BIOINDICATOR AND MOLECULAR-BASED WATER QUALITY ASSESSMENT OF SELECTED WATER BODIES IN PENINSULAR MALAYSIA

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BIOINDICATOR AND MOLECULAR-BASED WATER QUALITY ASSESSMENT OF SELECTED WATER BODIES IN PENINSULAR MALAYSIA

ABSTRACT

Water bodies are essential to every living organism. A number of attributes such as temperature, dissolved mineral contents, can be used to assess whether or not the water body is contaminated with bacteria. In addition, water quality can be assessed using both physico-chemical parameters and bioindicators. Water quality assessment was performed using physico-chemical and biological aspects in two water bodies of varying location namely Fraser's Hill and Pangkor Island. Between these two water bodies the physico-chemical properties that were found to be of significant difference was, amount of dissolved oxygen (DO), temperature (°C) and total suspended solid (TSS). There was no significant difference in the other tested parameters which were Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Ammonia Nitrogen (NH_3-N) and pH. Test conducted using aquatic water beetles as bioindicators found that Fraser's Hill had more diverse beetle families compared to Pangkor Island. On top of physico-chemical parameters and bioindicators, presence of bacteria was also studied using *16S* rDNA to evaluate presence of bacteria in different water locations in these two water bodies. Bacterial DNA was detected in all the water samples collected from Pangkor Island whereas only one sample was detected in the Fraser's Hill water sample. In conclusion, the water index (WQI) of the water bodies in Fraser's Hill and Pangkor Island showed Class I and Class II readings respectively in line with the distribution of water beetle and presence of bacteria.

Keywords: water beetle, diversity, abundance, water quality, bacteria *16S* rDNA

BIOPENUNJUK DAN PENILAIAN KUALITI AIR MELALUI KAEDAH MOLEKULAR DI SEMENANJUNG MALAYSIA

ABSTRAK

Air merupakan sumber kehidupan yang penting bagi semua kehidupan. Beberapa aspek boleh digunakkan untuk mengesahkan ketulenen sumber air samada ia telah di cemari oleh kehadiran kuman atau tidak. Tambahan juga, kualiti air boleh dikaji mengunakkan kedua-dua parameter kimia-fizik dan biopenunjuk. Dua sumber air yang berbeza lokasi telah dikaji untuk mengenalpasti tahap kualiti air iaitu Fraser's Hill dan Pulau Pangkor. Antara dua lokasi sumber air tersebut beberapa perbezaan dari segi sifat kimia-fizik iaitu, oksigen terlarut (DO), jumlah pepejal yang terapung dan suhu badan air (°C) didapati menunjukkan perbezaan ketara. Tidak terdapat perbezaan ketara dari segi sifat fizik-kimia yang dikaji dari segi keperluan oksigen biokimia (BOD), keperluan oksigen kimia (COD), kandungan ammonia (NH_3-N) dan pH. Kajian menggunakkan biopenunjuk kumbang air mendapati badan air di Fraser's Hill mempunyai kepelbagaian yang kerap dari segi spesis familia kumbang air berbanding dengan Pulau Pangkor. Selain daripada sifat kimia-fizik dan biopenunjuk, kehadiran bakteria juga telah dikenalpasti menggunakkan kaedah *16S* rDNA dalam kedua-dua badan air. DNA bakteria telah dikenalpasti di semua tempat kajian di Pulau Pangkor manakala, satu lokasi bakteria dikenalpasti di Fraser's Hill. Indeks Kualiti Air (WQI) di Fraser's Hill mengklasifikasikan sampel air tersebut dalam kategori kelas I manakala sampel air Pulau Pangkor menunujukkan air tersebut dikelaskan dalam kategori kelas II sejajar dengan pengagihan kumbang dan bakteria.

Kata Kunci: kumbang air, kepelbagaian, kelimpahan, kualiti air, bakteria rDNA *16S*

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LIST OF SYMBOLS AND ABBREVIATIONS

±	:	plus minus
≤	:	less than or/ and similar to
°C	:	Degree Celsius
µl	:	Microliter
g	:	Earth's gravitational acceleration
A260/A230	:	Absorbance at 260nm over substance at 230nm
A260/A280	:	Absorbance at 260nm over substance at 280nm
AGE	:	Agarose Gel Electrophoresis
BLAST	:	Basic Local Alignment Search Tool
bp	:	base pairs
BOD	:	Biochemical Oxygen Demand
COD	:	Chemical Oxygen Demand
DNA	:	Deoxyribonucleic acid
dNTP	:	deoxynucleotide triphosphate
DO	:	Dissolved Oxygen
DOE	:	Department of Environmental
E-value	:	Expectation value
max	:	Maximum
mg	:	Milligram
min	:	Minutes
N	:	Number
NH_3-N	:	Ammonia Nitrogen
nm	:	Nanometer
PCR	:	Polymerase Chain Reaction

pH	:	power Hydrogen
rDNA	:	Ribosomal DNA
RT	:	room temperature
s	:	Total number of taxa in the community
SDI	:	Simpson Diversity Index
sp	:	Species
TSS	:	Total Suspended Solid
WHO	:	World Health Organization
WQI	:	Water Quality Index

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of the study

The earth's rivers, lakes and oceans are mainly composed of a clear transparent chemical substance known as water. The chemical formula for water is H_2O which means this chemical molecule is made of two hydrogen atoms and one oxygen atom that are bonded by a covalent bond. Water exists in three states of matter which are liquid, solid and gas vapor however the term water strictly refers to the freely flowing liquid state of this particular substance that exist at a specific standard ambient temperature and pressure. Water that is safe to be consumed or to be used in the preparation of food without the risk of health problems is known as drinking water or also known as potable water (Greenwood & Earnshaw, 1997). Water is obviously important for living organisms to stay hydrated and to produce food (Atwood, 2017). The quality of water bodies reflects living activities occurring around its area.

Water bodies are essential to every living organism (Wada et al., 2017). In order to determine the quality of any given water body, a number of attributes such as temperature, oxygen availability, dissolved mineral contents, sediment and substrate type, the presence of pollutants such as pesticides, acidic materials and heavy metals, and whether or not the water body is contaminated with bacteria need to be assessed (Bartram & Balance, 1996; Toriman et al., 2012). These qualities are then compared to numeric principles to determine its safety level for a specific utilisation (Gail, 2001). Besides that, water quality can also be determined by using the diversity of the beetle taxa as a bioindicator (Heino, 2002; Briers & Biggs, 2003; Sánchez-Fernández et al., 2006).

A bioindicator refers to a living organism that is capable of indicating whether or not an environment is healthy (Li et al., 2010; Kamarudin et al., 2012). These living organisms serve as a 'marker' to an ecosystem due to their response to any given ecological pressures (McGeoch, 1998; Shahabuddin & Verma, 2003; Friberg et al., 2011).

Since different kinds of organisms require different amount of oxygen to survive, the presence or absence of certain types of organisms can be used to indicate the pollution level in the water (Holt & Miller, 2011; Hamza-Chaffai, 2014). In this approach, living organisms or their responses to the environment are observed to determine the quality of the aquatic environment they inhabit (Barbour & Paul, 2010). Water beetles refers to all aquatic beetles that inhabit stagnant water or slow moving water that are usually like ponds, dams, pools, lakes and the edges of streams (Miller, 2003; Heino et al., 2005). They are important elements in the lentic and lotic trophic webs that participates in the energy flow and nutrient cycling (Vanni, 2002). The families of beetles are Dytiscidae, Gyrinidae, Haliplidae, Noteridae, Amphizodiae, Hygrobiidae, Meruidae and Hydroscaphidae (Balke et al., 2005). These beetles are also important food source for other aquatic animals of the particular water resources. Microbial organisms, especially bacteria, for example, are used to monitor water quality as they possess many advantages over other organisms such as size and wide range of habitats (Figueras & Boreggo, 2010). The presence of bacteria correlates to the lower amount of dissolved oxygen in any water body (Dabkowski, 2012); thus, a lower amount of dissolved oxygen in water bodies reflects the level of pollution in the water. The study of environmental metagenomics also plays an important role in detecting the presence of bacteria in any water body.

Environmental metagenomics refers to the DNA-based microbial detection approach conducted on any given environment (Zeyaullah et al., 2009). In metagenomics, the collective genome (metagenome or microbiome) of coexisting microbes known as microbial species (Ghazanfar et al., 2010) is randomly sampled from the environment and subsequently sequenced (Schloss & Handelsman, 2003; Cardoso et al., 2012). By directly assessing the collective genome of co-occurring microbes, metagenomics has the potential to give a comprehensive view of genetic diversity, species composition, evolution and interactions in the environment of natural microbial communities (Simon

& Daniel, 2011). Generally, *16S* rDNA gene was amplified to assess bacterial diversity in metagenomic samples (Devine et al., 2012). Therefore, in this study, *16S* rDNA gene was isolated from metagenomic samples obtained from two water bodies to detect the presence of bacteria in the sampled water. This was done in order to meet the objective of this study which was to determine the water quality of two water bodies in Peninsular Malaysia i.e. the streams in Fraser's Hill and Pangkor Island. Besides that, the diversity of the water beetles and physical parameters of the water bodies were also assessed to determine the quality of the water bodies at both locations. Even though environmental issues in Malaysia, were subjected to the measures taken to address the problem have been inadequate and inconsistent thus far (Jahi, 2002). Studies related to Malaysian environmental issues are at present, very limited and positive effects to measure on riverine biota were rarely documented (Syafiuddin et al., 2018).

1.2 Objectives of the study

The overall aim of this study was to determine the level of water quality in Fraser's Hill and Pangkor Island using physical, chemical and biological indicators. The specific objectives were:

(i) to determine the Water Quality Index (WQI) of selected water bodies in Fraser's Hill and Pangkor Island;

(ii) to isolate and identify water beetle species present in the water bodies;

(iii) to optimize DNA isolation procedure from water-based metagenomic samples

(iv) to detect the presence of bacteria in the two water bodies via PCR using a universal *16S* ribosomal DNA primer pair.

CHAPTER 2: LITERATURE REVIEW

2.1 Water

Water is vital for the survival of human and virtually plays an important role for every living thing. The quality of water either for industrial purposes or for domestic usages is an important factor that need to be measured. According to Davis and De Weist (1996), the standard for drinking water has been set based on criteria, which includes, the colourless, and odourless. Besides that, the water must have taste; and should not possess any adverse physiological particles. The chemistry of water changes due to the presence of certain minerals and makes the water undesirable for consumption (Sauvant & Pepin, 2002). Serious environmental and health issues may rise when the water supply system is not coupled with appropriate sanitation (Ayodele & Olusiji, 2015). Therefore, there is a need to set a standard to measure water quality.

To date, water is often discussed from two perspectives, i.e, physical and normal forms. In the physical perspective, water is judged based on the colour, and is described as blue, green or grey water (Shiklomanov, 1993). In the normal perspective, water is considered as a foremost source of life and is the basic need for living beings (Breckenbridge, 2005). Water is mainly found on the earth's surface in liquid state under certain temperature and pressure, but it can also be in a solid or in gaseous state. The solid state of water can be found in the form of ice, glaciers and the gaseous state can be seen as vapor, cloud, fog and mist (APEC, 2011).

The Earth's surface is covered with nearly 71% of water and serves as an essential element for living beings. Earth's ocean comprises of nearly 97% of water and hardly 1.7% of water is available in ground water (Rahmanian et al., 2015; Nasirian, 2007). Moreover, 2% of Earth's water is in the form of ice or icebergs in Antartica and 0.001% is in the form of vapour in air (Rahmanian et al., 2015). Overall, rivers, lakes and the

water in atmosphere comprise of less than 0.3% (Bhat, 2014). A water molecule travels to different parts of the world due to its atmospheric circulation. The evaporation, transpiration, condensation, precipitation and run-off is a continuous cyclic process to reach the sea or ocean beds (Bhat, 2014). The water vapour redistributes the energy through evaporation and transpiration, which ultimately contributes to the precipitation on land. There is also a large amount of water stored into different types of minerals in hydrated forms (Gail, 2001). In nature, water is the available abundantly and is being preserved and maintained through rainfall, surface water, sub-surface water and ground water (Sharma, 2014).

Portable water is essential to all living organisms living and the quality of water plays an important role for survival (Nithyanandam et al., 2015). Safe drinking water or portable water is a precise necessary factor and its availability has improved quite a bit in the last few decades all over the globe (Shaheed et al., 2014). But still, basic facilities are lacking, such as access to safe drinking water and proper sanitation facilities (Onda et al., 2012). In addition, studies have estimated that by 2025, more than half of the world's population will be facing acute water shortage and water-based vulnerable diseases (Swain et al., 2010; Hunter et al., 2009). A report, issued by Natural Resource Defence Council Washington (NRDCW) in November 2009, suggests that by 2030, the water demand will exceed supply by 50% in some of the developing nations of the globe (Huang et al., 2015).

2.2 Water quality assessment

Maintaining good water quality is of major concern. A good water quality includes water that are free from environmental pollutions, microbes, degraded particles, and waste deposits (Efe, 2002b). Water quality determines the eminence of human life, it is very important for us to keep a constant watch on the quality of water (WHO, 2012). Assessment should be done based on temperature of water, minerals dissolved. Besides,

the presence of microbes are essential for maintaining the quality of human life (Kumar & Gopal, 2015). After investigating all the parameters, water samples can be compared to standard samples and specific guidelines in order to decide on whether or not the water sample can be used for specific purposes. The quality of water resources like lakes, oceans, rivers, rain water and ground water has to be preserved for the future (Shaheed et al., 2013). Not only it plays an important role in drinking water is also crucial in industrial applications (Gupta et al., 2015), agricultural, livestock production, food, cosmetics, pharmaceutical and many more (Cosgrove & Loucks, 2015). At present, the quality of water has deteriorated due to an increase in human population size and an escalation of the level of industrialisation and urbanisation (Mohamed et al., 2015). Therefore, it is very important to keep a constant watch on the quality of water, mainly in those areas where availability of quality water is very minimal.

The quality of a water body can be determined by assessing the following parameters namely physical parameter, chemical parameter and biological parameter. The physical and chemical components of a water sample can be determined by assessing the pH value, the amount of dissolved oxygen (DO), the degree of biological oxygen demand (BOD), chemical oxygen demand (COD), the total suspended solid, and the level of soil erosion (Santhosh et al., 2008; Lee et al., 2015). On the other hand, the biological parameters are studied by assessing the presence of pathogenic microbes and bioindicators like beetles (Wright et al., 2000).

2.2.1 Physical parameters

2.2.1.1 Temperature and pH

As temperature has an impact on both the chemical and biological characteristics of surface water, the level of dissolved oxygen (DO) in the water, photosynthesis rate of aquatic plants, metabolic rates of aquatic organisms and the sensitivity of these organisms to pollutions, parasites, and diseases are found to play a major role in water quality

(Wetzel, 2001). According to Driche et al. (2008), an increase of pH value in water bodies was due to the photosynthetic activities and consumption of oxygen (O_2) by algae present in water. According to the Department of Environment of Malaysia, the pH level in a range of 6.5 to 8.5 is acceptable for domestic water supply. Measuring the pH values in a water body acts as an indicator, since high and low pH can indicate water corrosion, metallic taste, or a slippery feel or a soda taste (Wurts, 2012).

2.2.1.2 Dissolved oxygen (DO)

Another important physical parameter that was usually used to assess quality is the amount of dissolved oxygen (DO). DO is a measure of the oxygen present in water (Carpenter, 1965; Antonopoulos & Gianniou, 2003). Fish and many aquatic animals depend on DO for their survival. DO establishes an anaerobic environment, a condition in which oxidised forms of many constituents are predominant in the water (Gupta et al., 2017). Anoxic conditions in water reduce oxidised forms of chemical species and frequently lead to the release of undesirable odours which results in the development of a toxic condition (EPA, 2012). DO is estimated in milligram per liter (mg/L) or parts per million (ppm). Generally, cold water and fresh water can hold more oxygen compared to warm water and salt water due to the solubility of oxygen which dissolves easily in cold water compared to warm water (Gandaseca et al., 2011). The DO level must not exceed 8 mg/L for the survival of aquatic fish in warm water (EPA, 2012).

Adequate DO (5.0 mg/L) is necessary for a good water quality and aerobic life forms; therefore, if DO level in water dropped below 5.0 mg/L level, aquatic organisms would become stressed (Robinson et al., 2004). As the concentration becomes lower, the stress becomes greater, and this may cause the fish in the river to die (Wang et al., 2003). The typical DO concentration levels observed in streams and rivers throughout the world are between 3 to 9 mg/L (Robinson et al., 2004).

2.2.2 Chemical Parameters

2.2.2.1 Biochemical Oxygen Demand (BOD)

Most of the water-borne bacteria, algae and other macro-organisms survive on the natural organic detritus and organic waste. These bacteria decompose organic materials using DO, thus reducing the level of DO in a water body. Biochemical Oxygen Demand (BOD) is a "measure of amount of oxygen that bacteria will consume while decomposing organic matter under aerobic conditions" (Sullivan et al., 2010). BOD is measured by incubating the samples for five days and the loss of oxygen from the beginning to the end of the test period will be measured. In general, unpolluted water bodies will have a BOD in value of 2 mg/L or less while the value for waste waters may go up to 10 mg/L or more. If effluents with high BOD levels were discharged into a river, bacterial growth will accelerate as they consume DO in the river (Kulshrestha & Sharma, 2006; Kumar et al., 2012). A typical concentration range of BOD for streams and rivers throughout the world is between 2 to 15 mg/L (Robinson et al., 2004).

2.2.2.2 Chemical Oxygen Demand (COD)

Chemical oxygen demand (COD) is defined as the total quantity of oxygen required to oxidise all the organic matter into carbon dioxide and water (Agbaire et al., 2009). COD values are always greater than BOD values, but COD measurements can be made in a few hours while BOD measurements take five days (Garg et al., 2010; Salih et al., 2013). The COD in unpolluted surface waters usually ranges from 20 mg/L or less to greater than 200 mg/L in waters receiving effluents (Agbaire et al., 2009; Garg et al., 2010). If any effluents with high BOD values were released into rivers or streams, the bacterial growth in those water bodies will get activated and consume more oxygen, leading to a reduction of DO in a river (Shah & Joshi, 2017).

2.2.2.3 Ammonia nitrogen

The growth of algae, bacteria and other plants is dependent on the nutrients available in water which is in the form of phosphorus and nitrogen. Fertilizers, septic water, waste water and wastes from pets or other animals are the typical source of these nutrients (Carpenter et al., 1998). However, excess amount of phosphorous in a water body can help in the excessive growth of vegetation and may reduce DO level (Smith & Schindler, 2009). Nitrogen (N_2), the other form of nutrients for bacteria, algae, etc., is found in a water body in the form of nitrate (NO_2), nitrite (NO_3), and ammonia (NH_4) (Baker et al., 2016). In aerobic waters, ammonia is converted to nitrate (Bristow et al., 2016). High levels of ammonia in water indicates low DO and an increased pH, which indirectly effects the aquatic organisms in a water body (Luo et al., 2014). The nitrogen concentration of more than in 0.5 mg/L in water is considered toxic and when consumed, may cause blue-baby syndrome in infants (Santamaria, 2006). The United States Environmental Protection Agency (EPA) had set a standard for nitrogen levels in water and suggested that the value should not be more than 10 mg/L (Mensinga et al., 2003). If the content of nitrogen in a drinking water exceeded their concentration, symptoms such as restlessness, dullness, weakness, muscle tremors, profuse salivation, vocalisation, lung edema, tonic-clonic convulsion and finally, death due to heart failure may be presented by a person who consumed it (Markesbery et al., 1984; Camargo & Alonso, 2006; Majumder et al., 2006; Ojosipe, 2007). The high level of nitrite with low amount of ammonia enhances self-purification activities of the surface water which increases the rate of nitrification-denitrification, and transformation process in river water (Li & Bishop, 2004). The different levels found in these parameters can determine if the water source is safe for human consumption or if the water source is harmful to human health (Onda et al., 2012).

2.2.2.4 Total Suspended Solids (TSS)

Total suspended solids (TSS) load is described as the concentration of solid-phase material suspended in liquid mixture of water sediment. It is usually expressed in milligrams per liter (mg/L) (Gray et al., 2000). High levels of TSS are usually considered as harmful to human life and may affect central nervous system causing dizziness, numbness, irritation on lips and face (Miller & Layzer, 2005). The presence of organic chemicals in a water body imparts an objectionable taste, odour and gives different colour to water (Avvannavar & Shrihari, 2008). TSS is an important factor to be taken into account during the measurement of pollution caused by urbanisation. Pollutants such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), phosphorous and organic compounds are adsorbed onto receiving water bodies of reservoirs which can indicate high TSS load (Rossi et al., 2005).

2.2.2.5 Water Quality Index (WQI)

Assessment of the quality of water is very important in order to make sure that the water is fit for consumption. To date, many methods have been established to assess water quality. One of the simplest methods to determine water quality is by conducting chemical analysis (Nithyanandam et al., 2015). Real-time monitoring of water quality can also be done to assess the purity level of a water body mainly by measuring the level of alkalinity, pH value, colour of water, taste and odour, radon, heavy metals, and the presence of hormones (Global Environment Centre, 2014). Water Quality Index (WQI) is a specific measurement which allows direct comparisons of the overall quality of different water sources even though the concentration range of the individual constituents may vary in any given water body (Stoner, 1978). This quality index was developed by Horton (1965) in the United States of America. The index was developed by selecting and assessing ten different water quality parameters such as the amount of DO, BOD, COD, pH value, the number of fecal coliforms, alkalinity and the level of chloride, nitrates, total phosphates

present in the sampled water (Nithyanandam et al., 2015). It is noteworthy that the weightage of each parameter reflects significantly on the WQI for the different uses of water. Although water quality index is widely used and accepted in the European, African and Asian countries (Horton, 1965), many modifications including one conducted by Alobaidy et al. (2010), were made to improve the safety of water (Bhargava et al., 1998). The modifications were made due to the nature of study which cannot assess water quality over a long span of time (Flotemersch et al., 2006). In this thesis, to overcome this inadequacy, a bioindicator-based approach was used to assess long term ecological changes in water bodies (Sharma et al., 2008).

2.3 Bioindicators

Bioindicators are living organisms like animals, plants, microbes or planktons that can be used to indicate the health of an ecological niche or a natural ecosystem (Holt & Miller, 2011; Parmar et al., 2016). The naturally prevailing bioindicators can act as tools for the detection of any changes in the environment or a society (Khatri & Tyagi, 2015).

For example, copepods and other small water crustaceans present in water bodies can be monitored for changes in physiological and biochemical parameters. When tropic conditions are favourable, the copepods are found abundant in water bodies like rivers, waterfalls, and lakes (Gonzalez et al., 2011). Many environmental factors promote the survival of bioindicator in an ecological area and allows the measurement of the effectiveness of reservoirs (Andrulewicz et al., 2008). The number of individuals of each bioindicator will reflect the ecological changes in environmental conditions of a locality (Fabricius et al., 2012). Changes in the environment are regularly attributed to anthropogenic disturbances (Tejerina-Garro et al., 2005). Today bioindicators are used to study all types of environments with all kinds of major taxonomic groups (Doughty 1994; El-Din et al., 2013). Bioindicator species successfully indicate a condition of the surroundings due to their mild tolerance to environmental variability (Holt & Miller,

2011). Biological indicators such as Ephemeroptera, Plecoptera, Mollusca, Trichoptera were often used to assess the quality of water (Figueras & Borrego, 2010). Amongst the many organisms used, aquatic beetles are found to be a good indicator for water quality assessments (Foster et al., 1989; Foster & Eyre, 1992; Sánchez-Fernández et al., 2004; Heino et al., 2005) and were used in different parts of the world for this purpose (Epele & Archangelsky, 2012; Barman & Gupta, 2015). It is noteworthy that the water beetles were used as bioindicators in Segura River Basin (Sánchez-Fernández et al., 2005) and Pangkor Island (Abdullah, 2006) to determine the quality of water in the water bodies.

2.3.1 Advantages of Bioindicators for Environmental Monitoring

Bioindicators are important elements in the lentic and lotic ecosystem that participate in energy flow and nutrient cycling. Besides, they are also an important food source for aquatic animals in water bodies. Thus, the community structure of aquatic insects depends on a range of entities such as water quality, type of substrate, particle size of sediment, water flow, sediment organic matter availability, oxygen concentration as well as environmental conditions surrounding the watercourse (Doughty, 1994).

Bioindicators utilise the biota to reflect the combined effects of both chemical pollutants and habitat alteration over time (Holt & Miller, 2011). Aquatic existence impacts their specific circumstance at some stages in their lifetime. For instance, these life forms can reflect on prior circumstances when conditions may have been more severe (Knoben et al., 1995). The duration of the past can be evaluated, and it relied upon the lifespan of the aquatic organisms under scrutiny. Therefore, the use of bioindicators is at a very basic level and is different from the classic measures of ecological quality and offers many advantages. First, bioindicators add a temporal component that relates to the life expectancy of an organism in a particular system, which permits the mix of present, past, or future ecological conditions (Mouillot et al., 2002). In addition, the tolerance range of bioindicators reflects the level of pollutants (Hamza-Chaffai, 2014). Another

advantage of bioindicators is their capacity to detect the biotic impacts of contaminations when many physical or chemical measurements are unable to do so (Hamza-Chaffai, 2014).

Since different kinds of bioindicators require different quantities of oxygen to survive, the presence or absence of certain groups of bioindicators may be used to indicate different water pollution levels. In this approach, water beetles or their responses to the environment were used to determine the quality of aquatic environment that they inhabit (Barbour & Paul, 2010). The low amount of dissolved oxygen in water bodies reflected the level of pollution in the aquatic ecosystem. According to studies by Stanley et al., (1994), Boulton (2003), and Bogan and Lytle, (2007), water beetles are good indicators as they can be used to assess the quality of the water bodies. The responses of insects to clean water are critical because they affect insect activity, distribution pattern and richness of species. Water beetles are considered as the best indicator group for selecting areas of high biodiversity in aquatic ecosystems due to their significant species richness patterns (Abellan et al., 2005). The total number and diversity of the aquatic beetles present at any given water body can be used to gauge the duration of pollution of that particular water body (Glastris et al., 2001). This is due to the fact that different aquatic beetle taxa have different living or survival requirements (Duran, 2006). The value of the unit of measurement for biodiversity depends on the uniqueness of the ecosystem with regards to the size of ecosystem. In addition, water beetles can also be used as the best indicator group for selecting areas for conservation (Sánchez-Fernández et al., 2006).

2.4 Water Beetles as Bioindicators

Fresh water invertebrates are extensively used to monitor the nutrients and pollutants in a conserved area and meet the criteria for the selection of an indicator taxa (Wright et al., 2000; Noss, 1990). Indicator taxa is the taxonomically stable invertebrates or bioindicators that are classified based upon their biology, stability, life cycle or life

history, geographical range, and their habitat (Sanchez-Fernandez et al., 2004). Water beetles are beetle species that inhabit stagnant water or slow-moving water such as ponds, dams, pools, lakes and the edges of streams (Miller, 2003). Water beetle species diversity changes upon slight changes occurring in an ecosystem (Dong et al., 2014) because they are sensitive to distinct level of pollutants. Their abundances reflect the levels of contamination in any given water body (Vazirani, 1977). The presence of these insects indicates that the water body contained an adequate level of oxygen. These insect families mainly eat debris and algae in the water which in turn, will keep the water body clean and clear (Prommi & Paayakka, 2015). The aquatic insects like water beetles are classified in the order of Coleoptera due to their tolerating nature to hydrological conditions (Ward & Neophytou, 1992).

2.4.1 Dytiscidae

The predaceous diving beetles, Dytiscidae belong to a diverse family which have the ability to adapt to different types of water bodies. The adult Dytiscidae are found in lentic ecosystems and are dependent of predation, stability, vegetation, pH and salinity (Fairchild et al., 2003). Figure 2.1 (a) and (b), depict the morphological structure of the species. The Dytiscidae are found at various lengths depending on the species (Larson & Labonte, 1994). This beetle family has an average length of 25 mm. The head and pronotum are dark brown and the elytra are yellow brown without yellow spots or markings. Dytiscidae have a visible scutellum that is completely covered by the corners of the elytra (Figure 2.1 a) (Friday, 1988). Both adult and larvae Dytiscidae attack a wide variety of small aquatic organisms, hence they are given the name predaceous beetle. All Dytiscidae are aquatic but these water beetles prefer stagnant water or slow-moving water such as ponds, dams, pools, lakes and the edges of streams (Miller, 2003). Dytiscidae are suitable for water quality monitoring programmes because they share the characteristics of macroinvertebrates such as crayfish, clams, snails, and worms that are also used for

the same programme (Chessman, 2003). These beetles are very sensitive to water pollution and are used as an effective bioindicator to assess water quality. Other beetles like Whirligig beetle larvae and riffle beetle larvae require dissolved oxygen in water, any slight variation in DO content can damage the larvae (da Rocha et al., 2010). Adult diving beetle use bubble mechanism of breathing during diving into the water. Among all other water beetle families this diving beetle is the most aggressive one when it comes to eating habits and prey anything in the water ranging from small fish to tadpoles, and glass-worms (Larson et al., 2000).

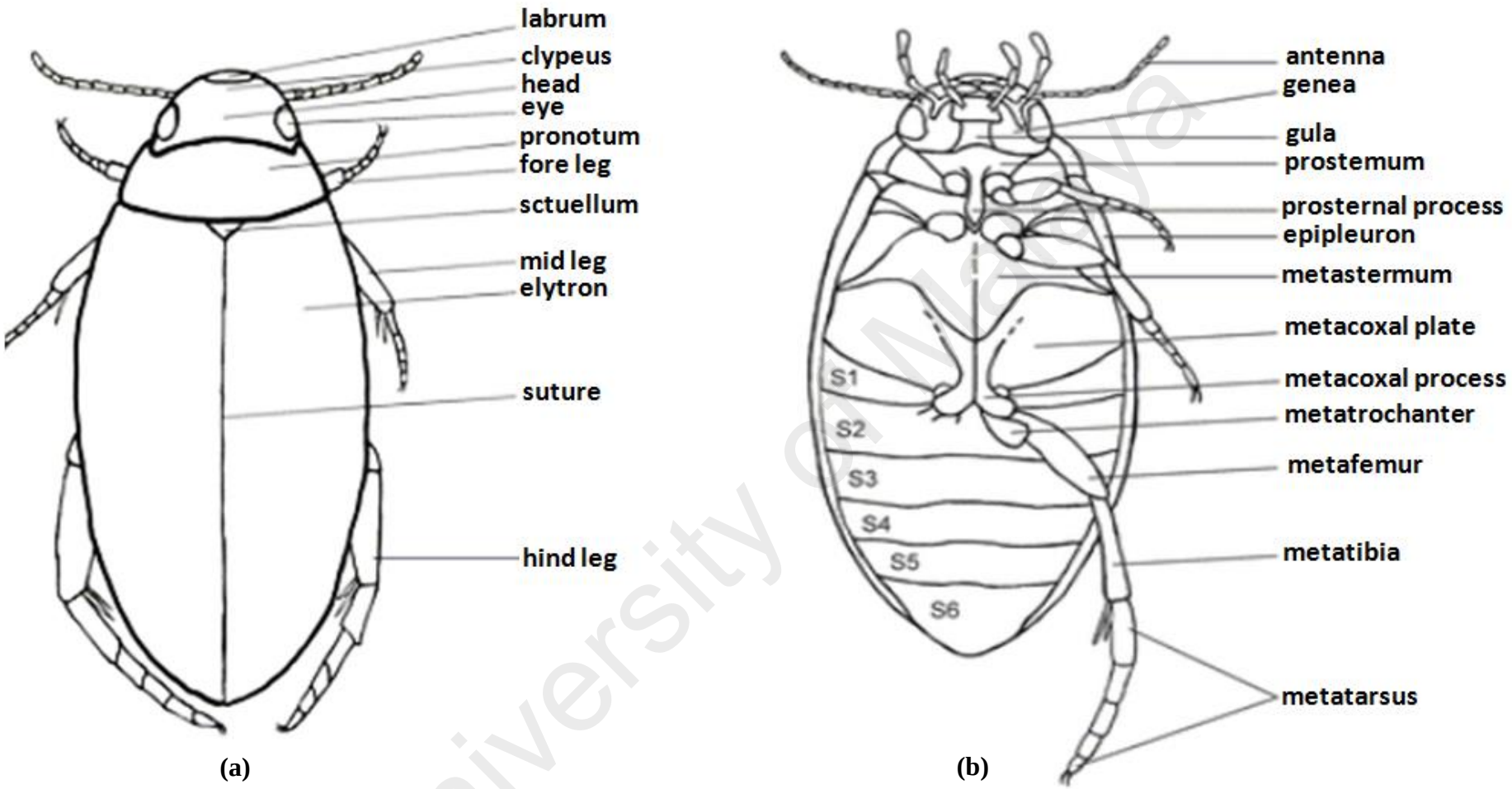


Figure 2.1: Morphological key of Dytiscidae in both (a) dorsal and (b) ventral adapted from Kehl (2014)**.**

2.4.2 Hydrophilidae

Hydrophilidae is known as the water scavenger beetles and were generally found in the shallower regions of wetlands. The body of this beetle group is streamlined and there are swimming hairs on the hind legs (Birmingham et al., 2005; Libonatti et al., 2011; Watts & Hamon, 2014). They are mainly distinguished by their long maxillary palpi that are longer than their antennas which functions as tactile (Fig 2.2 a, b). Hydrophilidae's bodies are either oblong or rounded with a size of 1-40 mm in length. The maxillary palpi are important to this beetle family because this structure enables them to breathe in water. Most Hydrophilidae have a Y-shaped impressed line on the vertex. The Hydrophilidae may superficially resemble Dytiscidae but the structure of their antenna allows them to be easily distinguished from Dytiscidae, where Hydrophilidae have clubbed antennae (da Rocha et al., 2010). Hydrophilidae are mostly aquatic but they have a subfamily called Sphaeridiinae which are found in dung, compost and decaying organic matters (Arnett & Thomas, 2000).

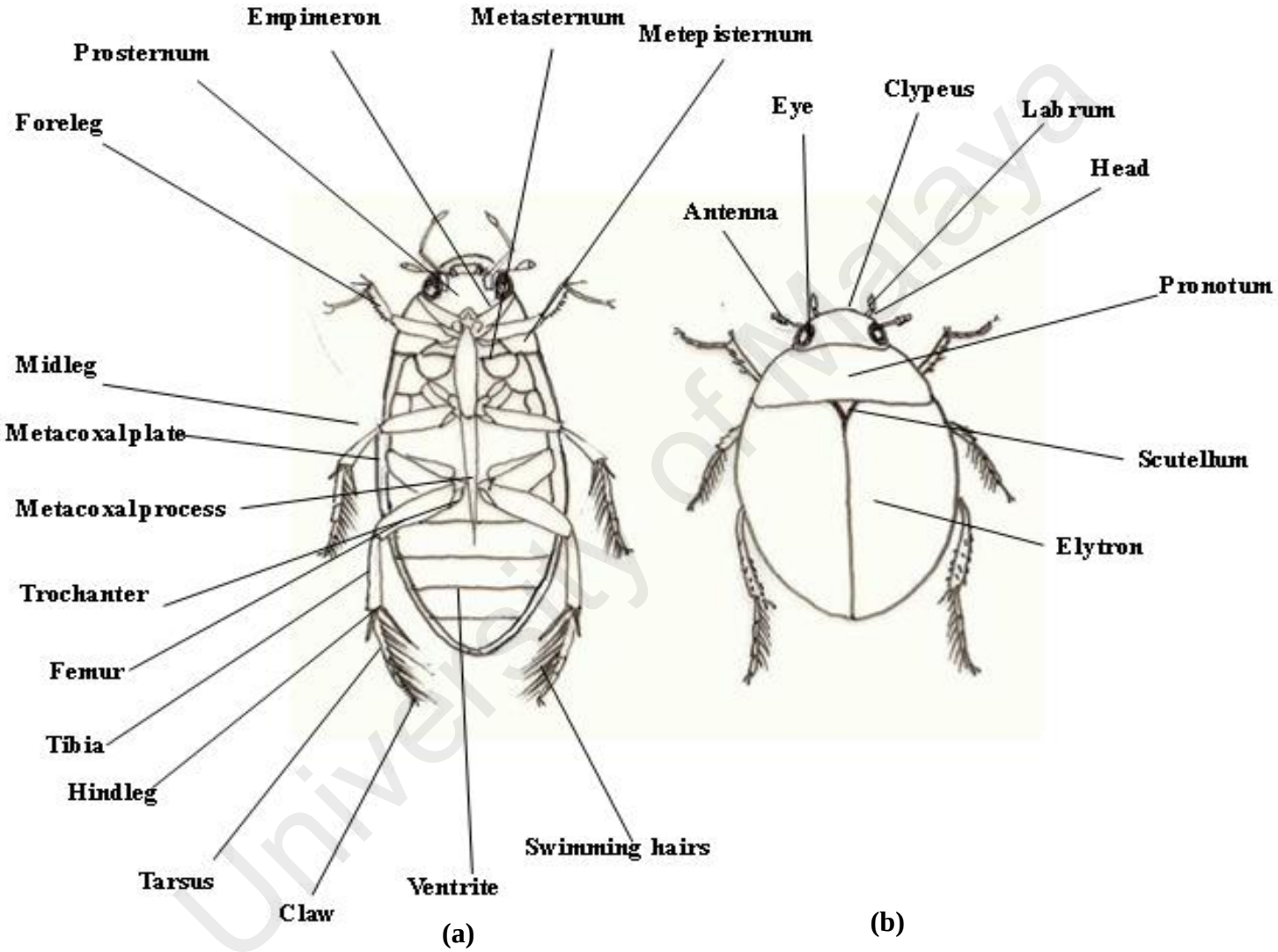


Figure 2.2: Morphological key of Hydrophilidae in both (a) ventral and (b) dorsal view

2.4.3 Gyrinidae (Whirligig beetles)

Gyrinidae is an air breathing beetle family. Their sensitivity to pollution is lower than that of riffle beetle and hence their presence in water body indicates the moderate level of water quality (Elliott, 2008). This is the smallest beetle species and can be eaten by other larger beetle families.

Whirligig beetles have an exceptional pair of divided eyes that allow them to see both upward into the air and downward into the water (Fig 2.3 a, b)(Jessup et al., 2002; Birmingham et al., 2005; Libonatti et al., 2011; Watts & Hamon, 2014). They are a group of sea bugs that have the ability to fly but invests a great part of its energy half submerged on water surfaces (Blagodatski et al., 2014). The female whirligig beetles store round and hollow eggs in parallel columns on submerged vegetation. The eggs are laid in small batches of a minimum of seven to a maximum of forty eggs. The eggs remain attached to the leaves of the underwater plants until they hatch. After hatching, the larvae move to the water surface. The larvae respire through lateral abdominal filaments and are predacious on insect larvae and nymphs (Arnett et al., 2002). Floating material and micro insects are the main food of Whirling Beetles. Due to this reason, the presence of this water beetles in a water body will keep the water surface clean. They prey by sensing the vibrations in the water line through their antennae (Lloyd et al., 2018). Adult beetles produce defensive secretions to protect themselves by releasing a smooth fluid (Voise & Casas, 2010). *Dinetus americanus* (a species in the Gyrinidae family), releases a liquid that smells like an apple (Bartlett & Stirling, 2003).

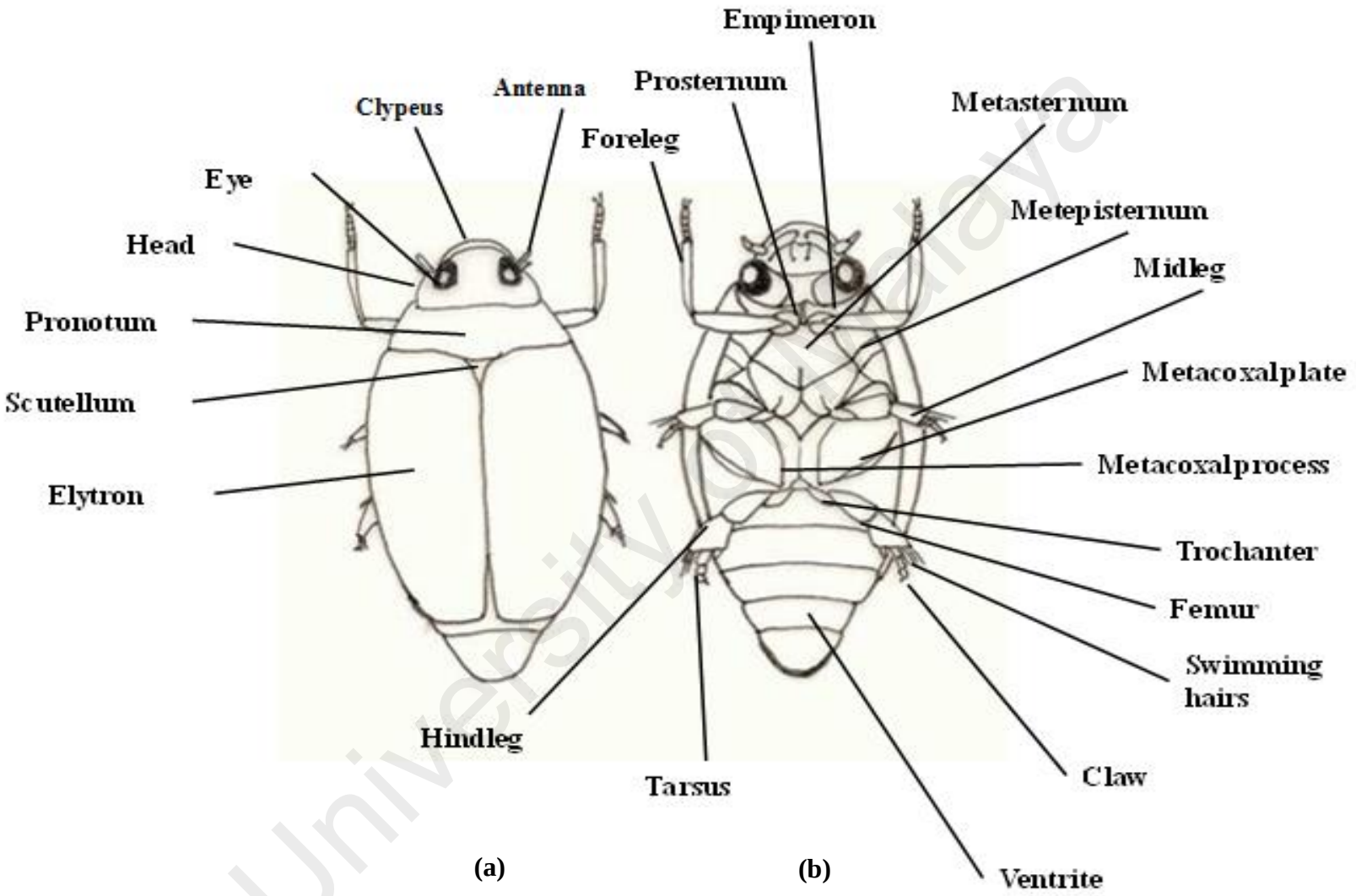


Figure 2.3: Morphological key of Gyrinidae in both (a) dorsal and (b) ventral view

2.5 Taxonomic key used for identification of water beetle families

In terms of taxonomic diversity, the term "water beetles", is represented in a wide range of aquatic and semi-aquatic habitats from damp moss, peaty pools, streams and canals to coastal rocky shores and salt marshes (Friday, 1988). The taxonomic key below that used as part of identification in the current study. This key further incorporates member of 14 beetle families belonging to 2 sub orders; Sub Family 1: Adephaga (= Hydradephaga); Sub Family 2: Polyphaga Gyrinidae; Haliplidae; Hygrobiidae; Noteridae; Dytiscidae. Hydrophilidae; Hydraenidae; Dryopidae; Elmidae (= Elminthidae); Scirtidae (= Helodidae); Heteroceridae; Limnichidae; Chrysomelidae (sub-family Donaciinae); Curculionidae (Friday, 1988).

1. On the underside of body, large plates cover base of hind legs……Haliplidae (crawling water beetle)

   Not as above……..……...……..Go to 2

2. Two pair of eyes, one pair on top and one pair on bottom of head……...…….……....…..Gyrinidae (whirligig beetle)

   Head has only one pair of undivided eyes…….....……...……...….Go to 3

3. Body is streamlined, hind legs have swimming hairs……..…..Go to 4

   Body is not very streamlined, no swimming hairs on hind legs

   …….…...Go to 5

4. Short, club-shaped antenna……...Hydrophilidae (water scavenger beetle)

   Longer antenna, not club-shaped …… Dytiscidae (predaceous diving beetle)

5. Club-shaped antenna has a cuplike segment at the base of club (in the middle of antenna).....Hydrophilidae (water scavenger beetle)

   Antenna in various forms but never with a cup-like segment at base of a club ……..…..……Go to 6

6. Antenna with comb-like club ……..Dryopidae (long toed or riffle beetle)

   Antenna in various forms but if club-shaped it is not a comb-like club ……..…….....Elmidae (riffle beetle)

2.6 Simpson Diversity Index (SDI)

Simpson Diversity Index (SDI) is a sum of squared proportions which takes into account the number of species present, as well as the relative abundance of each species. According to SDI, the increase of species richness and evenness is proportionate to the increase of species diversity (Pakulnicka et al., 2016). SDI is a statistical method which is used to evaluate a data group with different components, such as environmental stress, total number of existing organisms, changes in a population, and distribution of individuals (Barman & Gupta, 2015). In addition, SDI is also a measure of biodiversity of inhabitants in an ecology. This indexing method was invented by Simpson in 1949. The abundance of species with respect to the number of already existing species is accounted through this indexing method. Using this indexing process the richness of water beetles can be identified and analysed (Anderson & Crosby, 2018).

2.7 Abundance of Bacteria in Water Bodies

Rivers and lakes are more vulnerable to water contamination compared to coastal beaches (Department of Environment, 2008). This is because contaminants in coastal beaches are more rapidly diluted and dispersed by currents and large volumes of water. Besides water beetles and physical parameters, the presence of bacteria also plays a role

in determining the quality of water bodies such as beaches or other aquatic environment (Sobsey & Water, 2002). *Escherichia coli* and *Enterococci* are indicator bacteria that show the presence of faeces and an increased likelihood of water-borne pathogens Figueras & Borrego, 2010). If the amount of *E. coli* or *Enterococci* species in a water sample breaches the healthy guideline levels, the access to the water sources will be restricted (WHO, 2015). The maximum level stipulated in the guideline for freshwater beaches is 550 of *E. coli* per 100 milliliters while it is 280 of *Enterococci* species per 100 milliliters in coastal beaches (Harrington et al., 1993). The increasing number of bacteria in water has led to health risks and poor water quality level. A strong indication of richness in bacteria leads to low level of dissolved oxygen in the aquatic environment (Spietz et al., 2015).

2.8 Molecular Detection of Bacteria in Water Samples

2.8.1 Metagenomic Analysis

Metagenomics is a culture-independent and direct genetic analysis of microbes in their natural habitats (Handelsmann, 2004). Metagenomics enables the analysis of microbial functional gene composition which gives a broader description than phylogenetic studies (Thomas et al., 2012). The analysis is conducted based on the diversity of *16S* rRNA gene which was used to design universal primer pair in order to detect different bacterial species. This approach can also be used to detects, 'unculturable' bacteria that cannot grow under laboratory conditions (Spring et al., 2000). Metagenomics involves powerful genome sequencing approach which can sequence all types of bacteria present in an environmental sample. This applicability has resulted in the recent sharp increase of studies focusing on Metagenomic analysis of microbial communities as indicators of pollutions in a diverse set of environments such as freshwater, marine sediments and ocean ((Ashbolt et al., 2001; Cardoso et al., 2012).

The application of metagenomics in natural water systems is crucial in order to determine the effects of water quality due to the presence of microbial communities. Betaproteobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, and Alphaproteobacteria are cosmopolitan bacteria and are frequently retrieved from water systems (Newton et al., 2011). Tan et al. (2015) conducted a metagenomics analysis that was coupled to amplicon-based analysis and next-generation sequencing (NGS) approach to study microbial communities in freshwater samples of different land use in British Columbia. Besides, this metagenomics approach is frequently used to characterise the variations of microbial structure and its functions in raw and treated water in drinking water treatment plants (Chao et al., 2013). Metagenomics provides new insights into genetic network and potential biological processes associated with the molecular microbial ecology of microbial communities.

2.8.2 *16S* rDNA Sequence as a Bacterial Molecular Detection Marker

Among many molecular detection methods used for the identification of a bacterial species, *16S* rRNA is one of the gold standard method used with several universal primers (Griffen et al., 2012). A product of 1500 bp encoding for catalytic RNA as part of designed 30S ribosomal subunit can be amplified using the designed primer. This DNA fragment has the necessary properties that permits it to be the most frequently used marker (Lane et al., 1985; Mahmoud et al., 2015). Based on the gene sequence, a phylogenetic relatedness of an organism can be detected using bioinformatic tools (Srinivasan et al., 2015). The sequencing of *16S* rRNA gene can provide information related to its genus and species level (Clarridge, 2004). Cole et al. (2009) mentioned that *16S* rRNA databases contain nearly a complete length of sequences for a great number of strains and their taxonomic placements. This helped to identify a sequence from an unknown strain. Several morphological, biochemical and genetic characteristics have also been used to identify the constituents in the complex populations of microorganisms (Donelli et al.,

2013). The *16S* rDNA sequence divergence of different bacterial species has been exploited as an indicator of diversity. It is noteworthy that, PCR amplification of *16S* rDNA has been widely used as indicators of the presence of bacteria in water samples (Medlin et al,. 1988).

The broad-range PCR approach has been utilised to clone and sequence the *16S* ribosomal DNA (rDNA) for many years (Klindworth et al., 2012). Acinas et al. (2004) stated that *16S* ribosomal RNA (rRNA) genes are vital and at least a copy of it can be found in a genome. According to Woese (1987), rRNA is considered a perfect target for phylogenetic studies and taxonomic classification due to the universality of the genes.

In a metagenomics-based identification process, *16S* rDNA sequencing is used to identify whether or not the bacteria community consists of unusual genotypic profiles such as rare, slow-growing, uncultivable or culture-negative infectious species (Woo et al., 2008). The rRNA is considered as the most conserved gene (Clarridge, 2004; (Rajendhran & Gunasekaran, 2010). Portions of the rRNA sequence from distantly-related organisms are remarkably similar. Alternatively sequences from relevant organisms can be accurately aligned to ensure that the differences are easily measured (Boye et al., 1999). A large number of species have been identified using *16S* rRNA gene. Sequences from tens of thousands of environmental bacterial isolates are available in the GenBank (www.ncbi.nlm.nih.gov).

2.8.3 Polymerase Chain Reaction (PCR) of *16S* rRNA from Metagenomic Samples

In principle, any environment is amenable to metagenomics analysis provided that nucleic acids can be extracted from the sample material. Metagenomics transcends the culture requirements of microbes. In metagenomics, DNA is directly extracted from the environmental samples such as soil, seawater, acid mine drainage and etc. This is followed by the construction of metagenomic libraries by cloning large fragments of DNA in cloning vectors and screening of metagenomics library. The use of *16S* rDNA

amplicon sequencing allows the estimation of the abundance and diversity of bacteria, via the polymerase chain reaction (PCR) technique (Bartlett & Stirling, 2003; Lane et al., 1985; Huber et al., 2007). In the studies of metagenomics, the number of phylotypes in the *16S* rDNA sequences varied substantially for samples from different environments and geographical sites (Wang & Qian, 2009). The *16S* rRNA gene amplification and sequencing have been well documented as a useful tool for the detection and identification of bacteria (Bosshard et al., 2003; Drancourt et al., 2000; Mignard & Flandrois, 2006; Tang et al., 1998; Woo et al., 2008).

With PCR and two suitable primers trace amounts of DNA can be selectively multiplied. In principle, a single copy of the respective sequence in the assay can produce over a million-fold identical copies, which can then be detected and further analysed with the use of different methods (Schmitt et al., 2015).

CHAPTER 3: METHODOLOGY

3.1 Sampling Locations

This study was conducted in two localities in Peninsular Malaysia, namely Fraser's Hill (3.7140° N, 101.7350° E) and Pangkor Island (4.1312 N°, 100.331° E). Fraser's Hill is situated on the Titiwangsa Ridge in Pahang while Pangkor Island is situated off the coast of Perak. Both these locations are in Peninsular Malaysia.

3.2 Water Sampling

Sampling was carried out from January 2015 to December 2015 in Fraser's Hill and Pangkor Island, where samples were collected at four months interval. Water samples were isolated from three localities in Fraser's Hill, namely Jeriau Waterfall (N03° 42.018', E101° 45.100'), Lasak Trail (N03° 42.712', E101° 46.259') and Raub Trail (N03° 42.780', E101° 46.236'). From each sampling locations, seven sampling points were identified as replicates. Water samples for Pangkor Island were obtained from three localities, namely Pasir Bogak (N04° 13.894', E100° 33.160'), Teluk Nipah (N04° 14.906', E100° 33.065') and Teluk Cempedak (N04° 14.679', E100° 34.029'), where the seven sampling points were identified as replicates.

3.3 Water Quality Assessment

The quality of water sampled from the localities was assessed based on *in situ* water quality parameters such as biological oxygen demand (BOD), chemical oxygen demand (COD), dissolved oxygen (DO), temperature and total suspended solid (TSS). The diversity value of beetles collected as bioindicators and the presence of bacteria in the sampled water were also assessed. In order to identify the level of biodiversity of beetle in the sampling sites, Simpson's diversity index was used.

3.3.1 *In situ* Parameters

Water quality assessment was carried out by measuring the components listed by the Department of Environment Malaysia (2006), i.e. as water temperature, pH, dissolved oxygen (DO), total dissolved solid, salinity, biochemical oxygen demand (BOD), chemical oxygen demand (COD) and ammonia. These parameters were important information in order to determine Water Quality Index (WQI) as described by Department of Environment of Malaysia (2006) (Table 3.1). The range for each parameter was very important for WQI calculation by following the best fit equations for the estimation of various sub index values (Table 3.2). Temperature readings were obtained on site using mercury in glass thermometer. All other readings were also obtained on site using Horiba U-500 series equipment (Perera, 2005; Rizzi et al., 2017).

Table 3.1: Parameters assessed and water quality classification standard described by the Department of Environment of Malaysia (2006).

Parameters	Classes				
	I	**II**	**III**	**IV**	**V**
Ammonia Nitrogen (mg/L)	< 0.1	0.1 - 0.3	0.3 - 0.9	0.9 - 2.7	> 2.7
Biochemical Oxygen Demand (mg/L)	< 1	1 - 3	3 - 6	6 - 12	> 12
Chemical Oxygen Demand (mg/L)	< 10	10 - 25	25 - 50	50 - 100	> 100
Dissolved Oxygen (mg/L)	> 7	5 - 7	3 - 5	1 - 3	< 1
pH	> 7	6 - 7	5 - 6	< 5	> 5
Total Suspended Solids (mg/L)	> 25	25 - 50	50 - 150	150 - 300	> 300
Water Quality Index (WQI)	< 92.7	76.5-92.7	51.9-76.5	31.0-51.9	> 31.0

Table 3.2: Best fit equations for the estimation of the various sub index (SI) values from Department of Environment (2008) and Interim National Water Quality Standards for Malaysia.

SubIndex for DO (In % saturation)	**Range**
SIDO = 0	for $x \leq 8$
SIDO = 100	for $x \leq 92$
SIDO = $-0.395 + 0.030x^2 - 0.00020x^3$	for $8 < x < 92$
SubIndex for BOD	
SIBOD = 100.4 - 4.23x	for $x \leq 5$
SIBOD = 108* exp(-0.055x) - 0.1x	for $x > 5$
SubIndex for COD	
SICOD = -1.33x + 99.1	for $x \leq 20$
SICOD = 103* exp(-0.0157x) - 0.04x	for $x > 20$
SubIndex for NH_3-N	
SIAN = 100.5 - 105x	for $x \leq 0.3$
SIAN = 94* exp(-0.573x) - 5* I x - 2 I	for $0.3 < x < 4$
SIAN = 0	for $x \geq 4$
SubIndex for SS	
SISS = 97.5* exp(-0.00676x) + 0.05x	for $x \leq 100$
SISS = 71* exp(-0.0061x) + 0.015x	for $100 < x < 1000$
SISS = 0	for $x \geq 1000$

Table 3.2, Continued

Sublndex for pH	
SlpH = 17.02 - 17.2x + 5.02x^2	for x < 5.5
SlpH = -242 + 95.5x - 6.67x^2	for 5.5 ≤ x < 7
SlpH = -181 + 82.4x - 6.05x^2	for 7 ≤ x < 8.75
SlpH = 536 - 77.0x + 2.76x^2	for x ≥ 8.75

(x=concentration in mg/L for all parameters except pH and DO)

WQI= (0.22 x SI DO) + (0.19 x SI BOD) + (0.16 x SI COD) + (0.15 x SI AN) + (0.16 x SI SS) + (0.12 x SI pH) (1)

where,

SIDO = Sub lndex DO (% saturation)

SIBOD = Sub lndex BOD

SICOD = Sub lndex COD

SIAN = Sub lndex NH3-N

SISS = Sub lndex SS

SipH = Sub lndex pH $0 \leq WQI \leq 100$

The letter "x" in Table 3.2 represents the concentration of each parameter in mg/L except for pH. After each sub index for each parameter has been calculated, the values were substituted into equation 1 to calculate Water Quality Index, (WQI).

3.3.2 Water Beetles as Water Quality Bioindicators

3.3.2.1 Water Beetle Sampling in Water Bodies

Water beetles were collected using a sweep net as recommended by Arribas et al. (2012). Light trapping was also conducted as described in Abdullah (2008) and Abdullah et al. (2008). The former method was employed as the primary method for collecting aquatic beetles during the day while the latter was used at night. The aquatic net has a long-handle and a square-frame with a 30 cm mouth and a 1 mm mesh. The net was swung from side to side in a full 180° arc. One stroke was casually swept as walking through the stream. On the other hand, the light trap consists of a white mosquito net with the size of 1.8 m x 2.0 m x 2.0 m and is illuminated by a 160-watt mercury light bulb powered from a portable generator (Honda EU10i). Lighting hours were set for six hours from 1730-2330 hours.

3.3.2.2 Water Beetle Preservation and Species Identification

Water beetles were preserved in 70% alcohol and later pinned on an insect box with the size of 229 mm x 330 mm x 64 mm and dried at 40°C in oven. The specimens were identified by a staff, at the Department of Agriculture, Kuala Lumpur, Malaysia. Identification of specimens was conducted at family level using a number of taxonomic keys as described in Borror & Delong (1971), Cheng & Hashimoto (1978), Merritt & Cummins (1996) and Trivinho-Strixino & Strixino (2000). The specimens were photographed using Leica microscope model EZ4D which was attached to a digital camera.

3.3.2.3 Community Structure Analysis

The collected beetles were counted according to the number of individuals (N) and species (S). Then the diversity of the beetles was analysed using Simpson's Diversity index. Simpson Diversity Index (D) was used to determine the rarity (diversity) of the

species present on the sites. The Simpson's index is a measure of diversity, which takes into account both species richness and the abundance of the species present. In essence, it measures the probability that the two individuals randomly selected from an area will belong to the same species (Simpson, 1949).

Simpson's Diversity Index (SDI)

The Simpson's diversity index ('D'), was calculated using the following formula,

$$D = 1 - \sum_{i=1}^{s} pi^2$$

where 'Pi' is the proportion of individuals in the 'ith' taxon of the community and 's' is the total number of taxa in the community. This index places little weight on rare species and more weight on common species (Krebs, 1989). The index value ranges from 0 (indicating a low diversity level) to a maximum of 1.

3.3.2.4 Analysis of Variance (ANOVA) between the Two Water Bodies

In order to further strengthen the diversity and richness index of using Simpson's Diversity Index (SDI), ANOVA; $p \leq 0.05$ test was used. The advantage of using ANOVA was the ability of performing a variances analysis in a situation where two different group of observations (family and number of individual present in isolated locations) need to be compared. The observational data was required to be converted into normally distributed data then subsequently into diversity indices (Pallman et al., 2012). The statistical analysis was done with SPSS 16.0 statistical software. The SDI values were tested for significant differences for each of the localities using ANOVA. All of the determinants were calculated in triplicates, and the mean values were recorded.

3.3.3 Molecular-based Bacterial Detection Approach on Water Samples

3.3.3.1 Sample Collection

Five hundred millilitres of water sample were collected using a 500 ml black cap bottle. Water samples were collected from a total of six localities in both Fraser's Hill and Pangkor Island, namely Jeriau Waterfalls, Raub Trail, Lasak Trail, Pasir Bogak, Teluk Nipah and Teluk Cempedak. Note that triplicates of each sample were obtained for each locality. These samples were then labelled and kept in an ice box containing ice packs. All samples were later stored in -20 °C until future use.

3.3.3.2 Sample Preparation for Molecular-based Detection of Bacteria in Water Samples

3.3.3.2 (a) Preparation of Unfiltered Water Samples

The stored water samples in section 3.3.3.1 were thawed at room temperature and mixed thoroughly to obtain a homogeneous mixture. Subsequently, 25 ml of each of the stored water samples were transferred to a fresh 50 ml Falcon tube. The sample was either stored in -20 °C until future use or immediately subjected to the next analysis as described in Section 3.3.3.3. All the steps in this preparation were carried out under an aseptic environment.

3.3.3.2 (b) Preparation of Filtered Water Samples

The stored water samples in section 3.3.3.1 were thawed at room temperature and thoroughly mix in order to obtain a homogeneous mixture. Subsequently, 25 ml of each of the homogeneous mixture was filtered through a 20 micrometer sieve, and the flow-through was collected in a sterile 50 ml beaker. All the impurities that are larger than 20 μm remained in the residue part. The sample was later homogenised by gently shaking the beaker and later poured into a fresh 50 ml Falcon tube. The sample was either stored in -20 °C until future use or immediately subjected to the next analysis described in

Section 3.3.3.3. All the steps in this procedure were carried out under an aseptic environment.

3.3.3.3 Metagenomic DNA Isolation from Water Samples

Metagenomic DNA isolation from water samples was carried out following Kharbush et al., 2013 with minor modifications. Briefly, a homogeneous subsample of 15 ml water was poured into a fresh 15 ml Falcon tube once it was thawed from -20 °C. The sample was centrifuged at 10 000 x *g* for 5 minutes, and the resulting supernatant was discarded. Subsequently, the pellet was resuspended in 700 µl of sucrose lysis buffer (0.02 M EDTA, 0.4 M NaCl, 0.05M Tris-HCl and 2.57 g sucrose). The suspension was then transferred into a fresh 1.5 ml microcentrifuge tube. Later, 200 µg/ml Proteinase K were added to the sample in order to denature the proteins present in the sample before the sample was subjected to an incubation step at -80 °C for 16 hours. Then, the sample was subjected to two freeze-thaw cycles with one-hour interval for each cycle, followed by an incubation at 55 °C in a water bath for two hours. One volume of phenol was later added into the sample. The sample was later mixed by vortexing and centrifuged at 10 000 x *g* for 5 minutes at 4 °C. The resulting aqueous upper layer was then transferred into a fresh tube. The same centrifugation step was repeated after the addition of one volume of chloroform. The aqueous layer was then transferred into a fresh tube, and the DNA was precipitated using two volumes of 100 % ethanol. Subsequently, the sample was gently mixed and later centrifuged at 10 000 x *g* for 2 minutes at 4 °C. The resulting supernatant was later discarded, and the sample was left to dry at room temperature. In order to wash the pellet, 1 ml of 70 % ethanol was added. The sample was centrifuged at 5000 x *g* for 5 minutes at room temperature. The resulting supernatant was discarded, and the pellet was vacuum-dried for 10 to 20 minutes. The dried pellet was later dissolved in 30 µl mili-Q water and subjected to quality and quantity assessments. Next, the DNA sample was kept at -20 °C until future use.

3.3.3.4 DNA Quality Assessment

To prepare 1 % agarose gel, 0.5 g of agarose powder was weighed and boiled in 50 ml of 1 X Tris Borate EDTA (TBE; 445 mM Tris base, 445 mM borate and 10 mM EDTA). Subsequently, 1 µl/ml of Ethidium bromide was added. The mixture was boiled in a microwave oven at a medium high temperature until the agarose powder is completely dissolved (approximately 2 minutes). Then the solution was allowed to cool at room temperature. Next, the solution was poured into a gel casting tray. A comb was inserted onto the tray and the mixture was poured into the tray. The mixture was allowed to solidify at room temperature. Subsequently, the comb was carefully removed and the gel was placed in an electrophoresis tank where 1% TBE buffer was added to cover the gel to a depth of approximately 1 mm. After that, 5 µl of DNA sample were mixed to 1 µl loading dye before loading the sample into the wells. The sample was electrophoresed at 120 V for 25 minutes. Then it was viewed using a UV ray in a gel documentation system (Perkin Elmer, USA).

3.3.3.5 DNA Quantification

DNA quantification was carried out using a NanoPhotometer (Implen, Germany) according to the manufacturer's protocol. Independent multiple measurements of the optical density were carried out. A NanoPhotometer® P-Class Submicroliter Cell was inserted into the cell holder with the cell windows facing the light beam. The light beam was directed from right to left as indicated with small arrows. The Submicroliter Cell was inserted in the same direction. One microlitre of the sample volume was pipetted onto the centre of the measuring window and covered with a lid size 10 with the pathlength of 1 mm. Optical density (OD) measurement was then obtained. The lid was taken off and sample residues were removed from the measurement window and the mirror in the lid. Measurement window and mirror in the lid were cleaned well with a fluff-free tissue. Then, the cell was ready for the next sample.

3.4 Preparation of a Positive Control Sample for Bacterial Detection Analysis

A positive sample was prepared using DNA sample isolated from *Escherichia coli* strain JM109 as an indicator of successful amplification of *16S* rDNA region from a water sample.

3.4.1 DNA isolation from *Escherichia coli* strain JM109

DNA isolation from *Escherichia coli* was carried out as described in He (2011) with minor modifications. Briefly, 15 ml of the overnight *E. coli* culture (grown in Luria Broth medium, 10 g Tryptone, 5 g Yeast extract, 5 g NaCl) were transferred to a fresh 1.5 ml microcentrifuged tube and centrifuged at a maximum speed of 10 000 x *g* for 1 minute to pellet the cells. Then the resulting supernatant was discarded. The pellet was then resuspended in 600 ml lysis buffer (0.02 M EDTA, 0.4 M NaCl, 0.05 M Tris-HCl and 2.57 g sucrose) and the sample was then vortexed. After that, the sample was incubated for an hour at 37 °C. An equal volume of phenol was added to the sample and thoroughly mixed by vortexing. The sample was centrifuged at 10 000 x *g* for 5 minutes at room temperature. A white layer (protein layer) formed in the aqueous: phenol interface. The upper aqueous phase was then transferred to a fresh 1.5 ml microcentrifuged tube. To remove excess phenol, an equal volume of chloroform was added to the aqueous layer. Again, the sample was thoroughly mixed by vortexing. Then the tube was centrifuged at a maximum speed for 5 minutes. After that the aqueous layer was transferred into a fresh 1.5 ml Eppendorf tube. To precipitate the DNA, 2.5 or 3 volumes of cold 100 % absolute ethanol were added to the sample and gently mixed. Then the sample was incubated at -20 °C for 30 minutes or more. Subsequently, the sample was centrifuged at 5 000 x *g* for 15 minutes. The supernatant was then discarded, and the DNA pellet was rinsed with 1 ml of 70 % ethanol (stored at room temperature). Next, the sample was centrifuged at 17 900 x *g* for 2 minutes. The supernatant was carefully discarded, and the DNA pellet was air-dried. The DNA was then resuspended in 30 µl mili-Q water. Finally, the isolated

genomic DNA was subjected to quantitative and qualitative analysis as described in Sections 3.3.3.4 and 3.3.3.5.

3.4.2 Annealing temperature optimisation of *16S* rRNA PCR primers

The annealing temperature for the primers was optimised and the parameters that gave the most specific product were chosen to be used for subsequent PCR reactions. To optimize the annealing temperature, 12 different annealing temperatures ranging from 57 °C to 65 °C were tested using a gradient PCR thermocycler (Thermo Scientific Arktik, USA). The optimised annealing temperature was used for subsequent PCR reactions.

3.4.3 PCR Amplification of *16S* rDNA region from *E. coli*

PCR was carried out in order to amplify the *16S* rRNA gene from *E. coli* using a pair of *16S* universal primers as described in Kharbush et al. (2013). The primers were 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1522R (5'AAGGAGGTGATTCCAGCCGCA 3') targeting the 27th and 1522nd nucleotide of the *16S* rRNA gene respectively. A PCR product of approximately ~1500 bp was expected to be obtained. PCR was performed in a 25 µl reaction consisting of 1 X PCR Buffer, 1.5 mM $MgCl_2$, 2.5 mM of each dNTPs, 0.8µM of each primer, 1.0 U Taq polymerase, 25 ng/µl DNA and the mixture was topped-up with mili-Q water. The reaction is done in a sterile, 0.2 ml microcentrifuge tube. PCR amplification was performed in a thermocycler (Thermo Scientific Arktik, USA) with an initial denaturation step of 94 °C for 3 minutes, followed by 38 cycles of denaturation step at 94 °C for 1 minute, annealing at 57.9 °C for 1 minute and the final elongation step of 72 °C for 5 minutes.

3.4.4 DNA Purification of the *16S* rDNA fragment isolated from *E. coli*

The successfully amplified fragment was purified using QIA quick gel extraction kit (Qiagen, USA), following the manufacturer's protocol. Briefly, a fresh 1.5 ml microcentrifuge tube was first weighed. Later, the desired DNA band was excised from the agarose gel using a clean sharp scalpel and placed into a fresh 1.5 ml microcentrifuge tube (300 mg). Subsequently, three volumes of Buffer QG were added to one volume of gel slice (eg. 100 mg = 100 µl). The tube was then incubated at 50 °C for 10 minutes and vortexed every 2 to 3 minutes during incubation to aid gel dissolvement. A QIAquick spin column was placed in a 2 ml collection tube, and the sample was later applied to the column to bind the DNA. The tube was subjected to 17 900 x *g* centrifugation step for one minute at room temperature. The flow through was then discarded and the QIAquick column was placed back in the same collection tube. Then 0.5 ml Buffer QG were added to the column and centrifuged for one minute to remove all traces of agarose. Later 0.75 ml Buffer PE were added to the column and centrifuged for one minute. The flow through was discarded, followed by a 17 900 x *g* centrifugation step for another one minute. This was done in order to remove all Buffer PE residues. The QIAquick column was then placed in a fresh 1.5 ml microcentrifuge tube. Finally, 30 µl of mili-Q were added to the center of the column to elute the DNA, and the column was left to stand for one minute. The eluted DNA was either directly used for sequencing or stored in -20 °C for future use. Sequencing was done using *16S* rDNA primer pair used for PCR.

3.4.5 Sequence Analysis of *16S* rDNA fragment isolated from *E. coli*

The resulting chromatograms were first displayed using ChromasPro software (Technolysium Ltd.) to define the left and right trimming border. Basic Local Alignment Search Tool (BLAST) analysis was performed to verify whether the isolated sequence was indeed *E.coli 16S* rDNA sequence. The sequence similarity level with bacterial rDNA gene was assessed against other sequence available in the GenBank (http://www.ncbi. nlm.nih.gov/blast/Blast.cgi).

3.5 Detection of Bacterial DNA in Metagenomic Unfiltered Water Samples via PCR

Metagenomic DNA isolation of water samples was carried out as described in section 3.3.3.3. PCR was carried out as described in section 3.4.3 in order to amplify the *16S* rRNA gene from *E. coli* using a pair of *16S* universal primers as described in Kharbush et al., 2013.

CHAPTER 4: RESULTS

4.1 Water Quality Analysis

4.1.1 Physical parameters of Fraser's Hill and Pangkor Island

Based on the water quality classification standard described by the Department of Environmental pg 28, water quality index (WQI) of water bodies in Fraser's Hill and Pangkor Island was classified as a Class I and Class II quality, respectively (Table 4.1). Statistical analysis revealed that there was a significant difference for dissolved oxygen amount (Figure 4.1 d), mean temperature (Figure 4.1 f), and the amount of total suspended solid (Figure 4.1 g) between both localities.

Table 4.1: Water quality parameters and water quality index for Fraser's Hill and Pangkor Island

Parameter	Fraser's Hill	Pangkor Island
Temperature (°C)	20.75±2.50	24.36±2.62
Dissolved Oxygen (DO) (mg/L)	9.4±3.84	6.22±0.88
Biochemical Oxygen Demand (BOD) (mg/L)	0.4±0.64	0.41±0.80
Chemical Oxygen Demand (COD) (mg/L)	6.78±7.95	5.1±5.58
Total Suspended Solid (TSS) (mg/L)	0.01±0.01	1.35±1.56
Ammonia Nitrogen (NH_3-N) (mg/L)	0.05±0.05	0.04±0.07
pH	6.23±0.65	6.43±0.53
WQI	94.01	82.21
Class	**I**	**II**

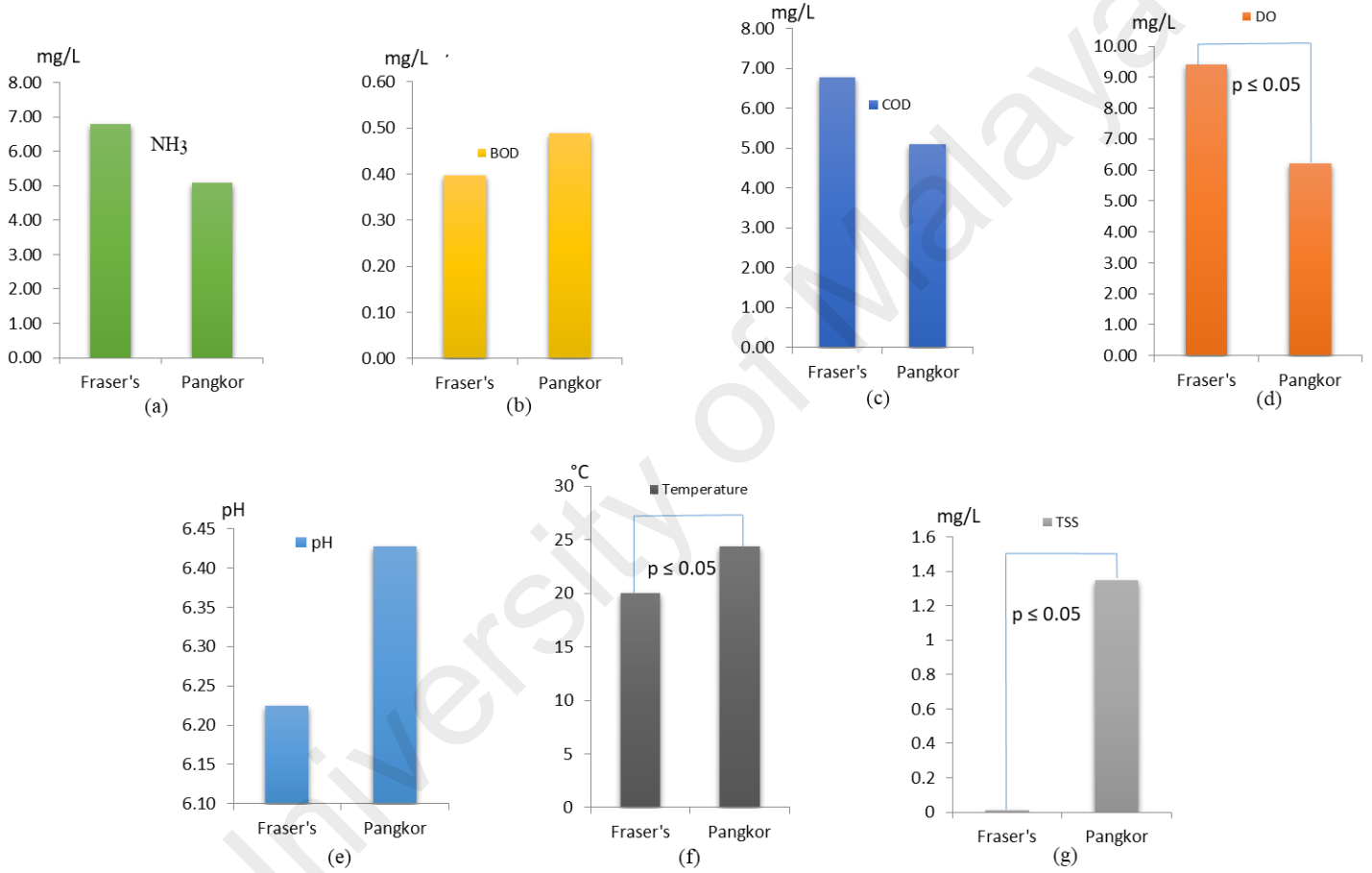


Figure 4.1: Comparison of physical parameters between waterbodies of Fraser's Hill and Pangkor (a) Ammonia Nitrogen; (b) Biological Oxygen Demand (BOD); (c) Chemical Oxygen Demand (COD); (d) Dissolved oxygen (DO); (e) pH; (f) Temperature; (g) Total Suspended Solid; in Fraser's Hill and Pangkor Island.

4.2 Diversity of Water Beetle from Fraser's Hill and Pangkor Island

A total of 112 individuals from three beetle families were isolated from Fraser's Hill, and 41 individuals of two families were sampled from Pangkor Island (Table 4.2). A total of seven species of water beetles from three families were isolated from the two localities. The total number of beetle individuals of family Gyrinidae in both the localities were 27. The highest number of beetles (76 individuals) isolated from both the localities came from family Hydrophilidae while only one beetle individual from family Dytiscidae was isolated from Fraser's Hill and none from Pangkor Island (Table 4.2). Family Dytiscidae species consist of *Cybister sugillatus* er, *Lacconectus corayi*, *Lacconectus krikkeni*, Family Gyrinidae; *Porrhorrhycus marginatus cast*; and Family Hydrophilidae consist of *Hydratus* sp, *Hydrophilus triangularis*, *Sternolophus rufipes*. Diversity index analysis revealed that water beetle species were significantly more diversed in Fraser's Hill indicating 0.93 Simpson Diversity Index (SDI) value compared to Pangkor Island with only 0.66 Simpson Diversity Index value ($p \leq 0.05$) (Figure 4.3).

Table 4.2: Number of individuals and the list of water beetle family and species collected from Fraser's Hill and Pangkor Island

		Fraser's Hill	**Pangkor Island**
Family	**Name of species**	**No. of Individual**	**No. of Individual**
Dytiscidae	*Cybister sugillatus* er	15	0
	Lacconectus corayi	14	0
	Lacconectus krikkeni	21	0
Gyrinidae	*Porrhorrhycus marginatus cast*	9	18
Hydrophilidae	*Hydratus* sp.	10	0
	Hydrophilus triangularis	23	10
	Sternolophus rufipes F	20	13
Total		**112**	**41**

(a)

(b)

(c)

(d)

(e)

(f)

(g)

Figure 4.2: An example of water beetle species from family Gyrinidae isolated from Fraser's Hill and Pangkor Island, (a) *Porrhorrhycus marginatus*, Family Dytiscidae; (b) *Cybister sugillatus*, (c) *Lacconectus corayi*, (d) *Lacconectus krikkeni*, and Family Hydrophilidae; (e) *Hydrophilus triangularis*, (f) *Hydratus* sp, (g) *Sternolophus rufipes*

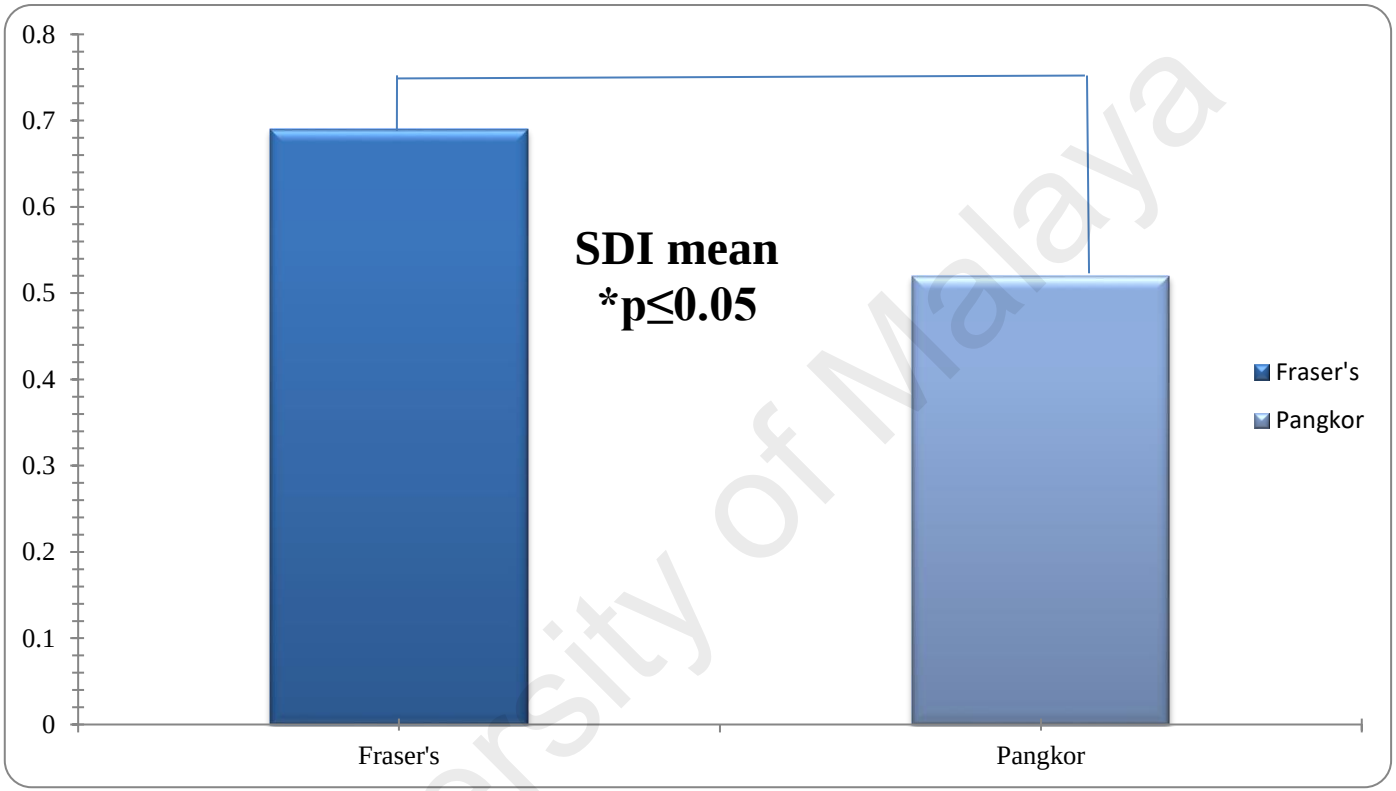
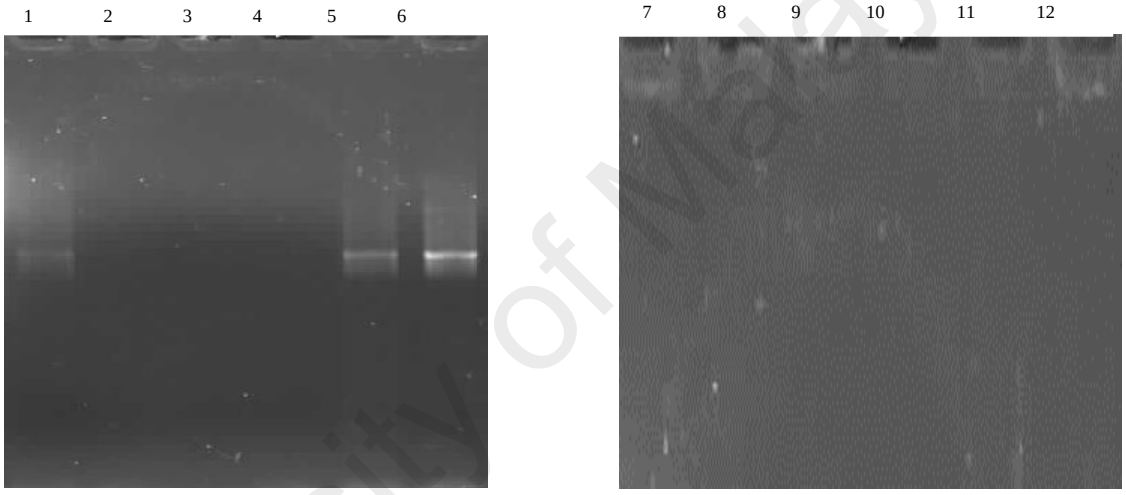


Figure 4.3: Significant Diversity Index difference was observed in the number of family and species of beetles isolated in both Fraser's Hill and Pangkor Island.

4.3 Molecular-based bacterial detection in metagenomic water samples

4.3.1 DNA-based detection of DNA Quality isolated from unfiltered and filtered water samples

A comparison made on DNA quality isolated from unfiltered (Figure 4.4 a) and filtered water samples (Figure 4.4 b) revealed that the concentration of isolated genomic DNA samples were relatively increased in unfiltered water samples compared to the filtered ones. Therefore, subsequent detection experiment on the presence of DNA in metagenomics water samples was made using unfiltered samples.



(a) (b)

Figure 4.4: Isolated genomic DNA collected from six different locations showed the presence of increased amount of DNA in (a) Unfiltered and (b) Filtered water samples. Lanes 1 & 7: Jeriao water sample, Lanes 2 & 8: Lasak Trail sample, Lanes 3 & 9: Raub Trail sample, Lanes 4 & 10: Pasir Bogak sample, Lanes 5 & 11: Teluk Nipah sample, and Lanes 6 & 12: Teluk Cempedak sample.

4.3.2 DNA Quantification and Quality Analysis of unfiltered water samples collected from Fraser's Hill and Pangkor Island

DNA quantification analysis revealed that the lowest DNA purity (A260/A280) value of 0.986 (Table 4.3) was isolated from Pasir Bogak metagenomic sample. DNA isolated from Jeriao water sample showed the highest purity value (A260/A280) of 1.222 (Table 4.3). The highest DNA concentration obtained was 30.5 ng/µl from Raub Trail while the

lowest was 14.1 ng/µl from Pasir Bogak. The ratio is appreciably lower than expected, indicating poor DNA quality obtained for isolated samples.

Table 4.3: Concentration and purity level of genomic DNA isolated from water samples obtained from the six sampling sites in Fraser's Hill and Pangkor Island

Sample	**DNA purity**		**DNA concentration**
	A260/280	**A260/230**	**(ng/µl)**
Jeriao	1.222	2.75	25.8
Lasak Trail	1.111	1.425	28.2
Raub Trail	1.182	1.182	30.5
Pasir Bogak	0.986	1.165	14.1
Teluk Nipah	1.011	1.431	28.2
Teluk Cempedak	1.172	1.165	24.3

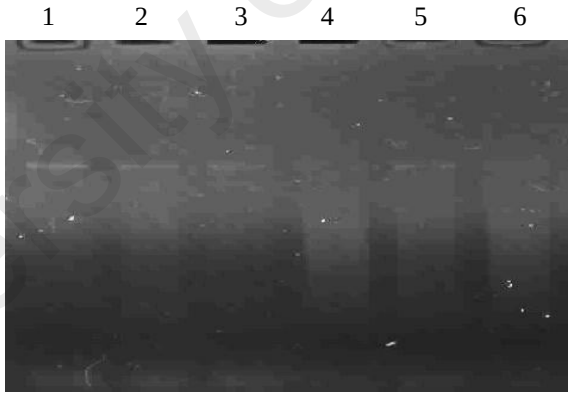


Figure 4.5: Qualitative analysis of unfiltered water sample from six sampling sites. DNA was present in all six water samples sampled from Fraser's Hill and Pangkor Island. Lane 1: Jeriao sample, Lane 2: Lasak Trail sample, Lane 3: Raub Trail sample, Lane 4: Teluk Nipah sample, Lane 5: Teluk Cempedak sample, and Lane 6: Pasir Bogak sample.

4.3.3 Preparation of positive control standard for the detection assay

4.3.3.1 Isolation of genomic DNA from *Escherichia coli*

Positive sample was prepared using DNA sample isolated from *Escherichia coli* strain JM109 (Fig 4.6) as an indicator of successful amplification of *16S* rDNA region from a bacterial sample. The obtained genomic DNA was found to be 601 ng/µl in concentration of high purity.

Figure 4.6: Agarose gel showing intact genomic DNA of *Escherichia coli*

4.3.3.2 Annealing temperature optimisation for the amplification of *16S* rDNA from universal primer

The Annealing temperature optimization experiment was conducted using *16S* rDNA universal PCR primer pair using *E. coli* DNA as the template described in (Kharbush et al., 2013). This experiment was performed using annealing temperatures that ranged from 57 °C to 65 °C (Fig 4.7). Non-specific bands were observed at lower annealing temperatures (Lanes 3 to 9, Figure 4.7) and more specific bands were obtained at higher temperature (Lanes 10 to 13). Temperature 61.6 °C (Lane 8) was chosen as the annealing temperature for subsequent reactions albeit the presence of a second band at 700 bp.

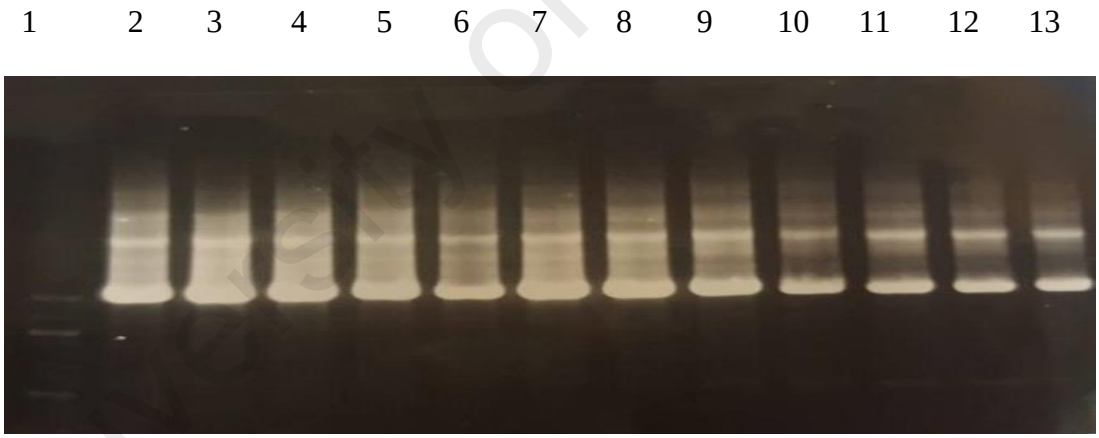
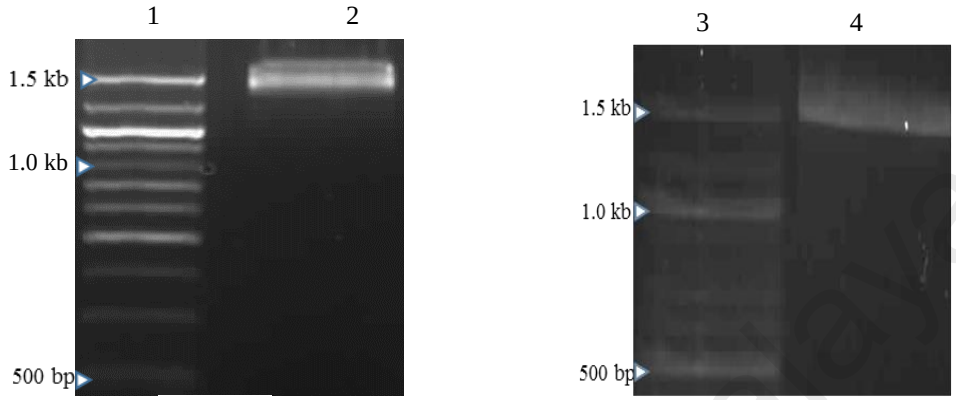


Figure 4.7: Annealing temperature optimisation experiment result showing the presence of multiple bands at temperatures 57°C to 61.6°C, and two specific bands at temperatures 62.8 °C to 65 °C using *16S* rDNA specific primer pair described in Kharbush et al. (2013). Lanes 1-12 *E. coli* at different annealing temperatures Lane 1:100 bp ladder (Promega, USA), Lane 2: 57.0°C, Lane 3: 57.2°C, Lane 4: 57.6°C, Lane 5: 58.1°C, Lane 6: 59.1°C, Lane 7: 60.2°C, Lane 8: 61.6°C, Lane 9: 62.8°C, Lane 10: 63.6°C, Lane 11: 64.4°C, Lane 12: 64.8°C, and Lane 13: 65.0°C.

4.3.3.3 DNA purification of the isolated *16S* rDNA from an *Escherichia coli* colony

DNA purification was successfully carried out. The purified DNA after post gel extraction (Fig 4.8 b) was fainter compared to pre gel extraction (Figure 4.8 a). The purified DNA was directly subjected to DNA sequencing.

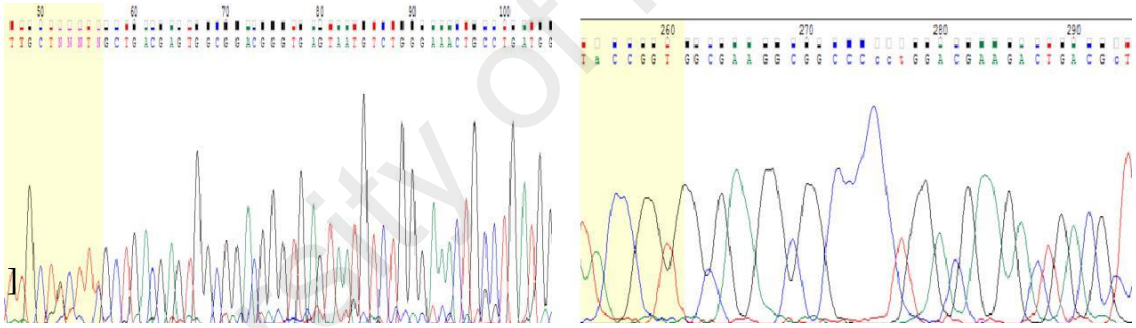


(a) (b)

Figure 4.8: Agarose gel electrophoresis analysis of *Escherichia coli* as a positive control sample (a) before and (b) after purification using QIAGEN Gel Extraction kit. Lanes 1 and 3: 500bp DNA ladder (Promega, USA), Lane 2 and 4: putative *16S* rDNA band.

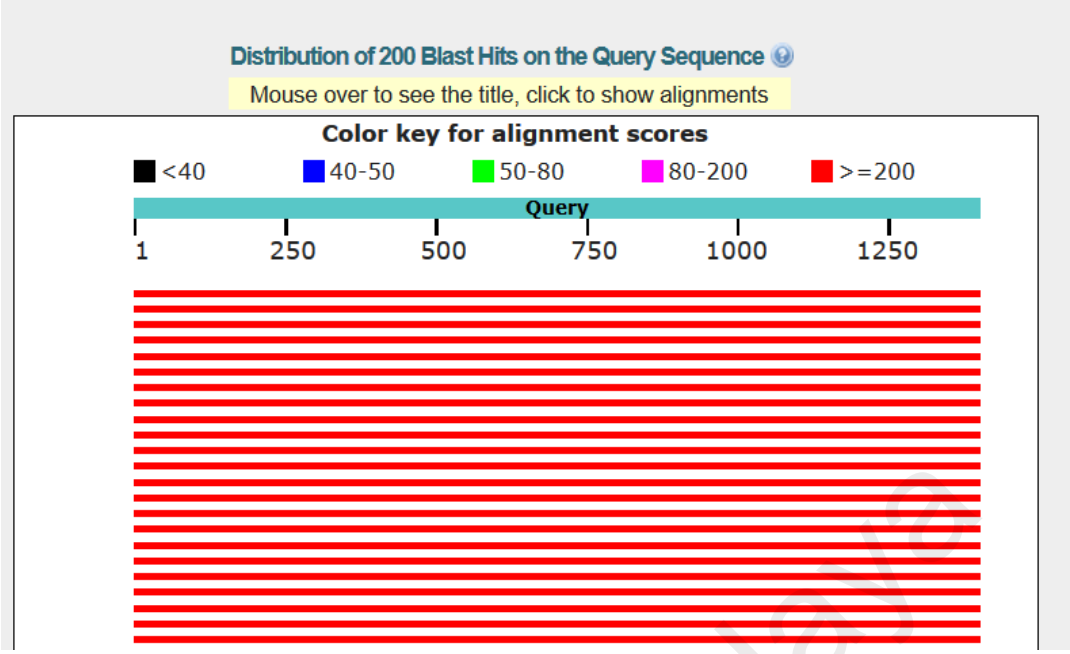
4.3.3.4 Sequence analysis of *16S* rDNA isolated from an *Escherichia coli* colony sample

The purified *16S* rDNA PCR amplicon from *E.coli* was further subjected to sequencing. Chromatograms obtained from amplification using both forward (Figure 4.9 a) and reverse (Figure 4.9 b) *16S* rDNA primers, suggested single nucleotide template signals. BLAST search analysis of the sequenced 1500 bp product isolated from *E.coli* showed 99% similarity with other *E.coli* strains available in the GenBank with an E-value of 0.0 (Fig 4.10). This result suggested that the primers designed for the amplification of *16S* rDNA had specifically amplified the *16S* rDNA region of *E. coli* and can be used as a positive control for bacterial detection experiment using metagenomic water samples.

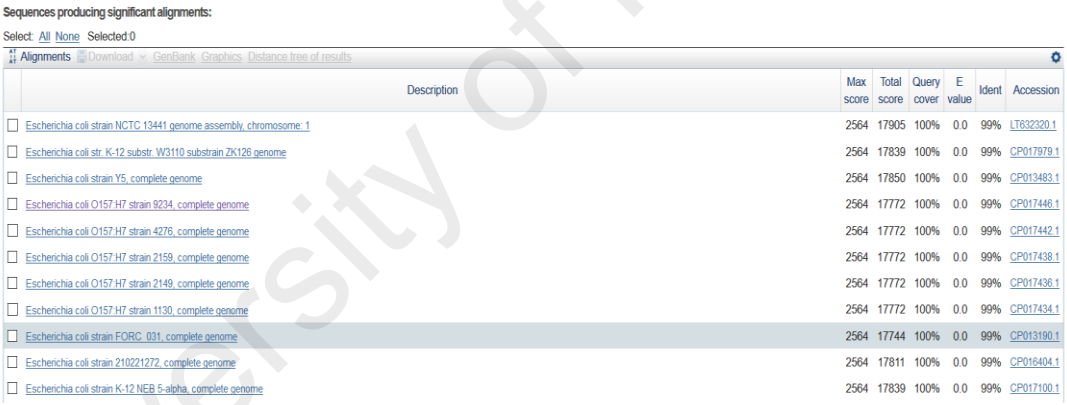


(a) (b)

Figure 4.9: Chromatograms showing partial (a) forward and (b) reverse sequencing reaction of *16S* rDNA fragment isolated from an *Escherichia coli* colony using a universal *16S* rDNA specific primer pair published by Kharbush et al. (2013).



(a)



(b)

Figure 4.10: BLAST result showing (a) the distribution of 200 BLAST hits of the 1500 bp query sequence and (b) sequence hits showing significant alignments with the query sequence in the GenBank with 99% sequence identity and an E-value of 0.

4.3.4 PCR-based Detection Assay from Six Metagenomics Water Samples Isolated from Fraser's Hill and Pangkor Island

PCR was performed on the metagenomic samples isolated from Fraser's Hill and Pangkor Island. An amplicon size of 1500 bp was observed (Fig 4.11) in water samples collected from Fraser's Hill (Lane 2, Jeriao) and three water samples (Lane 5, Pasir Bogak; Lane 6, Teluk Nipah; and Lane 7, Teluk Cempedak) from Pangkor Island. A positive control reaction showed a band of 1.5 kb. No bands were observed in negative control sample, confirming that PCR had successfully detected the presence of bacteria in the four water samples.

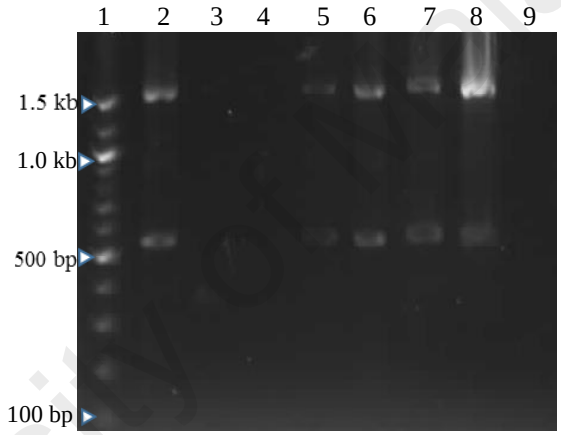


Figure 4.11: Agarose gel electrophoresis result showing amplification of 1500 bp for water samples using *16S* rDNA universal primer. Four out of the six water samples collected from the different locations showed positive reactions to PCR analysis. Lane 1: 100 bp DNA ladder (Promega, USA), Lane 2: Jeriao sample, Lane 3: Lasak trail sample. Lane 4: Raub trail sample, Lane 5: Pasir Bogak sample, Lane 6: Teluk Nipah sample, Lane 7: Teluk Cempedak sample, Lane 8: *Escherichia coli* as a positive control, Lane 9: Negative control.

CHAPTER 5: DISCUSSION

In the last decade, demand for fresh water has increased due to the increase of population size and industrial growth (Ramakrishnaiah et al., 2009). Since the last few decades, the aquatic ecosystem has continuously been suffering from the loss of biota (Harvell et al., 2002). The main cause for such biota loss is due to the growing human population and industrialisation that has generated various contaminants which leads to the reduction in the quality of water bodies (Bond et al., 2008). Such reduction was observed the assessment sites with moderate and strong industrial activities and the sites facing strong effluent eruption (Hoffman & Sgro, 2011). However, water quality in the fresh water rivers, waterfalls, and islands had been decreased due to pollutions caused by human activities (Fischer & Harris, 2003). The quality of water has deteriorated and has affected human and aquatic life enormously (Vitousek et al., 1997). Arnett et al. (2006) reported that a high distribution aquatic lives of fauna such as Hydrophilidae, Dytiscidae, and Gyrinidae correlate negatively with the occurrence of fishes in an aquatic environment.

In relation to this, assessment of water quality was performed in this study on physico-chemical and biological aspects in two water bodies namely Fraser's Hill and Pangkor Island. In the physico-chemical parameters, water quality was determined by measuring the presence of organic waste in the trails of waterfalls, and it was done by analysing water pH, ammonia nitrogen, dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solid (TSS), and temperature. Water Quality Index (WQI) is a new approach in assessing the overall quality of the water which involves all the chemical, and physical parameters (Yogendra & Puttaiah, 2008). As temperature has an impact on both chemical and biological characteristics of surface water, the level of DO in the water was expected to be affected due to photosynthesis of aquatic plants, metabolic rates of aquatic organisms and the

sensitivity of these organisms to pollution, parasite, and disease (Short & Neckles, 1999). In the current study, Fraser's Hill showed a significantly higher WQI value of 94.01 ($p \leq 0.05$) compared to Pangkor Island, with the WQI value of 82.2. This indicated that water quality in Fraser's Hill was cleaner and can be classified in Class I compared to that of Pangkor Island that can be classified in Class II (Table 4.1). On the other hand, the pH value, temperature, BOD, COD, TSS and NH_3-N content in both water bodies were within the permissible range. Salih et al. (2013) noted that pH and temperature showed a positive effect on BOD and COD of nearby areas of Mengkuang reservoir of Malaysia, suggesting that the suspended particles absorb sunlight and increase the temperature of water. In the same study, they have reported that the high BOD and COD values directly relate to the turbidity and coliform presence.

Biochemical oxygen demand (BOD) is regarded among the standard test for the presence of organic matter in water. In cases of high amounts of organic matter, this may result in pollution issues due to the presence of microorganisms that have the ability to biodegrade the organic material and therefore use the present oxygen and limit the amount of dissolved oxygen for the fish and other marine organisms (Collins et al., 2013). BOD corresponds to the requirement for oxygen, and in current study the BOD value of water sampled from Pangkor Island was higher than the value obtained for water sampled from Fraser's Hill, demonstrating that the need for oxygen is higher at the former rather than the latter. This information, comes along with the DO value, which showed high dissolved oxygen content for Fraser's Hill (Figure 4.1 d). This result is logical since the requirement for oxygen is pretty low in Pangkor Island when the bacteria presence is more prominent compared to Fraser Hills (Figure 4.4).

On the other hand chemical oxygen demand, regards the total amount of oxygen and does not include the differentiation between the biologically available and organically available oxygen (Collins et al., 2013). In principle such values are higher than BOD

values due to this lack of differentiation. In the current study, the COD values of both Pangkor Island and Fraser's Hill were higher than the BOD value (Figure 4.1 c). However, the COD value of water sampled in Pangkor Island was lower than the COD values of Fraser's Hill, an opposite pattern than the one seen with the BOD values (Figure 4.1 b) indicating that the water pollution in Fraser's Hill was at a more acceptable level compared to that of Pangkor Island.

Dissolved oxygen (DO) is another important parameter required to study the water quality in natural water resources (Robinson et al., 2004). An optimal DO is required for the survival of many microorganisms and aquatic species like fishes. In addition, DO establishes an anaerobic environment, a condition in which oxidized forms of many constituents are predominant in the water. In the current study, Fraser's Hill showed a significant high DO level compared to Pangkor Island (Figure 4.1 d), suggesting that Fraser's Hill water was a better option for drinking because it was free from organic pollutants. Yisa & Jimoh (2010) had reported that low DO values of any water body would directly relate to the presence of high organic pollutants and nutrients. However, adequate amount of DO is necessary for the growth of aquatic organisms and can be in the range of 3 to 9 mg/L (Robinson et al., 2004). The DO level in Fraser's Hill and Pangkor Island was within this range, amounting to 9.40 mg/L and 6.22 mg/L, respectively. Adequate level of DO as shown in the reading derived from the study correlates with the water quality in the sample study. Water quality can be further assessed based on the presence of certain aquatic insects which include Coleoptera, Trichoptera, Ephemeroptera and Plecoptera, and hence act as biological indicators (Ciparis et al., 2013; Merritt and Cummins, 1996). In our current study, a total of 155 beetle individuals were found in Fraser's Hill and Pangkor Island belonging to three different families, namely Dytiscidae, Gyrinidae and Hydrophilidae (Table 4.2). Since water beetles from, Dytiscidae family, have been reported as the most abundant species in the world (Jäch &

Blake, 2008), they were found present in every running and standing water body worldwide. Interestingly, no Dytiscidae was found in Pulau Pangkor (Table 4.2) indicating a disturbed aquatic environment. This claim was supported by the low level of DO observed in Pulau Pangkor compared to Fraser's Hill (Figure 4.1d). The same beetle family was found at Fraser's Hill which had a higher DO value. The presence of DO in water plays an essential role in the life cycle of aquatic organisms. Hoback and Stanley (2001) reported that the larva and pupa of aquatic insects need more oxygen and the insects sensitive to hypoxia cannot survive. In the current study, the DO level was found to be higher in Fraser's Hill compared to Pulau Pangkor. High amount of DO present in Fraser's compared to Pangkor Island where low DO primarily results from excessive algae and microbial growth caused by phosphorus thus indicating there is a significance difference in term of DO between both study sites. Excessive algae and microbial growth results in bacterial population increase in colony forms (Paerl et al., 2001).

Total suspended solid (TSS) parameter measures the total ions in water resources, and it ranges from 0.24 to 32 mg/L (Ittekkot, 1998). According to the current investigation, the waterfall area of the forest in Fraser Hills recorded a significantly lower TSS amount of 0.01 mg/L ($p \leq 0.05$) compared to the value obtained for Pangkor Island which was 1.35 mg/L (Figure 4.1 g). There was a high significant difference in the amount of TSS in Pangkor Island compared to Fraser's Hill. High TSS content indicating the solution contains high mineral level (Pyle et al., 2002), as a result from excessive sediment carried into streams and rivers from erosion of unstable streambanks, construction sites, agricultural activities, and urban runoff. Besides turbidity, beetles and bacteria are examples of suspended water solutes. The finding of the current study suggested that water body which contained a considerably higher TSS content (Pangkor Island: 1.35mg/L) affects beetles population considerably. TSS which correlates with the biotic (beetle and bacteria) presence in water bodies determine the structure of aquatic

communities in them. The substrate presence defines micro-distribution (Lloyd et al., 2018) in the environment. Therefore, aquatic beetles were considered as a good water quality indicator within faunistic communities (García-Criado et al., 1999). The lower TSS value in Fraser's Hill thus reflects a better micro-distribution for the beetle species growth and beetle families were isolated from this site in this study. Similarly, Marques et al. (2003) reported that mining activity in Troya Mina on the aquatic ecosystem of Basque County, Spain showed accumulation of heavy metals at the bottom sediments and heavy metals content in water columns correlate negatively with composition of water beetles. Neumann and Dudgeon, (2002) reported that the impact of agricultural activities on stream benthos in Hong Kong, China showed negative correlation to diversity of water beetles. Furthermore, TSS is directly related to the growth of bacteria, hence high values of TSS in any water body suggests the presence of coli forms (Sun et al., 2001). Our molecular-based bacteria detection experiment corroborate this claim. From PCR-based detection approach, a band of 1.5 kb was obtained in one out of three water samples isolated from Fraser's Hill and all water samples in Pangkor Island (Figure 4.1). The band indicated the presence of bacterial DNA was more prominent in water samples isolated from Pangkor Island compared to Fraser Hill. This may be due to the fact that Pangkor Island is more accessible to human activities compared to Fraser's Hill.

In the current study, no significant difference was recorded in the content of ammonia nitrogen. However, ammonia nitrogen (NH_3-N) was present in a variety of concentrations such as in many surfaces of water resources. Since it is a product of a microbiological activity, the presence of ammonia in natural water is regarded as sanitary pollution (Li & Bishop, 2004). Ammonium ion (NH_4+) present in the water gets oxidized by bacteria in the natural water into nitrite and nitrate. However, ammonia (NH_3), being a source of nitrogen also provides nutrients for algae and other plants, so it contributes to overload the natural system and causes pollution. The pH of water is directly related to

the presence of ammonium, which indicates that the higher pH value specifies the high levels of NH_3 in a water system (Camargo and Alonso, 2006; Majumder et al., 2006; Ojosipe, 2007). Water samples isolated from Fraser's Hill and Pangkor Island showed a very low level of NH_3-H (Figure 4.1 a). Acidity increases as pH values decreases, and alkalinity increases as pH values increases. Both the study sites (Fraser's Hill and Pulau Pangkor) falls under acidic category with no significance pH difference obtained from water samples between the two sites (Figure 4.1 e). Due to insignificant difference in the pH value found on both site it can be concluded that the beetles diversity population and bacteria could not be related in terms to the water acidity or alkalinity. Therefore, other parameters will be given a heavier weightage in the determination of water quality in this study compared to ammonia and pH.

During the study, water temperature at Fraser's Hill was significantly lower than the one sampled from Pangkor Island (Figure 4.1 f). This result was consistent with the DO measurements that show the same pattern as temperature. To be more accurate, this study shows that due to the higher temperature (24.36 °C) at Pangkor Island, the DO level decreased. The metabolic rate and the reproductive efficiency of water beetle was reported to be highly depended on water temperature (Miserendino & Archangelsky, 2006). With the increase in reproductive activity, it is obvious that the demand of oxygen increases as well, which may be a huge implication in cases of steam temperature where the diluted oxygen becomes limited (Schafer et al., 2006). The limitation in oxygen inhibits growth of water beetles substantiating the reason why water samples from Fraser's Hill has a lower temperature had a diverse water beetle population compared to that of Pangkor Island.

In the current study, a metagenomic approach was employed, where the DNA was extracted from the water samples. This experiment further corroborated, the increased species diversity in Class I Fraser's Hill was due to high water quality. By utilising the

16S rDNA method, the presence of the band observed in Pangkor Island was compared with the ones in Fraser's Hill, where the detection of *16S* rDNA band was observed in all three sites in Pangkor Island. *16S* rDNA universal primer was used to detect the presence of bacteria, and in this study the band was only present in Class II river (Figure 4.11). Four out of six studied sites were categorised as Class II in WQI which required conventional water treatment and conservation except for the samples from Lasak River and Raub River that were classified as Class I WQI which indicated that the water quality was adequately conserved. The use of *16S* rDNA sequencing revealed that Class II WQI had the presence of bacteria in the study site of both water bodies, supported by moderate SDI which indicated that the diversity of the water beetles was increasingly related to WQI. This substantiate the presence of a more diversed beetle family in Fraser's Hill compared to Pangkor Island (Figure 4.3). The results obtained from Simpson's Diversity Index are overall based on squared frequency values (Peet, 1974). As a result of this fact, species with low frequency often tend to be barely represented in the obtained values from Simpson's Index, which in turn may have some impact on the study, largely depending on its main purpose in question (Hill et al., 2003). The significance in Simpson Diversity Index ($p \leq 0.05$) corroborates the findings that the samples of beetles found in hill top (Fraser's Hill) was more diversed due to higher water quality compared to that of the coastal line regions (Pangkor Island).

CHAPTER 6: CONCLUSIONS

In conclusion, water samples obtained from Fraser's Hill was classified as a Class I quality with the WQI value of 94.01. The difference of water quality was significant between the two localities ($p \leq 0.05$) when water samples obtained from Pangkor Island can only be classified as a Class II quality with the WQI value of 82.2. This high water quality in Fraser's Hill correlated with beetle diversity in this locality. Significantly higher Simpson's Diversity Index value was obtained for water beetles species isolated from Fraser's Hill (SDI = 0.93) compared to that of found in Pangkor Island (SDI = 0.66). Molecular-based detection of bacterial DNA in water samples isolated from the two localities corroborated WQI and SDI data inferring that Fraser's Hill water samples were almost free from bacteria while all samples from Pangkor Island contained bacterial DNA.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Proceedings

Oral

Davindran, R and Fauziah Abdullah (2015) The Assessment of Stream and waterfall at Fraser's Hill using water beetle as indicator of Environmental Change. In Prosiding Saintifik Koridor Raub-Bukit Fraser 2015 (Seskorfra 2015) Persekitaran Lestari untuk Kesejahteraan Sejagat. Edited by Haja Maiddeen et al, Published by Faculty of Science, University Kebangsaan Malaysia, ISBN978-967-10417-3-4, pp. Universiti Kebangsaan Malaysia.