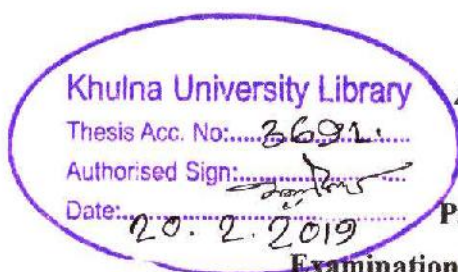


SCREENING OF SHRIMP FOR IDENTIFICATION OF PROHIBITED ANTIBIOTIC RESIDUE



A thesis

By

Papia Saha

Examination Roll No: MS- 170612

Session: 2017-2018



Fisheries and Marine Resource Technology Discipline

Khulna University

Khulna, Bangladesh

September, 2018

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A thesis submitted to Fisheries and Marine Resource Technology Discipline in
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In

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SCREENING OF SHRIMP FOR IDENTIFICATION OF PROHIBITED ANTIBIOTIC RESIDUE

Approved as to style and content

By


18.12.18

Dr. Ghausiatur Reza Banu

(Professor and Head)

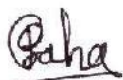
Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208
Bangladesh

September, 2018

DECLARATION
**SCREENING OF SHRIMP FOR IDENTIFICATION OF
PROHIBITED ANTIBIOTIC RESIDUE**

The results presented in the thesis with the above mentioned title 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.

Candidate



Papia Saha

Examination Roll No: MS- 170612

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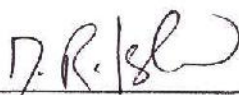
Co-Supervisor



Md. Jahidul Hasan

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

Supervisor



Md. Rashedul Islam

Associate Professor

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

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Abstract

The study was carried out to identify the existence of nitrofurans, chloramphenicol and tetracycline at different stages of shrimp muscle by using ELISA method. Antibiotic tested data of collected samples revealed that nitrofurans and chloramphenicol antibiotics were found in Shrimp muscle but for maximum samples the concentration were lesser in quantities. Only SEM was found above the cut off level in shrimp PL and PL after one week rearing in one farm of Khulna district and the concentration of SEM were 0.723 ppb and 0.920 ppb for shrimp PL and PL after one week rearing respectively. 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 muscle. Though SEM was found in PL and PL after one week in one farm of Khulna, the next stages (after one month & harvesting before one week) did not encompass harmful level of the residue. Other tested antibiotic residues comprised of AOZ, AMOZ, AHD and CAP were found below the cut off level. Present study did not reveal any big concern regarding presence of antibiotic residue in shrimp muscle; however, continuous monitoring is needed to reduce/prevent contamination of banned antibiotics in shrimp muscle.

CONTENTS

Contents		Page
Acknowledgement		i
Abstract		ii
Contents		iii
List of tables		iv
List of figures		iv-v
List of abbreviation		v
Chapter 01	INTRODUCTION	
	1.1 Background	1-3
	1.2 Objective of the study	4
Chapter 02	REVIEW OF LITERATURE	5-9
Chapter 03	MATERIALS AND METHODS	
	3.1. Sample Collection	10
	3.2. Study period	10
	3.3. Laboratory Procedure	10-21
	3.4. Data Analysis	22
Chapter 04	RESULT	23-45
Chapter 05	DISCUSSION	46-48
Chapter 06	CONCLUSION	49
Chapter 07	REFERENCES	50-54
Chapter 08	APPENDIX	55-57

List of Tables

Tables	Title	Page
1	Maximum and Minimum Concentration of Antibiotic Residue in different region	43
2	Average concentration of CAP	44
3	Average concentration of SEM	44
4	Average concentration of AOZ	45
5	Average concentration of AMOZ	45
6	Average concentration of AHD	45

List of figure

Figures	Title	Page
1	Concentration (ppb) of CAP in shrimp muscle (PL)	23
2	Concentration (ppb) of CAP in shrimp muscle (PL after 1 week)	24
3	Concentration of CAP in shrimp muscle (After 1 month)	25
4	Concentration of CAP in shrimp muscle (Harvesting before 1 week)	26
5	Concentration (ppb) of SEM in shrimp muscle (PL)	27
6	Concentration (ppb) of SEM in shrimp muscle (PL after 1 week)	28
7	Concentration of SEM in shrimp muscle (After 1 month)	29
8	Concentration of SEM in shrimp muscle (Harvesting before 1 week)	30
9	Concentration (ppb) of AOZ in shrimp muscle (PL)	31
10	Concentration (ppb) of AOZ in shrimp muscle (PL after 1 week)	32
11	Concentration of AOZ in shrimp muscle (After 1 month)	33
12	Concentration of AOZ in shrimp muscle (Harvesting before 1 week)	34
13	Concentration (ppb) of AMOZ in shrimp muscle (PL)	35
14	Concentration (ppb) of AMOZ in shrimp muscle (PL after 1 week)	36
15	Concentration of AMOZ in shrimp muscle (After 1 month)	37
16	Concentration of AMOZ in shrimp muscle (Harvesting before 1 week)	38

17	Concentration (ppb) of AHD in shrimp muscle (PL)	39
18	Concentration (ppb) of AHD in shrimp muscle (PL after 1 week)	40
19	Concentration of AHD in shrimp muscle (After 1 month)	41
20	Concentration of AHD in shrimp muscle (Harvesting before 1 week)	42

List of Abbreviation

Abbreviation	Elaboration
FIQC	Fish Inspection and Quality Control
DOF	Department of Fisheries
CAP	Cholramphenicol
TTC	Tetracycline
AOZ	3-Amino-2-Oxazolidinone
AMAZ	3-Amino-5-morpholino-methyl-1,3-Oxazolidinone
AHD	1-Aminohydroxy
SEM	Semicarbazide
ELISA	Enzyme Linked Immunosorbent Assay
MRPL	Minimum Required Performance Limit
MRL	Minimum Residual Limit
EU	European Union
NRCP	National Residue Control Plan
RASFF	Rapid Alert System for Food and Feed
ND	Not Detected

1.0 Introduction

1.1 Background

Shrimp, "The White Gold of Bangladesh" is the second largest export commodity of the country. In Bangladesh, shrimp farming is one of the most important sectors from the economical point of view and the position of this sector is second in terms of foreign currency earning. During the last three decades, this sector has drawn a special attention to the people due to its high export potential. Actually Bangladesh exports almost all shrimp to EU, USA and Japan (ASCC, 1995; Islam, 2008, Ahmed et al., 2008). In the export sector of Bangladesh, contamination with residues of banned carcinogenic antibiotic like nitrofurans metabolites, chloramphenicol and tetracyclines in frozen shrimp is a major concern of food safety. Though frozen shrimp sector represent the second largest exportable item contributing about 3.74% to gross domestic production (GDP) in 2013-2014 in Bangladesh (DoF, 2015).

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. Due to carcinogenic effect of nitrofurans, It has been declared completely unfit (zero tolerance limit) for human consumption (Finzi et al., 2005). 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 nitrofurans drugs (DoF, 2006). After ingestion, nitrofurans parent drugs are metabolized to furazolidone (AOZ), furaltodone (AMOX) 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, AMOX, AHD and SEM) bind with the protein in the body and remain for many weeks after treatment (Cooper et al., 2005).

Chloramphenicol (CAP) is a broad spectrum antibiotic that against gram-positive and gram-negative bacteria causes aplastic anemia. It has mentioned that chloramphenicol prohibited in USA and Japan and explicitly banned in Canada and European Union countries for its carcinogenic characteristics. However, it's used in shrimp culture of Latin America and Asia where shrimps are grown for export to USA, EU and Japan to control the diseases and also in

human and veterinary medicines (GEASAMP, 1997). Until now it's not possible to assess the CAP carcinogenicity due to lack of scientific information though CAP is treated as carcinogenic by IARC (International Agency for Research on Cancer) in human (Hanekamp et al., 2003).

Minimum Required Performance Limit (MRPL) of nitrofurans and chloramphenicol was established by EU for shrimp and prawn in 2003. MRPL of nitrofurans, especially furazolidone (AOZ), furaltadone (AMTZ), nitrofurantoin (AHD), nitrofurazone (SEM) was 1µg/kg whereas that of chloramphenicol was 0.3µg/kg. It indicates that designated laboratories must be able to detect nitrofurans and chloramphenicol at this concentration and importing countries will reject or destroy shrimp and prawn consignment above mentioned concentration level (Commission Decision 2003/181/EC). In 2008 and 2009, 18 and 44 consignments of Bangladesh respectively were rejected by EU due to presence of nitrofurans and chloramphenicol in shrimp and prawn muscle (DoF, 2014).

Tetracyclines (TCAs) are broad spectrum antibiotics that have been successfully used worldwide in both veterinary medicine and in aquaculture. Oxytetracycline (OTC) has been specially approved for use in dairy cattle and aquacultured cat-fish, salmon and lobster (US Code of Federal Regulation, 2003); this drug has 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 prevalence of TCA administration has raised concerns over the development of microorganism resistance to these drugs and also of human allergic reactions caused by the presence of TCA residues in food products as frequently consumed as shrimp and milk. 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).

In recent years, many Southeast Asian Countries especially Bangladesh has been facing difficulties to meet with the present food safety standards of the importing countries specially EU, USA and Japan. These countries have imposed a lot of non-tariff rules and regulations regarding food safety on the shrimp and prawn export of Bangladesh. To meet with the requirements, Government of Bangladesh (GOB) imposed a voluntary suspension on the export of prawn to EU countries for six months, executed from 20 May 2009 to 19 November 2009. To identify the sources of contaminations and prevent the entrance of banned antibiotics and chemicals in the shrimp value chain, Bangladesh Government has adopted many necessary steps.

These include implementation of training programs for the officials of Department of Fisheries (DoF) and the stakeholders of shrimp and prawn value chain, strengthening of monitoring activities under National Residue Monitoring Control Plan (NRCP), testing of the presence of banned antibiotics and chemicals in shrimp and prawn meat and different brands of feed and feed ingredients sample and adopting measures to implement traceability in the shrimp and prawn value chain in toto (DoF, 2009).

Therefore, a good analytical method is required to detect antibiotics in shrimp muscle and associated variables like feeds as well. Sensitive and rapid analytical methods have been developed to determine antibiotic residues in fish, such as microbiological assays (Kilinc, Meyer & Hilge, 2007), immunoassays (Kilinc & Cakli, 2008), liquid chromatography (Wen, Wang & Feng, 2006), capillary electrophoresis (Juan-García, Font & Picó, 2006), gas chromatography (Ding, Shen, Zhang, Jiang & Sun, 2005).

It is important to pay attention to this contamination because the potential hazards, such as allergies, toxic effects, acquisition of drug resistance in pathogens in the human body, as well as cancer (Martínez, 2009), are associated to the presence of these products in edible tissues. So, there is a global concern about the consumption of aquatic food containing low levels of antibiotics.

The aim of the present study was to apply ELISA method to the determination of Nitrofurans, Tetracycline and Chloramphenicol residues in Shrimp muscle. Intensive analysis, compilation, and interpretation of the collected data helped to understand the current situation of antimicrobial contamination in the shrimp industry of the country. The outcomes from this study would also be considered as a basis for further study of source identification of antibiotics in the shrimp value chain of Bangladesh.

1.2 Objective of the study

- To identify the existence of prohibited antibiotic residues in shrimp muscle (from larval stage to pre-harvesting adults) in the southwest region of Bangladesh.

2.0 Review of Literature

2.1 Act, rules and regulations

The constitution of Bangladesh (GB, 1972) gives more importance to food safety. Article 15 states "it shall be a fundamental responsibility of the state to secure the provision of the basic necessities of life including food" and article 18 states "the state shall regard the raising of the level of nutrition and the improvement of public health as moving its primary duties". Both articles indicate that the state must be ensured food safety in all levels of the state through legislation of appropriate laws.

The EU (Commission Regulation, 1995) mentioned that the use of nitrofurans for livestock production was completely prohibited in due to concerns about the carcinogenicity of the drug residues and their potential harmful effects on human health (Mccalla, 1983; Vroomen et al., 1990; Van Koten-Vermeulen, 1993). 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).

The EU established the maximum residue limit of Tetracycline & Oxytetracycline as 100 $\mu\text{g kg}^{-1}$ for muscle tissue, including salmonidae and finfish. Chromatographic analysis of tetracycline antibiotics in food was reviewed by Oka et al (2000).

EU (2003) amended Decision 2002/657/EC to set up Minimum Required Performance Limits (MRPLs) for certain residues in food of animal origin. In this amendment, it was mentioned that member states shall ensure the methods which used for detecting chloramphenicol, nitrofurantol metabolites and medroxyprogesterone substances meet the MRPLs set out in Annex II. As per Annex II, the MRPLs of nitrofurantol metabolites (SEM, AMOZ, AOZ and AHD), chloramphenicol and medroxyprogesterone are 1 $\mu\text{g/kg}$, 0.3 $\mu\text{g/kg}$ and 1 $\mu\text{g/kg}$ respectively. To protect human health, The European Union has established safe maximum residue limits (MRLs) for these drugs and other veterinary substances, for use as veterinary drugs in animal products entering in the human food chain. The use of veterinary drugs is regulated through EU Council

Regulation 2377/90/EC, which describes the procedure for establishing MRLs for veterinary medicinal products in foodstuffs of animal origin. Nitrofurans, for example furazolidone, had been widely used on food-producing animals, prior to their prohibition within the EU, because of their potential harmful effects on human health. Nitrofurans are included in Annex IV of the European Union Council Regulation that lists banned substances. For nitrofurans metabolite the MRPL was set at $1 \mu\text{g kg}^{-1}$ in aquaculture products.

Ioannis et al. (2005) mentioned that EU made several regulations and directives on food safety issues. Regulation EC 178/2002 states the general principles and obligations for exporting food to Europe. It has mentioned in the Article 11 of Regulation EC 178/2002 that the presence of chloramphenicol, nitroimidazoles, malachite green and nitrofurans residues excludes foods from exportation to EU. In addition to, Regulation EC 882/2004 explains the general procedures for official control, Regulation EC 852/2004 indicates the general hygienic requirements for all the food business operators and Regulation EC 853/2004 laid down additional specific requirements for food business related with foods of animal origin, including fishery products.

Legislating an act "Fisheries Hatchery Act, 2010" (GB, 2010) and a rules "Fisheries Hatchery Rules, 2011" (GB, 2011) Bangladesh Government has banned the use of chloramphenicol and nitrofurans in the production of shrimp and prawn PL (Post Larvae). Rule 11 (1) explicitly states that chemicals that mentioned in Group-A and Group-B of the Schedule 3 are prohibited totally in the hatcheries. On the other hand, Bangladesh Government has also banned the use of nitrofurans and chloramphenicol in fish feed and animal feed. Section 14(1) of "Fish and Animal Feed Act, 2010" (GB, 2011) states clearly that antibiotic, growth hormone, steroid and pesticide will not use in the fish and animal feed". The list of prohibited chemicals has mentioned in Group-A and Group-B of the Schedule 7 under "Fish and Animal Feed Rules, 2011".

2.2 Antibiotics and chemicals

GEASAMP (1997) mentioned that chloramphenicol and nitrofurantoin prohibited in USA and Japan and explicitly banned in Canada and European Union countries for its carcinogenic characteristics. However, these are used in shrimp culture of Latin America and Asia where shrimps are grown for export to USA, EU and Japan. GEASAMP also mentioned that chemicals applied in aquaculture farms in order to control the diseases. So, chemicals should be sold in the market with proper labeling-withdrawal period with reference to species and temperature, potential hazards to environment and human health (including treatment in case of accidental ingestion or contact), storage requirements and expiry date and disposal methods for unused products.

K. Holmstrom et al. (2003) found that about 74% shrimp farmers used antibiotics in the management of their ponds along the Thai coast. They used the antibiotics namely tetracyclines, quinolones, sulphonamides, chloramphenicol, gentamycin, trimethoprim, tiamulin and some unidentified antibiotics for preventive management and daily purpose. The authors assumed that the use of these antibiotics could develop antibiotic resistance amid the pathogens that caused infections of the cultured animals and humans.

Pfenning et al. (2003) reported that presence of chloramphenicol in animals is a burning question globally. Due to its fatal side effects, such as bone marrow depression, aplastic anaemia and other blood disorders, use of chloramphenicol was banned in food producing animals. But, it is finding in several foodstuffs including sea foods from Asia until now.

S. Graslund et al. (2003) documented the use of chemicals and biological products that were used in shrimp farming of Thailand. Farmers used on an average 13 types of chemicals and biological products namely soil and water treatment compounds, fertilizers, pesticides and disinfectants, antibiotics, microorganism products, immunostimulants, vitamins, feed additives and uncertain groups etc. The authors also mentioned that these products could have negative effects on the cultured shrimps, cause a risk of food safety, human and adjacent ecosystem.

FAO (2005) reported that chloramphenicol is a broad-spectrum antibiotic that used for treatment of serious infections in human body. But, it has not been possible to identify the safe dose of it due to the unpredictable effects of doses on different patients. That's why USA has prohibited its use in food-producing animals and animal food products.

Aftab Uddin et al. (2006) found that various types of chemicals were used in Bangladesh shrimp hatcheries to control the pathogens and increase the feeding efficiency so that mortality rate could reduce. Chloramphenicol, erythromycin, prefuran and oxytetracycline were used by 40, 25, 20 and 15% of the surveyed hatcheries in the broodstock tanks to prevent bacterial infections after eye stalk ablation whereas 31, 25, 12, 13 and 19% hatcheries used chloramphenicol, erythromycin, malachite green, furazolidone and neomycin sulphate in larval rearing tank for prophylactic and metaphylactic treatment.

EU (2007) reported that 90% shrimp farm of Bangladesh follows extensive culture technique where shrimps are not fed with formulated feed at all, but most of the farmers apply home-made mixture of rice bran and wheat bran where as in the rest 10% farm shrimps are feed with formulated feed during culture period.

Nowsad et al. (2009) investigated the source of nitrofurantoin in shrimp and prawn through questionnaire survey. The authors found that most of the hatchery operators and prawn farmers in Mymensingh district would not know about the fatal effects of nitrofurantoin where as those people of Cox's Bazar and Khulna would know about that of nitrofurantoin, but, they kept mum on this issue. In contrast, various types of antibiotics that sold in local market were found to be used in shrimp hatcheries. So, the authors concluded that both the shrimp/prawn hatchery and poultry/fish feed used in culture ponds might be the possible sources of nitrofurantoin in exportable shrimp/prawn products of Bangladesh.

Raffi et al. (2011) found chloramphenicol in farmed shrimp ranged from 0.02 to 0.3 µg/kg and in wild shrimp ranged from 0.01 to 0.04 µg/kg shrimp meat. The author assumed that it might be come from the released shrimps which were disease affected from the culture pond into the natural water body.

Paul et al. (2011) found that various types of drugs were used in different prawn (*Macrobrachium rosenbergii*) farms of north and south 24 Parganas districts of West Bengal, India namely chloramphenicol, nitrofurans, oxytetracycline and formalin etc. Most of the farms used chloramphenicol (40%) followed by oxytetracycline (23%), erythromycin (20%) and nitrofurans (10%).

3.0 Materials & Method

3.1 Sample Collection

Samples were collected from three different shrimp farming areas namely Shatkira, Bagerhat and Khulna and from each area five samples were collected randomly from five different farms. Firstly PL, then PL after one week feeding, after that one month later from stocking and finally one week before harvesting shrimp sample were collected from each farm. Thus, from shrimp farms, a total of 60 samples were collected. Then the samples were brought to the Quality Control laboratory, Boyra, Khulna. The sample was brought to the laboratory in iced condition.

3.2 Study period

The samples were collected from February- June, 2018.

3.3 Laboratory Procedure

3.3.1 Sample preparation for Nitrofurantoin

Frozen sample was kept in air to bring it normal temperature and washed with tap water. After washing head, shell and body appendages were removed from the body left for several minutes to air dry. Then the samples were chopped individually using a knife and taken into a blender to make paste. 1.0g of the paste was taken for the extraction procedure of Nitrofurantoin by using an electric weighing machine.

3.3.2 Required Materials for Nitrofurantoin

Materials Provided with Kit supplied by Quality Control Laboratory, 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 K₂HPO₄
4. Ethyl acetate
5. Hexane
6. Deionised water

Apparatus and Instrument

- Respective Microtitre Plate (e.g. AOZ/AMTZ/AHD/SEM)
- Micro plate reader equipped with a 450nm filter with a recommended reference filter of 630nm.
- Lint Free Tissue
- Water bath/Incubator
- N₂ 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 K₂HPO₄



3.3.3 Extraction Procedure

At first homogenized the each sample after remove head, shell and body appendages. Then weight 1g of homogenized sample in a 50 ml falcon tube and four 1g of previously analyzed blank tissue were used as two positive and two negative controls. Fortify two positive controls with 0.75 µg/kg SEM and 0.5µg/kg AHD, AOZ and AMOZ respectively. After that, 1g of homogenized sample was added with 4ml distilled H₂O, 0.5ml 1M HCl and 50 µl 10mM 4-Nitrobenzaldehyde. Vortex for 1 minute and incubated for 2 hours at 50°C by water bath. After incubation for 2 hours 5ml 0.1M K₂HPO₄, 0.4ml 1M NaOH and 6ml ethyl acetate were added and vortex for 1 minute. Then centrifuged the sample for 10 minutes at 4000rpm at 25°C and two layers were appeared. After centrifuge 3ml of the upper ethyl acetate layer of each sample was transferred into test tube and dry down at 60°C by N₂ evaporator. The residue was then dissolved in 1ml hexane and 1ml diluted Wash Buffer and vortex for 2 minutes. Then the sample was centrifuged for 10 minutes at 4000 rpm at 25°C. Lower phase was ready for application to microtitre plate.

3.3.4 Test 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 samples extract of the two tissues 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 3 times with 250 µl of 1X

the wash solution. 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. Enzyme reaction was stopped by the addition of 100 µl of Stop buffer. A colour change from blue to yellow was evident. Measure the optical density of each well at 450 nm and 630nm filter as the reference wavelength within 10 minutes of stopping the colour reaction.

3.3.5 Sample preparation for Chloramphenicol

Frozen sample was kept in air to bring it normal temperature and washed with tap water. After washing head, shell and body appendages were removed from the body. Then left for several minutes to air dry. After that the samples were chopped individually using a knife and taken into a blender to make paste. 3.0g of the paste was taken for the extraction procedure of Chloramphenicol by using an electric weighing machine.

3.3.6 Required Materials for Chloramphenicol (CAP)

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

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/N₂ 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

Preparation of Wash Buffer

Wash buffer were prepared by the dilution contents of wash buffer with 970 ml distilled H₂O.

Preparation of Spiking Solutions

A 100ng/ml chloramphenicol spiking material is already provided in the ELISA kit, there is 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

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.

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.

Isooctane: Chloroform

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

3.3.7 Extraction Procedure

At first 3g tissue was added with 6 ml ethyl acetate and homogenized in vortex for 1 minute. Then the mixture was centrifuged at 2000 rpm for 15 minute and two layers were appeared. 4 ml of upper phase were removed and reduced to dryness at 70°C under N₂ atmosphere. The residue was dissolved in 2 ml of isooctane/chloroform (2:3). Again vortex for 1 min as well as added 0.5

ml of diluted Tissue Extraction Buffer and vortex for 2 min. Then Centrifuged at 2000 rpm for 15 minutes. Upper phase was ready for application to microtitre plate.

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

3.3.9 Sample preparation for Tetracycline

Frozen sample was kept in air to bring it normal temperature and washed with tap water. After washing head, shell and body appendages were removed from the body left for several minutes to air dry. Then the samples were chopped individually using a knife and taken into a blender to make paste. 1.0g of the paste was taken for the extraction procedure of tetracycline by using an electric weighing machine.

**Materials Provided With Kit Supplied by Quality Control Laboratory,
Department of Fisheries, Khulna**

1. Oxytetracycline-coated Plate
2. Oxytetracycline Standard Stock
(450 ng Powder)
3. Empty Vials for Standards:
Negative control(*white CAP tube*)
0.15 ng/mL(*yellow CAP tube*)
0.375 ng/mL(*orange CAP tube*)
0.75 ng/mL(*pink CAP tube*)
1.5 ng/mL(*purple CAP tube*)
4.5 ng/mL(*blue CAP tube*)
4. Oxytetracycline Antibody
5. 100X HRP-Conjugated Antibody #2
6. Antibody #2 Diluent
7. 20X Wash Solution
8. Stop Buffer
9. TMB Substrate
10. Standard diluent
11. 5XOXTET extraction Buffer
12. 10XTET sample diluent

13. TET Balance Buffer Concentrate

14. Oxytetracycline Spiking Stock(2000ng Powder)

15. Chlorotetracycline Spiking Stock(2000ng Powder)

Chemicals not Provided in the Kit

Deionised water

Apparatus and Instrument

1. Micro plate reader (Biotek, Model ELx 800) equipped with a 450nm filter and a recommended reference filter of 630nm.

2. Lint Free Tissue

3. Eppendorf tube

4. 15 ml falcon tube

5. 50ml falcon tube

6. Pipettes: 20 µl to 200 µl, 100 µl to 1000 µl, 500 µl to 5000 µl, 1000 µl to 10000 µl

7. Plate sealer

8. Analytical Balance

9. Centrifuge

10. Vortex

11. Personal Computer with printer

Ready to Use Reagents

Ready to Use Reagents

a) 20X Wash Solution

Supplied ready to use. Stable up to expiry date when stored at +2°C to +8°C.

b) TMB Substrate

Supplied ready to use. Stable up to expiry date when stored at +2°C to +8°C.

c) Stop Solution

Supplied ready to use. Stable up to expiry date when stored at +2°C to +8°C.

Preparation of Oxytetracycline Work Standards

The oxytetracycline standard is provided as 450 ng stock. Add 1.5 mL of standard diluent to the stock vial and mix by vortexing for 1 minute to obtain 300 ppb stock vial. Vortex the solution vigorously for 2 minutes and leaves it at room temperature for 10 minutes. Repeat this procedure three times to ensure that all powder has dissolved. Transfer the solution to a dark plastic vial and store it at -20°C for any future use. To make working standards, serially dilute the 300 ppb standard stock vial with standard diluents. Mix well by vortexing each work standard tube for 30 seconds.

Preparation of 1X Wash Solution

Mix 1 volume of the 20X Wash Solution with 19 volumes of distilled water.

Preparation of 1X HRP-Conjugated Antibody -2

Mix 1 volume of the 100X HRP-Conjugated Antibody -2 with 99 volumes of Ab-2 Diluent.

Preparation of Spiking Solutions & Spiked Samples

The oxytetracycline/chlorotetracycline standard is provided as 2000 ng powder stock. To the powder stock add 1 mL of standard diluent to obtain 2000 ppb stock solution. Vortex the solution vigorously for 2 minutes and leaves it at room temperature for 10 minutes. Repeat this procedure three times to ensure that all powder has dissolved. Transfer the solution to a dark plastic vial and store it at -20°C for any future use.

It is good practice to have an intermediate step when spiking at lower values. Therefore, an initial dilution is used; this is made up in standard diluents from the kit.

Dilute stock solution

Take 100 µL of stock solution in 900 µL of standard diluents

→200 ppb Spiking Solution

The 200 ppb stock vial must be freshly prepared before used within the same day.

Spiking of the samples

Spike level 50 ppb

1 g Negative shrimp Matrix + 250 μ L of 200 μ g/mL Oxytetracycline / chlorotetracycline Spiking solution.

Preparation of 1X OXTET Extraction Buffer

Mix 1 volume of the *5X OXTET Extraction Buffer* with 4 volumes of distilled water.

Preparation of 1X TET Sample diluents

Mix 1 volume of the *10X TET Sample diluents* with 9 volumes of distilled water.

Preparation of 1X OXTET Sample balance buffer

Mix 1 volume of the *TET Sample balance buffer* concentrate with 1 volumes of distilled water.

3.3.10 Extraction Procedure

Homogenize the meat after remove head, shell & deveined of these shrimp. Weight 1.0g of homogenized shrimp in a 50ml falcon tube. . Weigh four 1 ± 0.1 g portions of previously analyzed blank tissue to be used as positive (2) and negative (2) controls. Fortify one positive control with Oxytetracycline and one with Chlortetracycline at 50 μ g/kg. Add 3mL of 1X OXTET Extraction Buffer to 1 g of homogenized shrimp. Vortex for 10 minutes in a multi-tube vortex or shake 30 minutes on a shaker. Centrifuge for 10 min at 4000 rpm. Transfer 200 μ L of supernatant to a new tube containing 25 μ L of 1X OXTET sample balance buffer. Swirl the tube. Then add 275 μ L of 1X TET sample diluents. Vortex for 1 minute. Dilute the sample 1+1 with 1X TET sample diluents. Vortex for 1 minute. The Solution is now ready for application to the microtitre plate.

3.3.11 Test procedure for ELISA

All reagents were brought to room temperature (20 to 25°C) before use (about 1 hour) and returned all reagents to 2-8°C after use. Then they were mixed by gently inverting or swirling prior to use, inserted sufficient number of wells a microwell holder and then recorded standard, blank, QC and sample positions. Then 75 µl was pipetted standards from lower to higher concentration into the appropriate wells of the microtitre plate and 75 µl sample extracts of the 2 tissue blank in duplicate and plated one set of duplicates 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 and pipetted 75 µl of extracted samples in to separate wells and then used a new pipette tip for each standard or sample. 100 µL of Antibody -1 was 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) and then washed the plate 3 times with 250 µL of 1X Wash Solution. After addition of 250µl wash buffer to the wells, the plate was incubated for 20-30 seconds; shook the plate gently before pouring out the wash buffer and then repeated this procedure for each of the three washes. After the last wash, the plates was inverted and gently tap the plate dry on paper towels. 150µL of 1X Antibody -2 was added in the solution and incubated the plate 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, incubate the plate for 20-30 seconds; the plate was shaken gently before pouring out the wash buffer. This procedure was repeated for each of the three washes. After the last wash, the plate was inverted and gently taps the plate dry on paper towels 100 µL of TMB was added to Substrate to each well. Time was counted the reaction immediately after adding the substrate. Then 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), 100 µL of Stop Buffer was added to stop the enzyme reaction. The optical density was measured of each well on a plate reader with 450 nm wavelength as soon as possible following the addition of Stop Buffer.

3.4. Data analysis

Graphical analysis was conducted on computer using Microsoft Excel software & SPSS software was used for one-way anova test.

4.0 Result

4.1 Result of CAP

4.1.1 Concentration (ppb) of CAP in shrimp muscle (PL)

The cut-off level of CAP (Chloramphenicol) in shrimp muscle is 0.109. In the present study in all the experimental sites concentration of CAP in PL were found below the cut-off level. The highest value of CAP in case of PL was found in station 5 of Khulna district and station 3 of Bagerhat district whereas the lowest value of SEM was found in station 2 of Khulna district and station 1 of Shatkhira district.

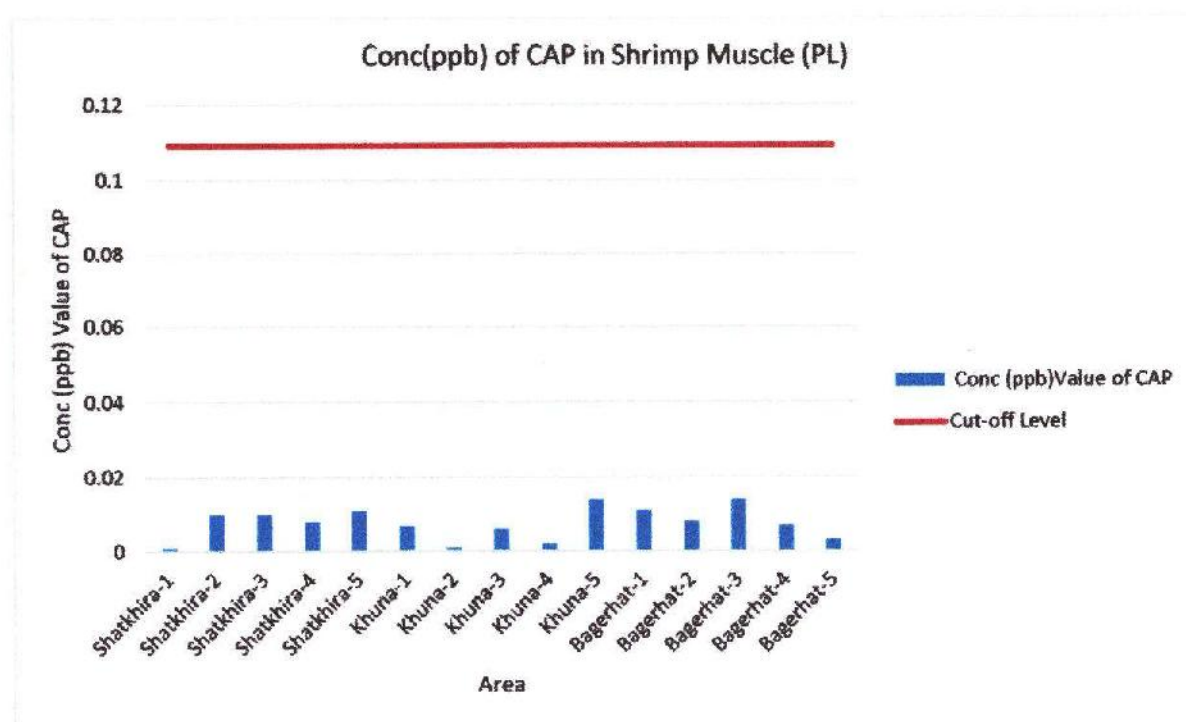


Fig.1. Concentration of CAP in shrimp muscle (PL)

4.1.2 Concentration (ppb) of CAP in shrimp muscle (PL after 1 week)

In all the experimental sites the concentration of CAP in shrimp muscle (PL after 1 week) was found below the cut-off level. The highest value of CAP in case of PL after one week was found in station 3 of Bagerhat district whereas the lowest value of SEM was found in station 2 and 4 of Khulna district.

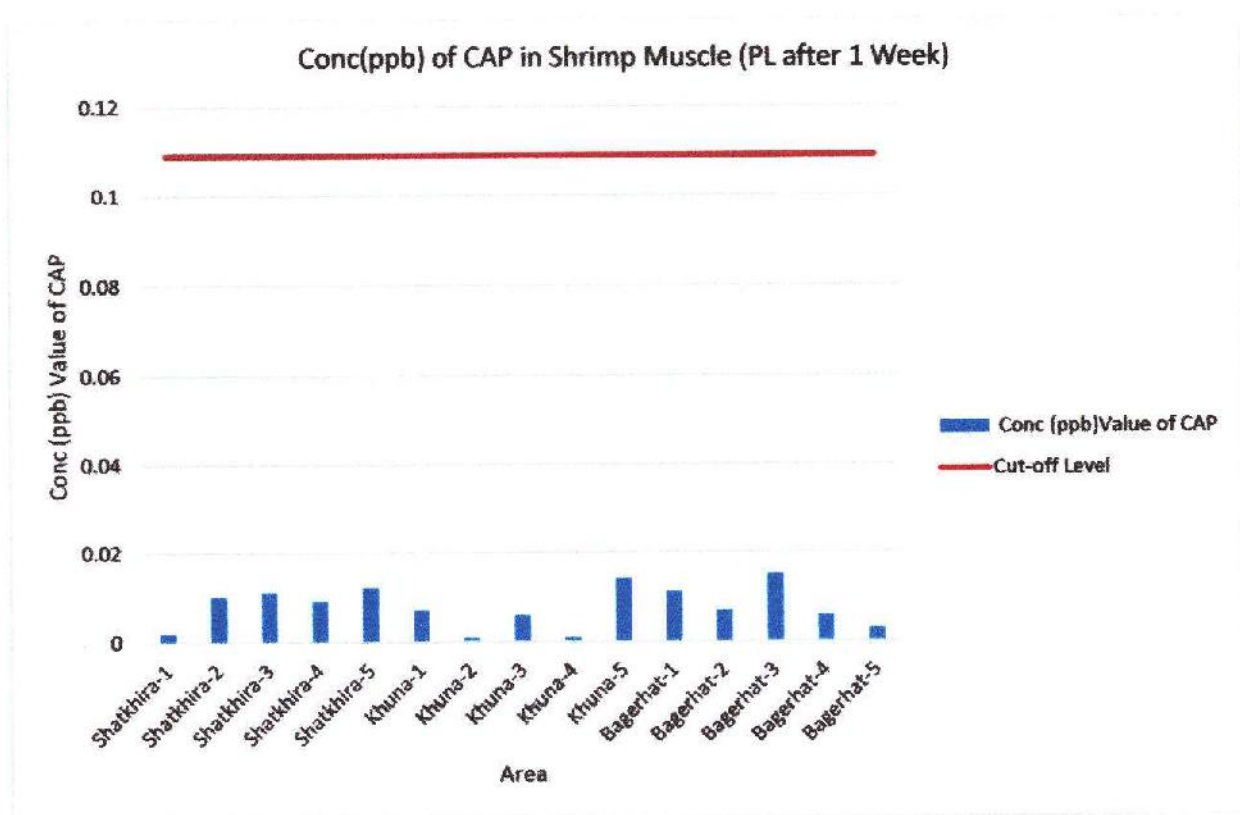


Fig.2. Concentration of CAP in shrimp muscle (PL after 1 week)

4.1.3 Concentration of CAP in shrimp muscle (After 1 month)

In all the experimental sites the concentration of CAP in shrimp muscle (After 1 month) was found below the cut-off level. The highest value of CAP was found in station 3 of Bagerhat district whereas the lowest value of SEM was found in station 2 of Khulna district.

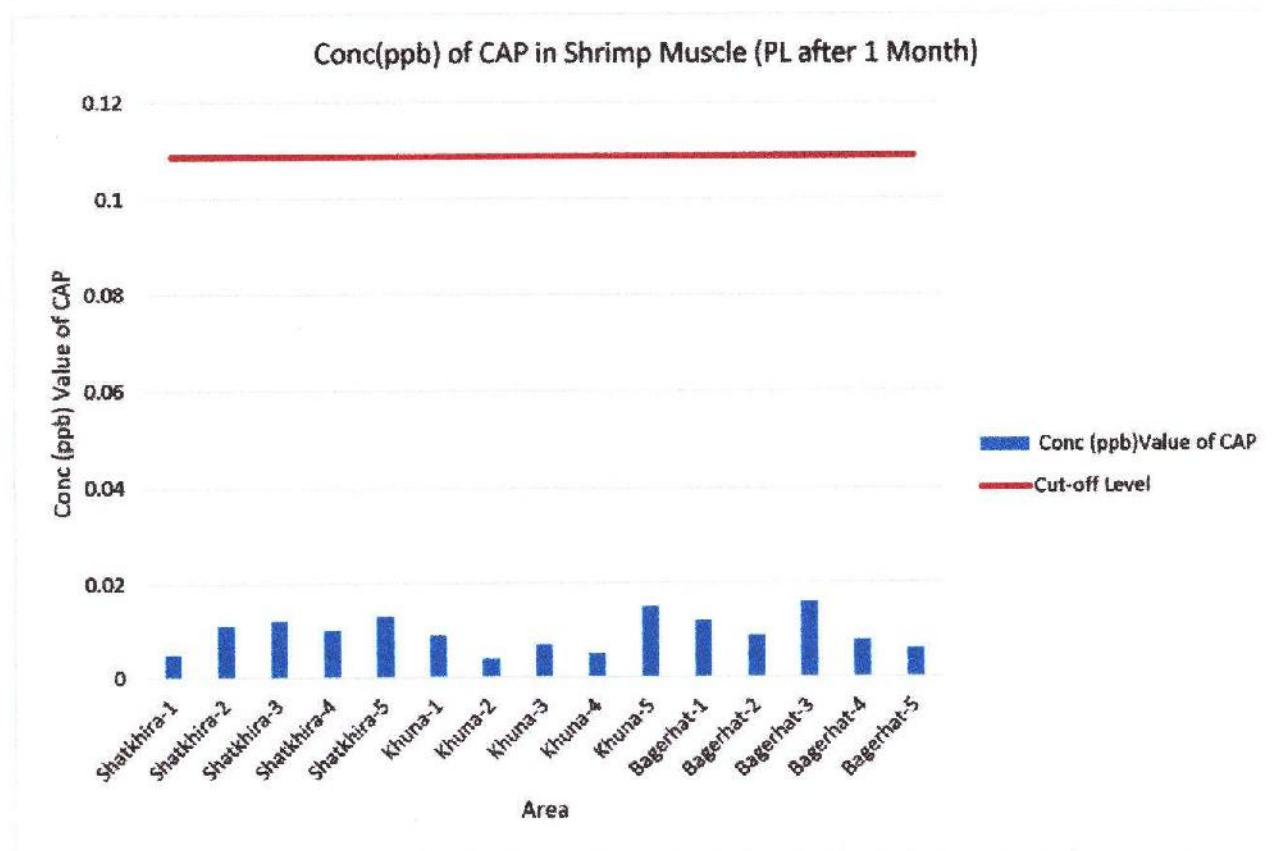


Fig.3. Concentration of CAP in shrimp muscle (after 1 month)

4.1.4 Concentration of CAP in shrimp muscle (Harvesting before 1 week)

In all the experimental sites the concentration of CAP in shrimp muscle (Harvesting before 1 week) was found below the cut-off level. The highest value of CAP was found in station 3 of Bagerhat district whereas the lowest value of SEM was found in station 2 of Khulna district.

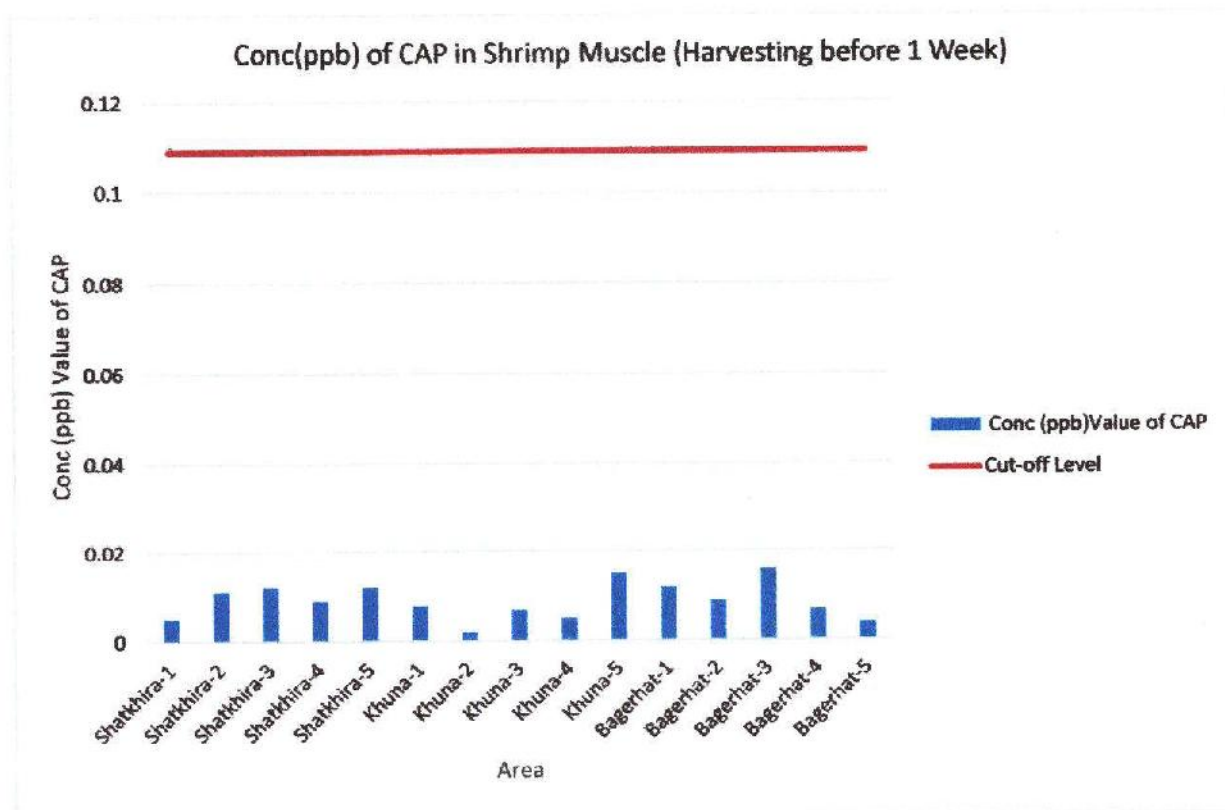


Fig.4. Concentration of CAP in shrimp muscle (Harvesting before 1 week)

4.2 Result of SEM

4.2.1 Concentration (ppb) of SEM in shrimp muscle (PL)

The cut-off level of SEM in shrimp muscle is 0.576. In the present study in all the experimental sites concentration of SEM in PL were found below the cut-off level except in the station 1 of khulna. The highest value of SEM in case of PL was found in station 1 of Khulna district and it was higher than the cut-off level whereas the lowest value of SEM was found in station 2 and 3 of Shatkhira district.

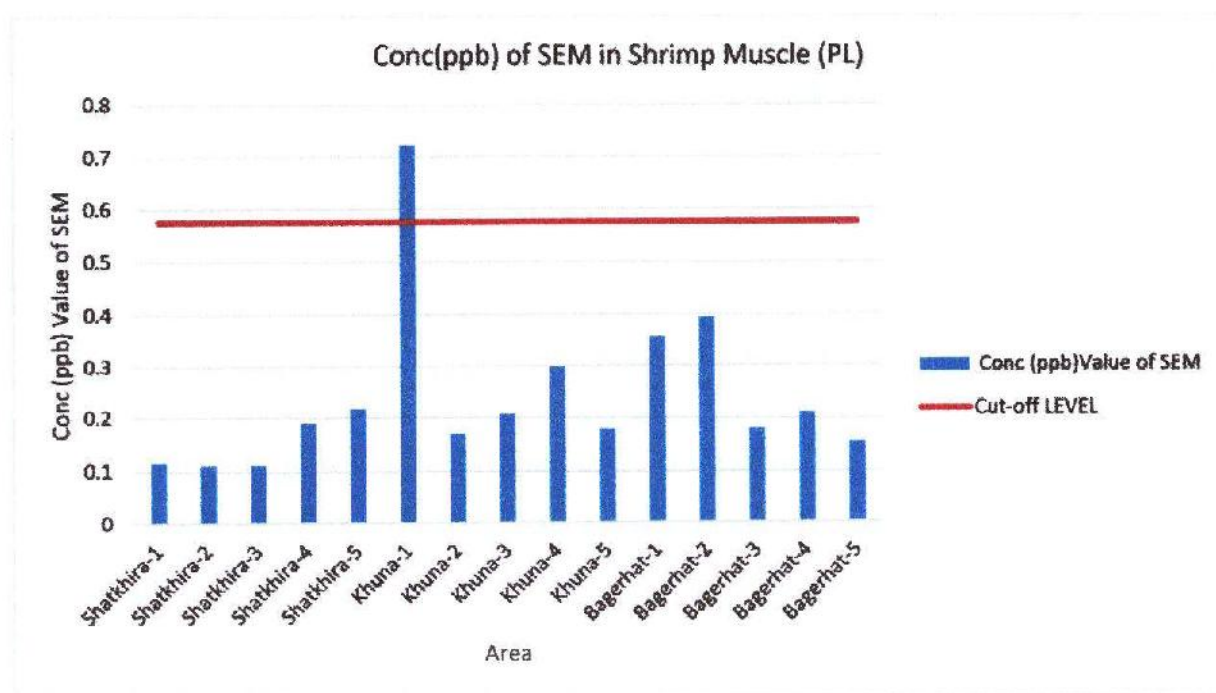


Fig.5. Concentration of SEM in shrimp muscle (PL)

4.2.2 Concentration (ppb) of SEM in shrimp muscle (PL after 1 week)

In all the experimental sites the concentration of SEM in shrimp muscle (PL after 1 week) was found below the cut-off level except in the station 1 of khulna. The highest value of SEM in case of PL after one week was found in station 1 of Khulna district and it was higher than the cut-off level whereas the lowest value of SEM was found in station 2 and 3 of Shatkhira district.

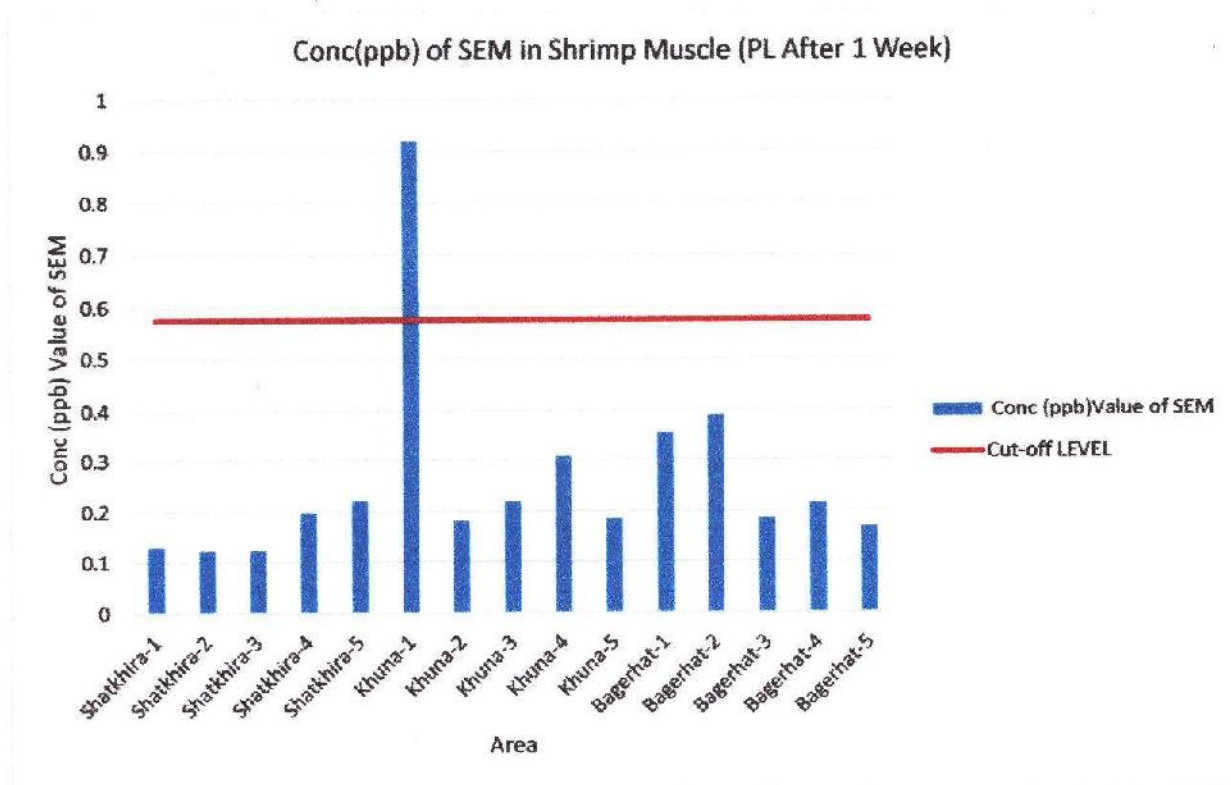


Fig.6. Concentration of SEM in shrimp muscle (PL after 1 week)

4.2.3 Concentration of SEM in shrimp muscle (After 1 month)

In all the experimental sites the concentration of SEM in shrimp muscle (After 1 month) was found below the cut-off level. The highest value of SEM was found in station 2 of Bagerhat district whereas the lowest value of SEM was found in station 1 of Khulna district.

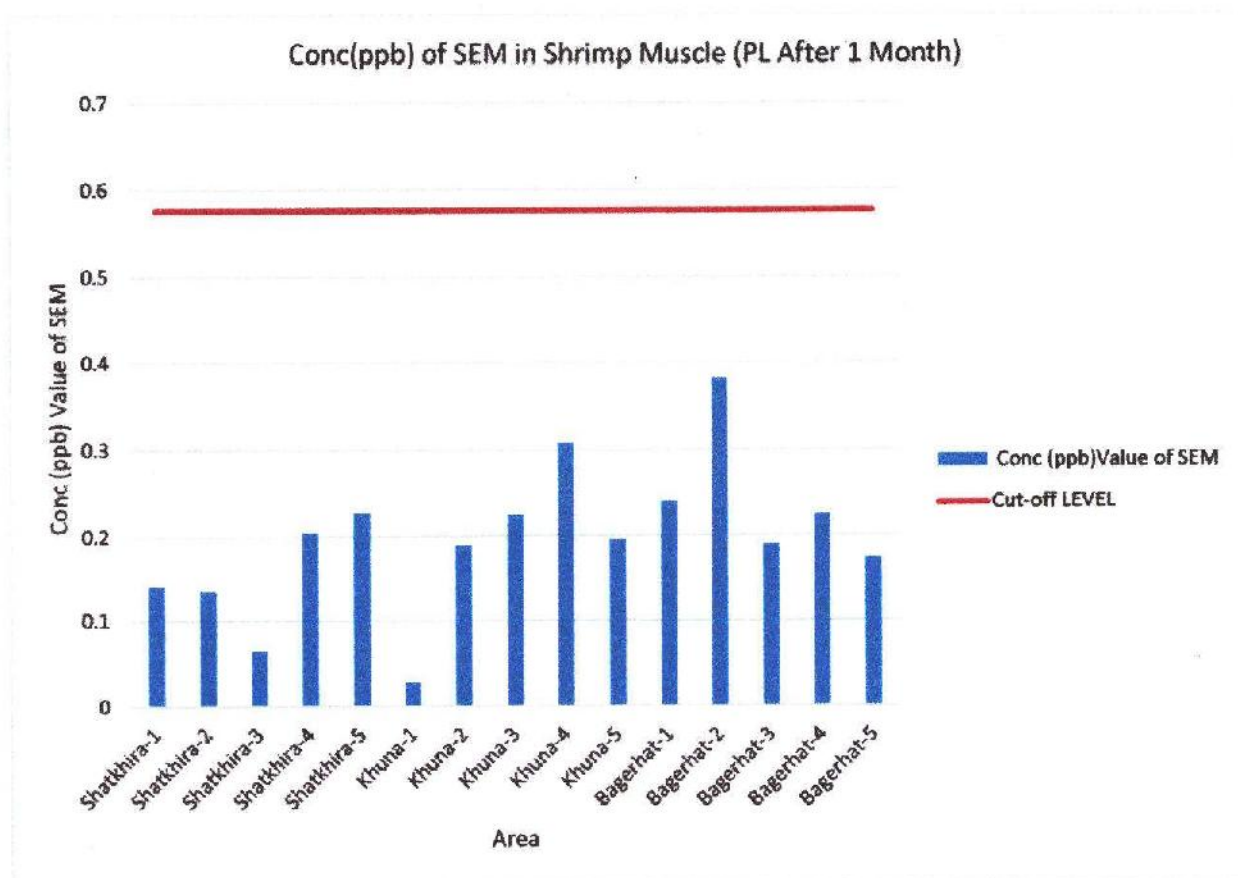


Fig.7. Concentration of SEM in shrimp muscle (after 1 month)

4.2.4 Concentration of SEM in shrimp muscle (Harvesting before 1 week)

In all the experimental sites the concentration of SEM in shrimp muscle (Harvesting before 1 week) was found below the cut-off level. The highest value of SEM was found in station 2 of Bagerhat district whereas the lowest value of SEM was found in station 1 of Khulna district.

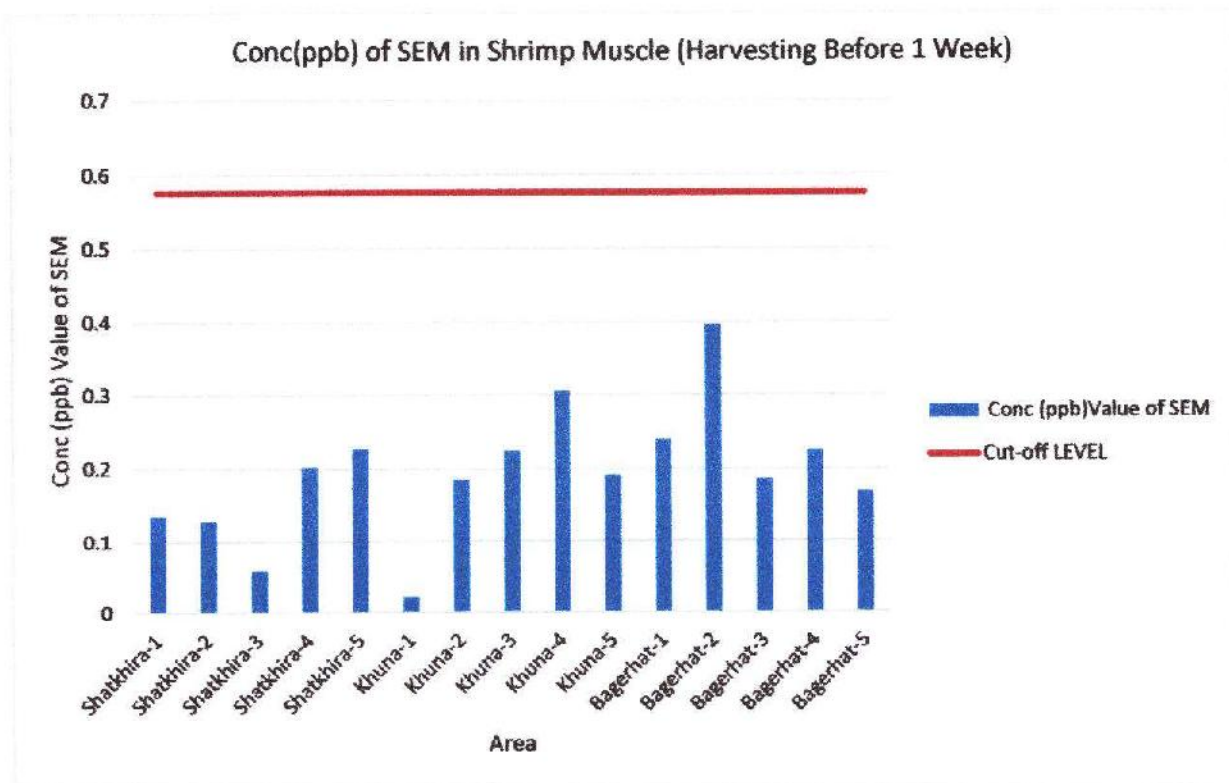


Fig.8. Concentration of SEM in shrimp muscle (Harvesting before 1 week)

4.3 Result of AOZ

4.3.1 Concentration (ppb) of AOZ in shrimp muscle (PL)

The cut-off level of AOZ in shrimp muscle is 0.371. In the present study in all the experimental sites concentration of AOZ in PL were found below the cut-off level. The highest value of AOZ in case of PL was found in station 5 of Shatkhira district whereas the lowest value of AOZ was found in station 4 of Khulna district.

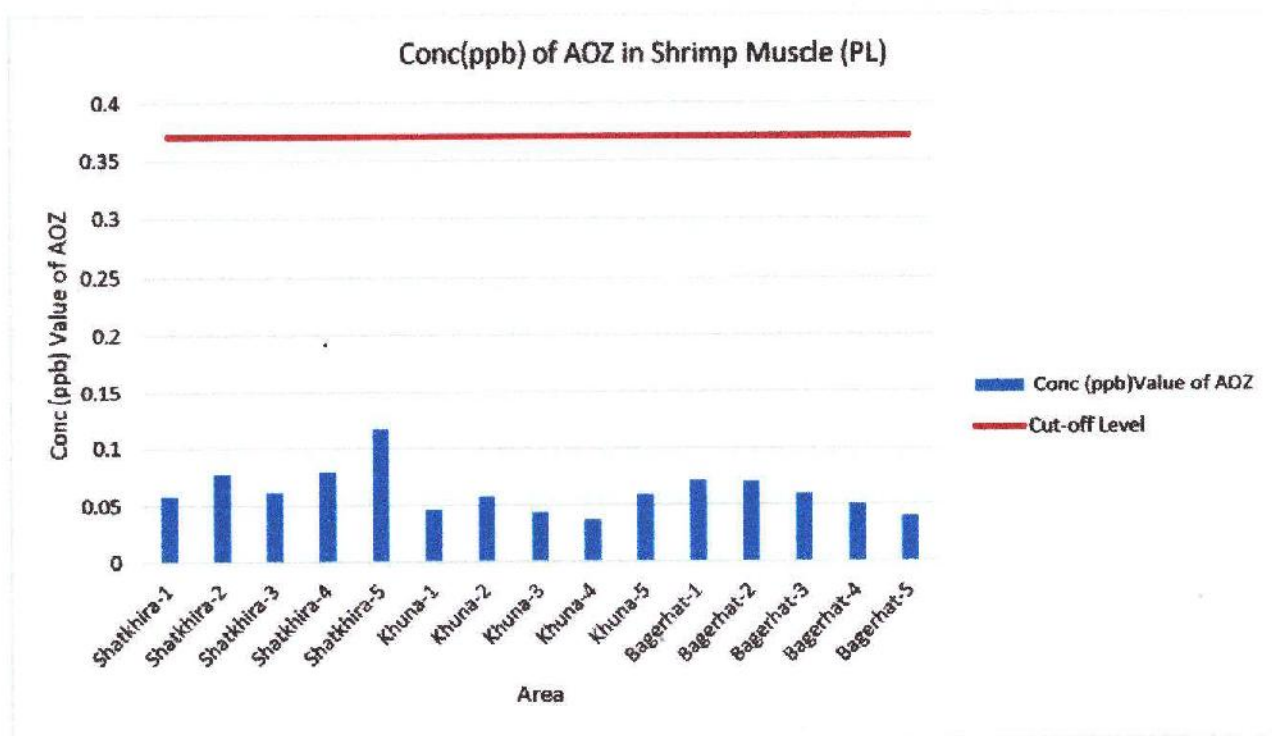


Fig.9. Concentration of AOZ in shrimp muscle (PL)

4.3.2 Concentration (ppb) of AOZ in shrimp muscle (PL after 1 week)

In all the experimental sites the concentration of AOZ in shrimp muscle (PL after 1 week) was found below the cut-off level. The highest value of AOZ was found in station 5 of Shatkhira district whereas the lowest value of AOZ was found in station 4 of Khulna district.

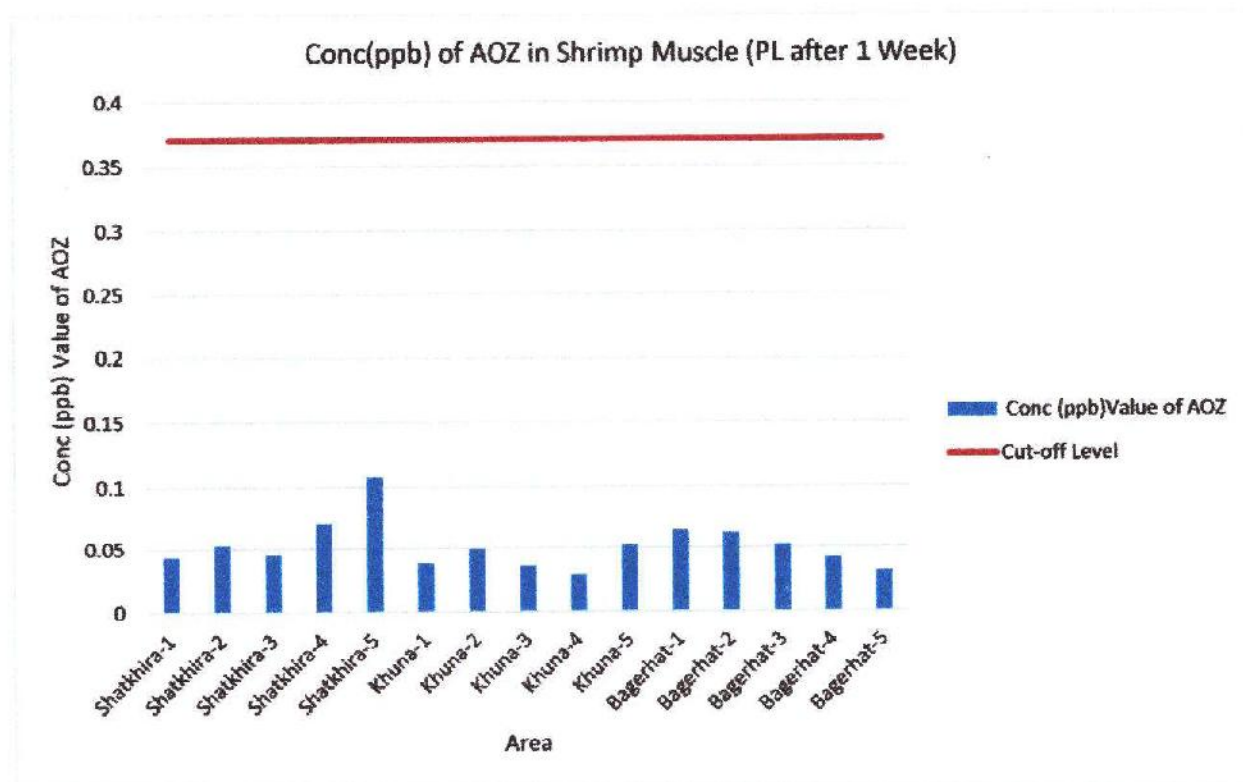


Fig.10. Concentration of AOZ in shrimp muscle (PL after 1 week)

4.3.3 Concentration of AOZ in shrimp muscle (After 1 month)

In all the experimental sites the concentration of AOZ in shrimp muscle (After 1 month) was found below the cut-off level. The highest value of AOZ was found in station 5 of Shatkhira district whereas the lowest value of AOZ was found in station 4 of Khulna district.

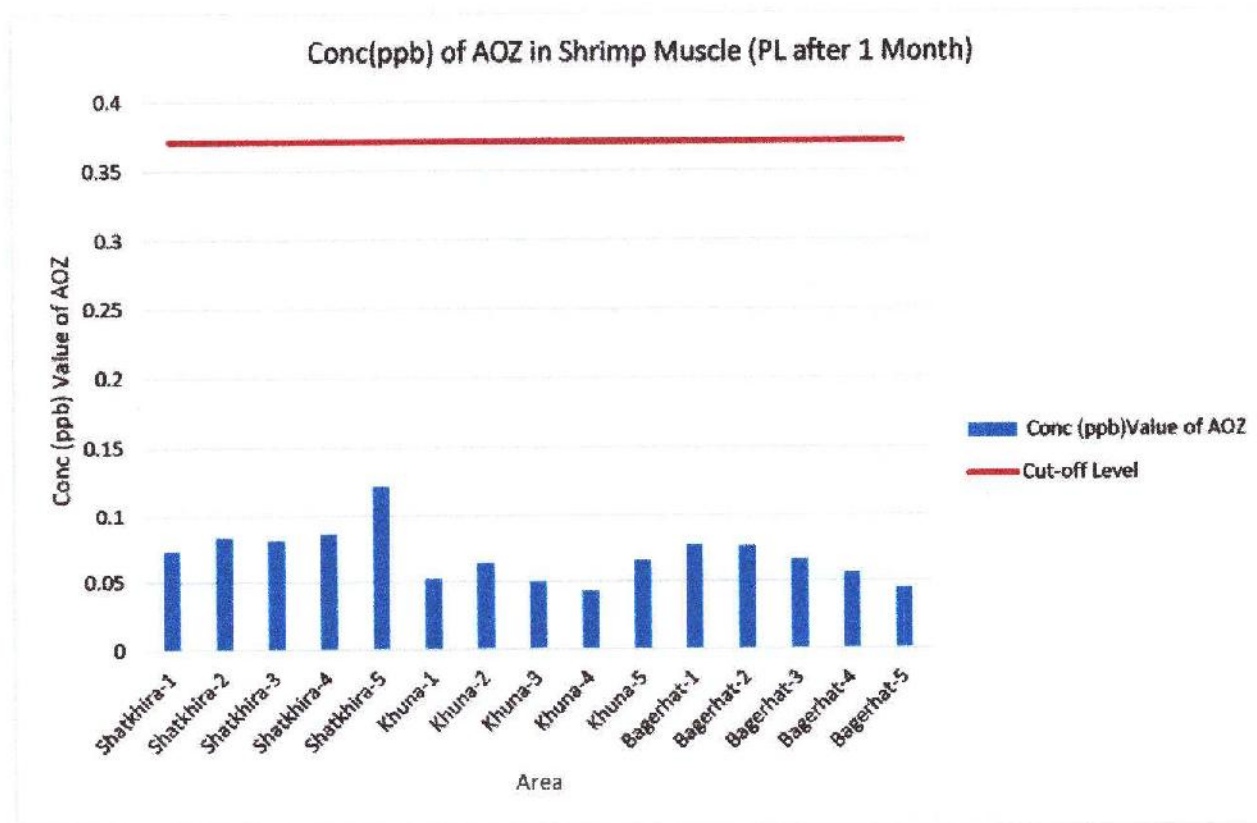


Fig.11. Concentration of AOZ in shrimp muscle (after 1 month)

4.3.4 Concentration of AOZ in shrimp muscle (Harvesting before 1 week)

In all the experimental sites the concentration of AOZ in shrimp muscle (Harvesting before 1 week) was found below the cut-off level. The highest value of AOZ was found in station 5 of Shatkhira district whereas the lowest value of AOZ was found in station 4 of Khulna district.

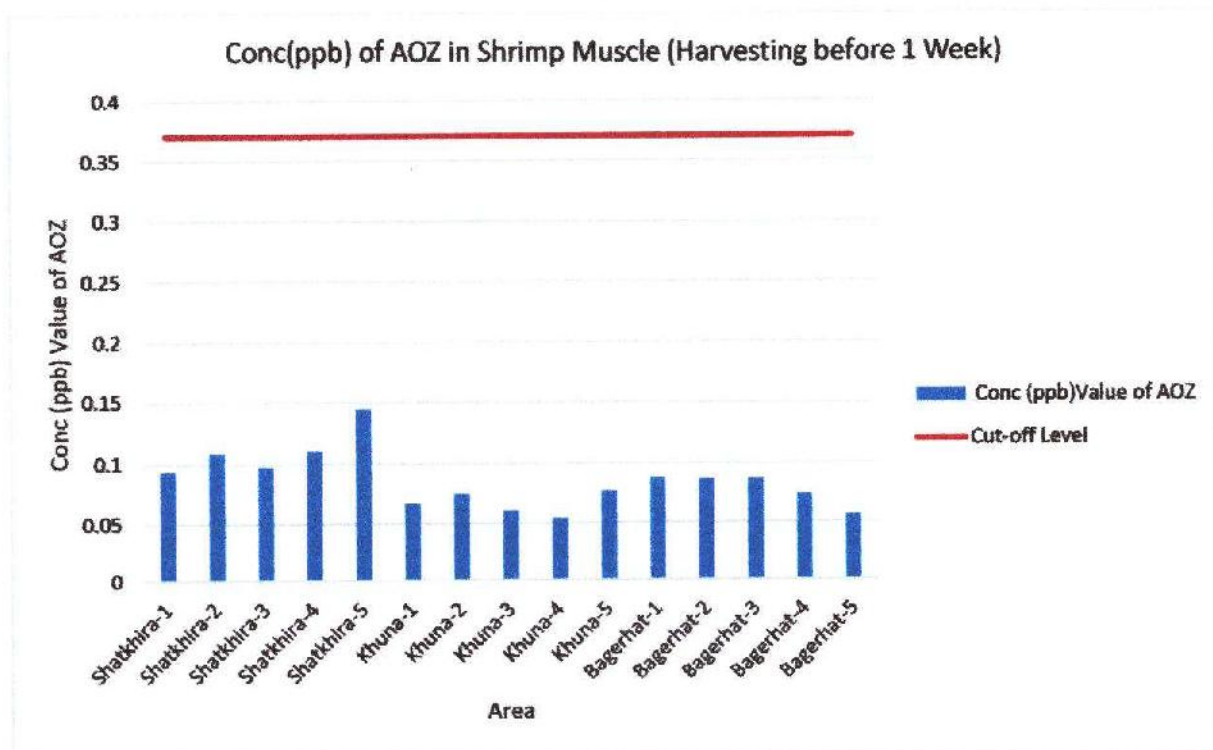


Fig.12. Concentration of SEM in shrimp muscle (Harvesting before 1 week)

4.4 Result of AMOZ

4.4.1 Concentration (ppb) of AMOZ in shrimp muscle (PL)

The cut-off level of AMOZ in shrimp muscle is 0.356. In the present study in all the experimental sites concentration of AMOZ in PL were found below the cut-off level. The highest value of AMOZ in case of PL was found in station 3 of Bagerhat district whereas the lowest value of AMOZ was found in station 5 of Bagerhat district.

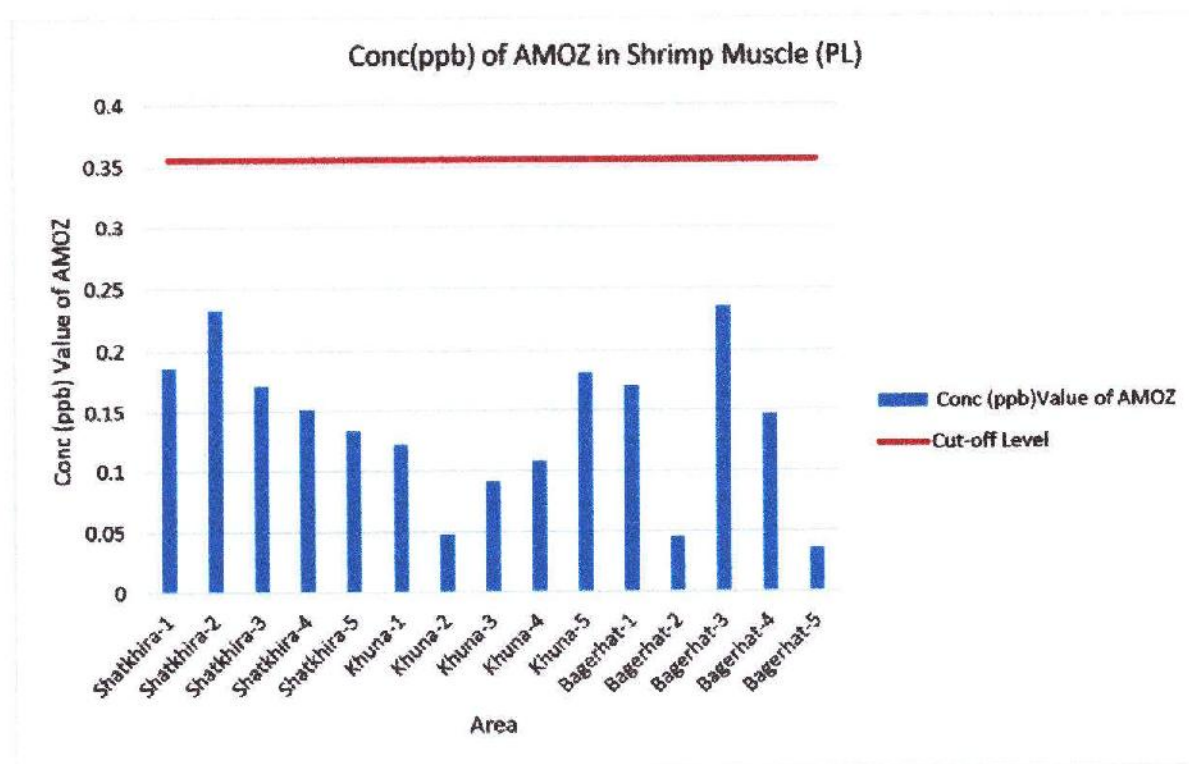


Fig.13. Concentration of AMOZ in shrimp muscle (PL)

4.4.2 Concentration (ppb) of AMOZ in shrimp muscle (PL after 1 week)

In all the experimental sites the concentration of AMOZ in shrimp muscle (PL after 1 week) was found below the cut-off level. The highest value of AMOZ was found in station 3 of Bagerhat district whereas the lowest value of AMOZ was found in station 5 of Bagerhat district.

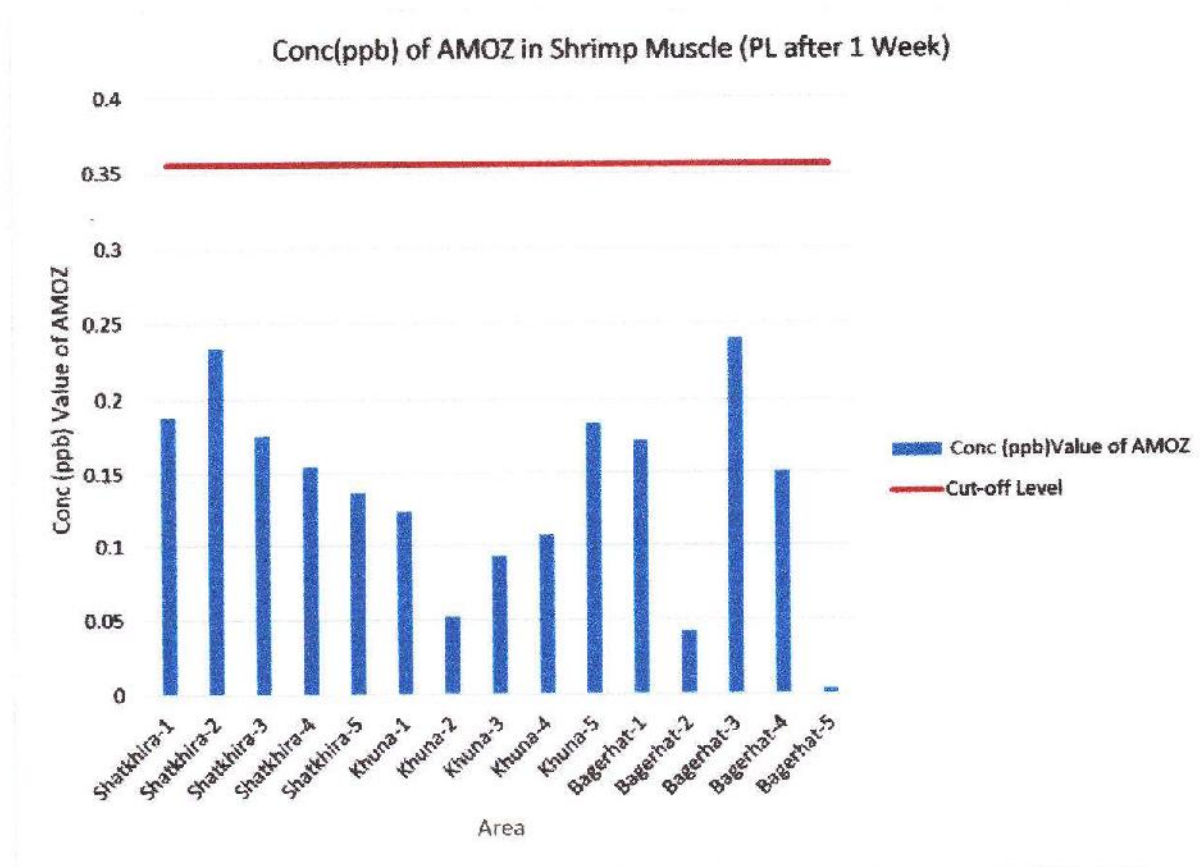


Fig.14. Concentration of AMOZ in shrimp muscle (PL after 1 week)

4.4.3 Concentration of AMOZ in shrimp muscle (After 1 month)

In all the experimental sites the concentration of AMOZ in shrimp muscle (After 1 month) was found below the cut-off level. The highest value of AMOZ was found in station 2 of Shatkhira district whereas the lowest value of AMOZ was found in station 2 of Khulna district.

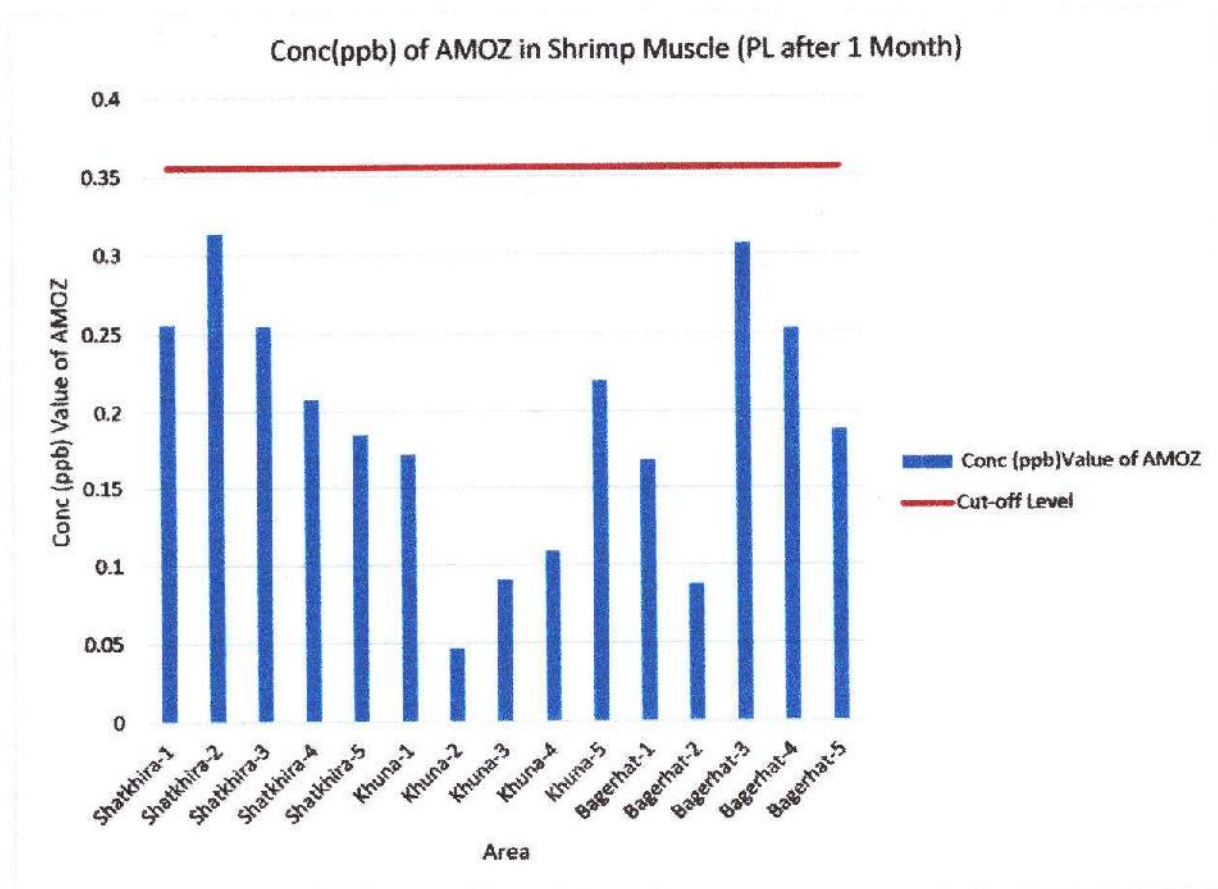


Fig.15. Concentration of AMOZ in shrimp muscle (after 1 month)

4.4.4 Concentration of AMOZ in shrimp muscle (Harvesting before 1 week)

In all the experimental sites the concentration of AMOZ in shrimp muscle (Harvesting before 1 week) was found below the cut-off level. The highest value of AMOZ was found in station 3 of Bagerhat district whereas the lowest value of AMOZ was found in station 2 of Khulna district.

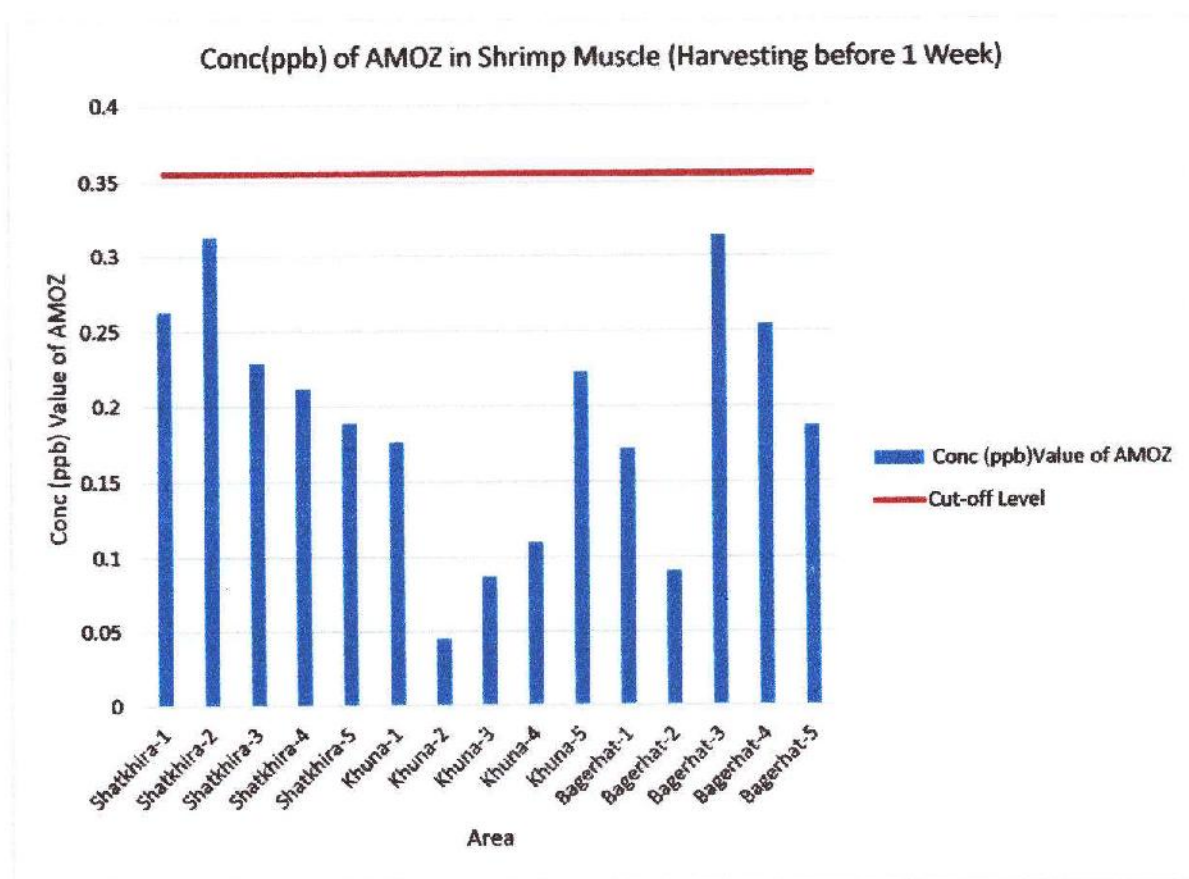


Fig.16. Concentration of AMOZ in shrimp muscle (Harvesting before 1 week)

4.5 Result of AHD

4.5.1 Concentration (ppb) of AHD in shrimp muscle (PL)

The cut-off level of AHD in shrimp muscle is 0.424. In the present study in all the experimental sites concentration of AHD in PL were found below the cut-off level. The highest value of AHD in case of PL was found in station 3 of Bagerhat district whereas the lowest value of AHD was found in station 2 of Khulna district.

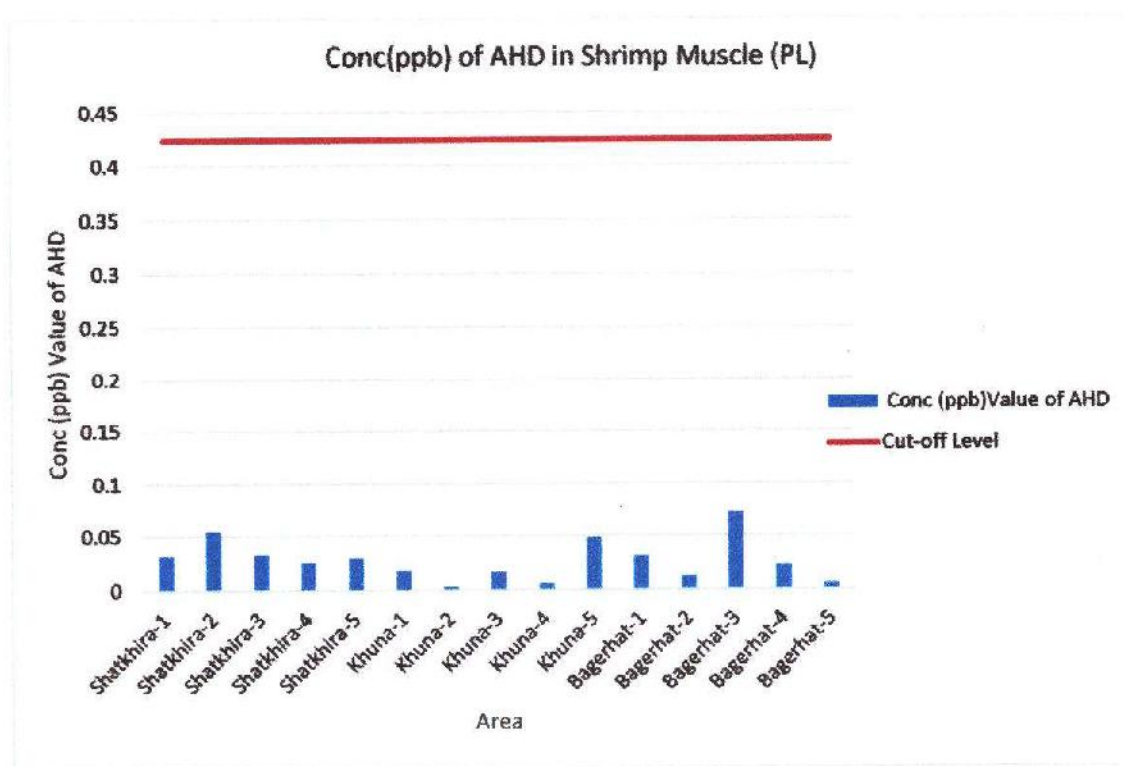


Fig. 17. Concentration of AHD in shrimp muscle (PL)

4.5.2 Concentration (ppb) of AHD in shrimp muscle (PL after 1 week)

In all the experimental sites the concentration of AHD in shrimp muscle (PL after 1 week) was found below the cut-off level. The highest value of AHD was found in station 3 of Bagerhat district whereas the lowest value of AHD was found in station 2 of Khulna district.

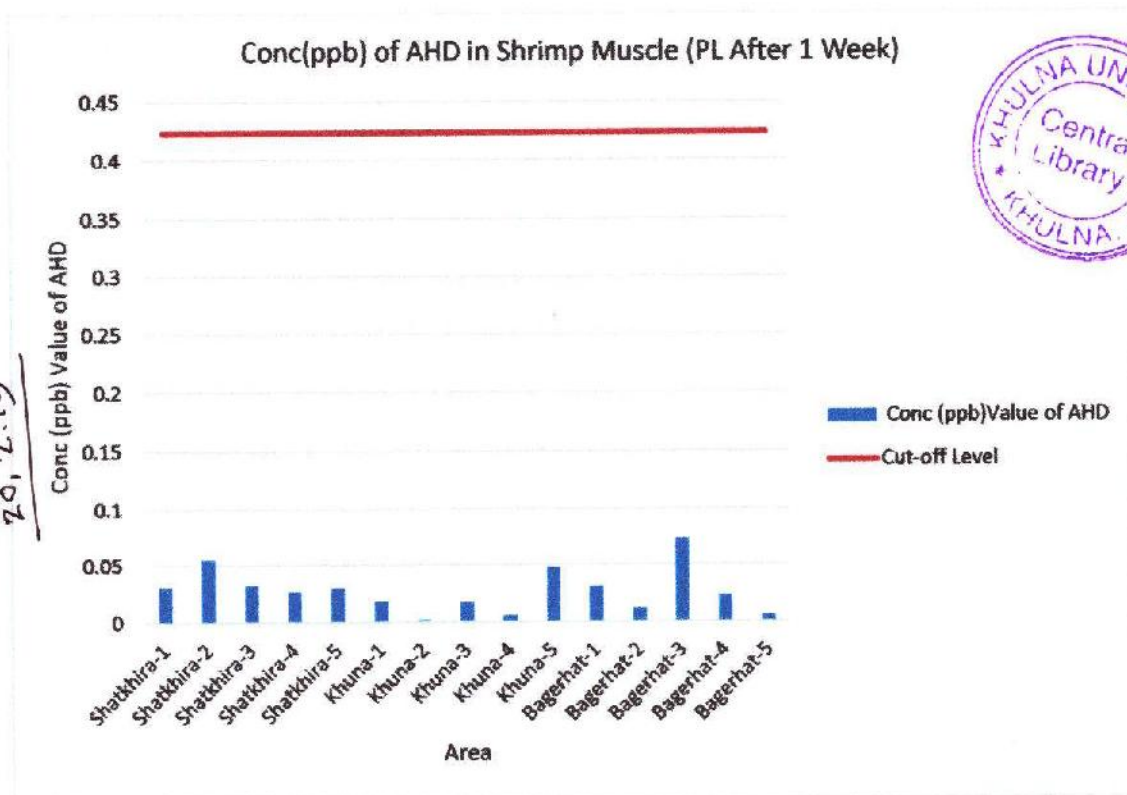


Fig.18. Concentration of AHD in shrimp muscle (PL after 1 week)

4.5.3 Concentration of AHD in shrimp muscle (After 1 month)

Like in shrimp muscle of PL after week, the concentration of AHD in shrimp muscle of after 1 month was found below the cut-off level in case of all stations. The highest value of AHD was found in station 3 of Bagerhat district whereas the lowest value of AHD was found in station 2 of Khulna district.

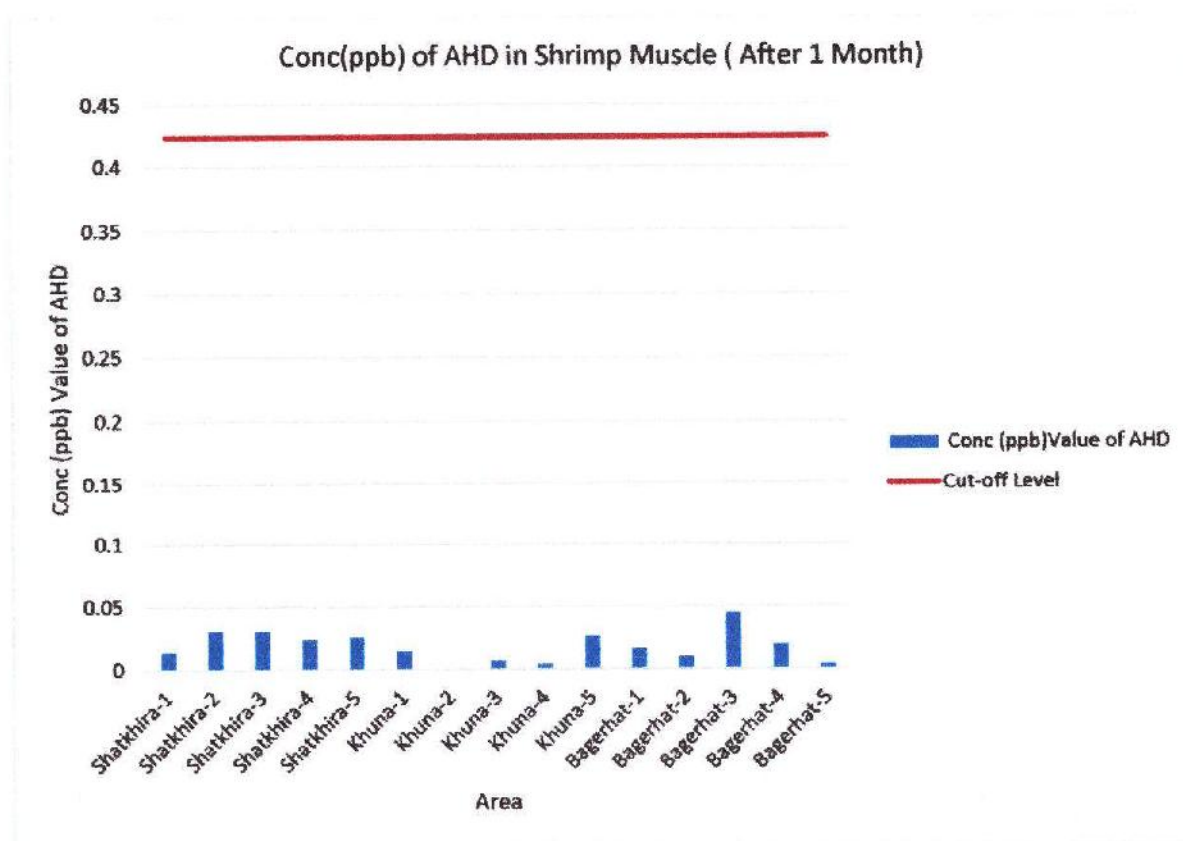


Fig.19. Concentration of AHD in shrimp muscle (after 1 month)

4.5.4 Concentration of AHD in shrimp muscle (Harvesting before 1 week)

In all the experimental sites the concentration of AHD in shrimp muscle (Harvesting before 1 week) was found below the cut-off level. The highest value of AHD was found in station 3 of Bagerhat district whereas the lowest value of AHD was found in station 2 of Khulna district.

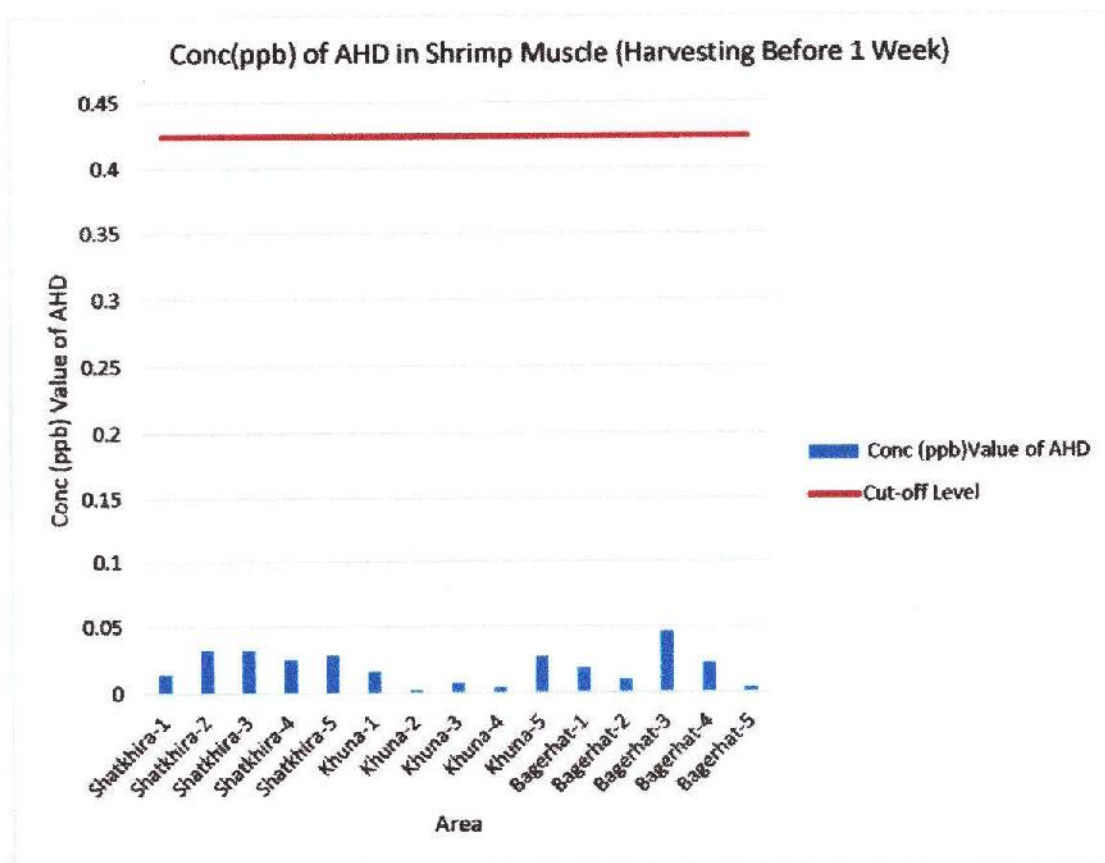


Fig.20. Concentration of AHD in shrimp muscle (Harvesting before 1 week)

4.6 Result of TTC

The value of TTC (Tetracycline) was too small that the machine could not detect it.

Table 1: Maximum and Minimum Concentration of Antibiotic Residue in different region

Concentration(ppb)		Stage-1(PL before stocking)	Stage-2(PL after one week)	Stage-3(one month after stocking)	Stage-4(one week before harvesting)
CAP	MAX	0.014	0.015	0.016	0.016
	AVG	0.0075	0.0076	0.0094	0.0089
AOZ	MAX	0.177	0.108	0.122	0.145
	AVG	0.0622	0.0545	0.0693	0.0856
AMAZ	MAX	0.236	0.240	0.314	0.314
	AVG	0.1375	0.1371	0.1907	0.1910
AHD	MAX	0.072	0.073	0.045	0.046
	AVG	0.0275	0.0274	0.0184	0.0191
SEM	MAX	0.723	0.92	0.381	0.396
	AVG	0.2414	0.2613	0.1952	0.1935

Average concentration of antibiotic residues in Shrimp muscle at different stages of culture period

Table 2: Average concentration of CAP in Shrimp muscle at different stages

Region	Average concentration of CAP			
	Stage-1(PL before stocking)	Stage-2(PL after one week)	Stage-3(one month after stocking)	Stage-4(one week before harvesting)
Shatkhirā	0.008	0.0088	0.0102	0.0098
Khulna	0.006	0.0058	0.008	0.0074
Bagerhat	0.0086	0.0084	0.0102	0.0096

Table 3: Average concentration of SEM in Shrimp muscle at different stages

Region	Average concentration of SEM			
	Stage-1(PL before stocking)	Stage-2(PL after one week)	Stage-3(one month after stocking)	Stage-4(one week before harvesting)
Shatkhirā	0.1494	0.1592	0.1544	0.1512
Khulna	0.3152	0.3626	0.1892	0.186
Bagerhat	0.2596	0.262	0.242	0.2434

Table 4: Average concentration of AOZ in Shrimp muscle at different stages

Region	Average concentration of AOZ			
	Stage-1 (PL before stocking)	Stage-2 (PL after one week)	Stage-3(one month after stocking)	Stage-4(one week before harvesting)
Shatkhira	0.0792	0.0642	0.089	0.1114
Khulna	0.049	0.0408	0.0548	0.0668
Bagerhat	0.0584	0.0584	0.064	0.0784

Table 5: Average concentration of AMOZ in Shrimp muscle at different stages

Region	Average concentration of AMOZ			
	Stage-1(PL before stocking)	Stage-2(PL after one week)	Stage-3(one month after stocking)	Stage-4(one week before harvesting)
Shatkhira	0.1754	0.178	0.2436	0.2412
Khulna	0.1102	0.1118	0.1278	0.1282
Bagerhat	0.1268	0.1216	0.2008	0.2036

Table 6: Average concentration of AHD in Shrimp muscle at different stages

Region	Average concentration of AHD			
	Stage-1(PL before stocking)	Stage-2(PL after one week)	Stage-3(one month after stocking)	Stage-4(one week before harvesting)
Shatkhira	0.0352	0.035	0.0252	0.0262
Khulna	0.0182	0.0182	0.0108	0.0112
Bagerhat	0.029	0.029	0.0192	0.02

5.0 Discussion

The study was conducted to observe the existence of antibiotic residue in shrimp muscle of different farms of Khulna, Shatkhira and Bagerhat region by using ELISA method. Screening test was conducted in the laboratory of FIQC in Boyra, Khulna.

From the study, it was found that the concentration of CAP in shrimp PL, PL after 1 week, after 1 month and harvesting before 1 week was below the cut-off level among the farms in the study areas. The concentration (ppb) of CAP in PL, PL after 1 week, after 1 month and harvesting before 1 week varies from 0.001-0.013 in Shatkhira region, in Khulna region it was 0.001-0.015 and in Bagerhat region it was 0.003-0.016 where the cut-off level is 0.109. Chloramphenicol is a broad-spectrum antibiotic that used for treatment of serious infections in human body. But, it has not been possible to identify the safe dose of it's due to the unpredictable effects of doses on different patients. That's why USA has prohibited its use in food-producing animals and animal food products (FAO, 2005).

The concentration (ppb) of SEM in shrimp PL and PL after one week in one farm of Khulna was 0.723 (Figure 4) and 0.920(Figure 5) respectively where the cut-off level is 0.576 that means it was higher than the acceptable limit. Though SEM was found in PL and PL after one week in one farm of Khulna, the next stages (after one month & harvesting before one week) did not contain harmful level of the residue. The others were below the cut-off level. The concentration (ppb) of SEM in shrimp PL, PL after 1 week, after 1 month and harvesting before 1 week varies from 0.059-0.228 in Shatkhira region, in Khulna region it was 0.022-0.92 and in Bagerhat region it was 0.156-0.396 where the cut-off level is 0.576.

The concentration of AOZ in shrimp PL, PL after 1 week, after 1 month and harvesting before 1 week was below the cut-off level among the farms in the study areas. The concentration (ppb) of AOZ in shrimp PL, PL after 1 week, after 1 month and harvesting before 1 week varies from 0.044-0.145 in Shatkhira region, in Khulna region it was 0.029-0.077 and in Bagerhat region it was 0.032-0.088 where the cut-off level is 0.371. Asian countries are frequently contaminated by AOZ and SEM (European Commission, 2002).

The concentration of AMOZ in shrimp PL, PL after 1 week, after 1 month and harvesting before 1 week was below the cut-off level among the farms in the study areas. The concentration (ppb) of AOX in shrimp PL, PL after 1 week, after 1 month and harvesting before 1 week varies from 0.044-0.145 in Shatkhira region, in Khulna region it was 0.029-0.077 and in Bagerhat region it was 0.032-0.088 where the cut-off level is 0.371.

The concentration of AHD in shrimp PL, PL after 1 week, after 1 month and harvesting before 1 week was below the cut-off level among the farms in the study areas. The concentration (ppb) of AHD in shrimp PL, PL after 1 week, after 1 month and harvesting before 1 week varies from 0.014-0.055 in Shatkhira region, in Khulna region it was 0.001-0.048 and in Bagerhat region it was 0.004-0.073 where the cut-off level is 0.424.

From the study it was found that among the 4 metabolites of Nitrofurantoin (SEM, AOX, AMOZ and AHD) only SEM was found higher than cut-off level and others including CAP were found but in lesser quantities. Nitrofurantoin and chloramphenicol used in hatchery and feed for microbial control (Aftab Uddin et al., 2006; Frappaolo et al., 1986; K. Holmstrom et al., 2003; Nowsad et al., 2009; Paul et al., 2009). Antibiotics are commonly used in aquaculture during the production cycle, both in the larval and growth phases. The use of antibiotics in aquaculture is associated with environmental and human health problems, including bacterial resistance, persistence of the disease in the aquatic environment, and effects on the biogeochemical composition of the sediment. The accumulation of antibiotic residues in the edible tissues of shrimp may also alter human intestinal flora and cause food poisoning or allergy problems (Ma et al., 2006). The tetracycline was not found in shrimp muscle. In shrimp PL and PL after one week from a farm of Khulna got higher quantities of SEM in their muscle and the other stages contain fewer quantities. It may be caused either by feed and/or environment or other sources. Trace amount of SEM was detected in meat of wild-caught prawn (*M. rosenbergii*) in Bangladesh where as high concentration of SEM (up to 100× higher) was detected in shell. So, the authors commented that SEM might be produced in shrimps naturally (McCracken et al., 2011). As most of the hatchery operators and shrimp/prawn farmers are not aware about the deleterious effects of nitrofurantoin, they often use such chemicals to reduce mortality of shrimp without considering the human health issue. Therefore, 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 nitrofurans in exportable shrimp/prawn products. Many unlabelled antibiotics were found to be used in the hatcheries (Nowasad et al., 2009).

The highest average concentration of CAP was found in stage 3 in Shatkhira, Khulna and Bagerhat region. The highest average concentration of SEM and AOZ was found in stage 2 and stage 4 in Shatkhira, Khulna and Bagerhat region respectively. The highest concentration of AMOZ was found in stage 3 in Shatkhira and stage 4 in Khulna and Bagerhat region. And the highest concentration of AHD was found in stage 1 in Shatkhira and stage 1 and 2 in Khulna and Bagerhat district. It was found that in most of the cases the average concentration of antibiotic residues in shrimp muscle increased gradually with the culture period. From this consequence, it could be said that probably feeding was responsible for increasing the concentration of antibiotic residue. Prawns raised in farms fed with commercial feed were contaminated with nitrofurans metabolites more than prawns raised in farms fed with home-made feed with organic and inorganic fertilizer only (Hassan et al., 2013).

But in the last stage (stage-4) the average value reduced in its concentration. It can be assumed that if the culture period could be able to increase, the average concentration would have reduced in the very minimum level. At present, to avoid sudden mortality at mature stage pre-matured/under grade shrimp is being harvested by the most of the farmers (DoF officials).

In 2011, Bangladesh government made a plan namely "National Residue Control Plan" (NRCP) to monitor fish and fishery products at different levels of production in regard to residues of undesirable substances especially nitrofurans and chloramphenicol residues in shrimp and prawn muscle. Under NRCP programme, the aim of the control on fish and fishery products is to assess compliance with the tolerance limits (for contaminants), maximum residue limits (MRLs for permitted substances), to reveal the illegal use of banned or unauthorized substances as well as to determine the origin of contaminant residue. In a word, the overall objective of this programme is to make the Bangladeshi fish and fishery products safe for consumers. At present, NRCP is being executed by the Department of Fisheries (DoF) under the Ministry of Fisheries and Livestock, Bangladesh. (DoF, 2012).

6.0 Conclusion & Recommendation

Antibiotic tested data of collected samples revealed that nitrofurantoin and chloramphenicol antibiotics were found in Shrimp muscle but maximum concentration is lesser in quantities. Only SEM was found in higher concentration in shrimp PL and PL after one week in one farm of Khulna district. Tetracycline was not detected in shrimp muscle. Increased aquaculture practice has resulted in increased levels of infections among species. Various classes of antibiotics exhibit activity against both Gram-positive and Gram-negative bacteria; therefore, they are widely used in aquaculture to treat or prevent diseases. Though in the present study most of the antibiotics residues were found at the very minimum level that is acceptable for human consumption, yet proper and advanced level management systems are the necessity to prevent future contamination in large scale. Policy makers, researchers and scientists should work together in addressing the issues of antibiotics use in aquaculture with the view to reduce the negative impacts.

The following strategies should adopt to reduce contamination:

- Intensive investigation on identification of sources liable to prohibited antibiotics contamination
- Should investigate withdrawal period of antibiotics and maintain it
- It is urgent need to bring all the shrimp hatcheries and shrimp farms under the government monitoring process to success the National Residue Control Plan (NRCP) program.

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8.0 Appendix

8.1 List of photographs





Laboratory Procedure

8. 2 Statistical test to observe any significance difference of concentration among the antibiotics

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CAP	Between Groups	.000	3	.000	.780	.510
	Within Groups	.001	56	.000		
	Total	.001	59			
AOZ	Between Groups	.009	3	.003	6.795	.001
	Within Groups	.025	56	.000		
	Total	.034	59			
AMOX	Between Groups	.043	3	.014	2.648	.058
	Within Groups	.303	56	.005		
	Total	.346	59			
AHD	Between Groups	.001	3	.000	1.413	.249
	Within Groups	.015	56	.000		

Total		.016	59			
SEM	Between Groups	.052	3	.017	.863	.466
	Within Groups	1.118	56	.020		
	Total	1.169	59			

Descriptive

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
CAP	PL	15	.00753	.004307	.001112	.00515	.00992	.001	.014
	PL after one week	15	.00767	.004562	.001178	.00514	.01019	.001	.015
	After one month	15	.00947	.003701	.000956	.00742	.01152	.004	.016
	Harvesting before one week	15	.00893	.004061	.001049	.00668	.01118	.002	.016
	Total	60	.00840	.004146	.000535	.00733	.00947	.001	.016
AOZ	PL	15	.06220	.020171	.005208	.05103	.07337	.037	.117
	PL after one week	15	.05180	.019538	.005045	.04098	.06262	.029	.108
	After one month	15	.06927	.020155	.005204	.05811	.08043	.043	.122
	Harvesting before one week	15	.08553	.024056	.006211	.07221	.09886	.054	.145
	Total	60	.06720	.023959	.003093	.06101	.07339	.029	.145
AMAZ	PL	15	.13747	.063251	.016331	.10244	.17249	.036	.236
	PL after one week	15	.13713	.068213	.017612	.09936	.17491	.004	.240
	After one month	15	.19073	.080571	.020803	.14611	.23535	.047	.314

	Harvesting before one week	15	.19100	.080788	.020859	.14626	.23574	.045	.314
	Total	60	.16408	.076629	.009893	.14429	.18388	.004	.314
AHD	PL	15	.02747	.019283	.004979	.01679	.03815	.003	.072
	PL after one week	15	.02740	.019518	.005040	.01659	.03821	.002	.073
	After one month	15	.01840	.012368	.003193	.01155	.02525	.001	.045
	Harvesting before one week	15	.01913	.012693	.003277	.01210	.02616	.002	.046
	Total	60	.02310	.016502	.002130	.01884	.02738	.001	.073
SEM	PL	15	.24140	.157246	.040601	.15432	.32848	.112	.723
	PL after one week	15	.26127	.198688	.051301	.15124	.37130	.123	.920
	After one month	15	.19520	.086471	.022327	.14731	.24309	.028	.381
	Harvesting before one week	15	.19353	.090336	.023325	.14351	.24356	.022	.396
	Total	60	.22285	.140787	.018176	.18648	.25922	.022	.920

