Screening and Identification of Water Microorganisms from Sungai Kelantan, Tanah Merah to Determine the Diversity of Microbiota

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DECLARATION

I declare that this thesis entitled "Screening and Identification of Water Microorganisms from Sungai Kelantan, Tanah Merah to Determine the Diversity of Microbiota" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : .....

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Date :

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This thesis is far being perfect, but expected that this report will be useful not only for the researcher, but also for the reader. For this reason, constructive thought full suggestion and critics are welcomed to make this dissertation better.

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LIST OF ABBREVIATIONS

AGE	agarose gel electrophoresis
CTAB	cetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DO	dissolve oxygen
DOE	Department of Environment
E.coli	*Escherichia coli*
EtBr	ethidium bromide
GTE	Glucose-Tris-EDTA
LB	Luria Broth
mg/l	milligram per litre
$MgCl_2$	magnesium chloride
MMD	Malaysia Meteorology Department
NaCl	Sodium chloride
OD	optical density
PCR	polymerase chain reaction
pH	potential Hydrogen
RNA	ribonucleic acid
rpm	rotation per minute
SLS	sodium lauryl sulphate
TAE	Tris-acetate-EDTA

TE	Tris-EDTA
UMK	Universiti Malaysia Kelantan
UV	Ultra Violet

LIST OF SYMBOLS

˚C	degree Celcius
%	percentage

PEMERIKSAAN DAN PENGENALPASTIAN MIKROORGANISMA DARI SUNGAI KELANTAN, TANAH MERAH UNTUK MENENTUKAN KEPELBAGAIAN MIKROOGANISMA.

ABSTRAK

Sungai diklasifikasikan sebagai ekosistem air tawar. Sungai mengandungi pelbagai jenis mikroorganisma. Sesetengah mikroorganisma air boleh menjadi indikator kepada tahap kualiti air sungai. Tujuan kajian ini adalah untuk mengkaji kepelbagaian mikroorganisma di Sungai Tanah Merah. Sampel air sungai telah diambil dan pengasingan mikroorganisma ini telah dilakukan dengan menggunakan pencairan bersiri dan kaedah contengan. Morfologi koloni bakteria yang tulen telah dilakukan dengan menggunakan kaedah pewarnaan gram bagi membezakan bakteria mengikut kumpulan mereka, gram positif dan gram bakteria negatif dan ia juga digunakan kerana ia merupakan ujian yang paling mudah untuk memerhatikan morfologi bakteria yang terdapat di dalam sungai air. Hasilnya, lima gram positif dan gram tiga bakteria positif dijumpai di dalam kawasan kajian. Bakteria tulen telah dikultur dalam Luria Broth. Kultur bacteria digunakan untuk pengekstrakan DNA untuk mengasingkan DNA dari sampel bakteria. Reaksi rantaian polimerase telah dijalankan untuk 30 pusingan. Hasilnya menunjukkan pelbagai *band* di dalam produk PCR yang menunjukkan bahawa sampel telah tercemar dengan bahan organik seperti garam. Walau bagaimanapun, morfologi bakteria tidak boleh digunakan untuk menentukan bakteria di peringkat spesies. Oleh itu, kajian lanjut mengenai kaedah berasaskan molekul mesti dijalankan.

SCREENING AND IDENTIFICATION OF WATER MICROORGANISMS FROM SUNGAI KELANTAN, TANAH MERAH TO DETERMINE THE DIVERSITY OF MICROBIOTA

ABSTRACT

River is classified as freshwater ecosystem which contains various type of microorganism. Some of water microorganism can be an indicator for river water quality. The aim of this study is to investigate the diversity of microorganism in Sungai Tanah Merah. The water samples were taken and isolation of these microorganisms had been done by using serial dilution and streaking method. The morphology of pure colony of bacteria was done by using gram staining method in order to differentiate the bacteria according to their group, gram positive and gram negative bacteria and it also used as it serve the simplest test to observe the morphology of bacteria present in river water. As a result, five gram positive and three gram positive bacteria found in that study area. Pure colony of bacteria was cultured in Luria Broth. Cultured bacteria were used for DNA extraction in order to isolate DNA from bacteria sample. Polymerase Chain Reaction (PCR) was conducted for 30 cycles. The result showed multiple bands in PCR products which indicate that the samples were contaminated with organic substance such as salts. However, morphological of bacteria cannot be used to determine bacteria at species level. Therefore, further study on molecular based method must be conducted.

CHAPTER 1

INTRODUCTION

1.1 Background of study

The Kelantan River basin has a maximum length and breadth of the catchment area is 150 km and 140 km respectively. According to Malaysia Meteorology Department (MMD), the Kelantan river is located in the northeastern part of Peninsular Malaysia between 4˚ 40’ and 6˚ 12’ N and 101˚ 20’ and 102˚ 20’ E. The river is occupied more than 85% of the state of Kelantan and is approximately estimated of having 248 km long and 11900 km^2 of drains area (Ahmad et al. 2009). It diverges into two rivers which are Galas and Lebir river that located near Kuala Krai where it approximately 100 km from river mouth. The main river from source to river mouth has four names which originated from Betis River (located 30 km from the source), the to the Nenggiri River, followed by Galas River before it meets the Lebir River to form Kelantan River. Commonly, Kelantan River has bank overflow during months of November to February as the northeast being affected by monsoon season. In dry season, months of March to May, the annual rainfall received by Kelantan River is ranging between 0 to 1750 mm and wet season is in mid-October to mid-January (Tan & Rohasliney, 2013). The annual flood commonly happen during the Northeast monsoon which brought heavy rainfall to east coast area especially Kelantan (Khan et al., 2014). The excessive water caused by heavy rainfall then flows into Kelantan River basin.

The river flows to several state capital which lies near the mouth of the river. The Kelantan River system flows northward passing through major towns such as

Kuala Krai, Tanah Merah, Pasir Mas and Kota Bharu, before finally discharging into the South China Sea. Water of the Kelantan River water is at present mainly used for four major irrigation schemes namely the Kemubu, Salor, Lemal and Pasir Mas, all of which lie in the lower reaches of the Kelantan River.

The level of turbidity in Kelantan River is higher due to high levels of suspended solid and siltation since early 1990s (Rohasliney et al., 2014). This problem caused by logging in upstream area (Ambak and Mohd Zaidi, 2010) and sand mining (Tan and Rohasliney, 2013). Based on Solid Suspended (SS) Index, Kelantan River has reported by Department of Environment (DOE) as a polluted river (DOE, 2009)

Tanah Merah River is one of the components of Sungai Kelantan. The catchment area of the river is covered about 884.14 km^2. The river is normally used by local people for domestic uses, transportation, agriculture, plantation irrigation, small scale fishing and sand mining. Tanah Merah River is located near the housing and industry. These areas are the major contributor to the degradation of water quality. This is due to a lack of adequate sanitation and waste removal facilities in the nearby settlements. This might also be caused by poor management and disposal of sewage which ultimately causing it to flow into the river.

1.2 Problem Statement

Some of microbes are useful as water indicator in order to identify the level of river health. For instances, *E. coli* is good indicator for faecal contamination in freshwater whereas *Enterococci* is the baseline indicator for marine water. There was no data on past research on water microbial in Sungai Kelantan in Tanah Merah. Thus, there is a need to identify the present microbiota in that area in order to plan for a proper mitigation. The overall objective of this research is to study the diversity of microbiota in Sungai Kelantan in Tanah Merah.

1.3 Objective

To determine the diversity of microorganism in Sungai Kelantan, Tanah Merah by using the morphological and molecular based method.

CHAPTER 2

LITERATURE REVIEW

2.1 Freshwater Ecosystem

Freshwater ecosystem such as river, lakes and groundwater has become the most essential requirement mechanism in our life. It is needed in order to act as drinking water, industrial activities and also agricultural practises. Moreover, freshwater ecosystem is one of the renewable resources that can be generated through biogeochemical cycle such as hydrological cycle. Hydrological cycle involve mainly three system encompassing atmosphere, lithosphere and also hydrosphere. Hydrological cycle has the ability to fertile soil, regulate nutrient and also involve mainly in regulation of our drinking water system.

The freshwater ecosystem is altered by several factors such as climate change and salinity. The increases level of salinity in freshwater might be influenced by several factors such as mining activity and irrigation (Arguelles et al., 2012). According to Doll and Zhang (2010), the effect of freshwater ecosystem and changes of flow river regime are due to increases of temperature. The structure and function of aquatic and riparian ecosystems for streams and river are determined by flow regime (Poff et al., 2009). Flow regime play an important roles in identifying the biotic composition, structure, function and diversity within river ecosystem and that river flow alteration may have strong impact on freshwater ecosystem (Doll and Zhang, 2010). Doll and Zhang (2010) were found that other biotic characteristic of freshwater ecosystem will influence the river flow alteration which is affecting the well-being of organism.

2.2 Environmental Microorganism

According to Chauhan et al. (2012), microorganism existing in every niche of our biosphere and occupies a peculiar place in the human view of life. Technically, the range size of microbes is about 0.2 to 200 μm in upper limit. It cannot be seen by naked eyes. Thus, microscope is needed to see them clearly.

Historically, microbes flourished for billions of years before any plants and animals is existed and the distribution of them continue to be most abundant form of life on earth (Gloria et al., 2012). All form of life originated from microbes. More than half of oxygen that human breathe in is generated by microbes. Besides, it also contributes greatly to the alteration of our climate. The microorganism can spread throughout the soil, water, air of our planet. They can create indirect changes of larger life forms and continue govern the lives and concerns of all living things.

The growth of microorganism relies upon specific chemical and physical factor such as pH, temperature and salinity. Specific microorganism can tolerate to specific condition in environment such as pH and temperature of water. As example *Lactobacillus* is able to live and growth in low pH condition where the other microorganism such as *E. coli* can survive in alkaline condition. The rate of growth and acceptability of microorganism to a certain type of environment depends on the level of its vulnerability.

In today's world, microbes play their importance in the viability of world ecosystem. Some of them either brings benefit or harms to environment. It can cause problem to environment and also can be a natural earth's remedy.

Microbes are progressively used for bioremediation to counteract the effect especially for water treatment. For instance, bioremediation process is used to clean

up some part of river where microbes are used to degrade flowing sewage to harmless carbon dioxide and water (Aggarwal, 2015). These products then can be used by plant and animal of their survival. In Strong and Burgess's study, microorganism is a tool for bioremediation process in order to clear up the polluted water and soil (Singh et al., 2013). The degradation of pollutant by bioremediation technology may be a beneficial waste as well as environmentally friendly alternative (Divya et al., 2015).

Microbes also hold a vital role for agriculture and industry. In agriculture, microbes are help in keeping the soil fertility and in crop protection (Deepak et al., 2014). It also reciprocates with plants which allow them to grow strong and increase yield production. According to Ahmad et al. (2011), recent developments in microbial and plant molecular biology have made it possible to develop transgenic plants with improved gene delivery systems.

Despites of their benefits, it also brings negative effect to environment. For example, *E. coli* can be pathogenic. The primary harmful effects of microbes upon existence and civilization are that an important cause of disease in animal and crop plants. Microbes are easy to be transmitted from one place to another. It often spoils food that later will cause a person to get food poison. The most pathogenic microbial in water are bacteria, protozoan parasite, fungi and viruses. Pathogenic bacteria are commonly found in faeces and wide variety can be present in wastewater due to contamination of fecal. Poor management of sewage has caused it to flows into water and led the growth of microorganism (Olaulu, 2014).

2.3 Changes and Trend of Microbial Diversity in Flood Area

The town of Tanah Merah in the state of Kelantan encountered with annual flooding due to Kelantan River bank overflowing which occurs at least once a year. Floodwater contaminated by microbes may contain bacteria, viruses, protozoa and helminthes. Exposure to this pathogen may cause illnesses ranging from mild gastritis to serious disease such as dysentery, infectious hepatitis and severe gastroenteritis (Chaturongkasumrit et al., 2011). The concentration of microbes in flood water depends on how many and what kind of sources contributed to the contamination, the volume of contaminants released and the degree of their dispersion in the environment and the level of treatment of the affected waste-water treatment facilities before flooding.

There are a few factors that contributed in flood disaster such as poor management of drainage system that can cause the excessive water in soil surface. Besides, improper plan for development is also one of the factors that assist flood to be occurred. It is because unplanned development can gives impact to climate change hence alter the rainy patterns (Basarudin et al., 2014)

According to Paradise (2014), flooding can cause the changes of microbial diversity because some of microbes would be more tolerant to halophile environment than other. The changes in microbial community are resulted from flash flooding that causes extremely large damage to ecological niches. In Jasosz and Davelos's study, due to their nature, microbial symbionts can alter host fitness by affecting its capability to survive, reproduce, compete, grow, or defends itself both positively and negatively (Paradise, 2014). According to Mhuantong et al. (2015), microbial variation in flood water affected by the decomposition of organic matter and other

biochemical activities in water, such as photosynthesis, recycling of nutrients, and sulfate/nitrate reduction and it closely related to the water properties. Even though the microbial community in flood water is temporally variable, but it gives a huge impact to human health and habitat.

A frequent and heavy rainfall will probably cause an increase number of pathogen contamination which will definitely impact the water quality thus it yield a higher number of microorganism in the water. A pathogenic microorganism found in water is harmful and capable of causing diseases in it hosts. Pathogenic microorganism in water is often associated with waterborne diseases. It can infect the human who consumed or exposed to contaminated water. A water-related disease has become a world major concern as it was globally estimated by World Health Organization (WHO) about 1.8 million people die annually (Forstinus et al., 2016). There a few example of waterborne diseases outbreak cause by intensive rainfall.

For instance, the most legendary case of waterborne disease occurred during 1993 in Milwauke, United States. The disease was caused by the *Cryptosporidium* cists present in water due to lack of clean drinking water and improper handling of food and water sources where related to the heavy rainfall and the serious surface runoff. Continuous heavy rainfall for several days leads to the spontaneous increasing number of people contaminated by harmful microorganism presence in water. The case has been labelled as the most serious waterborne disease contamination in the history of United States (Funari et al., 2012)

2.4 Microbes as River Health Indicator

According to Berg's study, to detect the presence of pathogen, indicators of microorganism have been used (Mishra et al., 2012). According to Baticna's study, microorganism such as *Escherichia coli (E. coli)* and total coliforms are indicator organisms of faecal contamination in water (Singh et al., 2013). From previous study by Sivaraja and Nagarajan (2014), indicator organisms are commonly used to assess the microbiological quality of surface waters and fecal coliforms are the most commonly used bacterial indicator of fecal pollution in India. The presence of fecal coliform bacteria in aquatic environments indicates that the water has been contaminated with the fecal material of anthropogenic or other animals. Fecal coliform bacteria can enter rivers through direct discharge of waste from mammals and birds, agricultural and storm runoff, and untreated human sewage. The presence of fecal coliform in water source gives highest potential health risk to individual that are exposed to it.

2.4.1 *Escherichia coli* (*E. coli*)

E.coli is categorised under faecal coliform. It is most preferred indicator compared to other faecal coliform organisms. Specifically, *E.coli* serve as an indication of fecal contamination, it is a natural and important part of bacteria flora in the gut of humans and animals (Olaulu, 2014). The use of *E.coli* as an indicator has been recommended by the United State Environmental Protection Agency (EPA) (Odonkor, 2013)

2.5 Morphological Characteristic and Classification of Bacteria

Bacteria are large group of single-celled microorganism. Their morphology is different from other microorganism such as eukaryotic cells or of the archaea which are bacteria-like microorganism that have their own unique characteristic. Typically, most of the bacteria are very small with a few micrometres in length. The size of bacteria ranges from 0.5 to 8µm (Gloria et al., 2012). The cell shape of bacteria has been also used in description and classification of bacterial species. They are exist in variety of shapes and the most common shapes are rod-like (bacillus) and spherical (coccus) (Huang, 2008). Bacteria are wide spread as they are existed in every habitat for example, growing in soil, water and even deep in Earth's crust. Bacteria play an important role in biogeochemical cycle such as nutrient cycling. Bacteria are varying in their morphology as stated in Figure 1.0, but they share one similar characteristic in term of their reproduction system which is binary fission. Binary fission is an asexual reproduction where one cell is divided into two new bodies. The growth of bacteria is affected by various factors, for instance temperature and pH. Bacteria lives best in temperature ranges from 25-40˚C. Extreme temperature will kill the microbes (Minh, 2006). In term of pH, bacteria grow best in range that close to neutral pH which are 6-8 pH. However, there are many bacteria that are tolerant to acidic or alkaline pH (Bradley, 2007).

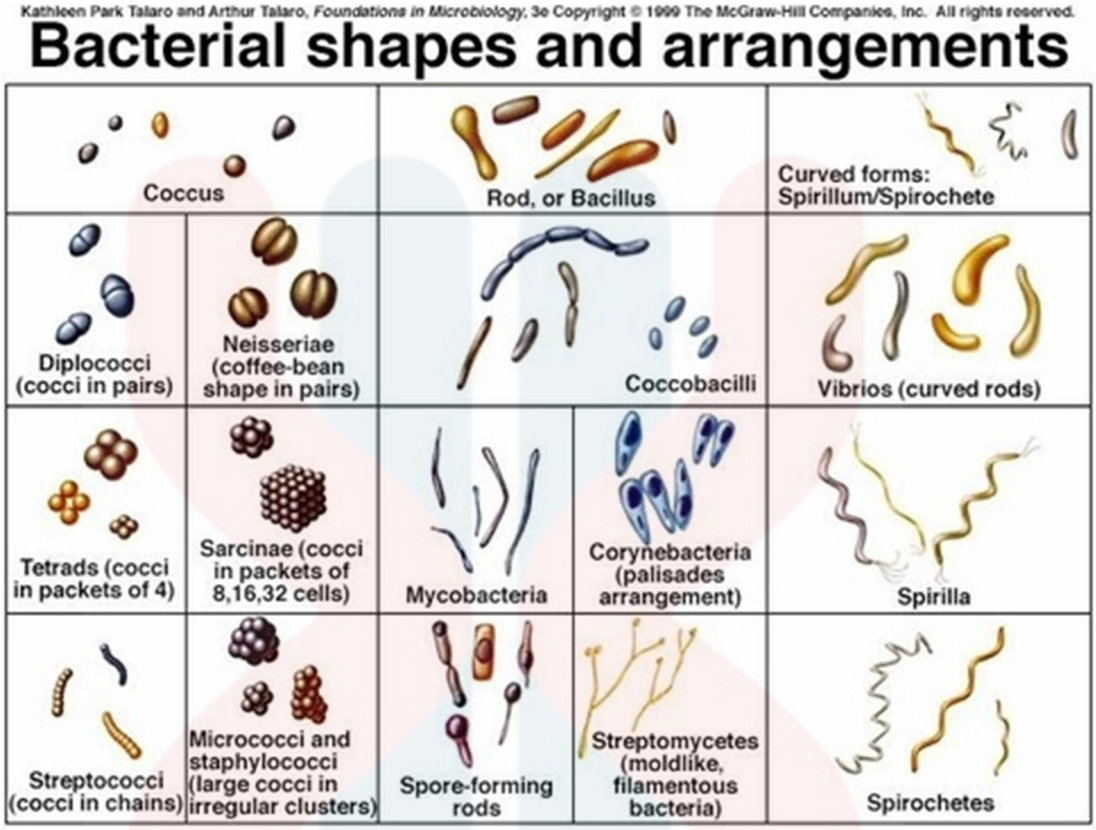

Figure 2.5 Bacteria shape and arrangement (Kathleen and Arthur, 1999)

2.6 Gram Staining

Gram Staining is one of the methods to identify the structure of the bacteria and allow distinguishing between different types of bacteria. It is widely used because it cost effective, easy and quick method. According to Srinivasan et al. (2012), the Gram stain is commonly used in epidemiologic and clinical studies. It involves smearing a sample onto a slide, staining the material using dyes that bind to bacterial cells, and visually inspecting under a microscope. Using this technique, Gram positive bacteria are stained purple/violet and gram negative stain red. Gram negative and Gram positive of bacteria can be identified according to their morphological characteristic, for example the outer membrane and the bacteria peptidoglycan layer. According to Julien et al., (2007), peptidoglycan is mostly found in Gram positive bacteria, in which it was measured for fairly close to half of

the mass of the cell wall. Gram positive bacteria have a thicker peptidoglycan layer outside the plasma membrane compared to Gram negative bacteria. Peptidoglycan forms a huge polymer that covered the cell. It consist strings of sugar molecule that linked together by chain of amino acid into a huge network. It helps to maintain the bacteria shape and can resist to high osmotic pressure (Julien et al., 2007).

As argued by Hassan and Peh, identification of *Lactobacillus acidophilus*, the Gram staining results indicated that the isolated bacteria could be identified through observing under light microscope and to determine the type of stain and its morphology of bacteria (Pyar et al., 2014).

The foremost limitation in Gram staining, it is complex methodology and tends to be error and can cause misinterpretation of the result (Ouyang et al., 2015). Certain gram positive bacteria might easily lose the stain and thus appear as a mixture of Gram negative and Gram positive bacteria or it known as Gram variable as stated in Figure 2. It is because of prolonged exposure to decolouring agent that eventually causes the removal of stains. Decolourization step is most crucial in performing Gram staining. Even Gram positive bacteria have possibility to appear in pink colour when it gets over-decolorized and Gram negative bacteria may appear as Gram positive bacteria when it under-decolorized (Sridhar, 2008).

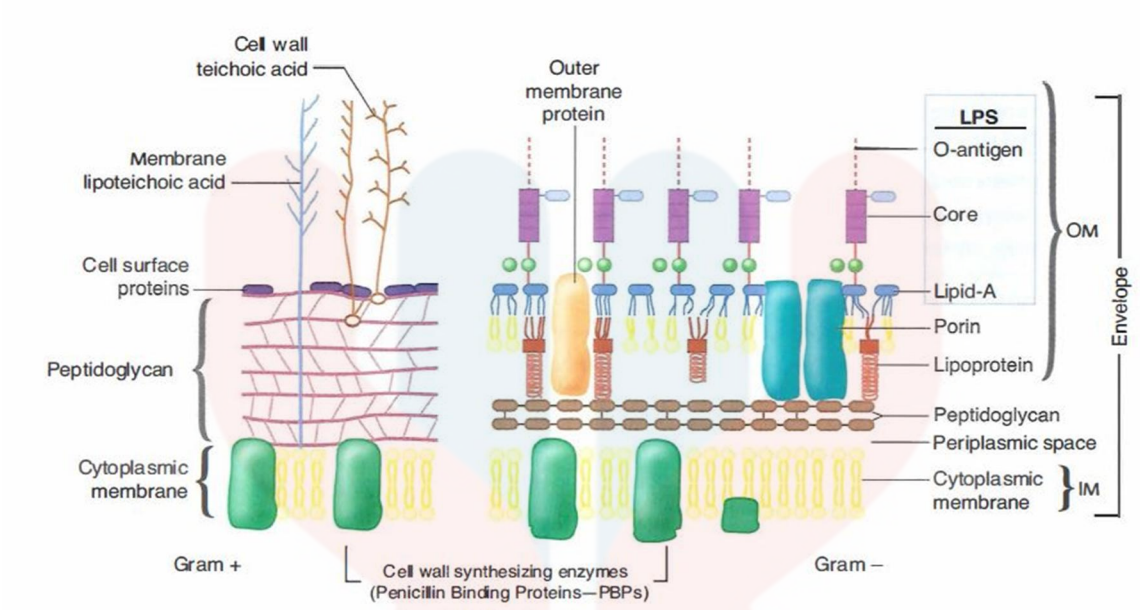

Figure 2.6: The cell wall of Gram positive and Gram negative bacteria (Tankeshwar, 2015)

2.7 DNA Extraction from Environmental Microbes

In Dahm's studies, the very first DNA extraction was done in 1869 by a Swiss physician, Friedrich Miescher (Tan, 2009). DNA extraction is the isolation of deoxyribonucleic acid (DNA) from the cells or viruses in which it normally resides. The extraction of DNA is pivotal to biotechnology. It is the starting point for various purposes, ranging from crucial research to general investigation and remedial decision-making. It is often used for downstream action in analysis process of detection of viruses or bacteria in the environment. Besides, DNA extraction also helps in diagnosing disease or genetic disorder (Amna, 2010).

The extracted DNA has proved cooperated to Polymerase Chain Reaction (PCR) amplification and restriction digestion (Deshmukh et al., 2007). The process of DNA extraction begins with the breaking down the bacterial membranes (Agata et

al., 2014). The association proteins in DNA may be degraded with addition of proteinase. DNA can be re-suspended in a buffer such as Tris or TE. To confirm the presence of DNA, electrophoresing on an agarose gel containing ethidium bromide will be conducted (Deshmukh et al., 2007)

2.8 Agarose Gel Elctrophoresis of DNA

Agarose gel electrophoresis (AGE) is the laboratory method used to separate DNA or RNA fragment (Lakshmi, 2011). Agarose is a natural linear polymer extracted from seaweed that forms a gel matrix by hydrogen-bonding when heated in a buffer and allowed to cool (Patricia and Silvia, 2012). According to Lee et al., (2012), prior current is applied, DNA is loaded into the wells in the gel in order to separate the DNA. AGE is a more effectual way of separating DNA fragment of various size ranging 100 bp to 25 kb. This process needs the aid of electric field to move the separation of nucleic acid molecules where negatively charged molecules migrate toward anode (positive) pole (Muhittin et al., 2012). When DNA is loaded into the wells and the current is applied, the DNA fragments will moves to positively charged anodes because DNA molecule possesses net negatively charged due to their phosphate backbone (Lee et al., 2012).

The movement of nucleic acid molecule is depends on their size where the smaller and shorter fragment will move faster and easily through the pores. Purposely, AGE technique is used to estimate DNA sizes using restriction enzyme digestion to analysed PCR product. The concentration of agarose influenced the migration of nucleic acid as the pores size is reduced (Nancy, 2009). In order to maintain the charge on nucleic acid, TBE or TAE buffer is normally used in this

technique (Lakshmi, 2011). Generally, TBE and TAE work for the same function but despite of their similarity, each of them has different properties in term of buffering capacity which may influenced the time taken to running AGE process (Bahaman et al., 2012). After the AGE process is done, the fragment of DNA molecule can be visualized under Ultraviolet light with the aid of Ethidium Bromide (Muhittin et al., 2012).

2.9 Polymerase Chain Reaction of Environmental Microbes

Polymerase chain reaction (PCR) is a new invented of molecular biology technique for DNA replication. According to Joshi et al., (2010), Polymerase Chain Reaction is used to amplify a single stranded of DNA and generating thousands to millions of target DNA sequences. The technique of PCR is widely used mainly by clinicians and researchers to diagnosis diseases, clone and gene sequencing (Garibyan et al., 2013) because it is quick, inexpensive and simple (Joshi, 2010).

Normally, 16S rRNA is selected as a primer in order to complete the PCR process of amplification. 16S rRNA is broadly used because it universally present in all bacteria. It is provide special signatures that it can be analysed to provide an identification of bacteria (Woo et al., 2008). The benefit of using 16S as a primer in PCR, it can be compared among all bacteria. Hence, the unknown bacteria profiles can be identified and categorised according to their taxonomy (Janda and Abbott, 2007)

In order to complete the reaction, there are three major steps that must be done which is denaturation, annealing and followed by extension. Denaturation is the process of splitting out DNA double stranded by breaking the hydrogen bonds that

holds the templates to produce two DNA single stranded DNA. After single strands DNA is produced, it will undergo annealing process which allow the primers to bind DNA. The extension is the process where new DNA is produces by Taq polymerase enzyme. Template DNA, primers, nucleotides and DNA polymerase are essentially needed in PCR (Garibyan et al., 2013). According to Rahman (2013), the advantages of performing PCR are it is able to diagnose many human diseases and it is important for genetic fingering and paternity test. Besides, since PCR techniques is highly sensitive, therefore contamination DNA prone to yield inaccurate result.

There are some limitations in PCR mentioned by Garibyan et al., (2011), the DNA polymerase used in this reaction is tend to be error and can bring on mutation in the fragment generated. Another limitation, PCR product may be altered by non-specific binding of primers to other similar sequences on the template DNA. Other than that, it has potential of contamination during the first amplified product is transferred into the tube in which the second amplification is performed (Patricia, 2012).

2.10 NanoDrop Spectrophotometer

The NanoDrop spectrophotometer are used to quickly and easily measure 0.5-2 μL droplets of proteins, DNA, RNA, and other biomolecules. This capability has become increasingly important as molecular techniques continually evolve to use smaller amounts of material for analysis. The NanoDrop 2000c determines the optimal path length automatically (1 mm to 0.05 mm), providing the most extensive range of possible protein concentration measurements without dilutions (Desjardins, 2009).

NanoDrop spectrophotometer used to measure the amount of ultraviolet radiation absorbed by the bases. Heterocyclic rings of the nucleotides aid the nucleic acid to absorb the ultraviolet. Pure nucleic acids typically yield a 260/280 ratio of ~1.8 – ~2.0. The ratio of DNA that is less than ~1.8 might indicate contamination protein and if the ratio of DNA is greater than ~2.0, it indicates the presence of RNA as contamination (Desjardins et al., 2010). The contamination of RNA can be removed by adding RNase enzyme during the purification process (Oxford Gene Technology, 2011). Any contamination in DNA would affect the overall result of this research.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling Site

The study was carried out in Sungai Kelantan at Tanah Merah. The water samples were collected approximately one metre from river bank.

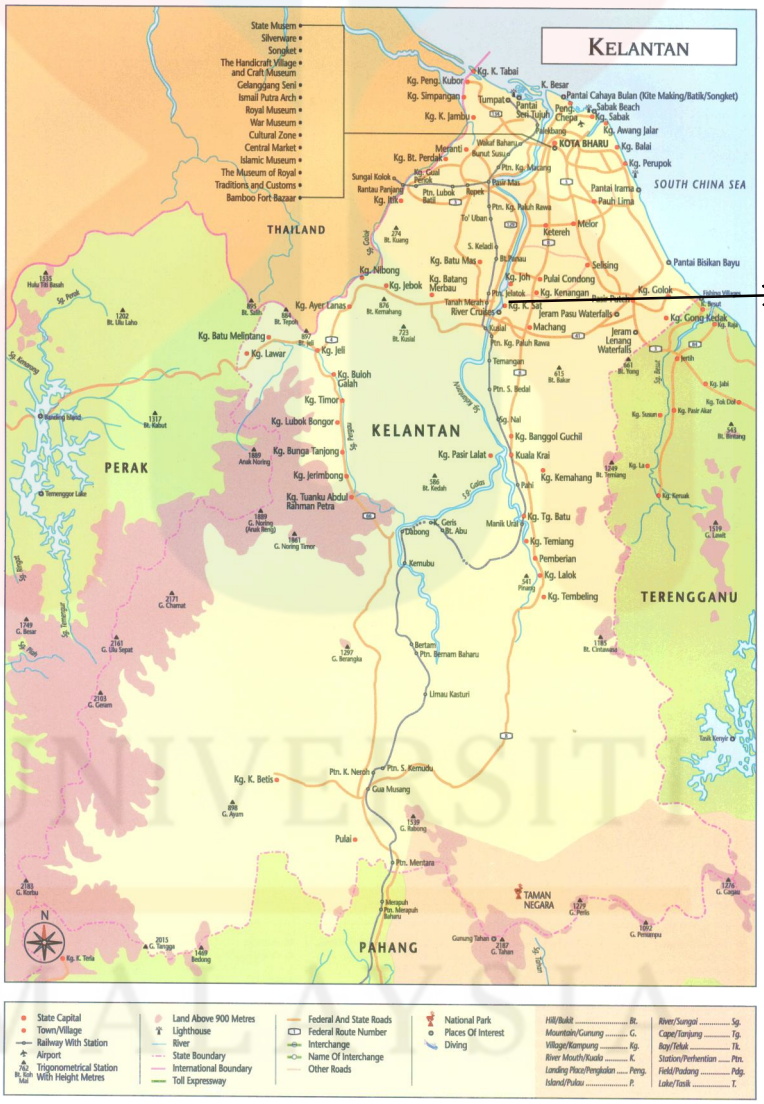

Figure 3.1 Map state of Kelantan (Wonderful Malaysia, 2007)

3.2 Water Sample Collection

The water samples were collected by using a water sampler. About 500 ml of water sample was taken in three different points at surface level, 50 cm and sediment and the water samples were immediately stored in ice box. After reaching Universiti Malaysia Kelantan (UMK), Jeli Campus, the water samples were kept in refrigerator in BAP 1.1 Laboratory at 4˚C till further processing.

3.3 Preparation of Nutrient Agar (NA)

The nutrient agar (Oxids brand) was weighed at 23.0 gram. The weighed agar powder was dissolved in one litre of distilled water using two litre conical flasks. The mixture was heated and stirred to fully dissolve all components. The dissolved mixture was autoclaved. After that, the mixture was allowed to cool until it falls to temperature approximately 45˚C - 50˚C. The nutrient agar mixture was swirled thoroughly in order to mix them up. Then, 30 ml of the solution was poured into petri dishes and these plates were kept in room temperature until solidified.

3.3.1 Glycerol Solution

Preparation of glycerol solution was done by mixing 20 ml of glyceric solution with 20 ml of distilled water. After that, the mixture solution was autoclaved at 121˚C for one hour and then stored at room temperature.

3.4 Serial Dilution of River Water

Initial dilution of river water was made by transferring one ml of river water sample to nine ml tubes containing distilled water to produce 10^{-1} dilution. Immediately, after 10^{-1} dilution was done, one ml of the solution was transferred to another tube containing nine mL distilled water. The steps were repeated until dilution 10^{-5} was produced.

3.5 Isolation of Bacteria Species from River Water Sample

From the diluted sample, 0.2 ml of sample from each diluted sample was spread for growth of bacteria on nutrient agar. After that, the master plate was incubated in incubator located at BAP 1.1 Laboratory at range of 30˚C - 37˚C for 24 hours. An individual colony was isolated from the master plate and was inoculated on separate nutrient agar plate for pure culture growth. After 24 hours incubation, the growth of colonies in the agar then was examined for their morphology using naked eyes (Panneerselvam et al., 2012).

3.5.1 Identification of Bacteria Morphology from River Water Sample

The shape, colour and arrangement of microbes were examined by using a light microscope range 4x, 10x, 40x and 100x. All the observation was recorded for morphology analysis.

3.6 Preparation of Luria Broth (LB)

One colony from each pure culture plate was added into 10 ml of Luria Bertani Broth in bijou bottle. The broth with bacteria culture was incubated in incubator shaker at 37˚C for 12 hours.

3.7 Preparation of Cetyltrimethylammonium bromide (CTAB)

Preparation of 100 ml of Cetyltrimethylammonium bromide (CTAB) buffer solution was done with 20 g of CTAB was dissolved in 86 ml of double distilled water. Then, 8.182 g of sodium chloride (NaCl) was added to the mixture solution. After that, 10 ml of 1.0M Tris (pH 8.0) was then poured into the solution and followed by adding 4 ml of 0.5M EDTA (pH 8.0). Lastly, the buffer was sterilised by autoclaving at 121˚C for one hour.

3.8 DNA Extraction from River Water Microorganism

3.8.1 Michele (2002)

DNA extraction of river water microbes was done by following method established by Michele (2002). One ml of cultured bacterial grown in Luria Broth medium was measured using spectrophotometer at 600nm (OD_{600}) in order to identify the active phase of the bacteria. Once the bacteria were in active phase, one ml of bacteria together with Luria Broth (LB) was transferred to 1.5 ml microcentrifuge tube. The suspended bacteria together with Luria Broth in a microcentrifuge tube were spun at 10000 rpm for one minute to form a compact pallet. The supernatant was discarded. The pellet was re-suspended in 560 µg of TE

buffer by repeated pipetting. Afterwards, 600 µl of CTAB buffer was added and vortexed to re-suspended the bacteria cell. The mixture containing buffer and pellet was incubated in water bath for 10 minutes at 65˚C. After incubation, 600 µl chloroform was added and mixed thoroughly by inverting the tube until the phases were completely mixed. Then, the sample was centrifuged at 14 000 rpm for five minutes and the supernatant was transferred to new microcentrifuge tube. Then, 60 µl of cold ethanol was added into supernatant and it was gently mixed in order to precipitate DNA. The mixture was centrifuged at maximum speed for 15 minutes. One ml of ethanol 70 % was added to wash away the salt from DNA pellet and the pellet were set to dry for approximately 12 hours at room temperature. The pellet was re-dissolved in 50 µl of TE buffer and stored at -20˚C till further processing. After DNA extraction was done, agarose gel electrophoresis was conducted to confirm the presence of bacteria DNA.

3.8.2 DNA extraction with Glucose-Tris-EDTA (GTE) and Proteinase K method (He, 2011)

DNA Extraction was done by transferring 1 ml of cultured colony into 1.5 ml microcentrifuge tube and it was spun in centrifuged at 2 000 rpm for 15 minutes. After centrifugation, the pellet was formed. The supernatant was removed and the pellet then washed twice by using one ml of Glucose-Tris-EDTA (GTE) pH 8.0 buffer and centrifuged at 10 000 rpm for five minutes. Then, the pellet in microcentrifuge tube was mixed with 200 µl GTE and the mixture was kept on ice for 15 minutes. After ice incubation, 50 µl of RNAse and 100 µl of lysozyme were added to the mixture nd the mixture was incubated at 37˚C for 2 hours. After

incubation, 50 µl of Proteinase K and 50 µl of 10% Sodium Lauryl Sulphate (SLS) were added and incubated at 50˚C for 30 minutes. After incubation, 200 µl of 0.5M Tris-EDTA (TE) pH 8.0 buffer and 500 µl of Phenol chloroform Isoamyl alcohol (25:24:1) were added into microcentrifuge tube and it was inverted for several times. Then, the mixture was centrifuged for 15 minutes at 10 000 rpm. After centrifugation, two layer mixtures were formed and the upper layer was transferred into a new tube. Next, 400 µl of 0.2M, pH 5.0 sodium acetate and 800 µl of cold isopropanol were added to the upper layer in the new microcentrifuge tube and the mixture was incubated at room temperature for 10 minutes before proceeding centrifugation process at 10 000 rpm for 15 minutes. The DNA pellet then was washed with 500 µl of 80 % cold ethanol by centrifuging at 10 000 rpm for five minutes. After centrifugation, the pellet was air dried and dissolved in 50 µl TE buffers (pH8.0) and kept at -20˚C. The presence of DNA was confirmed by conducting agarose gel electrophoresis.

3.8.3 Optimization of DNA Extraction with Glucose-Tris-EDTA and Proteinase K.

DNA extraction was used to isolate DNA from the cell. The extraction started with one ml of cultured colony was transferred into 1.5 ml microcentrifuge tube and it was spun in centrifuged at 13 000 rpm for 15 minutes. After centrifugation, the pellet was formed. The supernatant was removed and the pellet then washed twice by Glucose-Tris-EDTA (GTE) buffer and centrifuged at 10 000 rpm for five minutes. The pellet was added with 200 µl GTE and the mixture was kept on ice for 15 minutes. After that, 50 µl of RNAse and 100 µl of 0.25 mg/mL lysozyme were added

and the mixture was incubated at 37˚C for 2 hours. After incubation, 50 µl of Proteinase K and 50 µl of 10% Sodium Lauryl Sulphate (SLS) were added and incubated at 50˚C for 30 minutes. After incubation, 200 µl of Tris-EDTA (TE) buffer (pH 8.0) and 500 µl of Phenol chloroform Isoamyl alcohol (25:24:1) were added to the mixture and inverted for several times to mix well. Then, the mixture was centrifuged for 15 minutes at 10 000 rpm. After centrifugation, two layer mixtures were formed and about 150 µl of upper layer solution was transferred into a new tube. Next, 400 µl of sodium acetate (0.2 M, pH 5.0) and 800 µl of isopropanol were added and the mixture was immediately inverted for several times before incubated at room temperature for 10 minutes before it undergo centrifugation process at 10 000 rpm for 10 minutes. The DNA pellet then washed with 500 µl of 80% cold ethanol by centrifuged at 10 000 rpm for five minutes. After centrifugation, the pellet was air dried and dissolved in 50 µl TE buffer and kept at -20˚C. Then, electrophoresing on agarose gel containing ethidium bromide was conducted in order to confirm the presence of DNA.

3.9 Agarose Gel Electrophoresis of DNA sample from River Water.

By using method proposed by Paulse et al (2012), agarose gel 1.0 % was prepared by weighing out 0.8 gram of agarose into 250 mL conical flask. Then, 80 mL of 1xTAE was added into conical flask and swirled to mix. The mixture was placed in microwave for one minute to dissolve the agarose. The mixture was left at room temperature, 25˚C for five minutes to cool down. Then, the mixture was added with one µg of ethidium bromide (10 mg/ml) and swirled to mix. The agarose was poured into the tank and the comb was inserted into the gel. The agarose gel

electrophoresis was left to liquidified for at least 30 minutes with the lid on. Lastly, 1xTAE buffer was poured into gel tank to submerge the gel from two to five mm depth. The first well was filled with 6 µL of 1 kb DNA ladder and 6 µL of diluted DNA samples with loading dye were loaded in subsequence well. The power source was set at 80V for 30 minutes. The gel was placed on transilluminator and it was viewed under UV light.

3.10 Quality and Quantity Assessment of DNA of Cultured Bacteria.

NanoDrop spectrophotometer was used to measure the DNA extracted from microbes isolated from Kelantan River water concentration and the absorbance ratio (A260/A280), following the method established by Sambrook & Russell (2001). DNA concentration was estimated by measuring the absorbance at 260/280 nm.

3.11 Polymerase Chain Reaction (PCR) of Extracted DNA Samples

Following method established by Appalasamy (2012), PCR was performed by using PCR master mix and universal primer 16s RNA. The amplifications of DNA extracted from river water was done by preparing the master mix using 20 µl of DNA, 1x buffer, 2 µl of 25 mM magnesium chloride ($MgCl_2$) solutions, 1 µl of 0.2mM dNTPs in equimolar ratio, 0.5 µL of forward primers 5' –CGC TGG CGG CGC GTC TTA AA-3', 0.5 µl of reverse primer 5'-TTC ACC GCT ACA CCT GGA A-3', 5x Gotaq flexi buffer and 0.5 µl of Taq polymerase (Promega, US) and 16 µl double distilled water. About 2 µl of DNA template was inserted in PCR tube. PCR tube containing DNA template was then added with 23 µl of master mix. The polymerase chain reaction was performed by three main procedures in order to

complete the PCR that was carried out for 30 cycles. The amplified DNA was electrophoresed using 0.8% agarosel gel and run at 80V for 30 minutes.

Table 3.1 The table showed a main cycles condition that take place in order to complete the polymerase chain reaction (PCR)

Cycle	Temperature (˚C)	Time (minutes)	Number of cycles
Initial denaturation	95	2	1
Denaturation	95	1	30
Annealing	55	1	30
Extension	72	1	30
Final extension	72	5	1
Soak	4	Indefinite	1

CHAPTER 4

RESULTS AND DISCUSSION

Identification of water microorganism is the process of identifying the presences of microorganism in water. The water samples used in this experiment was taken in Sungai Kelantan which is located in Tanah Merah, Kelantan. In this study, two methods have been used in order to ensure the presence of microorganism in study area. The first method is called as morphological method where the morphology of microorganism is observed in term of their shape, arrangement and colour with aided of light microscope. Second method is molecular based method, where the microorganism presences in water were confirmed by using Agarose Gel Electrophoresis (AGE) and Polymerase Chain Reaction (PCR).

4.1 Physical Parameter of Tanah Merah River

4.1.1 Temperature

Collection of water samples were done in sunny day for all points. Therefore, the temperature recorded at surface, middle and sediment layer for all points were same, ±29˚C. The bacteria growth would work best at 37˚C as the mean of many mammals is 37˚C (Minh, 2006). The temperature of water can be affected by several factors. The most influenced factors are sunlight exposure, water flow and human intervention (Stefanie et al., 2012). Tanah Merah River has disturbed by human activities such as sand dredging which could alter the river temperature. Changes of temperature in river water may give a vital impact to several species in water

(Harriet, 2015). As some bacteria can only withstand at certain temperature. Thus, the changes of temperature in river may inhibit their growth.

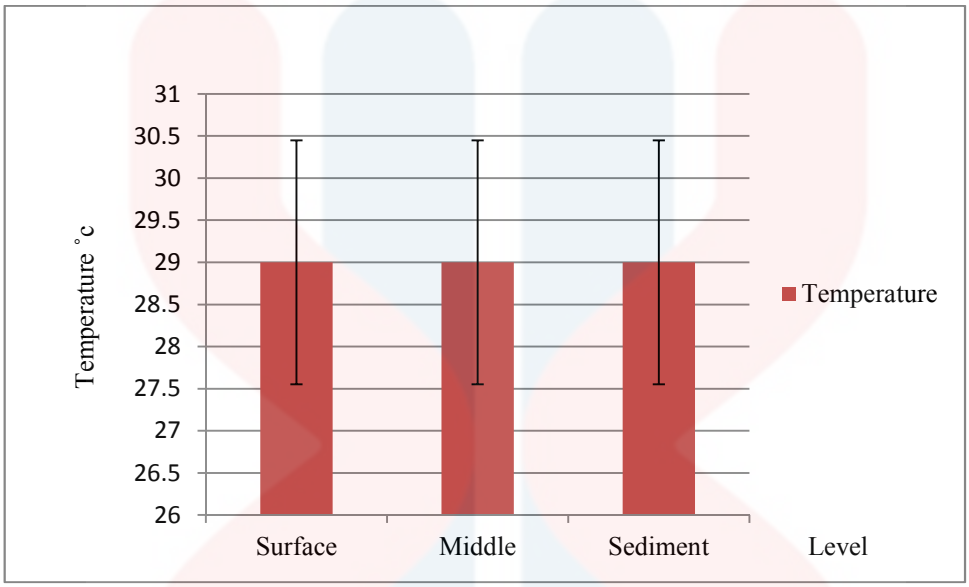

Figure 4.1 A graph bar of average temperature in Tanah Merah River. The reading was taken in a sunny day at three different points.

4.1.2 pH

The average pH reading of surface, middle and sediment were recorded 6.98, 6.96 and 6.94 respectively. pH reading at surface level was the highest and sediment was recorded as the lowest. The overall pH in all depth was in a suitable range which is not too acidic or basic. Since the average pH of surface, middle and sediment were acceptable for the survival of aquatic and microorganism, the presence of microbes in this river can be easily identified.

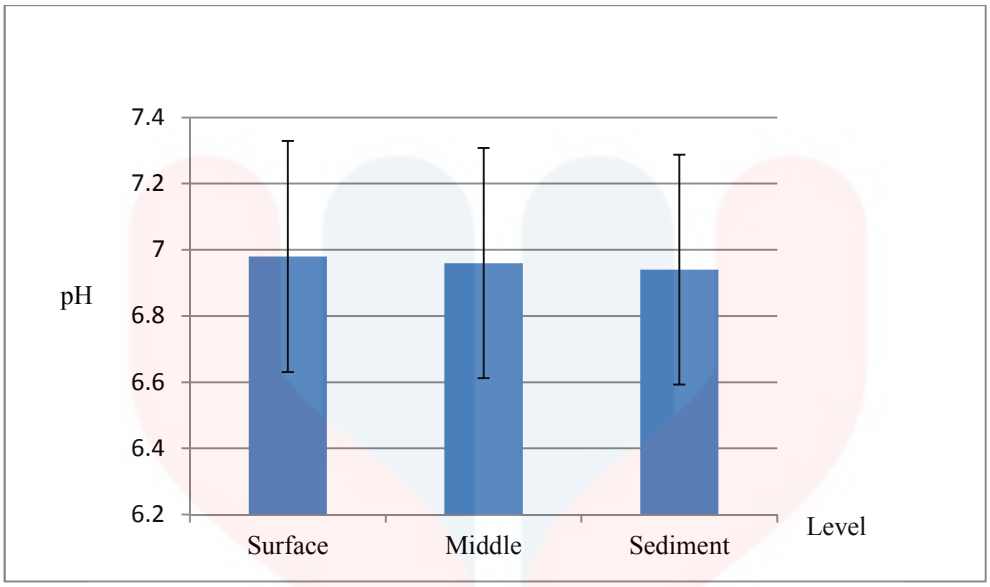

Figure 4.2 A graph bar of an average of pH reading at Sungai Tanah Merah. The reading was taken by using YSI parameter.

4.1.3 Dissolve Oxygen (DO)

Dissolve oxygen (DO) refers as the amount of oxygen presences in water. The sources of oxygen in water are either by direct absorption from atmosphere or from respiration of plants. Dissolve oxygen is one of the important elements to sustain the survival of aquatic life. Form the figure 4.3, surface level shown the highest DO reading, 6.16 mg/l while sediment is the lowest DO reading, 5.99 mg/l. The differences of DO reading is affected by the depth of the water. As water goes deeper, the reading of DO decreased (Ahmad et al., 2012). It is because deep water far from atmosphere. Therefore, it can only absorb least amount of oxygen.

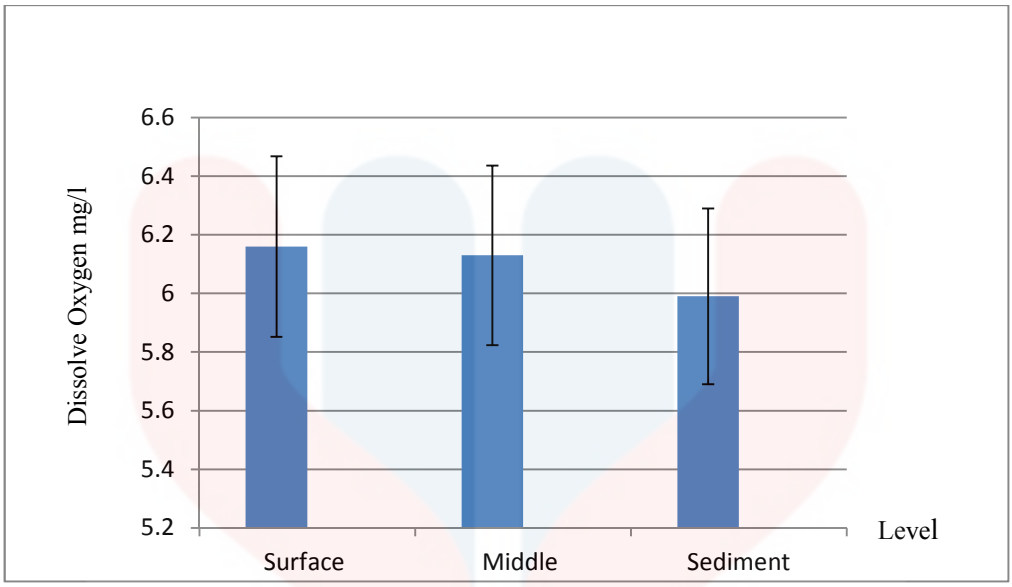

Figure 4.3 A graph bar represent the reading of Dissolve Oxygen (DO) in Tanah Merah River. The measurement of dissolve oxygen in river water was taken at three different levels. The amounts of dissolve oxygen presence in three different parts were almost the same.

4.1.4 Salinity

Salinity refers to the amount of salts dissolved in water. Usually, salinity is measured in part per thousand, ppt. The concentration of salinity in Tanah Merah River at all point is 0. Concentration of salinity influenced by a few factor such as evaporation rate as the evaporation process decreases the volume of water. Hence, increases the salinity (Yan et al., 2015). As the surface area of the river is lower, the evaporation is occurs at slow rate. Besides, salinity in river water is low because sodium chloride has dissolved through rocks and soils. Thus, only tiny fragments of salt pass through river water.

4.2 Determination of Bacteria Morphology Using Gram Staining Method

In this study, gram staining method has been used in order to categorize the microorganism according to their group, gram positive and gram negative microorganism. Beside of identifying microbe's grams, gram staining also used as it serves the fastest and simplest test to observe the morphology of microbiota such as colour and shape (Yuki et al., 2009).

This experiment is done by smearing a sample on glass slide. The smear of microorganism on slide were let air dried and heat fixed. Then, the drying smear was stain with a few drops of crystal violet dyes. Crystal violet dyes itself has positively charged particle that helps to bind with negatively charged particle presence at the cell wall of the microorganism. After one minute, the crystal violet was washed with tap water before adding iodine on the smear to form violet-iodine complex which helps to hold the dyes on slide. The slide was washed again and then it was decolorized by using acetone as it act as decolorizing agent. The decolourisation process must be done quickly and washed with tap water. Lastly, the smear was treated with a few drops of safranin and after one minute a slide was washed again with tap water. The excess water on slide was removed by using a tissue and it was air dried and heat fixed before it was observed under light microscope. The specimen under microscope was observed in magnification of 4x, 10x and 40x. Some of the microorganism was observed under oil-immersion lens which is 100x magnifications in order to see their shape and colour clearly.

According to figure 4.4 the total numbers of gram positive and negative have been recorded in surface, middle and bottom area of the river. A total of five out of eight samples in all areas were gram positive bacteria and only three samples shown

it belongs to gram negative bacteria. The highest numbers of gram positive bacteria recorded were in middle and bottom level of the river and no gram negative bacteria was recorded in middle area. By using gram staining technique, gram positive stain purple/violet and gram negative stain red. The gram positive bacteria retain crystal violet dyes as it has thick cell wall consisting peptidoglycan that able to resist the reaction of acetone. A thin peptidoglycan in gram negative microorganism was disrupted by alcohol in decolourisation process (Beveridge, 2009).

In figure 4.5, the data recorded show a slightly differences in number of bacteria in term of their shape. Referring to above figure, the blue colour chart indicated the number of coccus found in Tanah Merah River, the red colour chart represented the number of staphylococcus and the green colour chart designated to show the number of short bacillus presence in study area. Coccus is characterized as round shape cell, once in a while marginally levelled when they are contiguous each other. Staphylococcus is existed as the arrangement of coccus in grape-like bunches (Giancarlo, 2013) formed by unpredictable cell divisions. Short bacillus is stumpy and they look like coccus and bacillus. The shape of bacteria cell is maintained due to their rigid cell wall (Sagar, 2015).

During performing a gram staining test, several aspects must be taken into consideration to avoid errors in results. First of all, aseptic technique must be done properly in order to minimize the contamination on smearing slide. Besides, take a small amount of sample and ensure that the area of smearing is wide enough to avoid the overlapping between microorganisms. Other than that, decolourisation process must not exceed 30 seconds because over expose on alcohol (acetone) cause the

breaking of the cell wall. Thus, there is no remaining stain on slide that needs to be observed.

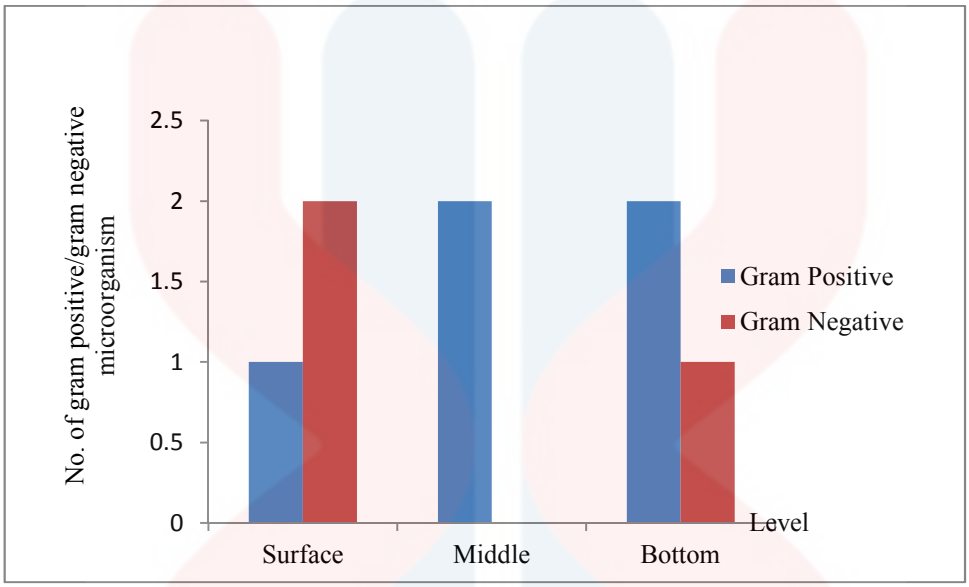

Figure 4.4 A diagram showed a number of Gram positive and negative bacteria presence in Tanah Merah River. The Grams of bacteria were determined by inspecting the colour of single colonies using light microscope. Purple colour bacteria indicate Gram positive while pink colour bacteria are Gram negative.

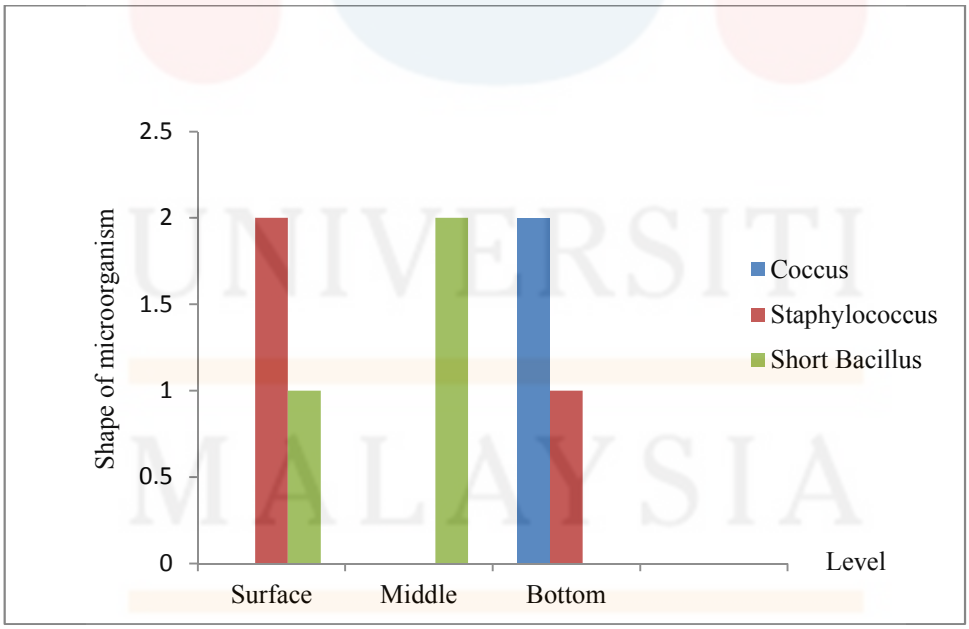

Figure 4.5 A graph bar represent shape of bacteria found in Tanah Merah River.

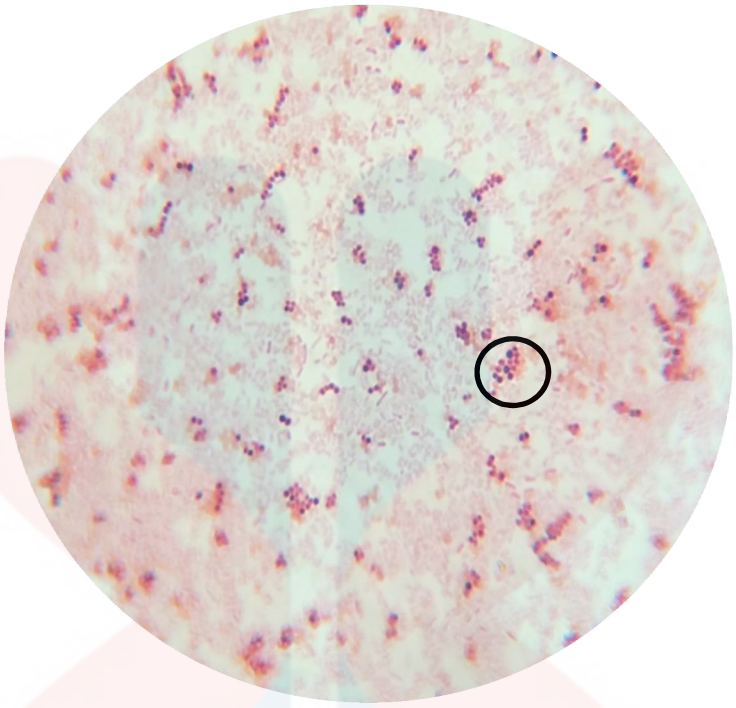

Figure 4.6 An image of gram negative staphylococcus bacteria found in Sg. Tanah Merah. The morphology of bacteria was observed under light microscope at 100x magnification.

Figure 4.7 An image of Gram positive short bacillus bacteria. The morphology of bacteria was inspected under light microscope at 100x magnification.

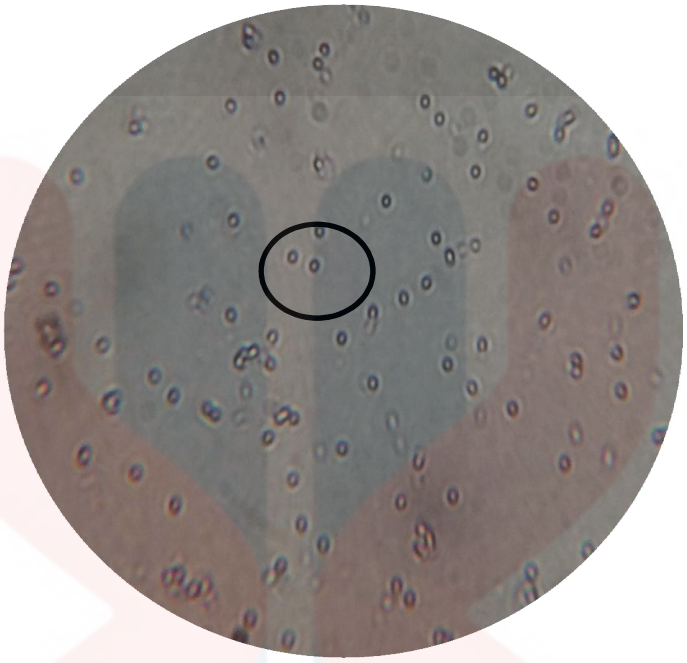

Figure 4.8 An image of Gram positive bacteria. The coccus shape of bacteria was observed by using microscope at 100x magnification.

4.3 Measurement of Bacteria Growth (OD_{600})

Bacterial development rate relies on upon a few components including medium, temperature, pH and so on. Growth of bacteria can be determined by measuring optical density (OD) which empowers a method for measuring the number of bacteria in a sample. The technique works by sending light through at a particular wavelength, for this situation 600nm (OD_{600}).

Before measuring the growth of bacteria, the bacteria samples were inoculated into a bijou bottle and the samples were incubated in incubator shaker at 30˚C 150 rpm for 24 hours. The growths of bacteria were determined by using spectrophotometer. Bacteria are at active phase when the absorbance readings were in 0.3-0.6A ranges (Guennadi et al., 2007). The reading of optical density (OD) was taken once the cultured bacteria in Luria Broth turned cloudy. Based on table 4.1,

there was no reading recorded at point 1 (middle) as no bacteria grow in nutrient agar. Therefore, no bacteria was cultured in Luria Broth. The highest absorbance reading was at point 1 surface, 0.532A and the lowest absorbance reading was at point 3 bottom, -0.049A. The negative value was obtained due to over-cultured in incubator shaker. Therefore, the bacteria in a sample were dead due to lack of nutrient and over heat exposure (Timothy et al., 2013).

Table 4.1 The table shown the reading of spectrophotometer at $OD_{600.}$ Absorbance at wavelength 600nm was used in order to determine the growth phase of cultured bacteria in Luria Broth (LB).

	Surface	Middle	Bottom
Point 1	0.532A	-	0.420A
Point 2	0.236A	0.072A	0.446A
Point 3	0.284A	0.229A	-0.049A

4.4 DNA Extraction for Environmental Microorganism in River Water

DNA extraction was done in order to break open the cell containing DNA. The extraction of DNA is important in further scientific investigation such as Polymerase Chain Reaction (Deshmukh, 2007).

DNA extraction involved four main steps which are breaking the open cell to release DNA, separating DNA from proteins and other debris, DNA precipitation and cleaning the DNA.

Using method established by Michele (2002), CTAB was used as it helps to lyse the bacteria cell. The mixture containing CTAB buffer was incubated in water

bath at 65˚C. The amount of DNA doubled when the suspended bacteria containing CTAB buffer is incubate at 37˚C compared to incubation at 65˚C as higher temperature for bacteria lysis could cause DNA degradation (Chen et al., 2010). Thus, affect the precipitation of DNA. Therefore, no result was obtained by using this method.

DNA extraction with GTE and Proteinase K was optimized due no bands were presence in previous result of Agarose Gel Electrophoresis (AGE). Experiment was begins with cultured bacteria in Luria Broth (LB) were spun in centrifuged for 15 minutes. The centrifugation speed was increased from 2 000 rpm to 13 000 rpm to form the pellet. In order to lyse the cell, detergent, Sodium Lauryl Sulphate (SLS) was added, Mark et al (2015) found that SDS used in DNA extraction to remove lipid membranes. The addition of Phenol Chloroform Isoamylalcohol (25:24:1) were resulted on obtaining two phases which the aqueous phase containing nucleic acid was upper and the lower phase containing RNA and lipid were lower. For DNA precipitation, sodium acetate was added to the solution as it helps to precipitate out the DNA from the cell (Lee et al., 2011). The DNA pellet was washed by 80% cold ethanol by centrifugation at 10 000 rpm for retrieval of the pellet. The pellet was air dried and dissolved in TE buffer in order to protect the DNA from degradation. The presence of DNA samples were confirmed by conducting agarose gel electrophoresis.

4.5 Agarose Gel Electrophoresis (AGE) from Water Microbes

Agarose Gel electrophoresis (AGE) was used in this experiment as it serve the method of separating and analysing DNA in order to determine the size of DNA fragment. The experiment was conducted by preparing an agarose powder mixed with 1xTris-Acetate-EDTA (TAE) buffer. Function of TAE buffer in AGE to maintain pH of DNA solution to neutral. Then the mixture was heated in microwave until it was completely melted. After that, ethidium bromide (EtBr) was added to gel as EtBr able to facilitate visualization of DNA. After cooling solution to about 60˚C, then it is was poured into casting tray containing a sample comb and it was allowed to solidify at room temperature. After solidify, the comb in a casting tray was removed carefully. The removal of comb leaves small wells in the gel. The samples containing DNA mixed with loading dye were then pipetted into wells. Loading dye helps the DNA to sink into the wells of the gel. The migration of DNA in gel was facilitated by electric current at 80V.

The separation of DNA was according to their size. The movement of DNA was towards positive poles as DNA was a negatively charge molecules due to its phosphate backbone (Timothy et al., 2007). The movement of large molecule DNA was slower than smaller DNA molecule since small molecule can fit the hole easily. The DNA movement across agarose gel was aided by applying the electric current. The result of this of this experiment was a series band where each band was contained DNA molecules of a particular size (Lee et al., 2012). The band furthest from the start of the gel was contain smallest fragment of DNA and the band closest to the start of gel was contained the largest DNA fragment. Figure 4.9 shows the

movement of DNA fragment along the gel. The both DNA samples were travelled at same distance as they have similar molecular weight.

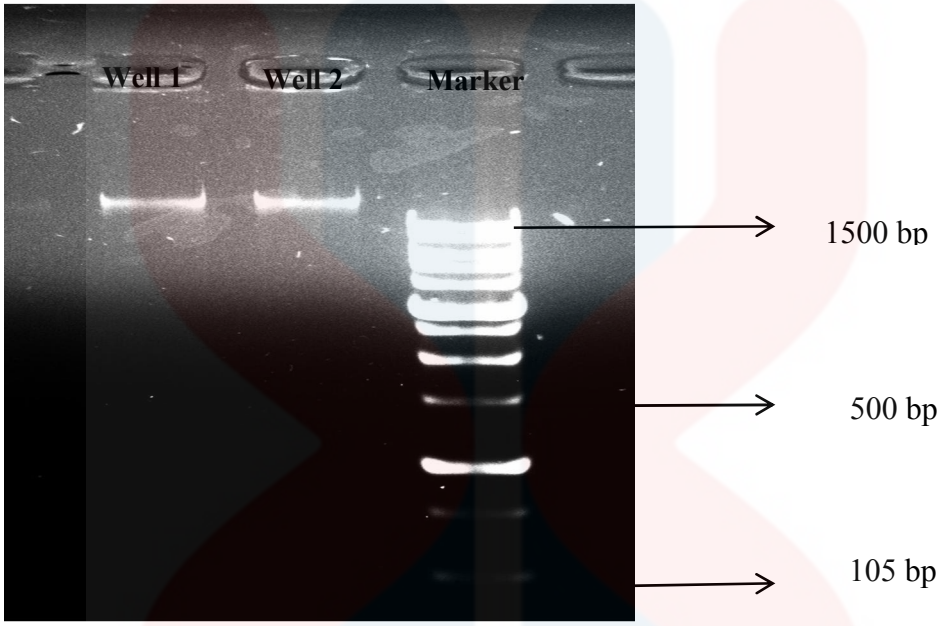

Figure 4.9 An image of a gel agarose electrophoresis. Before electrophoresis, Ethidium Bromide (EtBr) was added into the gel. The separation of DNA fragment was aided by electric current at 80 V for 30 minutes and the gel was visualized under UV light and the picture was taken with gel documentation system.

4.6 Absorbance Reading to Determine the Purity of DNA sample using Nanodrop spectrophotometer $OD_{260/280}$

In this research, NanoDrop spectrophotometer was used to determine the DNA purity which takes measurement at wavelength of 260 and 280 nm. The ratios between ~1.8-2.0 were indicated pure DNA. The determination of DNA purity is important for molecular biology application such as Polymerase Chain Reaction (PCR). Referring to table 4.2, $A_{260/280}$ ratios of all samples were below the range of pure DNA. Ratios below than the ranges were indicated that DNA samples contaminated with excessive salt (Carlos et al., 2001). The contamination in DNA samples degrades the quality of DNA. Impurities in DNA caused inaccurate result of

molecular biology such as PCR as contamination in DNA lowering the efficiency of PCR.

Table 4.2 Represented absorbance measurements of DNA samples. The purity of DNA was determined by using NanoDrop spectrophotometer at wavelength of 260 and 280nm.

	Surface	Middle	Bottom
Point 1	1.34A	-	1.33A
Point 2	1.01A	1.09A	1.16A
Point 3	0.99A	1.03A	1.37A

4.7 Polymerase Chain Reaction (PCR)

In this study, Polymerase Chain Reaction (PCR) was done to make multiple copies of DNA. There are three main procedures in order to complete the PCR that was carried out for 30 cycles.

The initial step is denaturation of DNA double stranded. It was accomplished by heating the DNA sample and caused the bond between nucleotide to break and produced two single stranded DNA. Next, temperature was reduce to 55˚C, 16s primer was bind to DNA template. Temperature was raised to 72˚C and nucleotide in the solution was added to the annealed primer to create a new strand of DNA.

Figure 4.10 represents the result of PCR product after running gel electrophoresis. Supposedly, electrophoresis of PCR product yields a single band of correct size. The result showed a multiple bands which indicates in non-specific binding. Inaccurate result of PCR result can be influenced by several factors such as DNA quality, temperature, concentration of primer and magnesium chloride. In this

case, the ratios of DNA sample at wavelength 260 and 280 nm were below 1.8-2.0. It showed that DNA samples were contaminated with organic substance such as salt. According to Jose et al (2012), $A_{260/280}$ ratios value below than 1.3 too poor to amplify. Thus, it caused failure in the result of PCR products.

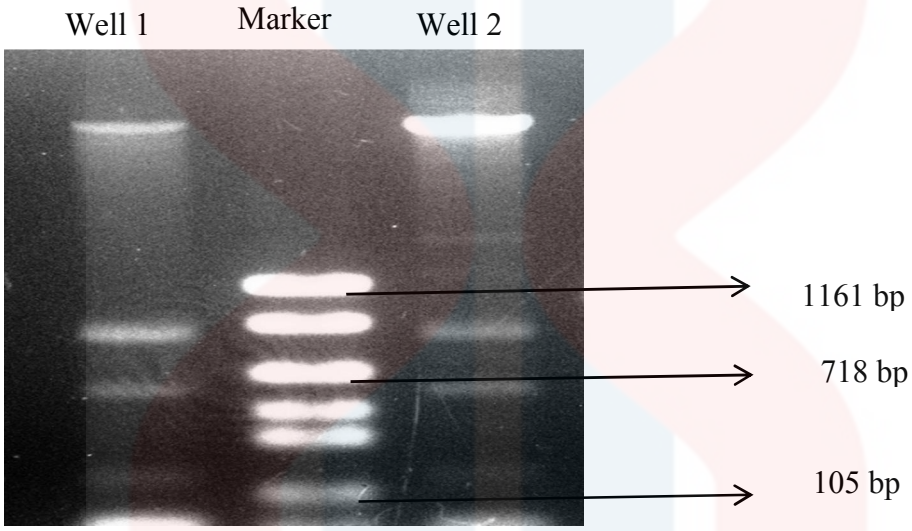

Figure 4.10 The image was represent the result of agarose gel electrophoresis (AGE) of Polymerase Chain Reaction (PCR) products. M is represented as Marker. Multiple bands of DNA fragment at well 1 and well 2 were clearly seen. The separation of bands appears when the gel was exposed under UV light.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Based on the result of the present study, it could be said that the diversity of microorganism in freshwater are very depending on the changes of the environment. The changes of environment conditions could disturb the growth of water microorganism.

According to the result of this study, microorganisms found in Tanah Merah River were mostly gram positive bacteria. Through the observation under light microscope, five of the isolation bacteria were gram positive and the other three samples were gram negative bacteria. The morphology of each bacteria was determined. There are three common shapes of bacteria that presence in Tanah Merah River which is coccus, staphylococcus and short-bacillus.

Due to presence of bacteria in Tanah Merah River therefore, further study on identification of bacterial individual species must be done in order to determine types of bacteria that is live in that river.

5.2 Recommendations

The used of selective and differential of media for bacteria growth is recommended since certain bacteria only growth best in certain media. Besides, the determination of bacteria morphology cannot be used in order to identify the type of bacteria found in Tanah Merah River. Therefore, more optimization in Polymerase Chain Reaction (PCR) is needed in future to avoid multiple bands in a result of PCR product.

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