ASSOCIATION BETWEEN PHYSICOCHEMICAL PARAMETERS AND THE PRESENCE OF *ESCHERICHIA COLI* IN CATFISH

Hasna Binti Parakkasi

(34866)

Bachelor of Science with Honours (Resource Biotechnology) 2015
Association between Physicochemical Parameters and the Presence of *Escherichia Coli* in Catfish

Hasna binti Parakkasi
(34866)

A Thesis Submitted in Partial Fulfilment of the Requirements of The Degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: Professor Dr. Kasing Apun
Co-supervisor: Dr. Lesley Maurice Bilung

Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2015
ACKNOWLEDGEMENT

Foremost, I would like thank to Allah, with His Permission, I manage to complete this project. I also would like to extend my sincere appreciation to my supervisor Prof. Dr. Kasing Apun and co-supervisor, Dr. Lesley Maurice Bilung for their dedicated supervision, guidance and encouragement throughout this study.

Besides that, I would like to express my sincere gratitude to Mr. Azis, lab assistant of Microbiology lab and all postgraduate students in Microbiology lab for their advices, knowledge and support in completing this project.

Last but not least, I am deeply grateful to my beloved family and friends for their care and continuous support throughout my studies in UNIMAS.

Thank you.
DECLARATION

I hereby declare that this thesis entitled 'Association between Physicochemical Parameters and the Presence of Escherichia Coli in Catfish' is based on my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

Name: Hasna binti Parakkasi
Matric No: 34866
Date: 24 June 2015
# TABLE OF CONTENTS

ACKNOWLEDGEMENT i
DECLARATION ii
TABLE OF CONTENTS iii
LIST OF ABBREVIATION iv
LIST OF TABLES v
LIST OF FIGURES vi
ABSTRACT vii
CHAPTER 1 INTRODUCTION 1
CHAPTER 2 LITERATURE REVIEW 4
2.1 Catfish 4
2.2 Aquaculture Industry 5
2.3 Physicochemical Parameters of Water 6
2.4 Microbe in Fish 6
2.5 Indicator Bacteria 7
2.6 E. coli 8
2.7 Pathogenic E. coli O157:H7 9
CHAPTER 3 MATERIALS AND METHODS 11
3.1 Sample collection 11
3.2 Physicochemical Analysis of Water 12
3.3 Microbial Analysis of Water and Catfish Samples 12
3.3.1 Sample Processing 12
3.3.1.1 Sample Enrichment 13
3.3.1.2 Serial Dilution 13
3.3.1.3 Standard Plate Counts 14
3.4 Gram Staining and Biochemical Tests 14
3.5 DNA Extraction 15
3.6 Detection of E. coli O157:H7 using Multiplex PCR 15
3.7 Agarose Gel Electrophoresis (AGE) 18
3.8 Statistical Analysis 18
CHAPTER 4 RESULTS AND DISCUSSION 19
4.1 Physicochemical Analysis of Water 19
4.2 Bacterial Counts of Water and Catfish Samples 21
4.3 Gram staining and Biochemical tests 23
4.4 Detection of E. coli using multiplex PCR 27
4.5 Statistical Analysis 33
CHAPTER 5 CONCLUSION 35
CHAPTER 6 REFERENCES 37
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOD</td>
<td>Biochemical oxygen demand</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EMB</td>
<td>Eosin-Methylene Blue</td>
</tr>
<tr>
<td>EPA</td>
<td>US Environmental Protection Agency</td>
</tr>
<tr>
<td>LB broth</td>
<td>Luria Bertani broth</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Product and Service Solution / Statistical Package for Social Science</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>Tris-acetate-EDTA buffer</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 2.3 Level of physicochemical suitable for freshwater fish rearing 6
Table 3.1 Samples taken from each sampling site 11
Table 3.6.1 Primers used for the detection of *E. coli* O157:H7 using Multiplex PCR 16
Table 3.6.2 The PCR mixture 17
Table 3.6.3 Cycling condition of PCR amplification for *E. coli* O157:H7 18
Table 4.1 Temperature, pH and DO, BOD level of water samples 19
Table 4.2 The bacterial count (CFU/mL) of each type of sample 22
Table 4.3 The results of Gram staining and biochemical tests performed 25
Table 4.4 Distribution of *E. coli* and *E. coli* O157:H7 in samples 28
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Taxonomy of catfish showing 4 of the 36 families</td>
<td>4</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Sample processing of catfish samples</td>
<td>13</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Growth of <em>E. coli</em> on EMB agar</td>
<td>24</td>
</tr>
<tr>
<td>4.3.2</td>
<td><em>E. coli</em> seen under microscope using 1000X magnification with oil immersion</td>
<td>24</td>
</tr>
<tr>
<td>4.3.3</td>
<td>Results of the biochemical tests performed</td>
<td>25</td>
</tr>
<tr>
<td>4.4.1</td>
<td>Result of the PCR assay</td>
<td>29</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Result of the PCR assay</td>
<td>30</td>
</tr>
<tr>
<td>4.4.3</td>
<td>Result of the PCR assay</td>
<td>31</td>
</tr>
</tbody>
</table>
Association between Physicochemical Parameters and the Presence of E. coli in Catfish
Hasna binti Parakkasi (34866)

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Catfish may carry pathogenic bacteria that can cause harm when consumed. Therefore, this study is aimed to determine the level of physicochemical parameters in water and the distribution of Escherichia coli in water and catfish samples. In addition, the study was also done to determine the occurrence of pathogenic E. coli O157:H7 in the samples. The study was done in one catfish farm and two wet markets. From each sampling site, 5 water and 5 catfish samples were collected. Therefore, a total of 45 samples composed of 15 catfish intestines, 15 catfish muscles and 15 water samples were used. Only water samples were subjected to physicochemical analysis while for microbial analysis, both water and catfish samples were analysed. Microbial analyses were done by using standard plate count followed by a series of biochemical tests and detection of E. coli O157:H7 using multiplex PCR. The results showed that the selected physicochemical levels were in Class I as according to the National Water Quality Standards for Malaysia. The total heterotrophic bacterial count of water, catfish intestines and muscles ranged from $1.77 \times 10^4$ CFU/mL to $6.26 \times 10^5$ CFU/mL which both in the catfish muscle. From the 45 samples, E. coli spp. was present in all samples except in 3 catfish muscles where no E. coli spp. can be found. However, pathogenic E. coli O157:H7 was not detected in any of the samples. This indicates that the catfish and water samples are free from pathogenic E. coli O157:H7.

Keywords: Escherichia coli, E. coli O157:H7, catfish, bacterial count, Polymerase Chain Reaction (PCR), water.

ABSTRAK


Kata kunci: Escherichia coli, E. coli O157:H7, Kiraan bakteria, Polymerase Chain Reaction (PCR), air.
CHAPTER 1
INTRODUCTION

Catfish is one type of fish commonly found in ASEAN countries especially in Malaysia (MoA Incorporated, 2015). It can be found mainly in freshwater habitat such as ponds and lakes as well as in rice field areas (Chong et al., 2000). Generally, catfish is considered safer and healthier to consumer (Norah, 2010). However, since catfish is an aquatic animal, it will provide risk as pathogenic bacteria such as *Escherichia coli* (*E. coli*) can be introduced to the catfish especially when it grows in contaminated water.

*E. coli* is a type of faecal coliform bacteria commonly used as pollution indicator in food or water (Del Rio-Rodriguez et al., 1997). *E. coli* being a faecal origin is usually found in gastrointestinal tract of humans and animals is mostly harmless and plays an important part of a healthy human intestinal tract (CDC, 2014). However, some *E. coli* are pathogenic and being the causative agent of many gastrointestinal disease (Baron, 1996). Pathogenic *E. coli* cause infection with diarrheagenic effects which can be divided into several categories according to the symptoms (Nataro & Kaper, 1998). The symptoms cause by pathogenic *E. coli* including traveler’s diarrhea (enterotoxigenic *E. coli*), hemorrhagic colitis and hemolytic-uremic syndrome (enterohemorrhagic *E. coli*), infants’ watery-diarrhea (enteropathogenic *E. coli*) and persistent diarrhea (enteroaggregate *E. coli*) (Nataro & Kaper, 1998).

Reports showed that food poisoning is always associated with *E. coli* infection (Del Rio Rodriguez, 1997). It is said that *E. coli* has been the most common causes of food poisoning in many countries including South America (Utsunomiya et al., 1995), Europe
An example of enterohemorrhagic serotype of the bacterium \textit{E. coli} is \textit{E. coli} O157:H7.

Infection by \textit{E. coli} O157:H7 can be triggered by many factors such as animal contact, water source and person-to-person transmission (WHO, 2011). Besides that, interaction of physicochemical parameters such as pH, temperature, biochemical oxygen demand level and water activity also plays a role in the survival and growth of the \textit{E. coli} O157:H7 (Buchanan & Doyle, 1997). Moreover, the composition and activities of microbial population in water is affected by the physicochemical conditions (Sigee, 2005). Previous study shown that, the quality of water from where the fish harvested was correlated with the microbial occurrence in fish (Sanyal \textit{et al.}, 2010). It was because coliform was not the normal floras of fish (Cohen & Shuval, 1973). This showed that the physicochemical characteristics also play a significant role in the presence of microorganisms in catfish.

Usually, \textit{E. coli} O157:H7 will show its pathogenic effects through consumption of contaminated food (Karch \textit{et al.}, 2005). The symptoms of the bacteria may need as short as 1 day or as long as 10 days before it shows, begin with mild belly pain or non-bloody diarrhea that can be worsen over several days (CDC, 2014). \textit{E. coli} O157:H7 affects human by producing Shiga toxin in the intestine which act systemically on sensitive cells in the brain, kidney and other organs (Gyles, 2007).

Nowadays, aquaculture industry is rapidly grown as seafood has high demand in both local and international markets (Khosravi \textit{et al.}, 2013). Malaysia, Thailand, Vietnam and China are the catfish exporters in Asia (Josupeit, 2008). Contamination of seafood with pathogenic \textit{E. coli} has been known to occur, much of which is due to contaminated water.
sources (Khosravi et al., 2013). However, there is only few research carried out on the presence of *E. coli* in poikilothermic animals such as fish even though *E. coli* being the most studied microorganism (Reynolds et al., 1978). This has raise concern about public health associates with catfish consumption.

Therefore, it is important to conduct this study as only few researches have been carried out on *E. coli* O157:H7 on catfish. The finding of this study was carried out through Colony Forming Unit (CFU) count by quantifying the viable cell number of *E. coli* spp. which appeared as black colonies with green metallic sheen on the EMB agar. Then, the *E. coli* spp. isolated from the Eosin Methylene Blue agar was subjected to a series of biochemical tests. The target genes, Shiga-toxin producing genes and genes encoded for antigen O157 and antigen H7 were detected by using multiplex Polymerase Chain Reaction (mPCR) which aid in amplifying DNA sequences. Both catfish and water samples for this study were taken from one catfish farm and two different wet markets.

This study was conducted with the following objectives:

1. To detect the level of physicochemical parameters of the water from the sites where the samples taken
2. To determine the concentration of *E. coli* spp. and detect the *E. coli* O157:H7 in both water and catfish samples in the selected study sites
3. To determine whether there exist any correlation between the physicochemical parameters and the concentration of *E. coli* spp.
CHAPTER 2

LITERATURE REVIEWS

2.1 Catfish

Catfish belongs to order Siluriformes or ray-finned fish. It got its name because it has whiskers or barbells which much like the common house cat. The characteristics of the barbells that located on area near the mouth often used to differentiate the catfish species. There are many different species of catfish (Figure 2.1). The commonly found are channel catfish (*Ictalurus punctatus*), blue catfish (*Ictalurus furcatus*) and flathead catfish (*Pylodictis olivaris*) (Bussolini & Byrum, 2011). They can be found mainly in freshwater habitat such as ponds and lakes as well as in rice field areas (Chong et al., 2000). Catfish was one of the major species consumed by Malaysian besides mackerel, shrimp, tilapia and squid (The Nation, 2014). Besides used as food source, high-value of fish gelatin can be produce by using catfish skin as raw material (Mahmoodani et al., 2014).

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Genus species</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siluriformes</td>
<td>Ictalurida</td>
<td><em>Ictalurus punctatus</em></td>
<td>Channel catfish</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ictalurus furcatus</em></td>
<td>Blue catfish</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ictalurus melas</em></td>
<td>Black bullhead</td>
</tr>
<tr>
<td></td>
<td>Ameiurida</td>
<td><em>Ameiurus catus</em></td>
<td>White catfish</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ameiurus nebulosus</em></td>
<td>Brown bullhead</td>
</tr>
<tr>
<td></td>
<td>Silurida</td>
<td><em>Pylodictis olivaris</em></td>
<td>Flathead catfish</td>
</tr>
<tr>
<td></td>
<td>Clariida</td>
<td><em>Silurus glanis</em></td>
<td>European catfish</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Clarius ganis</em></td>
<td>African catfish</td>
</tr>
<tr>
<td></td>
<td>Pangasiida</td>
<td><em>Pangasiodon gigas</em></td>
<td>Giant catfish</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pangasius bocourdi</em></td>
<td>Basa</td>
</tr>
</tbody>
</table>

Figure 2.1. Taxonomy of catfish showing 4 of the 36 families.

2.2 Aquaculture Industry

Aquaculture production is dominated by Asia where it contributes around 91% of the world’s total by volume and 82% by value (Yeoh et al., 2010). Besides that, Asian countries have highest variety of cultured species and listed among the top ten aquaculture producers in the world (Yeoh et al., 2010). High demand of catfish resulted in rapid growth of catfish aquaculture in Malaysia (Kechik, 1995). Besides that, catfish aquaculture is a profitable investment as it does not require high cost to start the catfish farming. Catfish can be reared in tanks, canvas, underwater cages or other vessels and in pond. Malaysia, Thailand, Vietnam and China are the catfish exporters in Asia (Josupeit, 2008). The catfish are export in a form of frozen fresh catfish, canned, fillet or even processed into snacks. In Malaysia, there were many areas reserved for the catfish farming. One of the examples is the Tanjung Manis halal hub in Sarawak which reared catfish in a large scale (Borneo Post, 2012).

Nowadays, catfish industry is gaining attention and had contributes significantly to the economies of the developing countries (Subasinghe, 2007). Malaysia has the potential to become a major country in the aquaculture industry in Asia Pacific if this sector is joins by more companies (Alongi et al., 2003). A direct employment to 89,453 fishermen and 21,507 fish culturists has been provided by fisheries sector in Malaysia (Yeoh et al., 2010). As for catfish, it has been successfully entered the international trade and had shown strong unit value and the trend increase year by year (Helga, 2006).
2.3 Physicochemical Parameters of Water

The composition and activities of microbial population in water is affected by the physical and chemical conditions (Sigee, 2005). Some organisms can survive in wide range of condition and some are very sensitive to change in conditions (Raibole & Singh, 2011). The changes in physical and chemical conditions cause the aquatic life became stressed and more prone to the infection of bacteria, fungus and other pathogens. Besides that, poor quality of water can increase the growth of pathogenic bacteria. The level of physicochemical conditions suitable for freshwater fish is shown in Table 2.3.

Table 2.3. Level of physicochemical suitable for freshwater fish rearing.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Suitable Level</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>29.4</td>
<td>(Wellborn, 1998)</td>
</tr>
<tr>
<td>pH</td>
<td>6-9</td>
<td>(Tucker &amp; D’Abramo, 2008)</td>
</tr>
<tr>
<td>Dissolved oxygen (mg/L)</td>
<td>5</td>
<td>(Patil et al., 2012)</td>
</tr>
<tr>
<td>Biochemical oxygen demand (mg/L)</td>
<td>3-6</td>
<td>(Bhatnagar et al., 2004)</td>
</tr>
</tbody>
</table>

2.4 Microbes in Fish

The microbial flora of freshly caught fish is largely a reflection of microbial quality of the water from where they are harvested (Noomissabegum and Kasturi, 2014). It is mainly because the range of bacterial genera isolated from fish is related to the aquatic habitat of the fish that varies with physicochemical factors and the bacterial load in the water (Cahill, 1990). Both studies done by Apun et al. (1999) and Odebiyi et al. (2013) found that bacteria can be found in the organ of fish such as skin surface and gills but it was primarily detected in the fish
intestines. Bairagi et al. (2002) and Saha et al. (2006) reported that bacterial species isolated from fish digestive system were mostly aerobic or facultative anaerobic. Moreover, the bacteria found in the digestive tract of fish were highly variable (Nieto et al., 1984). Fish intestines commonly had Gram positive bacterial species such as Bacillus sp., Listeria and Staphylococcus and Gram negative bacterial species such as Aeromonas hydrophila, Citrobacter freundii, E. coli, Enterobacter aerogenes, Klebsiella sp., Pseudomonas sp., and Vibrio anguillarums (Apun et al., 1999). Meanwhile, the gills of fish were dominate by Gram negative rods bacteria especially Cytophaga spp. although other bacteria such as Aeromonads, enterobacteria, Gram positive cocci, pseudomonads and vibrios have also been recovered from fish gills (Austin, 2006).

In catfish, many studies have been done to detect the occurrence of microorganism especially microbiological pathogens. For instance, microbial analyses of US catfish fillets has found the presence of Shigella, L. monocytogenes and Salmonella in the samples taken but no E. coli O157:H7 (McCaskey et al., 1998). Another study found that 21% of the domestic channel catfish and 41% of the Vietnamese basa were positive for Salmonella (Pal and Marshall, 2009).

2.5 Indicator Bacteria

Fish are susceptible to microbial contamination because it has soft tissues and live in aquatic environment. Bacteria can stay on the surface of the body or enter the fish body through the gills or skin (Shinkafi and Ukwaja, 2010). The fish might contaminate with pathogenic bacteria such Salmonella spp., Escherichia coli and Staphylococcus aureus which known to be the cause of enteric and other infectious disease thus human consumption or handled can result
in potential danger (Olayemi et al., 1991; Pal and Dasgupta, 1992). Therefore, indicator bacteria were used to detect the presence of faecal contamination in food and water (Anukool & Shivani, 2011). The occurrence of pathogenic bacteria in fish often detected by presence of indicator bacteria because they were more safe and easy to detect compared to the real pathogenic bacteria (USEPA, 2000).

Coliforms are the major microbial indicator of monitoring water quality (Brenner et al., 1993). One species of coliform is faecal coliform, a Gram negative, rod-shaped, facultative anaerobic and non sporulating bacterium which presence is associated with warm blooded animal pollution (Sanyal et al., 2010). Previous studies shown that, the quality of water from where the fish harvested was correlated with the microbial occurrence in fish (Sanyal et al., 2010). It was because coliform was not the normal floras of fish (Cohen & Shuval, 1973). One of the famous examples of faecal coliform is Escherichia coli (E. coli).

2.6 E. coli

E. coli was named after its founder Theodore Escherich, a paediatrician who discovered the bacterium in the late 1800's (Shulman et al., 2007). E. coli is a non-spore forming, rod shaped, Gram negative bacillus which is a normal inhabitant of the large intestine of vertebrates, including humans, cows, pigs and goats (Tortora et al., 2010). The optimum temperature for the growth of E. coli is at 37°C (Gadgil et al., 2005). The bacterium, E. coli form dark black colour with green metallic sheen colonies when grow on Eosin Methylene Blue (EMB) agar (Lal and Cheeptham, 2007).
Most *E. coli* strains are harmless and its presence served beneficial function in the body (Feng, 2001). The bacterium helps produce certain vitamins and breaks down otherwise indigestible foodstuffs (Tortura *et al.*, 2010). However, some strains of *E. coli* with different mechanisms of pathogenicity have developed the capability to cause disease that can affect a wide range of cellular processes (Kaper *et al.*, 2004). With the increase in international travel and trade globalization, diarrheagenic *E. coli* has become a worldwide public health threat. Fortunately, its identification has become easier with the development of diagnostic tools based on the detection of the virulence trait. For instance, Polymerase chain reaction (PCR) methodologies can identify pathogenic strains within hours (Rubino *et al.*, 2011).

### 2.7 Pathogenic *E. coli* O157:H7

*E. coli* O157:H7 is an enterohemorrhagic serotype of *E. coli* bacterium that can cause disease typically through contaminated food consumption (Karch *et al.*, 2005). *E. coli* O157:H7 has the ability to cause illness to humans resulting from its ability to generate numerous virulence factors, most notably Shiga toxin (Johannes, 2010). Shiga toxin (Stx) has multiple variants such as Stx1 and Stx2 that acts by inhibiting protein synthesis in endothelial and other cells (Sandvig, 2002). Endothelial cells line the interior surface of blood vessels, and are known to be extremely sensitive to *E. coli* O157:H7, which is cytotoxic to these cells (Sandvig, 2002). Besides Shiga toxin, *E. coli* O157:H7 also generate numerous other putative virulence factors. The virulence factors including proteins which aid in the attachment and colonization of the bacteria in the intestinal wall which can lyse red blood cells and liberate iron to help support *E. coli* metabolism (Welinder-Olsson and Kaijser, 2005).
Infection by *E. coli* O157:H7 in human is associated with wide range of clinical illness, including asymptomatic shedding, non-bloody diarrhoea, haemorrhagic colitis, hemolytic uremic syndrome, and death (Mead and Griffin, 1998). Usually, little or no fever is present and in most of the mild cases the illness resolves in five to ten days. Haemolytic uremic syndrome (HUS) mostly caused by *E. coli* O157:H7 infection is a condition where the patient suffered from acute renal injury, thrombocytopenia, and microangiopathic haemolytic anaemia (Karmali *et al.*, 1983).
CHAPTER 3
MATERIALS AND METHOD

3.1 Sample Collection

The samplings were carried out in three different sites within the months of February and March 2015. The selected locations consisted of two wet markets which were Samarindah market and Kota Sentosa market and one catfish farm which was located in Kampung Haji Baki, Kuching. A total of 24 water samples (9 water samples for Biochemical Oxygen Demand (BOD) analysis and another 15 water samples for microbial analysis) and 15 catfish samples were collected from the three selected sampling sites (Table 3.1). All water samples were kept in sterile bottles and stored in an ice box for transportation. Meanwhile, the catfish samples were kept in plastic bags containing water where the catfish taken. All samples were transported and analysed immediately upon arrival at the Microbiology Laboratory, UNIMAS.

Table 3.1. A number of samples taken from each sampling site

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of catfish samples (for microbial analysis)</th>
<th>No. of water samples</th>
<th>No. of water samples (for microbial analysis)</th>
<th>No. of water samples (for BOD analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samarindah market</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Kota Sentosa market</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Catfish farm</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>15</strong></td>
<td><strong>15</strong></td>
<td></td>
<td><strong>9</strong></td>
</tr>
</tbody>
</table>
3.2 Physicochemical Analysis of Water

Temperature, pH and dissolved oxygen (DO) of the water samples were taken at the sampling sites using portable pH meter (detect temperature and pH simultaneously) and DO meter respectively. Readings were taken at three different points and the average were calculated and recorded. Biochemical oxygen demand (BOD) measurements were tested in laboratory using APHA (1989) standard method. The water samples taken for BOD analysis from each site were divided into two parts. One part was tested immediately for DO. The other part was incubated in the dark at room temperature for 5 days and the amount of DO remaining was tested. The difference in oxygen levels between the water samples of the first part and second part were calculated in milligrams per liter (mg/L). The difference represents the BOD level as it is the amount of oxygen utilized by microorganisms to degrade the organic matter present in the water sample during the incubation period.

3.3 Microbial Analysis of Water and Catfish samples

3.3.1 Sample Processing

Sample processing was done using methods as mentioned by Apun et al. (1999). The water and catfish samples were processed and analyzed immediately upon arrival at laboratory (Figure 3.3.1). Each catfish were killed by performing euthanasia which did not cause any tissue damage. A total of 10 g (wet weight) of the muscle and 1 g of intestine in each catfish were isolated with sterile scalpel and used as samples for microbial analysis. Both catfish intestine and muscle were weighted separately using weighing balance before minced finely. As for the water samples, 10 mL of each water samples were used.
3.3.1 Sample Enrichment

Ten grams of catfish muscle and 10 mL of water were placed in stomacher bag with 90 mL of Luria Bertani (LB) broth. For the 1 g of catfish intestine, 9 mL of LB broth were placed in the stomacher bag. The catfish isolates and water samples were homogenized with the LB broth and incubated at 37°C ± 2 for 24 hours.

3.3.1.2 Serial Dilution

The sample mixtures were subjected to ten-fold serial dilutions in Phosphate Buffered Saline (PBS). One mL of the sample mixture was diluted with 9 mL of Phosphate Buffer Saline (PBS). The samples were diluted from 10⁻¹ until 10⁻³.
3.3.1.3 Standard Plate Counts

A total of 100 μL of diluted samples from 10^-2 and 10^-3 dilution tube were plated on Eosin-Methylene Blue (EMB) agar in duplicate. After that, the plates were incubated at 37°C ± 2 for 24 hours and observed for the formation of green metallic sheen colonies. The green metallic sheen colonies on the EMB agar were counted as it indicates positive growth of *E. coli*. The range of number of bacterial colonies counted was between 30-300 colonies and the Colony Forming Unit per microlitre (CFU/mL) was calculated. The CFU/mL of the bacteria was calculated using formula as mention by Reynold and Farinha (2005). The formula used was as follows;

\[
\text{Number of bacteria (CFU/ml) = } \frac{\text{Number of colonies}}{(\text{dilution factor } \times \text{volume plated})}
\]

Then, a single colony from the EMB agar was picked and striking technique was applied on a new EMB agar to get a single colony. The colony on the new EMB agar then was incubated at 37°C ± 2 for 2 hours. After incubation, single colony was picked from each plate and growth in a bijoux bottle contains 5 mL of LB broth and incubated again overnight at 37°C ± 2. After incubation, one inoculum of *E. coli* sp. from the bijoux bottle was streaked into slant agar and a portion were stored into glycerol stock for culture stock.

3.4 Gram-staining and Biochemical Tests

All isolates were tested through Gram-staining and a series of biochemical tests as recommended by Hemraj *et al.* (2013). The biochemical tests included were motility test and
IMViC test (Indole test, Methyl red test, Voges Proskauer test and Citrate test). *E. coli* pure cultures from previous studies done by Postgrad student in UNIMAS were used as positive control.

3.5 DNA Extraction

Bacterial DNA extraction was carried out using boiled cell method as mentioned by Apun et al. (2011). *E. coli* from the culture stock was isolated and cultured in 5 mL LB broth for 24 hours at 37°C ± 2 with agitation at 150 rpm. Then, the broth suspension (1 mL) that incubated for 24 hours centrifuged at 13,000 rpm for 5 minutes and pellet form was collected by discarding the supernatant. The pellet was re-suspended in 100 μL of distilled water and boiled for 20 minutes. The cells that lysed then cooled in ice for another 20 min and centrifuged at 13,000 rpm for 3 min. The final supernatant containing DNA was transferred to a new centrifuge tube, stored at -20°C. The final supernatant was used as DNA template in the multiplex PCR assay.

3.6 Detection of *Escherichia coli* O157:H7 using Multiplex PCR

A single tube reaction of Multiplex PCR assay was done to detect the pathogenic *E. coli* O157:H7 as done by Apun et al (2011). Four sets of synthesized primers were used in this study to detect the targeted genes. The targeted genes are responsible for producing Shiga-toxin genes and encoded genes for antigen O157 and antigen H7 (Table 3.6.1).