Antibiotic Residues and Dyes in the Prawn Culture Sector of Khulna Region

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DECLARATION

Antibiotic Residues and Dyes in the Prawn Culture Sector of Khulna Region

The results presented in this project 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

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Abstract

The present investigation was conducted to find out the status and trends of the presence of chloramphenicol, nitrofuran metabolites and dyes in freshwater prawn *Macrobrachium rosenbergii* in the Khulna region of Bangladesh. All data on chloramphenicol, nitrofuran metabolites and dyes presence in prawn muscles during 2013 to 2017 were collected from Fish Inspection and Quality Control (FIQC) Khulna wing of the Department of Fisheries. Presence of antibiotic residues in the prawn muscles was detected using ELISA screening and LC–MS–MS by the FIQC Wing of the Department of Fisheries. The study exposed less deliberate use of those banned antibiotics and dyes though those chemicals were detected in many *M. rosenbergii* in 2013, 2014 2015, 2016 and 2017 in both fresh muscles and pre-export consignments. The percentage of contamination with these banned antibiotics in *M. rosenbergii* samples detected by LC–MS–MS Confirmation test were 0.62%, 0.22%, 0.30%, 0.35% and 0.04% in 2013, 2014, 2015, 2016 and 2017 respectively while 0.01%, 0.06%, 0.16%, 0.03% and 0.11% samples were detected with dyes which were beyond Minimum Required Performance Limit (MRPL) or Maximum Residual Limit (MRL). The result of the present study also revealed that among four nitrofuran metabolites (AOZ, AMOZ, AHD, SEM), nitrofurazone was found more frequently but in smaller quantities in 2014 and 2017; while an increasing trend was observed on the presence of dyes in prawn of Bangladesh during 2013 to 2017 except 2016. Moreover, only 3 samples were contaminated with chloramphenicol beyond MRPL with a mean value of 0.22±0.01 and 0.39±0.00 ppb in 2013 and 2014. Finally, there has been a decreasing trend observed on the presence of nitrofuran and chloramphenicol in prawn of Bangladesh. On the contrary an increasing trend was noticed in case of dyes in prawn of Bangladesh.

No farmers deliberately used nitrofuran, chloramphenicol and dyes in their prawn farms. Though these banned items were detected in many the farmed prawns. More monitoring system should be conducted to the control illegal use of these veterinary drugs. To protect this all commercial feed should be strictly screened and any chance of deliberative, accidental or incidental leakage should be checked.

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List of abbreviations and non-English words

Abbreviation	Elaboration
AHD	1-aminohydantoin
AOZ	3-amino-2-oxazolidinone
AMOZ	3-amino-5-morpholino-methyl-1,3-oxa-zolidinone
CAP	Chloramphenicol
DOF	Department of Fisheries
EC	European Commission
et al.	et alli; Latin word meaning "and others "
EFSA	European Food Safety Authority
ELISA	Enzyme linked immuno-adsorbent assay
EU	European Union
FIQC	Fish Inspection and Quality Control
FMRT	Fisheries and Marine Resource Technology
GOB	Government of Bangladesh
KG	Kilogram
LC	Liquid chromatography
MS	Mass spectrometry
NRCP	National residue Monitoring Control Plan
PPM	Parts Per Million
RASFF	Rapid Alert System for Food and Feed
SEM	Semicarbazide
%	Percentage
MRPL	Minimum Required Performance Limit
PPB	Parts Per Billion

Chapter One

INTRODUCTION

Chapter 1

Introduction

1.1 Background

Aquaculture in Bangladesh has developed rapidly and provides income and quality protein for national and overseas consumers, e.g. 48,574 metric tons of prawn (*Macrobrachium rosenbergii*) were produced during 2016-17 (DOF, 2017). The rapid development and intensification of culture practices have been associated with serious disease outbreaks and economic losses. Ideally any therapeutic treatment of an infectious disease should be based on a correct diagnosis including identification of the pathogen involved before any treatment takes place. The reality for most aquaculture farmers in less developed countries like Bangladesh however, is quite different with limited availability and access to such diagnostic services. Farmers seem to base their choices of disease prevention and treatment measures on previous experiences, anecdotal advice from shops selling and distributing antibiotics, antimicrobials and other chemicals like dyes, and less often taking advice from veterinarians who do not have the capacity and knowledge to provide diagnostic services (Hossain *et al.,* 2018). However, there is limited knowledge on how farmers perceive and diagnose diseases and what determines their choice of type of treatment, e.g. use of antimicrobials, antibiotics and so on. 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.*, 2018).

In recent years, many Southeast Asian prawn/shrimp exporting countries have been facing difficulties in meeting the present food safety standards of the importing countries.

Because in many developed countries, consumer awareness of the potential health hazards of antibiotic residues, chloramphenicol and dyes in prawn and the desire of producers to avoid litigation, prompted the development of biological and chemical tests to monitor the presence of antibiotic residues, chloramphenicol and dyes and establish the identity of specific residues in positive prawn tissues. Since 2005, the number of notifications of Rapid Alert System for Food and Feed (RASFF) post forwarded by the European Commission (EC) has been increased against exported seafood of many countries, particularly for the *M. rosenbergii* (DoF, 2009). To meet the requirements, the Government of Bangladesh (GoB) imposed a voluntary suspension on the export of *M. rosenbergii* for six months to EU countries executed from 20 May 2009 to 19 November 2009. In order to identify the source of contamination and prevent the entrance of banned antibiotics in the shrimp value chain, the GoB has adopted various reforms in shrimp/prawn sector. These include implementation of training programs for the officials of the Department of Fisheries (DoF, 2009) and the stakeholders of prawn value chain, strengthening of monitoring activities under National Residue Monitoring Control Plan (NRCP), testing of the presence of banned antibiotics in prawn meat and adopting measures to implement traceability in the total shrimp value chain (DoF, 2009).

In 1995, the use of nitrofurans for 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). Whether these antimicrobials, antibiotics remain in the sediment or leach into the surrounding water of aquaculture, the end result is still selection of antimicrobial and antibiotic-resistant bacteria (Davies and Davies, 2010; Marshall and Levy, 2011; Buschmann *et al.*, 2012).

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). The half-life of this parent drug is 7–63 min. This instability results in rapid depletion of nitrofurans in blood and tissue (Nouws and Laurensen, 1990). However, the formed metabolites (AOZ, AMOZ, AHD and SEM) bind with the protein in the body and remain for many weeks after treatment (Cooper *et al.*, 2005). Therefore, tracing of nitrofuran metabolites in prawn muscle is important for adequate control of these contaminants. Since 2006, DoF has been involved in detecting nitrofuran drugs, other antibiotics, antimicrobials and dyes in prawn products.

In contrast to the large therapeutic arsenal to fight against mammalian diseases, the use of pharmaceutical substances is rather limited in scope in fish and seafood farming, and it has always been basically limited to some anesthetic substances and to anti-infective and antimicrobial agents against parasitic and microbial diseases. As a consequence, the unregulated use of dye chemicals from the family of the triphenylmethane dyes, malachite green (MG), a common commercial and inexpensive fabric dye, has developed and been used as a therapeutic multi-usage drug to globally reduce parasitic, microbial, and fungal diseases found in fish and seafood farming. MG has, for instance, been used both prophylactically and in the treatment of fungal infections for fish and eggs for more than 80 years. In the course of the 1980s, 1990s, and 2000s, many concerns were raised in regard to the toxicity of this substance, and different toxicological studies were carried out for MG and for some other similar dyes applied or potentially applied for their therapeutic qualities in fish farming. MG has now been banned in nearly all of the regions

of the world, including North America and Europe, but can still be present in various inappropriate fish farming practices around the world.

Recently, the Joint WHO/FAO Expert Committee on Food Additives (JECFA) has evaluated the risk for public health of the use of MG and crystal (gentian) violet (CV) in fish farming. The Codex Committee on Residues of Veterinary Drugs in Foods has recommended that competent authorities should not permit their use in food-producing animals including fish/seafood farming. This should therefore lead to an absence of detectable residues in products from this industry. However, they still appear to be present, probably because they are still widely used in the textile industry and elsewhere and are commercially available as inexpensive therapeutic chemicals for ornamental fish. In addition, the dyes are persistent in the sediment of water sources for aquaculture and will be absorbed and bioaccumulated in aquaculture tissues over time. As a result of these assessments and recommendations, several countries and the EU since 2004 have assigned a specific food safety concern to these substances and mandated that they should be actively controlled in food products and food trading derived from the fish and seafood farming industry.

There have been trade issues associated with certain dye compounds used as veterinary medicines, particularly with MG and its chemically related congeners in aquaculture. This chapter is intended to review these pharmacologically active dyes from their chemistry and toxicological concerns to their regulatory monitoring in aquaculture products due to their undesirable presence in aquaculture-sourced foods.

The present investigation is a follow up research undertaken to review and analyze the status and trends of the use of nitrofuran drugs and chloramphenicol (CAP), antimicrobials and dyes in the farmed prawns in the Khulna region of Bangladesh.

1.2 Objectives

- To find out the recent status and trends of the presence of nitrofuran and chloramphenicol in freshwater prawn *Macrobrachium rosenbergii* (De Man) in the Khulna region of Bangladesh.

- To track down the recent status and trends of the presence of dyes in freshwater prawn *M. rosenbergii* (De Man) in the Khulna region of Bangladesh.

Chapter Two

LITERATURE REVIEW

Chapter 2

Literature Review

Hartig *et al.* (2005) conducted research on nitrofurans. According to his report, nitrofurans are frequently employed in the poultry and fish production for their excellent antibacterial, pharmacokinetic and growth promoting properties. Due to a multi-stakeholder involvement in the chain of production, transportation, marketing and processing of fish and shrimp in Bangladesh, it seems increasingly difficult to identify the sources of antibiotic contamination. Such contamination in fish foods may occur from the water supply, feed, hatchery produced seed, etc. and by the primary producers and other traders in the handling and distribution chain and finally by the processors and packers. If such unethical contamination continued, rapidly expanding fisheries sector will be doomed, millions of people will be unemployed and finally, the county's economy will be devastated. Considering such backdrop the study was conducted to investigate the source of nitrofuran drugs in shrimp and prawn through participatory stakeholder based approach.

Chloramphenicol is an antibiotic that is not approved for use in food-producing animals and has been prohibited from extra-label uses in food-producing animals by the US and EU countries. It is suspected to be a carcinogen and may potentially affect the reproductive system in humans as cited by the US Department of Agriculture (2013).

In aquaculture, nitrofurans, as cited in the study of Hassan, *et al.* (2013), are commonly used as growth promoter and in the prophylactic and therapeutic treatments of bacterial and protozoan infections such as gastrointestinal enteritis, fowl cholera, and *coccidiosis* black heads. The use of nitrofurans for livestock production was completely prohibited in the EU due to concerns about the carcinogenicity of the drug residues and their potential harmful effects on human health.

Meanwhile, malachite green (MG) is a synthetic dye used to color fabric and paper. It has been used around the world in treating external fungal and parasitic infections on fish eggs, fish and shellfish. MG is an effective fungicide, especially as a general hatchery disinfectant. It is not registered for use in aquaculture and is prohibited for use in aquaculture products intended for human consumption.

The potential presence of these antibiotics and chemicals in major aquaculture products of the Philippines, particularly milkfish and shrimp, is one of the reasons that might be holding back the country from reaching its full potential in supplying the international market. "The country should be able to produce acceptable test certificates for antibiotic residues. This can be done now because we have the available Liquid Chromatograph/Mass Spectrometer/Mass Spectrometer (LC-MS-MS), the instrument required to produce the acceptable analysis," explained Dr. Santiago. LC-MS-MS is used in analytical chemistry as a powerful technique that has very high sensitivity and selectivity and so is useful in many applications. Its application is oriented towards the separation, identification, and quantification of chemicals of particular masses in the presence of other chemicals.

Given this, a project titled, "Monitoring of *Chloramphenicol, Nitrofuran Metabolites* and *Malachite Green* in Aquaculture Feeds, Bangus and Shrimps for Regulatory and Trade Purposes" is being implemented by RASL under the leadership of Dr. Santiago. RASL is the first laboratory in the entire UP System that is ISO-17025 accredited, the international standard for competence of laboratories.

Hassan *et al.,* (2013) conducted research on monitoring the presence of chloramphenicol and nitrofuran metabolites in cultured prawn, shrimp and feed in the southwest coastal region of Bangladesh. He reported that the nitrofuran antibiotics have been widely used for the treatment of infectious diseases in cattle, pigs, poultry and aquaculture.

Vass *et al.,* (2008) depicted nitrofuran parent drugs, furazolidone, nitrofurazone, nitrofurantoin and furaltadone and their related structures. These parent compounds metabolise rapidly after ingestion to form corresponding tissue bound metabolites (Nouws and Laurensen, 1990; McCracken *et al.*, 1995). *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. If nitrofuran remains in food, it causes mutagenesis, carcinogenicity and terato genesis (Ebringer *et al.*, 1982, Czeizel *et al.*, 2001). It has been reported that furazolidone overdose in mammals may not only cause muscle convulsions but also can lead to nerve breakage throughout the body (Tsaia *et al.*, 2009). Nitrofurazone can also inhibit sperm production in mice (George *et al.*, 1996). In both birds and mammals, nitrofurans are rapidly metabolized following oral administration and the

majority of a dose is quickly eliminated in the urine, but metabolites bindeasily to tissues and can persist at low concentrations for weeks following treatment (Craine & Ray, 1972; Vass *et al.*, 2008).

The rapid metabolism of nitrofurans has made screening for parent drugs difficult in food products, but the development of assays that can detect nitrofuran metabolites have been used to demonstrate the persistence of residues in egg yolk and albumen (McCracken *et al.*, 2001; Stachel *et al.*, 2006).As a result of their rapid metabolism, nitrofuran parent substances are not suitable for monitoring and typically the irmetabolites are analyzed. For example 3-amino-2-oxazolidinone (AOZ) is the metabolites of furazolidone, which can be released from bound tissues under acidicconditions and detected by reaction with 2-nitrobenzaldhydeto produce 2-nitrophenylmethylene-3-amino-2-oxazolidinone (NPAOZ) (Vass *et al.*, 2008), the occurring reaction is shown in Figure 2 (Chung-Wei Tsaia *et al.*, 2009).Because of the toxicity of nitrofuran, the European Union (EU) banned the use of nitrofuran antibiotics in food producing animals (Commission Decision 2003/181/EC, Cooper *et al.*, 2004) and set the minimum required performance limit (MRPL: "minimum content of an analyte ina sample, which at least has to be detected and confirmed") for the detection of nitrofuran residues (metabolites) in food of animal origin at 1µg/kg (Commission Decision 2003/181/EC). Thus, a sensitive and reliable method for the determination of nitrofuran at residual levels is very important.

In the pas tdecades, several analytical methods have been developed for the screening and quantitation of nitrofuran metabolites in foods and biological samples. Some methods already reported for the determination of nitrofurans e.g. detection of AOZ (Cooper *et al.*, 2004) by enzyme immunoassay (ELISA) has been used for the detection of AOZ (3-amino-2-oxazolidone) in prawn tissue, quantitative determination off our nitrofuran metabolites in chicken meat by HPLC-MSMS (Mottier *et al.*, 2005), analysis of matrix-bound nitrofuran residues in honey by HPLC-MSMS (Khong *et al.*, 2004).However, Ultra Performance LC (UPLC) and MS/MS (Quattro Premier XE) is a more sophisticated technique following a very effective isolation of analyte ions from the noise-producing matrix. Following the EU decision2002/657/EC the goal of present study is to optimize UPLC–MS/MS technique for a simultaneous method development, Validation and confirmatory analysis of nitrofuran metabolites(AMOZ, AOZ, AHD and SEM)) by assessing its derivatives in Katla Fish (*Katlakatla*) and Bagda shrimp (*Peniousmonodon*) by UPLC MS/MS(QPXE).

Studies examining the bioavailability of nitrofuran metabolites have demonstrated the possibility of residual transfer to secondary species. When rats were fed pig tissue containing radio-labelled (14C) furazolidone, 41% of the total amount consumed was made bio available to the rat. Bioavailability can occur through the ingestion of contaminated meat and animal products (such as eggs), even after cooking (Gottschall and Wang, 1995; McCracken and Kennedy, 1997), as well as by transfer to the progeny of hens (McCracken *et al.*, 2001, 2005a; Finzi *et al.*, 2005) emphasing the health risk for consumers.

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 authors determined that between 67% and 100% of the residues remained present in the tissue after cooking, frying, grilling, roasting and microwaving. Another study demonstrated that AOZ in egg was stable up to (at least) 12 months during storage at 4°C, and that 78% of AOZ occurs in the yolk as opposed to albumin (McCracken *et al.*, 2001).

Recently it was also found that 50% of total SEM residues in egg were found in the shell, which may be significant if an eggshell product reaches the consumer (McCracken and Kennedy, 2007).The opinion of the European Food Safety Authority (EFSA) on the presence of the nitrofurazone metabolite semicarbazide in food has been published (European Food Safety Authority, 2005). On the basis of the difference in magnitude between experimental animals and humans (including infants), as well as the use of sensitive methodology (i.e. intraperitoneal administration of medicine resulting in direct exposure of the uterus to high concentrations of chemicals), the EFSA concluded that the issue of carcinogenicity is not a concern for human health at the concentrations of SEM encountered in food (European Food Safety Authority, 2005). Although, it should be noted that nitrofurazone, nitrofurantoin, furaltadone and furazolidone are depicted on the State of California Proposition 65 Carcinogens List (US Environmental Protection Agency, 2008). The global nitrofuran crisis during 2002–2003 revealed frequent findings of tissue bound residues in poultry and aquaculture products imported to EU countries from Thailand, China, Taiwan, India, Vietnam, Ecuador and Brazil (Anon, 2008). Moreover, nitrofuran residues were also found in poultry and pork muscle produced in European countries such as Portugal, Italy, Greece,

Romania and Bulgaria (O'Keefe *et al.,* 2004). Later inspection by EU authorities, revealed nitrofuran contamination in products originating from over nine countries in 2007, the highest incidences being from India (37%), China (37%), Bangladesh (10%) and Thailand (5%) in a variety of products including shrimp, honey and canned meat (European Commission, 2008). Despite strict legislation banning its use in food animal production in the EU, nitrofurans continue to be used due to their effectiveness and availability, as is evident from the European Commission Rapid Alert System for Food and Feed (RASFF).

The RASFF, in place since 1979, provides regulatory authorities with an effective tool for the exchange of information regarding measures taken to ensure food safety in European Union countries (European Commission, 2008).The notifications made regarding prohibited nitrofurans are published in the RASFF Weekly Overviews in 2007 and 2008 (until week 37). It is evident that aquaculture products from Asian countries are frequently contaminated by AOZ and SEM. The highest concentration of AOZ was 150 μg/kg in frozen peeled black tiger shrimps from India. However, findings of nitrofurans at lower concentrations (10–63 μg/kg) were not rare.

The emerging issue of the presence of nitrofurazone metabolite SEM in edible tissue of non-animal origin has caused an increase in public awareness in recent years. From the total of nitrofuran metabolites notified by the RASFF, SEM was the highest with 48.9%, 60.9% and 71.0% of all nitrofuran notifications in years 2004, 2005 and 2006, respectively, although a decline in 2007 (31%) was evident (Commission Regulation, 2002). Product notifications for semicarbazide contamination have included not only food stuffs of animal origin such as aquaculture products (shrimp, prawn and crab), bovine and porcine tissue, poultry and chicken eggs but also in products such as baby food and flour (European Commission, 2008).

SEM in baby food have caused great concern for infant health and resulted in the development of appropriate detection methods (De Souza *et al.,* 2005; Ginn *et al.,* 2006). It was found that azodicarbonamide, a foaming agent used in gasket production decomposes into gases (primarily nitrogen and carbon dioxide) during the heat treatment process and can leave trace amounts of residues such as biurea, urazole, cyanuric acid and cyamelide (European Commission, 2003). Moreover, studies have confirmed the presence of SEM as a by-product of azodicarbonamide treated gaskets in jarred foods (Stadler *et al.,* 2004).

Potentially susceptible products include jams, honey, fruit juices, pickles, sterilised products, mayonnaise, mustard, and ketchup. Currently azodicarbonamide is suspended from use in EU countries (Commission Directive, 2004).

The formation of SEM during the baking of bread (Becalski *et al.*, 2004, 2006) and flour-coated poultry products (Hoenicke *et al.*, 2004) was also confirmed when the use of azodicarbonamide as an additive in flour was examined. SEM formation has also been observed in samples such as carrageen (a seaweed extract used as a food additive), starch and egg white powder treated with hypochlorite solutions containing 12% active chlorine (Hoenicke *et al.*, 2004). Hypochlorite is commonly used for carrageen bleaching or water disinfection and also as a disinfectant during egg breaking procedures (de la Calle and Anklam, 2005). SEM has also been found to occur naturally in particular crustaceans such as shrimp, prawn, and crab, generating queries over its suitability as a marker for detection purposes in these species (Pereira *et al.*, 2004; Saari and Peltonen, 2004; Hoenicke and Gatermann, 2006).

Several veterinary drugs are illegal for use in food-producing animals in the UnitedStates because of their toxicity to humans, their linkage diseases, and antibiotic to fatal resistance in human pathogens including Bacillus and Vibrio species. The Food and Drug Administration is responsible for the safety of all fish and fishery products entering the United States, but funding for testing is limited. State testing has repeatedly resulted in the detection of banned veterinary drugs. Current testing and enforcement may be insufficient. Contaminated product is still entering the country because exporting ountries often don't have sufficient resources for alternative means of combating disease.

Previous studies have focused on the use of veterinary drugs in aquaculture, including the history of their use, their impacts on the environment and human health, the toxicity of historically used antimicrobials, and alternatives to unsafe veterinary drugs. Methods have been developed for the rapid screening of animal and foodsamples using enzyme-linked immunosorbent assays (ELISA), and several methods have been developed for the detection of veterinary drug residues. The best and most2sensitive method is high-pressure liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS). The instrument significantly reduces background signal and the purpose of this study was to screen for and

confirm the presence of illegal veterinary drug residues in Prawn. Commercially available Prawn samples were tested for chloramphenicol, fluoroquinolones, malachite green, and nitrofurans, which are drugs that have high enforcement priority in the United States due to their adverse health effects. The methods used to confirm the presence of residues were procedures preferred by FDA laboratories for the detection of drug and chemical residues in food.

The general objective of the project is to generate a national database that regulatory agencies and aquaculture farmers will be able to use in controlling chemical residues in Prawns and milkfish production. "With the development of the capability to detect the levels of residues that is acceptable to the export market, our country can start to build up a national database on product contamination and, depending on the data that will be generated, we can institute control policies on the use of chemicals/drugs in aquaculture," added Dr. Santiago.

Chapter Three

MATERIALS AND METHODS

Chapter 3
Materials and Methods

3.1 Study area and period

The study was carried out in Khulna devision during August-December, 2017. The study area was selected because of its contributing role to prawn aquaculture and frozen fishery export. About 80% of total *M. rosenbergii* production are coming from this division which accounted for 70% of total shrimp/prawn export in 2009–2010 (DoF, 2011).

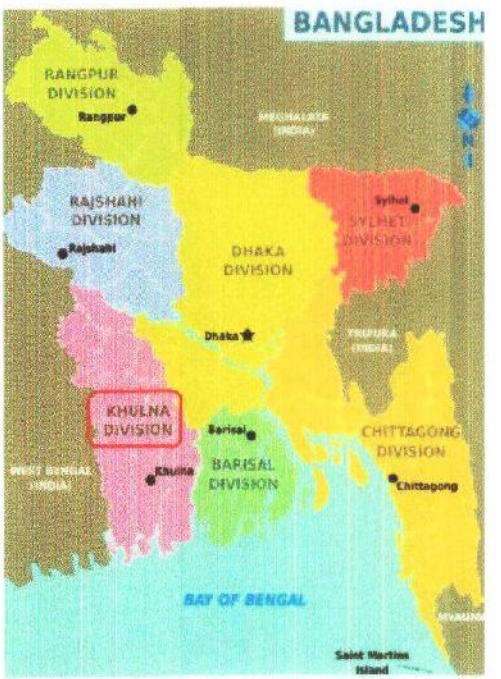

Fig. 1 Map of study area

3.2 Collection of data on antibiotic residue detection

All data on antibiotics presence in prawn muscles during 2013 to 2017 were collected from Fish Inspection and Quality Control (FIQC) Khulna wing of the DoF. Antibiotic residues present in the animal muscle were detected by the Fish Inspection and Quality Control (FIQC) wing of the DoF. For the detection of nitrofuran metabolites and chloramphenicol, FIQC followed the method of US FDA/CFSAN (2004) and Young (2003).

3.3 Statistical analysis

The collected data were scrutinized and summarized carefully and then they were tabulated. Data were analyzed using MS excel (2010) software and IBM SPSS Statistics (Version 20.0.0.0). One way ANOVA test was performed to identify any significant difference among year-wise collected data.

Chapter Four

RESULTS

Chapter 4
Results

4.1 Year wise trend of antibiotic and dyes contamination in prawn muscle

Year-wise trends of contamination of prawn by banned antibiotics and dyes during 2013 to 2017 are given in Table 1. In accordance with LCMS-MS Confirmation, out of 3209, 3187, 3035, 3112 and 2753*M. rosenbergii* samples tested in 2013, 2014, 2015, 2016 and 2017 a total of 20, 7, 9, 11 and 1 samples were detected with banned antibiotics above Minimum Required Performance Limit (MRPL) and or maximum residual limit (MRL), respectively while 1,2,5,1 and3 were detected with banned dyes above Minimum Required Performance Limit (MRPL) respectively.

Table: 01 Year wise trend in antibiotic and dyes contamination of prawn muscle

Sample/ Year	Antibiotics and dyes	No of tested sample					No of contaminated sample *				
Year		2013	2014	2015	2016	2017	2013	2014	2015	2016	2017
M. rosenbergii	Antibiotics	3209	3187	3035	3112	2753	20	7	9	11	1
							0.62%	0.22%	0.3%	0.35%	0.04%
	Dyes	3209	3187	3035	3112	2753	1	2	5	1	3
							0.01%	0.06%	0.16%	0.03%	0.11%

* All were above MRPL (Minimum Required Performance Limit) or MRL (Maximum Residual Limit); MRPL for nitrofuran metabolites= Zero Tolerance; MRL for CAP=0.3 ppb and Dyes= 1-2 ppb

The percent of contamination with banned antibiotics namely nitrofuran metabolites and chloramphenicol were 0.62, 0.22, 0.3, 0.35 and 0.04, respectively in 2013, 2014, 2015, 2016 and 2017. Although once decreased in 2014, the degree of non-compliance sharply increased again in 2015 and 2016 in freshwater prawn muscles (Table 1).Furthermore, the percent of contamination with banned dyes were 0.01, 0.06, 0.16, 0.03 and 0.11, respectively in 2013, 2014, 2015, 2016 and 2017. While an increasing trend was observed in the degree of non-compliance of dyes during the whole period. Although once decreased in 2016, the degree of non-compliance sharply increased again in 2017 in freshwater prawn muscles (Table 1).

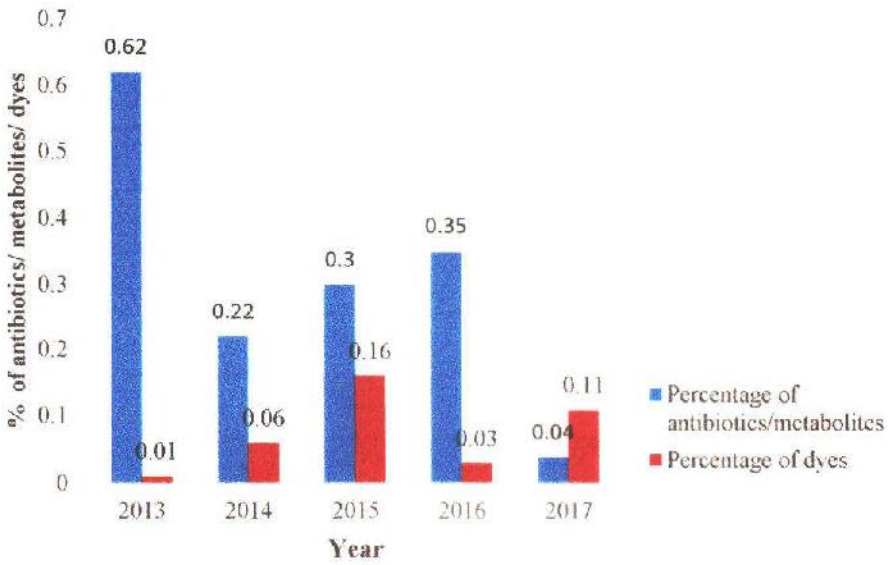

Fig 2. Year wise trend in antibiotic and dyes contamination of prawn muscle

4.2 Level of antibiotic and dyes contamination in prawn muscle

Different varieties of nitrofuran metabolites were found in prawn samples in different levels. In most cases more than one metabolite were found in one or more samples. The range, Mean±Stdv and % non-compliance of the detected levels in both ELISA Screening and LCMS-MS Confirmation test of nitrofuran metabolites, CAP and dyes is given in Table 2.

Table: 02 Level of nitrofuran metabolites, chloramphenicol and dyes (ppb) in prawn muscles

Year	Types of antibiotic /metabolites / dyes	ELISA Screening		LCMS-MS Confirmation			
		No. of contaminated sample*	% non-compliance	No of contaminated sample*	% non-compliance	Range (ppb)	Mean±Stdv (ppb)
2017	SEM	43	1.56	1	0.04	0.55-0.55	0.55±0.00
	AHD	1	0.04	ND	ND	ND	ND
	AOZ	ND	ND	ND	ND	ND	ND
	AMOZ	ND	ND	ND	ND	ND	ND
	CAP	ND	ND	ND	ND	ND	ND
	Dyes	14	0.51	3	0.11	1.09-1.23	1.18±0.08
2016	SEM	43	1.38	11	0.35	0.22-1.16	0.48±0.26
	AHD	ND	ND	ND	ND	ND	ND
	AOZ	ND	ND	ND	ND	ND	ND
	AMOZ	ND	ND	ND	ND	ND	ND
	CAP	ND	ND	ND	ND	ND	ND
	Dyes	2	0.06	1	0.03	1.21-1.21	1.21±0.00

Year	Types of antibiotic /metabolites / dyes	ELISA Screening		LCMS-MS Confirmation			
		No. of contaminated sample*	% non-compliance	No. of contaminated sample*	% non-compliance	Range (ppb)	Mean±Stdv (ppb)
2015	SEM	55	1.81	9	0.29	0.23-1.32	0.53±0.33
	AHD	3	0.10	ND	ND	ND	ND
	AOZ	ND	ND	ND	ND	ND	ND
	AMOZ	ND	ND	ND	ND	ND	ND
	CAP	3	0.10	ND	ND	ND	ND
	Dyes	8	0.26	5	0.16	0.69-1.31	0.95±0.28
2014	SEM	51	1.60	6	0.19	0.42-1.25	0.72±0.31
	AHD	12	0.38	ND	ND	ND	ND
	AOZ	ND	ND	ND	ND	ND	ND
	AMOZ	ND	ND	ND	ND	ND	ND
	CAP	06	0.19	1	0.03	0.39-0.39	0.39±0.00
	Dyes	9	0.28	2	0.06	1.46-1.73	1.59±0.19
2013	SEM	69	2.15	18	0.56	0.23-1.46	0.71±0.43
	AHD	7	0.22	ND	ND	ND	ND
	AOZ	ND	ND	ND	ND	ND	ND
	AMOZ	ND	ND	ND	ND	ND	ND
	CAP	8	0.25	02	0.06	0.21-0.22	0.22±0.01
	Dyes	13	0.41	01	0.03	1.89-1.89	1.89±0.01

* All were above MRPL (Minimum Required Performance Limit) or MRL (Maximum Residual Limit); MRPL for nitrofuran metabolites= Zero Tolerance; MRL for CAP=0.3 ppb and Dyes= 1-2 ppb; ND= Not Detected

4.2.1 ELISA Screening

A total of 3209 *M. rosenbergii* samples were tested for ELISA Screening, where 97 samples were found contaminated with banned antibiotics and dyes in 2013. Out of 97 prawn samples, SEM, AHD, CAP and dyes was detected in 69, 7, 8 and 13 samples.

In 2014, Out of 3187 prawn samples, 78 were found contaminated with banned antibiotics and dyes of which 51, 12, 06, 06 and 9 samples were detected with SEM, AHD, CAP and dyes respectively beyond MRPL or MRL.

However, 3035 prawn samples were tested during 2015, where 69 were detected contaminated with banned antibiotics and dyes of which 55, 3, 3 and 8 sample were found to be contaminate with SEM, AHD, CAP and dyes.

Sequentially, a total 3112 *M. rosenbergii* samples were tested where 45 were detected contaminated with banned antibiotics and dyes by ELISA Screening of which only 43 and 2 samples were detected with SEM and dyes contamination in 2016.

In 2017, out of 2753 sample which were ELISA Screened, 58 were detected to be contaminated with banned antibiotics and dyes in which 43, 1 and 14 were found to be contaminated with SEM, AHD and dyes.

4.2.2 LCMS-MS Confirmation

In 2013, out of 97 samples of *M. rosenbergii,* only 21 samples were confirmed to be contaminated with banned antibiotics and dyes by LCMS-MS Confirmation test where 18, 2 and 1 were contained SEM, CAP and dyes beyond MRPL or MRL. Range, mean±Stdv and % non-compliance of SEM, CAP and dyes were 0.23-1.46, 0.21-0.22 and 1.89-1.89 ppb; 0.71±0.43, 0.22±0.01 and 1.89±0.01 ppb; 0.56, 0.06 and 0.03 respectively (Table 2)(Fig 3).

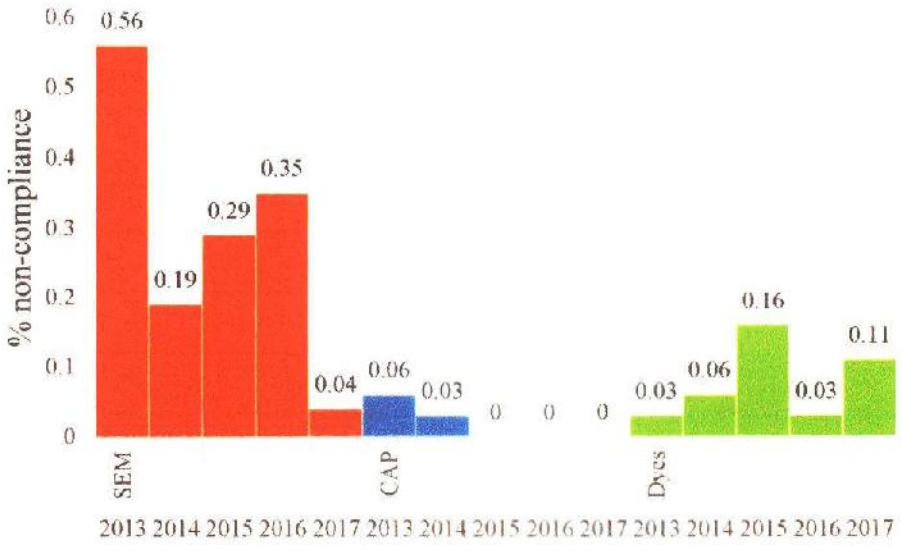

Fig 3. Year wise frequency of percentage of % non-compliance due to SEM, CAP and dyes contamination

In 2014, out of 78 samples of *M. rosenbergii,* only 9 samples were confirmed to be contaminated with banned antibiotics and dyes by LCMS-MS Confirmation test where 6, 1 and 2 were contained SEM, CAP and dyes beyond MRPL or MRL. Range, mean±Stdv and % non-compliance of SEM, CAP and dyes were 0.42-1.25, 0.39-0.39 and 1.46-1.73ppb; 0.72±0.31, 0.39±0.00 and 1.59±0.19 ppb; 0.19, 0.03 and 0.06 respectively (Table 2)

However, out of 69 samples of *M. rosenbergii,* 14 samples were confirmed to be contaminated with banned antibiotics and dyes by LCMS-MS Confirmation test where 9 and

5 were contained SEM and dyes beyond MRPL in 2015. Range, mean±Stdv and % non-compliance of SEM and dyes were 0.23-1.32 and 0.69-1.31 ppb; 0.53±0.33 and 0.95±0.28; 0.29 and 0.16 respectively (Table 2).

Sequentially, 45 samples of *M. rosenbergii were tested for* LCMS-MS Confirmation, where 11 and 1 samples were contaminated with banned SEM and Dyes beyond MRPL in 2016. Range, mean±Stdv and % non-compliance of SEM and dyes were 0.22-1.16 and 1.21-1.21 ppb; 0.48±0.26 and 1.21±0.00 ppb; 0.35 and 0.03 respectively (Table 2).

A total 58 samples of *M. rosenbergii* were tested for LCMS-MS Confirmation in 2017. Out of them only 1 sample was found to be detected with SEM while 3 were detected with banned dyes beyond MRPL. Range, mean±Stdv and % non-compliance of SEM and dyes were 0.55-0.55 and 1.09-1.23ppb; 0.55±0.00 and 1.18±0.08; 0.04 and 0.11 respectively (Table 2)

4.2.2.1 SEM

A total 18, 6, 9, 11 and 1 *M. rosenbergii* samples were detected with SEM with a mean residue of 0.71±0.43, 0.72±0.31, 0.53±0.33, 0.48±0.26 and 0.55±0.00 ppb in 2013, 2014, 2015, 2016 and 2017. The frequency of SEM contamination was decreased up to 2014, then again a sequential increasing trend was observed in SEM contamination level since 2016 (Fig 4 & 5).

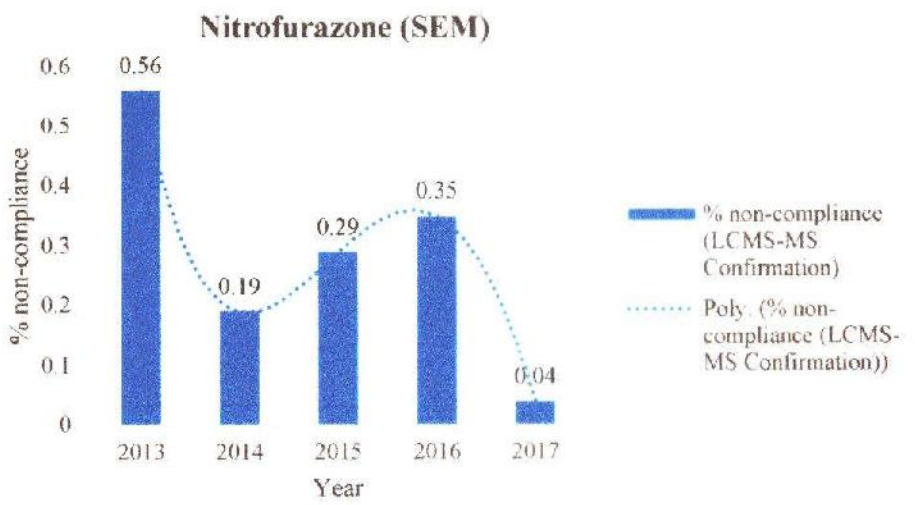

Fig 4. Frequency of percentage of % non-compliance due to SEM contamination

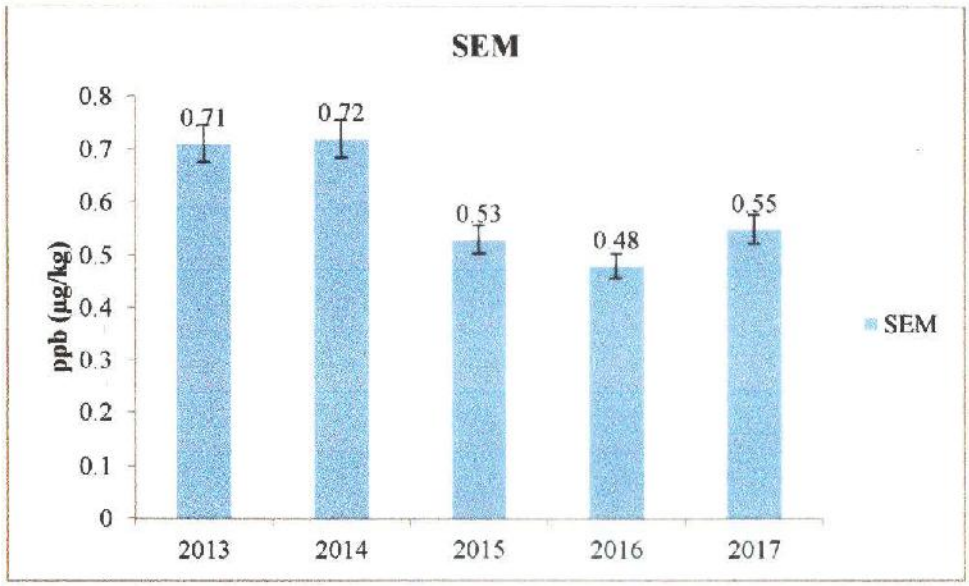

Fig 5. Year wise trend of mean±stdv SEM concentration of non-compliance prawn sample

4.2.2.2 CAP

In case of CAP, 2 and 1 samples were found to be contaminated with detected with CAP beyond MRPL with a mean value of 0.22±0.01 and 0.39±0.00 ppb in 2013 and 2014 (Fig 6 & 7). The frequency of CAP contamination was decreased up to 2014, then no sample was detected with CAP.

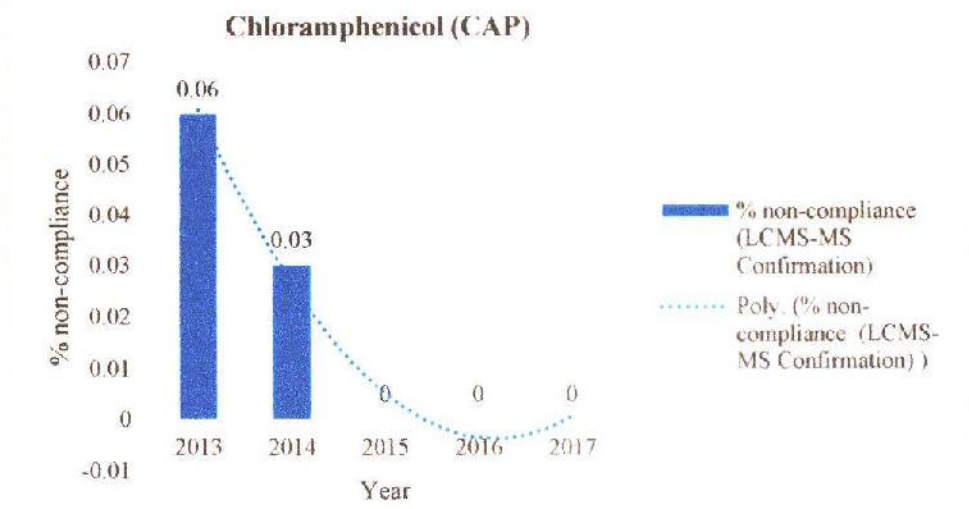

Fig 6. Frequency of percentage of % non-compliance due to CAP contamination

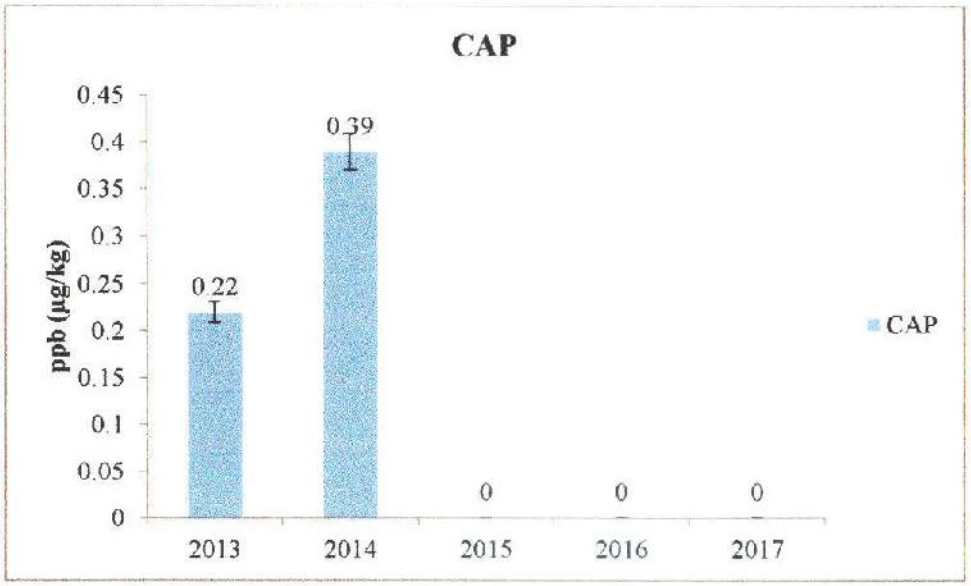

Fig 7. Year wise trend of mean±stdv CAP concentration of non-compliance prawn sample

4.2.2.3 Dyes

A total 1, 2, 5, 1 and 3 *M. rosenbergii* samples were detected with dyes with a mean residue of 1.89±0.01, 1.59±0.19, 0.95±0.28, 1.21±0.00 and 1.18±0.08 ppb in 2013, 2014, 2015, 2016 and 2017.The frequency of dyes contamination was increased since 2015, then fall in 2016 and again increasing trend was observed with high concentration in 2017 (Fig 8 & 9).

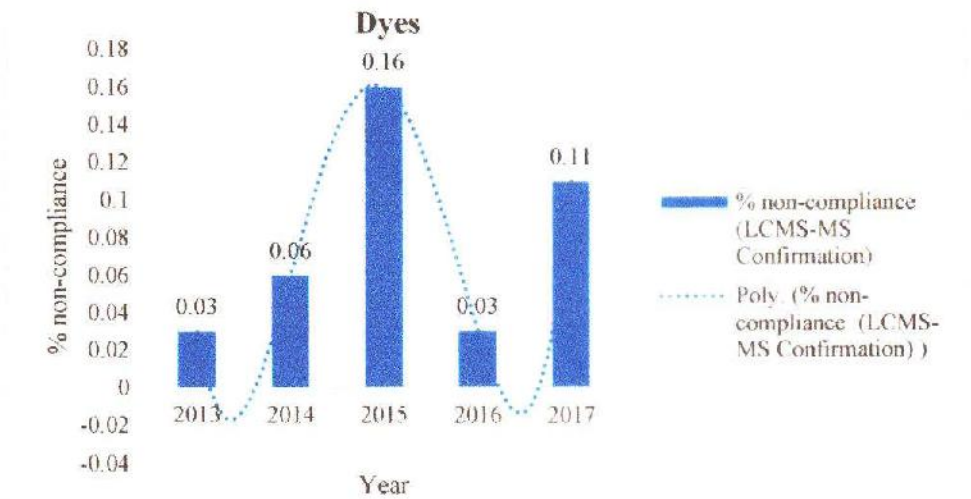

Fig 8.Frequency of percentage of % non-compliance due to dyes contamination

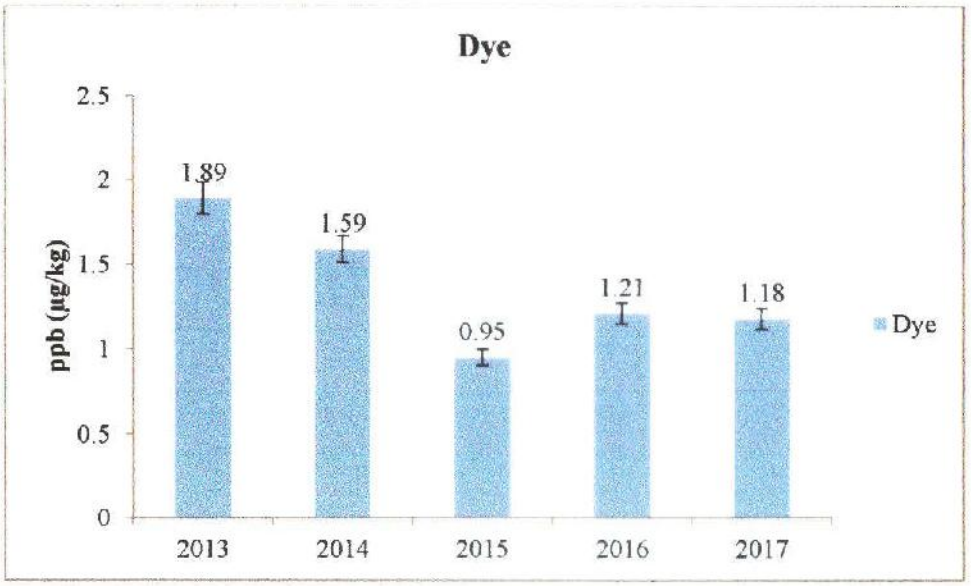

Fig 9. Year wise trend of mean±stdv CAP concentration of non-compliance prawn sample

Chapter Five

DISCUSSION

Chapter 5

Discussion

Antibiotic residue and dyes screening data of 2013, 2014, 2015, 2016 and 2017 when compared, some major remarked findings were come out. First notice was less deliberate use of those banned antibiotics and dyes though those chemicals were detected in many *M. rosenbergii* in 2013, 2014, 2015, 2016 and 2017 in both fresh muscles and pre-export consignments. Secondly, among four nitrofuran metabolites, nitrofurazone was found more frequently but in smaller quantities in 2014 and 2017; while an increasing trend was observed on the presence of dyes in prawn of Bangladesh during 2013 to 2017 except 2016. Thirdly, only 3 samples were contaminated with chloramphenicol beyond MRPL with a mean value of 0.22±0.01 and 0.39±0.00 ppb in 2013 and 2014. Thirdly, there has been a decreasing trend observed on the presence of nitrofuran and chloramphenicol in prawn of Bangladesh. Fourthly, no samples were found to be contaminated with AHD, AOZ and AMOZ. However, an important remarkable observation was pointed out, an increasing trend was noticed in case of dyes in prawn of Bangladesh. Above findings were more or less similar to the findings of Hassan *et al.* (2013), Pereira et al. (2004) and Saari and Peltonen (2004).

Pereira *et al.,* (2004) as well as Saari and Peltonen (2004) have also detected SEM in many crustacean muscles in Asian countries. However, the validity of SEM as a definite marker for veterinary antibiotic nitrofurazone has been questioned because of the discovery that SEM may arise from the sources other than veterinary antibiotics (Bock *et al.*, 2007).

There might be a possibility of SEM contamination from feed materials. Becalski *et al.* (2004) and Becalski *et al.* (2006) mentioned that SEM has been reported to be formed during the baking of bread. Addtionally, Hoenicke *et al.* (2004) reported that SEM presence in flourcoated poultry products. Moreover, Hoenicke *et al.,* (2004) observed SEM formation in starch and egg white powder as well as in carrageen, treated with hypochlorite solutions containing 12% active chlorine. There might be a possibility of SEM transformation from these sources into aquaculture water, sediments and animal muscles. Several reseachers mentioned that farmed crustaceans may often be contaminated by SEM naturally hence SEM naturally occurs in many crustaceans like shrimp, prawn and crab (Pereira *et al.,* 2004; Saari and Peltonen, 2004).

Poucke (2010) and Kennedy (2010) concluded that SEM was present as a natural component in the shell and the meat of all crustacean species from crabs to langoustines and shrimp. The metabolic route for its production in shrimp/prawn could not be confirmed, but a fundamental role in protein synthesis was thought to be one of the possibilities (Kennedy, 2010). This has generated queries over suitability of SEM detection as a test for the presence of nitrofuran in crustacean muscles (Hoenicke *et al.,* 2004). Kennedy (2010) mentioned that the SEM content in the inner core meat of prawn/shrimp, because the SEM detected in wild prawns was surface-associated and the SEM content in the inner meat was well below the MRL (1 ppb).

Cooper *et al.* (2004); Finzi *et al.* (2005); Szilagyi and de la Calle (2006) were reported that Furazolidone is used for the treatment of infectious diseases caused by *E. coli, Vibrio cholera* and *Salmonella* spp. in cattle, swine, fish, prawns, and poultry. Additionally, Draisci *et al.,* 1997 made a remark that it also to prevent and control bacterial and protozoan infections such as fowl cholera, coccidiosis blackheads and swine enteritis in poultry and pigs and mastitis in dairy cattle. While, McCracken and Kennedy (1997) and Cooper *et al.,* (2004) narrated that it is an important growth promoter in fish and poultry that promote weight gain and feed conversion efficiency. Hossain *et al.,* (2018) described the application of growth promoter in fish and shell fish health management in southwestern region of Bangladesh.

Present study it was also observed that SEM contamination although less in 2014 and 2017 was 0.56% and 0.29%, 0.35% in 2013, 2015 and 2016, respectively (Table 2). On the other hand, CAP contamination was 0.06% in 2013 but reduced to 0.03% in 2014 and became nil in 2015, 2016, 2017 (Table 2).

Moreover, it is a lamentable truth that, present study it was also revealed that an increasing trend of dyes contamination in prawn of Bangladesh, frequency of dyes contamination were 0.03%, 0.06%, 0.16%, 0.03% and 0.11% in 2013, 2014, 2014, 2015, 2016 and 2017 in Khulna region of Bangladesh. Verdon and Andersen (2017) reported that, dyes are primarily used as a treatment for fungal and external parasite infections in fish and to protect incubating eggs from fungus in aquaculture. Many of the dyes described from these chemical classes have antiseptic, antimicrobial, or other medicinal properties with uses in veterinary medicine. Many also have unique affinities for binding to different cellular components rendering these

therapeutic dyes excellent biological stains. Other dyes and pigment residues have been found in fish from environmental exposure to textile and manufacturing effluents15 as well as from food additives intentionally added to color seafood products.

Srivastava *et al.*, (2004) reported that malachite green is widely used in aquaculture as a parasiticide and in food. It controls fungal attacks, protozoan infections and some other diseases caused by helminths on a wide variety of fish and other aquatic organisms. However, the dye has generated much concern regarding its use, due to its reported toxic effects. The toxicity of this dye increases with exposure time, temperature and concentration. It has been reported to cause carcinogenesis, mutagenesis, chromosomal fractures, teratogenecity and respiratory toxicity. Histopathological effects of malachite green include multi-organ tissue injury. Significant alterations occur in biochemical parameters of blood in malachite green exposed fish. Residues of malachite green and its reduced form, leucomalachite green have been reported from serum, liver, kidney, muscles and other tissues as also from eggs and fry.

Several researchers reported that, the dyes residues in fish originate from its illicit use in fish farms or from environmental pollution as a result of its industrial applications and discharge of the waste water to the rivers and streams without any pre-treatment (Khodabakhshi and Amin, 2012; Pourreza and Elhami, 2010; Schuetze, Heberer, and Juergensen, 2008). The dyestuff is readily absorbed and distributed in different tissues of the exposed fish. It is extensively metabolized and transformed to a reduced colorless compound, leucomalachite green (LMG). However, Lee *et al.* (2010) was determinated of malachite green and crystal violet in processed shrimp products which also supported the recent findings as Srivastava *et al.* (2004), Bergwerff and Scherpenisse (2003), Fallah and Barani (2014).

As malachite green is not registered as a veterinary drug, its administration is not allowed in the European Union either. Nevertheless, the use of Malachite green, Leucomalachite green, Crystal violet, Leuco crystal violet had become an issue due to its toxicity effects. The ban on its use in aquaculture products should be ensured to avoid its potential adverse effects in humans. Therefore, more monitoring system as well as research should be conducted to the control illegal use of these veterinary drugs.

Chapter Six

CONCLUSION

Chapter 6

Conclusion

The objective of this research was to quantify veterinary drug residues in imported, farm-raised prawn and make this information available to the general public in order to evaluate the effectiveness of current imported seafood testing, thus contributing to the collective data on the topic. The use of unapproved veterinary drugs to treat prawn in aquaculture systems results in the accumulation of drug residues in the edible tissues of the prawn. The residue concentrations detected in this study are within the ranges described in the few published reports regarding veterinary drug residues in fish and prawn purchased at the retail level.

"Rapid Alert System for Food and Feed" from the data available, it appears that nitrofuran antibiotics are still used in some countries as growth promoters and prophylactic. The presence of nitrofuran residues in meat, aquaculture and other products originating predominantly from non-European countries has been well documented in recent years by the European agents because they are cheap and effective. Therefore, sampling procedures and monitoring plans for regulatory laboratories are necessary to ensure consumer safety. Present European legislation does not permit any confirmed concentration of nitrofuran residues in food commodities, although an MRPL of 1 μg/kg has been laid down by the European Commission for nitrofuran metabolites in edible tissues of animal origin. Detection of a parent nitrofuran or its metabolite below the concentration of 1 μg/kg requires enforcement action (product withdrawal, issue of alert notifications by the RASFF etc.) to be initiated. 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

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