SCREENING OF SHRIMP AND PRAWN FEEDS AS A SOURCE OF PROHIBITED ANTIBIOTICS

A thesis

By

Moshrefa Akther

Examination Roll No:MS- 170621

Session: 2016-2017

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Approved as to style and content

By

G. R. Banu
18.12.18

Dr. Ghausiatur Reza Banu

(Professor and Head)
Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208
Bangladesh

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DECLARATION

SCREENING OF SHRIMP AND PRAWN FEEDS AS A SOURCE OF PROHIBITED ANTIBIOTICS

The results submitted in this thesis are entirely of the candidate's own investigations and no part of the results has been accepted for any degree, nor is it being concurrently submitted for any degree.

Moshrefa

(Signature of Candidate)

Moshrefa Akther

Examination Roll No:MS- 170621

Session: 2016-2017

(Signature of Co-Supervisor)

Md. Jahidul Hasan

Fish Inspection & Quality Control Officer
FIQC, Department of Fisheries, Khulna

(Signature of Supervisor)

Md. Rashedul Islam

Associate Professor
Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208, Bangladesh

Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208, Bangladesh
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Moshrefa Akther

Khulna University

September , 2018

Abstract

Presence of antibiotics in farmed prawn and shrimp is a major concern in the export sector of Bangladesh. Due to detection of banned antibiotics consignments were rejected by the foreign buyers. The study was undertaken to analyze commercial feeds to identify the existence of nitrofurans, chloramphenicol and tetracycline. Antibiotic tested data of collected samples revealed that nitrofuran and chloramphenicol antibiotics were found in Shrimp feed but for maximum samples the concentration was lesser in quantities. The study revealed that feeds were contaminated with nitrofuran metabolites SEM (semicarbazide). Cut off level for SEM is 0.576 ppb thus indicates of its presence above acceptable limit in those two samples. Tetracycline was not detected in shrimp feed. Highest concentrations of SEM were found in CARE feed in Khulna region which was 1.992 ppb and was also present in Aman feed and concentration was 1.069 ppb. Other metabolites of nitrofuran AOZ, AHD and AMOZ were below cut off level. Present study did not reveal any big concern regarding presence of antibiotic residue in shrimp feed; however, continuous monitoring is needed to reduce/ prevent contamination of banned antibiotics in shrimp industry.

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List of Abbreviation

Abbreviation	Elaboration
FIQC	Fish Inspection and Quality Control
DOF	Department of Fisheries
CAP	Cholramphenicol
TTC	Tetracycline
AOZ	3-Amino-2-Oxazolidinone
AMOZ	3-Amino-5-morpholino-methyl-1,3-Oxazolidinone
AHD	1-Aminohydatoin
SEM	Semicarbazide
ELISA	Enzyme Linked Immunosorbent Assay
MRL	Minimum Residual Limit
EU	European Union

Chapter- 1

Introduction

1. Introduction

1.1. Background of the study

Shrimp farming has emerged one of the important economic activities in Bangladesh and become the second largest export industry after garments (Rahman et al., 2009). Frozen food export is the second largest export item of Bangladesh, earning about 400 millions of foreign currency yearly which is about 3% of total export and contributing 3.78% in GDP. Shrimp contains more than 80% of frozen food item. Bangladesh captured 2.5 % of world shrimp market (Uddin et al., 2013). Shrimp culture is of central importance to the fisheries sector in Bangladesh particularly in the context of export earnings. To maximize production, in the past 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). In meeting the present food safety standards of the importing countries many Southeast Asian prawn/shrimp exporting countries have been facing difficulties in recent years.

Presence of antibacterial metabolites like nitrofuran metabolites, chloramphenicol and tetracyclines in farmed prawn and shrimp is a major concern in the export sector of Bangladesh. Due to detection of these banned antibiotics, consignments are rejected by the foreign buyers for the last couple of years. About 100 shipments of frozen prawn products from Bangladesh were rejected by the European Union from 2005 to 2009 and as a result more than $ 500 million foreign currency earning deployed by Bangladesh (Nowsad, 2011).

On May, 2009 more than 50 fresh water prawn consignments from Bangladesh were rejected from different European countries, mostly from Belgium, due to the detection of toxic antibiotic, nitrofuran, in them. After the rejection of product Bangladesh Govt. had decided to stop export shrimp and prawn product for the 6 months suspension period till November 20, 2009. Abul Basher, Chief Executive Officer of Bangladesh Frozen Foods Exporters Association expressed his deep concern on impact of EU banned on exported prawn due to Nitrofuran. Due to the presence of nitrofuran in exported prawn on last May, 2009 as a result, thousands of workers of shrimp processing plants have been rendered unemployed since last one month as licenses of

three shrimp processing factories had been cancelled in Khulna region by EU rejected Bangladesh's $350 million seafood sector in (April 20, 2009) (Prabal Barua,2010).

As nitrofurans are used to protect infections against *Salmonella spp.* and *E coli.,* fowl cholera and coccidiosis black heads (Draisci et al., 1997) it is appearing as a serious blow to the steady growth of this sector. EU prohibited the use of nitrofuran in 1995 for livestock production (Commission Regulation, 1995) due to carcinogenic effect of nitrofuran, it has been declared completely unfit (zero tolerance limit) for human consumption (Finzi et al., 2005). 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). Fast growing frozen shrimp export sector is now at serious stake due to such zero tolerance policy of the EU (Commission Regulation,1995) and hard lining of the FDA on presence of nitrofuran drugs (Dof, 2006). It is well known that nitrofurans are metabolized rapidly in vivo and within 7 to 63 minutes results in the rapid depletion of nitrofuran in blood and tissue (Nouws and Laurensen 1990, McCracken et al., 1995). However, the form of metabolites 3- Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3- amino-2-oxazoilidinone (AOZ), 1-aminihydantoin (AHD) and Semicarbazide (SEM) bind to tissue proteins in the body for few weeks after treatment (Gikas et al., 1996; McCracken and Kennedy 1997; Cooper and Kennedy 2005).

Chloramphenicol is a potent, broad-spectrum antibiotic used only at therapeutic doses for treatment of serious infections in humans. Due to the unpredictable effects of doses on different patient populations, it has not been possible to identify a safe level of human exposure to chloramphenicol. Therefore, United States of America Federal regulations prohibit its use in food-producing animals and animal-feed products. Chloramphenicol (CAP) , has some activity against *Burkholderia pseudomallei* is frequently employed in animal production for its excellent antibacterial and pharmacokinetic properties. It leads to hematotoxic side effects and can cause aplastic anemia, leukemia, bone marrow suppression and gray baby syndrome in human , because of this side effects it has been banned in USA and EU. A 'zero tolerence' has been established by the Food and Drug Administration (FDA) for CAP residues in seafood like shrimps, crabs, crayfishes and other animal products.

Tetracyclines (TCAs), Oxytetracycline (OTC) etc. are also used in aquaculture. Oxytetracycline is widely employed to treat bacterial infections in aquaculture farms, such as vibriosis and furunculosis. Though they are specially approved for use in dairy cattle and aqua cultured cat-fish, salmon and lobster (US Code of Federal Regulation, 2003); these drug have not been approved by the U.S. for shrimp aquaculture although it is believed to be used widely in shrimp production outside the U.S. (M.C. Carson et al., 1998). The US FDA has set the tolerance for the combined residues of TC, OTC and CTC at 2μg/g in muscle (US Code of Federal Regulation, 2003). The EU residue tolerance for TC, OTC and CTC in muscle is 100ng/g (EMEA, 1995).

To identify the source of contamination and prevent the entrance of banned antibiotics is becoming main concern in this sector. Probable source can be shrimp feed and feed ingredients, seed sources, imported inputs for shrimp and fish feed production, indigenous inputs and drugs used in aquaculture and poultry industries. If sources of such contaminations are not identified, and required preventive measures are not taken in time, Bangladesh shrimp sector will face a setback leading to severe decline and the loss of competitiveness in export market.

1.2. Objective of the study

-To identify whether commercial fish feed as a source of prohibited antibiotics in shrimp and prawn sector of Bangladesh.

Chapter-2

Literature Review

2. Literature review

The contribution of Bangladesh shrimp is small in terms of its share of the international market (i.e. 2-3% of world production of farmed shrimp). It is the 12th largest cultured shrimp producer in the world. Third most important source of foreign exchange earnings amounted US$ 543.84 million in 2012-2013. Fisheries sector contributes 4.39% to GDP and 2.79% to foreign exchange earnings (Sea Food Export from Bangladesh and Current Status of Traceability).

According to Rahman et al., (2013), Shrimp and prawn together represented the second largest exportable items contributing to foreign exchange earnings of Bangladesh. Shrimp export and cultivation in Bangladesh had undergone rapid expansion over the last two decades. Shrimp and prawn together represented the second largest exportable items contributing to foreign exchange earnings of Bangladesh. Between 1983 and 2003 the volume of shrimp and prawn cultivated in inland aquaculture had increased more than 14 times.

According to 'Final report of a mission carried out in Bangladesh from 19 to 28 January 2010 by European Commission' it was found that the export of shrimp and fish represented 45% and 11%, respectively, of the value of all exported agricultural commodities. The EU was the main export market, followed by the USA. There were 71 fish/shrimp processing plants approved by the competent authority for export to the EU (44 in Khulna region, 23 in Chittagong region, four in Dhaka region) and 202,693 shrimp farms and hatcheries of which 179,325 had been registered by the Department of Fisheries to date.

Uddin et al., (2013), described that, Frozen food export was the second largest export item of Bangladesh, earnings about 400 millions of foreign currency yearly which was about 3% of total export where Shrimp contained more than 80% of frozen food item.

According to the findings of Akter, (2017), to economic growth and GDP the contribution of frozen shrimp was quite significant for Bangladesh and in the last few decades, and the contribution of frozen shrimp had occupied the 4th position (1st-RMG, 2nd Jute-goods, 3rd Tea) in the list of total exportable commodities of Bangladesh.

Aftabuddin et al., (2008), found that to control bacteria, fungi and protozoa in the shrimp hatcheries in Bangladesh A wide range of antibiotics and chemicals were used. It was found that Seventy percent of the surveyed hatcheries used formalin and 30% used malachite green to control parasites in broodstock holding tanks. Sixty percent surveyed hatcheries used formalin and 40% hatcheries used povidone iodine (PVP) as a disinfectant in broodstock before placed to the spawning tank. Forty percent of the hatcheries used the Ciprofloxacin, 30% used chloramphenicol, 15% used erythromycin, 10% used prefuran and 5% used oxytetracycline in broodstock maintenance to prevent possible bacterial infections after eye stalk ablation. The concentration of chloramphenicol used in Bangladesh shrimp hatcheries to control different bacterial diseases were three to four times higher than other countries (Ruangpanich 1988).

According to the study of Sheikh Aftab Uddin and Md. Abdul Kader, 2006, A variety of chemicals were found to be used in Bangladesh shrimp hatcheries for increased and controlled production of seed in hatcheries, increased feeding efficiency, improvement of survival rates and control of pathogens. To control all types of bacteria Chloramphenicol, Erythromycin, Oxytetracycline and Prefuran were found to be widely used with varying success. The antibiotics Chloramphenicol and Nitrofurans (Prefuran) were banned worldwide for use in food production because of their potentially serious side effects.

Chloramphenicol caused fatal aplastic anemia and Nitrofurans were classified as carcinogen (Graslund and Bengtsson, 2001).

Oxytetracycline was known to enhance the production of plasmid mediated resistance in aquatic bacteria (Shotts et al., 1976) and Erythromycin developed strains resistant (Primavera et al., 1993).

Dierberg and Kiattisimkul (1996) reported that Malachite green was a respiratory poison, persistent residues in tissues of seafood. Treflan a possible human carcinogen induced thyroid and liver tumours in mammals (Hurley, 1998).

Hassan et al., (2013), worked on the presence of chloramphenicol and Nitrofuran in cultured prawn, shrimp and feed and found that freshwater giant prawns were more frequently contaminated with nitrofuran and chloramphenicol than marine shrimps , levels of nitrofuran and chloramphenicol contaminations decreased in 2010 in both prawn and shrimp compared to 2008 and 2009, prawns raised in farms fed with commercial feed were contaminated with nitrofuran metabolites more than prawns raised in farms fed with home-made feed with organic and inorganic fertilizer only, and farms operated without feed or fertilizers had no such antibiotic contamination at all. They also found that some farms used veterinary medicines other than nitrofuran and chloramphenicol, like oxytetracycline for treatment of disease or other purposes. Shrimp/prawn raised without feed/fertilizers had no antibiotic contaminations.

Vass et al., (2008), described that in 1995 due to concerns about the carcinogenicity of residues in edible tissue Nitrofuran antibiotics, employed for the treatment of bacterial diseases in livestock production, were banned from use in the European Union (EU). Nitrofurans were commonly employed as feed additives for growth promotion, and mainly used for livestock (i.e. poultry, swine and cattle), aquaculture (i.e. fish and shrimp). Under EU regulation, countries with products intended for the EU are bound by the same regulations as locally produced food (Commission Decision, 2003), therefore food imported into the EU should be free of nitrofurans. The use of nitrofurans for livestock has also been prohibited in countries such as Australia, USA, Philippines, Thailand and Brazil (Khong et al., 2004).

Nowsad et al., (2009), found in their study, most of the hatchery operators and prawn farmers did not know about the devastating effect of nitrofuran on prawn. They also found through survey , 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. Many unlabelled antibiotics were found to be used in the hatcheries. Chloramphenicol, although well-known as a banned antibiotic for use in shrimp, was reported to be used in the hatcheries of Cox's Bazar.

According to Fabio Granados-Chinchilla and César Rodríguez , (2017), the use of tetracyclines as growth promoters was allowed in many countries . However, beginning from 1 January 2017, tetracyclines were no longer be allowed for use as growth promoters in USA. The use was restricted to therapeutic use only and subject to a veterinary feed directive.

Chapter-3

Methodology

3. Methodology

3.1. Study area and target groups

Three districts named Bagerhat, Satkhira and Khulna were selected for the present study and the areas were selected because of its contributing role to the shrimp and prawn aquaculture and frozen fishery export. Target groups of this study include feed manufacturers and prawn and shrimp producers who use the feeds of the target feed manufacturers. However, sample feeds were collected from five different farms of each district. The samples were collected from February- June, 2018.

3.2. Sample collection

Feed samples were collected from selected farms of three districts. For selecting the farms at first different areas of each district were selected considering continuous production of shrimp and then target farms were selected by applying simple random method from those selected areas of each district. In the selected farms four different types of commercial feeds were found to be used and these include Aman feed, PCF, Nutrition feed and Care feed. From each farm sample feeds were collected once only.

3.3. Analysis of feed samples

Feed samples were analyzed for screening test by validated ELISA (Enzyme Linked Immuno Sorbent Assay) machine (Biotek, Elx-800). Detailed procedure were given below:

3.3.1. Determination procedure of Chloramphenicol (CAP) in shrimp feed using ELISA

Required Materials for Chloramphenicol (CAP)

1. Diluent/Wash Buffer (Conc.)
2. Conjugate (Conc.)
3. Tissue Extraction Buffer (Conc.)
4. One Shot Substrate
5. Standards
6. Stop Solution
7. 100ng/ml chloramphenicol spiking material

Materials not provided with kit

1. Ethyl acetate
2. 2,2,4-Trimethylpentane (Isooctane)
3. Chloroform
4. Deionised water

Apparatus and Instrument

1. CAP Microtitre Plate
2. Micro plate reader (Biotek, Model ELx 800)
3. Lint Free Tissue
4. Water bath/N_2 Evaporator
5. Eppendorf tube
6. 10 ml test tube
7. 50 ml falcon tube
8. Pipettes : 10 µl to 1000 µl
9. Plate sealer
10. Analytical Balance
11. Centrifuge
12. Vortex
13. Blender
14. Personal Computer with printer

Ready to Use Reagents

1. One Shot Substrate
2. Standards
3. Spiking Material
4. Stop Solution

Preparation of Wash Buffer, Spiking Solutions, Spiked Samples, Conjugate, Tissue Extraction Buffer and other Reagent

(a) Preparation of Wash Buffer

Wash buffer were prepared by the dilution contents of wash buffer with 970 ml distilled H_2O.

(b) **Preparation of Spiking Solutions**

A 100ng/ml chloramphenicol spiking material was already provided in the ELISA kit, there was no need to make up any additional standard stock solutions. Therefore an initial dilution to 10ng was made up in diluted wash buffer from the kit.

10ng = 50µl of 100ng spiking material + 450µl of diluted wash buffer.

Preparation of spiked sample

0.15ng/g = 45µl of 10ng/ml spike + 3g negative sample

(c) Preparation of Tissue Extraction Buffer (Conc.)

Tissue Extraction Buffer was supplied as a concentrate. This buffer was prepared by the mixture of 0.5 ml tissue extraction buffer with the 200ml diluted wash buffer. The proportion were 1:400.

The diluted Tissue Extraction Buffer was used immediately.

(d) Preparation of Conjugate

Conjugate was supplied as a concentrate. Dilute the conjugate in two steps:

Dilution 1: 20 µl conjugate concentrate were added to 2 ml diluted wash buffer.

Dilution 2: From the dilution 1 25 µl were added to 5 ml diluted wash buffer. The diluted conjugate was used immediately just before ELISA plate preparation.

(e) Isooctane: Chloroform

40 ml of Isooctane and 60 ml of Chloroform were taken in 200 ml glass bottle and shacked well to mix them properly. The ratio was 2:3.

Extraction Procedure

At first 3g feed was added with 6 mlethyl acetate and homogenised in vortex for 1 minute. Then the mixture were centrifuged at 2000 rpm for 15 minute and two layers were appeared.4 mlof upper phase were removed and reduced to dryness at 70°C under N_2atmosphere. The residue were dissolved in 2 mlof isooctane/chloroform (2:3). Again vortex for 1 min as well as added0.5 mlof diluted Tissue Extraction Buffer and vortex for 2 min. Then Centrifuged at 2000 rpm for 15 minute. Upper phase was ready for application to microtitre plate.

Test procedure for ELISA

Before use (about 1 hours) all reagents were brought to room temperature (20 to 25°C). All reagents were returned to 2-8°C after use. All reagents were mixed by gently inverting or swirling prior to use. Sufficient number of wells were inserted into a microwell holder. Standard, blank, QC and sample positions were recorded. 25 µl standards were pipetted from lower to higher concentration into the appropriate wells of the microtitre plate supplied for CAP. 25 µl sample extracts of the 2 tissue blank in duplicate were pipetted . One set of duplicates was plated at the beginning and once at the end of the set. 25 µl sample extracts of the recoveries in duplicate was pipetted; plated them once at the beginning and once at the end of the set. 25 µl of extracted samples was pipetted in to separate wells ; a new pipette was used to tip for each standard or sample. 100 µl conjugate was pipetted to each plate. Microtitre plate was covered with adhesive film .The microtitre plate was gently tapped from side to side for a few seconds before incubating for 1 hour at room temperature (20 to 25°C) in the dark . Plate was inverted and tapped out liquid. Microtitre plate was washed 6 times with diluted diluent/wash buffer over a 10-15 minute period (ensuring that every well is filled). After final wash liquid was discarded and tapped onto tissue paper until completely dry. Immediately after washing, 125 µl of the one shot substrate solution was pipetted into each well. Microtitre plate was gently tapped from side to side and incubate the microtitre plate for 20 ± 2 mins. at room temperature (20 to 25°C) in the dark. By the addition of 100 µl of stop Solution per well colour reaction was stopped . A colour change from blue to yellow were evident. Measured the optical density of each well at 450 nm and 630nm filter as the reference wavelength within 10 minutes of stopping the colour reaction.

3.3.2. Determination procedure of Nitrofuran in shrimp feed using ELISA

Required Materials for Nitrofuran

Materials Provided With Kit supplied by Fish Inspection and Quality Control office, Khulna

1. Microtitre plate
2. Conjugate (Conc.)
3. Diluent/Wash Buffer (Conc.)
4. One Shot Substrate

5. Stop Solution
6. Standards
7. Conjugate (Concentrate)
8. 10mM 4-Nitrobenzaldehyde

Materials not provided with kit

1. 1M HCl
2. 1M NaOH
3. 0.1M K_2HPO_4
4. Ethyl acetate
5. Hexane
6. Deionised water

Apparatus and Instrument

- Respective Microtitre Plate (e.g. AOZ/AMOZ/AHD/SEM)
- Micro plate reader equipped with a 450nm filter with a recommended reference filter of 630nm.
- 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
- Personal Computer with printer

Ready to Use Reagents

1. One Shot Substrate
2. Standards

3. Spiking solution
4. Stop Solution
5. 10mM 4-Nitrobenzaldehyde (in DMSO)
6. Conjugate Diluent
7. 1 M HCl
8. 0.1M K_2HPO_4

Extraction Procedure

At first homogenized each feed sample. 1g of homogenized sample was weighted 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 0. 75µg/kg AOZ, AMOZ and 0.5µg/kg AHD,SEM respectively. After that, 1g of homogenized sample was added with 4ml distilled H_2O, 0.5ml 1M HCl and 50 µl 10mM 4-Nitrobenzaldehyde.Vortex for 1 minute and incubated for 2 hours at 50°C. After vortex for 2 hours 5ml 0.1M K_2HPO_4, 0.4ml 1M NaOH and 6ml ethyl acetate were added and vortex for 1minute.Then centrifuged the sample for 10 minutes at 4000rpm at 25°C and two layers were appeared. After10 minutes 3ml of the upper ethyl acetate layer of each sample was transferred into test tube and dry down 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 centrifuged the sample for 10 minutes at 4000rpm at 25°C.Lower phase was ready for application to microtitre plate.

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 inverted the plate 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 incubated the plate for 20 minutes at room temperature (20-25°c) in the dark. Colour reaction were stopped by the addition of 100 µlof Stop Solution per well. A colour change from blue to yellow were evident. Measure the optical density of each well at 450 nm and 630nm filter as the reference wavelength within 10 minutes of stopping the color reaction.

3.3.3. Determination procedure of Tetracycline in shrimp feed using ELISA

Required Materials for Tetracycline

1. Oxytetracycline-coated Plate
2. Oxytetracycline Standard Stock
3. Standards
4. Oxytetracycline Antibody#1
5. 100X HRP-Conjugated Antibody #2
6. Antibody #2 Diluent
7. Wash Solution
8. Stop Buffer
9. TMB Substrate
10. Standard diluent
11. Extraction Buffer
12. Sample diluents
13. TET Balance Buffer Concentrate
14. Oxytetracycline Spiking Stock
15. Chlorotetracycline Spiking Stock

Chemicals not Provided in the Kit

1. Deionised water

Apparatus and Instrument

- Micro plate reader (Biotek, Model ELx 800) equipped with a 450nm filter and a recommended reference filter of 630nm.
- Lint Free Tissue
- Eppendorf tube
- 15 ml falcon tube
- 50 ml falcon tube
- Pipettes : 20 µl to 200 µl, 100 µl to 1000 µl, 500 µl to 5000 µl, 1000 µl to 10000 µl
- Plate sealer
- Analytical Balance
- Centrifuge
- Vortex
- Personal Computer with printer

Ready to Use Reagents

1. Wash Solution
2. TMB Substrate
3. Stop Solution

Preparation of Oxytetracycline Work Standards

The oxytetracycline standard was provided as 450 ng stock . 1.5 mL of standard diluents was added to the stock vial and mix by vortexing for 1 minute to obtain 300 ppb stock vial. The solution was vortexed vigorously for 2 minutes and left it at room temperature for 10 minutes. This procedure was repeated three times to ensure that all powder was dissolved. The solution was transferred to a dark plastic vial and stored it at -20°C for any future use. To make working standards, serially dilute the 300 ppb standard stock vial with standard diluents. Each work standard tube was vortexed for 30 seconds.

Extraction Procedure

At first homogenized each feed sample. 1g of homogenized sample was weighted in a 50 ml falcon tube. Weigh four 1g of previously analyzed blank tissue were used as two positive and two negative controls. One positive control with Oxytetracycline and one with Chlortetracycline at 50 µg/kg was fortified. 3mL of 1X OXTET Extraction Buffer was added to 1 g of homogenized feed. Then it was vortexed for 10 minutes in a multi-tube vortex. It was centrifuged for 10 min at 4000 rpm. 200µL of supernatant was transferred to a new tube containing 25µL of 1X OXTET sample balance buffer. The tube was swirled. Then 275µL of 1X TET sample diluents was added. It was vortexed for 1 minute. The sample was diluted with 1X TET sample diluents. Then it was again vortexed for 1 minute.

Test procedure for ELISA

Before use (about 1 hours) all reagents were brought to room temperature (20 to 25°C). All reagents were returned to 2-8°C after use. All reagents were mixed by gently inverting or swirling prior to use. Sufficient number of wells were inserted into a microwell holder. Standard, blank, QC and sample positions were recorded. 75 µl standards was pipetted from lower to higher concentration into the appropriate wells of the microtitre plate. 75 µl sample extracts were pipetted of the 2 tissue blank in duplicate. One set of duplicates was plated at the beginning and once at the end of the set. 75 µl sample extracts of the recoveries was pipetted in duplicate; plated them once at the beginning and once at the end of the set.75 µl of extracted samples were pipetted in to separate wells ; used a new pipette tip for each standard or sample. 100 µL of Antibody -1was added and mixed well by gently rocking the plate manually for 1 minute. The plate was incubated for 55 minutes at room temperature (20 –25°C / 68 –77°F). The plate was washed for 3 times with 250 µL of 1X Wash Solution. After addition of 250µl Wash buffer to the wells, incubated the plate for 20-30 seconds; shaked the plate gently before pouring out the wash buffer. This procedure was repeated for each of the three washes. After the last wash, inverted the plate and gently tapped the plate dry on paper towels. 150µL of 1X Antibody -2 solution was added. The plate was incubated for 25 minutes at room temperature (20 –25°C / 68 –77°F).

The plate was washed for 3 times with 250 of µL 1X Wash Solution. After addition of 250µl wash buffer to the wells, incubated the plate for 20-30 seconds; shaked the plate gently before pouring

out the wash buffer. This procedure was repeated for each of the three washes. After the last wash, inverted the plate and gently tapped the plate dry on paper towels.

100 µL of TMB Substrate was added to each well. Time the reaction immediately after adding the substrate. The solution was mixed by gently rocking the plate manually for 1 minute while incubating . After incubating for 15 minutes at room temperature (*20 –25°C / 68 –77°F*), added 100 µL of Stop Buffer to stop the enzyme reaction. The optical density of each well on a plate reader was measured with 450 nm wavelength as soon as possible following the addition of Stop Buffer.

3.4. Data Analysis

One-way ANOVA and Microsoft excel were used for statistical analysis of antibiotic contamination for feed ingredients.

Chapter-4

Result

In case of PCF feed concentration of SEM was found above the cut off level for sample number 3 of Khulna region. However, for the rest of all experimental sites SEM concentration was found below the cut-off level. Maximum level was found in Khulna region scaled 1.539 ppb and minimum level was also in Khulna which was 0.046 ppb.

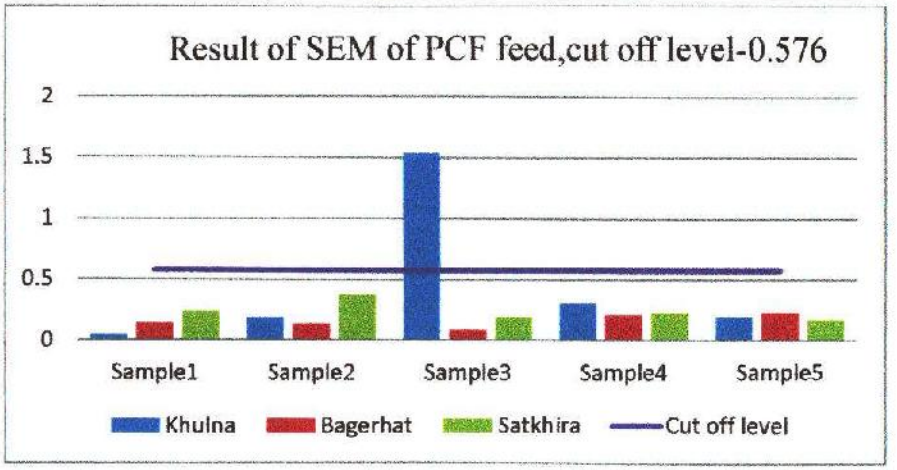

Fig.3. Concentration of SEM in PCF feed

4.4. Concentration (ppb) of SEM in Nutrition feed

In all the experimental sites the concentration of SEM was found below the cut-off level. The highest value of SEM was found in sample 2 of Satkhira district whereas the lowest value of SEM was found in sample 2 of Bagerhat district. Maximum level was 0.377 ppb and minimum level was 0.044 ppb .

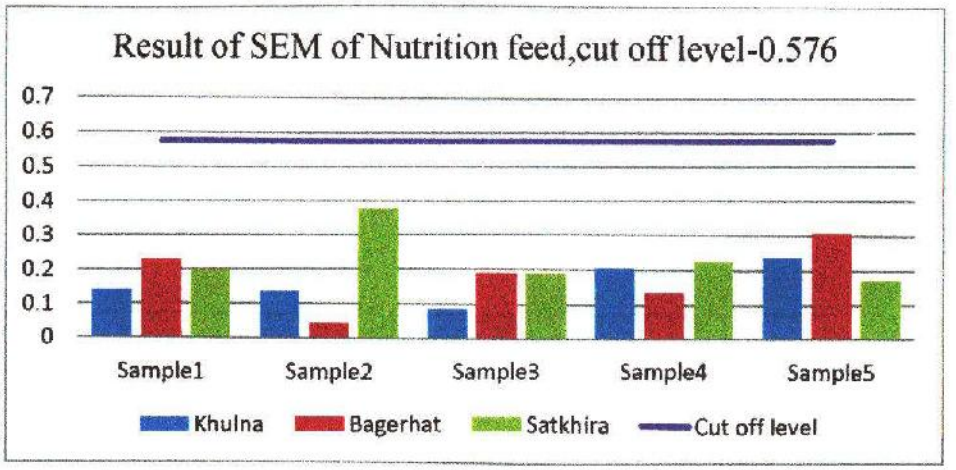

Fig.4. Concentration of SEM in Nutrition feed

Result of AOZ

4.5. Concentration (ppb) value of AOZ Aman feed

The cut-off level of AOZ in shrimp feed is 0.371. In the present study in all the experimental samples, concentration of AOZ in shrimp feed were found below the cut-off level. The highest value of AOZ in case of feed was found in sample 1 of Bagerht district whereas the lowest value of AOZ was found in sample 5 of Bagerhat district. Maximum level was 0.108 ppb and minimum level was 0.029 ppb.

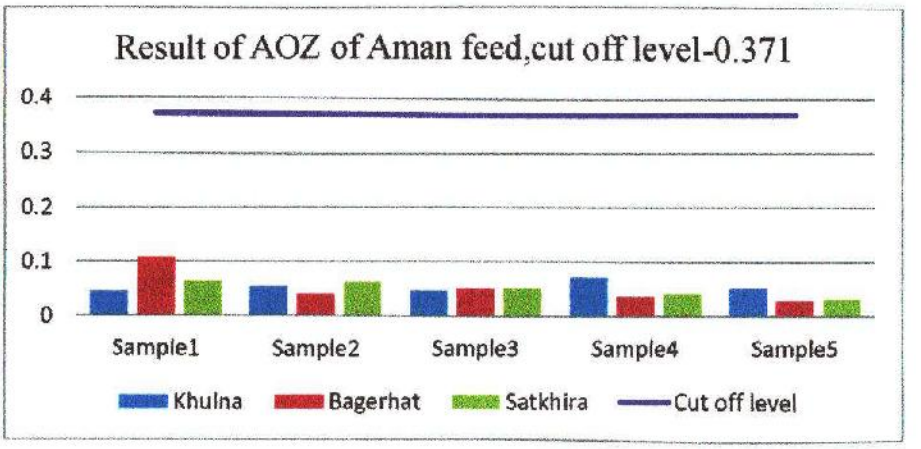

Fig.5. Concentration of AOZ in Aman feed

4.6. Concentration (ppb) of AOZ in CARE feed

Concentration of AOZ in CARE feed was below the cut-off level in all stations. Maximum value was 0.123 ppb was in sample 5 of Bagerhat district and minimum value was 0.042 ppb was in sample 4 in Khulna District.

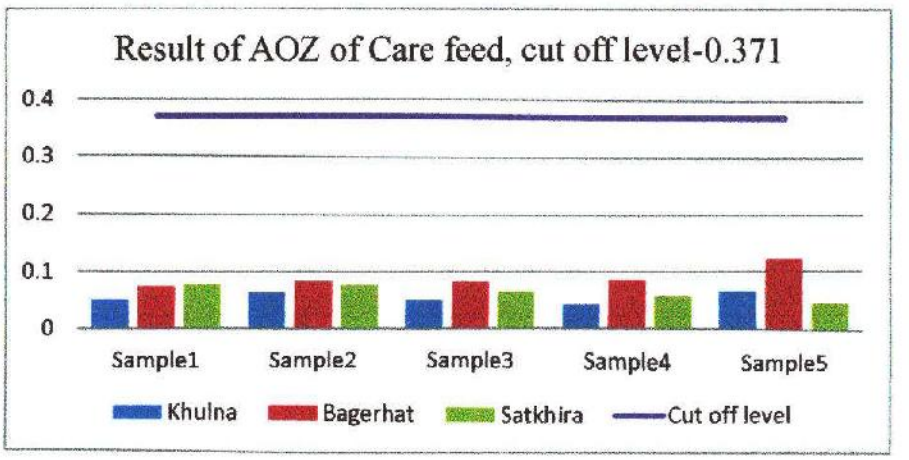

Fig.6. Concentration of AOZ in CARE feed

4.7. Concentration (ppb) of AOZ in PCF feed

In the present study in all the experimental samples, concentration of AOZ in shrimp feed were found below the cut-off level. Maximum value was 0.138 ppb which was in sample 1 of Bagerhat district and minimum value was 0.051 ppb in sample 5 of Bagerhat district.

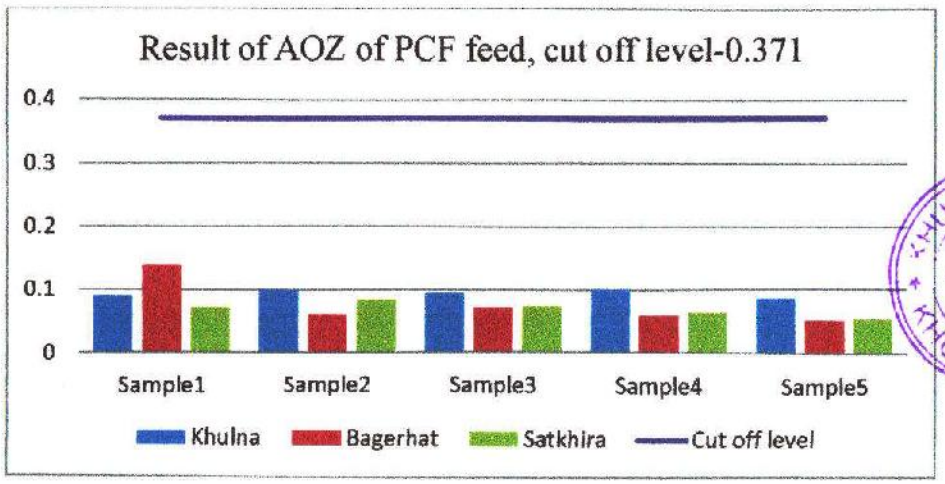

Fig.7. Concentration (ppb) of AOZ in PCF feed

4.8. Concentration (ppb) of AOZ in Nutrition feed

In the present study in all the experimental samples, concentration of AOZ in shrimp feed were found below the cut-off level. The highest value of AOZ in case of feed was found in sample 1 of Bagerhat district whereas the lowest value of AOZ was found in sample 5 of Bagerhat district. Maximum value was 0.116 ppb and minimum value was 0.037 ppb.

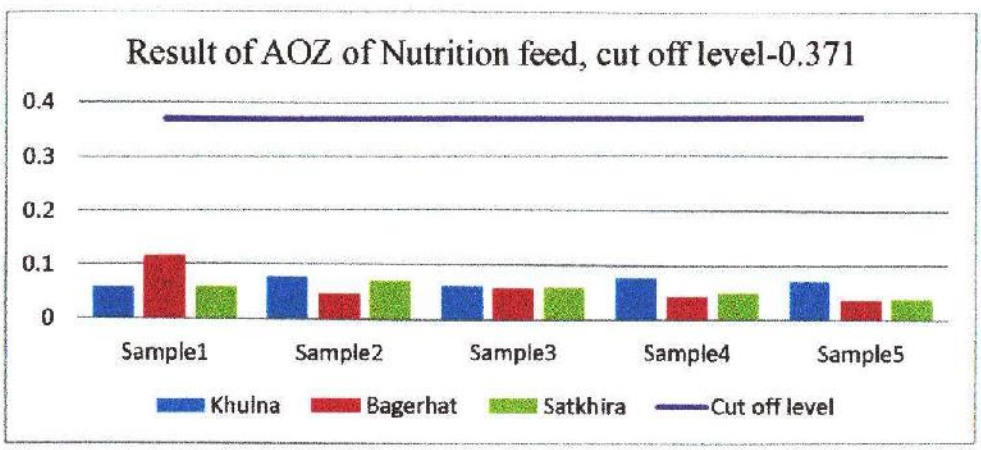

Fig.8. Concentration (ppb) of AOZ in Nutrition feed

Result of AHD

4.9. Concentration (ppb) value of AHD in Aman feed

The cut-off level of AHD in shrimp feed is 0.424. In the present study in all the experimental sites concentration of AHD in shrimp feed were found below the cut-off level. The highest value of AHD in case of shrimp feed was found in sample 3 of Satkhira district whereas the lowest value of AHD was found in station 5 of Satkhira district. Maximum value was 0.106 ppb and minimum value was 0.01 ppb.

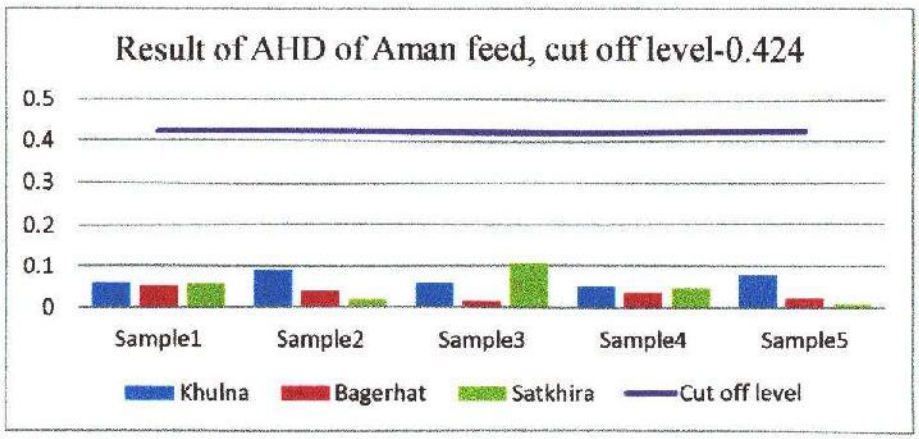

Fig.9. Concentration (ppb) of AHD in Aman feed

4.10. Concentration (ppb) value of AHD in CARE feed

Concentration (ppb) of AHD in CARE feed was below the cut-off level. Maximum value was 0.154 ppb in sample 3 in Satkhira and minimum value was 0.011 ppb in sample 5 of Satkhira.

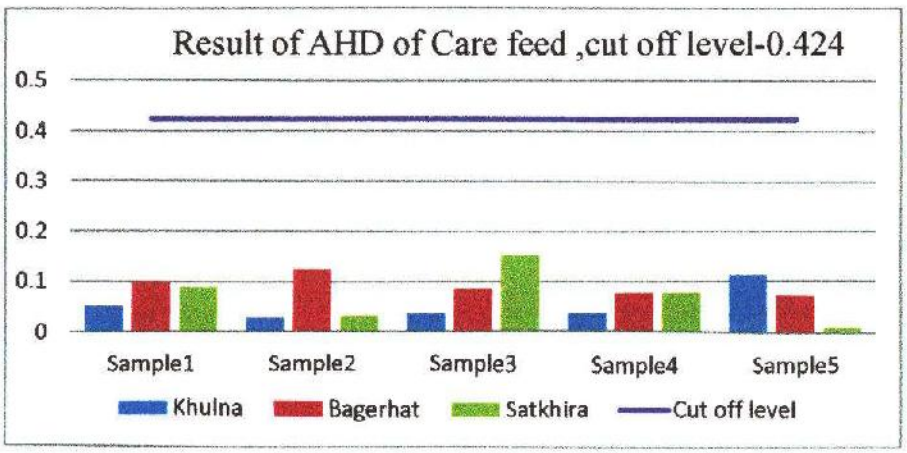

Fig.9. Concentration (ppb) of AHD in CARE feed

4.11. Concentration (ppb) value of AHD in PCF feed

In all the experimental sites the concentration of AHD in shrimp feed was found below the cut-off level. The highest value of AHD was found in sample 3 of Satkhira district whereas the lowest value of AHD was found in sample 5 of Satkhira district. Maximum value was 0.146 ppb and minimum value was 0.011 ppb.

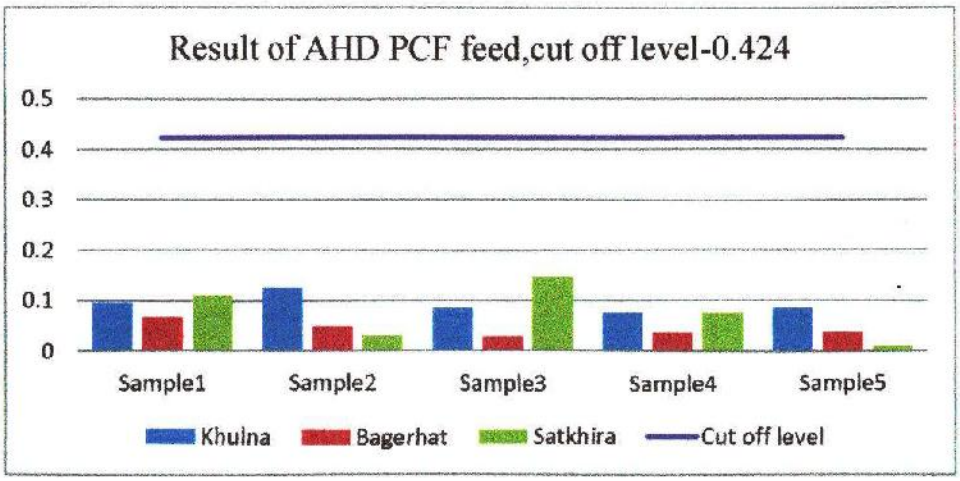

Fig.11. Concentration (ppb) of AHD in PCF feed

4.12. Concentration (ppb) value of AHD in Nutrition feed

Concentration (ppb) of AHD in Nutrition feed was below the cut-off level. Maximum value was 0.108 ppb and minimum value was 0.01 ppb.

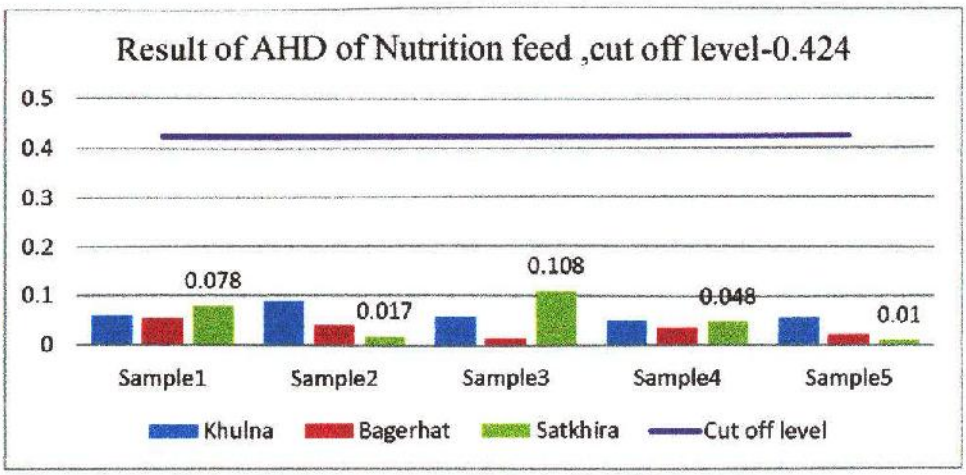

Fig.12. Concentration (ppb) of AHD in Nutrition feed

Result of AMOZ

4.13. Concentration (ppb) value of AMOZ in Aman feed

Cut off level of AMOZ for shrimp feed was 0.356. Concentration (ppb) of AMOZ in Aman feed was below the cut-off level. Maximum value was 0.234 ppb in sample 2 of Khulna district and minimum value was 0.047 ppb in sample 2 of Satkhira district.

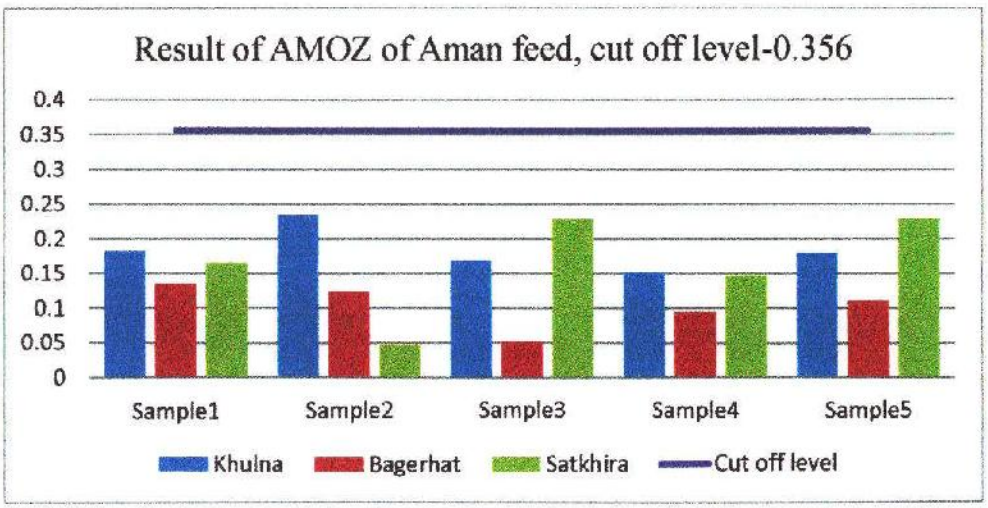

Fig.13. Concentration (ppb) of AMOZ in Aman feed

4.14. Concentration (ppb) value of AMOZ in CARE feed

In the present study in all the experimental sites concentration of AMOZ in Care feed were found below the cut-off level. The highest value of AMOZ in case of feed was found in sample 2 of Bagerhat district whereas the lowest value of AMOZ was found in sample 5 of Satkhira district. Maximum value was 0.191 ppb and minimum value was 0.009 ppb.

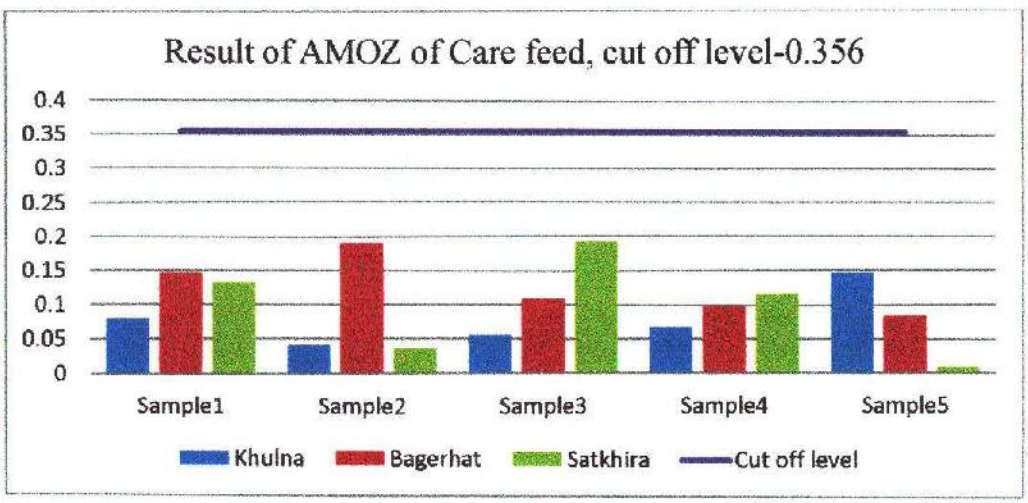

Fig.14. Concentration (ppb) of AMOZ in CARE feed

4.15. Concentration (ppb) value of AMOZ in PCF feed

Concentration (ppb) of AMOZ in PCF feed was below the cut-off level in all experimental sites. Maximum value was 0.2 ppb in sample 3 of Satkhira District and minimum value was 0.01ppb in sample 5 of Satkhira district.

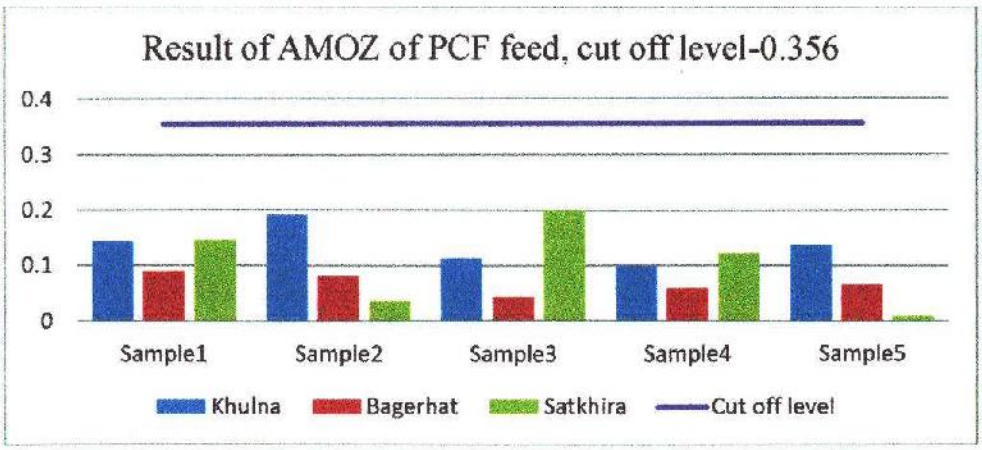

Fig.15. Concentration (ppb) of AMOZ in PCF feed

4.16. Concentration (ppb) value of AMOZ in Nutrition feed

Concentration (ppb) of AMOZ in Nutrition feed was below the cut-off level. Maximum value was 0.238 ppb in sample 2 of Khulna district and minimum value was 0.007 ppb in sample 5 of Satkhira.

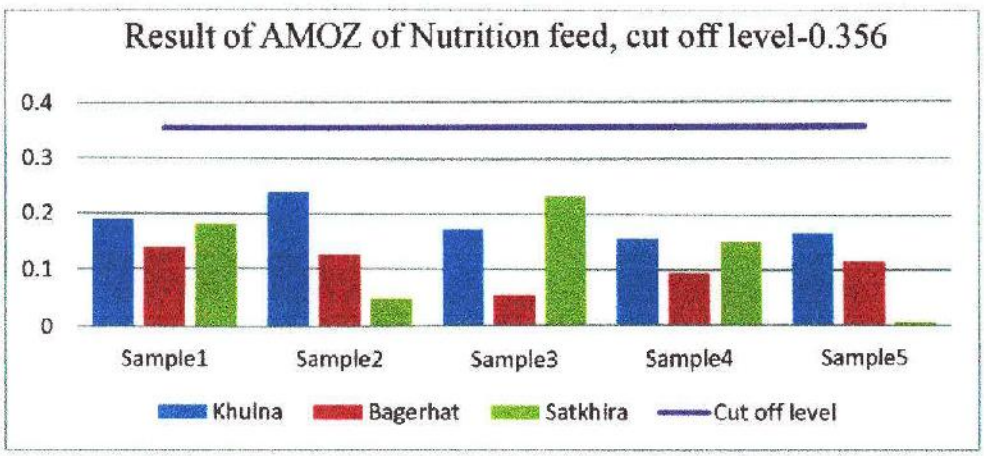

Fig.16. Concentration (ppb) of AMOZ in Nutrition feed

Result of CAP

4.17. Concentration (ppb) value of CAP in Aman feed

The cut-off level of CAP (Cholramphenicol) in shrimp feed is 0.109. In the present study in all the experimental sites concentration of CAP in feed were found below the cut-off level. The highest value of CAP in case of feed was found in sample 3 of Satkhira district and the lowest value was found in sample 3 of Bagerhat district. Maximum value was 0.015 ppb and minimum value was 0.002 ppb.

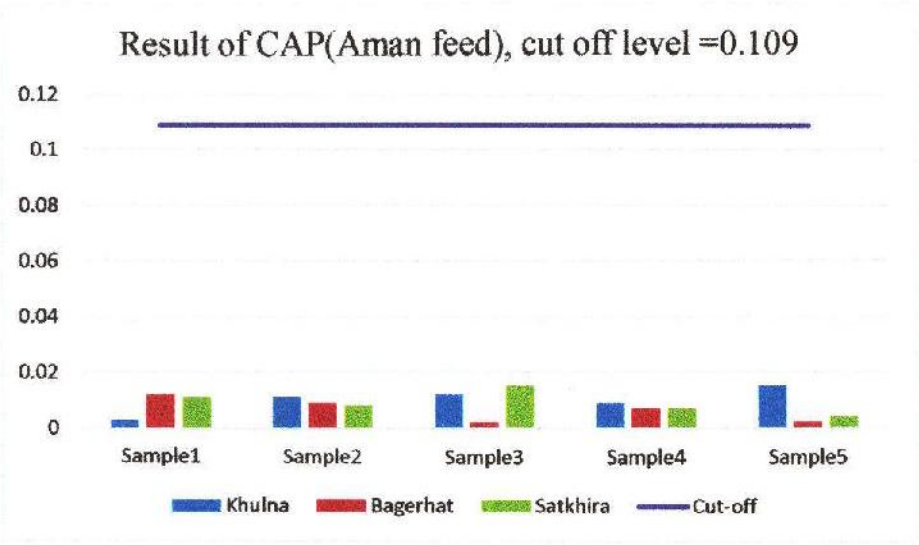

Fig.17. Concentration (ppb) of CAP in Aman feed

4.18. Concentration (ppb) value of CAP in CARE feed

Concentration (ppb) of CAP in CARE feed is below the cut-off level. Maximum value was 0.015 ppb in sample 3 of Satkhira District and minimum value was 0 in sample 1 of Bagerhat.

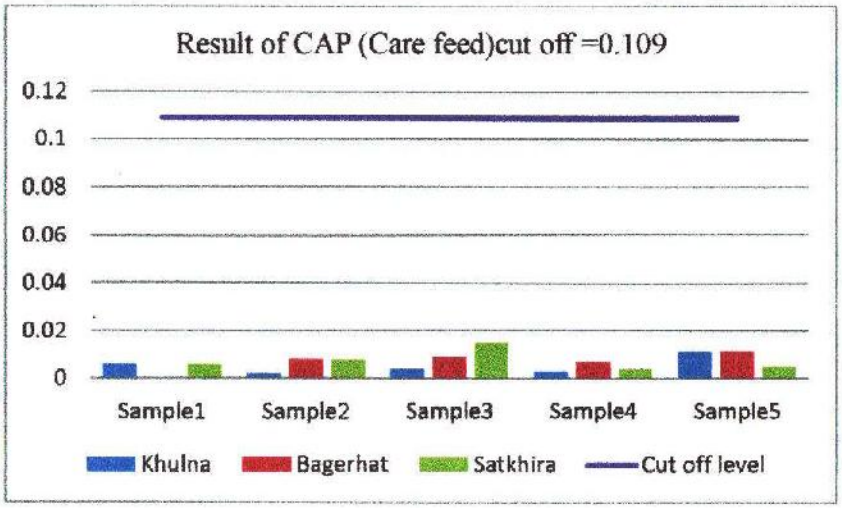

Fig.18. Concentration (ppb) of CAP in CARE feed

4.19. Concentration (ppb) value of CAP in PCF feed

Concentration (ppb) of CAP in PCF feed was below the cut-off level. Maximum value was 0.015 ppb in sample 3 of Satkhira and minimum value was 0.001 ppb in sample 1 of Khulna.

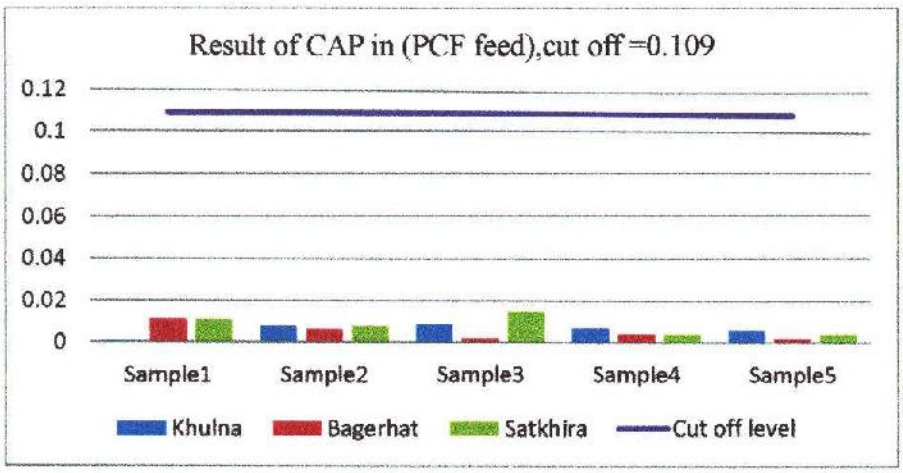

Fig.19. Concentration (ppb) of CAP in PCF feed

4.20. Concentration (ppb) value of CAP in Nutrition feed

Concentration (ppb) of CAP in Nutrition feed was below the cut-off level. Maximum value was 0.015 ppb and minimum value was 0.003 ppb.

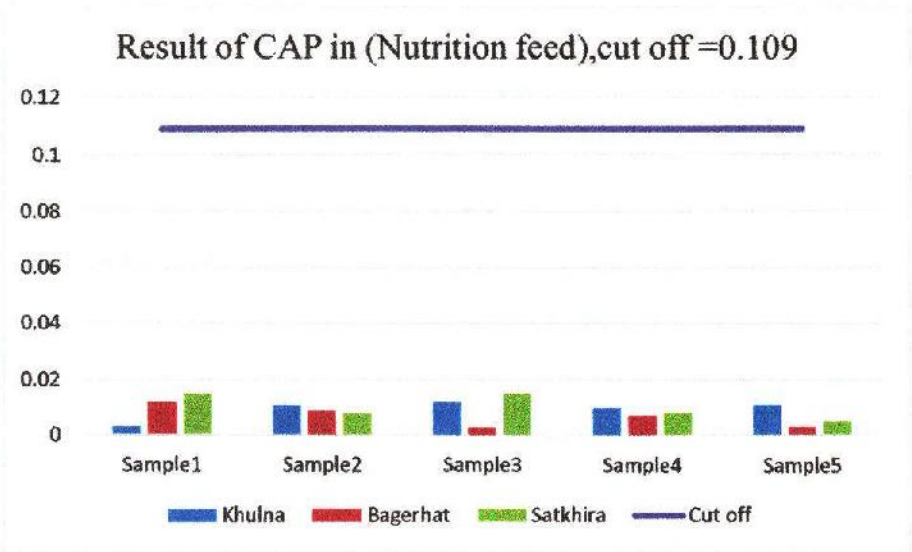

Fig.20. Concentration (ppb) of CAP in Nutrition feed.

Table 1. A summary table of concentration of Antibiotic residues in feed of different types

Concentration (ppb)		Aman feed	Care feed	PCF feed	Nutrition feed
SEM (cut off level-0.576)	Max	1.069	1.539	1.535	0.24
	Average	0.940	0.284	0.406	0.1918
	Std. Deviation	±.356	±.082	±.563	±.191
AOZ (cut off level-0.371)	Max	0.108	0.123	0.138	0.116
	Average	0.0522	0.0696	0.0799	0.0618
	Std. Deviation	±.052	±.0696	±.1918	±.0618
AHD (cut off level-0.424)	Max	0.106	0.154	0.146	0.108
	Average	0.049	0.072	0.0706	0.049
	Std. Deviation	±.0495	±.072	±.0706	±.049
AMOZ (cut off level-0.356)	Max	0.234	0.192	0.191	0.238
	Average	0.150	0.10	0.1024	0.1378
	Std. Deviation	±.150	±.100	±.102	±.1378
CAP (cut off level =0.109)	Max	0.015	0.015	0.015	0.015
	Average	0.008	0.0066	0.006	0.0088
	Std. Deviation	±.008	±.0066	±.0065	±.0088

Average concentration of antibiotic residues in four kinds Shrimp feed

Table-2. Average concentration of SEM in Shrimp feed

Region	Average concentration of SEM			
	Aman feed	Care feed	PCF feed	Nutrition feed
Khulna	0.3282	0.4548	0.7986	0.1618
Bagerhat	0.1818	0.1594	0.1916	0.1814
Satkhira	0.2412	0.24	0.2288	0.2322

Table-2. Average concentration of AOZ in Shrimp feed

Region	Average concentration of AOZ			
	Aman feed	Care feed	PCF feed	Nutrition feed
Khulna	0.0536	0.0548	0.0946	0.0696
Bagerhat	0.0524	0.0896	0.0758	0.0602
Satkhira	0.0506	0.0644	0.0694	0.0556

Table-2. Average concentration of AHD in Shrimp feed

Region	Average concentration of AHD			
	Aman feed	Care feed	PCF feed	Nutrition feed
Khulna	0.0676	0.0528	0.094	0.0628
Bagerhat	0.033	0.092	0.0434	0.0328
Satkhira	0.048	0.0726	0.0744	0.0522

Table-2. Average concentration of AMOZ in Shrimp feed

Region	Average concentration of AMOZ			
	Aman feed	Care feed	PCF feed	Nutrition feed
Khulna	0.1836	0.0774	0.137	0.185
Bagerhat	0.1034	0.126	0.0674	0.1048
Satkhira	0.1634	0.0974	0.1028	0.1236

Table-2. Average concentration of CAP in Shrimp feed

Region	Average concentration of CAP			
	Aman feed	Care feed	PCF feed	Nutrition feed
Khulna	0.01	0.0052	0.0062	0.0094
Bagerhat	0.0064	0.007	0.0062	0.0068
Satkhira	0.009	0.0076	0.0084	0.0102

Chapter-5

Discussion

6. Discussion

Feed samples of four company named Aman feed, CARE feed, PCF feed and Nutrition feed were collected from Khulna, Bagerhat and Satkhira districts. Five farms from each district and a total of 15 farms were selected for this study. From the test result some feed samples were found contaminated with antibiotics.

Contamination of commercial feed with Nitrofuran metabolites SEM was found as one the major findings of the present study. Residue range of SEM was found between (0.022-1.992) ppb. SEM levels of some feed crosses cut off level 0.576 ppb. Other metabolites AHD, AOZ, AMOZ were also found. Residue range of those metabolites was-AOZ (0.0290-.377) ppb, AHD (0.010-.154) ppb , AMOZ(0.0070-.238)ppb. These were found below cut off level. Other antibiotic CAP was also found. Its residue range was between (0-0.015) ppb. This was below cut off level 0.109 ppb. Level of Tetracycline was so low that the machine couldn't measure the range.

According to Hassan et al., 2013, the level of SEM in shrimp/prawn muscles was found to be increased, in contrary to the reduced levels of other metabolites. Nitrofurans might have transmitted to prawns and shrimps through ingestion of contaminated aqua feeds or ingredients used in feed. Naturally farmed crustacean flora may often be contaminated by SEM because SEM naturally occurs in many crustaceans like shrimp, prawn and crab (Pereira et al., 2004; Saari and Peltonen, 2004).

From data on post export rejection of Bangladeshi prawn revealed that most of the consignments were rejected only due to the presence of SEM metabolites. From different data of 2008, 2009 and 2010 it was found that SEM contamination although nil in 2008, it was found 37.21% in 2009 and 54.55% in 2010. On the other hand, AHD contamination was 80.95% in 2008 but reduced to 18.60% in 2009 and became nil in 2010 (Hassan et al., 2013).

The study revealed the presence of highest SEM Concentration in Khulna which was 1.992 ppb and this value was much higher than cut off level (0.562 ppb). In four samples SEM concentration crossed cut off level. The concentration was found 1.069 ppb in Aman feed , 1.535ppb and 1.992 ppb in two samples of Care feed and 1.539 ppb in PCF feed. From

comparison with previous data of 2008, 2009 and 2010 it was found SEM concentration was higher than before.

The study also revealed that other metabolites of Nitrofuran - AOZ, AHD and AMOZ was found below cut off level. CAP (chloramphenicol) was also found below of its cut off level 0.109 ppb.

From the study of Hassan et al., (2013), it was found prawns raised in farms fed with commercial feed were contaminated with nitrofuran metabolites more than prawns raised in farms fed with home-made feed with organic and inorganic fertilizer only. Farms operated without feed or fertilizers had no such antibiotic contamination at all.

From the examination of four types of commercial feed, three types of feed (Aman, Care and PCF feed) were found contaminated with SEM. It is a matter of disappointment for the export industry of Bangladesh. Cause of using antibiotics in feed could be its ability to maximize shrimp's/ prawn's growth. The other reason could be the ignorance of the manufacturer about the harmful effects of antibiotics.

European Commission, 2002, reported that aquaculture products from Asian countries are frequently contaminated by AOZ and SEM.

A consultant at the Bangladesh Shrimp Foundation- a multilateral facility to support industry-told that it was widely believed nitrofuran came to prawns from the feed, which are sourced from markets .Stakeholders said the health hazardous element comes from feeds, although the use of nitrofuran is prohibited in Bangladesh, with some suspecting that the element is coming through illegal channel of trade.

Recommendations

1. A detailed and consecutive investigation is needed whether shrimp & prawn feed can be a possible source of banned antibiotics.
2. A detailed investigation on the source of contamination of shrimp with banned antibiotics is essential and as a first step it prompts a systematic analysis of feed and feed ingredients used in the shrimp farming in Bangladesh.
3. Before using feed and feed ingredients for the production of shrimp/prawn test should be done to see whether it contains antibiotics or not.

Chapter-6

Conclusion

6. Conclusion

Data on antibiotic residue revealed that some commercial feed contaminated with nitrofuran metabolites SEM. Other antibiotics were below cut off level. Nitrofurans are a class of drugs typically used as antibiotics or antimicrobials. It has been prohibited from use in food-producing animals in most countries due to public health and safety concerns, particularly in relation to the carcinogenic potential of either the parent compounds or their metabolites. The use of these in food production is already banned in the United States, Australia, Canada, Japan, Singapore, Bangladesh, and the European Union because of a possible increased cancer-risk through long-term consumption. While importing feeds for animals, shrimps and prawn, provision for providing certificate guaranteeing the prawn and animal feeds to be nitrofuran-free has been made mandatory. As shrimp and prawn industry play an important role in export sector of Bangladesh, this sector requires special attention. The increasingly complex requirements for food safety assurance and traceability set by major export markets represent a threat to the trade of this significant sector. Several studies shows that percent of other nitrofuran metabolites except SEM has been decreased significantly. Strictly screening of all commercial feeds should be done and any chance of deliberative, accidental or incidental leakage should be checked.

Chapter-7

References

References

Aftabuddin, S., Kader, A., Kamal, A. M., & Zafar, M. (2009). Present status on the use of antibiotics and chemicals in shrimp hatcheries and grow-out ponds and their environmental implications in Bangladesh. *Aquaculture, Aquarium, Conservation & Legislation-International Journal of the Bioflux Society (AACL Bioflux)*, *2*(4).

Barua, P. (2010). Nitrofuran: Pull the Trigger to Safeguard the National Interest. *Aquanet Magazine*, (1), 20-22.

Cooper, K. M., McCracken, R. J., & Kennedy, D. G. (2005). Nitrofurazone accumulates in avian eyes—a replacement for semicarbazide as a marker of abuse. *Analyst*, *130*(6), 824-827.

Commission Regulation, 1995. 1442/95, 1995, 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 foodstuffs of animal origin. Official J. Eur. Communities L143, 26–30.

Dierberg, F. E., & Kiattisimkul, W. (1996). Issues, impacts, and implications of shrimp aquaculture in Thailand. *Environmental Management*, *20*(5), 649-666.

Draisci, R., Giannetti, L., Lucentini, L., Palleschi, L., Brambilla, G., Serpe, L., & Gallo, P. (1997). Determination of nitrofuran residues in avian eggs by liquid chromatography–UV photodiode array detection and confirmation by liquid chromatography–ionspray mass spectrometry. *Journal of Chromatography A*, *777*(1), 201-211.

DoF. 2006. Status of frozen food export. Department of Fisheries. Ministry of fisheries and livestock Bangladesh.

DoF, 2015. National Fisheries Week 2015. Department of Fisheries, Bangladesh.

European Commission, 2002. Commission Decision 2002/ 657/EC, 2002, of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official J. Eur. Communities L221, 8–36.

Finzi, J. K., Donato, J. L., Sucupira, M., & De Nucci, G. (2005). Determination of nitrofuran metabolites in poultry muscle and eggs by liquid chromatography-tandem mass spectrometry. *Journal of chromatography B*, *824*(1-2), 30-35.

Farhana akter.,2017. The export trend of shrimp industries in Bangladesh: an analysis.

Gräslund, S., & Bengtsson, B. E. (2001). Chemicals and biological products used in south-east Asian shrimp farming, and their potential impact on the environment—a review. *Science of the Total Environment*, *280*(1-3), 93-131.

Granados-Chinchilla, F., & Rodríguez, C. (2017). Tetracyclines in food and feedingstuffs: From regulation to analytical methods, bacterial resistance, and environmental and health implications. *Journal of analytical methods in chemistry, 2017.*

Hurley, P. M. (1998). Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. *Environmental health perspectives*, *106*(8), 437.

Hossain, M. S., Uddin, M. J., & Fakhruddin, A. N. M. (2013). Impacts of shrimp farming on the coastal environment of Bangladesh and approach for management. *Reviews in Environmental Science and Bio/Technology*, *12*(3), 313-332.

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

IMO/FAO/UNESCO-IOC/WMO/WHO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection. (1997). *Towards safe and effective use of chemicals in coastal aquaculture* (No. 65). Food & Agriculture Org..

Khong, S. P., Gremaud, E., Richoz, J., Delatour, T., Guy, P. A., Stadler, R. H., & Mottier, P. (2004). Analysis of matrix-bound nitrofuran residues in worldwide-originated honeys by isotope dilution high-performance liquid chromatography– tandem mass spectrometry. *Journal of agricultural and food chemistry*, *52*(17), 5309-5315.

Kennedy, G., 2010. Seafood Importers and Processor Alliance. Royal Flemish Academy of Belgium for Science and Arts. SIPA Press Release. 24th November, 2010.

Mohammad Chhiddikur Rahman, Md. Abdus Salam, Niaz Md. Farhat Rahman, Mohammad Mijanur Rahman and Md. Monir Hossain .,2013. Present Status and Potentiality of Shrimp in Bangladesh.

M.C. Carson., M.A. Ngoh., S.W. Hedley., J. Chromatogr.,1998. b712 (1998) 113.

McCracken, R. J., & Kennedy, D. G. (1997). Determination of the furazolidone metabolite, 3-amino-2-oxazolidinone, in porcine tissues using liquid chromatography-thermospray mass spectrometry and the occurrence of residues in pigs produced in Northern Ireland. *Journal of Chromatography B: Biomedical Sciences and Applications*, *691*(1), 87-94.

Nouws, J.F.M., Laurensen, J., 1990. Post mortal degradation of furazolidone and furaltadone in edible tissues of calves. Vet. Q. 12,56–59.

Nowsad, A. K. M., Islam, M. R., & Faruk, M. A. R. (2009). Studies on nitrofuran contamination in exportable shrimp and prawn products. *Progressive Agriculture*, *20*(1-2), 143-151.

Pereira, A. D. S., Donato, J. L., & 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*(1), 63-69.

Primavera, J. H., Lavilla-Pitogo, C. R., Ladja, J. M., & de la Peña, M. R. (1993). A survey of chemical and biological products used in intensive prawn farms in the Philippines. *Marine Pollution Bulletin*, *26*(1), 35-40.

Ruangpanich, N. (1988). Some problems in tiger shrimp hatcheries. *Brackish water Division, Department of Fisheries, Bangkok.*

Shotts Jr, E. B., Vanderwork, V. L., & Campbell, L. M. (1976). Occurrence of R factors associated with Aeromonas hydrophila isolates from aquarium fish and waters. *Journal of the Fisheries Board of Canada*, *33*(4), 736-740.

Saari, L., & Peltonen*, K. (2004). Novel source of semicarbazide: levels of semicarbazide in cooked crayfish samples determined by LC/MS/MS. *Food additives and contaminants* , *21*(9), 825-832.

Shamsuzzaman, M. M., & Biswas, T. K. (2012). Aqua chemicals in shrimp farm: A study from south-west coast of Bangladesh. *The Egyptian Journal of Aquatic Research*, *38*(4), 275-285.

U.S. Code of Federal Regulations, Title 21, Part 556, Sections 150, 500, and 720. U.S. Government Printing Office, Washington DC, 2003, (Chapter 1).

Uddin, M. B., Sultana, T., Rahman, M. M., Uddin, M. B., Sultana, T., & Rahman, M. M. (2013). Shrimp export marketing of Bangladesh. *Bangladesh Res Publ J*, *8*(2), 146-151.

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

Appendix

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
SEM	Aman	15	.28473	.356159	.091960	.08750	.48197	.046	1.539
	CARE	15	.19180	.082504	.021302	.14611	.23749	.044	.377
	PCF	15	.40633	.563248	.145430	.09442	.71825	.022	1.992
	Nutrition	15	.19180	.082504	.021302	.14611	.23749	.044	.377
	Total	60	.26867	.341318	.044064	.18049	.35684	.022	1.992
AOZ	Aman	15	.05220	.019480	.005030	.04141	.06299	.029	.108
	CARE	15	.06960	.020532	.005301	.05823	.08097	.042	.123
	PCF	15	.19180	.082504	.021302	.14611	.23749	.044	.377
	Nutrition	15	.06180	.019850	.005125	.05081	.07279	.037	.116
	Total	60	.09385	.072039	.009300	.07524	.11246	.029	.377
AHD	Aman	15	.04953	.027761	.007168	.03416	.06491	.010	.106
	CARE	15	.07247	.040276	.010399	.05016	.09477	.011	.154
	PCF	15	.07060	.039239	.010131	.04887	.09233	.011	.146
	Nutrition	15	.04927	.027963	.007220	.03378	.06475	.010	.108
	Total	60	.06047	.035266	.004553	.05136	.06958	.010	.154
AMOZ	Aman	15	.15013	.058423	.015085	.11778	.18249	.047	.234
	CARE	15	.10027	.054898	.014175	.06987	.13067	.009	.192
	PCF	15	.10240	.055677	.014376	.07157	.13323	.010	.200
	Nutrition	15	.13780	.066289	.017116	.10109	.17451	.007	.238
	Total	60	.12265	.061525	.007943	.10676	.13854	.007	.238
CAP	Aman	15	.00847	.004324	.001116	.00607	.01086	.002	.015
	CARE	15	.00660	.003906	.001009	.00444	.00876	.000	.015
	PCF	15	.00653	.003907	.001009	.00437	.00870	.001	.015
	Nutrition	15	.00880	.004039	.001043	.00656	.01104	.003	.015
	Total	60	.00760	.004081	.000527	.00655	.00865	.000	.015

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
SEM	Between Groups	.465	3	.155	1.356	.266
	Within Groups	6.408	56	.114		
	Total	6.873	59			
AOZ	Between Groups	.194	3	.065	32.353	.000
	Within Groups	.112	56	.002		
	Total	.306	59			
AHD	Between Groups	.007	3	.002	2.086	.112
	Within Groups	.066	56	.001		
	Total	.073	59			
AMOZ	Between Groups	.028	3	.009	2.724	.053
	Within Groups	.195	56	.003		
	Total	.223	59			
CAP	Between Groups	.000	3	.000	1.321	.277
	Within Groups	.001	56	.000		
	Total	.001	59			

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Feed	(J) Feed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
SEM	Aman	CARE	.092933	.123519	.875	-.23413	.42000
		PCF	-.121600	.123519	.759	-.44867	.20547
		Nutrition	.092933	.123519	.875	-.23413	.42000
	CARE	Aman	-.092933	.123519	.875	-.42000	.23413
		PCF	-.214533	.123519	.315	-.54160	.11253
		Nutrition	.000000	.123519	1.000	-.32707	.32707
	PCF	Aman	.121600	.123519	.759	-.20547	.44867
		CARE	.214533	.123519	.315	-.11253	.54160
		Nutrition	.214533	.123519	.315	-.11253	.54160
	Nutrition	Aman	-.092933	.123519	.875	-.42000	.23413
		CARE	.000000	.123519	1.000	-.32707	.32707
		PCF	-.214533	.123519	.315	-.54160	.11253
AOZ	Aman	CARE	-.017400	.016332	.712	-.06064	.02584
		PCF	-.139600*	.016332	.000	-.18284	-.09636

		Nutrition	-.009600	.016332	.935	-.05284	.03364
		Aman	.017400	.016332	.712	-.02584	.06064
	CARE	PCF	-.122200*	.016332	.000	-.16544	-.07896
		Nutrition	.007800	.016332	.964	-.03544	.05104
		Aman	.139600*	.016332	.000	.09636	.18284
	PCF	CARE	.122200*	.016332	.000	.07896	.16544
		Nutrition	.130000*	.016332	.000	.08676	.17324
		Aman	.009600	.016332	.935	-.03364	.05284
	Nutrition	CARE	-.007800	.016332	.964	-.05104	.03544
		PCF	-.130000*	.016332	.000	-.17324	-.08676
		CARE	-.022933	.012536	.271	-.05613	.01026
	Aman	PCF	-.021067	.012536	.343	-.05426	.01213
		Nutrition	.000267	.012536	1.000	-.03293	.03346
		Aman	.022933	.012536	.271	-.01026	.05613
	CARE	PCF	.001867	.012536	.999	-.03133	.03506
		Nutrition	.023200	.012536	.261	-.00999	.05639
AHD		Aman	.021067	.012536	.343	-.01213	.05426
	PCF	CARE	-.001867	.012536	.999	-.03506	.03133
		Nutrition	.021333	.012536	.332	-.01186	.05453
		Aman	-.000267	.012536	1.000	-.03346	.03293
	Nutrition	CARE	-.023200	.012536	.261	-.05639	.00999
		PCF	-.021333	.012536	.332	-.05453	.01186
		CARE	.049867	.021542	.107	-.00717	.10691
	Aman	PCF	.047733	.021542	.131	-.00931	.10477
		Nutrition	.012333	.021542	.940	-.04471	.06937
		Aman	-.049867	.021542	.107	-.10691	.00717
	CARE	PCF	-.002133	.021542	1.000	-.05917	.05491
		Nutrition	-.037533	.021542	.312	-.09457	.01951
AMOZ		Aman	-.047733	.021542	.131	-.10477	.00931
	PCF	CARE	.002133	.021542	1.000	-.05491	.05917
		Nutrition	-.035400	.021542	.363	-.09244	.02164
		Aman	-.012333	.021542	.940	-.06937	.04471
	Nutrition	CARE	.037533	.021542	.312	-.01951	.09457
		PCF	.035400	.021542	.363	-.02164	.09244
		CARE	.001867	.001478	.590	-.00205	.00578
	Aman	PCF	.001933	.001478	.562	-.00198	.00585
CAP		Nutrition	-.000333	.001478	.996	-.00425	.00358
	CARE	Aman	-.001867	.001478	.590	-.00578	.00205
		PCF	.000067	.001478	1.000	-.00385	.00398

		Nutrition	-.002200	.001478	.451	-.00611	.00171
		Aman	-.001933	.001478	.562	-.00585	.00198
	PCF	CARE	-.000067	.001478	1.000	-.00398	.00385
		Nutrition	-.002267	.001478	.425	-.00618	.00165
		Aman	.000333	.001478	.996	-.00358	.00425
	Nutrition	CARE	.002200	.001478	.451	-.00171	.00611
		PCF	.002267	.001478	.425	-.00165	.00618

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

SEM

Tukey HSD

Feed	N	Subset for alpha = 0.05
		1
CARE	15	.19180
Nutrition	15	.19180
Aman	15	.28473
PCF	15	.40633
Sig.		.315

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.

AOZ

Tukey HSD

Feed	N	Subset for alpha = 0.05	
		1	2
Aman	15	.05220	
Nutrition	15	.06180	
CARE	15	.06960	
PCF	15		.19180
Sig.		.712	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.

AMOZ

Tukey HSD

Feed	N	Subset for alpha = 0.05
		1
CARE	15	.10027
PCF	15	.10240
Nutrition	15	.13780
Aman	15	.15013
Sig.		.107

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.

AHD

Tukey HSD

Feed	N	Subset for alpha = 0.05
		1
PCF	15	.00653
CARE	15	.00660
Aman	15	.00847
Nutrition	15	.00880
Sig.		.425

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.

CAP

Tukey HSD

Feed	N	Subset for alpha = 0.05
		1
PCF	15	.00653
CARE	15	.00660
Aman	15	.00847
Nutrition	15	.00880
Sig.		.425

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.