*, 2013). In Bangladesh, there has been an increase in the production and consumption of freshwater fish and shrimp reared in aquaculture systems mainly the Nile tilapia *(Oreochromis niloticus)* and the shrimp *(Penaeus monodon)*. The yield of the Nile tilapia has been increased especially in the southwest region of Bangladesh.

Successful aquaculture is now depending on chemicals which have been used in various methods for centuries (Faruk *et al.*, 2008). Aquaculture drugs are significant components in health management of aquatic animals, soil and water management, improve aquatic productivity, disease management and growth promotion (Subasinghe *et al.*, 1996). To improve the production and quality of fishery products, veterinary drugs have been widely used for the treatment of parasitic and microbial diseases caused by the stressful conditions and high farming density in fisheries (Uchida *et al.*, 2016). Misuse and improper application of antibiotics without observing the required withdrawal period can result in high residue levels in fisheries and the environment (Barani & Fallah, 2015). These antibiotic residues in fishery products can contribute to the development of antibiotic resistance, which is a major concern for human and animal health worldwide (WHO, 2014).

Antibiotics are chemical substances produced by microorganisms that either destroy or inhibit the growth of other microorganisms. Antibiotics can be either broad spectrum, which means that they are active against a wide range of microorganisms. Narrow spectrum drugs target a specific group of microorganisms and are able to interfere with a metabolic process specific to those organisms. In general antibiotics work by: (i) preventing the synthesis of bacterial cell wall components (ii) damaging the bacterial cytoplasmic membrane; (iii) interfering with protein or nucleic acid synthesis.

In the past, to maximize production various antibiotics and chemicals like nitrofuran, chloramphenicol, malachite green, crystal violet, etc. were used in prawn/shrimp aquaculture.

both prophylactically and therapeutically (Nowsad, 2007; Shamsuzzaman and Biswas, 2012; Hossain *et al.*, 2013).

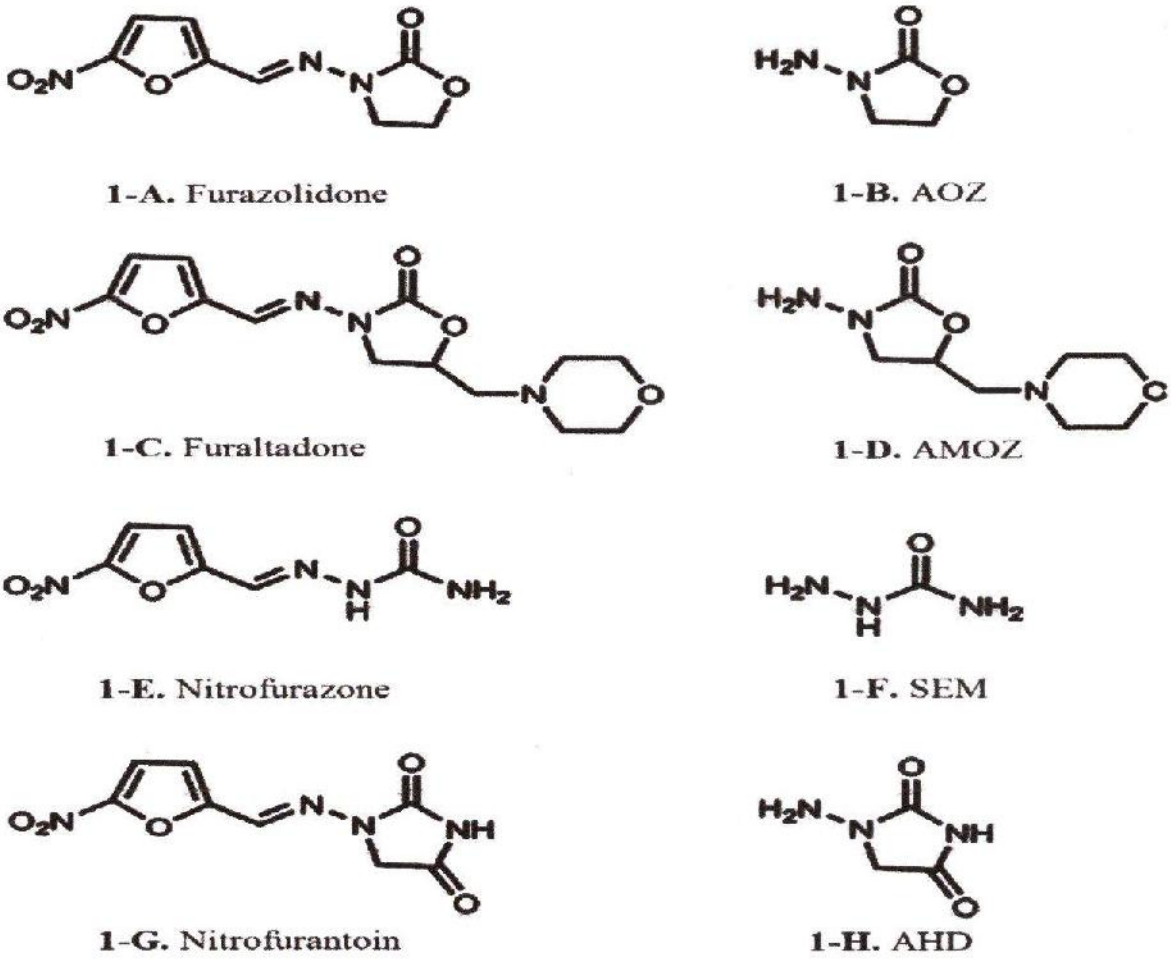


Fig. 1. A-H. Structures of four nitrofuran antibiotics (on the left side) and their respective free metabolites (on the right side).

In aquaculture, nitrofurans are commonly used as a growth promoter and in the prophylactic and therapeutic treatments of bacterial and protozoan infections such as gastrointestinal enteritis caused by *Escherichia coli.* and *Salmonella* sp. (Draisci *et al.*, 1997). In 1995, the use of nitrofurans in livestock production was completely prohibited in the EU (Commission Regulation, 1995) due to concerns about the carcinogenicity of the drug residues and their potential harmful effects on human health (Van Koten-Vermeulen, 1993). After ingestion, nitrofuran parent drugs are metabolized to furazolidone (AOZ), nitrofurazone (SEM),

furaltadone (AMOZ) and nitrofurantoin (AHD) rapidly and form corresponding tissue-bound metabolites (Nouws and Laurensen, 1990). However, their reside was remain in animal body for weeks, possibly months, as protein bound residues (Conneely, *et al.*, 2002)

Chloramphenicol is a broad spectrum antibiotic, often used in veterinary practice, including treatment of aquaculture species. World aquaculture production has grown considerably in the last decade and chloramphenicol is also often used prophylactically in these farming practices. This could result in the occurrence of chloramphenicol residues in commercial fish/shrimps and adverse reactions and side effects in humans have been extensively demonstrated. (Allen *et al.*, 1985).

The WHO and European Union have established a minimum required performance limit of 1 µg/kg for nitrofuran metabolites for edible tissues of animal origin (Commission Decision, 2003). They have a policy of zero tolerance toward the use of chloramphenicol and nitrofurans in food-producing animals. Chloramphenical and nitrofuran residues can be determined by methods with extremely expensive preparatory procedures such as LC-UV, LC-MS, or LC-MS/MS. In this study, enzyme immunoassay (ELISA) was used for assessment of residues which is simple, rapid, sensitive and cost-effective compared with traditional method.

Considering the above facts, the present study was conducted to identify different types of antibiotics used in aquaculture activities in south west region as well as to evaluate whether those items exist in appropriate limit or not.

1.1. Objective

To determine the residue level of nitrofurans and chloramphenicol in cultured tilapia in south west region of Bangladesh.

Chapter Two

Review of Literature

2. Review of Literature

Cháfer-Pericás *et al.* (2011) conducted on multiresidue determination of antibiotics in feed and fish samples for food safety evaluation. Comparison of immunoassay vs LC-MS-MS and found that 20 fish and 4 feed samples were analyzed in order to confirm the feasibility of the immunoassays for detecting antibiotic residues. Sulfonamide residues were not detected in any fish sample. Tetracycline residues were detected in some fish samples from marine farms, with total concentrations between 2.1 and 152 ng/g. In all cases, the obtained results correlated well with those achieved by LC-MS- MS. Therefore, the new methodology allows for food safety of the medicated fish.

Chico *et al.* (2008) experimented on high-throughput multiclass method for antibiotic residue analysis by liquid chromatography–tandem mass spectrometry and found that a simple and rapid method has been developed for the residue analysis of 39 antibiotics (tetracyclines, quinolones, penicillins, sulfonamides and macrolides) in foodstuffs of animal origin. The method combines an effective extraction technique, which uses water–methanol as extracting solvent, with ultra-high-pressure liquid chromatography–tandem mass spectrometry, allowing both confirmation and quantification in a single chromatographic run. The multiresidue method has been validated in chicken muscle matrix according to European Union Decision 2002/657/EC. It has been implemented as a routinemethod in a Public Health Laboratory, instead of the five plates test and LC methods previously used.

Degroodt *et al.* (1992) experimented on chloramphenicol and nitrofuran residue analysis by HPLC and photodiode array detection in meat and fish and described a chromatographic method for the analysis of residues of chloramphenicol and nitrofurans in meat and fish. Chloramphenicol, furazolidone, furaltadone and nitrofurazone were extracted from the tissues with ethyl acetate. The extract was purified with petroleum ether and n-pentane and then analyzed with a liquid chromatograph and a photodiode array detector using a Chromspher C-8 column. Chloramphenicol and nitrofurans were detected at 280 nm and 360 nm respectively. Blank samples fortified with chloramphenicol and nitrofurans at levels of 5, 10 and 20 µg/kg gave recoveries in the range of 69 to 88%. A quantitation limit of 1 µg/kg for nitrofurazone and fwazolidone and of 2 µg/kg for chloramphenicol and furaltadone in meat and fish has been reached.

Fernando *et al.* (2017) experimented on determination of nitrofuran metabolites in shrimp muscle tissue by liquid chromatography-photo diode array detection and described a High Performance Liquid Chromatography Diode Array Detection (HPLC-DAD) method to detect tissue bound nitrofuran metabolites, namely 3-amino-2-oxazolidinone (AOZ), 3- amino-5-morpholino-methyl-1, 3-oxazolidinone (AMOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD). The bound residues were hydrolysed and derivatized into the corresponding nitrophenyl derivatives (NPAOZ, NPAMOZ, NPSEM and NPAHD) with 2-nitrobenzaldehyde and analysed by HPLC-UV at 275 nm. The linearity of the photometric detector response to the four derivatized nitrofuran metabolites was verified using matrix matched calibration standards in the range of 1e20 mg/kg and the average correlation coefficients for NPAOZ, NPAMOZ, NPSEM and NPAHD were 0.9960, 0.9951, 0.9984 and 0.9993, respectively. The decision limit of the method was below the Minimum Required Performance Limit (MRPL) of 1 mg/kg, for all four nitrofuran metabolites, which makes it compatible with the EU requirements. This method has been successfully used with aquaculture and poultry products; however, currently it is validated according to EU guidelines only for shrimp muscle tissue.

Hassan *et al.* (2013) researched on monitoring the presence of chloramphenicol and nitrofuran metabolites in cultured prawn, shrimp and feed in the Southwest coastal region of Bangladesh and found that chloramphenicol and nitrofuran residues present in the animal muscles, feeds and feed ingredients were detected using LC–MS–MS by the Fish Inspection and Quality Control Wing of the Department of Fisheries. The study revealed that farmers did not deliberately use those banned antibiotics, but these chemicals were detected in many *M. Rosenbergii* and *P. monodon* samples in 2008, 2009 and 2010, in both fresh muscles, pre-export and post-export consignments. The percentage of contamination with this banned antibiotics in *M. rosenbergii* samples were 17.74%, 22.89%, 13.60% and in *P. monodon* samples were 12.65%, 15.79%, 11.85% in 2008, 2009 and 2010 respectively.

Holmström *et al.* (2003) researched on Antibiotic use in shrimp farming and implications for environmental impacts and human health and showed that a large proportion of shrimp farmers along the Thai coast used antibiotics in their farms. Of the seventy-six farmers interviewed, 74% used antibiotics in shrimp pond management. Most farmers used them prophylactically, someone a daily basis, and at least thirteen different antibiotics were applied. Many farmers were not well informed about efficient and safe application practices. A more restrictive use of antibiotics

could have positive effects for the individual farmer and, simultaneously, decrease impacts on regional human medicine and adjacent coastal ecosystems. It is likely that dissemination of information could contribute to a decreased use of antibiotics, without decreasing the level of shrimp production.

Le and Munekage (2004) conducted research on residues of selected antibiotics in water and mud from shrimp ponds in mangrove areas in Viet Nam and showed that TMP, SMX, NFXC and OXLA are found in all samples in both shrimp ponds and surrounding canals. The highest concentrations of TMP, SMX, NFXC and OXLA are 1.04, 2.39, 6.06, and 2.50 ppm in water samples; and 734.61, 820.49, 2615.96, 426.31 ppm (based on wet mud weight), respectively. The comparison of antibiotics residues between study sites and types of shrimp ponds will be discussed in this paper. The results also suggest that antibiotics residues may cause harmful effect on ecosystems in the study sites.

Santos and Fernando (2016) experimented on analytical strategies for the detection and quantification of antibiotic residues in aquaculture fishes: a review and stated that growth is associated with the implementation of intensive and semi-intensive production methods, with the use of antibiotics in order to prevent the emergence and spread of infectious diseases in fish. Fluoroquinolones, tetracyclines, and sulfonamides, among others, are widely used for this purpose. This practice constitutes a real public health concern, not only due to the presence of antimicrobial residues in edible tissues, which can cause allergic reactions in hypersensitive individuals, but also due to the emergence of bacterial resistance. Consequently, the European Union's Regulatory Agencies have established maximum residue limits and specific requirements regarding the performance of analytical methods.

Nowsad *et al.* (2013) experimented on studies on nitrofuran contamination in exportable shrimp and prawn products and found that a series of antibiotics sold in local veterinary drug shops of Cox's Bazar and Khulna were used in shrimp hatcheries. While conducting stakeholder based dialogue in Khulna and Cox's Bazar, hatchery operators were directly blamed by shrimp/prawn farmers, depot holders and processors for using nitrofuran drug. Many hatchery technicians mentioned that they used nitrofuran drugs in the hatcheries 5-6 years back, much before than it was declared banned on shrimp products, but at present they were not using it. While asking about the unlabeled drugs using in their hatcheries, they did not respond but mentioned the efficacy of nitrofuran in the survival of shrimp/prawn PL and promotion of their growth. From

the participatory stakeholder based study it was understood that both the prawn/shrimp hatchery and poultry feed/fish feed used as shrimp/prawn feed in grow out ponds might be the possible sources of contamination of nitrofuran drugs in exportable shrimp/prawn products.

Ramos *et al.* (2003) conducted research on determination of chloramphenicol residues in shrimps by liquid chromatography–mass spectrometry and showed that a liquid chromatographic method with mass spectrometric detection and identification (LC–MS) is presented for the determination of chloramphenicol (CAP) in shrimp tissues. Homogenized shrimp samples were extracted with phosphate buffer (pH 7.0). Clean-up was carried out on a C SPE cartridge. Chloramphenicol was determined by LC–MS-ESI in 18 negative modes. The column used was a Symmetry Shield with a mixture of acetonitrile–water (25:75) as mobile phase. 21 Shrimp samples were fortified at CAP levels between 0.2 and 50 ng/g with 5D-CAP as internal standard. At these levels, accuracies lay between 101 and 110% and between-day reproducibility's were lower than 7.1%, expressed as the variation coefficient of the mean. Limit of decision (CCa) was 0.02 ng/g. Limit of quantitation (LOQ) was 0.2 ng/g.

Monteiro *et al.* (2015) researched on multiresidue antimicrobial determination in Nile tilapia *(Oreochromis oiloticus)* cage farming by liquid chromatography tandem mass spectrometry. A method was developed and validated for simultaneous assessment of 12 drugs of different antimicrobial classes (chloramphenicol, florfenicol, oxytetracycline, tetracycline, chlortetracycline, sulfadimethoxine, sulfathiazole, sulfamethazine, enrofloxacin, ciprofloxacin, norfloxacin, and sarafloxacin) on the Nile tilapia's muscle *(Oreochromis niloticus)*. This study presents the development of a rapid method using ultrafiltration by Captiva cartridges and liquid chromatography tandem mass spectrometry triple quadruple (Agilent 6430 - Agilent Technologies) in a negative mode for florfenicol and a positive mode for the others. The sample pretreatment involves extraction with 5 g of muscle fish, 1 mL of 0.1 M Na2EDTA and 24 mL of acetonitrile: water (0.1% formic acid; 70:30), and purification by Captiva cartridges, followed by the determination of all compounds in a single run. Sulfadimethoxine-d6 was used as an internal standard to obtain more reliable results.

Samanidou and Evaggelopoulou (2007) experimented on analytical strategies to determine antibiotic residues in fish and resulted in the emergence of antibiotic-resistant bacteria in aquaculture environments, in the increase in antibiotic resistance in fish pathogens as well as in the transfer of these resistance determinants to human pathogens. Moreover, the use of large

amounts of antibiotics may lead to the presence of residual antibiotics in fish tissue and fish products. Fluoroquinolones, tetracyclines, penicillins, sulphonamides and other antibiotics, exhibiting activity against both Gram-positive and Gram-negative bacteria, are widely used for the treatment and prevention of diseases in fish. An extended and comprehensive review on the recent analytical methodologies concerning antibiotic residues in fish reported in the literature is provided in the present article. Emphasis is given on sample preparation regarding isolation and purification, chromatographic conditions and method validation according to legislation

Suseno *et al.* (2016) performed an experiment of elimination simulation of CAP in tiger shrimp (*Penaeus monodon*) and white shrimp (*Litopenaeus vannamei*). After 5 days of depuration process, the concentration of CAP in *P. monodon* decreased to 94.85% (muscle), 97.98% (cephalothoraxes), and 90.30% (exoskeleton). The elimination half-life of CAP in P monodon was 0.596 day in the muscle, 0.716 day in cephalothorax, and 0.437 day in exoskeleton. On the other hand, concentrations of CAP in *L. vannamei* decreased to 97.74% (muscle), 90.30% (cephalothoraxes) and 97.63% (exoskeleton). The elimination half-life of CAP in *L. vannamei* was 0.6624 day (muscle), 0.859 day (cephalothorax) and 0.796 day (exoskeleton). CAP was retained better by *P. monodon* compared to *L. vannamei.*

Tsai *et al.* (2010) researched on quantitative determination of four nitrofurans and corresponding metabolites in the fish muscle by Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry and found that this developed method carried limits of quantification lower than 10 μg/kg for the nitrofurans and 1.0 μg/kg for the metabolites. It was observed that the decision limit (CCα) ranged from 2.93 to 5.01 μg/kg for the nitrofurans and 0.19 to 0.43 μg/kg for the metabolites. The detection capability (CCβ) was between 3.62 and 6.20 μg/kg for the nitrofurans and between 0.23 and 0.54 μg/kg for the metabolites. The linear calibration curve parameters in the fortified fish muscle were between 1.0-100.0 μg/kg and 0.1-10.0 μg/kg for the nitrofurans and metabolites, respectively.

Vass *et al.* (2008) experimented on nitrofuran antibiotics: a review on the application, prohibition and residual analysis and provided an overview of nitrofuran toxicity, metabolism, and also specific aspects of legislation surrounding their prohibition. Special attention is devoted to semicarbazide - a nitrofuran metabolite and food contaminant. Analytical procedures for nitrofuran analysis in various matrices and validation requirements for screening and confirmation methods with respect to EU regulations are also reviewed.

Xu *et al.* (2005) performed research on residues of enrofloxacin, furazolidone and their metabolites in Nile tilapia *(Oreochromis niloticus)* and stated that enrofloxacin metabolized into ciprofloxacin in both Nile tilapia and *P. chinensis*, the maximal concentration of enrofloxacin in muscle, liver and plasma of Nile tilapia were 3.61 μg/g, 5.96μg/g, 1.25μg/ml respectively, and ciprofloxacin in muscle was 0.22μg/g. The maximal concentration of enrofloxacin and ciprofloxacin in *P. chinensis* were 1.68μg/g and 0.07μg/g respectively. The residues of furazolidone [3-(5-nitrofurfurylidenamino)-2-oxazolidinone] and its main metabolite 3-amina-2-oxazolidinone (AOZ) in Nile tilapia were first determined by HPLC/MS. Results showed that after oral dose of 30 mg/kg for 7 days, the maximum concentration of furazolidone in Nile tilapia was 413μg/kg after 6 h, whereas AOZ residue reached its maximum (31μg/kg) right after stopping treatment.

Pan *et al.* (2014) performed a study on determination of chloramphenicol, thiamphenicol, and florfenicol in fish muscle by matrix solid-phase dispersion extraction (MSPD) and ultra-high pressure liquid chromatography tandem mass spectrometry and developed a method for the determination of chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FF) in fish muscle. Muscle tissue was blended with octadecylsilyl-derivatized silica (C18). A column made from the C18/muscle tissue matrix was washed with n-hexane, after which CAP was eluted with acetonitrile/water (50:50, v/v) and partitioned into ethyl acetate. The final extract was evaporated, and reconstituted into methanol/water (10:90, v/v) for the analysis of UPLC MS/MS. The correlation coefficient (r^2) with each matrix-matched calibration curve is higher than 0.999. CCa and CCb of the fenicols upon the method were ranged from 0.02 to 0.06 mg/kg and 0.11-0.16 mg/kg respectively.

He *et al.* (2016) conducted on residues and health risk assessment of quinolones and sulfonamides in cultured fish from Pearl River Delta, China and stated that quinolones and sulfonamides were widely distributed in the cultured fishes. The concentrations of total quinolones ranged from 2.5 to 185.7μgkg−1 wet weight (w. wt.) while the concentrations of total sulfonamides ranged between >LOD and 140.5μgkg−1 (w. wt.). Higher levels of veterinary antibiotics (VAs) were found in freshwater fishes than marine fishes. The eel and bass contained the highest concentrations of total quinolones (185.7±19.9μgkg −1w. wt.) and total sulfonamides (140.5 ± 12.5μgkg−1 w. wt.) in the muscle tissue, respectively. The estimated daily intake (EDI) results showed that the contribution of investigated fishes to dietary intakes of quinolones and

sulfonamides were far below the acceptable daily intake (ADI). It would not seem to pose a risk to the public health. Due to the potential risk of antibiotics on the aquatic environment and human health, further investigation on the impact of these emerging pollutants is urgently encouraged.

Chapter Three

Materials and Method

3. Materials and Method

3.1. Sample Collection and Period of Sample Collection

Ninety samples were collected randomly from different Upazillas of Khulna, Satkhira and Bagerhat districts where 30 samples were collected from each district. The samples were directly collected from various fish farms during August-December, 2017. The weight of each fish sample was 130-150g. The samples were collected in polyethylene bags and kept in ice. Then the samples were brought to the Quality Control laboratory of Department of Fisheries in Boyra, Khulna and stored at -20°C.

3.2. Questionnaire Interview

To collect information about the status of feed type questionnaire interviews were performed during sample collection from the respective farmers. For that purpose, a structural questionnaire was developed to assemble information. The questionnaire is attached in Appendix.

3.3. Study Area

The study was conducted in different Upazillas of Khulna, Satkhira and Bagerhat district.

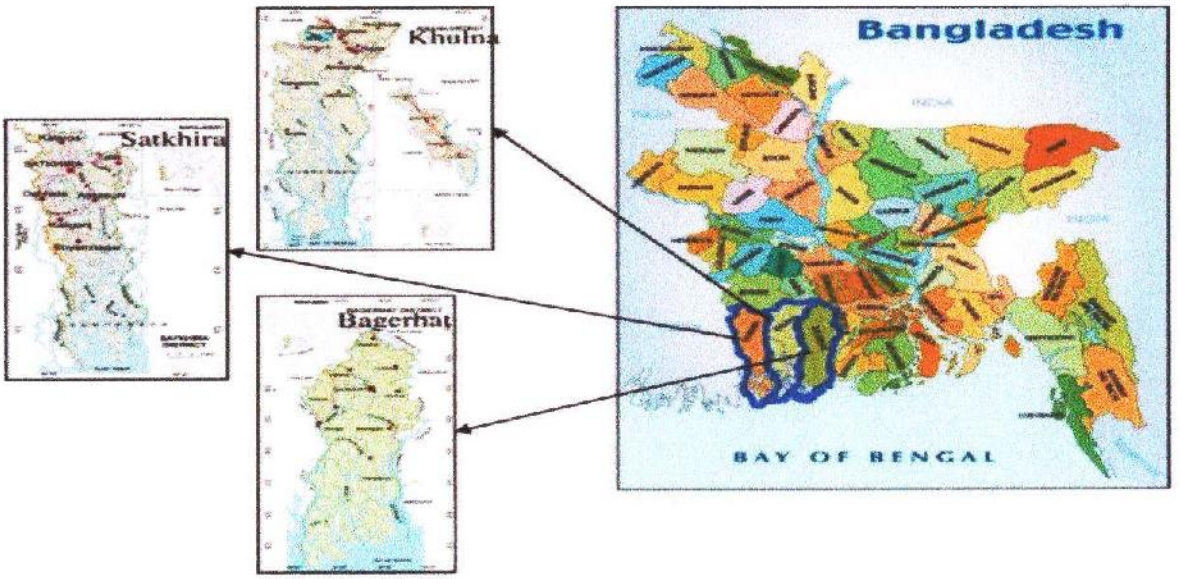


Figure 2: Study area in Khulna, Satkhira and Bagerhat district

3.4. Sample Preparation

Frozen sample was thawed and kept in normal temperature. Then the sample was washed with RO (Reverse Osmosis) water. Head, scale and body appendages were removed from the body. After air drying samples were chopped individually using a knife and then the sample was taken into a blender to make paste. From the paste 3.0g was taken for extraction procedure by using an electric weighing machine. Then the samples were taken to the laboratory for further preparation.

3.5. Laboratory Procedure

Nitrofuran metabolites and Chloramphenicol residues present in the shrimp muscles were detected using ELISA in the Quality Control Laboratory of the Department of Fisheries, Khulna, Bangladesh.

3.5.1. Required Materials for Chloramphenicol (CAP)

Materials were provided with Kit Supplied by Quality Control Laboratory, Khulna.

Diluent/Wash Buffer (Conc.), conjugate (Conc.), tissue extraction buffer (Conc.), one shot substrate, standards, stop solution and 100ng/ml chloramphenicol spiking material.

3.5.1.1. Apparatus and Instrument

CAP Microtitre Plate, micro plate reader (Biotek, Model ELx 800), lint free tissue, water bath/N_2 evaporator, eppendorf tube, 10 ml test tube, 50 ml falcon tube, pipettes: 10 µl to 1000 µl, plate sealer and analytical balance, centrifuge, vortex, blender and personal computer with printer.

3.5.1.2. Extraction Procedure

At first 3g tissue was added with 6 ml ethyl acetate and homogenized in vortex for 1 minute. Then the mixture was centrifuged at 2000 rpm for 15 minute and two layers were appeared. Four ml of upper phase were removed and reduced to dryness at 70°C under N_2 atmosphere. The residue was dissolved in 2 ml of isooctane/chloroform (2:3) and again vortexed for 1 min then 0.5 ml of diluted Tissue Extraction Buffer was added and vortexed for 2 min. The mixture was centrifuged at 2000 rpm for 15 minute and the upper phase was ready for application to microtitre plate.

3.5.1.3. Sample preparation procedure for ELISA

Before starting ELISA procedure all reagents were kept in room temperature (19-25°C) in order to minimize edge effects.

The assay was performed in duplicate following a layout prepared according to sample number.

Twenty five microliter (25 µl) standards from lower to higher concentration were pipetted into the appropriate wells of the microtitre plate (8x12). Then 25 µl sample extract of the 2 tissue blank were pipetted in duplicate. One set of blank duplicates at the beginning and once at the end of the set were plated. Again 25 µl sample extract of the recoveries were pipetted in duplicate, then plated them once at the beginning and once at the end of the set. Then 25µl of extracted sample were pipetted in to separate wells. New pipette tip was used for each standards or sample. 100µl conjugate were pipetted to each plate. Then the microtitre plate was covered with adhesive film. After that the microtitre plate was tappped from side to side for a few seconds before incubating for half hour at room temperature (+19 to + 25°C) in the dark. The plate were inverted and liquid were tapped out. Microtitre plate were washed 6 times with diluted wash buffer over a 10-15 minute period. After final washing liquid were discarded and tapped onto tissue paper until completely dry. Immediately after washing, 125µl of the one shot substrate solution were pipetted into each well. Microtitre plate were tapped from side to side and incubated the microtitre plate for 20 minutes at room temperature (+19 to +25°C) in the dark. Colour reaction were stopped by the addition of 100 µl of Stop Solution per well. A colour change from blue to yellow were evident. The optical density of each well at 450 nm and 630 nm filter as the reference wavelength were measured within 10 minutes of stopping the colour reaction.

3.5.2. Required Materials for Nitrofuran

Materials were provided with Kit supplied by Quality Control Laboratory, Khulna.

Microtitre plate, conjugate (Conc.), diluent/wash buffer (Conc.), one shot substrate, stop solution, standards, 10 mM 4-Nitrobenzaldehyde.

3.5.2.1. Apparatus and Instrument:s

Respective microtitre plate (e.g. AOZ/AMOZ/AHD/SEM), micro plate reader equipped with a 450nm filter with a recommended reference filter of 630 nm, lint free tissue, water bath/incubator, N_2 evaporator, eppendorf tube, 10 ml test tube, 50 ml falcon tube, pipettes: 10 µl to 1000 µl and 1ml to 10 ml, plate sealer, analytical balance, blender, centrifuge, vortex and personal computer with printer.

3.5.2.2. Extraction Procedure

At first each sample was homogenized after removing head, shell and body appendages. One gram of homogenized sample was weighed in a 50 ml falcon tube. Weigh four 1g of previously analyzed blank tissue were used as two positive and two negative controls. Fortify two positive controls with AOZ, AMOZ, AHD and SEM respectively at 0.5 µl/kg. After that, 1g of homogenized sample was added with 4ml distilled H_2O, 0.5ml 1M HCl and 50µl 10mM 4-Nitrobenzaldehyde.Vortex for 1 minute and incubated for 2 hours at 50°C. After vortexed 5ml 0.1M K_2HPO_4, 0.4ml 1M NaOH and 6ml ethyl acetate were added and vortex for 1minute.Then the sample was centrifuged for 10 minutes at 4000 rpm at 25°C and two layers were appeared. After 10 minutes 3ml of the upper ethyl acetate layer of each sample was transferred into test tube and dried at 60°C under N_2 atmosphere. The residue was then dissolved in 1ml hexane and 1ml diluted Wash Buffer and vortex for 2 minutes. Then the sample was centrifuged for 10 minutes at 4000rpm at 25°C.The lower phase was ready for application to microtitre plate.

3.5.2.3. Sample preparation procedure for ELISA

Before starting ELISA procedure all reagents were kept in room temperature (19-25°C) in order to minimize edge effects.

The assay was performed in duplicate following a layout prepared according to sample number.

100 µl standards from lower to higher concentration were pipetted into the appropriate wells of the microtitre plate supplied for AMOZ/AOZ/AHD/SEM. Then pipetted 100 µl sample extract of the two tissue blank in duplicate. One set of duplicates at the beginning and the once at the end of the set were plated. Again 100 µl sample extracts of recoveries were pipetted in duplicate and plated them once at the beginning and once at the end of the set. Then 100 µl of extracted sample were pipetted into separate wells. A new pipette tip for each standards or sample was used. 50 µl

of conjugate were added in each well and mixed well by gently rocking the plate for 1 minute. Then the plate was incubated for 30 minutes at room temperature (20-25°C). After 30 minutes the plate were washed 6 times with the wash buffer. After the last wash the plate was inverted and gently tapped the plate dry on paper towels. After drying 100 µl of one shot substrate were added and mixed the solution by gently rocking the plate for 1 minute. After that the plate was incubated for 20 minutes at room temperature (20-25°C) in dark. Colour reactions were stopped by the addition of 100 µl of stop solution per well. A colour change from blue to yellow was evident. The optical density of each well at 450 nm and 630nm filter as the reference wavelength was measured within 10 minutes of stopping the colour reaction.

3.6. Analysis of data

The collected data were scrutinized, summarized carefully and tabulated. Normality test was done to check the data after input the data in Microsoft Excel 2013. As the data were parametric, One Way Variance was conducted.

Chapter Four

Result

4. Result

4.1. Types of nitrofuran metabolites and chloramphenicol

Chloramphenicol (CAP) and four types of nitofuran metabolites namely furazolidone (AOZ), nitrofurazone (SEM), furaltadone (AMOZ) and nitrofurantoin (AHD) were found to be bind with the protein in the body muscle of tilapia.

4.2. Level of different metabolites in tilapia muscle

All the 90 samples of *O. niloticus* were detected with banned antibiotics below the level of maximum residual limit (MRL) (Table 1).

Table 1: Nitrofuran metabolites and chloramphenicol (ppb) in tilapia muscles.

Types of antibiotic/metabolites	No. of contaminated sample	Range in ppb	MRL
SEM	90	0.003-0.364	0.346
AHD	90	0.007-0.403	0.374
AOZ	90	0.005-0.054	0.355
AMOZ	90	0.002-0.037	0.348
CAP	90	0.007-0.029	0.108

4.3. Area wise level of different metabolites in tilapia muscle

The contamination of sample with banned antibiotics in Khulna, Bagerhat and Satkhira were 100, 100 and 100% respectively. Different types of nitrofuran metabolites were found in different animal samples at different levels. In most cases more than one metabolite was found in one or more samples. The range of the detected levels of metabolites in different areas are given in Table 2.

Table 2: Area wise range of the detected levels of nitrofuran metabolites.

District	No. of sample tested	No. of contaminated sample (Above MRL)	% contamination	Metabolite
Khulna	30	1	3.33	AHD
Bagerhat	30	2	6.67	AHD
Satkhira	30	1	3.33	SEM

In the present study, a total of 90 tilapia samples from three different districts were tested for all the nitrofuran and CAP residues, only 4 samples were found to be contaminated with banned antibiotics above maximum residue level. Among them 3 samples were positive with AHD metabolite and 1 sample was positive with SEM metabolite. SEM, AHD, AOZ, AMOZ and CAP were detected in all the samples and no samples were completely free from antibiotic residue. The study also revealed that more than 95% samples does not exceed the FIQC recommended acceptable limit.

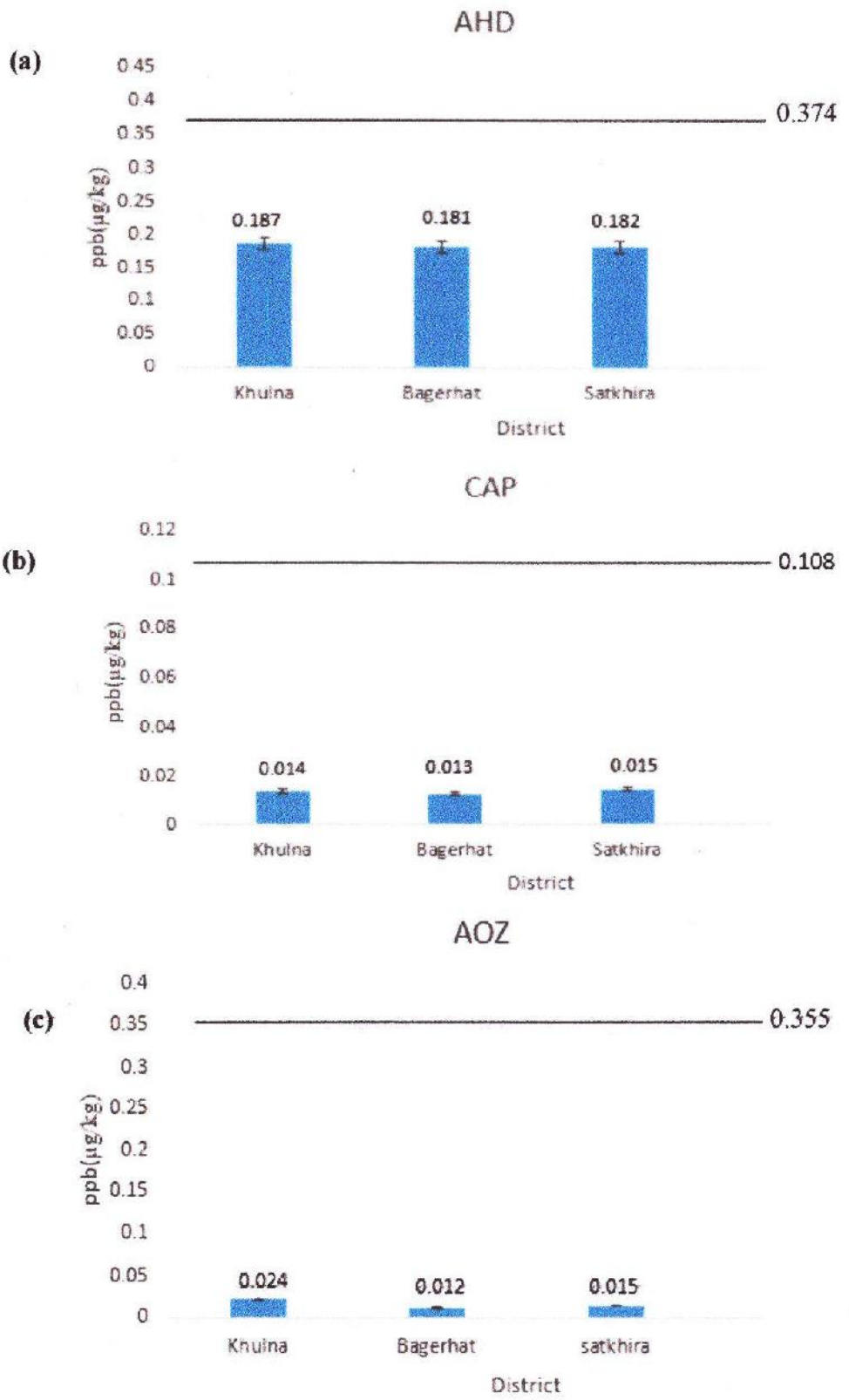
Area wise the mean value of nitrofuran metabolites and chloramphenicol (ppb) in tilapia muscles in different areas are presented in Table 3. It was evident that all nitrofuran metabolites like SEM, AOZ, AMOZ, AHD and CAP existed in all samples in which SEM was highest in Shatkhira district that was lowest in Khulna district. In case of AHD, highest residue were found in Khulna and lowest in Satkhira district. Additionally, AOZ was highest in Khulna district and lowest in Bagerhat district and AMOZ was highest in Bagerhat district and lowest in Satkhira district. CAP was found highest in Satkhira district and that was lowest in Bagerhat district.

Table 3: Area wise nitrofuran metabolites and chloramphenicol (ppb) in tilapia muscles.

District	SEM			AHD			AOZ			AMOZ			CAP		
	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL
Khulna	0.008-0.291	0.097±0.079[a]		0.067-0.403	0.187±0.072[a]		0.005-0.054	0.024±0.012[b]		0.002-0.037	0.011±0.007[b]		0.005-0.029	0.014±0.005[a]	
Bagerhat	0.005-0.339	0.092±0.099[a]	0.346	0.027-0.389	0.181±0.086[a]	0.374	0.005-0.022	0.012±0.005[a]	0.355	0.002-0.037	0.012±0.008[b]	0.348	0.007-0.025	0.013±0.004[a]	0.108
Satkhira	0.003-0.364	0.121±0.085[a]		0.054-0.249	0.182±0.058[a]		0.005-0.036	0.015±0.008[a]		0.002-0.019	0.007±0.004[a]		0.007-0.028	0.015±0.005[a]	

Different superscripts indicate significant difference at 5% level of significance.

4.4. Variation of different antibiotics in Khulna, Bagerhat and Satkhira districts



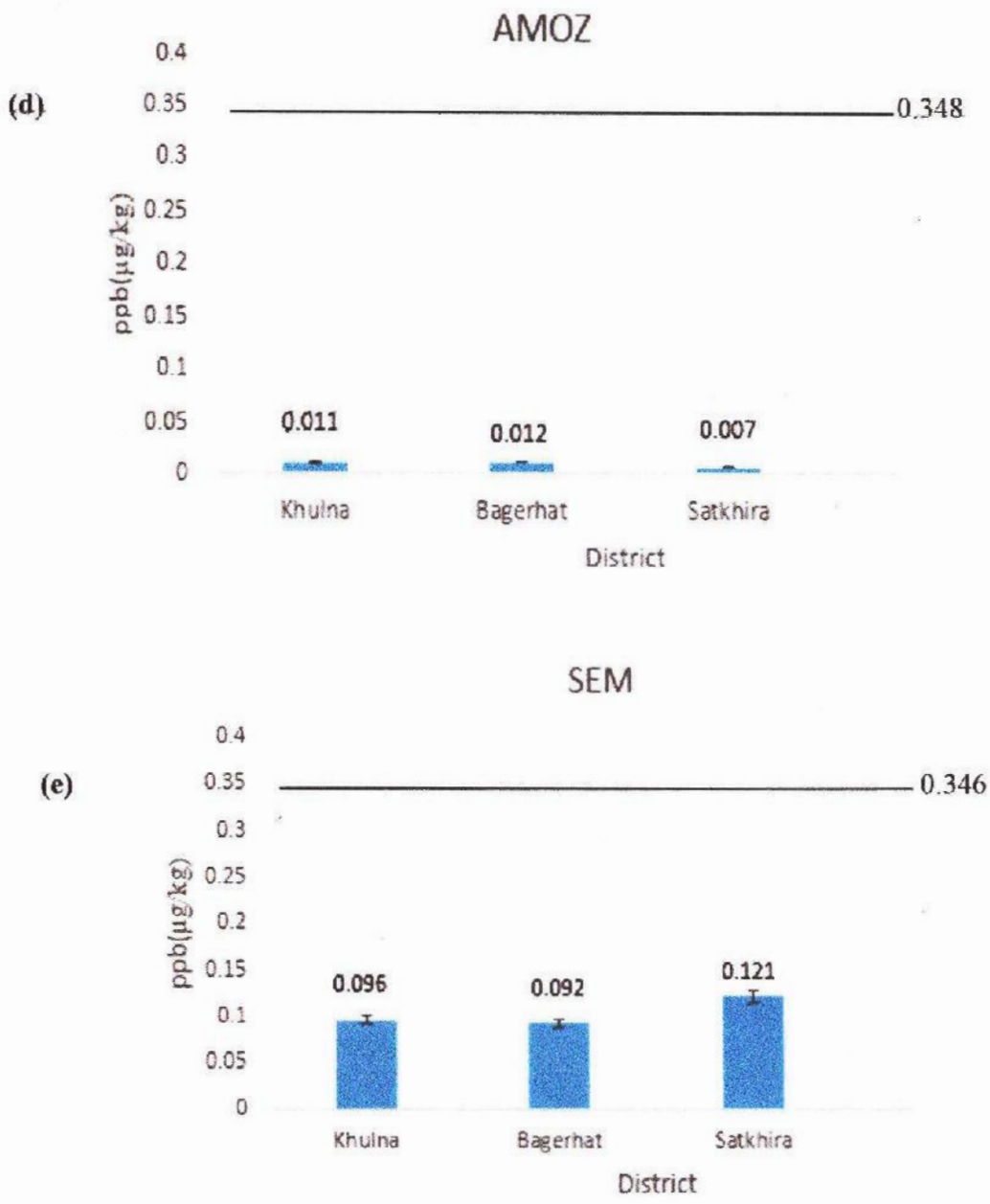


Figure 3: Variation of different antibiotics in Khulna, Bagerhat and Satkhira districts

4.5. Factors responsible behind antibiotic residue

There were several factors played an important role behind this and among them direct application of antibiotics in supplementary feed, use of commercial poultry feed, use of homemade feed with organic fertilizer like cow dung and inorganic fertilizers.

The questionnaire survey revealed that, in Khulna district, 40% farms used commercial feed, 23.33% farm used homemade feed with organic and inorganic fertilizers such as cow dung, poultry litter, urea, triple super phosphate and murate of potash, 16.67% farms used only organic and inorganic fertilizer and 20% farms operated without feed or fertilizer. In Bagerhat, 26.67% farms used commercial feed, 36.67% farms used homemade feed with organic and inorganic, 23.33% farms used only organic and inorganic fertilizer and 13.33% farms operated without feed or fertilizer. In Satkhira, 23.33% farm used commercial feed, 33.33% farms used homemade feed with organic and inorganic fertilizers, 16.67% farms used only organic and inorganic fertilizer and 26.67% farms operated without feed or fertilizer (Table 4) and the feeds those are frequently used in farms are presented in Table 5.

Table 4: Area wise percentage of different types of feed used in different farms.

Types of farm	% of farm applied		
	Khulna	Bagerhat	Satkhira
Commercial feed	40	26.67	23.33
Homemade feed with organic and inorganic fertilizer	23.33	36.67	33.33
Only organic and inorganic fertilizers	16.67	23.33	16.67
Without feed or fertilizer	20	13.33	26.67

Table 5: Name of the feeds that frequently used in different farms in three districts.

Feed Name	Company	Ingredients	Rate of feeding	Status
Tongway Tilapia Grower	Tongway Feed Mill company Ltd.	Fish meal, Soyabean meal, Meat and bone meal, Flour, Rice bran, Vitamin	2-3% of body weight.	Commercial feed
Tilapia Finisher	Spectra Hexa Feeds Ltd.	Fish meal, Wheat, Rice polish, Masterd oil cake, Soya meal, Meat and bone meal, Flour, Salt, D.C.P. Vitamin and Mineral premix.	2 times per day	Commercial feed
Floating Tilapia Grower	Unique Hatchery and Feeds Ltd.	Corn, Soya meal, Full fat soya, Vegetable protein, Fish meal, Flour, Rice polish, Deoiled rice bran, Amino acid, Vitamin, Mineral and Feed additives.		Commercial feed
Carp-Tilapia Pre Nursery powder Feed	SMS Feeds Ltd.	Fish meal, Soyabean meal, Flour, Fish oil, Rice bran, Amino acid, Vitamin and Mineral premix, Enzyme, Natural growth promoter.		Commercial feed
Floating Tilapia Feed	Paragon Group	Fish meal, Soyabean meal, Rice polish, Flour, Vitamin and Mineral premix.		Commercial feed
Floating Carp Fish Feed	Paragon Group	Fish meal, Soyabean meal, Rice polish, Flour, Vitamin and Mineral premix.		Commercial feed
Tilapia Grower	Lili Fish Feed Ltd.	Fish meal, Soyabean meal, Mustard oil cake, Corn, Rice polish, Meat and bone meal, Flour, Deoiled rice bran, Oil, Vitamin and mineral, Pellet binder, Salt and Enzyme.	5% of body weight per day.	Commercial feed

Table 5: Name of feed that frequently used in different farm in three districts:

Feed Name	Company	Ingredients	Rate of feeding	Status
Saudi-Bangla feed	Saudi-Bangla Fish Feed Company Ltd.	Fish meal, Fish oil, Soyabean meal, Gluten meal, Yeast meal, Wheat flour, Growth promoter.		Commercial feed
Supplementary feed		Rice bran, Wheat bran, Oil cake and occasionally incorporating Soybean meal and Fish meal.	Once or twice in a week	Homemade feed
Farm made feed		Rice bran, Wheat bran, Oilcake, Fishmeal, Flour, Maize, Oyster shell, Salt, Vitamin premix and Additives.	Once or twice in a week	Farm made feed

Chapter Five

Discussion

5. Discussion

The analyzed results showed antibiotic residues in the all sample but below MRL. It was aiso found that antibiotic concentrations widely varied in different area. Compared antibiotic residue monitoring data with the questionnaire survey, major observed findings were: (i) 3.33%, 6.67% and 3.33% sample of Khulna, Bagerhat and Satkhira district contaminated with AHD, AHD and SEM, ii) among the 4 nitrofuran metabolites, AHD was abundantly found (iii) tilapia raised in farms fed with homemade feed with organic and inorganic fertilizers were more contaminated with nitrofuran metabolites than the farms fed with commercial feed iv) CAP also widely distributed among samples. Many researchers in Asian countries have detected SEM in many crustacean muscles (Pereira *et al.*, 2004; Saari and Peltonen, 2004). Researchers reported that these type of metabolites sometimes naturally occur in samples in low concentration. Antibiotic residue present in muscle below shell occur due to nature while present in muscle near stomach due to feeding antibiotic. The most frequent administration route for antibiotics in white fish is oral, in which the antibiotic is incorporated into the feed with subsequent exposure to the extremely aggressive aquatic environment.

It has been found that, the residue of AHD was highest among 4 nitrofuran metabolites of three districts and 6.33% and 3.33% contaminated samples were detected in Bagerhat and Khulna districts with AHD metabolite above maximum residue level. But in Satkhira, 3.33% contaminated samples were detected with SEM metabolite.

In aquaculture a variety of feeds are used in Bangladesh including supplementary feed, farm-made feeds and industrially manufactured pelleted feeds. In general, extensive farmers mainly use supplementary feed (Mamun-Ur-Rashida and Belton, 2014). Homemade feed was usually prepared by rice bran, wheat bran, oil cake and occasionally by soybean meal and fish meal. It has been showed that, farms those used homemade feed with organic and inorganic fertilizers mostly used antibiotic intentionally or unintentionally in Bagerhat and Khulna district. In Bagerhat district, 36.67% farms were found which use homemade feed with organic and inorganic fertilizer and in Khulna district 23.33% farms were found to use homemade feed. In two cases, 6.66% and 3.33% contaminated fish were found in Bagerhat and Khulna district respectively. But in Satkhira district, 3.33% contaminated fish were identified which fed with commercial feed.

Farmers mainly use organic and inorganic fertilizers in three districts. The most widely used organic fertilizer was cow dung, which is relatively cheap and readily available in rural Bangladesh. The use of inorganic fertilizer is not widespread. It has been found that, in most cases, farmers often use inorganic fertilizers like urea, lime, murate of potash and triple super phosphate (TSP), which are usually used in combination with cow dung. But the questionnaire survey revealed that the use of poultry litter as organic fertilizer was common in three districts.

Antibiotic was frequently used in farms which reared fish with homemade feed containing organic and inorganic fertilizers. In this study, it was found that farms which used homemade feed with organic and inorganic fertilizer, 6.33% and 3.33% fish were found contaminated with AHD metabolite in Bagerhat and Khulna district. But in Satkhira district, 3.33% fish were found contaminated with SEM metabolite where commercial feed were used.

Nitrofurans parent compounds metabolize rapidly after ingestion to form corresponding tissue bound metabolites (Nouws and Laurensen, 1990; McCracken *et al.*, 1995). Due to this instability, effective monitoring of their illegal use has been difficult. The short *in vivo* half-life of the parent drugs (7 to 63 minutes) results in rapid depletion of nitrofurans in blood and tissue (Nouws and Laurensen, 1990). However, the formed metabolites (AOZ, AMOZ, AHD and SEM) bind to tissue proteins in the body for many weeks after treatment, making them more practical for monitoring public compliance of the EU ban (Hoogenboom *et al.*, 1991; Horne *et al.*, 1996; McCracken and Kennedy, 1997; Cooper *et al.*, 2005). Although the metabolism of nitrofurans is not well documented, a suggested mechanism is through cleavage of the nitrofuran ring, leaving the specific tail group covalently bound to tissue (Leitner *et al.*, 2001). *In vivo,* these metabolites can be released by natural stomach acids (Hoogenboom *et al.*, 1992); this fact is taken into consideration in the isolation of metabolites for residue analysis. The stability of metabolites during the storage and cooking of meat was demonstrated recently (Cooper and Kennedy, 2007). Eight months storage did not have a significant effect on the residual concentration of nitrofurans in incurred muscle and liver pig samples.

The use of drugs in the treatment of disease in finfish and shrimp is a common practice. In order to ensure that tissue residues are within tolerance when antibiotics are used in the treatment of disease in aquatic species, established minimum drug withdrawal times must be strictly adhered to prior to harvesting. The ideal aquaculture drug would be rapidly and highly absorbed. It would

distribute to desired tissues and have a relatively short half-life (Guarino *et al.*, 1988). Antibiotic therapy should always be used wisely to minimize the development of drug resistance in target pathogens (Frappaolo and Guest, 1986), a phenomenon well documented for pathogens of aquatic species (Bullock 1984; DePaola 1988; Ganzhorn 1985; Hastings and McKay 1987; MacMillan 1985; McPhearson *et al.*, 1991; Taylor, 1987). Development of drug resistance in bacteria is not only a human safety concern but may also contribute to the increased incidence and prevalence of chronic and recurrent forms of bacterial disease in aquatic species (Egidius and Anderson, 1979).

Many antibiotics are not biodegradable and persist in the surrounding environment, where they fight against bacteria that continue to develop resistance. Studies of shrimp ponds in Thailand, Vietnam, the Philippines and Mexico have found relatively high levels of bacteria that are resistant to antibiotics, especially *Vibrio* bacteria (Cabello, 2006). Any time you handle or eat raw or undercooked shrimp/fin fish, you run the risk of getting food poisoning. However, when the muscle of fish you eat were grown with large quantities of antibiotics, you take on the additional risk of getting food poisoning from antibiotic-resistant bacteria, which by definition is much more difficult to treat.

Chloramphenicol is a drug of last resort to treat typhoid fever and meningitis in humans. It is generally not used when less toxic drugs are available. Unfortunately, the drug is also used in industrial shrimp production. Although many countries restrict the direct application of chloramphenicol, it is still often applied illegally or indirectly by mixing it with the fish feed. According to analysis of FDA data Food & Water Watch obtained by submitting a Freedom of Information Act request, 39 shipments of shrimp failed import inspections due to the presence of chloramphenicol between 2003 and 2006. The drug is used sparingly in human medicine because it can cause aplastic anemia, a condition in which bone marrow stops producing red and white blood cells and platelets, which are essential for carrying oxygen and for a healthy immune system (Wallerstein *et al.*, 1969).

Aplastic anemia is often irreversible and fatal, and onset may occur three weeks to 12 months after exposure. Chloramphenicol is only partially deactivated by cooking. In a study, fish cooked for 30 minutes at 212° F still retained 71 percent of the antibiotic. (Shakila *et al.*, 2006). Even less chloramphenicol was destroyed when the fish was cooked for a shorter, more typical length of time.

The use of antibiotics in aquaculture is associated with environmental and human health problems, including bacterial resistance, persistence of the disease in the aquatic environment, and effects on the biogeochemical composition of the sediment. The accumulation of antibiotic residues in the edible tissues may also alter human intestinal flora and cause food poisoning or allergy problems (Ma *et al.*, 2006).

Chemical agents should only be applied if there is an appropriate diagnosis of the situation and always under previously established control protocols. According to Páez-Osuna *et al.* (2003) and Nakata *et al.* (2005), to evaluate the impact of antibiotic administration, the usage standards, the hydrology of the area, and the physical-chemical properties of the water should be known. Chávez- Sánchez and Higuera-Ciapara (2003) stated that best practices in aquaculture management should be prioritized to avoid the entrance of pathogens into cultivation systems, and antibiotics should only be administered as a last resort. The most frequent administration route for antibiotics in fish is oral, in which the antibiotic is incorporated in the feed with subsequent exposure to the extremely aggressive aquatic environment. Cabello (2004) opinion on this matter was for this reason, it is important that the antibiotic be contained within a pellet to maintain its stability and protect it from factors such as leaching and binding to trivalent and divalent cautions. It must be certain that the tilapia will eat the food when the antibiotic therapy is applied, because the disease will otherwise not be treated, the environment will be contaminated, and the emergence of bacterial resistant strains will be favored.

Additionally, Chávez and Montoya (2004) and Montoya (2002) reported that the water temperature of the farm ponds is a critical point to consider, because parameters such as the maximum concentration, distribution volume, and rate of elimination of the antibiotic may be affected. The pH, oxygenation, salinity, stage of disease, climatic changes, and presence of natural food in the ponds are other factors that affect antibiotic therapies among aquatic organisms. The use of pharmacological agents, antibiotics, and other chemical agents should be considered as methods of last resort in shrimp farming and aquaculture in general. None of the antibiotics is approved for use in shrimp in the United States. Furthermore accordance with FDA (2001), the medicated feed is used in an extra label manner only for treatment of minor species as defined in the Code of Federal Regulations (21 CFR 514.1(d) (1) (ii). In an aquatic species, the extra label use of medications added to feed in limited to products approved for use in other aquatic species.

Antibiotics must not be used as a preventative measure, since bacteria very rapidly develop resistance to them, leading to their ineffectiveness. Chemical agents should only be applied if there is an appropriate diagnosis of the situation and always under previously established control protocols.

Chapter Six

Conclusion

6. Conclusion

Overall, the occurrence of nitrofuran metabolites and CAP contamination in farm raised tilapia beyond acceptable limit except 3.33%, 6.67% and 3.33% samples of Khulna, Bagerhat and Satkhira district respectively. However National Residue Monitoring Plan should be intensified and farmers, feed processors and sellers should be aware and involved in NRMP programs through appropriate traceability network linkage. All commercial feeds should be strictly screened, any chance of deliberative, accidental or incidental leakage should be checked and public awareness should be created against illegal use of antibiotic. Regulatory authorities and producers are required to identify and eliminate the contamination source to ensure the chemical safety of foods available to the consumer.

Chapter Seven

References

7. References

Ahmed S., Arifur Rahman, A.F.M., Mustafa, M.G., Hossain, M.B. and Nahar, N. 2012. Nutrient composition of indigenous and exotic fishes of rainfed waterlogged paddy fields in Lakshmipur, Bangladesh. *World J. Zool,* 7, pp. 135-140.

Al Mahmud, N., Robiul Hasan, M.D., Hossain, M.B. and Minar, M.H. 2012. Proximate composition of fish feed ingredients available in Lakshmipur region, Bangladesh. *Am.-Eurasian J. Agric. Environ. Sci*, 12, pp. 556-560.

Allen, E.H. 1985. Review of chromatographic methods for chloramphenicol residues in milk, eggs, and tissues from food-producing animals. *Journal-Association of Official Analytical Chemists*, 68, pp. 990-9.

Barani, A. and Fallah, A. A. 2015. Occurrence of tetracyclines, sulfonamides, fluoroquinolones and florfenicol in farmed rainbow trout in Iran. *Food and Agricultural Immunology*, 26, pp. 420-429.

Bullock, G. 1984. Enteric red mouth disease of salmonids. US Fish Wildl Serv, Fish Disease Leaflet 67.

Cabello, F. C. 2004. Antibióticos y Acuicultura en Chile: consecuencias para la salud humana y animal. *Academia de Medicina, Instituto de Chile*, vol.132, pp. 1001-1006.

Cabello, F.C. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental microbiology*, *8*(7), pp.1137-1144.

Cháfer-Pericás, C., Maquieira, Á. Puchades, R., Miralles, J. and Moreno, A. 2011. Multiresidue determination of antibiotics in feed and fish samples for food safety evaluation. Comparison of immunoassay vs. LC-MS-MS. *Food Control*, *22*(6), pp. 993-999.

Chávez, S. M. C. and Montoya, R. L. 2004. Medidas de bioseguridad para evitar la introducción y dispersión de enfermedades virales en granjas camaronícolas, VII *Simposium Internacional de Nutrición Acuícola*, Hermosillo, Sonora. México.

Chávez-Sánchez, M. C. and Higuera-Ciapara, I. 2003. Manual de Buenas Prácticas de Producción Acuícola de Camarón para la Inocuidad Alimentaria. Servicio Nacional de

Sanidad Inocuidad y Calidad Agroalimentaria (SENASICA), ISBN 968-5384-04-5, Mazatlán, Sinaloa, México.

Chico, J., Rúbies, A., Centrich, F., Companyó, R., Prat, M.D. and Granados, M. 2008. High-throughput multiclass method for antibiotic residue analysis by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A, 1213*(2), pp. 189-199.

Commission Decision, 2003. Commission Decision 2003/181/EC of 13 March 2003 amending decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. *Off. J. Eur. Commun.* L, 71, pp. 17–18.

Commission Regulation, 1995, Commission regulation 1442/95 of 26 June 1995 amending Annexes I, II, III and IV to Regulation (EEC) No 2377/90 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in food stuffs of animal origin. *Official J. Eur. Communities*, L, 143, pp. 26-30.

Conneely, A., Nugent, A. and O'keeffe, M. 2002. Use of solid phase extraction for the isolation and clean-up of a derivatised furazolidone metabolite from animal tissues. *Analyst,* 127(6), pp. 705-709.

Cooper, K.M. and Kennedy, D.G. 2007. Stability studies of the metabolites of nitrofuran antibiotics during storage and cooking. *Food Additives and Contaminants,* 24, pp. 935–942.

Cooper, K.M., McCracken, R.J. and Kennedy, D.G. 2005. Nitrofurazone accumulates in avian eyes – a replacement for semicarbazide as a marker of abuse. *Analyst,* 130, pp. 824–827.

Degroodt, J.M., De Bukanski, B.W., De Groof, J., Beernaert, H. and Srebrnk, S. 1992. Chloramphenicol and nitrofuran residue analysis by HPLC and photodiode array detection in meat and fish. *Journal of Liquid Chromatography & Related Technologies*, *15*(13), pp.2355-2371.

Depaola, A., Flynn, PA., McPhearson, R.M. and Levy, S.B. 1988. Phenotypic and genotypic characterization of tetracycline and oxytetracycline resistant *Aeromonas hydrophila* from cultured catfish *(Ictalurus punctatus)* and their environments. *Applied and Environmental Microbiology,* pp. 54-1861.

Draisci, R., Giannetti, L., Lucentini, L., Palleschi, L., Bram-billa, G., Serpe, L. and Gallo, P. 1997. Determination of nitrofuran residues in avian eggs by liquid chromatography UV photodiode array detection and confirmation by liquid chromatography ion spray mass spectrometry. *Journal of .Chromatogrphy, A*, 777, pp. 201–211.

Egidius E., and Andersen K. 1979. Bath immunization- a practical and non-stressing method of vaccinating farmed sea rainbow trout (Salmo gairdneri Richardson) against vibriosis. *Journal of Fish Diseases*, 2, pp. 405-410.

Faruk, M.A.R., Ali, M.M. and Patwary, Z.P. 2008. Evaluation of the status of use of chemicals and antibiotics in freshwater aquaculture activities with special emphasis to fish health management. J. Bangladesh Agric. Univ, 6, pp. 381-390.

FDA, 2001. United States FDA. Aquaculture drugs (a chemical hazard). In: *Fish and fishery products hazards and controls guidance*. 3rd ed. Washington, DC: US FDA, Centre for Food Safety and Applied Nutrition, Office of Seafood, pp. 127-144.

Fernando, R.U.C.H.I.K.A., Munasinghe, D.M.S., Gunasena, A.R.C. and Abeynayake, P. 2017. Determination of nitrofuran metabolites in shrimp muscle by liquid chromatography-photo diode array detection. *Food Control, 72*, pp. 300-305.

Frappaola, P.J. and Guest, G.B. 1986. Regulatory status of tetracyclines, penicillin, and other antibacterial drugs in animal feeds. J An Sci 62(Suppl 3):86.

Ganzhorn, J. 1987. Aeromonas salmonicida, apparent drug resistance to ormetoprim-potentiated sulfadimethoxine. *American Fisheries Society, Fish Health Section Newsletter, 15*(4).

Guarino. A.M., Plakas, S.M., Dickey, R.W. and Zeeman, M. 1988. Principles of drug absorption and recent studies of bioavailability in aquatic species. *Vet Hum Toxicol*, 30(Suppll):41.

Hassan, M.N., Rahman, M., Hossain, M.B., Hossain, M.M., Mendes, R. and Nowsad, A.A.K.M. 2013. Monitoring the presence of chloramphenicol and nitrofuran metabolites in cultured prawn, shrimp and feed in the Southwest coastal region of Bangladesh. *The Egyptian Journal of Aquatic Research, 39*(1), pp. 51-58.

Hastings, T.S. and McKay, A. 1987. Resistance of Aeromonas salmonicida to oxolinic acid. *Aquaculture, 61*(3-4), pp. 165-171.

He, X. 2016. Residues and health risk assessment of quinolones and sulfonamides in cultured fish from Pearl River Delta, China. *Aquaculture*, 458, pp. 38-46.

Holmström, K., Gräslund, S., Wahlström, A., Poungshompoo, S., Bengtsson, B.E. and Kautsky, N. 2003. Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International journal of food science & technology*, *38*(3), pp. 255-266.

Honculada Primavera, J.1993. "A Survey of Chemical and Biological Products used in Intensive Prawn Farms in the Philippines." *Marine Pollution Bulletin*, 26(1), pp. 35-40.

Hoogenboom, L.A.P., Berghmans, M.C.J., Polman, T.H.G., Parker, R. and Shaw, I.C. 1992. Depletion of protein-bound furazolidone metabolites containing the 3-amino-2-oxazolidinone side-chain from liver, kidney and muscle tissues from pigs. *Food Additives and Contaminants*, 9, pp. 623–630.

Hoogenboom, L.A.P., Tomassini, O., Oorsprong, M.B. and Kuiper, H.A. 1991. Use of pig hepatocytes to study the inhibition of monoamine oxidase by furazolidone. *Food and Chemical Toxicology*, 29, pp. 185–191.

Horne, E., Cadogan, A., OKeeffe, M. and Hoogenboom, L.A.P. 1996. Analysis of protein-bound metabolites of furazolidone and furaltadone in pig liver by high-performance liquid chromatography and liquid chromatography mass spectrometry. *Analyst*, 121, pp. 1463–1468.

Hossain, M.B., Amin, S.M.N., Shamsuddin, M. and Minar, M.H. 2013.Use of aqua-chemicals in the hatcheries and fish farms of Greater Noakhali, Bangladesh, Asian J. Anim. Vet. Adv, 8, pp. 401-408.

Le, T.X. and Munekage, Y. 2004. Residues of selected antibiotics in water and mud from shrimp ponds in mangrove areas in Viet Nam. *Marine pollution bulletin*, *49*(11-12), pp. 922-929.

Leitner, A., Zollner, P. and Lindner, W. 2001. Determination of the metabolites of nitrofuran antibiotics in animal tissue by high-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 939, pp. 49–58.

Ma, D., Hu, Y.; Wang, J., Ye, S. and Li, A. 2006. Effects of antibacterial use in aquaculture on biogeochemical processes in marine sediment. *The Science of the Total Environment*, vol. 367, No.1, pp. 273-277.

MacMillan, J. 1985. Infectious diseases. In: Tucker CS Channel Catfish Culture. *Elsevier Science Publishers*, Amsterdam, Netherlands, pp. 405-496.

Mamun-Ur-Rashid, M., Belton, B., Phillips, M. and Rosentrater, K.A. 2014. Improving aquaculture feed in Bangladesh: From feed ingredients to farmer profit to safe consumption. WorldFish, Penang, Malaysia. Working Paper: 2013-34.

McCracken, R.J., Van Rhijn J.A. and Kennedy, D.G. 2005b. The occurrence of nitrofuran metabolites in the tissues of chickens exposed to very low dietary concentrations of the nitrofurans. *Food Additives and Contaminants*, 22, pp. 567–572.

McPhearson, R.M., DE Paola, A., Zywno, S.R., Motes, M.L. and Guarino, A.M. 1991. Antibiotic resistance in gram-negative bacteria from cultured catfish and aquaculture ponds. *Aquaculture*, pp. 99-203.

Monteiro, S.H. 2015. Multiresidue antimicrobial determination in Nile tilapia (*Oreochromis Niloticus*) cage farming by liquid chromatography tandem mass spectrometry. *Aquaculture*, 447, pp. 37-43.

Montoya, V. N. 2002. Residuos de antibióticos en camarones: límites residuales y detección de anfenicoles, In. *Centro Nacional de Acuicultura e Investigaciones Marinas Edgar Arellano*, 54.

Nakata, H., Kannan, K., Jones, D. P. and Giesy, P. J. 2005. Determination of fluoroquinolones antibiotics in wastewater effluents by liquid chromatography-mass spectrometry and fluorescente detection. *Chemosphere*, vol. 58, pp. 759-766.

Nouws, J.F.M. and Laurensen, J. 1990. Post mortal degradation of furazolidone and furaltadone in edible tissues of calves. *Veterinary quarterly*, 12, pp. 56–59.

Nowsad, A.K.M., Islam, M.R. and Faruk, M.A.R. 2013. Studies on nitrofuran contamination in exportable shrimp and prawn products. *Progressive Agriculture*, *20*(1-2), pp. 143-151.

Nowsad, A.K.M.A. 2007. Final Report on Studies on Nitrofuran Contamination in Exportable Shrimp and Prawn Products. Bangladesh Fisheries Research Forum (BFRF), Dhaka.

Páez-Osuna, F., Gracia, A., Flores-Verdugo, F.,Lyle-Fritch, L. P., Alonso-Rodriguez, R., Roque, A. and Ruiz Fernandez, A. C. 2003. Shrimp aquaculture development and the environment in the Gulf of California ecoregion. *Marine Pollution Bulletin*, vol.46, No.7, pp. 806-815.

Pan, X. D. 2014. Determination of chloramphenicol, thiamphenicol, and florfenicol in fish muscle by matrix solid-phase dispersion extraction (MSPD) and ultra-high pressure liquid chromatography tandem mass spectrometry. *Food Control*, 52, pp. 34-38.

Pereira, A.S., Donato, J.L.and De Nucci, G. 2004. Implications of the use of semicarbazide as a metabolic target of nitrofurazone contamination in coated products. *Food Additives and Contaminants*, 21, pp. 63–69.

Ramos, M., Munoz, P., Aranda, A., Rodriguez, I., Diaz, R. and Blanca, J. 2003. Determination of chloramphenicol residues in shrimps by liquid chromatography–mass spectrometry. *Journal of chromatography, B, 791*(1-2), pp. 31-38.

Saari, L. and Peltonen, K. 2004. Novel source of semicarbazide: levels of semicarbazide in cooked crayfish samples determined by LC/MS/ MS. *Food Additives and Contaminants*. 21, pp. 825–832.

Samanidou, V.F. and Evaggelopoulou, E.N. 2007. Analytical strategies to determine antibiotic residues in fish. *Journal of separation science, 30*(16), pp. 2549-2569.

Santos, L. and Ramos, F. 2016. Analytical strategies for the detection and quantification of antibiotic residues in aquaculture fishes: A review. *Trends in Food Science &Technology*, 52, pp. 16-30.

Shakila, R. 2006. "Stability of chloramphenicol residues in shrimp subjected to heat processing treatments." *Food Microbiology*, 23, pp. 47-51.

Shamsuzzaman, M.M. and Biswas, T.K. 2012. Aqua chemicals in shrimp farm: a study from south-west coast of Bangladesh, *Egypt. J. Aquat. Res*, 38 (4), pp. 275-285.

Siddiq, M.A., Miah, M.I., Ahmed Z.F. and Asadujjaman M. 2013. Present status of fish, fishers and fisheries of Dogger Beel in Hajiganj Upazilla, Chandpur, Bangladesh. *Journal of Aquatic Science*, 1, pp. 39-45.

Subasinghe, R.P., Barg, U. and Tacon, A. 1996. Chemicals in Asian Aquaculture: Need, Usage, Issues and Challenges. In: Use of Chemicals in Aquaculture in Asia, Arthur, J.R. (Ed.). Southeast Asian Fisheries Development Center, Aquaculture Department, Iloilo, Philippines, pp: 1-6.

Suseno, H., Hudiyono, S. and Muslim, M. 2016. Elimination of chloramphenicol by tiger shrimp *(Penaeus monodon)* and white shrimp *(Litopenaeus vannamei)*. *HAYATI Journal of Biosciences*, *23*(3), pp. 117-120.

Taylor, P. 1987. Antibiotic resistance to Romet-30 in bacterial infections of catfish. *American Fish Fisheries Society, Fish Health Section Newsletter*, *15*(4).

Tsai, C.W., Tang, C.H. and Wang, W.H. 2010. Quantitative Determination of Four Nitrofurans and Corresponding Metabolites in the Fish Muscle by Liquid Chromatography-Electrospray Ionization-Tandem. *Journal of Food and Drug Analysis*, *18*(2).

Uchida, K., Konishi, Y., Harada, K., Okihashi, M., Yamaguchi, T. and Do, M. H. N. 2016. Monitoring of antibiotic residues in aquatic products in urban and rural areas of Vietnam. *Journal of Agricultural and Food Chemistry*, 64, pp. 6133-6138.

Van Koten-Vermeulen, J.E.M. 1993. Report of the 40th meeting of the joint FAO/WHO expert committee on food additives (JECFA). World Health Organization, Geneva, p. 85.

Vass, M., Hruska, K. and Franek, M. 2008. Nitrofuran antibiotics: a review on the application, prohibition and residual analysis. *Veterinarni Medicina*, *53*(9), pp. 469-500.

Wallerstein, R.O. 1969. "Statewide study of chloramphenicol therapy and fatal aplastic anemia." *Journal of the American Medical Association*, pp. 208-2045.

World Health Organization. 2014. Resistance: Global Report on Surveillance. Retrieved from *http://www.who.int/drugresistance/documents/surveillancereport/en/*.

Xu, W. 2005. Residues of enrofloxacin, furazolidone and their metabolites in Nile .tilapia (*Oreochromis niloticus*). *Aquaculture*, 254, pp. 1–8.

Appendix

Appendices

Appendix 1: Questionnaire

Basic information

1. Name of District:
2. Name of Upazilla:

Farm/Hatchery Information:

1. Name of the farm/ghar/hatchery:
2. Name and Age of farmer :
3. Education level:
4. Contact:

No	Name of antibiotic	Stage	Types of feed	Method of application	Duration of application	Source	Fin fish/Shrimp	Types of Farm
1								
2								
3								
4								

Appendix 2

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CAP	Between Groups	.000	2	.000	.770	.466
	Within Groups	.002	87	.000		
	Total	.002	89			
AOZ	Between Groups	.002	2	.001	12.807	.000
	Within Groups	.007	87	.000		
	Total	.009	89			
AMOZ	Between Groups	.000	2	.000	3.103	.050
	Within Groups	.004	87	.000		
	Total	.005	89			
AHD	Between Groups	.001	2	.000	.053	.948
	Within Groups	.466	87	.005		
	Total	.466	89			
SEM	Between Groups	.016	2	.008	1.050	.354
	Within Groups	.678	87	.008		
	Total	.694	89			

CAP

Tukey HSD[a]

Districts	N	Subset for alpha = 0.05
		1
Bagerhat	30	.01367
Khulna	30	.01423
Satkhira	30	.01533
Sig.		.444

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

AOZ

Tukey HSD[a]

Districts	N	Subset for alpha = 0.05	
		1	2
Bagerhat	30	.01277	
Satkhira	30	.01517	
Khulna	30		.02393
Sig.		.558	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

AMOZ

Tukey HSD[a]

Districts	N	Subset for alpha = 0.05	
		1	2
Satkhira	30	.00763	
Khulna	30	.01080	.01080
Bagerhat	30		.01213
Sig.		.209	.753

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

AHD

Tukey HSD[a]

Districts	N	Subset for alpha = 0.05
		1
Bagerhat	30	.18133
Satkhira	30	.18290
Khulna	30	.18727
Sig.		.947

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

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SEM

Tukey HSD[a]

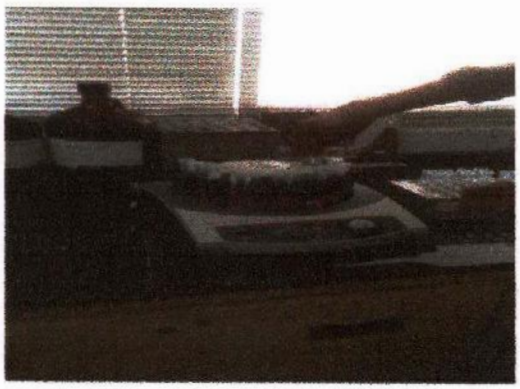
Districts	N	Subset for alpha = 0.05
		1
Bagerhat	30	.08960
Khulna	30	.09667
Satkhira	30	.12107
Sig.		.355

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

Photo Gallery

Plate 1: Sample and data collection from Satkhira and Khulna district



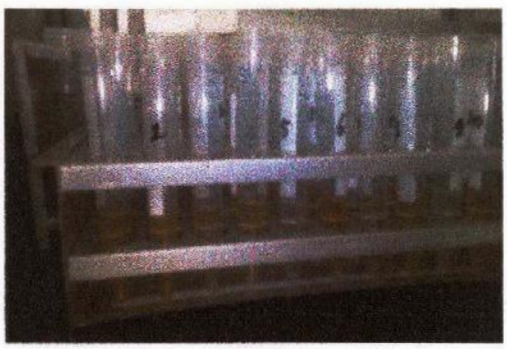


Plate 2: Processing of sample for ELISA in FIQC laboratory

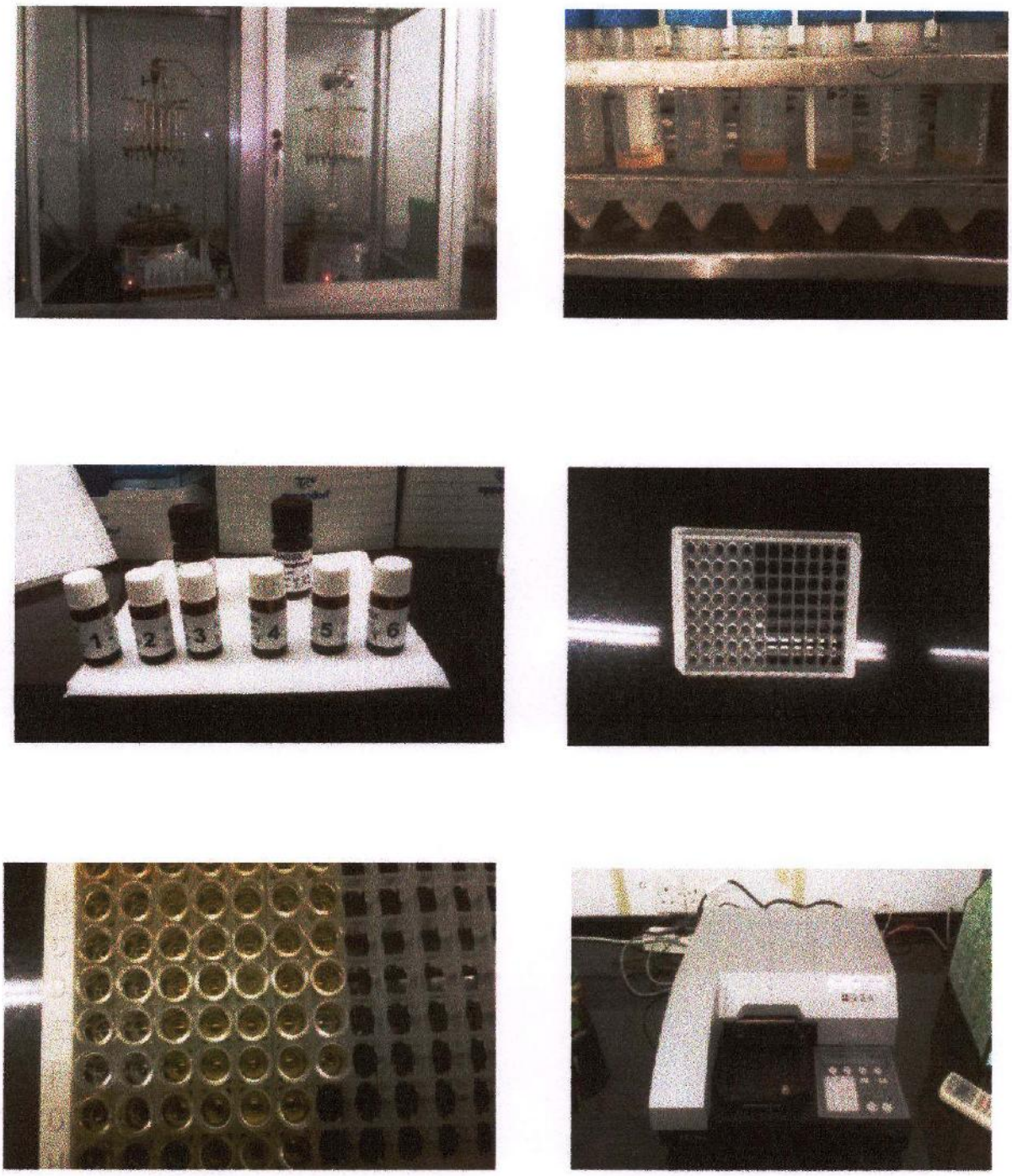


Plate 3: Sample after Kit operation and an ELAISA in FIQC laboratory