DETERMINATION OF WATER MICROORGANISMS DIVERSITY IN SUNGAI KELANTAN, KOTA BHARU

by

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DECLARATION

I declare that this thesis entitled "Determination of Water Microorganism Diversity in Sungai Kelantan, Kota Bharu" 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 : _____

Name : _____

Date : _____

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DETERMINATION OF WATER MICROORGANISM DIVERSITY IN SUNGAI KELANTAN, KOTA BHARU

ABSTRACT

Sungai Kelantan is the biggest and longest river in Kelantan State. Objective of this research is to determine water microorganism diversity in Sungai Kelantan, Kota Bharu using river water sample. Diversity determined by variety of morphology and abundance of both grams. The water sample took at three points of different depths which were at the surface, in the middle and at bottom. The water quality of Sungai Kelantan, Kota Bharu was determined by using YSI-85 multiparameter. Type of gram and shape of microorganisms' morphology recorded to determine diversity of microorganism in Sungai Kelantan, Kota Bharu. DNA extraction protocol used TE buffer, CTAB buffer, Proteinase K, lysozyme, chloroform, cold ethanol and 70% ethanol. Products of DNA extraction then undergo agarose gel electrophoresis for 30 minutes at 80 volt to ensure presence of DNA. DNA then amplified by Polymerase Chain Reaction. The PCR results have multiple bands because of over amplification. It can be overcome by increasing annealing temperature. The diversity of water microorganism of Sungai Kelantan is diversed based on the abundance of shape, margin, elevation, appearance, optical density and pigmentation. Grams also showed diversity of water microorganism by variety of different shape microorganisms found in Sungai Kelantan, Kota Bharu.

PENENTUAN KEPELBAGAIAN MIKROOKGANISMA AIR DI SUNGAI KELANTAN, KOTA BHARU

ABSTRAK

Sungai Kelantan adalah sungai terbesar dan terpanjang di Negeri Kelantan. Objektif kajian ini adalah untuk menentukan kepelbagaian mikroorganisma air di Sungai Kelantan, Kota Bharu menggunakan sampel air sungai. Kepelbagaian ditentukan oleh morfologi dan jenis Gram. Sampel air diambil pada tiga titik kedalaman yang berbeza iaitu di permukaan, di tengah-tengah dan pada dasar sungai. Kualiti air Sungai Kelantan, Kota Bharu ditentukan menggunakan YSI-85 parameter-pelbagai. Jenis gram dan bentuk morfologi mikroorganisma direkodkan untuk menentukan kepelbagaian mikroorganisma di Sungai Kelantan, Kota Bharu. DNA pengekstrakan protokol yang digunakan TE, CTAB, Proteinase K, lysozyme, kloroform, etanol sejuk dan 70% etanol. Produk pengekstrakan DNA kemudian menjalani elektroforesis gel agarose selama 30 minit pada 80 volt untuk memastikan kehadiran DNA. DNA kemudian dilipatkaligandakan oleh PCR. Produk dari PCR mempunyai banyak band kerana terlebih dilipatgandakan. Cara mengatasinya dengan menaikkan suhu pemanasan yang pertama. Kepelbagaian mikroorganisma air Sungai Kelantan ditentukan berdasarkan variasi bentuk, margin, ketinggian, rupa, ketumpatan optik dan pigmentasi. Gram menunjukkan kepelbagaian mikroorganisma air dalam bentuk yang berbeza yang ditemui di Sungai Kelantan, Kota Bharu.

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µm	micrometre
µmol	micromole
A260	Absorbance of 260
A280	Absorbance of 280
Abs	Absorbance
AGE	Agorose Gel Electrophoresis
ATGC	Adenine, Thymine, Guanine and Cytosine
cm	centimeter
CTAB	cetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside 5'-triphosphate
E.coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
etc	et cetera
kb	kilobase pair in duplex nucleic acid, kilobase in single-stranded nucleic acid

km	kilometer
LB	Lysogeny Broth/ Luria Broth/ Luria Bertani
L	litre
M	molar
m	metre
mg	milligram
$Mg2^{+}$	magnesium
$MgCl_2$	Magnesium Chloride
min	minutes
mL	milliliter
Mm	millimolar
mm	millimetre
mmol	millimolar
NA	nutrient agar
NaCl	Sodium Chloride
NaI	Sodium Iodide
ng	nanogram
nm	nanometer
OD	optical density
Organisms/ ml	organisms per mililitre

PCR	Polymerase Chain Reaction
pH	potential hydrogen (capacity of hydrogen)
RNA	ribonucleic acid
rpm	revolution per minute
rRna	ribosomal ribonucleic acid
sp	species
spp	referring to all species in that given genus
TAE	Tris-acetate- ethylenediamine tetra-acetic acid
TE	Tris- ethylenediamine tetra-acetic acid
Tris	Tris (hydroxymethyl) aminomethane
TSS	Total Suspended Solid
UV	ultraviolet
V	voltage

LIST OF SYMBOLS

°C	Degree Celsius
°F	Degree Fahrenheit
%	Percentage
>	More than
=	Equal to

CHAPTER 1

INTRODUCTION

1.1 Background of study

Sungai Kelantan is the biggest and longest river in Kelantan State. According to Ibbit *et al.* (2002) the overall length of Sungai Kelantan is 150 km while its width is 140 km. Towns such as Kuala Krai, Kota Bharu, Pasir Mas and Tanah Merah are along Kelantan River based on research by Ibbit *et al.* (2002). The other three rivers in North Kelantan (Pengkalan Datu, Pengkalan Chepa and Kemasin River) are small rivers that are less than 10 km long. These rivers disgorge within the area of Kelantan Delta Plain. Besides these rivers, a lot of small artificial drainage system can be found in the whole Kelantan Delta. It is used for agricultural purpose (Ibbit *et al.*, 2002).

Malaysian Drainage and Irrigation Department (2000) has classified flood in Malaysia into flash flood and monsoon floods. The difference between these two types of flood is the rate of time taken by the flood to become normal. Monsoon flood usually last until a month or even more than a month while flash floods only required several hours to become normal according to Noorazuan (2006). Flood that has occurred at Sungai Kelantan is monsoon flood. Northeast monsoon that occurs from November to March brought heavy rainfall that causes flood (Khan e*t al.*, 2014). It occurred every year thus it is categorized as an annual flood (Khan *et al.*, 2014). On December 2014 and early January 2015 monsoon flood has occurred at Kelantan involving almost all districts in Kelantan especially Kota Bharu and Manek Urai.

The Sungai Kelantan flows with fresh water. According to Sigee (2004) freshwater environments showed variety especially by physical and chemical features that lead to different microbial existence. One of the most crucial physical of freshwater is lentic and lotic systems. Lentic system is standing waters such as ponds, lakes, marshes and other enclosed system while lotic system is flowing water such as rivers, estuaries, and canal. Sungai Kelantan is lotic system as the rivers flow along Kelantan state.

Microorganisms may be defined as those organisms that are not visible to the bare eye and requiring a microscope for detailed observation. These biotas have a size range (maximum linear dimension) up to 200 mm and vary from viruses, through bacteria and archaea, to microalgae, fungi, and protozoa (Sigee, 2004). According to Theresa (1999) the most common shapes of microorganisms are rod-like, called the bacillus or spherical called cocci. Bacteria also form spirals and corkscrews, cocoa, commas, and branch structure. They also may form multi-cell form by joined together such two joined cocco called diplococci while a chain of cocci called streptococci.

The most common microorganisms that will be found in freshwater are amoeba, euglena, pseudomonas and ciliates. Amoeba can be pathogenic and non-pathogenic microorganisms, that it does not harmful to other living organisms especially human. Non- pathogenic microorganisms usually found everywhere in the environment however some of the species may mutant to become pathogenic. Euglena and ciliates is mobility microorganism and moves by different means. Pseudomonas is pathogenic microorganism and harmful to other living things.

1.2 Problem statement

Past few years floods occur at Kelantan state and the water level increases about ground level and microorganisms from soil, agriculture activity, waste from crops, waste from sewage and drainage may be carried away by water flow into Sungai Kelantan There are limited studies on flood occurred in Kelantan and there is no previous research about microorganisms in Sungai Kelantan. Thus, this is the first study on microorganisms profiling via molecular based identification that will be conducted using water sample from Sungai Kelantan. This research will help to find the diversity of microorganisms at Sungai Kelantan, Kota Bharu.

1.3 Objective

1. To determine water microorganism diversity in Sungai Kelantan, Kota Bharu using river water sample.

CHAPTER 2

LITERATURE REVIEW

2.1 Description of Sungai Kelantan and Its Uses

The Sungai Kelantan has been used by people for many purposes such as household uses, irrigation of plantation and agricultural activity, fishing in small scale and sand dredging. The Sungai Kelantan water has been turbid since the early 1990s because of the high amount of suspended solids and siltation that caused by activities done by Kelantan state people. There is even logging activities in the upstream areas of Lojing Highlands (DOE 2009a; Ambak & Zakaria, 2010) and sand mining activities that lead more pollution flows into Kelantan River. There are approximately 128 sand mining operations along the Kelantan River from Kuala Krai to Tumpat (Ambak *et al.,* 2010). The volume of sand mining activity along the Kelantan River increases each year because of the high demand of sand for industry and construction and high demand of urbanization in Kelantan state especially around Kota Bharu town.

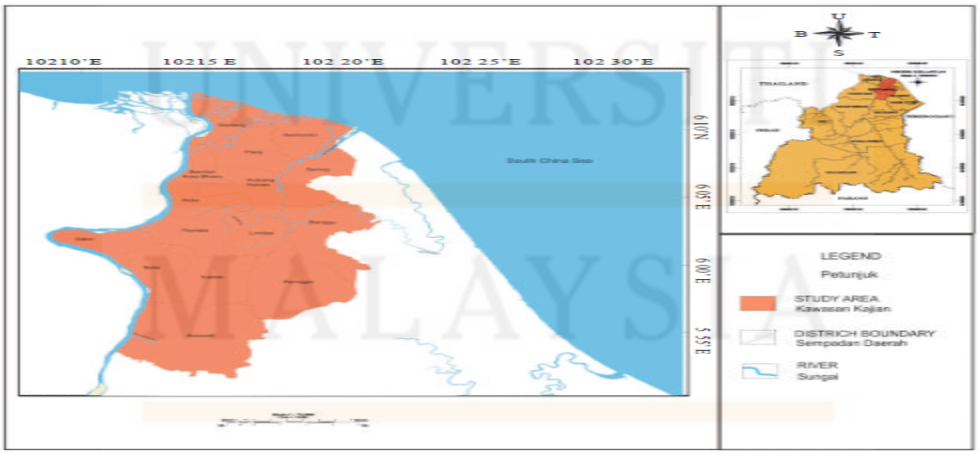

Figure 2.1: Location map of the flooded area in Kelantan state (DSM, 2005)

According Khan *et al*. (2014), Kota Bharu is located in Kelantan state at the east coast of Peninsular Malaysia with the latitude of 06°10'N and the longitude of 102°20'E. The study area, Kota Bharu is one of the main districts in Kelantan and become the capital city of Kelantan which was the main location of commercial center and also government and private office. The total land area of Kota Bharu is about 394 km^2. It consist of fourteen major districts which was Badang, Kemumin, Panji, Bandar Kota Bharu, Sering, Kota, Kubang Kerian, Banggu, Pendek, Kubang Kerian, Banggu, Pendek, Limbat, Peringat, Beta, Kadok and Ketereh (Figure 2.1).

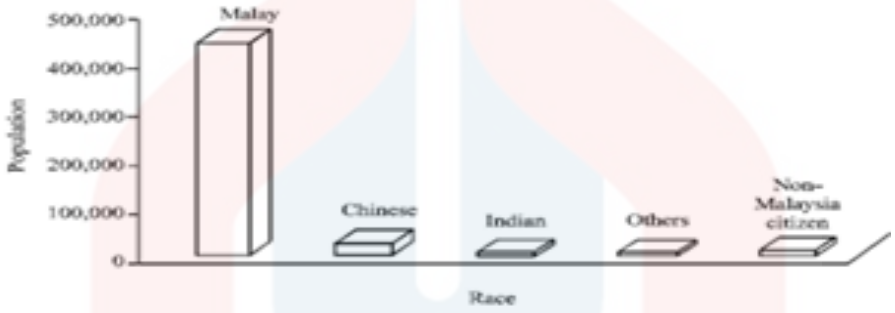

Figure 2.2: Population in Kota Bharu based on demographic distribution (DSM, 2010)

The rapid development of Kota Bharu raises the city as it becomes the main focus of people all over the world. Kota Bharu is categorized as the highest population district in Kelantan because of its role as the main city of Kelantan. It is recorded that in the year of 2010, the total population in Kota Bharu reaches 468,438 people from different race such as Malay as the majority, Chinese, Indian and other non-citizen and the number keep increasing year by year (Figure 2.2). The major land use in the study area is mainly agriculture and paddy is the main crops grown here other than mixed horticulture, rubber and orchards (Khan *et al.*, 2014)

The main river channel in Kota Bharu is Kelantan River which occupies approximately 12,700km^2 or 85% of the state. The platform of Kelantan river which is the major river is floodplain area (Khan *et al.*, 2014).

2.2 Annual Flood

Flood in Kota Bharu is mainly caused by heavy rainfall brought by the Northeast monsoon because of the location of Kelantan. Kelantan state is situated at the North-East corner of Malaysia that facing the South China Sea. Thus, it is subjected to North East monsoon and flood has been occurring on a yearly basis typically during the month of November and December (Hoong, 2007). Several years before Sungai Kelantan overflow and sunk almost half of Kota Bharu town especially low ground area.

Main cause of flood occurred in Kota Bharu is heavy rainfall brought by the monsoon. Kota Bharu flooded is annual occasion as it happens every year during the monsoon season. The Northeast monsoon occurs from November to March every year (Figure 2.3) causing heavy rain to fall at Peninsular Malaysia especially Kelantan, Terengganu and Pahang. Due to its geographical, Kota Bharu has become extremely vulnerable to monsoon flood every year. The unprecedented in November, 2005 which was triggered by monsoon, has been described as one of the worst natural flood in the history of Kota Bharu before the recent flood on 2014 (Khan *et al.*, 2014).

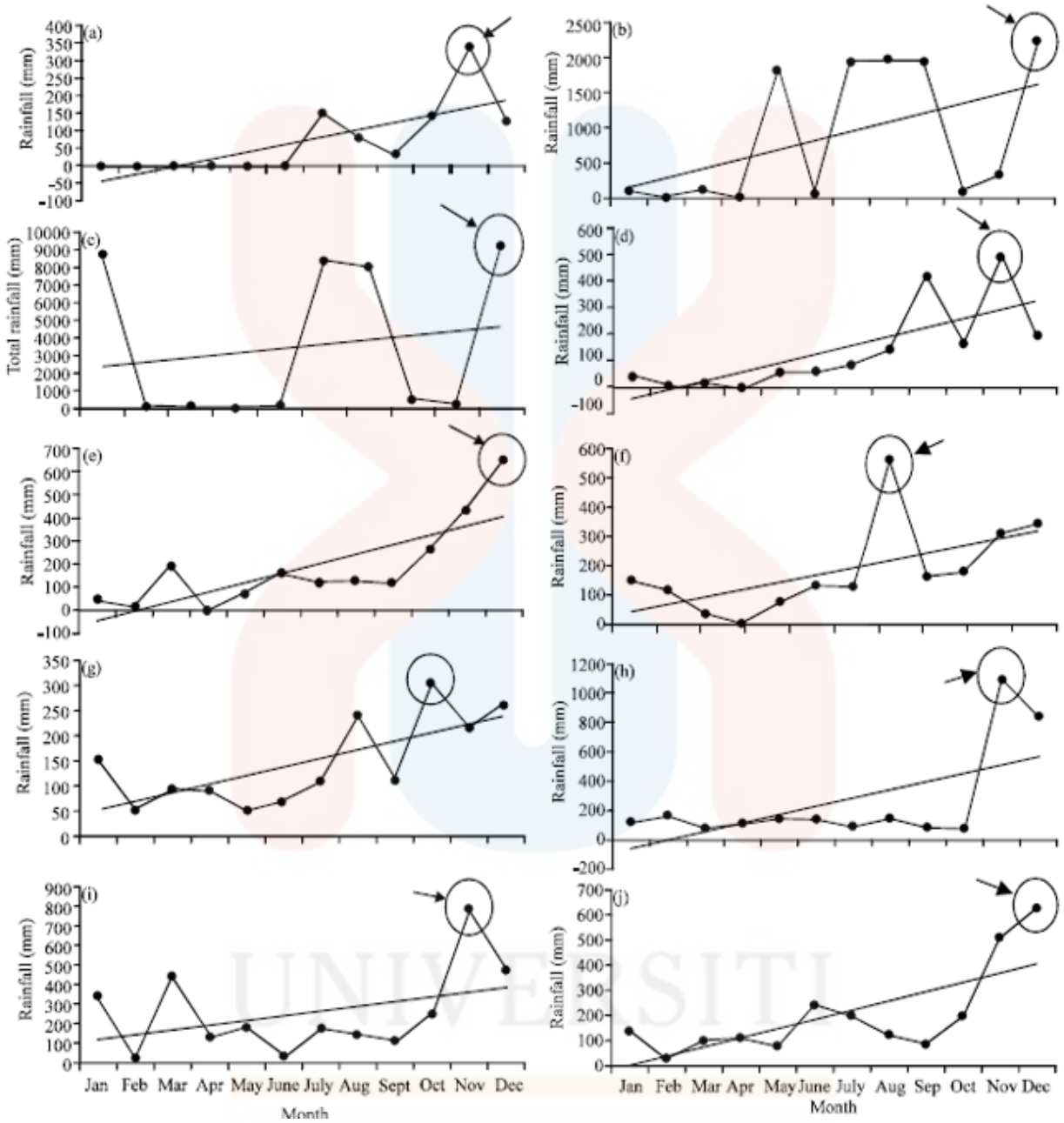

Figure 2.3 (a-j) Rainfall data from rainfall station in Jeti Kastam, Kota Bharu (DDIM, 2011), Rainfall distribution (a) 2001, (b) 2002, (c) 2003, (d) 2004, (e) 2005, (f) 2006, (g) 2007, (h) 2008, (i) 2009 and (j) 2010

In 1926, severe flood damages almost all state in Peninsular Malaysia including Kota Bharu, Kelantan. In a decade and more, in 1988 monsoon flood caused 55 lives loss. Hence, government improves in precaution and mitigation to overcome flood (Khan *et al.*, 2014). The latest disastrous flood occurred on November to December 2014.

2.2.1 Effect of flood to Kelantanese people and their lives

Mooney (2010) stated that flood cause depletion of food, fresh water, and shelter that flood lead to humanitarian crisis. For rural area, half of whom have been left homeless, and children are at risk of starvation. People were struggling to cope with lack of food and disease. Children under the age of 5 years are at risk of severe malnutrition.

Based on the latest government census on 2010, the sum of people living near Kelantan River Basin is approximately 2.5 million people in 1990. Between 1957 and 1990, these floodplain areas had rapid population growth of about 513.3 percent. This means Kelantanese are highly vulnerable to the risk from floods. Thus, the issue is related to the floods frequency often pose the risk of loss of life and destruction of property in any one year in Kelantan River Basin (Tuan & Hamidi, 2013).

Frequent flooding can cause millions of dollars in property damages and loss of lives. A natural disaster can be perceived as a problem or a phenomenon that is capable of causing damage to properties and loss of lives and its occurrence is normally sudden and without warning (Tuan & Hamidi, 2013).

2.3 Freshwater Microorganisms

Pelagic and benthic communities develop at lotic systems. Developments of pelagic and benthic communities depend on size and flow of river (Sigee, 2004). Bacteria that present on the surface, and in subsurface regions, of stream-bed sediments are involved in a number of key ecosystem processes – including the breakdown (mineralization) of organic matter, assimilation of inorganic nutrients, and acting as a food source for consumer organisms is called benthic bacteria (Sigee, 2004).

Recent studies by Findlay *et al.* (2003) on chemical composition at freshwater, showed the differences of labile (easily assimilable) and recalcitrant (poorly assimilable) carbon sources in promoting bacterial community responses such as oxygen consumption, productivity, extracellular enzyme activity, and community composition. Optical microscope can observe the smallest organism which is the bacteria. In present of larger quantities of waters rich in decomposing organic substances there will be a lot of bacteria found. Unicellular organism is made up of only one cell thus the bacteria is unicellular organisms (Carboni, 2006).

2.3.1 Different freshwater environments affects microorganism presence

Freshwater can be divided into several parts, one of it is springs. At spring the major group of organisms that will be found are photosynthetic bacteria and algal with communities ranging from 10^2 to 10^8 organisms/ml. The highest concentration of the presence of them are along the shallower edges of the spring and in association with rock surfaces, where light is available and inorganic nutrients are in highest concentrations. Although heterotrophs are also present, numbers are usually low (10^1 to 10^6 organisms/ml) because dissolved organic matter is low (Sandrin *et al*., 2006). According to Sandrin *et al. (*2006), at rivers, microorganism presences usually near their life source. Streams contain mostly primary producer communities, especially when light can penetrate to the bottom of the stream. The nature flows of the stream water column causing attachment of photosynthetic organisms with biofilms. The constant water movement of stream make phytoplankton cannot form spatially stable populations.

According to Findlay (2010), most of these patterns probably are driven by variation in strong environmental selectors. For example, soil and stream pH affect bacterial communities (Fierer *et al*., 2007), and stream water chemistry has large effects on both bacterial and fungal colonizers of leaf litter (Harrop *et al*., 2009). Examples from the tundra of North America (Crump *et al*., 2007) and high-elevation lakes (Reche *et al*., 2005) indicate that dispersal limitation can cause bacterio-plankton communities to differ among lakes. Perhaps the best summary of present state of knowledge is that some strong selectors (pH and organic matter attributes) clearly exist, but these selectors are not universal drivers of real-world patterns in microbial composition.

Dispersal limitation might be important, but when dispersal limitation is critical might not be a simple question of geographic distance (Reche *et al*., 2005). According to Sandrin *et al*. (2006) as the stream develops (progresses away from the source) and becomes larger, it tends to accumulate dissolved organic matter from surface runoff and sediments. The increase in dissolved organic matter limits the penetration of light and consequently begins to limit photoautotrophic populations. In general, the concentration of heterotrophs in streams and rivers ranges from 10^4 to 10^9 organisms/ml, with microbial numbers increasing as dissolved organic matter increases. Because of their flow patterns, stream and river waters are for the most part well aerated (Harrop *et al*., 2009). Therefore, heterotrophic populations are predominantly aerobic or facultatively aerobic.

Another example for freshwater will be lakes. According to Sandrin *et al*. (2006) lakes contain extensive primary and secondary producer populations that interact dynamically. The littoral zone (if there is one) has a high primary activity where the planktonic community is composed of algae (major) and cyanobacteria (minor). Microbial processes in freshwater are variable in spatial and temporal; in response to variance in nutrient presence (Dodds *et al*., 2000), temperature (Boyero *et al*., 2011), organic matter quality or quantity (Gessner and Chauvet, 1994), hydrological influence (Valett *et al*., 1997), or usage of land (Mulholland *et al*., 2008). Microbial diversity might also correspond to the variables. While phytoplankton usually major in the limnetic habitat because of wavelength and penetration of light is high varies with depth (Lydia, 2015).

2.3.2 Differences between freshwater and marine environment and the diversity of microbes in these environment

2.3.2.1 Differences between freshwater and marine environment

Sandrin *et al*. (2006) have stated that marine environment and freshwater environment are different in term of the chemical. In term of chemical marine environments are characterized by the salinity and tolerance toward salinity (Frank *et al*., 2006). Sandrin *et al*. (2006) also give differences of marine and freshwater environment physically. Oceans are large, deep (up to 11,000 m), and very active bodies of water with considerable mixing occurring especially in surface water layers. Freshwater environments are either fairly static (lakes) or running (rivers and streams). Lakes vary in depth from a few to >1000 m and similarly vary considerably in size.

2.3.2.2 Differences of microorganisms diversity in freshwater and marine environment

According to Sandrin *et al*. (2006) both freshwater and marine environments have diverse microorganism presences. Environmental condition thus triggers the composition and species availability of microbial communities and can be main forces of diversification (Horner *et al*., 2004). Lakes contain primary and secondary producers that interact dynamically. Heterotroph concentrations vary with neuston, the thermocline, and the upper layer of the benthos. Whereas in ocean, the highest microbial concentration is at neuston layer (Horner *et al*., 2004).

As in lake environments, the vertical distribution of the heterotrophs shows an increase at the thermocline. At greater depths, the numbers of heterotrophs quickly diminish until, at a depth of 200 m, concentrations are very low. Heterotrophs increase again immediately above the ocean floor (Sandrin *et al.*, 2006). Microbial processes in stream and marine ecosystems are variable in spatial and temporal in response to elements availability (Dodds *et al*., 2000), temperature (Boyero *et al*., 2011), organic matter quality or quantity (Gessner and Chauvet, 1994), hydrological influence (Valett *et al*., 1997), or usage of land (Mulholland *et al*., 2008). For this reason, bacterial numbers are uniform at all depths except when the weather is very calm for long periods of time. In addition, seasonal fluctuations occur in coastal bacterial numbers, which are not observed in the open ocean (Sandrin *et al.,* 2006). In general, there are two times of the year when there is an increase in bacterial populations in coastal waters, late spring to early summer and late summer to early fall, times when the phytoplankton are most active (Frank *et al*., 2006).

When flood occurs, the river water mixed with microorganisms at land and flows back into river. This action causes mixture of microorganisms from land with water microorganism. Therefore, the diversity of the microorganisms changed according to the microorganism tolerance. Certain microorganism can withstand abruptly change of temperature some cannot survive.

2.3.3 Blue-Green Algae

Referring to Figure 2.4, Blue- Green Algae which also known as *Cyanophyceae* is the first organisms to appear on Earth and able to produce their own food by means of photosynthesis. This organism is in between Bacteria and Protists due to their cellular organisation. They are also called as Eubacteria. They can easily found around filamentous algae such as Spirogyra (ACSF, 2014). This organism is usually found in freshwater and it is non-motile organisms. Figure 2.4 showed example of Blue-green algae under light microscope for 10x100 magnifications.

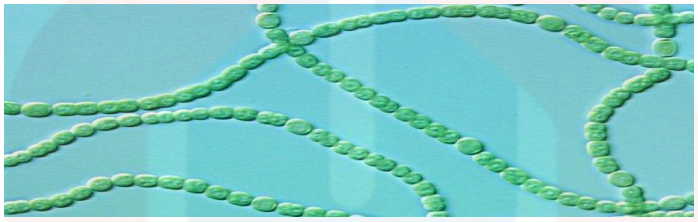

Figure 2.4 Blue- green algae. (ACSF, 2014)

2.3.4 Ankistrodesmus

According to Millan (1957), Ankistrodesmus are fusiform organism with chloroplast. Its chloroplast can elongate until its apex. The chloroplast is capable of shifting position within the cell and circulated by granules in bead form around the equator of the Ankistrodesmus. Chains of granules ordinarily extend from the edge of the pyrenoid diagonally across the cell to the opposite wall, in the direction of either apex.

Many conspicuous granules, crystals within vacuoles, etc., are usually present. The protoplast usually does not extend to the apices (Millan, 1957). This algae is unicellular needle-like cells tapering at both ends as shown as Figure 2.5. They often cluster together in bundles. This kind of algae usually found in the plankton of nutrient-rich lakes and ponds. It's often abundant in sewage ponds. Ankistrodesmus are often the cause of green dis-coloration in ornamental ponds. Figure 2.5 showed Ankistrodesmus picture under microscope.

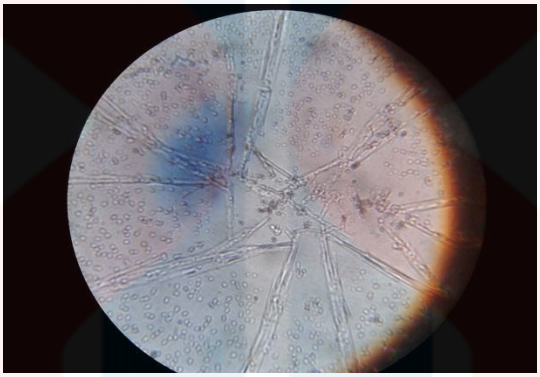

Figure 2.5 showed picture of Ankistrodesmus under 10x 100 magnification.Picture taken from water samples of Sungai Kelantan, Kota Bharu at Laboratory bap 1.1, UMK Jeli

2.3.5 *Escherichia coli*

Theodor Escherich, a German bacteriologist discovered *Escherichia coli* in 1885. Since then, *E. coli* has been commonly used for biological lab experiment and research. *E. coli* as shown as Figure 2.6 is rod-shaped facultative (aerobic and anaerobic growth) gram-negative

bacteria, which can be found in feces of animal, intestines of warm blood organism, and even at hot springs. The optimum temperature for their growth is at 37°C (Jacques & Ngo, 2004). *E. coli* has no spores. Thus, it can be killed by boiling or sterilization by alcohol. The presence *E. coli* in human large intestines help digestion processes, food breakdown and absorption, and vitamin K production. *E. coli* can also be found in environments at a higher temperature, such as on the edge of hot springs (Jacques & Ngo, 2004).

E. coli also can be used as an indicator in the field of water purification. The *E. coli* index can indicate a number of human feces in the water. The reason why *E. coli* is used as an indicator is due to a significantly larger amount of *E. coli* in human feces than other bacterial organisms (Jacques & Ngo, 2004).

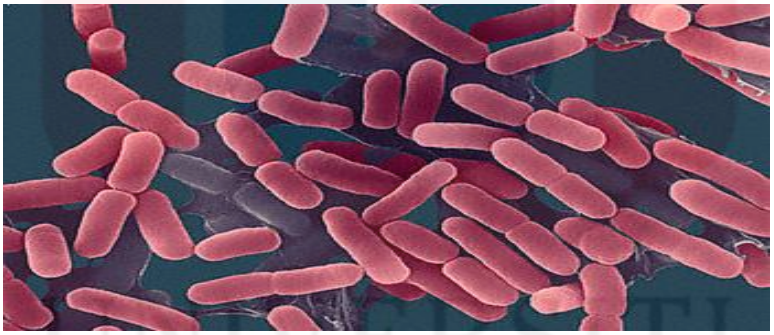

Figure 2.6 *E.coli*. (Dennis Kunkel Microscopy, Inc., 2013)

2.3.6 Amoeba

Amoeba as shown in Figure 2.7 is irregular in shape and unicellular organisms. Amoebae is mobile organisms that can move slowly by expanding its pseudopodia first in one direction and then in another. Amoebas can be found at bottom of the water body, on decomposing leaves and on spirogyra. Some amoeba is non-pathogenic while some is pathogenic (Egmond, 1995).

Example of pathogenic amoeba is *Naegleria fowleri* that cause amoebic meningitis that can lead to death. Presence of amoeba indicated water with thermal pollution. Water with thermal pollution usually comes from factories and industry, power plant station and household. The polluted water will enhance growth of amoeba as the thermal water act as incubator thus the concentration of amoeba will be high. Usually drinking water will be checked for the presence of amoebas because pathogenic amoeba can cause health problems in human (Carboni, 2006).

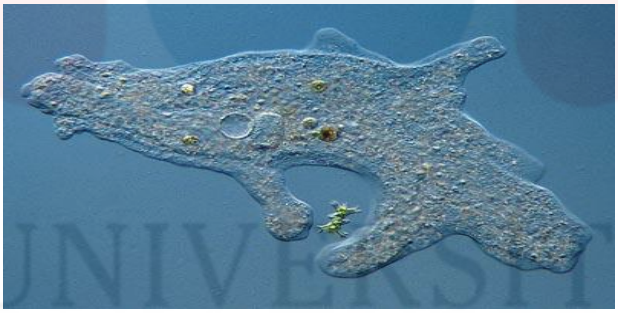

Figure 2.7 Amoeba. (Microscopy-UK, (Egmond,1995))

2.3.7 Euglena

Referring to Figure 2.8, organisms which move by means of flagella and also photosynthetic is called Euglena. Flagellum will vibrate as the organism moves. Euglena is indicator for organic pollution however it is non-pathogenic organisms. According to The Florida State University (2015), organic pollutant such as nitrogen acts as fertilizer for algae growth thus attracting presence of euglena. This organism accumulated when there are a lot of algae to feed on. Thus, high concentration of euglena also means high number of algae in the water bodies.

Euglena characteristics are large number of chloroplasts in its bodies and an orange coloured stigma that is sensitive to light and helps the Protist to locate more illuminated places. The body of Euglena has some helicoidal striping and is very mobile (The Florida State University, 2015).

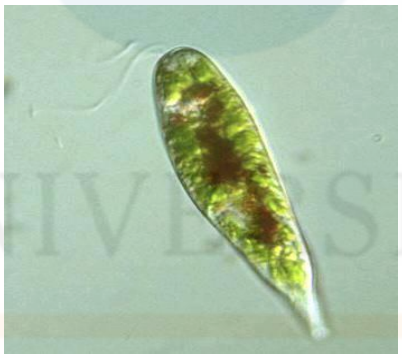

Figure 2.8 Euglena. (The Florida State University, 2015)

2.3.8 Ciliates

Another microorganism that usually found in freshwater is ciliates (Carboni, 2006). It is non- pathogenic expect for several species and one of the species is *Balantidium coli. Balantidium coli* if present in human small intestines can lead to diarrhea. Ciliates act as indicator for organic pollution and associated with sewage and waste treatment process and effluents. The crucial feature of ciliates is its body covered with many cilia according to Microbus, (2006). The cilia functions like fan and moves to help ciliates mobility. Ciliates feed on bacteria, on other Protists and on organic detritus.

The most popular ciliate is the paramecium as shown in Figure 2.9 that look likes a slipper, but unlike the slipper paramecium is always in motion, occupied in an incessant search for food. This organism is able to swim using the hundreds of cirri and also to walk by moving its ventral cirri one at a time. Coleps is a ciliate with a typical barrel form that swims quickly although it sometimes lingers around food (Microbus, 2006).

Figure 2.9 Showed example of ciliate which is Paramecium. (Microbus, 2015)

2.4 Morphology identification of microorganisms via observation

Morphology of the microorganisms growth can be seen and categories into shape, margin, elevation, size, texture, appearance, pigmentation, and optical property. For shape, it can be circular, rhizoid, irregular, filamentous and spindle. Microorganism margin can be entire, undulate, lobate, curled, rhizoid and filamentous. The elevation can be flat, raised, convex, pulvinate and umbonate. In term of the size it can be punctiform, small, moderate and large. While for the texture, it can be smooth or rough. The appearance of microorganism can be glistening (shiny) or dull. Microorganism also can have pigment for example purple, red or yellow and non-pigment such as cream, tan, and white. The optical property of microorganism can be opaque, translucent or transparent.

According to Tshikhudo *et al.* (2013), the conventional methods such as observation of the morphology of bacteria remain reliable for bacterial species identification. But, the conventional method has some weakness. The method requires time and lab work. Besides that, different environmental situation may produce false results. Moreover, a pure culture is required to identify the microbes however fast result is impossible and some bacteria cannot be culture. Technology development helps to overcome the problem. Christopher & Bruno (2003), stated identification via morphology still has significant taxonomic value although the method has little traits and variation among them.

Cultural characteristics can be identified by observation of microbial growth on media. Microbial colony has own unique characteristics than can be use to identified them. Colony characteristics or new species can be discovered by their appearances (Christopher & Bruno, 2003).

Shape of colony or pure culture also used to recognize microorganisms species (Cabeen & Wagner, 2005). Cocci (round in shape), bacilli (rod-shaped) and spirilli (spiral-shaped) said as the most common shape of microorganism found (Cambray, 2006).

2.4.1 Observation of microorganism morphology using microscope

Staining of microorganism and viewed under light microscope done to identify bacteria morphology (Bergmans *et al.*, 2005). Antonie van Leeuwenhoek (1632-1723) was the first man to observe bacteria under a microscope. Staining of bacteria helps to appear their cellular structure because bacteria are colorless. First step to identify bacteria are morphology observation and Gram stain. This step is reliable morphological feature for identifying and classifying bacteria species.

Conventionally, light microscopy was used to identify colonies of bacteria and their morphologies. The weakness of the light microscope was its often insufficient resolution to project bacterial images for clarity of identification (Tshikhudo *et al.*, 2013). Light microscope also lower in magnification as its magnification only up to 10x 100 and needed oil for this purposes. As technology become more develops there are several other types of microscope than can be used to see microscopic organism such as electron microscope. Electron microscope used the principle of beam of electron to see image whereas light microscope use principles of light. Electron microscope enables more magnification because electron have shorter wavelength than light (Peck, 2013).

2.5 Gram staining

Danish bacteriologist, named Hans Christian Gram in 1882 has created the Gram staining method based on research by Xu (1997). It is almost always the first test performed for the identification of bacteria.

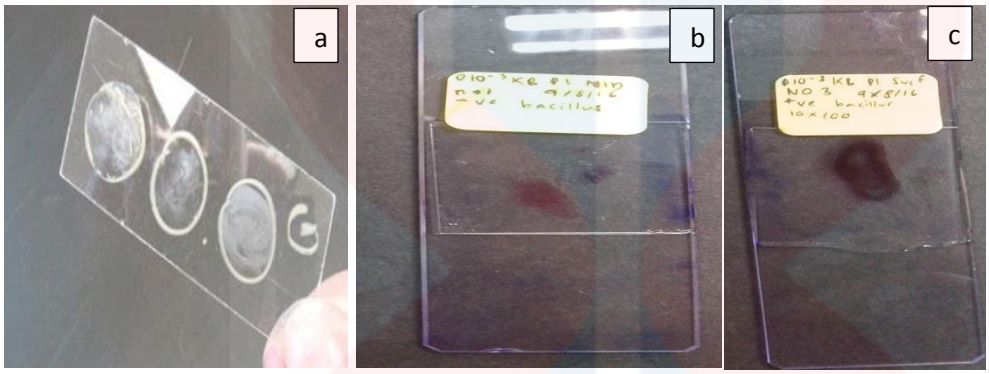

Figure 2.10 (a-c) :2.10a showed slide without stain, 2.10b showed slide with stain of pink while 2.10c showed slide of purple stain. Picture taken on 8 August 2016.

Figure 2.10a showed slide without any stain. Crystal violet is the primary stain for this method. Methylene blue also can be used as primary stain but usually people use crystal violet. The microorganisms with thick cell wall will retain the purple colour of crystal violet and appear purplish-brown under microscope as shown at Figure 2.10c. This method classified microorganisms into Gram positive or Gram negative. Gram negative microorganisms have thin cell wall that made up by peptidoglycan and takes colour of safranin which is pink (Xu, 1997).

Xu (1997) stated that iodine is used as mordant so that the colour of crystal violet will not be washed away. Iodine also functions as fixer to the dye. The step of draining the slide with microorganisms with acetone is function as decolorized. Acetone will dissolves the lipid layer from the gram-negative cells.

Smith (2010) stated that Gram's positive bacteria have thick cell wall thus it close its pores during dehydration. Thus, the colour of crystal violet remained in its cell wall. However if the rate of time of acetone applied to slide too microorganisms will be colorless. According to Crown (2015) Gram-variable may happen as the staining will be mix with each other producing wrong result of Gram stain. Gram- variable is the situation where the microorganisms have both staining which are crystal violet and safranin on their cell wall (Xu, 1997).

Basic fuschin will be applied as counterstain to gives colour to Gram negative bacteria which is reddish-pink. However, most laboratories applied usage of safranin instead of fuschin. Safranin gives out pink colour to the Gram negative bacteria as shown at Figure 2.10b. (Crown, 2015) But in some species application of safranin is not reliable, such as *Haemophilus* spp., *Legionella* spp., and some anaerobic bacteria. The polychromatic nature of the Gram stain enables determination of the size and shape of both Gram-negative and Gram-positive bacteria (Xu, 1997). There is wide range of staining method available and with appropriate used of dyes will enable different part of microorganisms structure to be stained. Parts of microorganisms that can be stained are capsules, flagella, granules, and spores can. Visualization of bacteria under microscope can be helped by applying staining techniques (Smith, 2010).

Mycobacteria, rickettsia and spirochetes need special stains to visualize them as Gram stain cannot help to visualize their cellular cells. As the technology developed some researchers founds modifications of the Gram stain that allow morphologic analysis of eukaryotic cells in clinical specimens (Xu, 1997).

2.6 Endospore staining

Several scientists including Perty, Pasteur, Koch, and Cohn conducted study and concluded that endospore microorganisms have refractile bodies because common dye such as carmine or carbol fuchsin cannot be used to stain them (Crown, 2015). Endospore microorganisms have unique resistance which they are resistance towards thermal. This resistance characteristic of endospore has been found after researcher try to overcome endospore microorganisms presence via sterilization, prevent infection and limit contamination of foods (Hussey & Zayaitz, 2007).

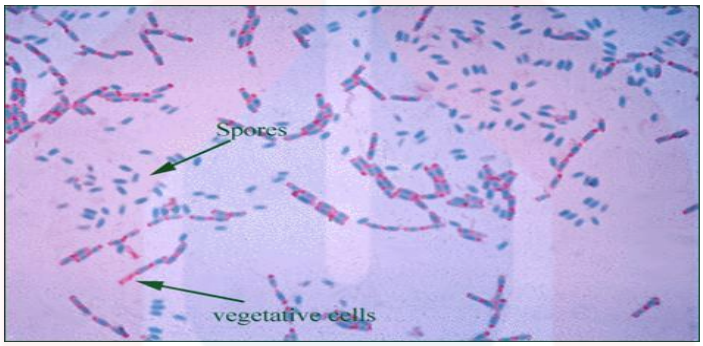

Figure 2.11: Endospore under microscope. (Faculty of Ivy Tech, 2013)

Vegetative cells has red colour while endospores and free spores appeared green or blue as shown as Figure 2.11. Dorner's method in 1933 was modified by Shaeffer and Fulton to faster the process however problem with heating with a Bunsen burner arise. Dorner and Shaeffer-Fulton additional modification developed when study conducted and resulting in identification of ways to stain endospore. This additional method enable to make staining and viewing spores occur quickly, easily, with less mess, and with sharp contrast (Hussey & Zayaitz, 2007).

The endospores refractility can be seen by simple staining or Gram staining. Nowadays, endospore stain is basic step in bacteria recognition and few genera of endospore bacteria must be known as for unknown samples of microorganism (Smith, 2010).

George Knaysi in 1941 proposed staining method that gives identification by layer individually. Later on around 1950s and 1960s several researchers found that several endospores microorganisms content high levels calcium than did the vegetative cells. Some endospores microorganism has dipicolinic acid and has small amount of water content (Crown, 2015). Endospore microorganism can be found in soil, freshwater or marine saprophytes. Examples of endospore microorganisms are Bacillus and Clostridium. Pathogenic endospore bacteria like *Bacillus anthracis* cause anthrax. Older bacillus showed more spores formation than younger one because of lacking in nutrients and competition.

2.7 Polymerase Chain Reaction of Microorganism

Nishiguchi *et al.* (2002) published paper about DNA isolation preparations for animals such as vertebrates and insects, microscopic organisms such as protozoa and extremely small animals. The range of animals and microscopic organisms is too wide thus Nishiguchi *et al.* (2002) published report that only focuses on several protocols that have been developed for rapid and efficient isolation of DNA. In 1984, a Nobel laureate biochemist named Kary Mullis developed Polymerase Chain Reaction (PCR). Development of PCR was founded when researcher found thermophiles and their biological activity of DNA polymerases at extreme temperature.

Usually DNA polymerases (enzymes that make new DNA) work only at low temperatures. DNA is tightly coiled in low temperature that causing polymerases has low chance to make new DNA. However the bacteria that live in hot springs enable to withstand high temperature up to 100°C that normal DNA will denatured in this temperature (Guruatma & Khalsa, 2010).

According to Guruatma & Khalsa (2010) factors that can optimize results of PCR are annealing of temperature and Magnesium concentration. Mg2+ concentration must be about 1.5mM to 3mM to get optimum results of PCR. According to Woo *et al.* (2008), 16S rDNA sequencing provides accurate identification of isolates with a typical phenotypic characteristic unlike phenotypic identification that affected by the presence or absence of non housekeeping genes or by variability in expression of characters.

DNA fragments can be detect by using real-time PCR methods via an on-line fluorescent detection system (Heijnen & Medema, 2006). Heijnen & Medema (2006) reported that detection of STEC O157 on DNA isolated from water samples is fast and quantitative technique when using real PCR. Real time PCR enables high recoveries of pure DNA and low concentration of PCR inhibitor but need to require easy DNA extraction of samples. Meanwhile, DNA extracted technique requires lab work and need optimization. This can be avoided by culture enrichment. Culture enrichment will increase number of target cell during the growth phase. Thus, sensitivity becomes more and can proceed to real time PCR without requirement of high quality of DNA extraction.

During enrichment step, cell growth can be monitored by real time PCR. This cause crucial data about culturing the detected cells can be collected and the implications for health risks of the detected pathogens (Heijnen & Medema, 2006). Detection method was developed during this time (Frahm & Obst, 2003). It combine culture enrichment with PCR detection (culture-PCR) resulting in sensitive and specific detection of culturable *E. coli*. However, this method was only possible by conducting multiple tests on serial dilutions of the samples and most probable number (MPN) need to be known.

2.8 Luria Broth for microorganism cultivation

Guiseppi Bertani in 1952 created Luria Broth recipe when he tried to optimize formation of plaque on *Shigella* indicator strain (Bertani, 2004). Currently LB used as bacterial culture medium but originally it is in bacteriophage genetics fields. According to Bertani (2004), LB has been said to be short form for "Luria Broth", "Luria-Bertani" medium, and "Lennox Broth"; however, the acronym means "Lysogeny Broth". Originally LB is used to develop *Shigella* growth and in bacteriophage genetic fields however nowadays LB has become liquid medium for the growth of *Escherichia coli* and other related enteric species.

According to Maria & Liao (2013) LB also widely use in the molecular biology field as culture medium for facultative organisms. Maria & Liao (2013) also stated that undergraduate microbiology teaching labs also used LB as media to culture microbes.

2.9 Optical density reading for cultured microorganism

According to Matlock *et al.* (2011) optical density will be used to measure bacteria culture via spectrophotometer and usually, OD_{600} will be used to determine the optimal time at which to harvest and prepare component cells. The concentration of bacteria in a suspension can be measured in a spectrophotometer and the means is called as optical density. In spectrophotometer when visible light passes through a cell suspension the light will be scattered thus greater scatter indicates that more bacteria or another material is present thus concentration of bacteria is high. Spectrophotometer used wavelength to measure amount of light scatter. Wavelength is vary depend on the particular type of cell at different phase of their growth. Generally, the mid-log phase of growth will be used Matlock *et al.* (2011).

2.10 Description of Spectrophotometry reading

According to Lin *et al*., (2010) DNA or RNA concentration can be read by using spectrophotometry and analyzing their purity. Usually wavelengths that will be used are 260 nm and 280 nm because this wavelength enable gather of further information. Biomolecule concentration of a solution and its ubiquitous property was determined by using spectrophotometric analysis (Trumbo *et al*., 2013). At 260 nm wavelength, the purines and pyrimidine in nucleic acids naturally absorb light. For DNA the formula used is concentration (µg/ml) = Abs260 ×50 and these values are called conversion factors. However, other substances with same wavelength can effects the reading of spectrophotometry. This condition can be overcome by selection of ratios and background corrections.

Trumbo *et al*. (2013) also describing the explanation about direct UV measurement A260/A280 Ratio. The A260/A280 ratio is the most common purity check of DNA. Maximum absorption at 280 nm showed protein contamination. Good level of purity of DNA ratio 260/280 nm must be grater or equal to one point eight while reading lower than that showed impurities of the sample. A260/A230 Ratio also can indicate contamination if there is increase in absorbance because it affects the 260 nm reading for DNA. The region of absorbance of peptide bonds and aromatic side chains is at 230 nm (Myers *et al*., 2013). Several buffer components possess high absorption at 260 nm and therefore can alter the results of spectrophotometer reading. According to observation by General Electric Company, (2012), EDTA buffer in concentrations above ten mM posses that characteristic and may alter end results of the reading.

General Electric Company, (2012) also stated that absorption at 230 nm can showed presence of contaminants in a sample, like presence of proteins, phenol, or urea. Pure sample indication of an A260/A230 ratio results will be two or above. Absorption at 320 nm can be resulted from light scatter caused by particles or to a precipitate in the sample and also dirty or damaged cuvettes (Trumbo *et al*., 2013). Increased in scattered light may also can cause by contaminations with chaotropic salts such as NaI. Thus to avoid this kind of reading, any interference from light scatter must be removed, interference from the cuvette, or in cases where a blanking plate is used to target the light beam through the sample. When using small volume cells or specialist small volume, spectrophotometers background correction is particularly useful (Lin *et al*., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.0 Study area for water microbial sampling

This experiment was conducted by collecting water sample at different depth in Sungai Kelantan, Kota Bharu. The sampling area chosen was located 69.4 km from Jeli. Figure 3.1 showed location of sampling in Sungai Kelantan, Kota Bharu. Orange circles on the Google Map denote the placed where water samples of Sungai Kelantan, Kota Bharu taken. The GPS of the location was 6°06'51.4"N 102°13'42.2"E, 6°06'51.4"N 102°13'42.2"E and 6°06'51.4"N 102°13'42.2"E respectively for P1, P2 and P3.

Figure 3.1 Location of sampling site. Sources from Google map 2016. Accessed on November 10, 2016.

3.1 River water collection at Sungai Kelantan, Kota Bharu

Water samples were collected one meter from the river bank and two meters deep. Water sampler was used to collect water samples at Sungai Kelantan, Kota Bharu river about one Litre at surface and middle area. For bottom samples, PVC pipe (3m) was used to take sediment along with water. The water samples were collected in triplicate at three different points. The water samples were collected at three points of different depths on the surface (10cm), in the middle (50 cm) and at bottom. Water samples collected in duplicate and labelled as point A or point B, and divided into surface, middle and bottom. This labelling applied for all three points. All the samples taken were filled in 50 ml falcon tubes and then immediately put into icebox filled with ice. The water qualities of Sungai Kelantan, Kota Bharu were determined by using YSI-85 multi-parameter and pH meter in term of dissolve oxygen (DO), salinity, pH, and temperature. The parameters were measured in triplicate and the data obtained were recorded.

3.2 Nutrient Agar preparation and cultivation of water microbial

Nutrient agar (NA) was used for microbial culture in this study and 23 gram of NA powder was mixed with one liter of distilled water in the one litre Scott bottle. Liquid NA was sterilized by autoclaving at 121°C with pressure 1.5 atm for 15 min and allowed to cool until it reaches a temperature of 45 - 50°C. The sterile NA was poured into petri dishes and let to solidify in room temperature inside laminar flow. Within 24 hours of taking river water samples from Sungai Kelantan, Kota Bharu serial dilution conducted by diluting river water samples of Sungai Kelantan, Kota Bharu from point B for each point. River water samples of point A of Sungai Kelantan, Kota Bharu were kept in chiller of - 4°C at Bap laboratory 1.1, UMK Jeli.

3.2.1 Serial dilution of water samples from Sungai Kelantan, Kota Bharu

Serial dilution prepared by pipetting nine ml of sterile distilled water into five pieces of 15ml Falcon tube. All five of the 15ml falcon tube labelled with dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} respectively for each tube. Firstly one ml of river water of Sungai Kelantan, Kota Bharu pipetted into nine ml of sterile distilled water.

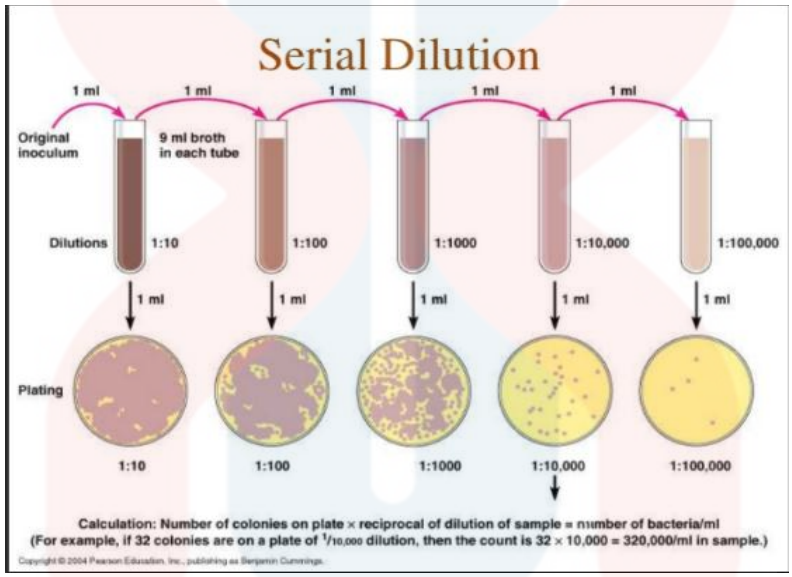

Figure 3.2 Serial dilution processes from water samples. (Tortora, 2004)

Mixing of river water sample of Sungai Kelantan, Kota Bharu and steriled distilled water was done by pipetting in and out several times. Figure 3.2 showed steps for serial dilution. The first falcon tube labelled as 10^{-1}. Then one ml of dilution 10^{-1} pipetted into second falcon tube labelled 10^{-2} and the step continuously with the rest of 15ml falcon tube until serial dilution of 10^{-5}. About 100 µl of river water of Sungai Kelantan, Kota Bharu taken from serial dilution of 10^{-5} used for spread plate method.

3.2.2 Culturing of bacteria using spread plate method of serial dilution from water samples of Sungai Kelantan, Kota Bharu

Spread plate method was used to spread diluted river water sample on steriled NA plate inside laminar flow of Bap Laboratory 1.1, UMK Jeli. Before using laminar flow, it was steriled by spraying 70% ethanol and UV light was switched on to steriled all the apparatus that need to be used. Hockey stick was steriled by flaming it before used to spread the dilution of river water samples from Sungai Kelantan, Kota Bharu and after done spreading for each plate. Each plate was labelled according to its point such as P1 Surface, P1 Middle and P1 Bottom and so do the rest labelled according to their point. Date and dilution also included on the plate.

Spread plate method used hockey stick to spread the river water sample from Sungai Kelantan, Kota Bharu onto sterile NA plate. Plates then incubated a day at 37°C in incubator at Bap Laboratory 1.1, UMK Jeli. All the samples of serial dilution of river water of Sungai Kelantan, Kota Bharu were kept in chiller at -4°C at Bap Laboratory 1.1, UMK Jeli. Morphology of growth of microorganism observed and recorded. After three days some plate of spread plate of serial dilution of 10^{-5} has no microorganism growth thus serial dilutions of 10^{-3} were used to make another culture of microorganism and the steps repeated until recording of microorganism morphology observation for serial dilution of 10^{-3}. After 24 hours the growth of microorganism on NA plate was observed and after three days NA plates with growth microorganism taken out from incubator and were put on sterile laminar flow.

3.2.3 Pure culture initiation using streaking method of microorganisms from water samples of Sungai Kelantan, Kota Bharu

Colony of each microorganism growth on NA plate that was incubated for 24 hour in incubator at temperature 37°C at Laboratory 1.1, UMK Jeli was circled. Isolation of single colony was done by streak plate method to obtain pure culture. Inoculum loop was sterilized by flame until it turned red and cooled before starting to inoculate microorganisms. Colony of growth microorganisms from NA plate was chosen and sterilized inoculum loop used to pick single colony and streaked onto new NA plate. The new NA plate with streaked microorganism labeled according to its master plate.

3.2.4 Morphology observation of microorganisms growth and isolated from river water samples of Sungai Kelantan, Kota Bharu

The NA plate with microorganisms incubated in the oven for 37°C at Bap Laboratory 1.1, UMK Jeli. Bacterial growth started to become visible in about two to three days or more depend of the microorganism's species. Morphology of the microorganism grows on plate which were visible by naked eyes were then categorized into shape, margin, elevation, size, texture, appearance, pigmentation and optical property as shown in Figure 3.3. The morphology of streaked microorganism once again viewed to ensure the new microorganism that grows was pure culture.

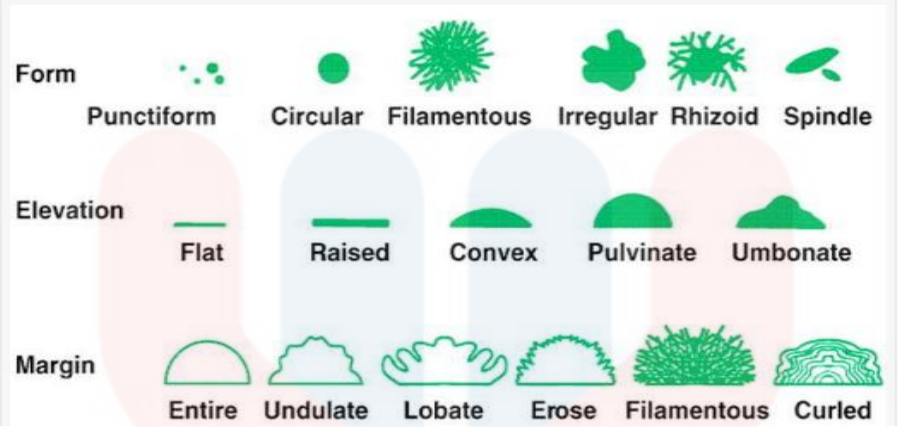

Figure 3.3 shows the classification of morphology observation of microorganism. (Medical-Labs, 2014)

3.3 Preparation of buffer for DNA Extraction of water microorganisms of Sungai Kelantan, Kota Bharu

3.3.1 Preparation of CTAB Buffer

This experiment required 100ml CTAB buffer, hence it was prepared by adding two gram of CTAB powder in 86 ml of double distilled water, 8.182 gram of NaCl, 10 ml of Tris (pH 8), and four ml of EDTA (pH 8). All this materials added together and stirred by magnetic stirrer and hot plate before autoclaved at 121°C with pressure one point five atm for 15 minutes.

3.3.2 Preparation of TE Buffer

TE buffer prepared by adding one ml of Tris (pH 8) with 0.2 ml of EDTA (pH 8) and mixed with distilled water up to 100 ml. The mixture was then stirred by magnetic stirrer to mix before autoclaved at 121°C with pressure one point five atm for 15 minutes.

3.4 Preparation of buffer for AGE of DNA samples extracted from water microorganisms of Sungai Kelantan, Kota Bharu

3.4.1 Preparation of TAE Buffer

TAE buffer was prepared by making 50x stocks solution by mixing 242 gram of Tris (pH 8), 57.1 ml of acetic acid, 100 ml of 0.5M EDTA(pH 8) and dissolve into one liter by distilled water. One times TAE prepared by diluting 50x TAE by 20 ml of 50x TAE added to 980 ml of distilled water. The mixture was then stirred by magnetic stirrer to mix before autoclaved at 121°C with pressure one point five atm for 15 minutes.

3.5 Culturing of pure culture of bacteria in Luria Broth

Using modified method by Nishigushi *et al*. (2002) microorganisms were grown in Luria Broth for 12 hours. The reading of optical density was taken at 600 nm (OD_{600}) to observe the active phase of microorganisms after 12 hours of incubation in incubator shaker.

Luria broth was prepared by adding 35.6 gram of LB powder into one liter distilled water and autoclaved at 121°C with pressure one point five atm for 15 minutes. About 10 ml of Luria broth was added into Bijou bottle. Steriled inoculum loop was used to select single colony which was then added into Luria Broth solution. Incubator shaker with 200 rpm and temperature of 37°C was used to incubate the microorganisms for 12 hours and more depend on the microorganism's OD reading. Microorganism that reached OD of 0.3A to 0.4A was taken out from shaking water bath and put into glycerol solution to prepared glycerol stock. The OD reading that reached over the optimum reading (0.3A to 0.4A) diluted using Luria Broth solution until reached the desired reading. Glycerol solution was prepared by adding 200 ml of glycerine added into 200 ml of distilled water. The ratio of glycerine and distilled water was 50:50. The glycerol stocks was then stored in -20°C freezer at Laboratory of Technology Microbes, UMK Jeli.

3.6 Determination of water microbial diversity using molecular based identification

3.6.1 DNA isolation of water microorganism from Sungai Kelantan, Kota Bharu

DNA isolation from river water microbe sample was done following modification from method developed by Nishiguchi *et al.* (2002). About 200 µL of microorganism culture in Luria Broth at OD exceeded 0.3A was spin down and then the upper phase containing media was discarded. About 570 µL of previously prepared TE buffer with pH8 was added to the pelleted cells. The pellets were re-suspended by repeated pipetting.

Previously prepared CTAB buffer with amount 600 µL was added into mixture of solution of TE and microorganism. About five µL of Proteinase K (20mg/ml) was added into solution. The solution then was incubated at 65°C for 10 min. About 600 µL chloroform was added and mixed well in the solution. The pellet was centrifuged at 14,000 rpm for five minutes and about 150 µL of supernatant (aqueous) was transferred to a new tube. The step was repeated with chloroform when there was presence of white protein layer.

About 600 µL of cold ethanol was added and mixed gently until the DNA precipitates. The pellet then was centrifuged for 15 minutes at 14 000 rpm and cold ethanol was removed. The remaining salts from DNA extraction was washed away by using one mL of 70% ethanol. The pellet was centrifuged at 10 000 rpm for two minutes, and the remaining ethanol was discarded. Air dried the DNA extracted for 10 minutes was proceeded. The pellet of extracted DNA then was resuspended in 50µL of TE buffer and was kept at -20°C in freezer at Laboratory of Technology Microbes, UMK Jeli.

3.6.1.1 Optimization of DNA isolation from water microorganism isolated from Sungai Kelantan, Kota Bharu with Proteinase K, and lysozyme

About 200 µL of microorganism culture grow in Luria broth at OD above 0.3A was spun down for ten minutes at 10 000 rpm and the upper phase containing media were removed. About 570 µL of previously prepared TE buffer (pH8) was added to the pelleted cells. The pellet was re-suspended by repeated pipetting. Previously prepared 600 µl CTAB buffer was added into the solution. The solution then was pipetted slowly to mix.

About 50 µL of Proteinase K (20 mg/ml) was added into solution and mixed by repeated pipetting. About 10 µL lysozyme (100 mg/ml) was added and slowly inverted the tube. The tube then was rested in ice for 10 minutes. Before incubated at 65°C for 30 min in water bath, the solutions were inverted slowly for 50 times. At every 10 minutes, the tubes were inverted about 20 times. About 600 µL chloroform was added into and mixed well by inverting 30 times and kept on ice for five minutes.

The pellet was centrifuged at 14,000 rpm for 10 minutes and about 450 µL of supernatant (aqueous) was transferred to a new tube. About 600 µL of cold ethanol was added and mixed gently until the DNA precipitates. The pellet was then centrifuged for 15 minutes about 14 000 rpm and cold ethanol was removed. The remaining salts from DNA were washed away by using one mL of 70% ethanol. The pellet was centrifuged for 10 000 rpm about two minutes, and ethanol was discarded. The extracted DNA was air dried for 20 minutes in laminar flow and soft tissue was used to absorb visible ethanol. The pellet then was re-suspended in 50µL of TE buffer and kept at -20°C in freezer at Laboratory of Technology Microbes, UMK Jeli.

3.6.2 Agarose Gel Electrophoresis (AGE) of DNA extracted from water microorganism of Sungai Kelantan, Kota Bharu

Agarose gel of 1% concentration was prepared by weighing 80 gram of agarose into a 250 mL conical flask. About 79.2 mL of one times TAE was added into agarose and mixed. The solution was microwaved for about one minute in a microwave oven to dissolve the agarose throughly. The gel was left to cool to room temperature on the bench for 15 minutes.

About three µL of Ethidium bromide (10 mg/mL) was added into the liquid agarose and swirled to mix. The gel was then poured slowly into the tank. A comb was inserted into the gel and the gel then left to solidify at room temperature. One time TAE buffer was poured into the gel tank to submerge the gel to two to five mm depth. Single well on the gel was filled with six µL of marker / DNA ladder (one kb) and the rest of the wells were loaded with one µL of loading dye plus five µL of DNA each. AGE was run at 80 V for 30 minutes. The gel with DNA then was placed on the UV transilluminator for viewing. UV Gel- Doc System was used to document the AGE of microbial DNA isolated from water samples of Sungai Kelantan, Kota Bharu.

3.6.3 Polymerase chain reaction (PCR) of 16s DNA region of water microorganism from Sungai Kelantan, Kota Bharu

Polymerase Chain Reaction was conducted using PCR master mix and 16s primer. The sequence for 16s primer used was forward 5'-CGC TGG CGG CGC GTC TTA AA-3' while for 16s reverse was 5'-TTC ACC GCT ACA CCT GGA A-3'**.** Master mix was prepared by adding double distilled water, 25mM Magnesium chloride ($MgCl_2$), dNTPs, five times green Gotaq flexi buffer, forward and reverse primer and Taq polymerase (Promega). About 16µL of double distilled water was pipetted into microcentrifuge tube. Next, two µL of $MgCl_2$ was added, followed by one µL of dNTPs. Then 2.5 µL of 5x green Gotaq Flexi buffer was added into the solution. About 0.5 µL of forward primer was added and followed by 0.5 µL reverse primer. Taq polymerase was added finally about 0.5 µL into the solution.

About two µL template (DNA) was inserted into PCR tube and labeled. About 23µL of master mix was pipetted into PCR tube with template. Polymerase chain reaction used three main cycles. This experiment was conducted for 30 cycles. Table 3.1 showed the detail of PCR that was conducted using DNA extracted from microorganism in Sungai Kelantan, Kota Bharu. Agarose gel electrophoresis was conducted as stated at 3.6.2 before viewed the amplified DNA. PCR product was kept in -20°C in freezer at Laboratory of Technology Microbes, UMK Jeli.

Table 3.1: Thermal cycling condition for GoTaq® DNA Polymerase mediated PCR amplification

Step	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

4.0 Isolation of water microorganisms from water samples of Sungai Kelantan, Kota Bharu

Physical parameters such as colour of river water of Sungai Kelantan, Kota Bharu, turbidity of river water of Sungai Kelantan, Kota Bharu and odour at the sampling site were observed. Figure 4.1 showed picture of Sungai Kelantan, Kota Bharu sampling site. The colour of Sungai Kelantan, Kota Bharu is reddish brown colour like "tea tarik". The turbidity of river water of Sungai Kelantan, Kota Bharu is very cloudy as the bottom of the river cannot be seen with naked eye. The odour around the sampling site was unpleasant. All of this could be due to human activity such as agricultural, sand dredging, sewage flowing nearby and wastes from residential area and shopping complex.

Agricultural activities use herbicides and pesticides. Both of this product mixed with soil and flows altogether into Sungai Kelantan, Kota Bharu during raining and watering. This hence caused Sungai Kelantan, Kota Bharu colour to become cloudy and causing the river water becomes cloudy. Colour of Sungai Kelantan, Kota Bharu changes to becomes turbid and lost its clarity and disturbance toward aquatic ecosystem occurred because of sand dredging activity by human. Heavy machines used to dredge sand from bottom of the river disrupt natural activity in Sungai Kelantan, Kota Bharu. Disturbance toward the physical occurred when the sand was dredge from the bottom of river upwards. Suspended solid amount increases along river water turbidity causing failure of light penetration from the Sun into the river bottom (Supriharyono, 2004).

Water quality of Sungai Kelantan, Kota Bharu degraded when the turbidity is high. Clarity of river water decreases and thus depleting amount of light require by aquatic plants rate of photosynthesis and disrupt primary production rates. High turbidity of river also will affect the population of fish in Sungai Kelantan, Kota Bharu (Gubbay, 2003). Through out this study, there were six microorganisms found on surface area, for middle area there were eight microorganisms while at the bottom there were 13 microorganisms found. It can be said that turbidity did not affect presence of microorganism at Sungai Kelantan, Kota Bharu. Since most microorganisms did not need light to conduct photosynthesis to make food, the microorganisms diversity were not affected by the turbidity (Gubbay, 2013).

Other water quality disturbance and pollutant such as excavation machinery and transportation oil wastes and spillage and water pollution problems (Phua *et al*., 2004). Food chain of aquatic ecosystem of Sungai Kelantan, Kota Bharu such as benthic organism and mammals are disrupted by sand mining activity (ECD, 2001). Sand mining damages benthic habitats; suspension feeders, such as sponges and hydrozoans may become clogged by the suspended solid or becomes stressed because the feeding and ecosystems disrupted by anthropogenic activity (ECD, 2001).

Benthic organisms are food for aquatic animals thus loss of them will affects the food chains balance (Gubbay, 2003). Throughout this study there was abundance of soil microorganism in the water samples of Sungai Kelantan, Kota Bharu. This may due to loss of benthic organisms that fed on microorganism in sediment layer.

High content of fine sediment and organic matter also contribute to high turbidity. Aquatic ecosystem may be affected by this condition. Primary production of ecosystem will decreases when light penetration is low as it cannot tolerate the turbidity. Wastes from residential areas flows via pipeline into Sungai Kelantan, Kota Bharu causing unpleasant smell around the Sungai Kelantan, Kota Bharu area.

The effluent from livestock farms, heavy precipitation, organic contamination and agriculture, and road runoff in which high suspended matter content may be the cause of the colour of Sungai Kelantan, Kota Bharu to turn cloudy as shown in Figure 4.1. Turbidity could also result from increases organic matter deposition. Clarity and turbidity of river water are related as high clarity thus low turbidity. High amount of sediments can be proved by the darkness colour of water.

Figure 4.1: Picture of Sungai Kelantan, Kota Bharu that showed high turbidity which is indicated by the intensity of river colour. Picture taken on 31.7.2016

Table 4.1: Comparison of parameters at three points of sampling at Sungai Kelantan, Kota Bharu. Parameters taken were temperatute, pH, salinity and dissolve oxygen (DO) at three levels of Sungai Kelantan which was surface, middle and bottom.

Point Parameter	Surface	Middle	Bottom
Temperature	28.2 ± 0	28.2 ± 0	28.2 ± 0
Ph	6.86 ± 0.05859	6.85 ± 0.07767	6.80 ± 0.13229
Salinity	0.1 ± 0	0.1 ± 0	0.1 ± 0
Dissolve oxygen	5.47 ± 0.26652	5.16 ± 0.44677	4.38 ± 0.13317

According to Table 4.1 the readings of temperature and salinity of Sungai Kelantan, Kota Bharu were the same for all the triplicate points. The reading for temperature of Sungai Kelantan, Kota Bharu was 28.2°C whereas the reading of salinity of Sungai Kelantan, Kota Bharu was 0.1 ppt. The temperature was in range of normal freshwater temperature reading which was between 25°C to 30°C. The reading of salinity of Sungai Kelantan, Kota Bharu resulted like that due to its location at downstream. As Sungai Kelantan, Kota Bharu is downstream all the river water of Sungai Kelantan flows there thus brings the sedimentation into Sungai Kelantan, Kota Bharu causing the sedimentation to coagulate there. In addition, river water from Sungai Kelantan, Kota Bharu was also used for agriculture activities. Leakage from agricultural activities which could have contained nitrogen, sodium, ammonium and other chemical may have caused the rise in its salinity along time. The pH of Sungai Kelantan, Kota Bharu was 6.6 to 6.9 that basically the pH is suitable for aquatic life to live within Sungai Kelantan, Kota Bharu. The common pH reading of freshwater is ranges from 6.5 until 7.5.

The dissolved oxygen in Table 4.1 showed all the points of Sungai Kelantan, Kota Bharu were adequate for the planktons and aquatic life to survive. Aquatic plant photosynthesis process causes dissolved oxygen amount increases. Oxygen from troposphere diffuse into surface water becomes dissolve oxygen. Aquatic animal and used in the degradation of organic matter in river water and benthos organisms at the sediments consume dissolved oxygen. The gaps of DO reading may occur at surface and bottom level because of high abundance of benthos organisms than presence of aquatic plants in the surface level. In addition, surface level is more close to atmosphere and oxygen is absorbed much higher than bottom level. The DO reading may also resulted from presence of other non-microbial organisms such as zooplankton and benthos organisms. Table 4.2 showed existence of other organisms besides microbial organism.

Table 4.2: Freshwater organisms found in water samples of Sungai Kelantan, Kota Bharu at Point1, Point 2 and Point 3.

Point	Point 1	Point 2	Point 3
Organisms found			
Ankistrodesmus	/	/	/
Cyclotella	/		
Daphnia	/		
Actinastrum		/	
Stigeolonium		/	
Nematode	/		/
Plosterium			/
Insect	/		/
Unknown	/	/	/

Table 4.2 showed existence of freshwater organism in Sungai Kelantan, Kota Bharu by conducting wet mount and mostly the organism presence is algae. Polluted and clean water can becomes habitat of algae and thus algae presence can contribute to water quality determination. Besides that, according to Jafari & Gunale (2006) algae can tolerate to pollutant such as toxic material hence used to investigate pollution rate in river. Disturbance at lower level of food chain can collapse the whole ecosystem (Jafari & Gunale, 2006). Palmer's (1969) reported that Oscillatoria, Euglena, Chlamydomonas, Cyclotella, Stigeoclonium, and Ankistrodesmus are the species of algae found in organically polluted waters. Gunale & Balakrishnan (1981) also reported the same species found in polluted water. Noel & Rajan (2015) recently reported that the genera also founded by they in polluted river.

Oscillatoria, Euglena, Chlorella and Ankistrodesmus are commonly found in heavily polluted waters (Ratnasabapathy, 1975). Mclean (1974) as well as Gunale & Balakrishnan (1981) has stated that Stigeoclonium can be used as indicator for organic pollution and have high tolerance towards heavy metals. All these reports agreed that presence of algae such as Ankistrodesmus, Cyclotella, and Stigeoclonium showed that the water bodies are polluted with organic material and also called sewage pond water bodies.

Optical density reading at 600 nm for isolated microorganism from Sungai Kelantan, Kota Bharu were summarised in Table 4.3 and 4.4. Spectrophotometry with wavelength of 600nm used to measure growth rate of cultured microorganism from Sungai Kelantan, Kota Bharu. Reading that above 0.2 A showed bacteria at its active phase.

Table 4.3 showed optical density reading of cultured microorganism in Luria Broth from Sungai Kelantan, Kota Bharu between Point 1, Point 2 and Point 3 at surface and middle. NO OD means that the microorganism did not reach optimum OD even after a week incubated and thus discarded. Dash (-) means no microorganisms incubated in LB solution.

Point	1	2	3	1	2	3
Plate Number	Surface	Surface	Surface	Middle	Middle	Middle
1	0.095 A	0.056 A	0.323 A	0.327A	0.109 A	NO OD
2	0.355A	-	0.072 A	0.366A	NO OD	0.074 A
3	0.046 A	-	-	NO OD	0.325 A	-

Table 4.4 showed the optical density reading of cultured microorganism in LB from Sungai Kelantan, Kota Bharu between Point 1, Point 2 and Point 3 at bottom. NO OD means that the microorganism did not reach optimum OD even after a week incubated and thus discarded. Dash (-) means no microorganisms incubated in LB solution.

Point	1	2	3
Plate Number	Bottom	Bottom	Bottom
1	0.357 A	0.381 A	0.300 A
2	0.380 A	0.380 A	NO OD
3	-	NO OD	0.207 A
4	-	NO OD	-
5	-	0.358 A	-
6	-	0.101 A	-
7	-	0.095 A	-

According to Friedrich (2010) precaution that need to be taken when using spectrophotometry are using clean and dry cuvette and setting of blank must be at 0A and confirmed again with sterile LB (media) and make sure that cuvette in the same orientation. The optimum reading of optical density to indicate microorganism at active phase is between 0.2A to 0.6A which is in between exponential phase to stationary phase. The growth rate of microorganism is exponential and divided into several phases such are lag, exponential, stationary and die-off phase as shown in Figure 4.2 (Friedrich, 2010).

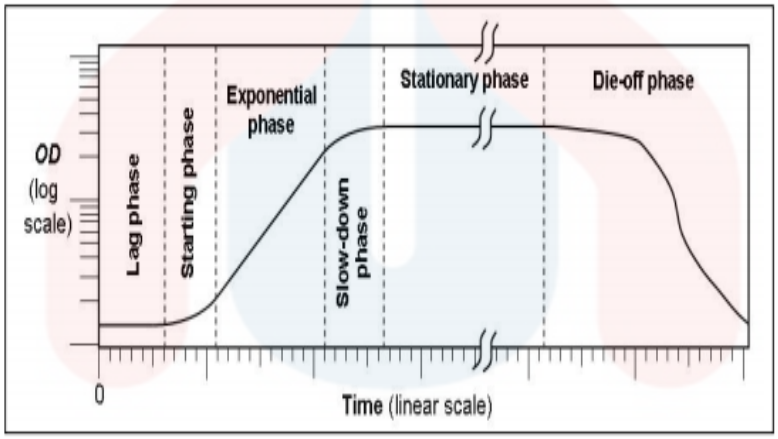

Figure 4.2: Growth phase of microorganism which are lag phase, exponential phase, stationary phase and die-off phase (Friedrich, 2010).

According to Friedrich (2010) the longest phase is stationary phase because the microorganism is finally establishing its life. Growth limiting factor such as depletion of nutrient such as oxygen level and accumulation of toxin that inhibit microorganism's growth determine stationary phase. The die-off phase is hardly to predict because it depend on the rate of the microorganisms growth and its nutrient content inside the medium.

4.1 Determination of microorganism diversity based on morphology observation of microorganisms found in Sungai Kelantan, Kota Bharu

This result showed that water microorganism at Sungai Kelantan, Kota Bharu was diverse because all morphology has many variations. Table 4.5 showed comparison between morphology observation of microorganism cultured at Point 1, Point 2 and Point 3 from river water microorganism of Sungai Kelantan, Kota Bharu. At each point there were three different depth taken which were surface, middle and bottom.

According to Table 4.6 there were differences between morphology of microorganism at different depth which were surface, middle and bottom. The interpretation of morphologys can be categorized to several morphology which are form, elevation, margin, surface, opacity and chromogenesis (Theresa, 1999). Form is the basic shape of the colony while elevation is the cross section of the colony. Margin is the magnified shape of edge of the colony. The surface means the colony appearance while opacity means the light penetration towards the colony. Chromogenesis is pigmentation that gives colour to the colony (Theresa, 1999). The differences of morphology can due to its niche and food source (Findlay *et al*., 2003). Microorganisms that were found at the bottom can be said as sediment microbes that fed on benthos organisms (Sigee, 2004).

Table 4.5: Comparison of morphology observation of microorganism found at Point 1, Point 2 and Point 3 in Sungai Kelantan, Kota Bharu

Point	P1	P2	P3
Morphology observation			
Form			
-Irregular	3	14	6
-Circular	4	1	4
-Filamentous	2	0	1
Elevation			
-Raised	9	14	11
-Flat	0	0	1
Margin			
-Filiform	1	0	1
-Entire	4	5	7
-Undulate	3	9	1
-Lobate	1	0	2
Surface			
-Glistening	2	2	4
-Smooth	5	9	3
-Dull	1	0	3
-Rough	0	1	1
Opacity			
-Translucent	4	7	5
-Opaque	4	5	6
Chromogenesis			
-Cream	7	11	9
-Purple	1	0	0
-Yellow	0	1	2

Table 4.6 Showed morphology observation of different depth which were at surface, middle and bottom of river water microorganism of Sungai Kelantan, Kota Bharu

Depth	Surface	Middle	Bottom
Morphology observation			
Form			
-Irregular	3	4	9
-Circular	2	2	4
-Filamentous	1	2	0
Elevation			
-Raised	6	8	13
-Flat	0	0	0
Margin			
-Filiform	2	0	0
-Entire	2	4	5
-Undulate	2	3	8
-Lobate	0	1	0
Surface			
-Glistening	1	2	4
-Smooth	3	4	8
-Dull	2	0	1
-Rough	0	1	0
Opacity			
-Translucent	4	5	3
-Opaque	2	3	10
Chromogenesis			
-Cream	6	6	12
-Purple	0	1	0
-Yellow	0	1	1

Figure 4.3 showed the comparison between present of Gram positive and Gram negative microorganism at Sungai Kelantan, Kota Bharu using Gram staining method. Gram positive microorganism is non-pathogenic whereas Gram negative microorganism is pathogenic. According to Tortora, (2004) there are several differences of Gram positive and Gram negative bacteria. One of the differences is thickness of peptidoglycan wall (cell wall) where Gram positive have thick layer but Gram negative have thin layer. This explains the reason of Gram positive stain remains purple while Gram negative take colour of Safranin which is pink.

Gram negative consider as pathogenic because it has outer membrane and lipopolysaccharide, it also has high resistance of lysozyme and penicillin and also having both endotoxins and exotoxins (Tortora, 2004). Gram positive were mainly found at the three level of depth. Usually Gram positive bacteria are not harmful to human but certain species.

Reported by Mark & Tratter (2007) there are several Gram positive genera that are harmful to human. There are Streptococcus, Staphylococcus and bacillus. Streptococcus is chain of coccus while Staphylococcus is clumped of sphere shape bacteria. Rod shapes microbes that can produce spores such as Clostridium and Bacillus while species that cannot produce spore are Corynebacterium and Listeria (coccobacillus) (Mark & Tratter, 2007). According to Sahebnasagh *et al*. (2011) Bacillus is facultative anaerobe whereas Clostridium is an obligate anaerobe. Plant diseases can also be caused by Gram positive microbes from Rathybacter, Leifsonia, and Clavibacter genera. Mhairi (2015) reported that some Gram-positive bacteria are also harmful to babies and even can lead to death of the victim.

Sagar (2015) stated that certain species of microbes can produce exotoxins that are heat labile protein and some other species can produce endotoxins that are heat stable lipopolysaccharide. Lipopolysaccharide is protein that forming cell wall of Gram negative microbes. This protein liberate upon cell lysis and death of microorganisms (Sagar, 2015). Sungai Kelantan, Kota Bharu have more abundance of Gram positive microorganism than Gram negative. Mostly the Gram positive microorganisms found in Sungai Kelantan, Kota Bharu are in bacillus shape except for two other shapes which are spirilium and streptobacillus.

While the entire Gram negative microorganism found at Sungai Kelantan, Kota Bharu is bacillus in shape. Pictures of the Gram positive and Gram negative microorganisms of Sungai Kelantan, Kota Bharu can be referred at Appendices.

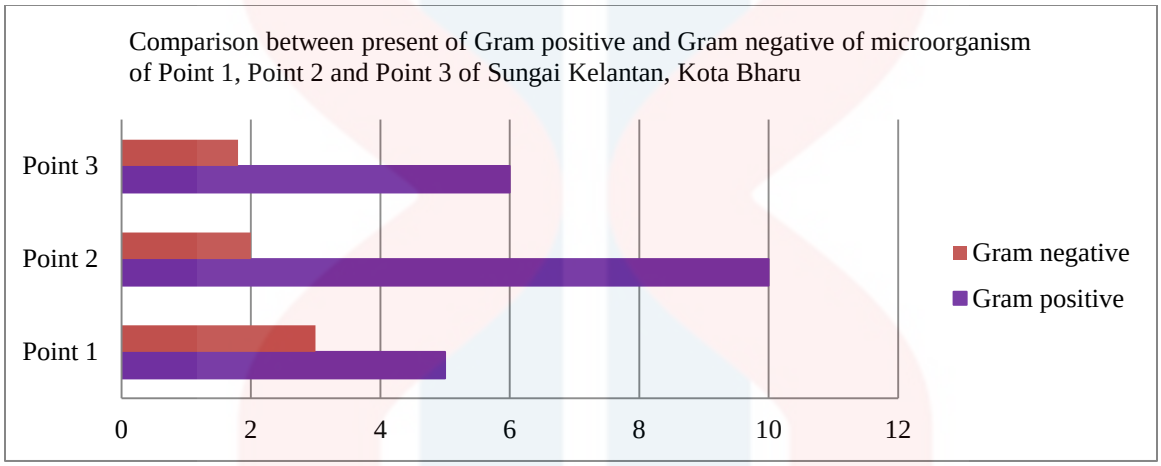

Figure 4.3: Comparison between present of Gram positive and Gram negative microorganism of Point 1, Point 2 and Point 3 of Sungai Kelantan, Kota Bharu

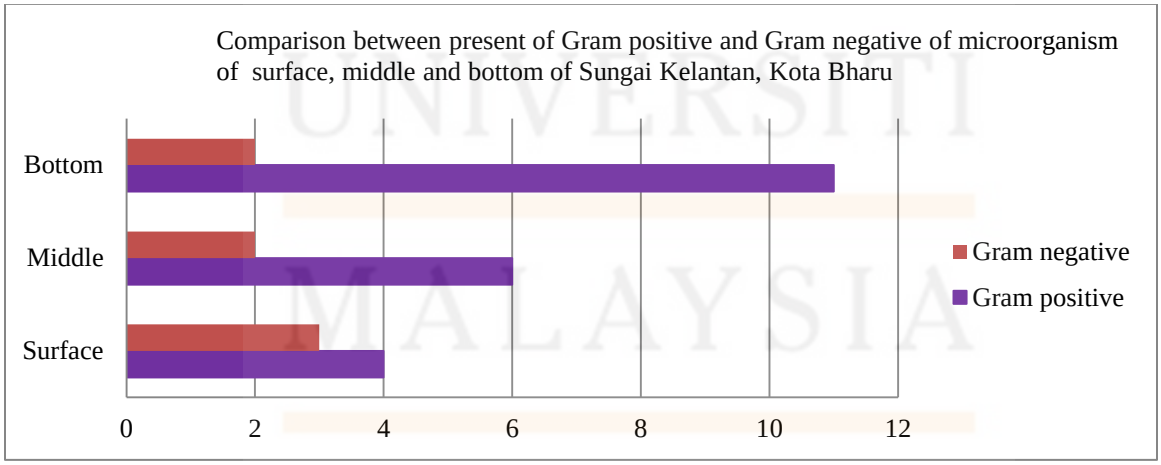

Figure 4.4: Comparison between present of Gram positive and Gram negative microorganism of surface, middle and bottom of Sungai Kelantan, Kota Bharu

4.2 Determination of microorganism diversity based on molecular based identification of microorganisms found in Sungai Kelantan, Kota Bharu

For DNA extraction chemicals and buffer that used are CTAB, TE, lysozyme, Proteinase K, chloroform, cold ethanol and 70% ethanol. CTAB function is to disrupt membrane and release DNA. While, TE buffer used to protect DNA from degraded and act as storage for DNA. Lysozyme used to break down cell wall of microorganism and Proteinase K function is to breaks down protein and degrades nuclease or other enzyme presence (Nishiguchi et al., 2002).

Whereas chloroform function is to solubilize lipid and protein and remove them from DNA while cold ethanol used to separate and precipitate DNA. In the other hand, 70% ethanol used to wash away salts from DNA. After DNA extraction method was done there was no DNA fiber found but still proceed for agarose gel electrophoresis step.

It can be said that Proteinase K and lysozyme are needed in this DNA extraction step. Although there was no presence of DNA fiber there was still probability that the DNA was there. Thus step AGE must be conducted to view existence of DNA. Steps of inverting the centrifuge tube, pipetted in and out and also flick seem to be not important however actually these steps are crucial to ensure that the solution reacted well with each other.

For AGE process this experiment used TAE and ethidium bromide. According to Guo & David (2009) separation of nucleic acid such as DNA and RNA can be done by using TAE buffer. TAE made up of Tris that act as cation while EDTA serves as cations and prevent DNA from molecular secondary structures. Fluorescent tag such as Ethidium bromide enables viewing of DNA. AGE have several application. In this experiment, AGE used to estimate DNA molecules size and analyzing PCR amplified product.

Guo & David (2009) stated there are several things that influence movement of DNA in well which are DNA's molecular weight, power of machine, agarose concentration, buffer used and visualization. The most crucial factor is the DNA size as smaller molecules travel fast and far (Guo & David, 2009).

Power of machine also important in getting good results as the voltage high the faster the DNA travel but too much heat will melting the agar. High voltages usage also decreases the resolution of the agar (above about 5 to 8 V/cm) (Guo & David, 2009). In term of concentration of AGE powder indicated that diluted concentration produces high movement speed of DNA transfer (Brody *et al.,* 2004) and the distance between DNA bands of a given length is determined by the percent agarose in the gel. High concentration of AGE powder suitable to be run with pulsed field electrophoresis (PFE), or field inversion electrophoresis (Brody *et al.* 2004).

Usually 1% agarose used in many research but 0.7% is good separation or resolution of large five to 10kb DNA fragments whereas 2% concentration used for small 0.2 to one kb fragments and 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case (Guo & David, 2009). Brody *et al.* (2004) stated that the most common buffers for agarose gel are TAE (tris acetate EDTA), TBE (Tris/Borate/EDTA) and SB (Sodium borate). TAE usually used because it has highest resolution for larger DNA.

Lastly, the element that needs to be considered is the visualization of DNA bands under UV light. Dye usually used to make band visible such as Ethidium bromide. Any band containing more than ~20ng DNA becomes visible when under UV light.

EtBr is dangerous because it is carcinogenic and safer dye can be used such as Gel Stare dye (Brody *et al.*, 2004). Gel usage benefit to user as it is easy to pour, not harmful to samples and samples can be recovered. However, gels can melt during electrophoresis and the buffer can wear off. Gels can be stored under chiller at 4°C in plastic bag. All the ethidium bromide contaminated waste must needs to be disposed through chemical and biological safety and need to wear double gloves when handling AGE (Guo & David, 2009). The AGE conducted was using one percent concentration (80 g AGE powder/ 79 ml one time TAE).

Figure 4.5 showed no presence of DNA fragment only markers were visible due to incomplete extraction method DNA. The DNA extraction method did not apply Proteinase K and lysozyme. The absence of both enzymes DNA cannot be extract properly as lysozyme used to break down cell wall of microorganism and Proteinase K used to breaks down protein and degrade nuclease or any other enzyme presence (Brody *et al*., 2004).

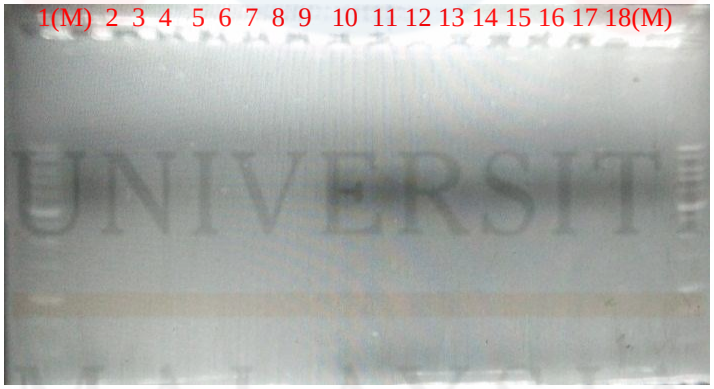

Figure 4.5: 1% AGE was run with 80 volt power for 45 minutes. The result from AGE of DNA from water microorganism of Sungai Kelantan, Kota Bharu was no presence of DNA band. Well 1(M) and well 18(M) were loaded with one kb lambda marker.

Figure 4.6 showed DNA fragment of microorganisms from Sungai Kelantan, Kota Bharu. However the concentration of the DNA is very low as the band is not shown clearly. Well number 2(M) was served as marker. Ladder used was 100 bp ladder in this experiment. As shown in Figure 4.6 the DNA band of water microorganisms of Sungai Kelantan, Kota Bharu was very dim. According to Brody *et al.* 2004 any band containing more than ~20ng DNA becomes distinctly visible.

Hence, water microorganisms of Sungai Kelantan, Kota Bharu really have DNA concentration less than 20 ng and explained the dim apperances of DNA band from Sungai Kelantan, Kota Bharu when viewing under ultraviolet. This prediction supported by nanodrop spectrophotometry reading at A260/A280 at Table 4.7.

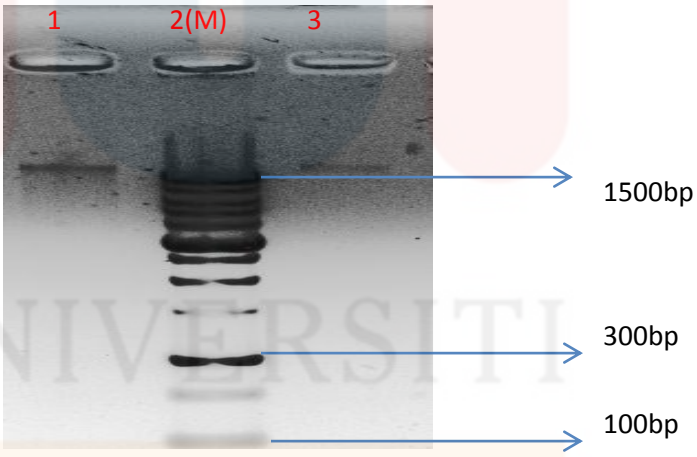

Figure 4.6 showed DNA fragment resulted from DNA extraction from microorganism of Sungai Kelantan, Kota Bharu. Well 1 and well 3 were DNA band for samples from Sungai Kelantan, Kota Bharu. Well 2(M) was filled with marker. Comparison between DNA band of water microorganism of Sungai Kelantan, Kota Bharu and 100 bp ladder. The DNA mass was stated as 72.5 (ng/ 5µl). Thus one µl of sample of Sungai Kelantan, Kota Bharu predicted to has 14.5 nanogram DNA. 1% AGE was used and ran for 30 minutes by using 80 volt power.

Figure 4.7 showed that DNA fragment of water microorganism of Sungai Kelantan, Kota Bharu from DNA extraction. According to Brody *et al.* 2004 any band containing more than ~20ng DNA becomes distinctly visible. This explained the dimness of DNA band of water microorganism of Sungai Kelantan, Kota Bharu on AGE when viewed on ultraviolet. This prediction supported by Nanodrop spectrophotometry reading at A260/A280 at Table 4.7.

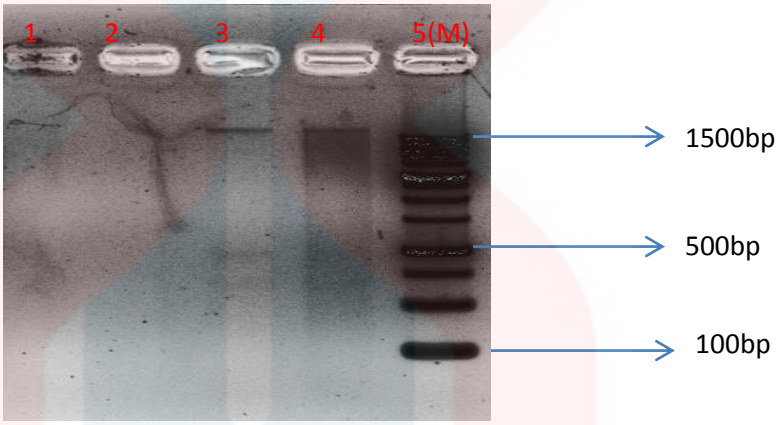

Figure 4.7 showed DNA band of water microorganism of Sungai Kelantan, Kota Bharu from DNA extraction. It showed comparison between DNA band of water microorganisms from Sungai Kelantan,Kota Bharu and ladder lambda 1kb. The band of DNA were really light at well 3 and well 4 and no band at all on well 1 and 2. Well 5(M) served as marker. DNA mass was stated as 42 (ng/ 5µl) thus it was predicted one µl of sample of Sungai Kelantan, Kota Bharu has 8.4 nanogram of DNA. This explained the dimness of DNA band. 1% AGE was used and ran for 30 minutes by using 80 volt power.

Table 4.7 showed DNA concentration reading of water microorganism at Sungai Kelantan, Kota Bharu from Nanodrop spectrophotometry that conducted in this experiment. According to Thermo Fisher Scientific (2010) the cause of negative values of P3 Bottom no 1(Figure 4.5, well 1) based on Figure 4.7 was dirty pedestal. It may also happen because using blank with higher absorbance than selected samples. The solution is wiping the pedestal with soft lens tissue and make new clean blank (Thermo Fisher Scientific, 2010).

Table 4.7 showed DNA concentration of water microbes from Sungai Kelantan, Kota Bharu reading from Nanodrop spectrophotometry

DNA extraction from samples	DNA concentration (ng/ µl)	A260/A280 (A)
P1 Surf no 1(Figure 4.5 well 3)	5.094	1.41
P1 Middle no 1(PCR sample)	11.829	1.79
P1 Bottom no1(no age run)	114.643	2.04
P1 Bottom no 2(Figure 4.6 well 3)	4.610	1.44
P2 Bottom no 1(Figure 4.6 well 4)	8.954	1.54
P2 Bottom no 5(Figure 4.6 well 2)	0.789	0.59
P3 Bottom no 1(Figure 4.6 well 1)	-2.512	2.04
P3 Bottom no 3(Figure 4.5 well 1)	11.126	1.72

According to Janda & Abbot (2007) 16S rRNA gene sequences commonly used to study bacteria phlogeny and taxonomy. 16s was used because of its presence in almost all microorganisms as multi gene family or operons. Patel (2001) stated that 16S rRNA gene (1,500 bp) is large thus it is suitable for informatics purposes and research. Lorenz (2012) stated that the PCR reaction results can be adjusted by changing variables such as increasing reagent concentrations and adding cycling affect the outcome of the amplicon profile. The best way to optimize PCR reaction is to changing its annealing temperature.

However, verification of human error must be taken into account and confirming all reagents was added to a given reaction and no contamination during process and no presence of primer dimers (Petti, 2007). Roche (2006) stated that nonspecific product of annealing and primer elongation events are called primer dimer. Primer dimer occurred when PCR reagents are mixed at room temperature. Nonspecific amplification can occurred when one primer elongated by wrong nucleotide. Primer dimer interrupting formation of specific PCR product and causing inefficient amplification thus lead to unsuccessful PCR.

Figure 4.8 showed PCR product of water microorganism of Sungai Kelantan, Kota Bharu were at well 3 and 4. Based on Figure 4.8 there were a lot of bright band. These results may caused by over-amplification of the PCR product. According to Palumbi *et al.* (2002) the alternatives for optimizing PCR are diluting the PCR product as a template or conducting new PCR reaction at >55°C or raising the annealing temperature.

Reducing the template amount, annealing at temperature of two to five degree Celsius, reducing the number of cycles, reducing $MgCl_2$ concentration and testing primer to ensure its specific gene is correct are optimization that can be done to overcome multiple band problem according to Palumbi *et al.* (2002). Based on Figure 4.8 the PCR products from river water microorganism of Sungai Kelantan, Kota Bharu were located lower from its supposedly position. This may due to the AGE machine error.

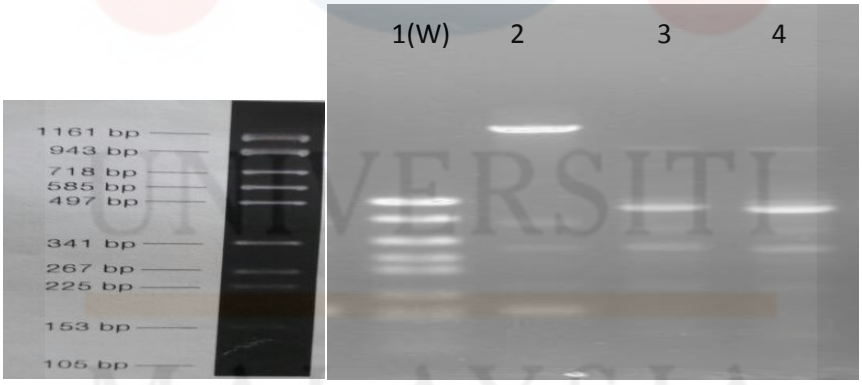

Figure 4.8 showed DNA concentration of one kb ladder and band of DNA of water microorganism from Sungai Kelantan, Kota Bharu from PCR product. It showed presence of multiple bands. 1% AGE was used and ran for 45 minutes by using 80 volt power. Well 1(W) was loaded with marker.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Determination of water microorganism diversity in Sungai Kelantan, Kota Bharu based on morphology observation and Gram stain

Throughout this research it can be found that water microorganism diversity could be determine by its morphology distribution and abundance of gram negative and gram positive microorganism present in Sungai Kelantan, Kota Bharu. Morphology of microorganisms was determined in term of form, elevation, margin, surface appearance, opacity and chromogenesis. Based on this finding within this research, it can be said that water microorganism of Sungai Kelantan, Kota Bharu was diverse. From 27 morphology observation of microorganims there are three types of form found which were irregular, circular and filamentous. At Point 1 there were 3, at Point 2 there were 14 while at Point 3 there were 6. For circular there were 4, 1 and 4 following Point 1, Point 2 and Point 3. Whereas for filamentous only found at Point1 which was 2 and Point 3 only one. There were two elevations found which were raised and flat. Point 1 have 9 raised, Point 2 have 14 raised while Point 3 have 11 raised and only one flat. There were four margins found which were filiform, entire, undulate and lobate. At Point 1 there were one filiform and lobate while entire were 4 whereas undulate were 3. At Point 2 there were only 5 entire and 9 undulate were found. At Point 3 there were one filiform and undulate found whereas 7 for entire whereas lobate found were 2.

For surface there were four types found which were glistening, smooth, dull and rough surfaces. Point 1 have 2 glistening, 5 smooth and 1 dull types of surfaces. Point 2 have 2 glistening, 9 smooth, and one rough types of surfaces. Point 3 has several surfaces found which were 4 glistening, 3 smooth and dull and one rough surface. For opacity there were only two type found which were translucent and opaque. Point 1 has 4 opaque and translucent while Points 2 has 7 translucent and 5 opaque whereas Point 3 have 5 translucent and 6 opaque. For chromogenesis there were three which were cream, yellow and purple. Points 1 have one cream and purple colour. Point 2 have 11 cream and one yellow microorganism. While Point 3 has 9 cream and 2 yellow microorganism.

Gram stain was conducted to distinguish between gram positive and gram negative microorganisms. The finding of this stain was presence of both grams from river water samples of Sungai Kelantan, Kota Bharu. There were several shape found during this research such gram positive with bacillus shape, gram positive with spirilium shape, gram positive with steptobacilli shape whereas small bacilli for gram negative shape, long bacilli for gram negative shape. Picture of both Grams inserted at Appendices. This showed water microorganism from river water sample of Sungai Kelantan, Kota Bharu was diverse. From 27 microorganism growth in this experiment the result for staining are at Point 1 Gram positive found was 5 while Gram Negative is 3. At point 2, 10 Gram positive and 2 Gram Negative. While at Point 3 Gram positive is 6 and Gram Negative is 1.

5.2 Recommendations

It is recommended for future study of this topic molecular based identification method is used for recognition of microorganism species level. After polymerase chain reaction (PCR) step, the next steps will be purification of PCR and final steps before species level recognition is DNA sequencing. More optimization in DNA extraction and PCR method is needed in further studies. Human error and systematic error must be reduced to get optimum results. It is also recommended for future study of this topic to use selective medium such as Mac Conkey agar or EMB agar. Usage of selective medium will enable selected colony to grow on it.

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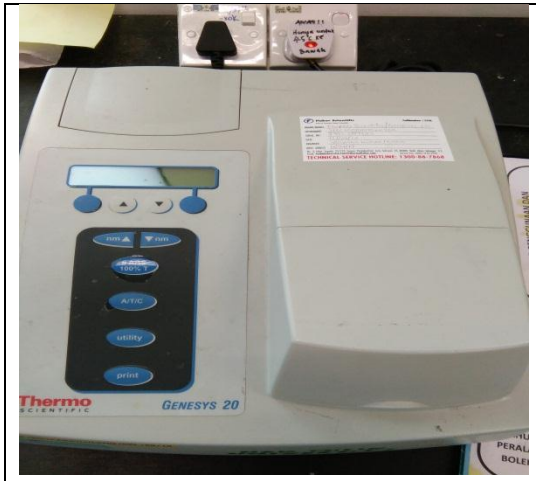
APPENDICES

Machines used in this research



Spectrophotometry

Shaking water bath

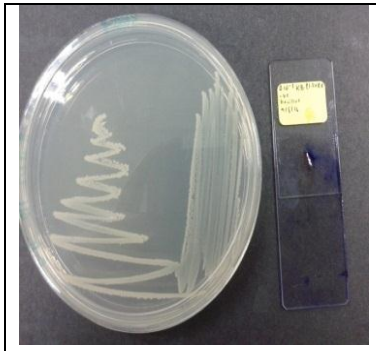
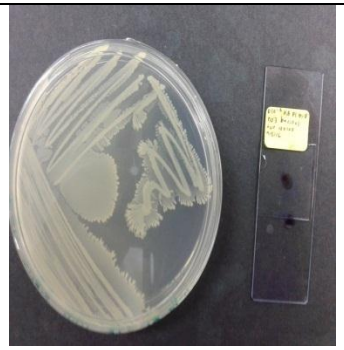
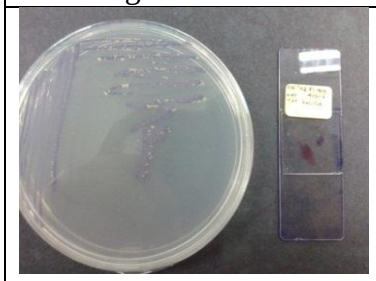
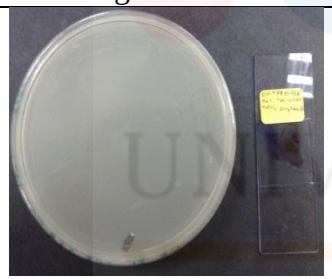
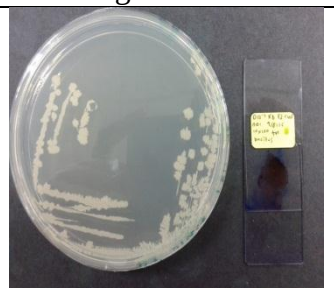
Centrifuge machine

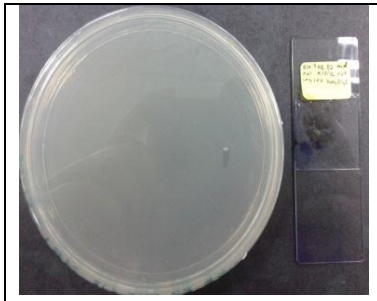
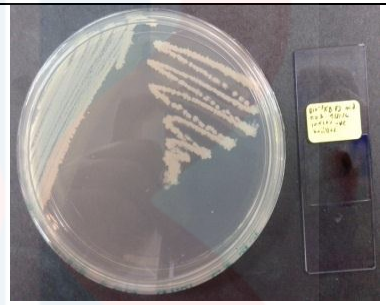
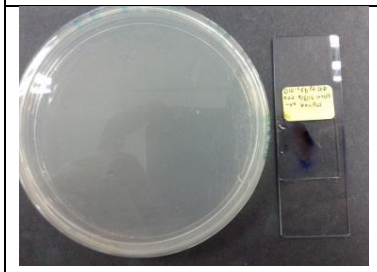
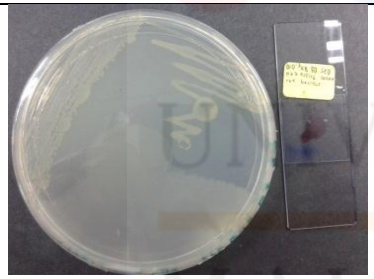
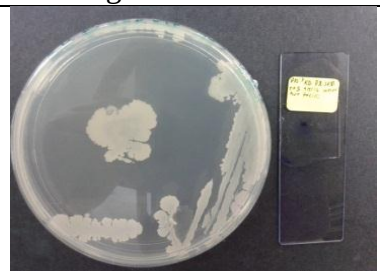
Nano drop spectrophotometry

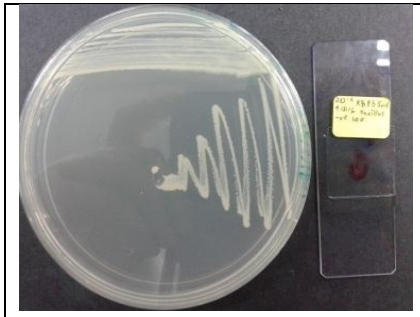
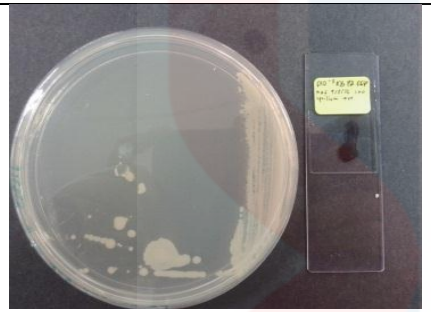
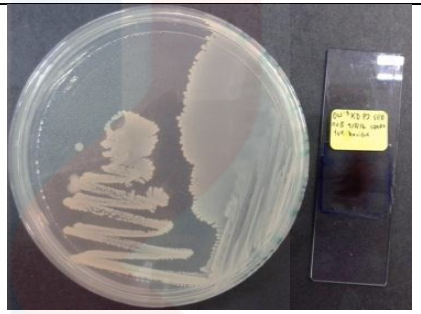
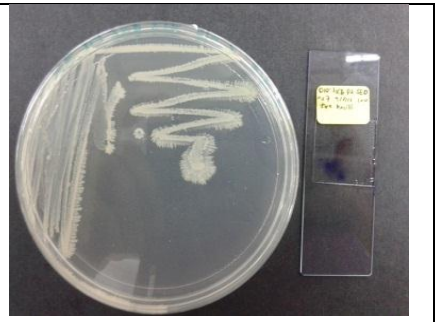
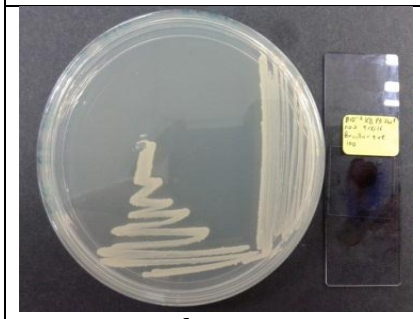
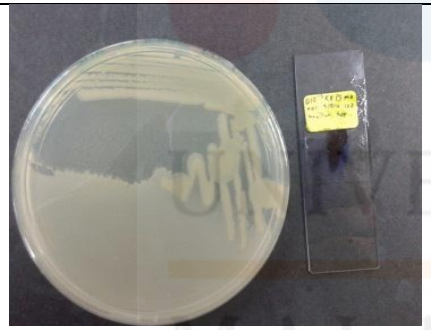
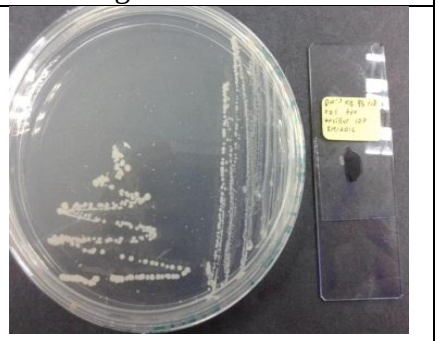
PCR machine

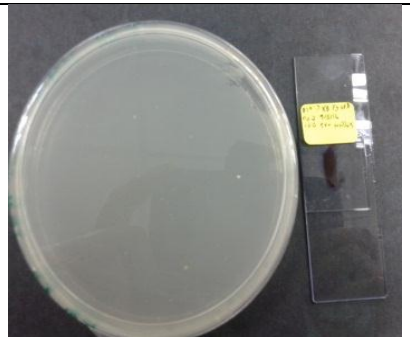
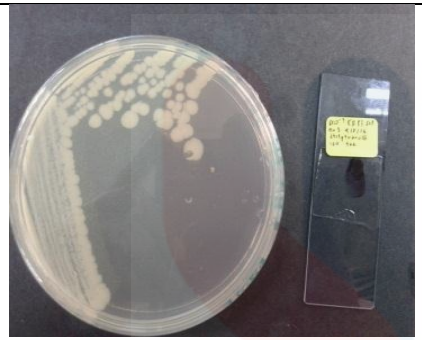
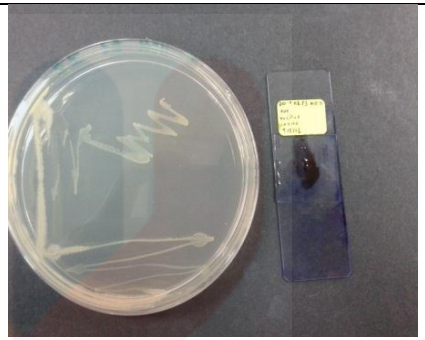
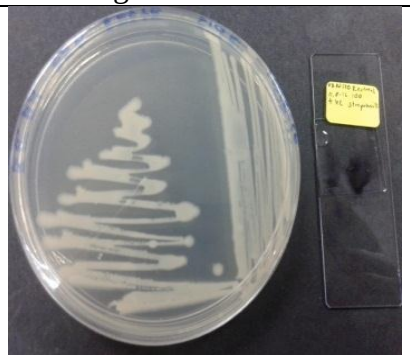
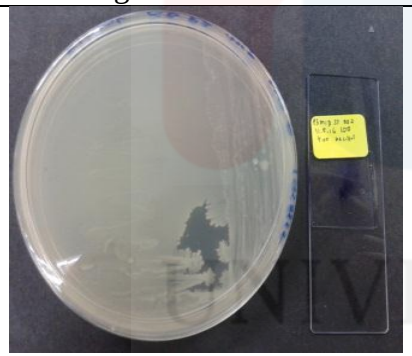
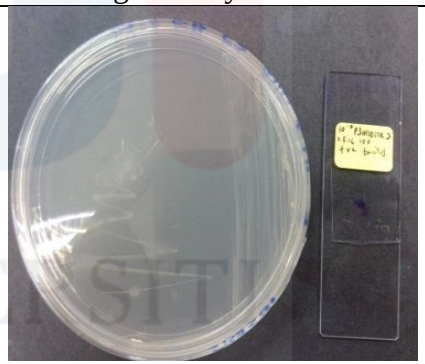
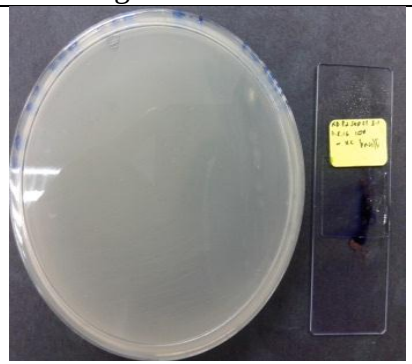
Incubator shaker

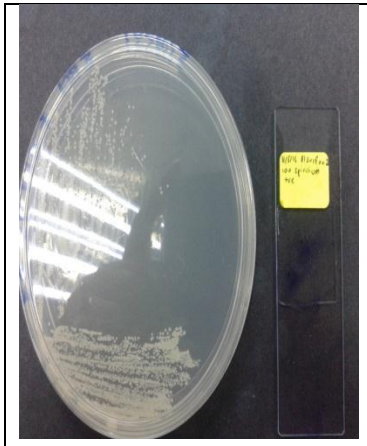
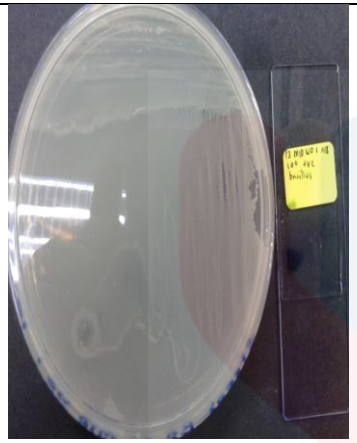
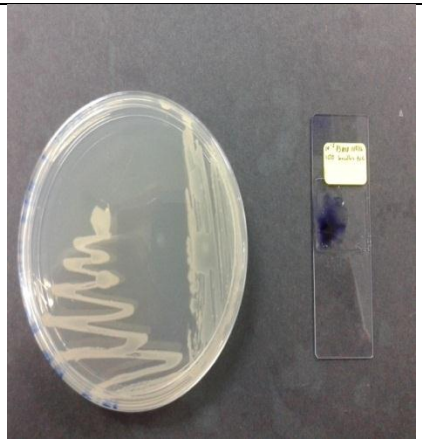
Morphology observation of water microorganism of Sungai Kelantan, Kota Bharu

 Form: Irregular Elevation: Raised Margin: Filiform Surface: Glistening Opacity: Translucent Chromogenesis: Cream	 Form: Irregular Elevation: Raised Margin: Entire Surface: Smooth Opacity: Translucent Chromogenesis: Cream	 Form: Irregular Elevation: Raised Margin: Undulate Surface: Dull Opacity: Opaque Chromogenesis: Cream	 Form: Filamentous Elevation: Raised Margin: Undulate Surface: Smooth Opacity: Translucent Chromogenesis: Cream
 Form: Circular Elevation: Raised Margin: Entire Surface: Glistening Opacity: Opaque Chromogenesis: Purple	 Form: irregular Elevation: flat Margin: entire Surface: dull Opacity: translucent Chromogenesis: cream	 Form: Irregular Elevation: Raised Margin: Entire Surface: smooth Opacity: opaque Chromogenesis: cream	 Form: Irregular Elevation: Raised Margin: Undulate Surface: Smooth Opacity: Translucent Chromogenesis: Cream

 Form: Irregular Elevation: flat Margin: undulate Surface: smooth Opacity: translucent Chromogenesis: cream	 Form: Irregular Elevation: raised Margin: Filiform Surface: dull Opacity: translucent Chromogenesis: Cream	 Form: Irregular Elevation: raised Margin: undulate Surface: smooth Opacity: translucent Chromogenesis: cream	 Form: irregular Elevation: raised Margin: undulate Surface: smooth Opacity: opaque Chromogenesis: cream
 Form: irregular Elevation: raised Margin: undulate Surface: smooth Opacity: opaque Chromogenesis: cream	 Form: irregular Elevation: raised Margin: entire Surface :glistening Opacity: translucent Chromogenesis: yellow	 Form: irregular Elevation: raised Margin: undulate Surface: smooth Opacity: opaque Chromogenesis: cream	 Form: irregular Elevation: raised Margin: undulate Surface: smooth Opacity: opaque Chromogenesis: cream

 Form: circular Elevation: raised Margin: entire Surface: smooth Opacity: opaque Chromogenesis: cream	 Form: circular Elevation: raised Margin: entire Surface: glistening Opacity: translucent Chromogenesis: cream	 Form: irregular Elevation: raised Margin: undulate Surface: smooth Opacity: opaque Chromogenesis: cream	 Form: Irregular Elevation: raised Margin: undulate Surface: smooth Opacity: translucent Chromogenesis: cream
 Form: Irregular Elevation: raised Margin: undulate Surface: dull Opacity: translucent Chromogenesis: cream	 Form: irregular Elevation: raised Margin: undulate Surface: glistening Opacity: opaque Chromogenesis: cream	 Form: irregular Elevation: raised Margin: entire Surface: rough Opacity: opaque Chromogenesis: cream	 Form: circular Elevation: raised Margin: entire Surface: glistening Opacity: opaque Chromogenesis: cream

 Form: irregular Elevation: flat Margin: undulate Surface: glistening Opacity: translucent Chromogenesis: cream	 Form: circular Elevation: raised Margin: entire Surface: dull Opacity: opaque Chromogenesis: cream	 Form: irregular Elevation: raised Margin: entire Surface: glistening Opacity: translucent Chromogenesis: yellow	 Form: Filamentous Elevation: raised Margin: Filiform Surface: dull Opacity: translucent Chromogenesis: cream
 Form: irregular Elevation: raised Margin: entire Surface: dull Opacity: opaque Chromogenesis: cream	 Form: irregular Elevation: raised Margin: lobate Surface: dull Opacity: translucent Chromogenesis: cream	 Form: irregular Elevation: raised Margin: entire Surface: glistening Opacity: translucent Chromogenesis: yellow	 Form: irregular Elevation: flat Margin: entire Surface: dull Opacity: translucent Chromogenesis: cream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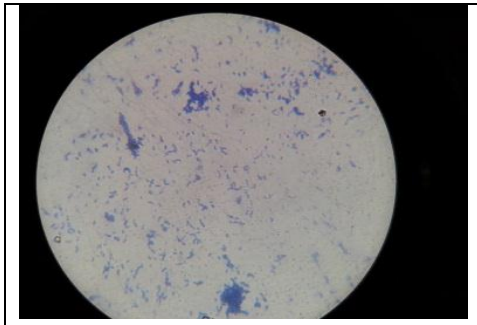
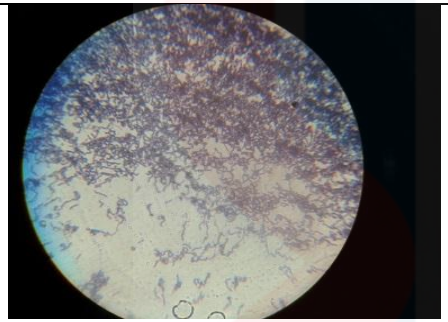
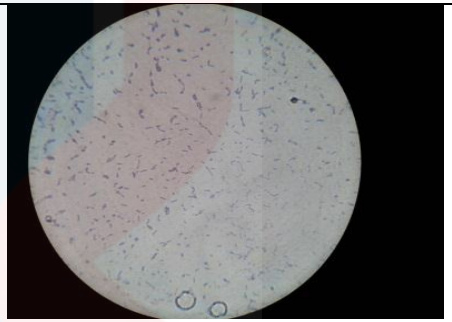
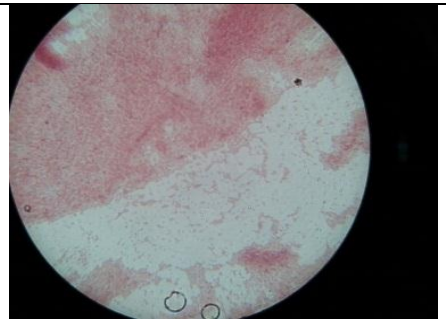
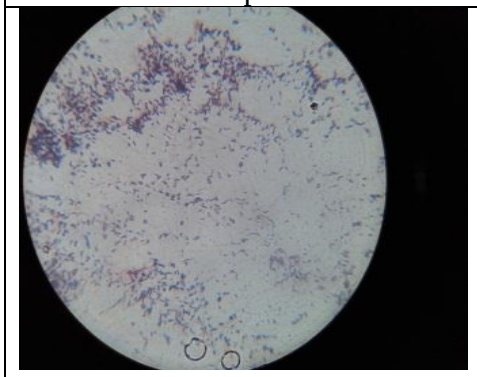
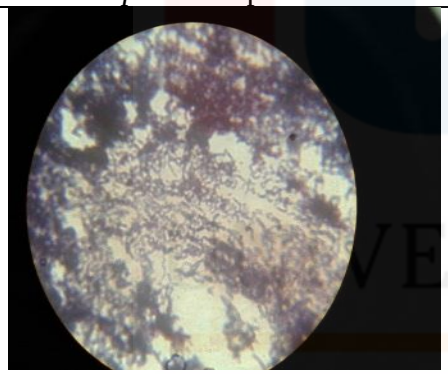
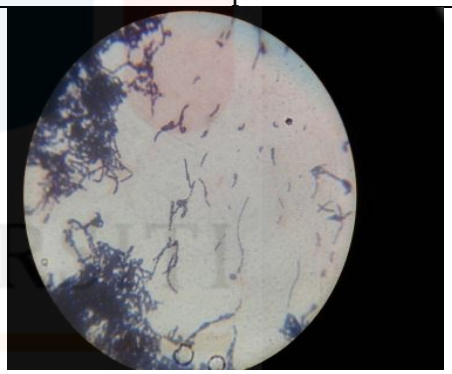
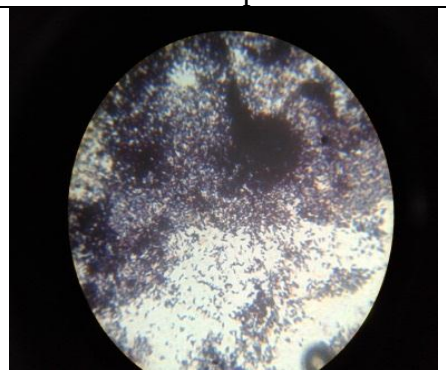
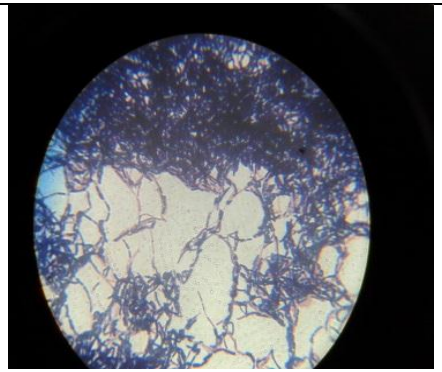
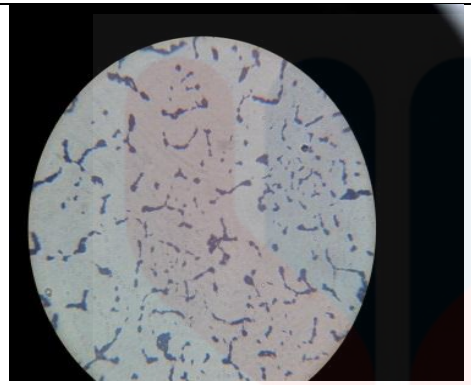
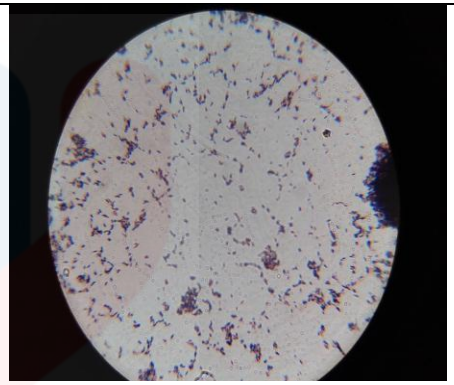
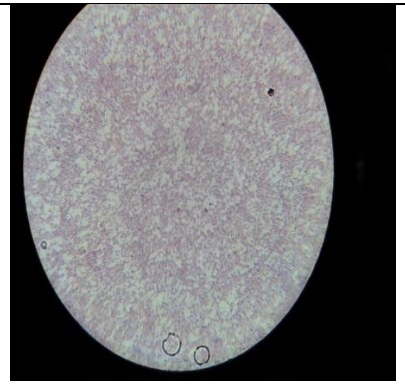
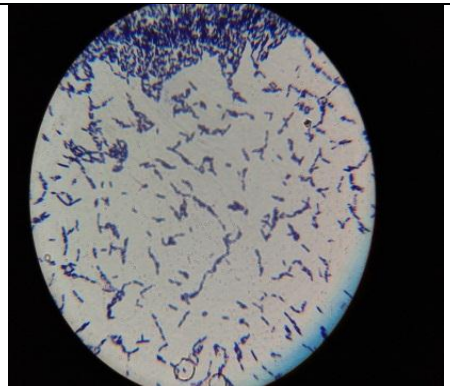
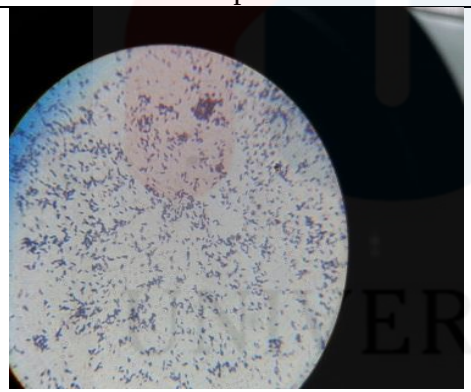
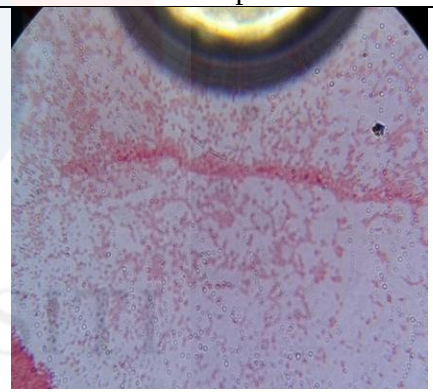
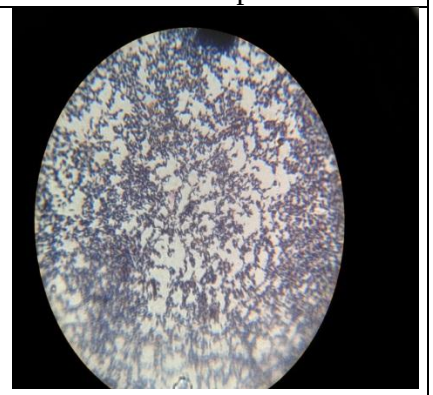
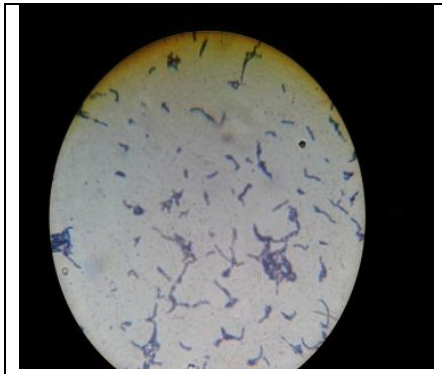
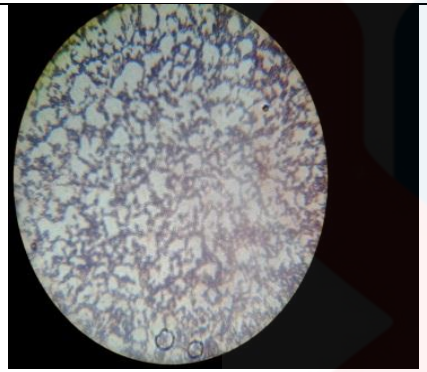
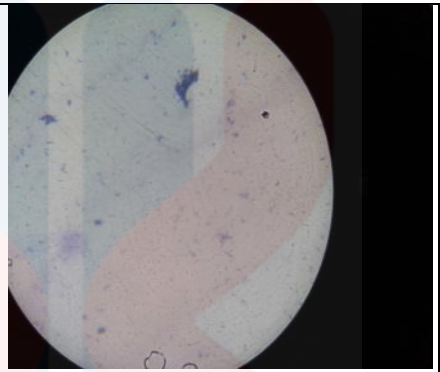
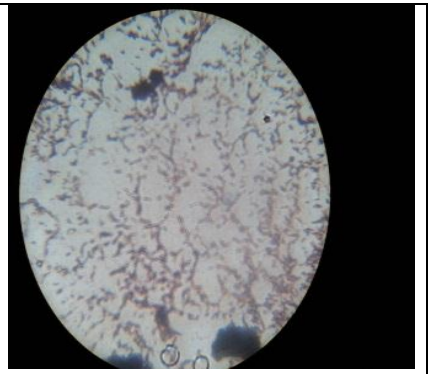
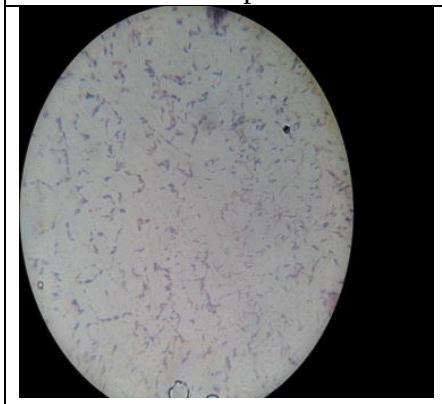
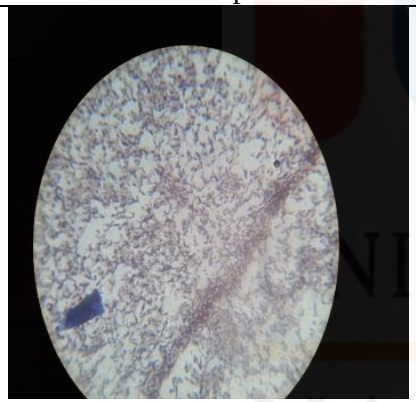
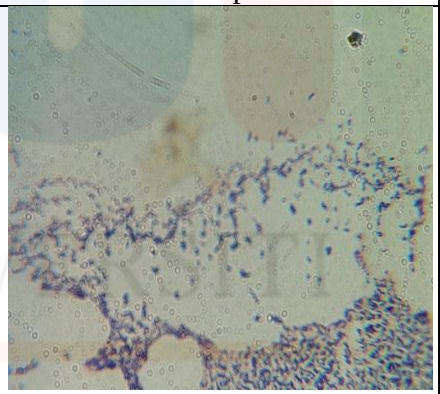
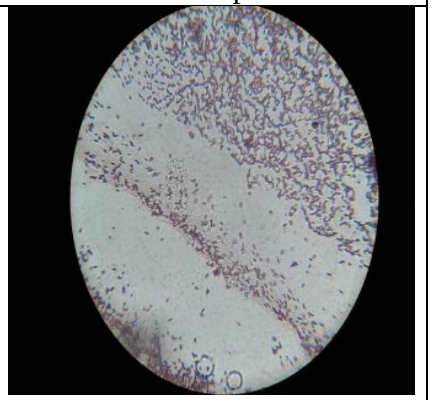
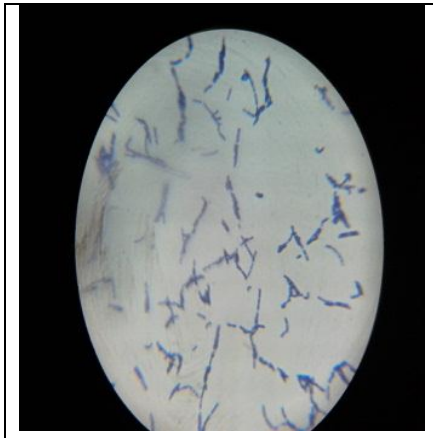
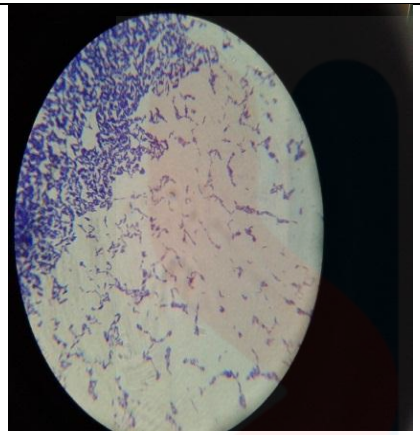
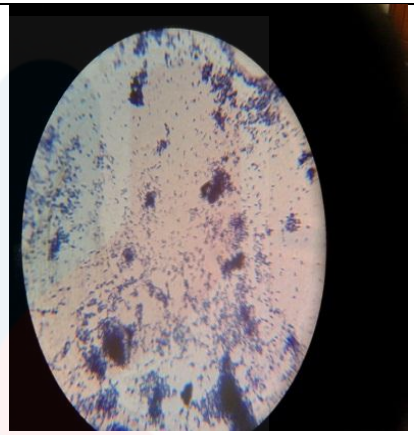
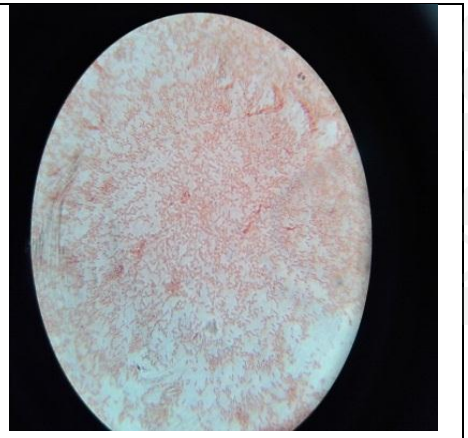
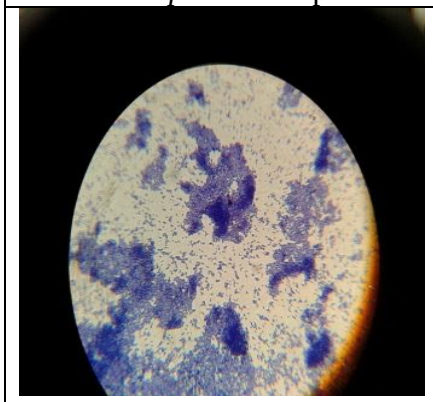
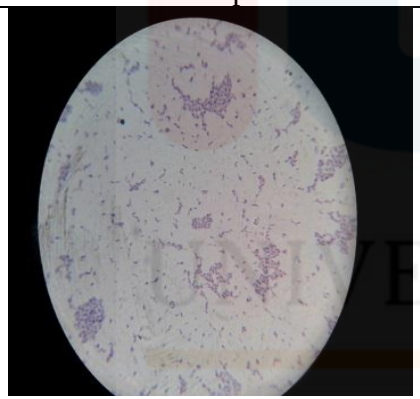
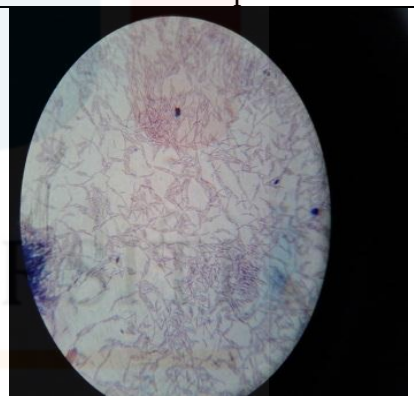
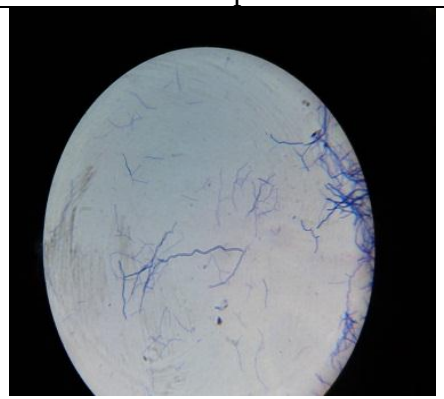
Gram staining of water microorganism of Sungai Kelantan, Kota Bharu

 Point: Point 2 Bottom species 5 Gram: Positive Shape: Small Rod Genus: *Bacillus* sp.	 Point: Point 2 Bottom species 6 Gram: Positive Shape: Sprilium Genus: *Spirilium* sp.	 Point: Point 2 Bottom species 8 Gram: Positive Shape: Rod Genus: *Bacillus* sp.	 Point: Point 3 Surface species 1 Gram: negative Shape: Small Rod Genus: *Bacillus* sp.
 Point: Point 2 Bottom species 7 Gram: Positive Shape: Rod Genus: *Bacillus* sp.	 Point: Point 3 Bottom species 1 Gram: Positive Shape: Small Rod Genus: *Bacillus* sp.	 Point: Point 2 Bottom species 4 Gram: Positive Shape: Streptobacilli Genus: *Streptobacilli* sp.	 Point: Point 3 Middle species 1 Gram: Positive Shape: Small Rod Genus: *Bacillus* sp.

 Point: Point 3 Bottom species 3 Gram: Positive Shape: Streptobacilli Genus: *Streptobacilli* sp.	 Point: Point 3 Bottom species 2 Gram: Positive Shape: Small Rod Genus: *Bacillus* sp.	 Point: Point 1 surface species 1 Gram: Positive Shape: Small rod Genus: *Bacillus* sp.	 Point: Point 1 Middle species 1 Gram: Negative Shape: Rod Genus: *Bacillus* sp.
 Point: Point 1 surface species 3 Gram: Positive Shape: Rod Genus: *Bacillus* sp.	 Point: Point 1 Middle species 2 Gram: Positive Shape: Rod Genus: *Bacillus* sp.	 Point: Point 1 surface species 2 Gram: Negative Shape: Small rod Genus: *Bacillus* sp.	 Point: Point 1 Middle species 3 Gram: Positive Shape: Small Rod Genus: *Bacillus* sp.

 Point: Point 1 Bottom species 1 Gram: Positive Shape: Rod Genus: *Bacillus* sp.	 Point: Point 2 Surface species 1 Gram: Positive Shape: Small Rod Genus: *Bacillus* sp.	 Point: Point 2 Middle species 1 Gram: Positive Shape: Small Rod Genus: *Bacillus* sp.	 Point: Point 2 Middle species 3 Gram: Negative Shape: Small Rod Genus: *Bacillus* sp.
 Point: Point 2 Bottom species 3 Gram: Positive Shape: Small Rod Genus: *Bacillus* sp.	 Point: Point 2 Bottom species 2 Gram: Negative Shape: Rod Genus: *Bacillus* sp.	 Point: Point 3 middle species 1 Gram: Positive Shape: Small rod Genus: *Bacillus* sp.	 Point: Point 3 surface species 1 Gram: Negative Shape: Small rod Genus: *Bacillus* sp.

 Point: Point 2 Bottom species 4 Gram: Positive Shape: streptobacilli Genus: *Streptobacilli* sp.	 Point: Point 3 middle species 1 Gram: Positive Shape: Rod Genus: *Bacillus* sp.	 Point: Point 3 middle species 2 Gram: Positive Shape: Small rod Genus: *Bacillus* sp.	 Point: Point 2 Bottom species 3.1 Gram: negative Shape: Small rod Genus: *Bacillus* sp.
 Point: Point 3 middle species 2 Gram: Positive Shape: Small rod Genus: *Bacillus* sp.	 Point: Point 3 middle species 1 Gram: Positive Shape: Small rod Genus: *Bacillus* sp.	 Point: Point 2 Bottom species T3 Gram: Negative Shape: Long rod Genus: *Bacillus* sp.	 Point: Point 1 surface species 2 Gram: Positive Shape: Spirilium Genus: *Spirilium* sp.