Assessment of Microbiological Activities of *Empuraau* Fish (*Tor Tambrades*) of Local Fish Farm

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LIST OF ABBREVIATIONS

µl  microliter
ml  milliliter
ºC  Degree Celsius
API  Analytical Profile Index
BOD  Biochemical Oxygen Demand
DO  Dissolved Oxygen
TAN  Total Ammonia Nitrogen
ddH₂O  Sterilize Double Distilled Water
DNA  Deoxyribonucleic Acid
EtBr  Ethidium bromide
NaCl  Sodium chloride
rpm  revolution per minute
PCR  Polymerase Chain Reaction
TAE  Tris Acetate EDTA
UV  Ultraviolet
V  Voltage
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Assessment of Microbiological Activities of Empurau Fish (Tor Tambrades) of Local Fish Farm

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ABSTRACT

Enterobacteriaceae is a member of gram-negative bacilli and contain more than 30 genera. They act as the main indicator for the bacteriological water quality. Study was conducted to determine the occurrence of Enterobacteriaceae family in Empurau (Tor Tambrades) pond at Indigenous Fish Research & Production Centre (IFRPC) in Tarat, Sarawak in which this it could reflect the level of microbe found in the pond. Studies also been conducted for detection on any possible pathogenic bacteria that might cause infectious disease in fish pond. Besides that, studies were conducted for determining the relationship between physicochemical parameter and microbiological parameter with the development of the fish in the pond. Water sample from the pond were collected and analyzed for the presence of Enterobacteriaceae. Sample plated on Nutrient Agar and colonies formed were tested on gram staining. Colonies with Gram-negative were further analyzed with (GTG)\(_5\) PCR. The phylogenetic tree was constructed based on the DNA fingerprinting to illustrate the relationship among the bacteria isolates. 11 randomly picked samples were tested with API 20E kit for identification of the bacteria

Key words: Enterobacteriaceae, (GTG)\(_5\) PCR analysis, API 20E kit,

ABSTRAK

Enterobacteriaceae merupakan bakteria “gram-negatif, mempunyai lebih dari pada 30 genera dan merupakan salah satu penanda utama untuk mengkaji kualiti air dari sudut mikrobiologi. Kajian dijalankan untuk melihat tahap kelangsungan spesis Enterobacteriaceae dalam kolam ikan Empurau (Tor Tambrades) di (IFRPC) Tarat, Serian Sarawak serta membantu untuk memahami kadar mikrob dalam kolam. Selain itu, kajian dijalankan untuk mengenalpasti mikrob yang mempunyai potensi untuk bertukar menjadi pathogen yang mungkin akan menyebabkan penyebaran penyakit dalam kalangan ikan dikolam tersebut dan untuk mengenalpasti hubung kait antara parameter dari sudut kimia fizikal dan juga mikrobiologi terhadap tahap pembangunan ikan dalam kolam tersebut. Sampel air dibawa ke makmal dan diuji atas agar nutrien dan koloni bakteria yang tumbuh di uji dengan ujian “gram staining”. Koloni gram- negatif di analisis dengan PCR (GTG)\(_5\) dan pokok “phylogeny” dihasilkan. 11 sampel dipilih secara rawak dan di uji dengan kit API 20E untuk mengenalpasti jenis bakteria tersebut.

Kata kunci: Enterobacteriaceae, analisa (GTG)\(_5\) PCR, kit API 20E.
CHAPTER I
INTRODUCTION

*Tor tambroides* or locally known as *Empurau*, is one of the most valuable and high commercial value fish species which belongs to *Cyprinidae* (carps) family. *Tor* have been identified as one of the indigenous species (Abdullah *et al.*, 2011). The members of the *Tor* family which can be found in Malaysia are *Tor tambroides* (Kelah), *Tor tambra*, and *Tor douronensis* (Semah). According by Pisolkar and Karamchadini (1984) and Talwar and Jhingran (1992), *Tor* inhabits both river and freshwater lakes but swims upstream to rapids within the rocky bottom to breed (Misieng *et al.*, 2011).

Although it is valuable and priced in Malaysia, *Tor* species has a very higher demand. It is said that a *Tor* that weight four kilogram could reach RM 2,800 – RM 3,000. According to Ng (2004), the price would increase more due to the recognition of *Tor* as an excellent game fish. Tor also has it potential as an ornamental fish due to its attractive coloration on their bodies. Tor also been recognized to have high potential in aquaculture (Misieng *et al.*, 2011).

According to Geldrich and Clarke (1966), the bacteriological qualities of the water reflect the bacterial flora of the fish (Apun *et al.*, 1999). *Tor* has a very bright potential in aquaculture sectors. Fish farmers should be able to understand the importance of managing the proper bacteriological quality of water, since bacteriological quality play an important role in the spreading of fish farmed disease. Robert *et al.* (1975) said that, infectious disease is one of the factors that affect the feasibility of fish farming and can destroy the economical status of the aquaculture sector (R.J. Roberts, 1976). Besides that,
Reichenbach-Klinke (1973) stated that, bacterial flora of the fish is the primary significant source of occupational disease on fish handlers (Apun et al., 1999).

According to Guo et al. (1988), there are close relationship between bacteria and many ecological factors in fish ponds. Some of the factors are the dissolved oxygen, suspended matter, organic detritus, transparency and nutrient salt. These factors show either positive or negative correlation in the pond management. Therefore, it is obvious that, a pond management had a very strong influence on bacterial number in the pond ecosystem (Apun et al., 1999).

The availability of information on microbiological aspect of fish grown in freshwater are still lacking. According to Geldrich and Clarke (1996), the bacteriological quality of the water from the originated area of the fish could signify the bacterial flora of the fish. To a certain extent, level of contamination of the water by enteric bacteria could be identified (Apun et al., 1999).

Not many research has studies has conducted to know the suitable environment for Tor habitats, however it is said that Tor need a very clean environment to live in. Thus assessment such as water quality and microbiological quality need to be done to determine the criteria for the suitable environment for Tor species.

Setting the criteria need to be done after the assessment of parameter both from the physicochemical parameter and microbiological parameter. Both this parameter plays a crucial role in identifying and setting the indicator for the Tor most suitable quality development and habitual environment.
Physicochemical analysis such as determination of hydrogen ion concentration (pH), temperature, level of Dissolved Oxygen (DO), level of Biochemical Oxygen Demand (BOD) and the Total Ammonium Nitrogen (TAN) content are correlated with the type of microorganism that presence. While the analysis of microbiological indicator will enhance the understanding of microbiological activity occurs in the aquatic environment. Further analysis on molecular techniques will be able to detect the presence of pathogenic bacteria which it can help to reduce the incidence of infectious disease spread in the Tor habitat.

The objectives of this study are to detect the level of the microbe in the fish farm and as well as to detect the pathogenic bacteria that might cause a spreading of infectious diseases in fish farm which incautiously lead to zoonosis. This is done by characterisation of microbes using PCR techniques. Besides that, this study was done to observe the relationship between physicochemical parameter and microbiological parameter to the development of the fish.
CHAPTER II

LITERATURE REVIEW

2.1 Tor species

Tor species are known as Mahseer or commonly known as Empurau, Kelah or Pelian and Semah. The Tor species belong to Cyprinidae (carp) family. Most common Tor species found in Malaysia are Tor tambroides, Tor douronesis, and Tor tambra. Tor has been identified as one of the indigenous species and high valued species that inhabit upland rivers (Kiat, 2004) (Abdullah et al., 2011).

According to Sungam et al. (2004) the number of Tor species has been on decline due to significant degradation in their natural habitat due to deforestation and agricultural development. As the demands of Tor species are high, it leads to overfishing of the species. According to Ng (2004) this scenario mainly occur due to that Tor specie are recognized as excellent game fish. It is also a high quality ornamental fish because it possesses good attractive coloration (Misieng et al., 2011).

Figure 2.0: Kelah (Tor tambroides)
2.2 Influence of physicochemical factors on the development of microorganisms

Physical and chemical factors affect the growth of aquatic microorganism in a multitude of ways, in which they may act with or against each other. The influences not only affected the sizes and composition of the microbial populations, but also the morphology and physiology of the individual bacteria and fungi (Rheinheimer, 1991).

Changes in metabolism, cell morphology and reproduction system may also occur in some species due to the changes in temperature, salt concentration or pH value. The concentration of some nutrients and active substance such as carbohydrates and vitamin may be too low or too high for some or even for all microorganisms.

2.2.1 Hydrogen Ion concentration (pH)

The hydrogen ion concentration (pH) plays a significant role in the aquatic organism environment as the concentration range of pH is typically pH 4 – 9. Any pH marked out of the range of standard pH may result in the imbalance of the ecosystem. It will cause not only the physiological change but also morphological changes such as irregular shape of the organism, swelling and also branching.
2.2.2 Temperature

One of the factors that affect the life process of microorganism is temperature. Microorganism such as bacteria, cyanobacteria and fungi can grow within the temperature of -10°C and +90°C. According to Ingraham (1962) and Precht (1973), within this range, the temperature affect growth rate, the nutritional requirements and to a lesser extent, the enzymatic and chemical composition of the cell (Rheinheimer, 1991).

There are four general effect of influactuation of temperature of water towards the aquatic ecosystem (Bryne 1998):

a) Increased in the temperature will increased the metabolic rate of an organism due to the enzymatic reaction. This will result in consumption of O₂ faster than usual rate. Some of the organism may survive, however most of organisms will start to break down. This will eventually raise the Biochemical Oxygen Demand (BOD) level.

b) Oxygen solubility decreases with the water temperature increase. Thus oxygen level will drop further.

c) The concentration of CO₂ will increase gradually as the level of O₂ decreases. Accumulation of CO₂ will lower the O₂ limit which the fish can tolerate.

d) Toxicity of any poisonous substances is correlated with the changes of temperature. The more the temperature increase, the more the toxicity of poisonous substance increase.
2.2.3 Turbidity

The life of microorganisms or especially aquatic microorganism is affected by the turbidity of the water. This is due to the seston, the total of living and dead material suspended in the water, which finally leads to the formation of sediment. According to Dietrich *et al.* (1975) seston consist of the following three components (Rheinheimer, 1991):

1. The fine particles of mineral material which originate on land and are transported from land to the water.

2. Detritus consisting of inorganic and predominantly organic material finely ground up.

3. The plankton which plant and animal creatures of small sizes floating in the water.

Many microorganisms feed on seston as substrates. A surface flora of numerous bacteria and fungi is carried by detritus particles. Detritus particles in particular frequently carry a surface flora of numerous bacteria and fungi (Rheinheimer 1991).

Increases in the amount of suspended organic matter will eventually increase the turbidity accompanied by a vigorous rise of the bacterial counts. This comparison between the turbidity measurements and the total microbial could thus permits some conclusion with respect to the kind of substance responsible for the turbidity.
### 2.2.4 Dissolved oxygen (DO)

The amount of oxygen dissolved in the solution of stream is known as dissolved oxygen (DO). The saturation point of the dissolved oxygen saturated in the solution is determined by four ways such as the solubility of the gas, the partial pressure of the gas in the atmosphere, temperature and also the impurities concentration in the water such as suspended solid. (Tchobanoglous *et al.*, 2004).

According to Smith (2004), the warmer water has a lower saturation point compared to the cooler water, and also for the water that flowing from the higher velocities abilities to hold DO are higher compared to the water running slower.

DO require for the process of respiration and decomposition. Since DO is slightly soluble in water, it becomes the important parameter for aerobic microorganism as well to other form of aerobic life in the aquatic environment. (Smith, 2004).

### 2.2.5 Biochemical Oxygen Demand (BOD)

Biochemical Oxygen Demand (BOD) is the amount of O$_2$ required by aerobic microorganisms to decompose the organic matter in a sample of water and act as an indicator to oxygen stress in the consequence of organic pollution. Theoretically, BOD$_5$ takes an infinite remaining time to complete because the rate of biochemical oxidation is assumed to be proportional to the amount of organic matter (Tchobanoglous *et al.*, 2004).
2.2.6 Total Ammonia Nitrogen (TAN)

NH₃ is the form of toxic ammonia which is toxic to fresh water organism at the concentration ranging from 0.53 to 22.8mg/L. However this toxicity level is depending both on pH and temperature. As both the pH and temperature decrease, the toxicity increases.

Excess amount of ammonia level may harm the aquatic life as it may direct to the changes in tissues of gills, liver and kidney. Aquatic organisms such as fish may experience such of symptoms as loss in equilibrium, hyper excitability, increased in respiratory activity and O₂ uptake and increased heart rate. Some of the experimental data shows that the ranges of ammonia that can lead to lethal were ranging from 0.2 to 2.0mg/L.
2.3 *Enterobacteriaceae spp*

*Enterobacteriaceae* spp. is a family of gram-negative bacilli and non-sporing bacteria. They are aerobic microorganism but under certain environment they can be function as facultative anaerobic. Peritichous flagella in *enterobacteriaceae* spp. aid in motility except for *Shigella* and *Klebsiella* which both of this microorganism are non-motile. *Enterobacteriaceae* spp. are capsulated and possess complex cell wall and they also comes with fimbriae (pili).

*Enterobacteriaceae* spp. contains more than 30 genera and 120 species that normally inhabit the intestines of human and animal. Since, some of organism in this group could cause primary infection on human gastrointestinal tract, therefore they are known as enteric. Furthermore, members of this family are the major contributor for opportunistic infection such as septicaemia and pneumonia (Alvin Fox, 2011).

As some of *enterobacteriaceae* spp. could be found in water sample, they act as the main indicator for the bacteriological water quality since they are group of coliform bacteria.

2.4 Zoonosis

Zoonoses are defined as an infectious disease of animal that can be transmitted to man. While according to The World Health Organization, they defined zoonoses as “these disease and infections which naturally transmitted between vertebrate animals and men.

CDC,(2006) reported that more than 100 zoonoses are recognised and most cases reported that the route of transmission are mainly from animal that have closed contact
with human. Besides that, there are over 500 different pathogens reported which are transmitted from animal to human (Corey, 1990).

2.5 (GTG)$_5$ PCR application

The determination of genetic diversity of different isolates through DNA fingerprinting could be identified by the application of (GTG)$_5$ PCR technique. (GTG)$_5$ PCR technique enable to determine the origin of bacteria and their relatedness. Application of oligonucleotide primer (GTG)$_5$ is necessary for the purpose of obtaining amplified PCR product (Matsheka et al., 2005). Multiple banding pattern are observed from the AGE performed for (GTG)$_5$ PCR. These bands represent the profiles of diverse isolates. Techniques of plotting the dendogram implement the phenotypic characteristic of isolates. The evaluation for determining the genetic diversity distance represent the clustered can be done from the dendogram. Repetitive extragenic palindromic PCR coupled with (GTG)$_5$ PCR was able to determine the source of fecal pollution by E.coli (Mohapatra et al., 2008). According to DeVuyst (2008), (GTG)$_5$ PCR techniques is a rapid and excellent genotypic tool for enterococci and lactobacilli typing. Based on the molecular typing of environmental enterobacteriaceae spp. isolates, it was reported that (GTG)$_5$ is the most appropriate method to be applied (Mohapatra et al., 2007). (GTG)$_5$ is also known as the cost-effective and easy to performed techniques for epidemiology studies (Matsheka et al., 2005).
3.1 Sample collection and sample processing.

Sample collection was conducted at Indigenous Fisheries Research & Production Centre (IFRPC) in Tarat, Serian Sarawak on 22\textsuperscript{nd} February 2012. Sample collection was done at seven randomly picked Empurau ponds. Water samples were collected using sterile 50ml Falcon tube, respectively at 2 points; the inlet and the outlet based on (Huys, 2003) methods with slight modification.

The pH and temperature of the pond water was measured using pH and temperature probe (Hanna Ltd), while the dissolved Oxygen (DO) was measured using DO Meter. Water sample from each point of inlet and outlet were poured into new sterile 50 ml Falcon tube and mixed. Date and time of sampling were labelled at the same time of sample collected. Samples were kept at 4 °C and were transported on the insulated double walled container to the laboratory for further analysis.
3.2 **Total Ammonia Nitrogen (TAN) determination**

Water samples were tested with Hach test kit. The water samples from each pond were tested according to the Hach Test Kit Manual.

3.3 **Total Plate Count**

1 ml of homogenized water sample was pipetted into 9 ml of distilled water and was labelled as dilution $10^{-1}$. Later 1 ml from dilution $10^{-1}$ was pipetted into 9 ml of distilled water and labelled as dilution $10^{-2}$. Each sample of 100 µl from $10^{-2}$ was then spread out onto non-selective Nutrient Agar (Oxoid Ltd) and incubated at 37 °C for 24 hours for the purpose of plate count. Single colonies formed were counted as one after overnight incubation.

3.4 **Bacterial isolation and identification**

3.4.1 **Bacterial isolation**

Following the incubation from the Nutrient Agar, an isolate was selected and streak on the Eosin Methylene blue (EMB) agar (Oxoid Ltd) using sterile loop to isolate pure colonies of presumptive *Enterobacteriaceae*. *E. coli* appear as blue-black colonies with metallic sheen on EMB agar and other bacteria such as *Shigella*, *Salmonella*, and *Proteus* will appear amber or transparent (colourless). Pure isolate
were kept on Nutrient Agar (Oxoid Ltd) slant in bijou bottle as stock and working culture.

3.4.2 Gram Staining.

A gram staining procedure was carried out based on standard recommended by the Bergey’s Manual of Systematic Bacteriology (1984) (Krieg et al., 1984). A total of 30 different pure isolates from the stock culture were inoculated and streaked onto Nutrient Agar (Oxoid, Ltd) by using a sterilized inoculating loop to obtain a single colony. After an overnight incubation, a single colony from the sample was picked and smeared onto microscope slide. The bacteria smear was then heat-fixed and placed on staining rack. The first staining is done by smearing the slide with crystal violet for 1 minute. Next, the slide was washed up with tap water and the excess water was drain off. This was followed by flooding the slide with iodine for 1 minute and later was rinsed with distilled water. Then the slide was briefly stained with 95% ethanol to decolorize the bacterial smear for about 30 second. The 95% ethanol was then completely rinsed off from the slide. Finally the smear was counterstained with safranin for about 30 second. After 30 second, the slide was completely rinsed off with distilled water. Slide was observed under a microscope at magnification of 100 x. Enterobacteriaceae spp. bacterial smear should appear pinkish as enterobacteriaceae spp. is gram negative bacteria while slide that appear with blue-purplish colour are identified as gram-positive bacteria.
3.5 DNA Extraction

Extraction of DNA was conducted based on boiling method describe by Freschi et al. (2005) with few modification in which the time for boiling was decreased to 10 minute, while the time for chilling the extraction on ice was decreased to 5 minutes. Bacteria were grown overnight in 3000 µl Luria Bertani (LB) broth (1st BASE) at 27 °C – 30 °C in the shaker (New Brunswick Scientific Exccea E10 Platform Shaker) at 12000 rpm. About 1500 µl of the overnight culture was transferred into a sterile 1.5 ml microcentrifuge tube and was centrifuged at 10000 rpm for 5 minutes. After that supernatant formed was discarded. Next, the remaining 1500 µl of the overnight bacteria culture was re-harvested using the same 1.5 ml microcentrifuge tube followed by centrifugation at 10000 rpm for 5 minutes. The supernatant formed was discarded. Later, 500 ml of sterile ddH2O was added to the pellet and was vortexed to resuspend the pellet. The suspension was boiled for 10 minutes and immediately placed the suspension on ice for 10 minutes. The supernatant was transferred to 0.6 ml tube and stored for PCR analysis.

3.6 (GTG)$_5$ PCR Fingerprinting

(GTG)$_5$ PCR was conducted according to Matsheka et al. (2005). The primer used was (GTG)$_5$ with the sequence of:

\[(GTG)_5 : 5'\text{-}GTG\text{ GTG\text{ GTG\text{ GTG\text{ GTG}}-3'}\]