Distribution of Water Microbes from Three Different Depths of Sungai Kelantan at Kuala Krai

By

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DECLARATION

I declare that this thesis entitled “title of the thesis”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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DISTRIBUTION OF WATER MICROBES FROM THREE DIFFERENT DEPTHS OF SUNGAI KELANTAN AT KUALA KRAI

ABSTRACT

A huge flood hit the Kelantan State, especially at Kuala Krai district in 2014. The flood normally hit the eastern of the Peninsular Malaysia every end of year. This natural disaster could cause changes in the environment condition and alter the ecology of the involved area. The diversity of microorganism in flood water may change due to the huge flood. By using the morphology identification method, this study shows that two types of bacteria shapes were found predominantly in the Sg. Kelantan river water in Kuala Krai. Bacteria *Bacillus* and *Coccobacillus* that were found in the river water samples were identified using the morphology observation. For the Gram Stain, Gram positive bacteria and Gram negative bacteria ware found in surface layer of the river. However, only Gram positive bacteria were found in the middle layer of river. The Gram negative bacteria were more abundant than the Gram positive bacteria at the bottom of river. By using the molecular method, this study shows the present of water microbe DNA band on the agarose gel by using the optimization of DNA extraction with standard CTAB method plus proteinase K and lysozyme. Overall the upper stream of Sg. Kelantan consists of the water microbe mostly from Gram positive bacteria with *Bacillus*-rod shape. However, more data of molecular bacteria are needed to be able to identify the diversity of microbe, at species level that is present in Sg. Kelantan at Kuala Krai.

TABURAN MIKROB AIR DARI TIGA KEDALAMAN YANG BERBEZA DARIPADA SUNGAI KELANTAN DI KUALA KRAI

ABSTRAK

Banjir besar telah melanda Negeri Kelantan, terutamanya di Daerah Kuala Krai pada tahun 2014. Banjir biasanya melanda pantai timur Semenanjung Malaysia setiap hujung tahun. Bencana alam ini boleh menyebabkan perubahan pada alam sekitar dan mengubah ekology kawasan yang terlibat. Kepelbagaian mikroorganisma di dalam air banjir mungkin berubah disebabkan oleh banjir besar. Dengan menggunakan keadah pengenalan morfologi, kajian ini menunjukkan bahawa terdapat dua jenis bentuk bakteria kebanyakannya ditemui di dalam Sg. Kelantan di Kuala Krai. Bakteria *Bacillus* dan *Coccobacillus* yang dijumpai di dalam contoh air sungai telah dikenalpasti melalui pemerhatian morfologi. Bagi pewarnaan Gram, bakteria Gram positif dan bakteria Gram negatif telah dijumpai di lapisan permukaan sungai. Walaubagaimanapun, hanya bakteria Gram positif sahaja yang terdapat di lapisan tengah sungai. Bakteria Gram negatif adalah lebih banyak berbanding bakteria Gram positif di bahagian dasar sungai. Secara keseluruhan, aliran air di hulu Sg. Kelantan mempunyai mikrob air yang kebanyakannya dari bakteria jenis Gram positif dengan bentuk rod *Bacillus*. Dengan menggunakan kaedah molekular, kajian ini menunjukkan kehadiran *band* DNA mikrob air pada gel agarose dengan menggunakan kaedah pengoptimumkan pengekstraktan DNA dengan kaedah CTAB standart ditambah dengan proteinase K dan lysozyme. Walaubagaimanapun, lebih banyak data molekul bakteria diperlukan untuk mengenalpasti kepelbagaian mikrob, di peringkat spesies yang terdapat di dalam Sg. Kelantan di Kuala Krai.

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µm	micrometre
µmol	micromole
AACC	American Association for Clinical Chemistry
AGE	agarose gel electrophoresis
BLAST	Basic Local Alignment Search Tool
cm	centimetre
CO_2	carbon dioxide
CTAB	cetyltrimethylammounium bromide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside 5'-triphosphates
EDTA	ethylenediamine tetra-acetic acid
kb	kilobase pair in duplex nucleic acid, kilobases in single-stranded nucleic acid
km	kilometre
km^2	square kilometre
LB	Luria-Bertani
M	molar
mg	milligram
MIC	minimum inhibitory concentration
min	minute

ml/mL	millilitre
mm	millimetre
mmol	millimole
NA	nutrient agar
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometre
OD	Optical density
PCR	polymerase chain reaction
RM	Ringgit Malaysia
RNA	ribonucleic acid
rpm	revolution per minute
rRNA	ribosomal RNA
RT	room temperature
sp	species
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
V	voltage
WHO	World Health Organization

LIST OF SYMBOLS

%	percentage
ºC	degree Celsius

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Kelantan is situated in north-east of Peninsular Malaysia. The Sungai Kelantan is the major river in Kelantan. It is uniquely the only river in South-Asia that flow northwards (Ambak *et al.,* 2010). The Galas River (Sungai Galas) and the Lebir River (Sungai Lebir) near the town Kuala Krai are the two main rivers that contributed in formation of the Sungai Kelantan. The length of the main river from the point to the estuary is about 450 km and is fed by more than 180 streams and flow a catchment area about 11900 km^2. The river flows past four main town such as Kuala Krai, Tanah Merah, Pasir Mas, and Kota Bharu, the capital that located near the mouth of river, before it reach into the South China Sea (Ambak *et al.,* 2010). The Sungai Kelantan from the long time has been contribute to local people daily life activity such as in domestic uses, agriculture, transportation, irrigation and sand mining activities (Tan *et al.,* 2013). Sungai Nenggiri, one of the streams of Sungai Kelantan, flow along 51 km from Hulu Kelantan to Kuala Krai and was once the main communication route for neighbouring communities including for the 138 indigenous people posts along the river (Daily Express, 2015).

In December 2014, the great flood that hit Kelantan has left behind a mark of destruction to the Kelantan State. The heavy rain fallen had cause several soil erosion and untold damages to agriculture land. The effect from that nature disaster, the flood had changed the environment and ecology of the involved area. According to Shamshuddin *et al.* (2016), "the lower reaches of the river were heavily silted with infertile materials considered unfit for crop productions". Due to the great flood that

called “Bah Kuning” by the local people, the flood had caused Malaysia bear an economic loss approximately RM 2.856 billion and almost 300,00 people throughout the country were affected and the worst area that hit by the flood was the Kuala Krai district in Kelantan (Shamshuddin *et al.*, 2016).

Flood water are likely contained many pollutants and may have many pathogenic bacteria that consequently rise the human health risk (Veldhuis, Clemens, Sterk & Berends, 2010). The diversity of microorganism in flood water may change due to the stage of flood that hit Kelantan, especially at Kuala Krai because the flood at that area took a long time to recover. Microorganism could from sewer, flashed from soil, or open channel. According to Zummy *et al.* (2015), the most types of bacteria found in flood water in Pahang are *Shigella flexneri, Salmonella typhimurium,* and *Escherichia coli*.

1.2 Problem Statement

The end of the year 2014, floods has hit the state of Kelantan for more than two weeks. The river flows northward, and it overflow passing through the important towns including the Kuala Krai that cause the flood. Therefore, various types of microorganism from the terrestrial, agriculture industry, domestic waste and others will flow together into the river stream. Therefore, realizing the restriction of the previous studies, this research focused at the Sungai Kelantan in order to identify the diversity of microorganism in that river.

1.3 Objective

1. To culture, isolate and identify the microorganism in Sungai Kelantan from river water samples collected in the Kuala Krai using morphology method.

CHAPTER 2

LITERATURE REVIEW

2.1 Sungai Kelantan (Kelantan River)

Sungai Kelantan flows in the province of Kelantan in north-eastern Peninsular Malaysia (Waterware, 2016). According to Ambak *et al.* (2010), "Sungai Kelantan is a unique river in South-east Asia since it is the only river that flow northwards". The Sungai Kelantan from the long time has contribute to local people daily life activity such as in domestic uses, agriculture, transportation, irrigation and sand mining activities (Tan *et al.*, 2013). Figure 2.1 shows the whole map of Kelantan River.

Figure 2.1: The map of Kelantan River. The river flow from the upper stream at the Kuala Krai and flow northward pass through the Temangan, Machang, Tanah Merah, Pasir Mas, and Kota Bharu area before reach the sea. (Source: Google Map, 2016)

2.2 Flood and Its Effect in Kelantan

"Heavy rain falling on the land at the upper reaches of Kelantan River, Malaysia, on December 2014, had resulted in several soil erosion and untold damage to croplands" (Shamshuddin *et al.*, 2016). According to Shamshuddin *et al.* (2016), the lower reaches of the river were heavily silted with infertile materials considered unfit for crop productions. The flood had caused Malaysia bear an economic loss approximately RM 2.856 billion and almost 300,000 people throughout the country were affected and the worst area that hit by the flood was the Kuala Krai district in Kelantan (Shamshuddin *et al.*, 2016).

According to Zainal and Sunitha (2015), more than 200, 000 people were affected by this floods that has been depicted as the worst floods in decades and cause 21 people were died in this natural disaster. The figure 2.2 shows a view of the affected area in Kuala Krai due to flood in 2014.

Figure 2.2: Impact of flood in Kuala Krai (Source: Berita Harian, 2014)

2.3 Freshwater

According to Okafor (2011), freshwaters are determined as natural waters possess less than 1000 mg per liter of salt content. It is can classifiable into

atmospheric, surface and underground water. Each types of nature freshwater possess a unique microbial ecology. Surface waters may have either freshwater or saline water. Rivers, stream, ponds, wetlands and lakes are the examples of surface freshwater, and it contains large amount and variety group of microorganisms (Okafor, 2011).

Freshwater environment can separate into two groups, flowing water and standing waters. The flowing water includes lotic system are canals, estuaries and river while standing water includes lentic system like ponds, lakes, marshes and other enclosed water body (Alexopoulos *et al.* 2002).

2.3.1 Bacteria in Freshwater System

Bacteria are prokaryotic microorganism that usually cannot be seen by naked eyes and their sizes are in microns. According to Bridle (2014), a selection of waterborne bacteria pathogens, as identified by the World Health Organization (WHO), is *Campylobacter jejuni*, *Escherichia coli*, *Legionella* species, *Pseudomonas aeriginosa, Salmonella thyphi, Salmonella enterica*, *Shigella* and *Vibro cholera.* The most types of bacteria found in flood water in Pahang are *Shigella flexneri, Salmonella typhimurium,* and *Escherichia coli* (Zummy *et al.*, 2015).

According to Okafor (2011), the Gram positive bacteria like *Actinobacteria* are abundance in freshwater. Due to limitation in methods of cultural bacteria, only a few bacteria that Gram positive were found and most of it is Gram negative bacteria in freshwater.

There are varies of nature bacteria flora in the freshwater. There are huge amount of heterotrophic bacteria and small amount of chemoautotrophic or photoautotrophic bacteria (Okafor, 2011). In the oligotrophic surface water like

spring water, population of bacteria for the non-bentic population mostly of Gram-negative, non-spore forming rod bacteria, especially *Flavobacterium* and *Achromobactor*. The bacteria like *Pseudomonas, Proteus, Bacillus*, and *Eutrobacteriaceae* become important due to the rise of eutrophication. The bacteria include *Vibrio* and *Actinomycetales* usually encountered. In general, bacteria at planktonic or tectonic are the Gram-negative (Okafor, 2011).

According to Donderski & Wilk (2001), sediment at the bottom of the river or bentic level, contain huge number of psychrophilic and faecal heterotrophic bacteria. The presence of this bacteria and coliform bacteria, faecal bacteria, faecal *streptococci* and sulphur reducing *Clostridium* rods bacteria indicate of faecal contamination. At the bentic level, most bacteria are the Gram-positive bacteria include spore-forms and cocci, *Clostridium* and pleomorphic form like *Arthrobacter* and *Nocardia*. The photosynthetic bacterial is the example of the chemoautotrophic bacteria that found in the river (Okafor, 2011).

2.4 Morphology of Bacteria

There is variety of diversity in bacteria kingdom, with a wide range of morphologies shown by different type bacteria. Bacteria can be classified into two groups, Gram-negative and Gram-positive (Bridle, 2014). The morphology of bacteria can be identified by the size, shape and arrangement. Prokaryotes are the smallest organisms and their range from 0.5-2.0 μm in diameter. For the shape, usually bacteria present in three basic shapes: spherical (coccus), rod like (bacillus) and spiral (vibrio, spirillum and spirochete). In addition to characteristic of shapes, majority of bacteria also found in variety arrangements of groups cells such as pair (diplo-) and chain (strepto-) (Black, 2008). Figure 2.2 show the bacteria cell morphologies, arrangements and colours of the colony.

According to Okafor (2011), freshwater bacteria were able to culture and simply grouped freshwater into: i) fluorescent group; ii) chromogenic bacteria including violet, red, and yellow forms; iii) coliform group; iv) Proteus group; v) non-gas forming, non-chromogenic, non-spore-forming rods which do not produce Proteus-like colonies and may not acidify milk and liquefy gelatin; vi) aerobic spore-forming rods; and vii) white, yellow and pink cocci.

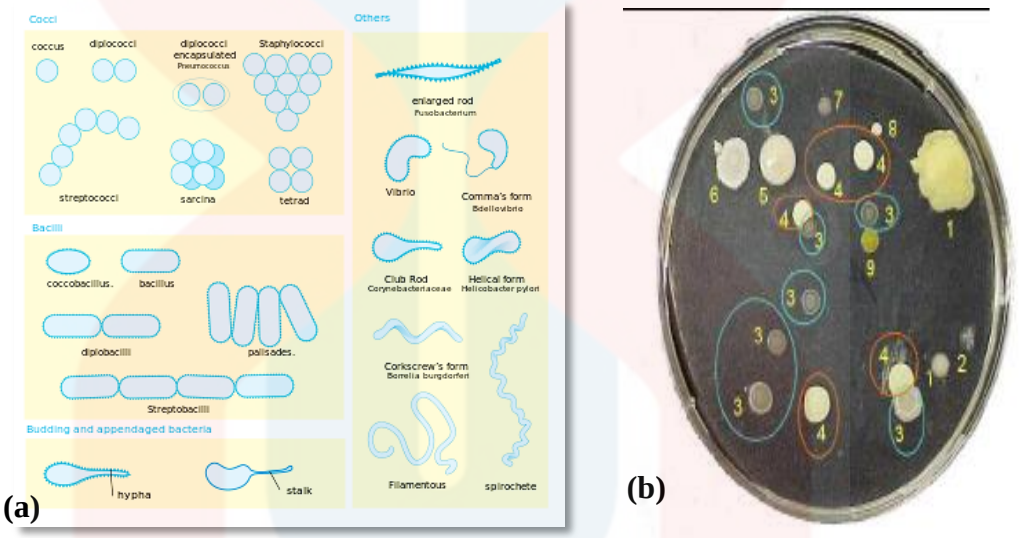

Figure 2.3: **(a)** Bacteria cell morphologies, arrangements (Source: Ruiz, 2006) and **(b)** Colours of the bacteria colony in nutrient agar. Colonies in every circle have the same type of morphology identification (Source: Reynolds, 2011)

2.5 Physiological Basis of Aquatic Microbial Ecology

There are a few factors that influence the nature distribution of bacteria in the freshwater. According to Lindstrom *et al*. (2005), the size of habitat, water retention time, ultraviolet radiation intensity, amount of organic matter supply, composition of phytoplankton, predation of protistan and matazooplankton, water chemistry especially the pH and the water temperature are the some component that affecting the type community of bacteria in freshwater.

According to Okafor (2011), most of microorganisms in water are usually does not exist in pure culture and they tend to exist as mixed culture. The condition of

environment can influence the type of abundance of organism in given environment at given time. The abundance of organism can be influence by some factor like nutrient availability, pH, oxygen tension, temperature, depth of water, flow velocity in moving water, and the light.

For the nutrient availability, quality of water is depends on the amount of nutrient that flow into that system. The rise of nutrient concentration in water system will caused eutrophication (Schinder & Vallentyne, 2008). At the pH 7 and above, it is the suitable condition for the bacteria, for example at pH 8 for *Vibrio* sp. The bacteria will grow rapidly when the system is aerated wisely and breakdown the organic material into CO_2 and water the produce new cells. The anaerobic bacteria developed under the anaerobic condition (Ishoj & Desforges, 2009).

The type of bacteria also influence by the depth of water. Every level of water becomes niche for different type bacteria. Generally, most bacteria are found in the upper layer of water than bottom layer. For example, the cocci bacteria are usually found at benthic whereas at the tectonic are Gram-negative mobile rods bacteria (Okafor, 2011).

2.6 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a laboratory technique that is used to produce a huge number copy of shorts section of DNA from a very small sample of genetic material. This process is called amplification of DNA and it enables specific genes of needed to be detected or measured (AACC, 2015). PCR is a method used to duplicate DNA unique sequences into identical copies DNA, which will then be used to determine the identity of the with a very high probability sample (a specific person, animal, or pathogenic organism) (Zipser, 2015).

In the past, the molecular techniques have shown improvement in overcoming the limitation of conventional phenotypic procedure for identification of bacteria phenotypes. PCR is very sensitive in reaction and allows quantitation or identification of bacteria at species level. By using the DNA based assay, one can easily trace bacterial strains and directly from small amount of cultured bacteria cells, in the same time increasing the sensitivity and reduce the time require for identification. PCR has particularly used in this regard, which relies on primer sequences designed to facilitated bacteria identification at any level of specificity like the strain, species or genus (PREMIER Biosoft, 2016).

In previous study by Miskin *et al.* (1999), PCR is one of the possible techniques to obtain information directly from microbe without cultivation process. By isolating the nucleic acid in PCR, it is able to assess the microbial population in environment through culture- independence method and followed by the amplification and sequencing of bacterial 16S rRNA genes.

2.7 16S rRNA Gene Region Sequencing

The 16S ribosomal RNA (rRNA) sequencing is a common amplicon sequencing method that is used to identify the bacteria that present in the environmental samples and this method is a well-established for studying phylogeny and taxonomy of samples from complex microbiomes or environment that are hard and difficult to study (Schmidt *et al.*, 1991).

According to Vetrovsky & Baldrian (2013), the 16S ribosomal RNA (rRNA) played important role as important target of study in ecology of bacteria. It is used to determine phylogenetic relationships between taxonomy, quantify the relative

abundance of taxonomy at various ranks and explore the diversity of bacteria in environment.

This 16S rRNA genetic marker is used for a number of reasons. It is present in almost all bacteria, often exists as multigene family and the function of the 16S rRNA has not change over time hence this gene is large enough for identification purpose (Janda & Abbott, 2007). According to Janda & Abbott (2007), the 16S rRNA gene sequencing informatics most attractive uses is to provide genus and species identification for isolates that do not fit any recognized biochemical profile. The limited number of studies to date suggest that the cumulative results of 16S rRNA gene sequencing provides genus identification in most cases (>90 %) but less so with regard to species (65 to 83 %), with from one to 14 % of the isolates remaining unidentified after testing.

2.8 Optical Density at 600nm (OD_{600}) for Monitoring Bacterial Growth

The optical density (OD) measurement of bacteria culture is the one of the technique that widely used in microbiology. According to Matlock *et al.* (2011), the measurement of bacteria culture is actually based on the amount of light scattered by the culture rather than the amount of light absorbed. The bacterial OD_{600} is used to monitor bacterial growth using the spectrophotometer.

The most common used of OD_{600} is to determination and standardization of the optimal time to induce a culture during bacterial protein expression protocol, to determine and standardize the inoculum concentration for minimum inhibitory concentration (MIC) experiments and to determine of the optimal time at which to harvest and prepare component cells (Matlock *et al.*, 2011).

2.9 Luria-Bertani (LB) Broth for Cultivation of Microbe

Luria-Bertani broth is rich medium that permit fast growth and good growth yield for many species of bacteria and widely used by the bacteriologists (Sezonov *et al.*, 2007). The growth in LB broth is carbon limited because it is contains enough amounts of all the essential inorganic nutrients (Sezonov *et al.*, 2007). According to Sezonov *et al.* (2007), the LB broth does not contain fermentable sugar that is utilizable by *E. coli*. This broth contain a low concentration of sugars which are decreased at an OD_{600} of 0.3, that forcing cell metabolism to switch to using amino acids as a carbon source.

2.10 CTAB Buffer for Microbe DNA Extraction

CTAB extraction buffer is a widely used reagent for the DNA isolation. It is effective in eliminating polysaccharides and polyphenols by employing the cationic detergent CTAB (cetyltrimethylammounium bromide) and Polyvinylpyrrolidone, the polyphenol binding agent (Ops Diagnostics, 2016). In previous study by Rajagopal *et al*. (2014), CTAB was used for preparation of competent bacteria cells (*E. coli* and *Bifidobacterium* sp.) and yeast (*Kluyveromyces lactis*). CTAB buffer permits efficient uptake of plasmid DNA and ligation-reaction product by rapid and routinely yields 10^5-10^9 transformants per microgram of plasmid DNA.

2.11 Gel Electrophoresis for Separation of DNA Molecule

According to National Diagnostics (2011), in a gel of electrophoresis, the DNA molecule experiences electric force proportional to its effective charge and the strength of electric field. Due to the fractional resistance of the matrix, the molecule move with the constant velocity.

Gel electrophoresis is a method to separate the mixture of DNA, RNA or protein defer to molecular size. The DNA molecule will be separated by an electrical field through an agar gel that contains tiny pores. The molecules move through the pores in gel at different speed that related to their length. Therefore, the short DNA sequence will travel greater distance rather than the long sequence through the gel (National Diagnostics, 2011).

2.12 The Staining of Microorganisms

According to Othman (n.d), the bacterial morphology could be examined in two ways. The first way is by observing the living, unstained organism which is used to observe bacterial motility. The second way is by observing dead cells that one stained with dyes. Almost all living bacteria are colorless, and do not present sufficient contrast with the water in which they are suspended to be clearly visible. By staining the bacteria, it makes them contrast in color with surrounding and more readily visible.

Different strains could be used to identify certain internal structure of the cells, which would otherwise pass unseen. Furthermore, in order to use the oil-immersion objective of microscope and thereby obtain the greatest degree of magnification, it is convenient to use stained method rather than wet-mounts. Although bacteria do not appear clearly different from their surroundings, they differ markedly by chemical staining. The chemical stain or dyes generally react with the bacteria cell but not with the background. The main advantage of staining is that it enables bacteriologist to use higher magnification, provide differentiation between various morphological types of microorganisms and it permit a study of internal structures of bacterial cells such as cell wall, or nuclear bodies (Othman, n.d).

Gram stain is a very important differential staining techniques used in the initial characterization and classification of bacteria in Microbiology. Gram staining identify bacterial in specimen and culture by their Gram reaction like Gram positive and Gram negative and also morphology (cocci or rod). Figure 2.3 show the structure of bacterial cell wall for Gram positive and Gram negative bacteria type.

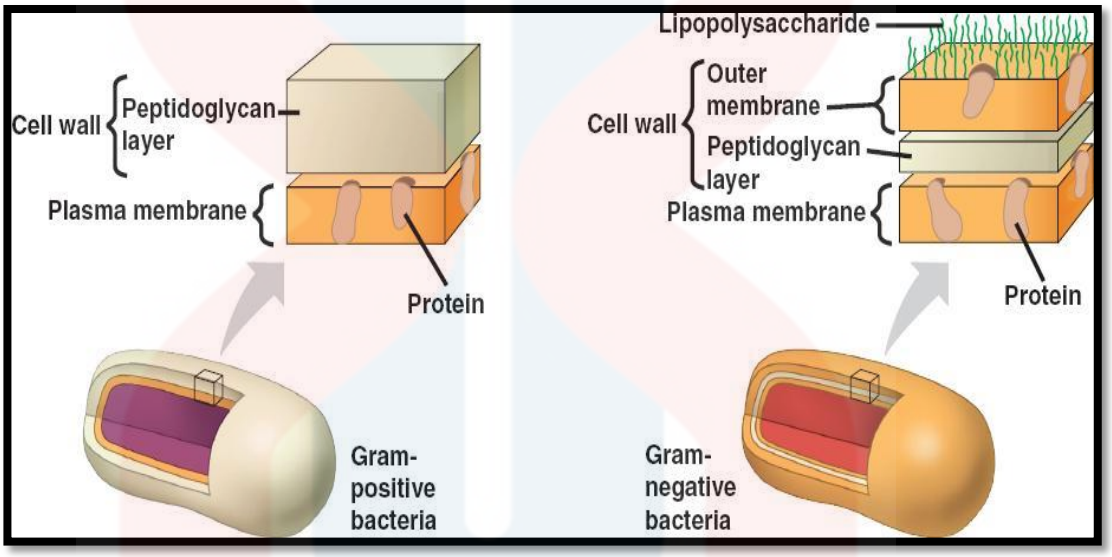

Figure 2.4: The structure of bacterial cell wall for Gram positive and Gram negative bacteria type

(Sources: Nicer Tutor, 2016)

The difference between bacteria Gram negative and Gram positive cell wall composition accounts for the Gram Staining differences. The Gram positive cell wall consists of thick layer of peptidoglycan with numerous teichoic acid cross linking which resists the decolorization.

According to Acharya (2015), in Gram staining, crystal violet (CV^+) in aqueous solution dissociates into CV^+ and Cl^- ions that penetrate through the wall and membrane of both Gram positive and Gram negative cells. The CV^+ interacts with negatively charged components of bacterial cells and staining the cells purple. The iodine (I^- or I^{3-}) was added and interacts with CV^+ to form large crystal violet iodine (CV-I) complex within the cytoplasm and the outer layer of the cell.

According to Black (2008), ethanol or acetone solution act as decolorizing agent that interact with the lipids of the membrane of both Gram positive and Gram negative bacteria. The lipopolysaccharide layer (outer membrane) of the Gram negative bacteria is removed from the cell and leaving the peptidoglycan layer exposed. Gram negative bacteria poses thin layer of peptidoglycan than Gram negative bacteria. During the decolorisation, Gram negative bacteria cell walls become leaky and allow the large amount of CV-I complex to be washed from the cell. By addition of ethanol, highly cross-linked and multi-layered peptidoglycan of Gram positive bacteria is dehydrated and traps the huge amount of CV-I complexes in the cell (Medicinehack, 2015). As a results, the Gram positive bacteria remain purple colour, while the Gram negative bacteria become colourless and revealed as red colour when the positively charge dye safranin is added as a counterstain. Therefore, the Gram positive bacteria is stain purple colour and Gram negative is stain in red or pink colour (Acharya, 2015). Figure 2.5 show the procedure of Gram staining.

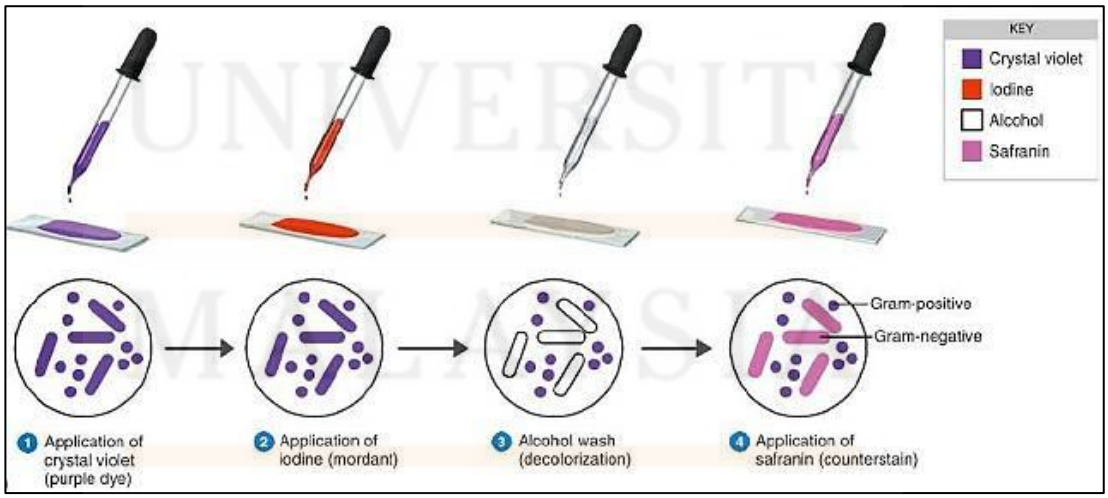

Figure 2.5: Gram staining procedure that involve the application of crystal violate as purple dye, iodine as a mordant, alcohol to decolorization and safranin as a counterstain. (Sources: Medicinehack, 2015)

CHAPTER 3

METHOD AND MATERIAL

3.1 River Water Samples Collection at Sungai Kelantan, Kuala Krai

Following method established by Paulse *et al.* (2012), 100 ml river water samples were collected in sterile 50 ml falcon tube by using water sampler. The water samples were collected from three points at the area of Tangga Krai, Kuala Krai. The distance between each point was one meter and the water samples were taken in three different depths; on the surface, middle (50 cm) and at the bottom of river with sediment for every point. The samples were stored in ice box (4 °C) to maintain the cold temperature. This water samples were collected on sunny day. Figure 3.1 shows Tangga Krai which located near the Kuala Krai Police Station. The geographical coordinate are 5.5307° N, 102.1965° E.

Figure 3.1: Tangga Krai (*white mark area*) (Source: Google Map, 18 November 2016)

3.1.1 Physical Parameter of Water Quality Measurement

The physical parameter of water quality such as pH, temperature, dissolved oxygen, and salinity reading was collected by using the YSI multi-parameter (Xylem, USA) in triplicate at point one, two and three for every layer of river, surface, middle and bottom.

3.2 Preparation of Nutrient Agar Plate, 50 % Glycerol Solution and 2x CTAB Buffer.

3.2.1 Nutrient Agar Media preparation, Nutrient Agar Plate and 50 % Glycerol Solution Preparation

Following methods by Brown (2009), 28 gram of nutrient agar (NA) powder (Oxoid, UK) was weighed on a balance and was added into the one liter of distilled water. The mixture was heated while stirring using the magnetic stirrer to fully dissolve all components and the pH was checked using pH meter and adjusted by adding HCl. The dissolved mixture was autoclaved at 121 ^{o}C for 15 minutes at 15 psi. The autoclaved mixture was allowed to cool but not solidify. The 30 ml of nutrient agar mixture was poured into each petri dish and leaved to solidify in sterile condition. After the nutrient agar become solid, the petri dishes were sealed into plastic bag and kept in 4 ^{o}C chiller till further used.

For 50 % glycerol solution, 20 ml glyceric acid was mixed with 20 ml of distilled water and mixed well. After that, the solution was autoclaved at 121 ^{o}C for 15 minutes at 15 psi and kept in room temperature.

3.2.2 2X CTAB Buffer (100 ml) Preparation

Cetyltrimethylammounium bromide (CTAB) powder was weighed for 2.0 gram and was dissolved in 100 ml of distilled water. After that, 8.182 gram of NaCl, 10 ml of 1 M Tris stock solution and 4 ml of 0.5 M EDTA stock solution was added into the CTAB solution. The mixture was autoclaved at 121 ^{o}C for 15 minutes at 15 psi.

3.3 Dilution and Culture of River Water Samples on Nutrient Agar

Water samples of Sg. Kelantan River were diluted in serial dilution tube that contain sterile distilled water (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}), followed by the 0.1 ml (100 µl) of 10^{-5} prepared serial dilutions into NA plates by spread plate technique. The plate was incubated at range of 30-37 ^{o}C for 24 hours in Memmert lab incubator.

3.4 Isolation of Bacteria Colony from Nutrient Agar Plates

Colonies formed on each Nutrient Agar plates that were inoculated with diluted Sungai Kelantan river water sample were examined visually with naked eye and a single colony was chosen for isolation and pure culture initiation. Individual colonies were streaked onto Nutrient Agar plate and incubated at range 30-37 ^{o}C for 24 hours until pure cultures were obtained by using streak plate technique. For morphology identification, the pure culture samples goes through the Gram staining process. Gram-stained samples were viewed with light microscope for preliminary identification of microbes using the morphological characteristics.

3.4.1 Cultivation of Microbe using Luria-Bertani (LB) Broth

About 35.6 gram of LB powder was mixed with one liter of distilled water and mixed well. The 10 ml of dissolved mixture was transfer into Bijou bottle and autoclaved at 121 ^{o}C for 15 minutes at 15 psi than kept in room temperature until used. For cultivation of bacteria, a single colony of pure culture bacteria was inoculated into LB broth and was incubated in incubator shaker for 12 hours at 300 rpm. The 12 hours cultured bacterial were observed their optical density by using the spectrophotometer at OD_{600} to monitor bacterial growth at active phase (0.3 A– 0.4 A). About 0.5 ml of 12 hours Luria Broth cultured bacteria was added into the 0.5 ml of 50 % sterile glycerol solution in the sterile two ml of centrifuge tube. This 50 % glycerol stock culture was stored in 4 ^{o}C for future study.

3.5 DNA Extraction of Sungai Kelantan River Microbe

3.5.1 DNA Extraction with Standard Cetyltrimethylammounium Bromide (CTAB) Method

Using modification method by He (2011), the 12 hours bacteria cultures (grown in Luria Broth medium) were measured the optical density at 600 nm (OD_{600}) to determine the active phase and one ml of bacteria culture at active phase was transferred to an one two ml centrifuge tubes and centrifuged at 10, 000 rpm for one minute to pellet the cells. The supernatant was discarded. The cell pellet was re-suspended in 560 µl TE buffer and 600 µl lysis buffer (CTAB buffer) and vortexed to completely re-suspended bacteria cell pellet. After that, the tubes containing buffer plus pellet were incubated 10 minutes at 65 ^{o}C in water bath. About 600 µl of

chloroform was added to the tubes and mixed well by inverting the tube until the phases are completely mixed and centrifuge at 14,000 rpm for five minute.

The upper aqueous (supernatant) layer was carefully transferred to a new sterile centrifuge tube by using micropipette. The 60 µl of cold ethanol (stored ethanol at -20 ^{o}C freezer) was added into the supernatant and was mixed gently to precipitate the DNA. The tube was centrifuged at maximum speed for 15 min. The supernatant was discarded and the DNA pellet was rinsed with one ml of 70 % ethanol (stored ethanol at room temperature). The mixture was centrifuged at maximum speed for two minutes and the supernatant was carefully discarded and the DNA pellet was air-dried. The DNA was re-suspended in 50 µl TE buffer (He, 2011) and stored in -20 ^{o}C till next step.

The extracted DNA samples was electrophoretically analysed on 1 % molecular grade agarose gel (Paulse *et al.*, 2012).

3.5.2 Extraction of DNA Using Standard CTAB Method plus Proteinase K

Using modified method by He (2011), the 12 hours bacteria cultures (grown in Luria Broth medium) were measured the optical density at 600 nm (OD_{600}) to determine the active phase and one ml of bacteria culture at active phase was transferred to an one two ml centrifuge tubes and centrifuged at 10,000 rpm for one minute to pellet the cells. The supernatant was discarded. The cell pellet was re-suspended in 560 µl TE buffer, 80 µl lysis buffers (CTAB buffer) and five µl of 20 mg/ml Proteinase K and vortexed to completely re-suspended bacteria cell pellet. After that, the tubes containing buffer plus pellet were incubated 10 minutes at 65 ^{o}C in water bath. About 80 µl of chloroform was added to the tubes and mixed well by

inverting the tube until the phases are completely mixed and centrifuge at 14,000 rpm for five minute.

The upper aqueous (supernatant) layer was carefully transferred to a new sterile centrifuge tube by using micropipette. The 60 µl of cold ethanol (stored ethanol at -20 ^{o}C freezer) was added into the supernatant and was mixed gently to precipitate the DNA. The tube was centrifuged at maximum speed for 15 min. The supernatant was discarded and the DNA pellet was rinsed with one ml of 70 % ethanol (stored ethanol at room temperature). The mixture was centrifuged at maximum speed for two minutes and the supernatant was carefully discarded and the DNA pellet was air-dried. The DNA was re-suspended in 50 µl TE buffer (He, 2011) and stored in -20 ^{o}C till next step.

The extracted DNA samples was electrophoretically analysed on 1 % molecular grade agarose gel (Paulse *et al.*, 2012).

3.5.3 Optimization of DNA Extraction with Standard Cetyltrimethylammounium Bromide (CTAB) Method without Proteinase K

Using modification method by He (2011), the 12 hours bacteria cultures (grown in Luria Broth medium) were measured the optical density at 600 nm (OD_{600}) to determine the active phase and 200 ml of bacteria culture at active phase was transferred to an one two ml centrifuge tubes and centrifuged at 10,000 rpm for one minute to pellet the cells. The supernatant was discarded. The cell pellet was re-suspended in 560 µl TE buffer and 600 µl lysis buffer (CTAB buffer) and vortexed to completely re-suspended bacteria cell pellet. After that, the tubes containing buffer plus pellet were incubated 30 minutes at 65 ^{o}C in water bath. About 600 µl of

chloroform was added to the tubes and mixed well by inverting the tube until the phases are completely mixed and centrifuge at 14,000 rpm for 15 minute.

The 100 µl of upper aqueous (supernatant) layer was carefully transferred to a new sterile centrifuge tube by using micropipette. The 100 µl of cold ethanol (stored ethanol at -20 ^{o}C freezer) was added into the supernatant and was mixed gently to precipitate the DNA. The tube was centrifuged at maximum speed for 15 min. The supernatant was discarded and the DNA pellet was rinsed with 600 µl of 70 % ethanol (stored at room temperature). The mixture was centrifuged at maximum speed for two minutes and the supernatant was carefully discarded and the DNA pellet was air-dried. The DNA was re-suspended in 100 µl TE buffer (He, 2011) and stored in -20 ^{o}C till next step.

The extracted DNA samples was electrophoretically analysed on 1 % molecular grade agarose gel (Paulse *et al.*, 2012).

3.5.4 Optimization of DNA Extraction with Standard Cetyltrimethylammounium Bromide (CTAB) Method plus Proteinase K and Lysozyme

Using modification method by He (2011), the 12 hours bacteria cultures (grown in Luria Broth medium) were measured the optical density at 600 nm (OD_{600}) to determine the active phase and 200 ml of bacteria culture at active phase was transferred to an one two ml centrifuge tubes and centrifuged at 10,000 rpm for 10 minute to pellet the cells. The supernatant was discarded. The cell pellet was re-suspended in 560 µl TE buffer and 600 µl lysis buffer (CTAB buffer), 50 µl of 20 mg/ml Proteinase K and 10 µl of lysozyme. The mixtures which contained buffers,

Proteinase K and lysozyme in tube were inverted more than 30 times to completely re-suspended bacteria cell pellet. After that, the tubes containing buffer plus pellet were incubated 30 minutes at 65 ^{o}C in water bath and the tubes were shake every 10 minute. About 600 µl of chloroform was added to the tubes and mixed well by inverting the tube 10 times until the phases are completely mixed and resting in ice for five minute before centrifuge at 14, 000 rpm for 15 minutes.

The 100 µl of upper aqueous (supernatant) layer was carefully transferred to a new sterile centrifuge tube by using micropipette. The 100 µl of cold ethanol (stored ethanol at -20 ^{o}C freezer) was added into the supernatant and was mixed gently to precipitate the DNA. The tube was centrifuged at maximum speed for 15 min. The supernatant was discarded and the DNA pellet was rinsed with one ml of 70 % ethanol (stored at room temperature). The mixture was centrifuged at 14, 000 rpm for two minutes and the supernatant was carefully discarded and the DNA pellet was air-dried for 10-15 minutes. The DNA was re-suspended in 50 µl TE buffer (He, 2011) and stored in -20 ^{o}C till next step.

The extracted DNA samples was electrophoretically analysed on 1 % molecular grade agarose gel (Paulse *et al.*, 2012).

3.5.5 Agarose Gel Electrophoresis of DNA Isolated from River Water Microbe

The extracted DNA samples were electrophoretically analysed on 1 % molecular grade agarose gel (Paulse *et al.,* 2012). Using modification method by Lewis (2015) and Paulse *et al.* (2012), 0.8 gram of agarose powder was weighed into 250 mL conical flask. Then, 79.22 mL of 1×TAE buffer was added into conical flask and swirled to mix. The agarose mixture was placed in microwave for about one

minute to dissolve agarose powder. The mixtures was left at 25 ^{o}C (room temperature) for five minutes to cool down about 60 ^{o}C. After the agarose mixture cool a little, three µl of ethidium bromide (10 mg/mL) as added and swirled to mix. The agarose gel was poured slowly into the tank and the comb was inserted into the gel. The agarose gel electrophoresis set up in tank was left to set for around 30 minutes. The 1×TAE running buffer was poured into the gel tank to submerge the gel from two to five mm depth. For sample preparation, each five µl DNA sample was mixed with one µl of loading dye. The first well was loaded with six µl marker (1 kb DNA ladder). The DNA samples with loading dye were loaded in the subsequent lane. The power source was set at 80 Volt for 30 minute. The gel was placed on the transilluminator and was viewed under the UV light.

3.6 Determination of Extracted DNA Purity and Concentration using Nanodrop Optical Density

Following method Yeates *et al.* (1998), to evaluate the purity of the extracted DNA, the absorbance ratio was determined by using nanodrop spectrophotometer (DeNovix, USA) at 260 nm (A_{260}) for DNA and 280 nm (A_{280}) for protein. For pure DNA, the value is 1.8, while values above 1.8 considered as RNA contamination and below 1.8 was considered as protein contaminated DNA preparation (McLennan *et al.*, 2013).

3.7 Amplification of 16S DNA Region from Isolated DNA Microbe

Using modificated method published by Appalasamy *et al.* (2012), the polymerase chain reaction amplification was performed by using the universal

primers 16S gene. The sequences of the primer are, for forward: 5'–CGC TGG CGG CGC GTC TTA AA-3' and for reverse: 5'-TTC ACC GCT ACA CCT GGA A-3'. The preparation of DNA amplification contained two µl of DNA, 2.5 µl 5 x buffer, two µl of 25 mM $MgCl_2$ solution, one µl of 10 mM dNTPs, 0.5 µl of 100 ng forward primers, 0.5 µl of 100 ng reverse primers and 0.5 µl of 5 u/µl polymerase GoTaq (Promega, US) and 16 µl double distilled water. The amplification was carried out using Eppendorf AG Thermal Cycler. The amplified DNA was electrophoresed using 1 % molecular grade agarose gel and run 80 V for 45 minutes. The agarose gel was stained with 1 % ethidium bromide and was de-stained in sterile distilled water. The gel was viewed and documented by using Gel XR System (Bio-Rad). Table 3.1 show the PCR cycle profile.

Table 3.1: Thermal cycling condition for GoTaq® DNA polymerase-mediated PCR amplification (Promega, 2014)

Step	Temperature	Time	Number of Cycle
Initial Denaturetion	95 ^{o}C	2 minutes	1 cycle
Denaturation	95 ^{o}C	1 minute	
Annealing	55 ^{o}C	1 minute	30 cycles
Extension	72 ^{o}C	1 minute	
Final Extension	72 ^{o}C	5 minutes	1 cycle
Soak	4 ^{o}C	Indefinite	1 cycle

After the PCR with extracted microbe DNA, the process was continued with AGE method as stated in section 3.5.5.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Determination of Physical Parameter of Water Quality Measurement in Sungai Kelantan

Figure 4.1, show the average data of water quality in river water from three points at different level. Water samples were collected in the early morning around 8.30 a. m to 9.30 a. m, at different level and at three points.

The temperature for every layer was average at 28.4 ^{o}C and pH reading for surface to bottom showed average between 6.65 and 6.87. The pH of freshwater changes when layering (stratification) occurs. The pH near the surface was usually higher at range 7.5 – 8.5 and pH at bottom layer was at range 6.5-7.5 (Fondriest Environmental, 2016). According to Lenntech (2013), most of the freshwater rivers usually have a pH of 6-8 depending on the surrounding soil and bedrock. The high temperature reduced the oxygen level in water and the pH become lower due to carbon dioxide influence. Therefore, the solubility of oxygen reduced as the temperature increased.

The salinity reading that measured on site showed the average was 0.0 ppt and this happened because of the sampling site was located at the upper stream of river and there was no infiltration with seawater. The bicarbonate ratios usually higher in the freshwater while sodium and chloride concentration greater in seawater (Paytan, 2006).

The dissolved oxygen (DO) showed the average reading at the surface was 5.98 mg/l, at the middle layer was 5.70 mg/l and at the bottom was 1.22 mg/l. DO at the bottom have the lower reading because of due to the biological activity in the water and low water flow. Microorganism required dissolved oxygen to decompose the organic material at the bottom of river. The DO level at the surface was much higher than the others layer because of human activity like boating that can aerate the water and increased the DO concentration.

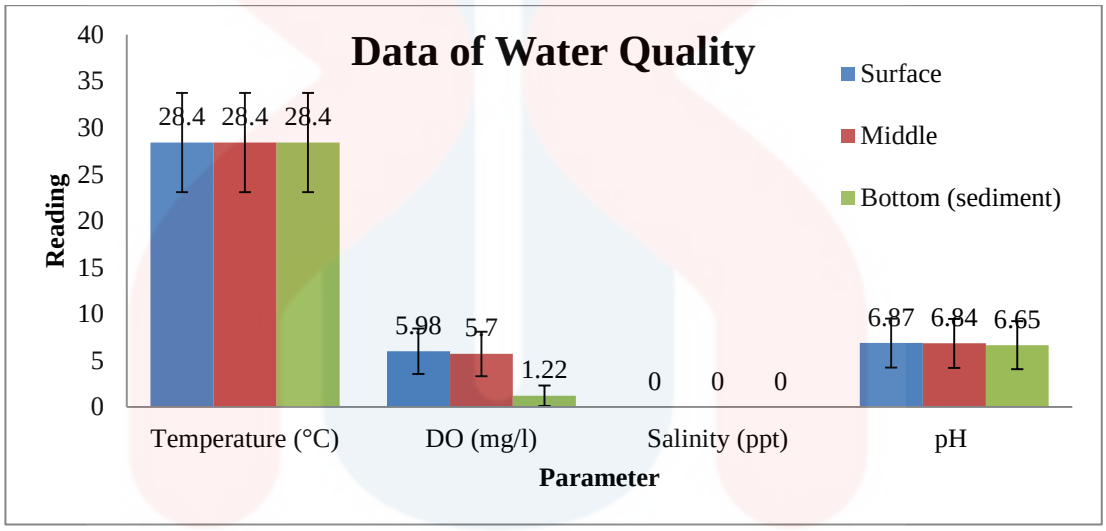

Figure 4.1: The average data of physical parameter of water quality in river water from three points at surface, middle and bottom layer of Kelantan River in Kuala Krai. Error bars indicate standard deviation of average data.

4.2 Determination of Bacteria Morphology View and Gram Stain by Using the Staining Method

According to Balachandar & Vendan (2007), taxonomy refer to the science of classification, which includes identification to identify the individual, nomenclature to naming the individual and systematic arrangement of any group of organism to an arrange the individual by group. Morphological characters was one of conventional method to classify bacteria based on the cell shape, size and structure, cell arrangement, occurrence of species structures, developmental forms, staining

reaction, mortality and flagella arrangement. Figure 4.4 show the results of morphology view and Gram stain of water microbe samples from Sg Kelantan (Tangga Krai, Kuala Krai). Figure 4.2 show the measured data of bacteria that present at every layer of the Sg. Kelantan River. At the surface of river, the number of Gram negative and Gram positive bacteria were the same amount, while at the middle layer, only Gram positive was detected. At the bottom layer of river, Gram negative bacteria were the most abundant bacteria in river water. Figure 4.3 show the diversity of bacteria that present in the river water at different depth of the river. There were two shapes of bacteria, *Bacillus* and *Coccobacillus* bacteria, and the *Bacillus* bacteria was the most abundant than the *Coccobacillus*.

In the middle layer of river, there was no growth of Gram negative bacteria. This was because of some bacteria cannot be cultured in the laboratory condition. According to Wade (2002), not all bacteria in sample can be growth in in-vitro culture condition and some of the bacteria depend on each other to growth. For example *Bacteroides forsythus,* it is Gram negative bacteria implicated in periodontitis that growth well in co-culture with other organism. The other reason some bacteria cannot be culture because the absence of required nutrient in culture medium and this medium itself can be toxic to the bacteria. Besides that, the target bacteria inhibit by the other bacteria in the sample (Wade, 2002).

The distribution of bacteria in the river may influence by the depth of the river. At the surface of river water, it consist of highest oxygen concentration that suitable for the obligate aerobes bacteria. According to Prescott *et al.* (1996), oxygen is needed by the obligate aerobes bacteria to grow. This bacteria use oxygen to generate energy by oxidized substrate. While at the bottom layer of river, the oxygen concentration is at the lowest rate and this condition is suitable for the obligate

anaerobes bacteria. According to Brooks *et al.* (2007), oxygen is poison to obligate anaerobes bacteria. This because obligate anaerobes bacteria is sensitive and have low tolerance to oxygen. In the middle layer of river mostly the facultative anaerobe bacteria. According to Hogg (2005), with or without oxygen, facultative anaerobe bacteria still can grow because they can produce energy by aerobic respiration with the present of oxygen and also able to undergo the fermentation or anaerobic respiration when absence of oxygen. This facultative anaerobe bacteria is usually gather at the surface or middle of river because they can generate more energy by performing the aerobic respiration rather than fermentation or anaerobic respiration.

According to Xiang *et al*. (2005), at different depth within the glacier, there were clear difference in predominant aerobe bacteria isolated in ice core, indicate that different type of bacteria deposit on ice surface under different condition. With this explanation, the distribution of bacteria from layered ice core reflects the distribution of bacteria in river water.

According to Bridle (2014) and Zummy *et al.* (2015), most of bacteria that found in the freshwater are the Gram negative and rod shape bacteria. Due to limitation in method of cultural bacteria, only a few of bacteria can grow in the NA and the other cannot be cultured because of unfavourable condition for bacteria. Figure 4.3 show the presence of the Gram negative of coccobacillus bacteria in water indicated the contamination of water. The infected animal contaminated the water with bacteria through the contact (Center of Disease Control and Prevention, 2015).

The Gram negative bacteria on the surface of water show the presence of the coliform bacteria. The coliform bacteria include fecal coliform bacteria and non-fecal coliform. Fecal coliform bacteria have two types, pathogen and non-pathogen

species. The contamination of river water indicated by the presence of fecal coliform bacteria in water that transmitted by the human or animal fecal materials (New Hampshire Department of Environment Services, 2011).

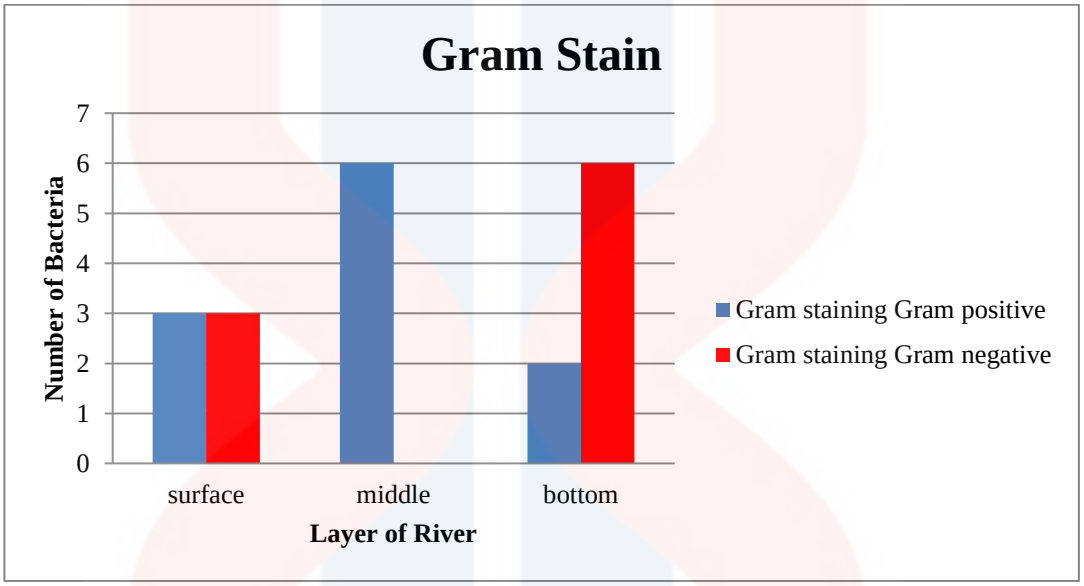

Figure 4.2: Numbers of bacteria at every layer based on the Gram staining. On the surface of river, sample for Gram positive and Gram negative bacteria have same amount. In the middle of river, present only Gram positive bacteria. At the bottom, Gram negative bacteria more abundant than Gram positive bacteria.

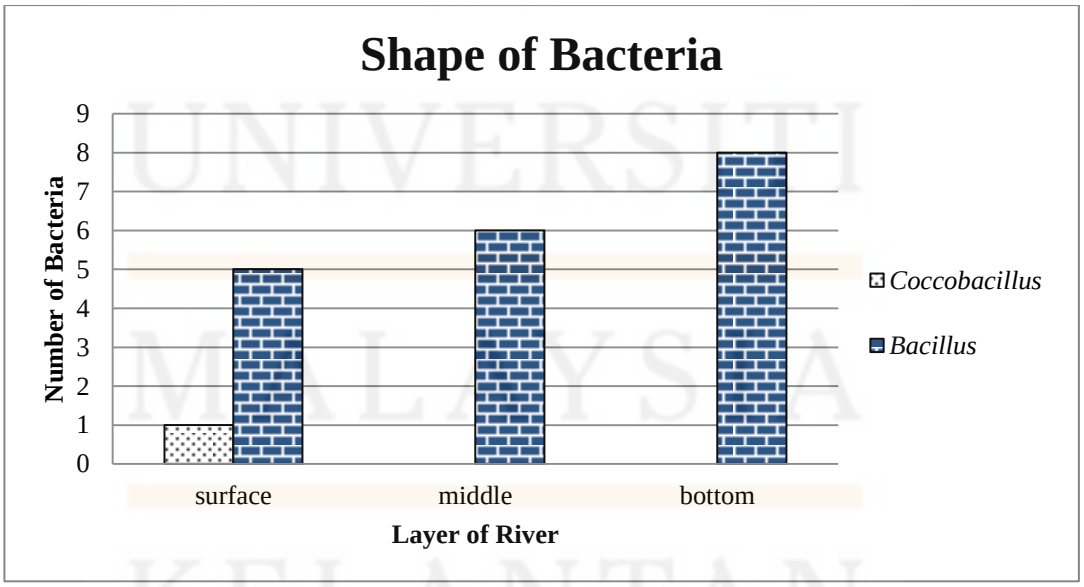

Figure 4.3: Diversity of bacteria at different layer of Sungai Kelantan at Kuala Krai. Every layer composed of *Bacillus* bacteria while *Coccobacillus* only found at the surface of river.

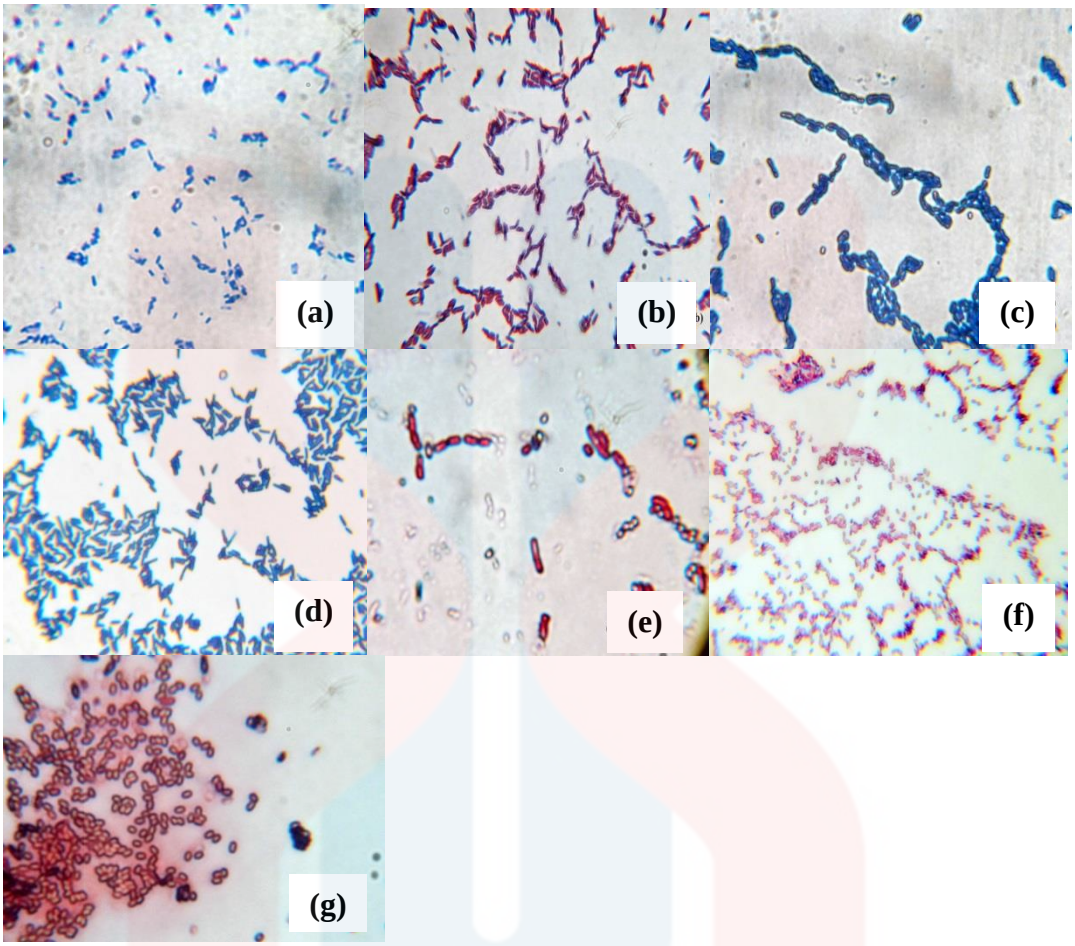

Figure 4.4: The morphology view and Gram stain of water microbe samples from: Sungai Kelantan (Tangga Krai, Kuala Krai). **(a)** Gram positive *bacillus* arranged in single cell (100×10 magnification) sample from bottom layer. **(b)** Gram negative *bacillus* arranged in side by side (palisades) (100×10 magnification) sample from bottom layer. **(c)** Gram positive *bacillus* arranged in chains (100×10 magnification) sample from surface layer. **(d)** Gram positive *bacillus* arranged in side by side (palisades) (100×10 magnification) sample from surface layer. **(e)** Gram negative *bacillus* arranged in chains (100×10 magnification) sample from bottom layer. **(f)** Gram negative *bacillus* arranged in single (100×10 magnification) sample bottom layer. **(g)** Gram negative *coccobacillus* arranged in pairs (100×10 magnification) sample from surface layer.

4.3 DNA Extraction of Water Microbe Isolated from River Water of Sungai Kelantan

DNA extraction was the removal of deoxyribonucleic acid (DNA) from the cells. In diagnostic process, extraction of DNA was the early step to detect the bacteria in the environment (Rice, 2016).

By using the optimization of DNA extraction with standard cetyltrimethylammounium bromide (CTAB) method plus Proteinase K and

lysozyme, four steps were used to remove and purify the DNA from the rest of the cell. The first step was the cell containing the DNA interest was lysis. To lyse the cell, lysis buffer like lysozyme, CTAB and proteinase K were added to the samples. Lysozyme was functioning to damage the bacteria cell walls by attacking the peptidoglycan, proteinase K used for destruction of protein in cell cultured cell for released the DNA. CTAB buffer was the main component of buffer for the extraction of DNA. It functions to remove the membrane lipids and promote cell lysis (Clarke, 2009).

According to Rice (2016), DNA associated protein, as well as other cellular protein may be degraded with the addition of protease K. To avoid the degradation from happened, TE buffer was added to protect the DNA from degraded. When the sample was centrifuged with addition of chloroform, the proteins were remains in the organic phase and the DNA was found at the interface between two phases.

For the precipitate steps, DNA was precipitated by mixing with cold ethanol and centrifuging. The cold ethanol serve as wash to remove the salt previous added, and DNA was insoluble in the alcohol and was come out of solution (van Oss, 1989). The DNA was wash again with 70% of ethanol and centrifuge for retrieval of cell pellet. After pouring the ethanol off the pellet and drying, DNA was re-suspended in a TE buffer. The presence of DNA was confirmed by electrophoresis on an agarose gel that consisted of EtBr that react with the DNA, and observe under the UV light (Rice, 2016).

By using the DNA extraction methods that stated in section 3.5.1 (DNA extraction with standard CTAB method by He (2011)), section 3.5.2 (DNA extraction with standard CTAB method by He (2011), plus proteinase K) and section

3.5.3 (optimization of DNA extraction with standard CTAB method by He (2011), without proteinase K), there were no band formed on the gel that represents the DNA of microbe. According to Fatima *et al.* (2014), various parameters such as incomplete cell lysis and DNA degradation may influence the effectiveness of DNA extraction procedure. This was because the low yield of DNA would result in less diversified pool of templates. This related to the DNA extraction protocols because it was necessary to choose the method that suitable to the characteristic of the sample and the intended follow up applications (Hwang *et al.*, 2012). According to Hwang *et al.* (2012), the resistance to cell lysis treatments may influence by the difference of cell wall component of bacteria. The Gram positive bacteria have thicker cell wall compared to the Gram negative bacteria. Therefore, to improve the cell lysis, lysozyme was added into the treatment to damage the bacteria cell wall and proteinase K to digest the protein and eliminate the contamination. Besides that, the hot detergent like SDS and chemical lysis can be added as the additional treatment (Hwang *et al.*, 2012).

Figure 4.5, 4.6 and 4.7 represent results from the agarose electrophoresis of water microbe DNA extraction by using method as stated in section 3.5.4. After the separation, the clearly define band were visible as the results of DNA fragment. To determine the sizes of the sample bands, the DNA ladder (marker) should be separated to a degree. According to Lee *et al.* (2012), agarose gel electrophoresis is the most effective method of separating the DNA fragments of different sizes ranging from 100 bp to 25 kb. The agarose polymers associate non-covalently and form a network of bundle and function as gel molecular sieving property of pore sizes. During the agarose gel electrophoresis, DNA was loaded into the well in the gel and a current applied. The phosphate backbone of the DNA is negatively

molecule charged, therefore the DNA fragment moved to the positively charged anode when placed in the electric field (Lee *et al*., 2012). The rate of movement of DNA molecule through the gel was determined by the size of DNA molecule, agarose concentration, DNA conformations, voltage applied, the present of ethidium bromide (EtBr), type of agarose, and electrophoresis buffer. The DNA molecules were separated by size and form the pattern that the distance traveled proportionally to the molecular weight.

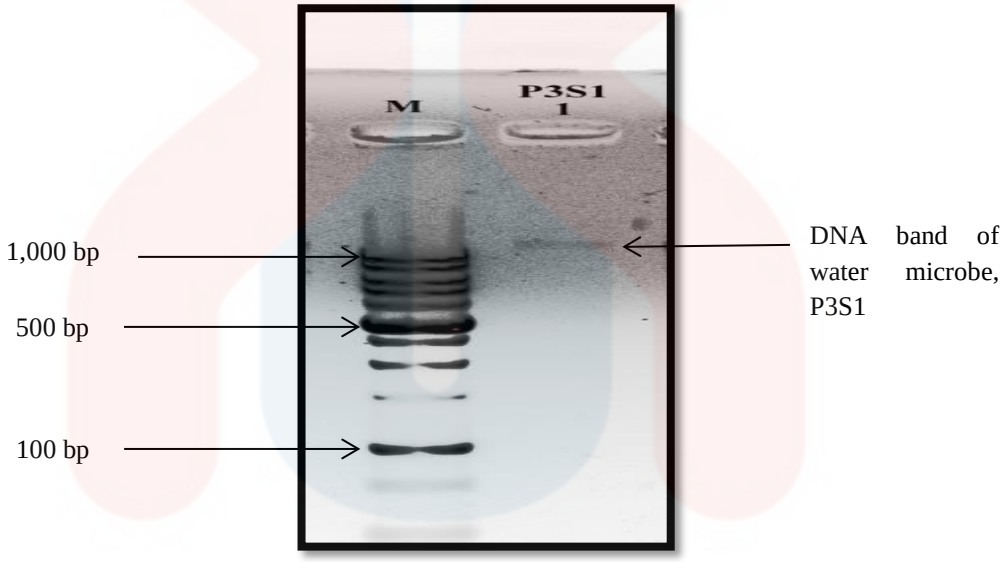

Figure 4.5: EtBr was stained DNA sample of water microbe on 1 % denaturing agarose with 1 kb DNA ladder as a marker. Fragment of extracted DNA of water microbe (1: Sample P3S1 and M: 1 kb DNA ladder). Electrophoresis show single band on top of gel.

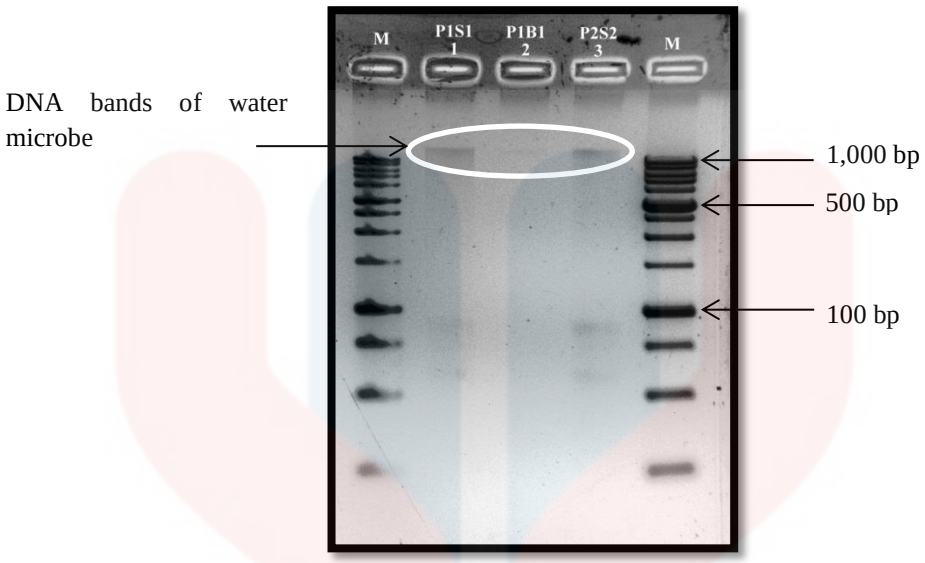

Figure 4.6: EtBr was stained DNA sample of water microbe on 1 % denaturing agarose with 1 kb DNA ladder as a marker. Fragment of extracted DNA of water microbe (M: 1 kb DNA ladder, 1: Sample P1S1, 2: Sample P1B1 and 3: Sample P2S2).

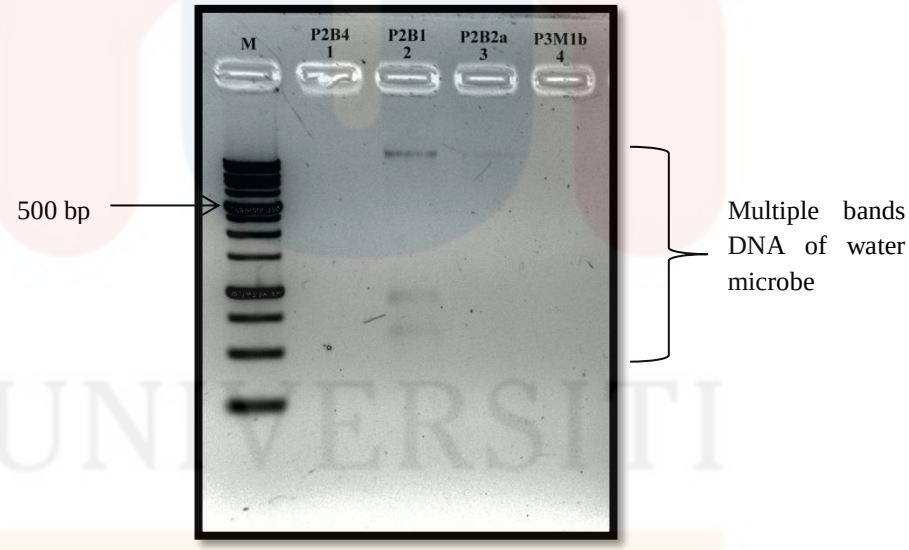

Figure 4.7: EtBr was stained DNA sample of water microbe on 1 % denaturing agarose with 1 kb DNA ladder as a marker. Fragment of extracted DNA of water microbe (M: 1 kb DNA ladder, 1: Sample P2B4, 2: Sample P2B1, 3: Sample P2B2a and 4: P3M1b). Well no. 2 show several band on gel, well no. 3 show slightly single band while well no.1 and no. 4 there were no band.

The DNA concentration of the water microbe sample was measured by examining the absorbance of the sample at 260 nm and the amount of DNA was calculated with 1.0 A_{260} unit equal to 50 µg/mL. The absorbance at 260 and 280 nm

was taken for determine the purity of the extracted DNA. The $A_{260/280}$ ratio as 1.8 was for a pure sample of DNA, where the $A_{260/280}$ ratio lower than 1.8 show the DNA preparation was contaminated with protein (Sambrook & Russell, 2001). The sample P1B1 and P3M1b not yielded an amount extracted of DNA that was significantly lower than the other sample (Table 4.1). These two samples of DNA extraction has lower $A_{260/280}$ ratio, thereby indicate that the extracted DNA was contaminated with protein. Table 4.1 shows the purity ratios of water microbe DNA samples. The purity ratios with concentration less than the sample type specific limit specified 20 ng/µL were not reliable indicator of purity due to the relative flatness of the spectrum (DeNovix, 2014).

Table 4.1: The concentration of extracted water microbe DNA by using the nanodrop spectrophotometer

DNA extraction sample	Concentration of DNA (ng/µL)	$A_{260/280}$
P1S1	6.939	2.20
P1B1	0.620	1.04
P2S2	7.010	1.83
P2B1	7.266	1.98
P2B2a	1.682	2.07
P2B4	-0.357	19.37
P3S1	5.318	2.21
P3M1b	0.175	0.68

4.4 Polymerase Chain Reaction (PCR) of Microbial DNA for 16S Gene Amplification

The 16S rRNA gene amplification was carried out using modified method in Appalasamy *et al.* (2012). In brief, GoTaq Flexi DNA Polymerase (Promega, US) was used for polymerase chain reaction (PCR) amplification and the thermal cycle conditions applied include the process of initial denaturation, denaturation, annealing, extension and final extension. The initial denaturation of DNA polymerase activation step first cycle at 95 ^{o}C for two minutes is to ensure the target DNA was completely denatured. The steps followed by 30 cycles of denaturation at 95 ^{o}C for one minute, annealing at 55 ^{o}C for one minute, and an extension at 72 ^{o}C for one minute. On completion of 30 cycles, the final extension at 72 ^{o}C for five minutes was applied (Promega, 2014). The PCR product was separated by the agarose gel electrophoresis and was visualize with ethidium bromide (EtBr). Figure 4.8 showed the AGE result of PCR product.

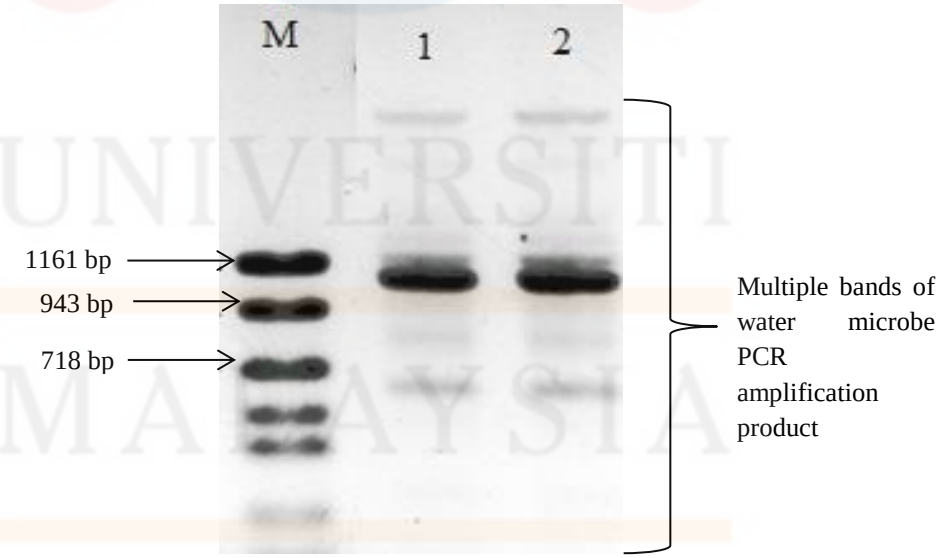

Figure 4.8: Visualization of PCR amplification products of water microbe DNA isolated by method using the 16S rRNA. The bands show the amplification of multiple band in PCR product of DNA microbe, sample P3S1 on the 1 % of agarose gel. (M: PCR marker, no 1 and 2: sample P3S1)

When the electrophoresis machine was loaded with gel, it created a charge different between the top of gel and the bottom of gel. The PCR product that loaded into the wells at the top of gel was migrated from top toward the bottom of the gel due to charge applied. The gel acts as sieve to trap DNA particles as they pass through. The band that close to the top of gel show the large particles get trapped and the smaller particle moved down through the gel. The ladder contain of specific size that separated out from largest to smallest in predictable pattern of bands (Lee *et al*., 2012).

This PCR product was unable to undergo the purification and sequencing of DNA that used to construct the library. In the Figure 4.8, multiple bands were detected under UV-transilluminator. According to National Diagnostics (2011), multiple bands from PCR product indicate multiple priming site for the primers within the target DNA and by rising the hybridization temperature, the multiple bands can be eliminated. In theory, 16S RNA amplification using microbe DNA should result in single band. However, the result of PCR obtain in this does not follow the above statement. This could because of the non-specific amplification product and the primer was complimentary to each other, especially near the 3'-end of primer. Besides that, there was contamination by another RNA or DNA during the preparation of PCR amplification (Sambrook & Russell, 2001).

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

By using the morphology identification method, this study showed the presence of a diverse of bacteria community and the distribution of various species in the water sample from Sg. Kelantan at Kuala Krai. The isolated bacteria were affiliated with shapes of *Bacillus* and *Coccobacillus*. At every layer of river (surface, middle and bottom), there were different types of bacteria that niche on it. The agarose gel electrophoresis had shown the present of DNA bacteria with the appearance of band on the gel. However, more data of molecular bacteria are needed to be able to confirm and identify the diversity of microbe that present in Sg. Kelantan at Kuala Krai.

5.2 Recommendations

For the recommendation, the study need to be proceed with the purification, sequencing and compared the sequence in database to identify the microbe based on the homology in database at National Center for Biotechnology Information (NCBI). Due to limitation of bacteria growth, metagenome method can be used to identify all the bacteria in Sungai Kelantan river water and it can show fast result without undergo the cultivation of bacteria in laboratory.

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