DEGRADATION OF MICROPLASTICS BY FORMULATED BACTERIAL CONSORTIUM ISOLATED FROM MANGROVE AREAS IN PENINSULAR MALAYSIA

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DEGRADATION OF MICROPLASTICS BY FORMULATED BACTERIAL CONSORTIUM ISOLATED FROM MANGROVE AREAS IN PENINSULAR MALAYSIA

ABSTRACT

Regardless of its importance as an ecosystem, coastal mangroves have historically been favoured as dumping sites for numerous waste, including plastics. This study was aimed to investigate the ability of bacteria isolated from mangrove areas to degrade selected microplastics in laboratory condition. Physico-chemical parameters such as dissolve oxygen (DO), pH, temperature, salinity and biochemical oxygen demand (BOD) of water samples collected from mangrove areas were analyzed, to correlate with the microbial abundance in the areas. Potential degrading bacteria microbial consortium was inoculated in Bushnell Haas broth containing selected microplastics (sole carbon source). The medium was incubated in a shaker at 28°C for 30 days and the weight reduction of microplastics was recorded. In total, there are 38 species of bacteria isolated from mangroves sediment. It was found that microbial abundance at Matang Mangrove, Perak recorded the highest number of bacteria with 3.7 x 10^7 CFU/ml. Positive correlation was shown between microbial abundance with DO and BOD. After 30 days of exposure, polyethylene was reduced by 27.9%, polyethylene terephtalate by 24%, polypropylene by 19.5% and polystyrene by 15%. The change in the peak of FTIR confirmed the degradation potential of microplastics by these bacteria. The results revealed that the consortia isolated from mangrove sediment have the potential to degrade selected microplastic, thus can be used to bioremediate microplastics in a mangrove environment ecosystem.

PENGURAIAN MIKROPLASTIK OLEH KONSORTIUM MIKROB YANG DIPENCILKAN DARI KAWASAN BAKAU DI SEMENANJUNG MALAYSIA

ABSTRAK

Hutan paya bakau mempunyai banyak kepentingan terhadap ekosistem. Namun paya bakau juga telah menjadi tapak pembuangan banyak sisa, termasuk plastik. Kajian ini bertujuan untuk mengkaji keupayaan bakteria yang diasingkan daripada kawasan hutan bakau untuk mengdegradasikan mikroplastik terpilih dalam keadaan makmal. Parameter fizik-kimia seperti oksigen terlarut, pH, suhu, kemasinan dan permintaan oksigen biokimia telah dianalisis dari sampel air yang diambil dari kawasan bakau untuk mendapatkan korelasi bakteria di kawasan tersebut. Degradasi diuji dengan menggunakan konsortium mikrob dan media Bushnell Haas yang mengandungi mikroplastik terpilih sebagai sumber karbon tunggal. Media tersebut telah diinkubasi di dalam shaker pada suhu 28°C selama 30 hari dan pengurangan berat mikroplastik direkodkan. Secara keseluruhan, terdapat 38 spesis bakteria yang telah diasingkan dan Paya Bakau Matang, Perak telah mencatatkan jumlah bakteria tertinggi dengan 3.7×10^7 CFU / ml. Hubungan yang positif telah ditunjukkan antara bilangan mikrob dengan oksigen terlarut dan BOD. Selepas 30 hari pendedahan, berat polietilena telah berkurangan sebanyak 27.9%, polietilena tereftalat sebanyak 24%, polypropylene sebanyak 19.5% dan polistirena sebanyak 15%. Perubahan di puncak FTIR mengesahkan potensi penguraian plastik ini. Hasil kajian menunjukkan bahawa konsortium diasingkan daripada sedimen bakau mempunyai potensi untuk degradasi microplastik terpilih, yang boleh digunakan untuk bioremediasi mikroplastik dalam ekosistem persekitaran bakau.

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LIST OF SYMBOLS AND ABBREVIATIONS

BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
DO	Dissolve Oxygen
DOE	Department of Environment
EPA	Environmental Protection Agency
FTIR	Fourier Transform Infrared Radiation
GESAMP	Group of Experts on the Scientific Aspects of Marine Environmental Protection
PE	Polyethylene
PET	Polyethylene Terephtalate
PP	Polypropylene
PS	Polystyrene
PUR	Polyurethane
PVC	Polyvinyl chloride
SPI	The Society of Plastic Industry
WWF	World Wildlife Fund

LIST OF APPENDICES

CHAPTER ONE: INTRODUCTION

1.1 Mangroves

According to Kathiresan and Bingham (2001), mangroves are woody plants which mainly grow along the coastal lines. They are most found in warm, humid climate, particularly the sub-tropical and tropical latitudes. In addition, they usually grow between the latitude of 25°N and 25°S and they exist as low shrubs in unfavourable conditions and in favourable condition it can reach over 40 m in height (Chong, 2006).

Mangrove is able to survive in extreme conditions such as extreme tides, high salinity, high temperatures, strong winds, muddy sediment and also anaerobic soils (Kathiresan & Bingham, 2001). The unique root systems, leaf structures and special bark help the plant to adapt in the environment. The root help mangrove tree to anchor the soft mud and enhance its stability to face the water current. This unique characteristic allows them to grow along coasts and river mouths, where no other trees can grow.

Tan and Basiron (2000), reported that in Malaysia, mangrove forest are mainly found along sheltered coastlines protected from strong waves and cover an area of approximately 577,558 ha. According to Chong (2006), mangrove distributions in Peninsular Malaysia primarily are located on its west coast facing the Malacca Straits, while mangrove forests on its east coast facing the South China Sea are small and mainly restricted to river mouths (Figure 1.1).

Manwhile, Abd. Shukor (2004), reported that about 88,667 ha of mangrove area had been gazetted as forest reserve with the biggest area of mangrove forest reserve are Perak (43,502 ha), Johor (17,029 ha), Selangor (15,090 ha) and Kedah (7,949 ha).

Figure 1.1: Distribution of mangrove in Peninsular Malaysia.
(Peninsular Malaysia Mangroves online mapping, 2011)

Mangrove forests are highly productive and are biologically important which comprise of diverse type of woody plant and the muddy sediment make it a unique habitat for various group of invertebrate and also the nursery for fish juveniles (Kamaruzaman, 2013). Mangrove ecosystems are highly loaded with sulphur, nitrogen and organic matter, which can be utilised by living microorganisms. Mangrove provide wide range of ecosystem services and play an important roles to human society and coastal marine system as its provide unique ecosystem commodities.

In this study, sampling was carried out at six mangrove sites located in Peninsular Malaysia. The sites are Matang mangrove Forest, Cherating Mangrove, Serkam Mangrove, Tanjung Piai Mangrove, Sedili Besar Mangrove, and Pasir Puteh Mangrove.

1.1.1 Matang Mangroves, Perak

The Matang Mangrove Forest Reserve (4°49'9.82"N, 100°40'28.93"E) is located in the administrative districts of Larut, Matang and Selama in Perak. They are located on Peninsular Malaysia north-western coast of. There are 19 independently gazetted forest reserves with 40,466 hectares of forest areas, excluding major waterways (Azahar *et al*., 2003). More than 85% of Matang mangroves are tidal swamp which flooded daily and occasion wash only occur during the highest spring tide (Azahar *et al*., 2003).

1.1.2 Serkam Mangroves, Melaka

Serkam Mangroves (2°10'1.91"N, 102°23'11.34"E), is located in Jasin District, Malacca. Land-use within three kilometres radius around this mangrove forest was includes oil palm and other plantations, and residential areas, some of which become the main sources of pollution into to nearby rivers and mangrove area. Boating activities are carried out daily for fishing and ecotourism purposes.

1.1.3 Tanjung Piai, Johor

Tanjung Piai (1°19'36.89"N, 103°26'45.57"E) is one of the five Ramsar sites in Malaysia, and is a widely known natural attraction with high ecotourism potential. It also has a high socio-economic value for fisheries. Moreover, these mangroves in Southwest Johor creates natural barrier protecting the inland villages and agricultural lands from storms and tsunami.

1.1.4 Sedili Besar, Johor

Sedili Besar is located at (1°55'19.82"N,104°5'17.35"E), on the eastern side of Johor in Malaysia. This area comprises of two rivers, Sungai Sedili Kecil and Sungai Sedili Besar. Both rivers have ecotourism potential and have both aesthetic and recreational

values, where tourists can take boat rides along to see the pleasant scenery along the rivers. However, agricultural activities and village settlements had reduced the area of the freshwater swamp forest in and around these two rives.

1.1.5 Pasir Puteh, Kelantan

Pasir Puteh Kelantan (5°51'11.14"N, 102°31'13.77"E) is a town, district (jajahan) and parliamentary constituency in Kelantan, Malaysia. There are three mixed mangrove areas that cover 15.8 ha in pasir Puteh (Zailani, 2009). Two areas are on the left of the river and one is on the right river this area might not be influenced by tide every day. Soils of the area are trade up of sand, silt and clay.

1.1.6 Cherating Mangroves, Pahang

Pahang mangrove forest, in Kuantan, covers an area of 343 ha, between the coordinate of (4°7'49.04"N, 103°23'37.13"E). Its location is within the Kuantan estuary, and irrigated by the Kuantan River that flows out to the South China Sea. This area is exposed to semidiurnal tides that lie in wet tropical area. Nurfathiah *et al*. (2014), described that Pahang mangrove forest is surrounded by the brackish water ecosystem, provides natural resources for various microorganisms.

1.2 Importance of mangrove

Mangroves have a crucial role in protecting the nature and the ecosystem such flourish in salty muddy sediment flooded by sea waves during high tide. During the Asian tsunami on 26 December 2004, area in Malaysia which were protected by a thick belt of mangroves suffered very little damage as mangroves absorbs the destructive energy (Dahdouh *et al*., 2005). Mangroves trees act as the defence shield for wave and wind and they protect the coastline from erosion (Spalding *et al.,* 2014).

According to Polidoro *et al.* (2010), Malaysia's mangroves are rich in species diversity as compared to mangrove in tropical Africa and Australia. Mangroves act as the reproduction habitat for various prawns, crab, fishes and other marine organisms which are necessary to support a feasible fishing industry. In addition, WWF reported that there is about 50% of mangrove on west coast of Peninsular Malaysia having a fish landing activities.

A past study by Kathiresan and Bingham (2001), proved that the presence of mangroves can reduce the amount of carbon dioxide in the atmosphere via photosynthesis. The absorption of carbon dioxide from the atmosphere would reduce the global warming and the green house effect.

Mangrove sediments are able to retain nutrients in the soil for the use of other organisms. According to Fujimoto (2000), each year, a 20-year old mangrove forest can store up to 11.6 kg m^{-2} of carbon and the C burial rate of 580 g m^2. Consequently, planting mangroves is beneficial as it helps in the process of stabilizing the atmospheric carbon to by controlling climate change.

The ability to retain nutrient in sediment depend on the characteristics of the sediment and the sites flow patterns. Kaly *et al*. (1997), in their study stated that mangrove systems assist in recycling of carbon, sulphur and nitrogen. In addition mangrove is the only system that recycles sulphur efficiently in nature for the utilisation of other organisms (Kathiresan, 2001).

Besides retaining nutrients, mangrove extracts have been used in native medicine. Kathiresan and Bingham (2001), stated in their research that the mangrove extracts have a potential for the treatment of serious disease such as AIDS. While the leave of

Bruguiera sp. is used to reduce blood pressure, and the bark of *Rhizophora sp*. able to cure antidiarrhoea, astringent and antemetic activities.

In term of bioremediation, there is a lot of potential microbial isolated from mangrove sediment. Kato *et al*. (2001), reported that microorganisms that have potential to utilise and degrade contaminants present originally in contaminated sediment as it have ability to adapt and survive in unfavourable condition.

1.3 Mangrove issue

Regardless of the importance and benefit of mangroves towards the ecosystems, it has been indiscriminately exploited with irresponsible management practices. This include unsustainable forestry, land reclamation, as well as, agricultural and aquaculture activities. Spalding *et al.* (2014), reported that mangrove land conversion for economical and development purpose have resulted in the damage of mangroves ecosystems area since 50 years ago and the rate of mangrove disappearance are exceeding 1% per year in many developing countries such as India, Pakistan, and Cambodia.

Due to the ability of mangroves to fix and retain large quantity of carbon, mangroves disappearance may give significant effect on the world's carbon resources. According to Cebrain (2002), the loss of approximately one-third of the global mangroves population has consequently caused the net loss of 3.8×10^{14}g C stored as mangrove biomass. This is due to the uncontrolled large scale development and also industrial activity which give impact to natural environment.

As a result of urban development and also anthropogenic activities, mangrove environment experienced significant direct contaminant input particularly with plastics. According to Kathiresan and Bingham (2001), the coastal mangroves have become a

favoured dumping sites for solid waste disposal especially plastics. Fauziah *et al.* (2015) reported that, a total of 2542 pieces (265.30 gm^{-2}) of small plastic debris were collected from six Malaysia beaches with the greatest quantity was found in Kuala Terengganu.

Ryan *et al*. (2009), stated that sources of plastics litters that pollute the shoreline are resulted from marine and terrestrial sources. Plastic marine debris is readily transported by tides and currents across large expanses of the ocean, before they were accumulated in coastal areas that are associated with restricted water movement. Terrestrials sources of plastics come from drainage systems and anthropogenic activities all along the coastline such as, shipping ports, harbours, fishing and recreational activities. Nur & Jeffrey (2014), reported in her study that The Pasir Ris mangrove which situated in one of the largest recreational areas in Singapore had a lot of debris including of food wrappers, plastic bags, plastic bottles and drink cartons found between the aerial roots of the mangrove plants. Similar types of debris were also observed at Sungei Buloh, which is heavily visited by both tourists and local people.

Microplastics have been discovered widely in the natural environment, most notably in coastal sediments and oceans around the world. High concentrations of microplastics of up to 2175 particles per kg of dry weight sediments have been documented in the coastal regions of the Mediterranean Sea (Vianello *et al*., 2013). Although plastics are commonly deemed as biochemically inert (Roy *et al*., 2011), plastic additives incorporated during manufacturing change the property and increase plastic life by increasing its resistance to natural degradation (Browne *et al*., 2007).

Barnes *et al.* (2009), reported that such additives are hazardous to the environment. This is because they can delay plastic degradation and release potentially toxic chemicals into the marine environment. In addition, microplastics raise concerns over their effect to the biota. This is because the small size of the plastics makes them easier to be

ingested by marine organisms (Barnes *et al*., 2009) and enter the marine food chain. Consequently, ingesting plastics might risk the survival rate of these marine organisms.

This is supported by Betts (2008), that stated that the small size of the microplastics increases the likelihood for marine organisms to mistake the plastics for food and consequently, ingest them. There are increasing numbers of studies reporting microplastic accumulation within the food chain of marine biota. These lower tropic level organisms ingesting the plastics due to their lack of capacity to distinguish between food and plastic compounds. Consecutive paragraphs discuss the characteristics of plastics.

1.4 Plastic debris

Rios *et al*. (2007), described plastics as artificial organic polymers. They originated from the monomers polymerisations which are extracted from gas or oil. The characteristics of these materials such as lightweight, durable, strong and cheap contribute to the high demand for plastic products throughout these last three decades. One of feature is their flexibility of plastics making make the best material to be used for the production of a massive range of products.

Despite of all their benefits, Barnes *et al*. (2009), argued that plastics have a higher resistance towards natural degradation. Plastic do not easily degrade due to its chemical structure. They may break down, but only into smaller pieces. The majority ends up in landfill and marine ecosystems which may take thousand years to break down and decompose.

According to Andrady *et al.* (2011), plastic degradation in the environment can be divided into four mechanisms, thermooxidative, photodegradation, hydrolytic and biodegradation by microorganisms. Bioremediation is done to boost up the naturally

occurring degradation by providing the optimum condition which is a cost-effective treatment and with a logistically favourable clean-up technology (Margesin & Schinner, 2001).

1.5 Problem Statement

There is a growing environmental concerns on the use of minute plastic granules called the 'microplastics'. These smaller plastic granules have been used in cosmetics scrubber, air-blasting, and other industries. In addition, microplastics are produce by breaking down larger plastic (Ryan *et al*., 2009). Consequently, microplastics were reported to have toxicological impact on marine organisms. Laboratory studies conducted show that microplastics particles could be mistaken as food, and there is a risk that they might be ingested by marine organisms (Van & Janssen, 2014).

The concern about the dangerous side of macroplastic towards marine environment is due to plastic are no more inert in environment but it can intrude the food chain of marine life and when ingested it will be retained by marine organisms that normal absorption into certain tissues may not take place.

Browne *et al.* (2008) reported that microplastic might remain in the digestive tract if they are ingested. They can also be digested through the process defecation, as well as being transferred into the tissue of the body through the epithelial lining of the gut. In this light, Van Franeker *et al.* (2011), stated that plastic polution affect different organisms such as marine mammals, birds and reptiles.

There are reports indicating that microplastic particles have been found in the system of marine organisms like fishes, lobsters, sea cucumbers, mussels and oysters (Possatto *et al*., 2011). According to Ward *et al*. (2009), this will give harmful toxicological effects to the organism as it transfer to higher trophic levels.

These particles will enter the food chain of marine invertebrates easily via ingestion. Table 1.1 presents a list of laboratory experiments which reported microplastics ingestions by marine organisms, including invertebrates, echinoderm larvae, and zooplankton (Cole *et al*., 2011).

Table 1.1: Marine biota's uptake of microplastics.

Type of organisms	**Size of Microplastics (µm)**	**Technique of Identification**	**Author (s)**
Copepods (*Acartia tonsa*)	7–70	Microscopy	Wilson (1973)
Echinoderm larvae	10–20	Video observation	Hart (1991)
Trochophore larvae (*Galeolaria caespitosa*)	3–10	Microscopy	Bolton and Havenhand (1998)
Scallop (*Placopecten magellanicus*)	16–18	Detection of ^{51}Cr labelled particles	Brillant anf MacDonald (2002)
Amphipod (*Orchestia gammarellus*) Lugworm (*Arenicola marina*) Barnacle (*Semib balanoides*)	20–2000	Dissection and wormcase examination	Thompson *et al*., (2004)
Mussel (*Mytilus edulis)*	2–16	Dissection and fluorescence microscopy	Browne *et al*., (2008)
Sea cucumbers	Various	Excrement analysis	Graham and Thompson (2009)

Thus, necessary actions need to be taken in order to curb the introduction of microplastic into the marine food chain. This can be achieved either by preventing

microplastic from entering the coastal and marine environment, or the removal of the element from its current sites.

1.6 Objectives of the Study

This research aims to isolate the potential microplastic degradable bacteria from selected mangrove in Malaysia. In order to perform study, the following objectives are established:

i. To analyse the water quality of the selected mangrove areas.

ii. To correlate microbial abundance with water quality of the area.

iii. To isolate, screen, and identify microbes with the ability to degrade microplastics, and

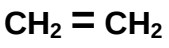
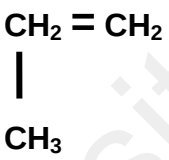
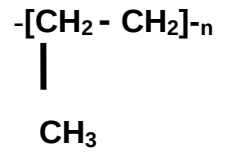
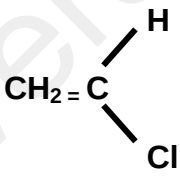
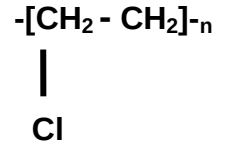
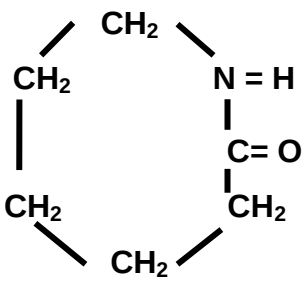
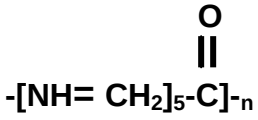
iv. To investigate the ability of isolated bacteria to degrade selected microplastic under laboratory condition.

CHAPTER 2: LITERATURE REVIEW

2.1 Plastics

Plastics are defined as polymers that can be shaped into different size and shapes upon heating (Joel, 1995). Plastics are made from the polymerisation of monomers, which are extracted from gas and oil (Rios *et al*., 2007). The monomer units made up of organic carbon-based molecules or element such as oxygen, sulfur, and nitrogen. The plastic is differentiated by its property, such as type of element, their proportion and the placement of monomer as shown in Table 2.1.

Table 2.1: Examples of some common plastics and their monomers (Wiley, 2001)

	Monomer		**Polymer**
Ethylene		Polyethyelene (PE)	
Propylene		Polypropylene (PP)	
Vynylchloride		Polyvinylchloride (PVC)	
Caprolactame		Poly (E-Caprolactame) (PA6)	

Plastic can be divided into two categories which are thermoplastics and thermoset plastic (Thakur & Nayak, 2012) in which thermoplastic have more flexibility and

versatility as it will return to its original form when it is heated. These types of plastic are used in various applications such as fibers and films as shown in Table 2.2.

Table 2.2: Properties and principal uses of thermoplastic (BBC, 2014)

Name of thermoplastic	**Characteristics**	**Primary uses**
Polyamide (Nylon)	Creamy coloured, strong , relatively hard, highly resistant to wear, able to self-lubricate, good resistance to machines and chemicals	These thermoplastics are mainly used to manufacture gear wheels, casings for power tools, hinges for small cupboards, bearings, clothing, and curtain rail fittings.
Polymethyl methacrylate (Acrylic)	Hard, stiff ,durable and polish well as well as having good machinability however, these plastics can scratch easily and brittle if used in small sections. They are good insulator for electrics and machines.	Mostly used to produce storage box covers, aircraft windows and canopies, basin, signs, car light covers, and bath.
Polypropylene	Light, tough, hard but scratches easily, It has high resistance to work fatigue and chemicals	Primary used to produce containers with built-in hinges, laboratory or medical equipment, string and plastic seats.
Polystyrene	Light, hard and stiff, but quite brittle. Often transparent and has good resistant to water/	Plastic containers, boxes and packaging of toys, particularly model kits.
Low density polythene (LDPE)	Tough, but highly flexible, and fairly soft. Has good chemical resistance and good electrical insulators	Primary used for packaging particularly for making toys, packaging films, bags and bottles.
High density polythene (HDPE)	Hard, and stiff. Can be sterilised	Household equipment, plastic bottles, tubing.

Second category of plastic is thermoset plastic. in which it cannot cannot return to its original form as it will hold its shape in long term once it has been hardened Table 2.3 shows some of the principle uses and properties of thermoset plastic.

Table 2.3: Properties and principal uses of thermoset plastic (BBC, 2014)

Name	Properties	Principal uses
Epoxy resin	Hard, brittle unless reinforced; can be good electrical insulator, good resistance to chemicals.	Mostly used as adhesives, Casting and encapsulation, and to bond other materials
Melamine formaldehyde	Strong, and can be stiff and hard; strong, chemicals and stain resistance	Can be used as laminates for work surfaces as well as for tableware and electric insulators.
Polyester resin	Can be a good electrical insulator, and has good chemical resistance. Can be stiff, hard but brittle unless laminated,	Casting and encapsulation and bonding of other materials
Urea formaldehyde	Good electrical insulator, can be hard, strong but brittles.	Adhesives, control knobs, electrical fittings and handles and

2.2 Types of plastics

According GESAMP (2015) there are various types of plastics that were produced worldwide, however the market were dominated mostly by polypropylene, polyethylene, polyethylene terephthalate and polystyrene.

2.2.1 Polypropylene

Polypropylene expressed as CnH_2n and it is a linear hydrocarbon polymer which each carbon atom is attached to a methyl group as shown in Figure 2.1 (Colin, 2015). Polypropylene has low density which is between 0.895 and 0.92 g/cm^3 (Tripathy, 2001).

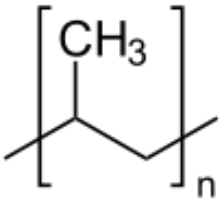


Figure 2.1: Structure of polypropylene (Colin, 2015)

Kumar *et al*. (2013), reported that the demand for polypropylene between 2004 and 2012 increased at 4.4% per year. It is used in variety of application as shown in Figure 2.2.

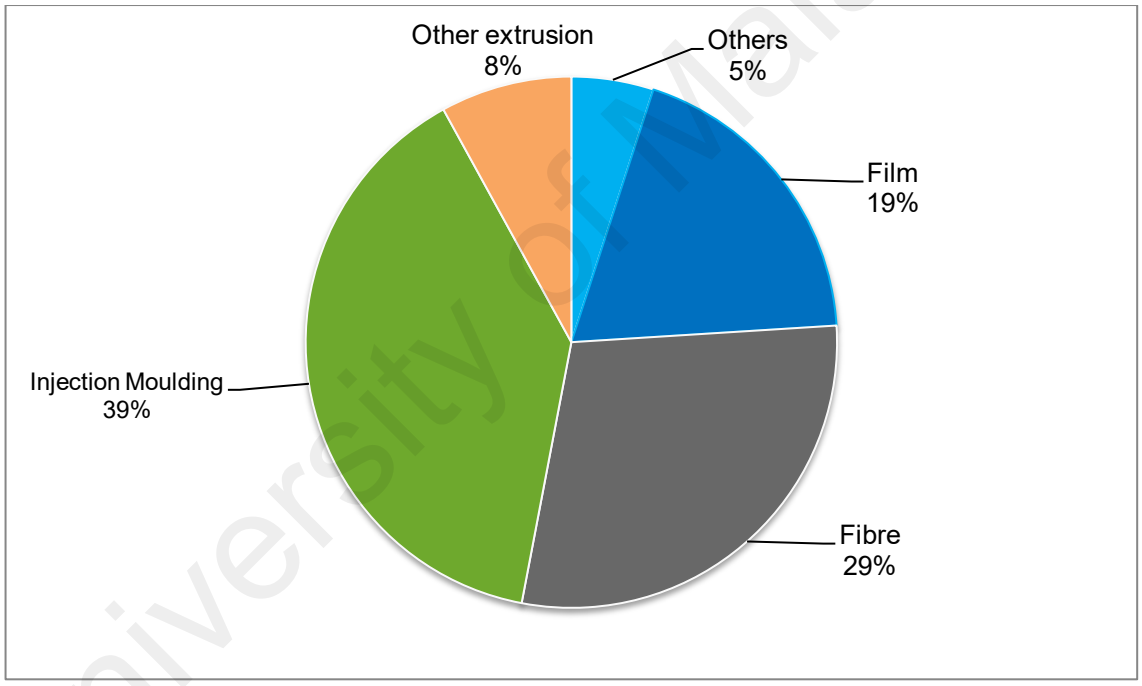


Figure 2.2 : Global polypropylene demand according to its applications, 2015 (Thammanayakatip, 2016)

Polypropylene is the most widely used thermoplastic due to its properties such as it is flexible for molding, low cost of production, an excellent resistance to acid and bases, and good fatigue resistance (Maddah, 2016). In addition, it can resist high temperature which make it suitable for item that need to be sterilised frequently (Asmita *et al.,* 2015). In addition, the rate of recycling for polypropylene is below 1% due to the difficulty to separate polypropylene from contamination and removing taint and odor (Iwan *et al*.,

2012). So that, exploration of new economically feasible technology to make this waste reusable is of great importance.

2.2.2. Polystyrene

Polystyrene is defined as a synthetic aromatic polymer consist of covalently bound units of styrene monomer known as C_8H_8n and the chemical structure is shown in Figure 2.3.

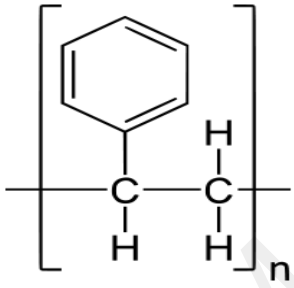


Figure 2.3: Structure of polystyrene (Colin, 2015)

There are three types of polystyrene such as extruded polystyrene, extruded polystyrene foam, and polystyrene foam (Atiq *et al.,* 2010). It is unstructured, linear polymer, low melting point and high molecular weight. Polystyrene In unprocessed form, is clear, brittle and it is often combined with other materials to obtain desired properties (Nicolas *et al*., 2016).

Polystyrene is a polymer that can be used for many purposes. It has been used to manufacture a range of products in foamed and rigid forms. It demonstrates exceptional physical and processing properties, hence, making it one of the most used form of plastic (Meenakshi *et al*., 2001). It is used in packaging, electronics, medical application, craft, manufactured items and constructions.

Meanwhile, The Society for Plastics Industry (SPI) has given the code number 6 for polystyrene which reflects how difficult it is to recycle the plastic (Aminudin *et al.,*

2013). In general, polystyrene is considered as difficult, or almost impossible to recycle. Tulio (2015), claimed that it is not economical to collect polystyrene due to the low density of the polystyrene foam. In response to these negative environmental and health impacts, over 100 cities around the world have moved to prohibit polystyrene foam in their communities. As an example, New York City, had enacted a ban on single-use polystyrene foam that took effect on July 1, 2015 .

2.2.3 Polyethylene

Generally, polyethylene is a long chain carbon atoms, and each carbon atom is attach with two hydrogen molecule. It can be divided into low and high density polyethylene which is differentiated by the structure. Low density of polyethylene have branched structure, cheaper, flexible and easier to cut. On the other hand, high density polyethylene have a linear structure which make it more stable than the branched polyethylene and is always milky white in color (Majid *et al*., 2010). Chemical formula for polyethylene is C_2H_{4n} and the structure is shown in Figure 2.4.

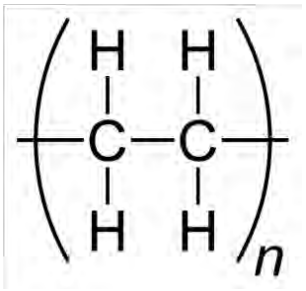


Figure 2.4: Structure of polyethylene (Colin, 2015)

Polyethylene is inexpensive and can be moulded, extruded and casted into different shapes. These features make it popular in construction as polyethylene is a dimensionally material that is stiff, hard, stiff, strong and absorbs little water (Cole, 2011).

Most polyethylene materials are colourless, and highly transparent, however, thicker materials might be opaque and off white in colour. Moreover, it has high chemical resistance against oils, greases and acids, as well as having good gas barrier properties (Plastic Europe, 2016).

Plastics Europe (2016), reported that polyethylene is most common form of polymer manufactured in the world and each year, its total production reaches over 90 million metric tons. Polyethylene is most commonly used in the production of grocery bags, shampoo bottles, and children's toys (Roy *et al.,* 2011). Technology advances have progressively improve polyethylene functions, thus, it has become one the most efficient naturally derived (petroleum and natural gas) products (Plastic Europe, 2016).

There is no doubt that polyethylene is an important and valuable material in industry. Polythene and plastic waste are found to accumulate in the environment, posing a major ecological threat. They are found to be considered non-degradable, once it enters the environment it has been found to remain there indefinitely. Widespread studies on the biodegradation of plastics have been carried out in order to overcome the environmental problems associated with synthetic plastic waste.

2.2.4. Polyethylene Terephthalate

Polyethylene terephthalate (PET) comprises of polymerised units of the monomer ethylene terephthalate, with repeating $C_{10}H_8O_4$ units. According to Fries (2013), the polyethylene terephthalate is polymer, which is produced by combining two monomers, modified ethylene glycol and purified terephthalic acid. PET can be manufactured through the process of terephthalic acid and ethylene glycol polymerisation. According to UNEP (2016), terephthalic acid is a crystalline solid derived from xylene while ethylene glycol is a colourless liquid derived from ethylene.

Ji (2013), described PET as member of the polyester polymers family, which is strong, stiff synthetic fiber in form of a plastic resin. Figure 2.5 illustrates the chemical structure of PET.

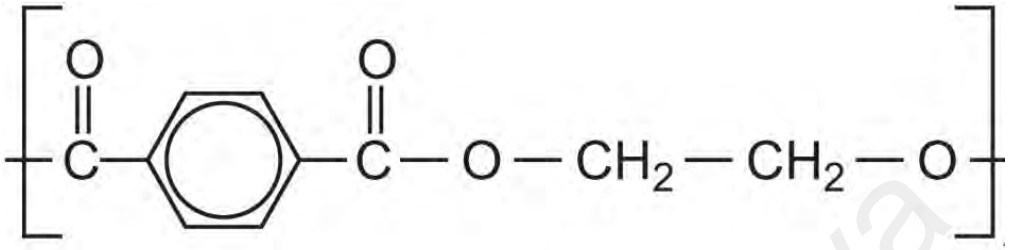


Figure 2.5: Structure of polyethylene Terephthalate (Ji, 2013)

PET has the code of 1 in its recyclability, indicating it is easy to recycle. It is one of the commonly recycled forms of plastic. As it is an exceptional water and moisture barrier properties, PET is commonly used for soft drinks bottles and for durable containers that are high-impact resistant (Hopewell *et al*., 2009). Aside from storing soft drinks, PET bottles are widely used to store edible oils, peanut butter, mouthwash, and as cereal box liners.

Furthermore, Arena (2003), described that modified PET can be heated in a microwave or in a conventional oven at 180°C for 30 minutes, making it ideal as a material for microwave food trays.

2.3 Overview of plastic production and consumption

Plastics have been chosen as packaging materials and replace cellulose-based products. This is due to their better physical and chemical properties such as versatility, lightweight, flexible, durable and relatively inexpensive. Most industries use plastic as part of their production as shown in Table 2.4. Commonly, it is used as packaging material, agricultural film, disposable diaper backing, and fishing nets.

Table 2.4: Percentage of plastic consumption by different sector (Plastic Europe, 2016)

Sector	**Consumption**
Packaging	39.6%
Building and construction	20.3%
Household and consumer products	21.7%
Automotive	8.5%
Electrical and electronic	5.6%
Agricultural application	4.3%

Plastic Europe (2016), reported that the yearly plastic production has gradually increased to 299 million tonnes in 2013 from 1.5 million tonnes in reported in the 1950s. These illustrates that the demand for plastic is increasing by 4% every year. Table 2.5 shows the market demand for plastics in Europe.

Table 2.5: Percentage of market demand and common applications of some plastics (Plastics Europe, 2016)

Types of Plastics	**Percentage of market demand**	**Common applications**
Polyethylene (PE)	17.2%	Bottles, plastic bags, gear, pipes for fish farming, cages
Polypropylene (PP)	19.2%	strapping, rope, gear, bottle caps
Polystyrene (expanded) (EPS) * (part of PS %)	12.1%	Bait boxes, floats, cups , expanded packaging
Polystyrene (PS)	7%	Utensils, containers, packaging
Polyvinyl chloride (PVC)	10.3%	Film, buoys, pipes and containers
Polyurethane (PUR)	7.5%	Insulation
Polyethylene terephthalate (PET)	7%	Bottles, strapping, gear
Others	19.77%	-

Due to the mass production and heavy use of plastics, the plastic debris load in the environment has increase rapidly. To address increasing concerns over plastic marine debris, it is crucial to understand its pollution status and its valuable information, such as plastic type, abundance, size, and source, is necessary to develop an efficient management strategies

2.4 Plastic Disposal

As the world's population continues to grow, the amount of garbage produced are increasing too. EPA reported that 12 % of total municipal solid waste is made up by plastics. Hopewell *et al*. (2009), suggested three primary methods in plastic handling. These methods are recycling, landfill and incineration. However, all of these methods have their own advantages and limitations.

2.4.1. Landfill

Landfill sites have been the repositories of human garbage. It is the common method to dispose garbage. However some countries face a problem of limited space for landfill. Landfills is known as the most cost effective way to dispose garbage as compared to other waste management (Steven & Daniel, 2014).

However, even though some plastic wastes are recycled, most of them will be dumped in landfill. Galen (2010), reported that 20-25% of landfill weight is plastic and it will takes a long time to breakdown and degrade in natural environment and potentially leak pollutant into soil and water (Heudorf *et al.,* 2007). This is supported by (Webb *et al.,* 2013) which stated that plastic wastes in landfills could take more than 20 years to degrade, due to the limited oxygen content in landfills.

Andrady (2011), explained that plastics in landfills have shown limited degradation because the thermooxidative degradation, and the anaerobic conditions in landfills only further the limit their rates of degradation.

A major disadvantage in burying plastic is that because material flow for plastics is linear rather than cyclic, hence, none of the resources used to produce the plastic could be recovered (Teuten *et al*., 2009). Leachate and toxins produce in landfill may have the potential to pollute groundwater and soil. In addition, landfill release methane and greenhouse gases as a byproduct of trash decomposer, which can contribute to health problem and climate change.

2.4.2. Incineration

Other than landfill, incineration is usually practiced to dispose plastic waste. By burning the plastic, it will overcomes the limitations of space in landfill (Webb *et al.,* 2013). Plastics are particularly attractive for burning, as they are made from petroleum and give out a lot of energy when burned. The energy can be used to heat homes or generate electricity.

However this method has drawbacks. One of the most prominent effects is the release of toxic substances into the atmosphere as a result of burning the plastics. Consequently, this will increase the production of carbon dioxide which will lead to global warming. Some toxic gases may be released during the burning unless they are incinerated at consistently high temperature. The presence of halogenated additives and PVC is common in mixed plastic wastes, increasing the risks of releasing other polychlorinated biphenyls, dioxins, and furans into the environment (Gilpin *et al.,* 2003).

In this light, researchers strive to develop better plastic recycling process in response to the substantial environmental effects of disposing plastics through landfills and burning.

2.4.3. Recycling

Reduce, reuse and recycle are the most appropriate ways to decrease the generation of plastic wastes, and to stop plastic wastes from being dumped in landfills. Plastic are persistent in nature as it will take a long time to degrade which mean it can sit in a landfill for a thousand years. Benefit of Recycling (2010), reported that 7.4 m^3 of spaces in landfill can be saved by recycling plastic for landfill.

They are many ways to recycle plastic materials, and the complexity of the recycling process depends on their properties. For instance, rigid containers make out of a single polymer would be simpler and less costly to recycle compared to multi-layer and multi-component packages (Andrady *et al*., 2011).

Recycling consists of various steps that include collecting, sorting, cleaning, size reduction and separation, as well as compatibilisation. These are done to decrease contamination from incompatible polymers. Thermoplastics, such PET, polyethylene and polypropylene, can potentially be recycled mechanically.

Meanwhile, thermosetting polymers such as epoxy resin and unsaturated polyester could not be mechanically recycled, however, they be pulvarised ad their size can be reduced to fine particles or powders to be used as filler materials (Rebeiz & Craft 1995). Each polymer has a different percentage of plastic recovered for recycling as shown in Table 2.6 and 2.7.

Table 2.6 : Quantities of plastic generated and recycled in MSW from 1960 to 2012 in USA (Environmental Protection Agency (EPA), 2014).

Year	Generated (1000t)	Recovery (1000t)	Recovery Rate
1960	390	-	-
1970	2900	-	-
1980	6830	20	0.30%
1990	17 130	370	2.20%
2000	25 550	1480	5.80%
2005	29 380	1780	6.10%
2008	30 260	2140	7.10%
2010	31 290	2500	8.00%
2011	31 840	2660	8.40%
2012	31 750	2800	8.80%

Table 2.7 :Percentages of plastics recovered for recycling from municipal solid waste facilities in the United States (World Centric, 2017).

Resin type	Percent Recovered
PET	20.7%
HDPE	11.3%
PVC	0%
LDPE/LLDPE	5.1%
PP	0.9%
PS	0.8%
Other	7.2%
All plastics	7.1%

Recyclability is identified through the labels in plastic products. The label shows the number surrounded by the recycling symbol. Each number indicates the category of resin used to produce the plastic as well as the recyclability of the products, as shown in Table 2.8.

Table 2.8: The Society for Plastics Industry (SPI) code.

	Used For	**Recyclability**	**Health**
Plastic 1 - Polyethylene Terephthalate (PET)			
	• Beverage bottles including such as for water and soft drink. • Detergent and cleaning containers • Food containers and bottles	Pet bottles can be recycled into: Polyester fabrics, carpets, bumper car filling and fiberfill for sleeping bags and jackets.	-
Plastic 2 - High Density Polyethylene (HDPE)			
	• water and milk jugs as well as plastic bags • Containers for household products like shampoo, laundry detergents, shampoo, as well as for motor oils•	Clear HDPE containers can be easily recycled to make new containers, while coloured HDPE can be converted into rope, pipes, lawn and garden edging, plastic lumber, and toys.	-
Plastic 3 - Polyvinyl Chloride (PVC or C)			
	•plastic squeeze bottles, cooking oil and transparent food packaging such as cling wrap • Window, door frames home siding and flooring	It is hard to recycle PVC as it contains a lot of additives. The disposal of PVC can result in potentially harmful substances.	Harmful chemical such as Lead, and Dioxins can cause diseases such as cancer, birth defects, and genetic mutation.

Table 2.8, continued

	Used for	**Recyclability**	**Health**
Plastic 4 - Low Density Polyethelene (LDPE)			
	• Bottles • Packaging for frozen food, and bread. • Plastic bags and wraps.	LDPE is not normally recycled.	-
Plastic 5 - Polypropylene (PP)			
	• Food containers • Disposable diapers • Outdoor carpet • House wrap	PP is not easily recycled depend on type and plastic grade.	-
Plastic 6 - Polystyrene (Ps)			
	• CD cases • Disposable cutlery from formed polystyrene (styrofoam) , and rigid polystyrene • Packaging and containers for food • Egg cartons • Insulation for buildings	Recycling polystyrene is possible but not economical.	Polystyrene can release styrene into the environment, and cause harmful effects to the kidney, liver, red-blood cells, and stomach

Table 2.8, continued:

	Used for	**Recyclability**	**Health**
Plastic 7 - Mixed (other)			
	• Medical storage • 5-gallon water containers • electronics • lids • baby bottles • bottles • clear plastic cutlery	Mixed resin plastics not usually recycled.	Polycarbonate plastic releases bisphenol A (BPA), which is known as Endocrine disruptor.

While some plastic waste is recycled, the majority of the plastics are left on sea and land over time fragment into smaller particles when exposed to the elements until they end up as microplastics (Claire, 2017). The presence of microplastics in the marine environment poses a great threat to the entire ecosystem. Considerable immediate reductions in the quantity of waste entering natural environments could be achieved by better waste disposal and material handling.

2.5 Microplastic

The Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) has defined microplastics as plastics with a maximum size of 5mm (Bowmer & Kershaw, 2010). Microplastics have been discovered widely in the natural environment, most notably in coastal sediments and oceans around the world (Mohamed Nor & Obbard, 2014). Hence, microplastic is of particular concern due to its abundance, and its persistence in the environment, which makes it a ubiquitous in nature.

Microplastic can be divided into primary microplastics and secondary microplastics Cole *et al*. (2011) described primary plastics as consist of microscopic sized plastic fragments. These types of plastics are commonly found in micro-beads in facial-

cleansers and cosmetics (Norwegian Environment Agency, 2014) as well as a material for air-blasting media (Table 2.9). The presence of microplastic fragments in the environment has raised particular environmental concerns. This is due to the minute size of the particles and their ubiquitous nature (Thompson *et al*., 2007).

Table 2.9: Microplastics contents in selected personal care products (Norwegian Environment Agency, 2014)

Type of product	**Microplastics Weight (mg)**	**Particle size (mm)**	**Plastic type**
Facial cleanser	1.62-3.04	0.1-0.2	PE
Hand cleanser	0.18-6.91	0.1-0.2	PE
Shaving foam	0.1-2	0.005- 0.015	PFTE
Tooth paste	0.1-0.4	0.04-0.8	PES
Facial Scrub	0.4-10.5	0.04-0.8	PE

Meanwhile, secondary microplastics comprise of tiny plastic fragments. According to Ryan *et al*. (2009), these fragments originated from breakdown of larger plastic debris, on sea and on land. Browne *et al.* (2008), suggested that this fragmentation is caused by the reduction of plastic debris structural integral due to the effects of physical, biological and chemical processes over time. Most microplastics found in the oceans are secondary plastics as a result of mesoplastic degradation and fragmentations. They can be divided into strands, hard, film and foam (Wessel *et al.*, 2016) shown in Figure 2.6.

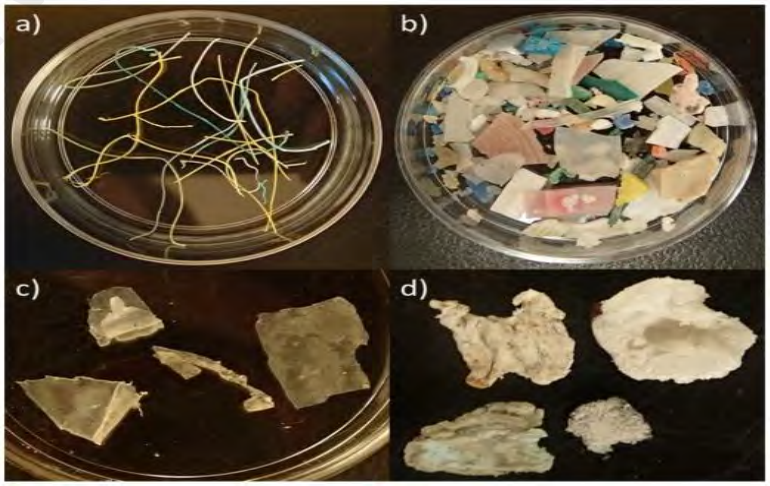


Figure 2.6 : Examples of the shapes of microplastics, a)strands, b)hard, c)film, d)foam (Wessel *et al*., 2016).

Both microplastic types (primary and secondary) exist in marine ecosystems at high concentrations. It has been estimated that about 245 tonnes of microplastics are produced each year which end up in water bodies where they become ingested and incorporated into the bodies and tissues of marine organisms (Morris, 2015).

High concentrations of microplastics of up to 2175 particles per kg of dry weight sediments have been documented in the coastal regions of the Mediterranean Sea (Vianello *et al.,* 2013). Small pieces of floating plastics in the surface ocean were first reported in the scientific literature in the early 1970s (Carpenter & Smith, 1972), and later publications described studies identifying plastic fragments in birds in the 1960s (Harper & Fowler, 1987). This situation will become progressively worse to the marine environment if there are no action taken to solve this problem.

2.6. Sources of microplastic in Coastal and marine environment

The ocean is increasingly clogged by marine litters, which are generated by irresponsible disposal of wastes either directly or indirectly into seas and oceans (Ryan *et al*., 2009). Much concern has been given over plastic contamination in the natural environmental, both among the general public and researchers.

The presence of marine plastic debris pollutes the environmental and has many well documented hazardous effects on wildlife living at sea and along the coastal areas (Hammer *et al*., 2012). However, there is still a lack of precise knowledge about the quantity, sources, transport, accumulation and fate of plastics in the oceans.

Each year, the increasing number of land and sea based activities such as fisheries, shipping and irresponsible waste disposal have resulted in the abundant of plastic debris on the ocean. In this light, plastic is one of the most common materials used all around

the world, and plastic debris have been found as far as the Artic and Antarctic regions, indicating that plastic wastes can drift far from their sources (UNEP, 2014).

Meanwhile, the origin of plastic wastes varies for each region, for example in East Asian region, shipping and fisheries are the primary contributors for debris, while mass tourism in the Mediterranean has been reported to contribute debris to the southern North Sea (Kershaw *et al*., 2011).

The occurrence of marine debris and its potential to cause harm has resulted in it being recognised as a global problem and their persistence continues to increase as they seem to be extremely difficult to remove manually because of small size and less visibility. In general, most forms of plastic debris enter the oceans due to improper disposal of wastes, where at times, plastics and other wastes are disposed directly in to the sea (Barnes *et al.,* 2009).

According to (Norwegian Environment Agency, 2014) during national beach clean-up 2014 at Norway, plastics accounted for 68% of all items, excluding styrofoam. About 80% of plastic debris found at sea are results of land-based activities, particularly in densely populated or industrialised areas.

This debris get into the marine environment as a result of excessive use of plastic bags, disposal of solid wastes and littering (Derraik, 2002). Thus, utilising microbes for the degradation of microplastics is a promising and environmentally safe action to reduce the debris in marine environments. Table 2.10 shows the number of debris and litters found during beach clean-ups.

Table 2.10 : Total numbers of litter items counted in the national beach clean-up (Norwegian Environment Agency, 2014)

Types of Plastic	**Number (1.000)**	**Details**
Undefined	130	Come from different sources Different forms of plastics
Styrofoam	27	Debris related to fisheries such as from fish storage boxes
Ropes <50 cm	23	Debris related to fisheries such as debris from fish farming and shipping
Plastic and metal bottle caps.	19	Mostly come from Norwegian made bottles, as well as bottles from foreign countries
Beverage bottles	14	Mostly from Norwegian brands as well as from other foreign brands
Cigarettes, snuffs	11	Might come from sewage and boats
Food packaging	10	Mainly the residual wastes from fishing boats, shipping, and land based activities. Brand name/ label might indicate origin
Plastic bags	9	Partly from residual waste, from fishing boats, shipping and land based. The brand names or label indicate their origins.
Rope > 50 cm	9	Debris commonly related to fisheries, fish farming and, shipping activities.
Building materials	3	This type of debris originated from Norway and other foreign countries.

Each year, more than six million tonnes of rubbish ended up in the ocean, almost 80% of these wastes are plastic, and approximately 10% are fragmented plastic bags (Wabnitz & Nichols, 2010). As more than 50% of the world's population are residing within fifty miles of the coastline, Moore (2002), stipulated that plastics could enter the marine environment through rivers and wastewater-system, as well as, being blown off the shore.

This is supported by Kershaw (2011) who claimed that plastics get into the marine environment through poor waste management, rivers, or by being thrown into the ocean. Other researchers, like Redford *et al*. (1997), found that huge quantities of raw manufacturing materials found on beaches or at sea are from the accidental spillage during the waste handling and other processes. Furthermore, Browne *et al.* (2009), claimed that other land based sources for debris are leachates from landfill, effluent and wastewater.

Other common source for plastic debris in the marine environment is fishing gear (Andrady *et al*., 2011). These include lost or discarded fishing gears such as plastic monofilament line and nylon netting. As they are practically buoyant, they are able to drift at variable depths in the oceans. In recent years, it was estimated that almost 700,000 tonnes of fishing gears have been lost at sea, and this contributes to almost 10% of the total amount of marine debris (Good *et al.,* 2010).

Furthermore, these discarded fishing items such as nylon netting float and monofilament lines at particular depths ocean depth, resulting in "ghost fishing", causing accidental entanglement of marine lives (Mouat & Lozano, 2009). Table 2.11 presents the types of plastic waste cused by fisheries and fish farming activities.

Table 2.11: Plastic waste from fisheries and fish farming activities, Norway (Norwegian Environment Agency, 2014)

Applications	Total waste amount (Tonnes)	Recycled amount (Tonnes)	Potential risk for littering
Fish farming rings (PE)	7 000	500	These wastes have medium to low pollutant risks. This is because they are seldom discarded due to their value and the high cost for collection these equipment are often being stored or reused for other uses.
Feeding pipes (PE)	800	150	These wastes have medium to low pollutant risks. Due to the high cost for collection, these equipment are often being stored or reused for other use.
Nets, fish farming (PA)	2 500	1500	They have medium/low risk for littering, as when the nets are delivered for net- washing, some damaged nets might get lost
Ropes (PP)	3000	600	They have medium risk of pollutants. They are often lost or discarded , as there is no regular take back system due to their lower value.
Nets, trawls for fisheries (diverse)	2000	650	They have medium risk of pollutants as they are often lost or discarded into the ocean, and there is no regular take back system. Their presence increase the risk of ghost fishing
A range of floatation devices	200	-	They have medium to high: risk of pollutants, as they tend to get ost or discarded in the ocean. They have no regular take back system and are difficult to recycle
Total	15 500	3500	

Marine litter threatens biodiversity, health and economy. Considerable immediate reductions in the quantity of waste entering natural environments could be achieved by better regulation of waste disposal and material handling.

In addition, Lee *et al.* (2013) observation of debris in North South China Sea reported that a majority of floating and beached plastic debris are remnants of coastal or land based activities. The most common items collected during Ocean Conservancy's annual International Coastal Cleanup were remarkably consistent; cigarette butts topped the list, while plastic items comprise of 83% of other items (Thompson, 2009). Table 2.12 shows the abundance of microplastics found in sediments all across the world.

Table 2.12: Worldwide abundance of microplastics in sediments (Van *et al.,* 2015)

Continent	**Location**	**Location specification**	**Particle size**	**Measured abundance**	**Reference**
Africa	Canary Island	Beach	1 mm – 5 mm	<1 ->100g/L	Baztan *et al.,* (2014)
Americas	Hawaii	Beach	1 mm-15 mm	541- 18,559 items/260 L	McDermid and McMullen., (2004)
	US	Florida subtidal	250 mm- 4 mm	116 - 215 items/L	Graham and Thompson, (2009)
		Maine subtidal		105 items/L	
	Brazil	Beach	2 mm – 5 mm	60 items/m2	Ivar do Sul *et al*., (2009)
	Brazil	Beach	0.5 mm - 1 mm	200 items/0.01 m^2	Costa *et al*., (2010)
			1 mm - 20 mm	100 items/0.01 m^2	
	Hawaii	Beach	250 mm - 10 mm	0.12% -3.3% plastic by weight	Carson *et al*., (2011)
	Brazil	Tidal plain	1mm - 10 cm	6.36-15.89 items/m^2	Costa *et al*., (2011)
	Chile	Beach	1 mm - 4.75 mm	<1 - 805 items/m^2	Hidalgo-Ruz and Thiel, (2013)
	Quebec	River sediment	400 µm - 2.16 mm	52 - 13,832 beads/m^2	Castaneda *et al.,* (2014)
	Nova Scotia	Beach	0.8 µm - 5 mm	20 - 80 fibres/10 g	Mathalon and Hill, (2014)

Table 2.12, continued

Continent	Location	Location specification	Particle size	Measured abundance	Reference
Asia	Singapore	Beach	1.6 µm - 5 mm	0 - 4 items/ 250 g dry	Ng and Obbard (2006)
	India	Ship-breaking yard	1.6 µm - 5 mm	81.4 mg/kg	Reddy *et al*., (2006)
	South Korea	High tide line	2 mm - 10 mm	913 items/m^2	Heo *et al*.,(2013)
	India	Beach	1 mm - 5 mm	10 - 180 items/m^2	Jayasiri *et al*., (2013)
	South Korea	Beach dry season	1 mm - 5 mm	8205 items/m^2	Lee *et al*., (2013)
		Beach rainy season		27,606 items/m^2	
	Singapore	Mangrove	1.6 µm - 5 mm	36.8 items/kg dry	Nor and Obbard, (2014)
	NW Pacific	Deep sea and trench	300 µm - 5 mm	60-2020 items/ m^2	Fisher *et al.,* (2015)
	South Korea	Beach	50 µm – 5 mm	56 – 285 673 items/ m^2	Kim *et al*., (2015)
Europe	UK	Beach	1.6 µm - 5 mm	0.4 fibres/50 mL	Thompson *et al.,* (2009)
		Estuary		2.4 fibres/50 mL	
		Subtidal		5.6 fibres/50 mL	
	Sweden	Subtidal	2 µm - 5 mm	2 - 332 items/100 mL	Noren, (2007)
	UK	Beach	1.6 µm - 1 mm	<1 - 8 items/50 mL	Browne *et al*., (2008)
	UK	North Sea beach	38 µm - 1 mm	0.2 - 0.8 fibres/50 mL	Browne *et al.,* (2008)
		English Ch. beach		0.4 - 1 fibres/50 mL	
	Belgium	Harbour	38 µm - 1 mm	166.7 items/kg dry	Claessens *et al*., (2011)
		Continental Shelf		97.2 items/kg dry	
		Beach		92.8 items/kg dry	
	Portugal	Beach	1.2 µm - 5 mm	133.3 items/m^2	Martins and Sobral, (2011)

Table 2.12, continued

	Location	**Location specification**	**Particle size**	**Measured abundance**	**Reference**
	Germany	Urban beach	1 mm - 15 mm	5000 - 7000 items/m^3	Ballent *et al*., (2012)
		Rural beach		150 - 700 items/m^3	
	Germany	Tidal flat	1.2 µm - 5 mm	0 - 621 items/10 g	Liebezeit and Dubaish (2012)
	Italy	Sub-alpine lake	9 µm - 5 mm	1108 items/m^2	Imhof *et al*., (2013)
	Greece	Beach	1 mm - 2 mm	57 - 602 items/m^2	Kaberi *et al.,* (2013)
			2 mm - 4 mm	10 - 575 items/m^2	
	Belgium	High tide line	38 µm - 1 mm	9.2 items/kg dry	Van Cauwenberghe *et al*., (2013)
		Low tide line		17.7 items/kg dry	
	Italy	Subtidal	0.7 µm - 1 mm	672 - 2175 items/kg dry	Vianello *et al*., (2013)
	Germany	Beach	<1 mm	1.3 – 2.3 items/kg dry	Dekiff *et al*., (2014)
	Slovenia	Beach	0.25 - 5 mm	177.8 items/kg dry	Laglbauer *et al*., (2014)
		Infralittoral		170.4 items/kg dry	
Worldwide		Deep sea	5 µm - 1 mm	0.5 items/cm^2	Van Cauwenberghe *et al.,* (2013)

The statistics of microplastic distribution in the world's aquatic environment is very troubling as the concentrations are very high, hence creates a concern especially as it relates to impact of such enormous distribution on aquatic life.

For the majority of these studies the main focus was not to assess the occurrence and abundance of these pellets, but rather to evaluate the contaminant load present on these pellets. Indeed, their size, long environmental persistence and worldwide distribution, make them especially suitable for chemical analysis (Mato et al., 2001).

Even though plastics are generally deemed as biochemically inert (Roy *et al*., 2011), plastic additives are often inserted into plastics during manufacturing process to improve their features such as to extend the plastic life, enhance resistance to damages from microbial degradation and heat oxidation (Browne *et al*., 2007). The addition of such additives raises environmental concerns as they can extend plastic degradation times and may release potentially toxic chemicals to the marine environment (Barnes *et al*., 2009).

The high usage of plastics and the improper plastics waste management cause the accumulation of this waste on land and marine environment. If ingested, macro and microplastics could possibly bring adverse health complications to organisms (Fendall & Sewell, 2009). Such adverse effects include reduced, decreased steroid hormone levels, feeding stimuli, inhibition of gastric enzyme secretion, the blockage of the intestinal tract and delays in ovulation, and consequently failure to reproduce (Wright *et al*., 2013).

The microplastics is harmful to marine organisms as they concentrate and transfer chemicals from the water to the marine life via ingestions (Jayanthi *et al*., 2014). Plastics could be mistaken as food sources that it might be ingested by marine life like turtles, mussel, oyster, fishes and seabirds.

2.7 Environmental impact of plastic pollution

Most plastics are not biodegradable, and they will remain in the environment for decades. For instance, plastic film containers have a long lifetime while plastic bottles is claimed to last indefinitely. Furthermore, plastics are lightweight with high resistant to moisture. This allows them to float easily in water for long distances.

In the marine environment, organic pollutants can attach to the plastic surfaces, and plastics floating in the oceans and transport them through ocean currents. Past studies had reported that plastic particles at sea contain high levels of organic pollutants.

According to Mato (2001), studies had consistently found the presence of organic pesticides, such a dichlorodiphenyltrichloroethane (DDT), polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs) and bisphenol A (BPA) found toxic chemicals, such as polychlorinated biphenyls (PCBs), and nonylphenol (NP), on plastic debris samples taken from the ocean. This situation will deteriorate marine water quality and threatened microbial abundance in marine environment.

Prabhakar *et al.* (2016), stated that each year, plastics cause the death of up to 100,000 sea mammals, countless fishes and 1 million sea birds. Many of these animals died as they become entangled in nylon ropes, plastic strapping and plastic six-pack rings. In this light, Cole *et al*. (2011) mentioned the effect of the presence of plastic debris at sea has long been debated by environmentalists and researchers, macroplastics have been responsible for the injury and death of fishes, marine mammals, reptiles and fish as they accidentally ingest the food and become entangled in the plastic (Mouat & Lozano, 2009). Furthermore, Gregory (2009) depicted that floating plastic debris, can transport non-native marine species to new habitats and sinking plastic debris can smother the seabed, cutting gas-exchanges and creating artificial hard-ground,

Studies show that compared to adult birds, young birds ingests a predominantly higher amount of macroplastic and microplastic debris (Acampora *et al.,* 2014). Macroplastic and microplastic particles have also been detected in the system multiple fish species across the world, including the North Pacific Ocean (Jantz *et al.,* 2013), the South Atlantic Ocean (Dantas *et al*., 2012), the Mediterranean Sea (Romeo *et al.,* 2015), and the North Sea. Seabirds and fishes are not the only the species facing the problem of

plastic ingestion, sea turtles are also reported to die from entanglement and ingestion of marine plastic debris. Schuyler *et al.* (2014), projected that cases of plastic ingestion by green turtles had increased by nearly 20% from 1985 to 2012. This situation will seriously impact aquatic life, hence a suitable strategies should be developed and new regulations are to be set up to control the manufacture, sale and distribution of plastics in order to avoid exceeding critical environmental threshold concentrations.

It is assumed that most of this debris was ingested during predation activities. Studies such as Da Silva Mendes *et al*. (2015), reported that the most plastic particles ingested by these animals are white or transparent, indicating the possibility that plastics might be mistaken for jellyfish. The potential pathways of plastic debris transportation and its biological interactions is shows in Figure 2.7.

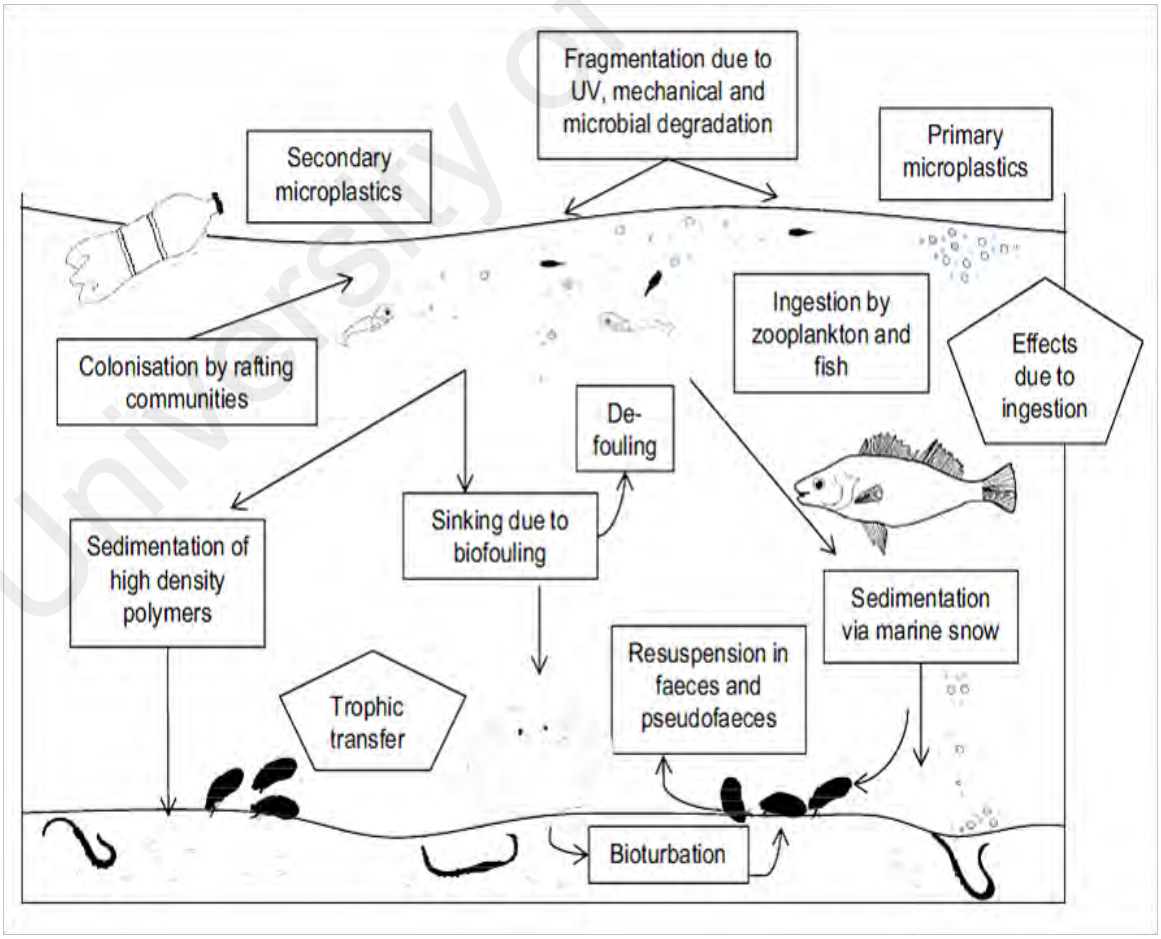


Figure 2.7: Interaction between marine organisms and plastic debris (Moret, 2010)

Moreover, Valavanidis and Vlachogianni (2014), reported the plastic ingestion cases reported between 2000 and 2010 involved more than 48 cetacean species, including whales and dolphins. This figure is 11 times higher than what recorded between the 1960s and 1970s. Plastic ingestion has become a major threat for marines mammals as dolphin and whale hunting had been predominantly outlawed as a measure to protect these endangered species. Plastic debris have become the most prominent hazards to them.

One such cases was reported by Derraik (2002), where a west Indian manatee, an endangered species was found dead in Florida as a result of the digestive tract blockage after ingesting a large piece of plastic. A recent study by Stephanis *et al.* (2013), reported that the death of sperm whales in the Mediterranean Sea could be attributed to either starvation or gastric rupture as the result of the ingestion of plastic debris. Besides direct mortality, there are many cases of sub-lethal plastic consumption by animals which lead to trophic or physiological impacts (Hirai *et al*., 2011). As ingested microplastics remain and accumulate in the digestive tract, and consequently, lead to health issues such as reduced energy reserves and internal blockages (Wright *et al.,* 2013).

Researchers have raised concerns related over the probable ingestion of microplastics by marine animals, Mato *et al*. (2001) reported that microplastics can absorb harmful chemicals up to one million times higher than ambient seawater, and these chemicals can be transferred to the host organisms biological tissues through ingestion (Teuten *et al*., 2009). This pollutant could give negative impact to environment since the chemicals such as addictives, monomer and by-product that are bound to particles may leach to the marine environment .

Table 2.13 shows the frequency of occurrence of different polymer types in 42 studies of microplastics debris sampled from marine sediments or at sea (Hidalgo-Ruz *et al.,* 2012).

Table 2.13 : Frequency of occurance different types of microplastics sampled at sea or in marine sediments in 42 studies (Hidalgo-Ruz *et al*. 2012).

Marine species	Plastics exposures	References
Suspension- and depositfeeding bivalves.	The particle-feeding bivalves show the ability for parparticle selection.	Ward and Shumway (2004).
Mussel (*Mytilus edulis)* Oyster (*Crassostreavirginica)*	10-um, non-fluorescent polystyrene beads.	Ward and Kach (2009).
Sea cucumbers (*Echinodermata* *Holothuroidea*)	Deposit and suspension feeding sea cucumber ingest small plastic fragments along with sediments (15-25 mm); during feeding trials, they ingested between 2 and 20-fold more plastic per individual (PVC fragments) and between 2- and 138-fold more nylon line than expected.	Graham and Thompson (2009)
Mussel (*Mytilus edulis*)	Initial experiments showed that microplastic particles accumulated in their guts; then, they are treated with seawater with microplastics (3.0 or 9.6 µg). These particles moved from the gut to the circulatory system within 3 days, and remained there for over 48 days.	Browne *et al*., 2008
Norwegian lobster (*Nephrops norvegicu*)	In an experimental setup, fishes with strands of polypropylene rope were fed to the Nephrops . Consequently, the study found that the plastic particles were ingested, but not excreted.	Murray and Cowie (2011)

Table 2.13, continued

Marine species	Plastics exposures	References
Green algae *(Scenedesmus)*	Nano-sized plastic beads and the adsorption of nano plastics.	Bhattacharya *et al.,*. 2010
Mussel (*Mytilus edulis*)	Digestive gland vacuoles in mussels absorb 1-80 m microplastics , and this is linked to granulocytoma formation	Bowmer and Kershaw (2010)
Bacteria, eukaryotes and archaea	Biofilm colonisation of polyethylene (LDPE).	Harrison *et al.,* 2014
Microbial biofilm	For 3 weeks, there was a colonisation of the microbial biofilms on 2 cm x 2 cm polyethylene films in seawater, this coincides with the significant changes in the PE physio -chemical properties of and more neutral buoyancy of the films.	Lobelle and Cunliffe (2011)

Based on the data and result that shown above, the presence of microplastic fragments in the environment has raised particular environmental concerns. It does not only cause aesthetic issue, conseuently can cause the decline of coastal economies for the lack of tourism activities and the increased costs of clean-ups.

Communities around the world are becoming more aware of negative impact of discarded plastic to the environment. Consequently, several prominent campaigns to curb microplastic problem, such as "Beat the Microbead" movement have been initiated. This campaign calls for the removal of plastic particles from personal care products (Jorgensen *et al*., 2015).

National and state level actions have also been taken as a measure to mitigate the negative environmental effects of microplastics. Rebecca (2016), reported that Illinois became the first U.S. state to ban cosmetics with microplastics while the Microbead-

Free Waters Act of 2015 was proposed by New Jersey Congressman Frank Pallone, which was enacted after being signed on December 28, 2015 (Hollman, 2013). The Act calls for a national ban on the productions and sales of products with microbeads by 2018. The increased knowledge on the detrimental effects of microplastics on the environment has driven many environmental groups to advocate the removal and ban of microplastics from various products.

Widespread studies on the degradation of plastics have been carried out in order to overcome the environmental problem associated with synthetic plastic waste. Consecutive paragraph discuss the pathway of plastic degradation.

2.8 Plastic Degradation

2.8.1. Photodegradation

Degradation is defined as a chemical change that reduces the average molecular weight of a polymer. Andrady (2011), reported that most plastics break down slowly through a combination of photodegradation, mechanical abrasion, and oxidation. Photo degradation is the alteration of materials by light. UV light from the sun provides the activation energy required to initiate the absorption of oxygen atoms of the polymer resulting to plastic fragmentation (Reisser, 2013).

Photodegradation is one of the important component of natural degradation. It can occur directly or indirectly in marine environment. Andrady (2011), reported that photodegradation is an effective mechanism for polypropylene and polyethylene degradation upon exposure to air when lying on a beach surface. On the whole, this is an incredibly lengthy process and insignificant in marine environment due to negligible rate of hydrolysis of most plastics in the ocean and limited of sunlight exposure and oxygen availability in seawater.

2.8.2. Thermal degradation

Thermal degradation is a degradation process resulted from overheating at high temperature. The properties of the polymer change as the component chain of polymer backbone will separate and go through molecular scission, before reacting with one another (Shah *et al.,* 2008). Thermal degradation involved chemical reactions which can cause changes to physical and optical properties, which are related to the initially specified properties (Andrady *et al*., 2008). Olayan *et al*. (1996) claimed that this process generally involves changes to the polymer's molecular weight and molecular weight distribution. Typical property changes include chalking, colour changes, cracking reduced ductility and embrittlement, and general reduction of other desirable physical properties. However, this process is taking much timw and becomes difficult when it takes place underwater due to the lower temperatures of the ocean.

2.8.3. Biodegradation

Currently, much research has been focused on the biodegradation method to overcome contaminant in environment. Biodegradation involves a process of breaking down organic substances into smaller compounds through the help of living organisms. In this light, Zheng *et al*. (2005) described that the natural degradation of plastic can cause the plastic to break into smaller pieces, consequently, polymer chains will have sufficiently low molecular weight to enable them to be metabolised by microorganisms.

During the biodegradation process, the plastic matters could be converted into minerals. Andradry *et al*. (2011), described that the carbon in the polymer chains will be converted to carbon dioxide or incorporated into their biomolecules. As a result, the aerobic biodegradation will produce carbon dioxide and water while the anaerobic biodegradation will produce water and methane (Vijay, 2015).

However, the entire process of biodegradation is very slow and in an unfavourable condition as it can take 50 years or more for plastic to fully degrade (Muller *et al*., 2012).

Gopferich (1997), suggested abiotic hydrolysis as the most suitable reaction that can help simulate the environmental degradation of synthetic polymers. Figure 2.8 illustrates the general mechanism of plastic biodegradation under aerobic conditions, as reported by Mueller (2003).

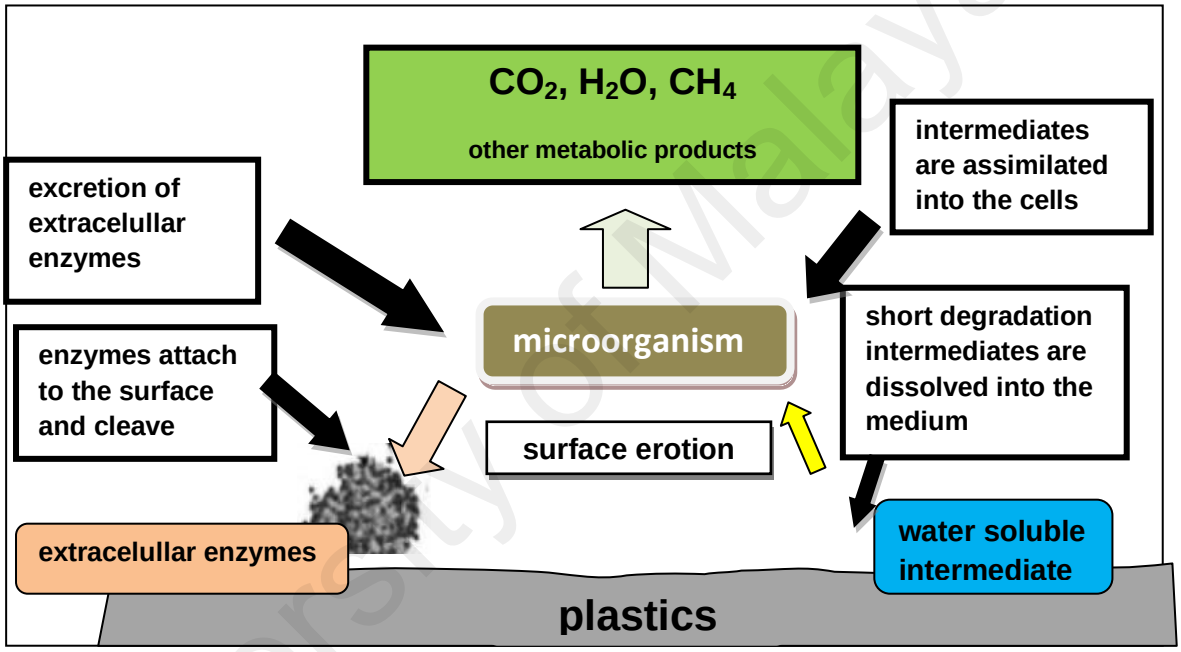


Figure 2.8 . General mechanism of plastic biodegradation under aerobic conditions (Mueller, 2003)

During the process of degradation, the polymers are converted into monomer, before they are being mineralized; as mentioned by Shah *et al*. (2008), the polymer need to be depolymerised into smaller monomers before they can be absorbed and biodegraded within the microbial cells. The microbial will start growing and it obtains carbon sources from plastic polymers as it latches on to the surface. During the primary degradation, low-molecular weight fragments oligomers, dimers or monomers formation is caused by the main chain cleaves (Komer *et al*., 2005). As a result, the breakdown fragments need to be fully used by the microorganisms need so that any

potential health environmental and risk do not occur. There are many research on biodegradation of polymer worldwide as shown in Table 2.14. It shows that bacteria are very opportunistic and can invade and adapt in any environment. Biodegradtion of microplastics is a promising and environmentally solution.

Table 2.14: Past research of plastic biodegradation

Plastic	Microorganism	Reference
Polyethylene	*Brevibacillus borstelensis*	Hadad *et al*. (2005)
	Rhodococcus rubber	Sivan et al. (2006)
		Gilan *et al*. (2004)
	Penicillium simplicissimum	YK Yamada-Onodera *et al.* (2001)
Polyurethane	*Comamonas acidovorans TB-35*	Akutsu *et al*. (1998)
	Curvularia senegalensis	Howard (2002)
	Fusarium solani	
	Aureobasidium pullulans	
	Cladosporium sp.	
	Pseudomonas chlororaphis	Zheng *et al.* (2005)
Polyvinyl chloride	*Pseudomonas putida AJ*	Anthony *et al.* (2004)
	Ochrobactrum TD	
	Pseudomonas fluorescens B-22	Mogil`nitskii *et al*. (1987)
	Aspergillus niger	
Plasticised polyvinyl chloride	*Aureobasidium pullulans*	Webb *et al*. (2000)
BTA-copolyester	*Thermomonspora fusca*	Kleeberg *et al*. (1998)

Biodegradation is determined by a wide range of factors. These factors include nature of pre-treatment, type of organism and polymer characteristic. In this light, the mechanism of biodegradation is influenced by the plastic's physical and chemical properties of plastics influence. In this regards, polyesters with side chains are commonly be less prone to assimilation compared to than those (Shah, 2008). Moreover, the morphology of the polymers significantly influence the biodegradation rate while the molecular weight is crucial for biodegradability as it determines various physical properties of the polymer; for instance, the increase of the polymer's molecular weight will consequently , decrease its degradability (Hadad *et al*., 2005).

The crystallinity degree is a significant factor that can affect biodegradability. This is because enzymes mainly attack a polymer's amorphous domains (Gilpin *et al.,* 2003). Furthermore, Gu *et al*. (2000), stipulated that the molecules in the amorphous region are loosely packed, making them more exposed degradation, meanwhile, the polymers' crystalline part shows more resistant compared to the amorphous region.

The higher exposure to UV radiation and mechanical erosion makes biodegradation in sediment to be more significant than in water (Gregory & Andrady, 2003). On the other hand, plastic degradation rate in sediment can be mostly negligible. This is the mechanical and chemical labile minerals like clays and feldspars are easily washed out into the ocean (Derraik *et al*., 2002).

Plastic biodegradation occurs actively in a range of soil conditions, based on the properties of the soil as different microorganisms would be responsible for the process of degradation in each type of soil, these microorganisms often have their own ideal growth conditions in the soil. Complete degradation refers to the destruction of the polymer chain and its complete conversion into small molecules such as carbon dioxide or methane is also called mineralisation (Mato *et al*., 2001).

The first reports on the presence of the microbial microplastics colonisation in seawater indicated a 'rod shaped Gram negative bacteria' with the size from ~0.5 mm polystyrene spherules (Thompson *et al*., 2009), and diatoms on plastic fragments in the Sargasso Sea (Derraik *et al*., 2002). Furthermore, studies on culture based seawater microcosm studies have shown microbials that are attached to polyethylene terephthalate and PET bags (Morishige *et al*., 2007). Chee *et al*. (2010) reported that there are over 90 genera of micro-organisms, including bacteria and fungi that have the capability to degrade plastics. These include *Ralstonia eutropha*, *Pseudomonas* sp., *Bacillus megaterium*, *Halomonas* sp and *Azotobacter***.**

There is huge potential utilize microbes to degrade plastic. Using microbes to degrade microplastics will enhance biodegradation without causing any harm to the environment (Bhardwaj *et al*., 2012). Therefore, identifying microbes that can degrade microplastics is a promising and environmentally safe strategy to facilitate natural bioremediation and influence the cleaning of natural ecosystems without imposing adverse impacts

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Area

Sampling was carried out at six mangrove sites located in Peninsular Malaysia. The sites are Matang mangrove Forest, Cherating Mangrove, Pahang, Serkam Mangrove, Melaka, Tanjung Piai Mangrove, Johor, Sedili Besar Mangrove, Johor and Pasir Puteh Mangrove, Kelantan as shown in Figure 3.1

Location	Coordinates
Matang Mangroves, Perak	4°49'9.82"N, 100°40'28.93"E
Serkam Mangroves, Melaka	2°10'1.91"N, 102°23'11.34"E
Tanjung Piai, Johor	1°19'36.89"N, 103°26'45.57"E
Sedili Besar, Johor	1°55'19.82"N,104°5'17.35"E
Pasir Puteh, Kelantan	5°51'11.14"N, 102°31'13.77"E
Cherating Mangroves, Pahang	4°7'49.04"N, 103°23'37.13"E

Figure 3.1 : Location of sampling sites

3.2 Sampling Methodology

3.2.1. Water collection

Water samples were collected 10cm from the surface of each sampling points. Prior to sample collection, LDPE bottles were washed with 10% nitric acid followed by at least three times washing with distilled water. Before taking the water samples, the bottles were rinsed with the sample prior to collection, capped and placed in a cooler box filled with ice at 4°C to minimize microbial activity to be transported back to the laboratory. The sampling bottles were labeled with dates and sampling source. The samplings were done in triplicates. Temperature, conductivity, pH and salinity of the water samples were measured *in-situ* by using multiprob meter (model YSI 556). In the laboratory, turbidity reading of the water is recorded from spectrophotometer (HACH PROGRAM). COD analysis was done using the standard method HANNA vial kit, and for BOD analysis, the bottles were incubated at 20^{o}C for 5 days before BOD values were calculated. All analyses were carried according to the APHA standard methods (1998).

3.2.2 Sediment collection

Samples of sediment were taken from three different points using a quadrat of 10cm X 10 cm with a three-layer system. All samples were collected during low-tide from areas with a lot of plastic waste (marine debris). The sediments were kept in sterile bottles with saline water. In the laboratory, the sample bottle was placed on a shaker for 24 hours to homogenize the mixture, prior to further analysis.

3.3 Analysis of samples

3.3.1 Isolation of bacteria from the sediment and microbial enumeration

The total count of bacteria and isolation of plastic degrading bacteria was carried out using sediment from three different depth (0-3 cm, 3-6 cm, 6-9 cm) sampling sites. Serial dilution was done by diluting 1g of sediment to 10^{-5} dilutions and inoculated in the Mineral Salt Media (MSM) agar plate. The composition of MSM used was as follows: K_2HPO_4, 1 g; KH_2PO_4, 0.2 g; NaCl,1 g; $CaCl_2.2H_2O$, 0.002 g; Boric Acid, 0.005 g; $(NH_4)2SO_4$, 1 g,; $MgSO_4.7H_2O$, 0.5 g; $CuSO_4.5H_2O$, 0.001 g; $ZnSO_4.7H_2O$, 0.001 g; MnSO4.H2O, 0.001 g and $FeSO_4.7H_2O$, 0.01g per litre distilled water.

The plates were incubated at 30°C for 24 hour and observed for microbial growth. Single colonies were then re-streaked onto fresh nutrient agar (NA) to obtain pure culture for further analysis.

3.3.2 Screening of potential plastic degrading strain (Bushnell's Haas Agar)

Bushnell Haas Media were used to screen for potential microplastic degradation microbial. This media contained all nutrients necessary for bacterial growth, except for a carbon source. Each individual isolate was grown in Bushnell Haas Agar infused with specific plastic polymers act as carbon sources and incubated for 30 days at room temperature. The potential isolate that able to grow in the medium and produce clear zone were selected for further analysis (Kannahi and Sudha, 2013).

3.3.3. Microbial Formulation for consortium

All potential microplastic degrading bacteria were combined together for the bioremediation purpose into a single microbial cocktail. Each type of microbes were grown separately until it reach 1.3 Abs at 600nm and the microbes are equally mixed to

a desired amount (Emenike *et al.,* 2016). Bushnell Haas Media was used for screening purpose and nutrient broth is used to grow the microbes for consortium. Incubation condition: 29°C at 150 rpm. The microbial is let to growth until stationary phase before using for treatment.

3.3.4 Screening of potential plastic degrading strain (Bushnell's Haas broth)

The consortium were inoculated in Bushnell's Haas medium supplemented with plastic sample (polystyrene, polypropylene, PET, and polyethylene) as the sole source of carbon at a final concentration of 0.1% (w/v). The media were incubated in shaker at 37^{o}C for 30 days. The optical density of the medium was checked each at 24 interval hour and recorded.

Pre-weighed 0.25g disinfected plastic were aseptically added to 250 ml culture broth Bushnell's Haas medium. The plastics were incubated with culture medium and it was shaken at 125 rpm for one month at 37^{o}C. As control each types of plastic films was added in uninoculated Bushnell's Haas medium. At the end of 30 days, the plastics were harvested. They were washed with 70% ethanol and distilled water to remove as much cell mass as possible from the residual film before being dried for 24 hours at 45^{o}C. Surface changes and weight loss of plastic materials were determined.

3.3.5 Fourier transform infrared spectroscopy (FTIR) Analysis

Fourier transform infrared spectroscopic FTIR studies were conducted to detect any changes in the structure of plastic before and after the treatment. FTIR Spectroscopy analysis is used to detect the degradation of plastic components in microbial media based on their changes in the functional group. The wavelength used to the detection is ranged from 400 to 4000 nm.

3.4 Identification of bacteria

All of microbial are identify using Biolog. However some of the bacterial that are not able be identified using Biolog are proceed with sequence analysis and blast method.

3.4.1. BIOLOG

Bacterial isolates were identified based on to their morphological, Gram stain and biochemical characteristics using Bergey's Mannual of Systematic Bacteriology as well as by using Biolog Bacterial Identification Test which uses Biolog Gen III Protocol. The identification system used standardised micro method using 94 Biochemical tests. The Omnilog Database collection is the Microbial Identification system software used to identify the bacteria.

3.4.2 Sequence analysis and blast

Amplified DNA was sequenced based on 16s rRNA. Basic Local Aligment Search Tool (BLAST) is used to detect the genus and species of the samples. BLAST performs its alignment by matching up each position of search sequences to each position of the sequence in the database. The lower the E-value, the more similar the sequence found in the database to the query sequence.

3.4.3 Statistical analysis

One way analyses of variance (ANOVA) between the parameter are done using SPSS 16. The result of $p<0.05$ indicated there is significant differences between the variables.

CHAPTER FOUR: RESULT AND DISCUSSION

4.1 Water Quality Analysis

4.1.1 Temperature

The water temperature at six sampling sites ranged from 30.1°C to 33°C. The highest temperature was observed at Pasir Puteh with 33°C, while the minimum temperature was recorded in Tg. Piai with 30.1°C (Table 4.1). This temperature profile is common in a typical sub-tropical aquatic system (Wahid *et al*., 1995).

Table 4.1 : Descriptive of temperature value of the sampling sites

Sampling Sites	Mean (°C)	Std. Deviation (°C)	Std. Error (°C)	95% Confidence Interval for Mean		Minimum (°C)	Maximum (°C)
				Lower Bound (°C)	Upper Bound (°C)		
Matang	30.800	0.200	0.115	30.303	31.297	30.600	31.000
Serkam	31.200	0.520	0.300	29.909	32.491	30.900	31.800
Tg Piai	30.100	0.656	0.379	28.471	31.729	29.400	30.700
Sedili Besar	31.900	1.500	0.866	28.174	35.626	30.400	33.400
Cherating	30.700	1.153	0.666	27.835	33.565	29.600	31.900
Pasir Puteh	33.000	1.353	0.781	29.640	36.361	31.700	34.400

Based on ANOVA, there are no significant different of temperature recorded between each sampling sites (ANOVA P > 0.05) (Table 4.2).

Table 4.2: Anova analysis (Temperature) between sampling sites

ANOVA					
Temperature	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15.925	5	3.185	3.107	.050
Within Groups	12.300	12	1.025		
Total	28.225	17			

The changes of temperature are influenced by sampling time, weather condition, and rainfall. This result is comparable with the previous study conducted by Gandaseca *et al*. (2011) of Miri Mangroves Forest Sarawak recorded range of 27°C to 32°C which is almost similar result to this study.

In addition, according to Kathiresan and Bingham (2001), to maintain the ecological activity, mangrove water temperature must surpass 24°C in the warmest month. This is because extreme change of temperature will give an impact on the biological, chemical and physical process in water bodies. The temperatures recoded in this study are within the acceptable standard of National Water Quality Standards, Malaysia (NWQS)

4.1.2 pH

In water quality assessment, pH is an important variable as it affects many biological and chemical processes within the body of water. Tg. Piai and Sedili Besar record a slightly acidic pH while pH in Matang, Pasir Puteh, Serkam and Sedili Besar are neutral with mean values varied from pH 6.62 to 7.57 (Table 4.3). The pH variation between each sampling sites is significantly different (ANOVA $P < 0.05$) (Table 4.4).

From the result, Tg Piai and Sedili Besar which are located at the southern part of Peninsular Malaysia recorded a slightly acidic pH. This change in pH of seawater indicates the present of certain pollutants that may come from tourism activities and urbanisation processes within the area.

Table 4.3 : Descriptive of pH value of the sampling sites

Sampling Sites	pH Mean	pH Std. Deviation	pH Std. Error	95% Confidence Interval for Mean		pH Minimum	pH Maximum
				pH Lower Bound	pH Upper Bound		
Matang	7.067	0.058	0.033	6.923	7.210	7.000	7.100
Serkam	7.570	0.062	0.036	7.415	7.725	7.500	7.620
Tg Piai	6.750	0.145	0.084	6.389	7.111	6.610	6.900
Sedili Besar	6.620	0.425	0.245	5.564	7.676	6.200	7.050
Cherating	7.620	0.020	0.012	7.570	7.670	7.600	7.640
Pasir Puteh	7.550	0.474	0.274	6.371	8.729	7.010	7.900

Table 4.4 : Anova analysis of pH between sampling sites

ANOVA					
pH	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.977	5	.595	8.222	.001
Within Groups	.869	12	.072		
Total	3.846	17			

The result of current study were similar with research done by Shamila (2012), in which the pH of coastal water at Johor Strait recorded slightly acidic value, pH 6.33. Newton *et al*. (2014), reported that the present of sulphur-reducing bacteria, and the acidic clays make pH of mangrove become acidic.

This result comparable with findings of some researchers that recorded pH 8.1 in mangrove during the raining season, which may be contributed by local effluent discharge that flow in the mangrove area (Mohammad *et al.,* 2014).

River water has pH range from pH 6.5 to pH 9 at day time which makes it the most suitable condition for aquatic life (Gandaseca *et al.,* 2011). Extremely high and low pH value is very harmful to marine environment (Gandaseca *et al*., 2011). The limit set by DOE (2006) of standard pH for seawater is from 6.5 to 8.5. All of the sampling sites were within the recommended value.

4.1.3 Salinity

Result of the analysis shows that the salinity ranged from 21.7 ppt to 37.8 ppt (Table 4.5). Mangroves grow in areas with salinity of surface water ranging from 0 to 40 ppt (Hutchings & Saenger, 1987). The salinity variation between each sampling sites was found to be significantly different (ANOVA $P < 0.05$) (Table 4.6).

Table 4.5 : Descriptive of salinity value of the sampling sites

Sampling Sites	Mean (ppt)	Std. Deviation (ppt)	Std. Error (ppt)	95% Confidence Interval for Mean		Minimum (ppt)	Maximum (ppt)
				Lower Bound (ppt)	Upper Bound (ppt)		
Matang	24.400	0.500	0.289	23.158	25.642	23.900	24.900
Serkam	28.300	0.361	0.208	27.404	29.196	27.900	28.600
Tg Piai	21.767	0.651	0.376	20.150	23.383	21.100	22.400
Sedili Besar	31.900	1.500	0.866	28.174	35.626	30.400	33.400
Cherating	31.000	0.529	0.306	29.686	32.315	30.400	31.400
Pasir Puteh	37.800	1.587	0.917	37.857	45.743	37.600	40.600

Table 4.6 : Anova analysis for salinity

ANOVA					
Salinity	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	737.316	5	147.463	151.158	0.00000000021
Within Groups	11.707	12	.976		
Total	749.023	17			

The highest salinity was observed at Pasir Puteh, Kelantan with 37.8 ppt while the lowest was recorded in Matang with 24.4 ppt. This is because Kelantan located at the east coast of Peninsular Malaysia surround by South China Sea which is the salinity concentration is more higher compared to Strait of Malacca. Previous study conducted by Hidayah (2014), near Pahang also show high amount of water salinity which was ranged from 32 ppt to 35 ppt. Hao *et al.* (2009), stated that the salinity of seawater is approximately 35% ppt, tending to be lower in tropical waters.

4.1.4 Turbidity

The turbidity values varied from 10.1 to 29.3 NTU (Table 4.7). Matang recorded the highest turbidity reading as compared to other sites. The turbidity variation between each sampling sites was significantly different (ANOVA $P < 0.05$) (Table 4.8). Matang recorded the highest turbidity perhaps due to the presence of debris from nearby area. Fawaz (2013), stated that higher turbidity is caused by the presence of suspended particles such as plankton, silt, organic matter, clay, and other microscopic or decomposers organisms in water. Turbidity is one of the indicators of polluted water.

Table 4.7 : Descriptive of turbidity value of the sampling sites

Sampling Sites	Mean (NTU)	Std. Deviation (NTU)	Std. Error (NTU)	95% Confidence Interval for Mean		Minimum (NTU)	Maximum (NTU)
				Lower Bound (NTU)	Upper Bound (NTU)		
Matang	29.300	0.173	0.100	28.870	29.730	29.100	29.400
Serkam	13.200	0.200	0.115	12.703	13.697	13.000	13.400
Tg Piai	22.100	0.755	0.436	20.225	23.976	21.400	22.900
Sedili Besar	10.100	0.700	0.404	8.361	11.839	9.600	10.900
Cherating	15.233	1.069	0.617	12.577	17.890	14.300	16.400
Pasir Puteh	12.900	0.500	0.289	11.658	14.142	12.400	13.400

Table 4.8 : Anova analysis for Turbidity

ANOVA					
Turbidity	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	777.496	5	155.499	369.747	0.0000000000010
Within Groups	5.047	12	.421		
Total	782.543	17			

Oil spill from the boat and contaminants from the human activity probably are contributing to the high turbidity in Matang. Chew and Chong (2011), also had reported that estuaries Matang has highly turbid water (>30 NTU). According to DOE Malaysia, the acceptability of water for domestic use range from 5 to 25 NTU. All the results obtain from this study are within the acceptability.

4.1.5 Dissolved oxygen

The average value of dissolved oxygen at sampling sites range from 5.04 to 9.55 mg/L (Table 4.9). The variation between each sampling sites was significantly different (ANOVA $P < 0.05$) (Table 4.10).

Table 4.9 : Descriptive analysis dissolved oxygen of the sampling sites

Sampling Sites	Mean (mg/L)	Std. Deviation (mg/L)	Std. Error (mg/L)	95% Confidence Interval for Mean		Minimum (mg/L)	Maximum (mg/L)
				Lower Bound (mg/L)	Upper Bound (mg/L)		
Matang	5.040	0.841	0.485	2.952	7.128	4.220	5.900
Serkam	8.950	0.727	0.420	7.145	10.755	8.310	9.740
Tg Piai	9.700	0.200	0.115	9.203	10.197	9.500	9.900
Sedili Besar	9.550	0.328	0.189	8.736	10.365	9.250	9.900
Cherating	6.030	0.895	0.517	3.807	8.253	5.130	6.920
Pasir Puteh	7.440	0.062	0.036	7.285	7.595	7.390	7.510

Table 4.10 : Anova analysis for dissolved oxygen

ANOVA					
DO	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	56.621	5	11.324	31.062	0.000002
Within Groups	4.375	12	.365		
Total	60.996	17			

Tg Piai recorded the highest amount of dissolved oxygen, which indicates that the availability of oxygen is high in the water body. In contrast, Matang record the lowest amount of dissolved oxygen with 5.04mg/L. This is because of the present of excessive organic matter such as sewage lead to the high oxygen demand. Oxygen demand increase with high content of organic material and other contributor such as fertiliser, animal farm and sewage (Gandaseca *et al.,* 2014). The result of dissolve oxygen obtained from this study is much higher than that of Toriman (2013), which recorded dissolved oxygen the range within 3.37 mg/L - 3.89 mg/L. Olatoyo (2004), stated that minimum 5mg/L is needed for supporting marine life, whereas, the functioning and survival of biological communities are adversely affected by the oxygen concentrations below 5mg/l may and oxygen concentration of 2 mg/l may lead to the death of most fishes. According to DOE and NWQS Malaysia, 5-7mg/L of DO is required for optimum fish health.

4.1.6 Biochemical Oxygen Demand (BOD_5)

The mean BOD_5 value ranged from 1.58 mg/L to 6.04 mg/L is shown in Table 4.11. There is a significant different variation between each sampling sites (ANOVA $P < 0.05$) (Table 4.12). Amadi (2010), stated that BOD_5 presents the amount of the biodegradable organic substances, and the amount of oxygen required in the decomposition of organic matters.

Table 4.11 : Descriptive biological oxygen demand value of the sampling sites

Sampling Sites	Mean (mg/L)	Std. Deviation (mg/L)	Std. Error (mg/L)	95% Confidence Interval for Mean		Minimum (mg/L)	Maximum (mg/L)
				Lower Bound (mg/L)	Upper Bound (mg/L)		
Matang	6.040	0.066	0.038	5.877	6.203	5.970	6.100
Serkam	3.343	0.501	0.289	2.100	4.587	2.930	3.900
Tg Piai	1.580	0.243	0.140	0.976	2.184	1.420	1.860
Sedili Besar	3.810	0.390	0.225	2.841	4.779	3.420	4.200
Cherating	5.720	0.236	0.137	5.133	6.307	5.500	5.970
Pasir Puteh	4.440	0.201	0.116	3.941	4.939	4.300	4.670

Table 4.12 : Anova analysis for BOD

ANOVA					
BOD	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	40.476	5	8.095	86.360	0.000000006
Within Groups	1.125	12	.094		
Total	41.601	17			

According to Water Quality Standards for Coastal Waters Marine Outfalls EPA (1986), to ensure that the water is free from pollution caused by sewage and other decomposable wastes, BOD_5 should not more than 5 mg/L in any time. However, Matang and Cherating recorded BOD_5 above than 5mg/L. The sites surround by residential area may contribute to the high value of BOD_5 in these two sites. To support, Ling *et al*. (2010), also had reported high BOD_5 in residential area.

4.1.7 Chemical Oxygen Demand (COD)

The reading of COD for water sample is ranged from 843 mg/L to 1015mg/L (Table 4.13).

Table 4.13 : Descriptive analysis chemical oxygen demand of sampling sites

Sampling Sites	Mean (mg/L)	Std. Deviation (mg/L)	Std. Error (mg/L)	95% Confidence Interval for Mean		Minimum (mg/L)	Maximum (mg/L)
				Lower Bound (mg/L)	Upper Bound (mg/L)		
Matang	816.3	4.509	2.6	805.1	827.5	812.0	821.0
Serkam	1015.0	1.000	0.6	1012.5	1017.5	1014.0	1016.0
Tg Piai	973.0	3.000	1.7	965.5	980.5	970.0	976.0
Sedili Besar	716.7	1.115	0.7	713.8	719.5	716.0	718.0
Cherating	1012.0	4.359	2.5	1001.2	1022.8	1007.0	1015.0
Pasir Puteh	721.0	4.000	2.3	711.1	730.9	717.0	725.0

The variation between sampling sites was significantly different (ANOVA $P < 0.05$) (Table 4.14).

Table 4.14 : Anova analysis for COD

ANOVA					
COD	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	291574.000	5	58314.800	93.905	0.000000003
Within Groups	7452.000	12	621.000		
Total	299026.000	17			

From the results, Sedili Besar shows the lowest COD reading as compared to other sites. According to Water Quality Standards For Coastal Waters Marine Outfalls EPA (1986), COD for seawater should not exceed more than 120 mg/L.

The value of COD from sampling sites exceeded the standard limit probably because of the presence of pollutant in the water. The higher level of COD indicated the higher pollution of water (Noraini *et al.,*2010).

4.1.8 Comparison between geographical region

Peninsular Malaysia is surrounded by two sea areas, Straits of Malacca at the west coast and South China Sea at the east coast area. Matang, Sekam and Tanjung Piai are located on the West coast of Peninsular Malaysia whereas Sedili Besar, Cherating and Pasir Puteh located on East coast of Peninsular Malaysia. Table 4.15 shows the overall results for these six sampling sites.

Table 4.15: Water quality results for West Coast and East Coast

Sampling Sites		**Temper a-ture (°C)**	**pH**	**Salinity (ppt)**	**Turbidity (NTU)**	**DO (mg/L)**	**BOD_5 (mg/L)**	**COD (mg/L)**
West Coast	**Matang**	30.8	7.067	24.4	29.3	5.04	6.04	816.3
	Serkam	31.2	7.57	28.3	13.2	8.95	3.34	1015
	Tg Piai	30.1	6.75	21.7	22.1	9.7	1.58	973
East Coast	**Sedili Besar**	31.9	6.62	31.9	10.1	9.55	3.81	716.7
	Cherating	30.7	7.62	31.0	15.2	6.03	5.72	1012
	Pasir Puteh	33.0	7.55	37.8	12.9	7.44	4.44	721

One way ANOVA statistical analysis were done to compare the significant different between the result of water quality obtained from west coast and east coast. There are significant different ($P< 0.05$) between the geographical region for salinity, turbidity, BOD_5 and COD, whereas there are no significant different for temperature, and dissolved oxygen.

Comparing the geographical sites, west coast water are more turbid than east coast. This probably because west coast has more are large scale projects and industries which contributed to the pollution. Faridah (2013) reported that sediment from developing activities in Taiping that flowed into the Matang mangrove areas thus worsen the pollution and turbidity level. Turbid water can reduce growth rate of marine life as it block the sunlight and lead to reduction in photosynthesis activity in Tg Piai also recorded a high turbidity level because it is located near Tanjung Piai Resort and Tanjung Piai Restaurant. The wastewaters from resort and restaurant activity flow to the water thus contributed to higher reading of turbidity level. Salinity is higher along the east coast due to the influenced of marine water from the South China Sea. COD and BOD_5 value in the water depends on contamination source that could originated from rivers passing through industrial areas along the west coast, while water in the east coast might be exposed to wastes from crude oil exploration and refinery as well as industrial activities (Pawar *et al*., 2013).

4.2. Microbial abundance

Microbial populations correspond to the environmental changes. Thus the pollution states of coastal marine environments can be measured through the change in sediment microbes. There have been considerable amount of studies reporting correlation between microbial community structure to the types and concentrations of pollutants in marine sediments (Gordon *et al.,* 2006).

High number of microbes indicate that the high nutrient flow in the area are helping the bacterial colonies to flourish. This is in agreement with research done by Ho *et al.* (2007), that number of bacteria available in a nutrient rich area is due to the present of organic matter. The condition is suitable for the microbes to fluorish.

Sediment from six selected mangroves in Peninsular Malaysia was analysed for their microbial abundance. It was found that, Matang Mangrove have the highest number of microbial count with 3.7 x 10^7 CFU/ml, followed by Cherating 1.4 x 10^7 CFU/ml, Serkam 0.7x10^7 CFU/ml, Sedili Besar 1.0 x 10^7 CFU/ml, Tg Piai 0.4 x 10^7 CFU/ml, Pasir Puteh 0.5x10^7 CFU/ml (Figure 4.1).

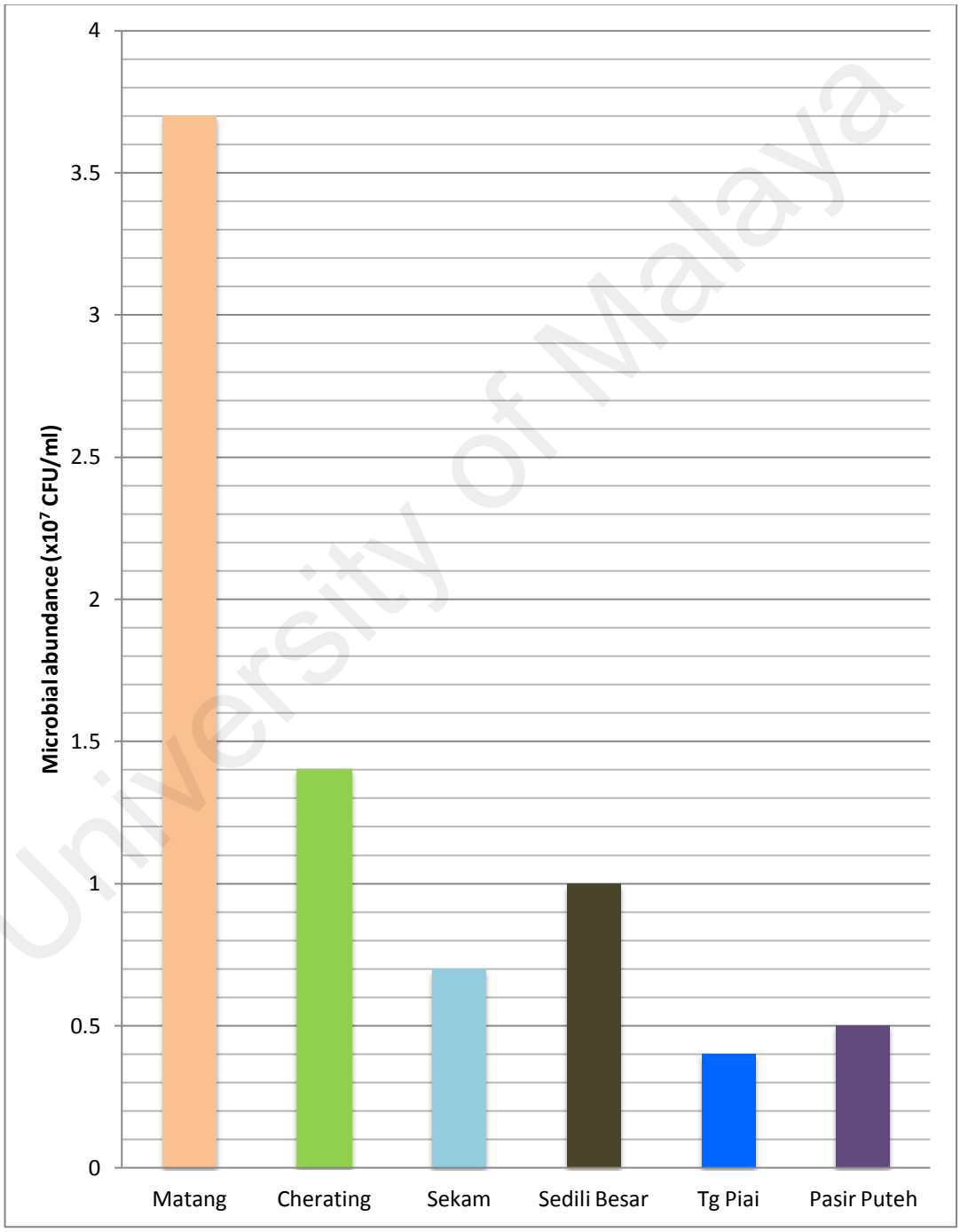


Figure 4.1 : Microbial abundance (Based on sampling sites)

Statistical analysis was done to identify the influence of some water quality parameters on microbial abundance. No correlation was found between turbidity and microbial abundance, $r^2 = 0.4962$ (Figure 4.2). The abundance of bacteria keep increasing with the increase in turbidity. High turbidity is the result from contaminants that polluted the water. Some of them are rich in nutrient, which enhance the growth of microbial. Keegan (2012), stated that the higher the intensity of scattered light, the higher is the turbidity due to the presence of microscopic organisms.

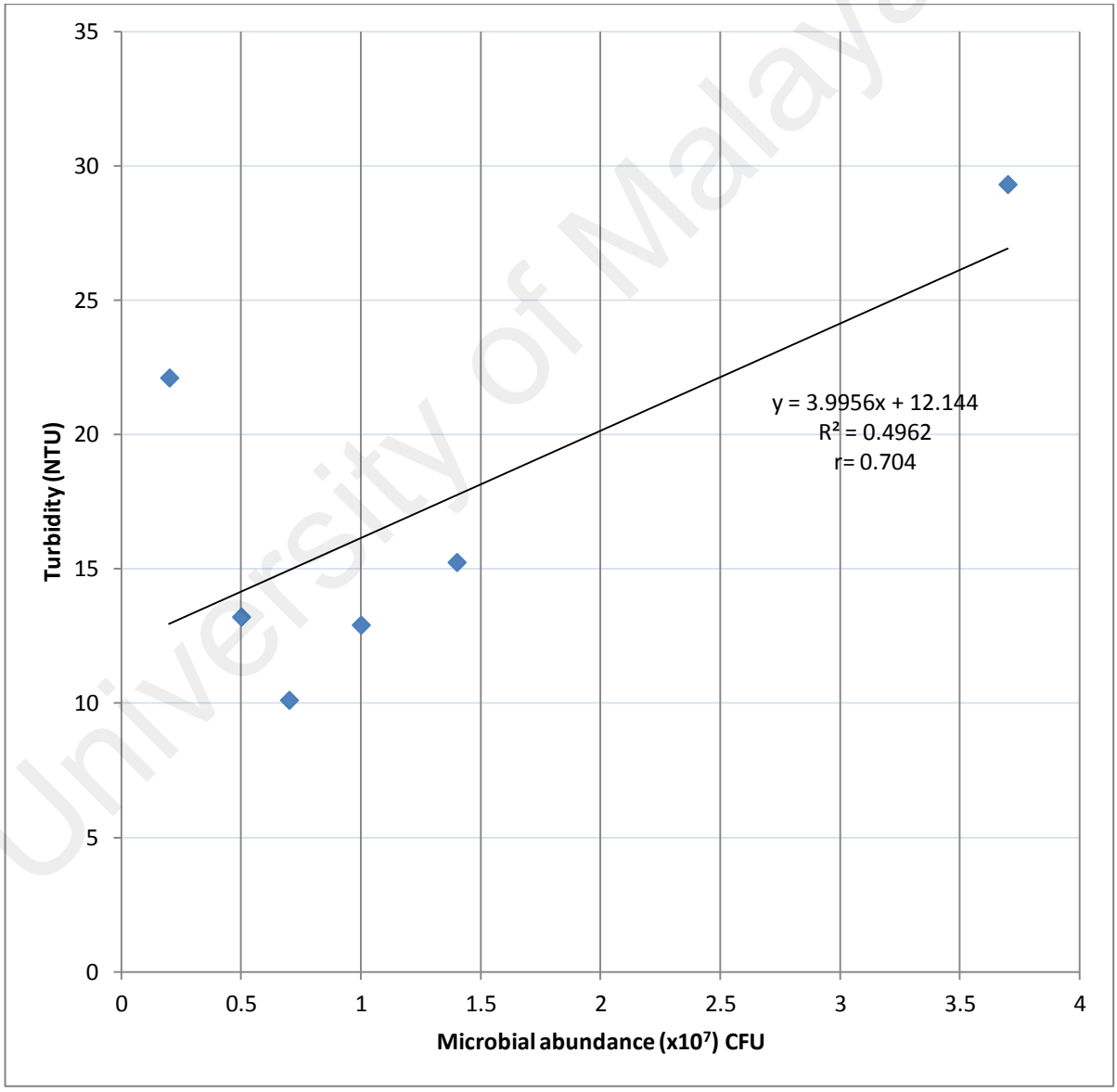


Figure 4.2 : Correlation of microbial abundance and turbidity

The availability of dissolve oxygen influenced microbial abundance. There is correlation between microbial abundance and dissolve oxygen, $r^2 = 0.7612$ (Figure 4.3).. Previous study done by Rachel *et al.* (2015), also resulted with strong correlation between microbial abundance and dissolved oxygen.

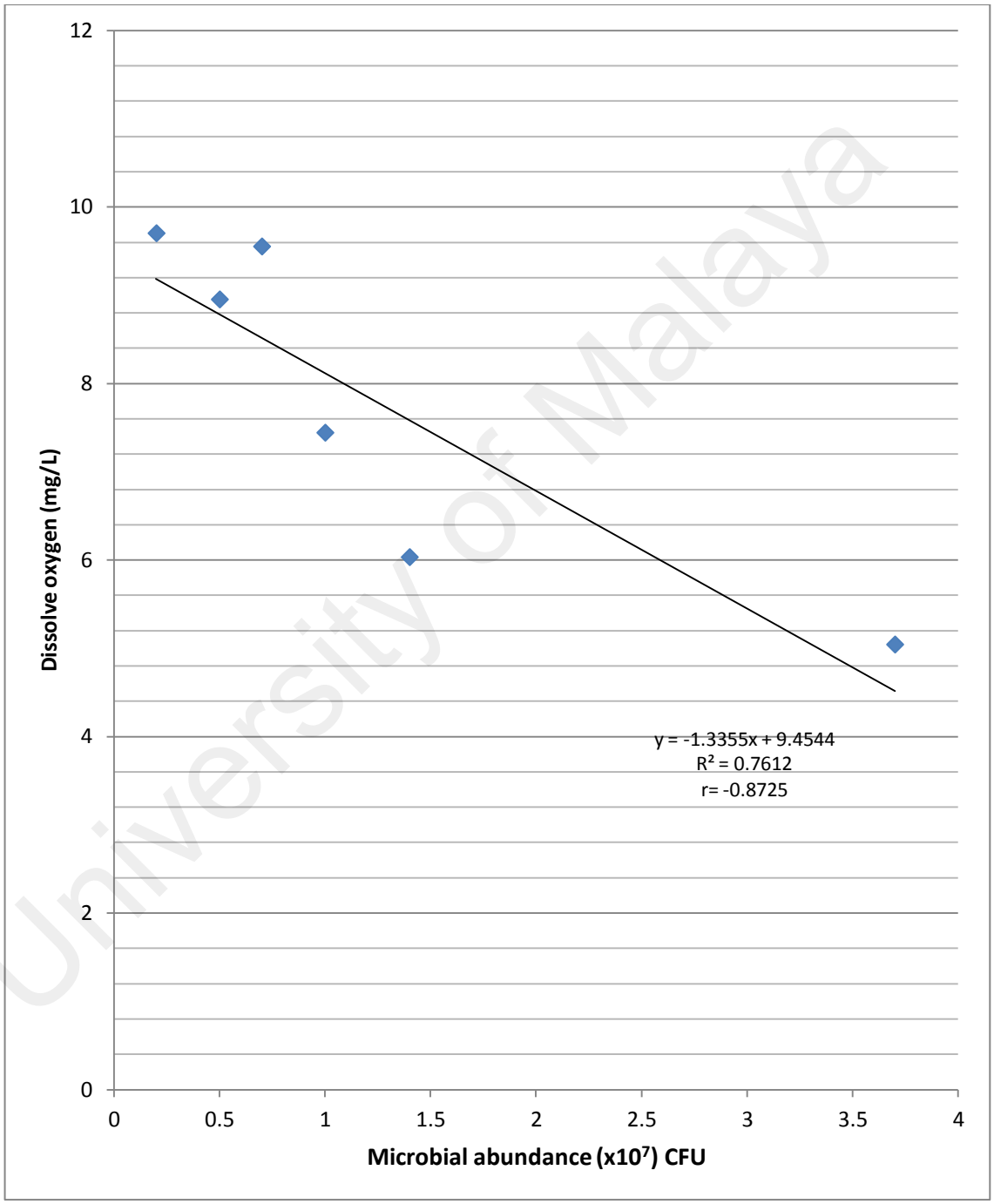


Figure 4.3 : Correlation of microbial abundance and DO

Positive correlation is found between microbial abundance and BOD, $r^2 = 0.6315$ (Figure 4.4). High BOD indicated that the presence of a high number of organic waste. Microorganisms like bacteria decompose organic waste and they will start the process to break down the waste when organic matter such as dead plants, leaves, sewage, or even food waste are present in the water In this case, the biochemical demand for oxygen will be high since the microbes would need oxygen to multiply. It was supported by previous study done by Barnes (1998), stated that an effluent showing high BOD levels will increase bacterial growth in the river and the presence of bacteria will decrease the river's oxygen level.

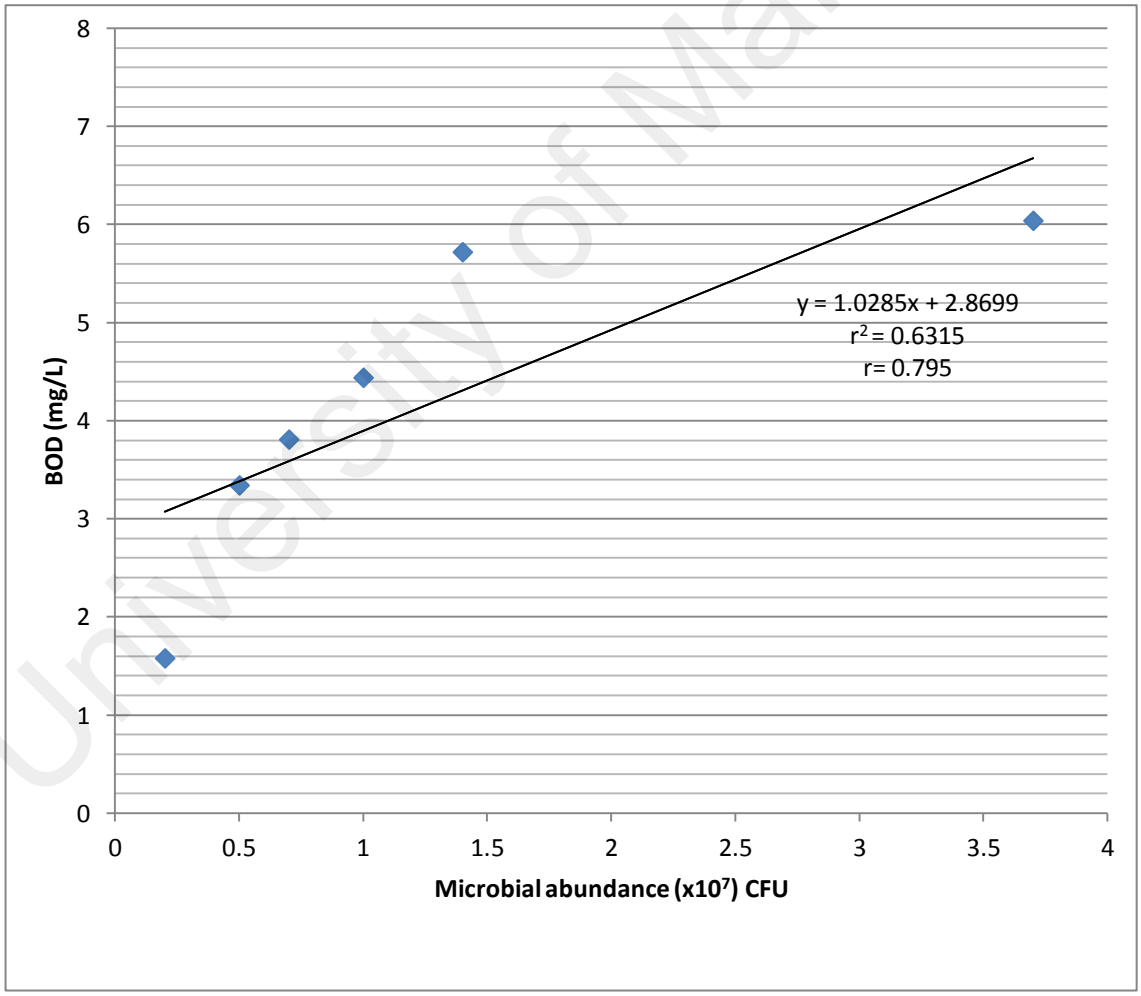


Figure 4.4 : Positive Correlation of microbial abundance and BOD_5

In addition, the microbial abundance of three different layers namely layer 1 (0-3cm), layer 2 (3-6cm) and layer 3 (6 cm to 10 cm) was analyzed. Figure 4.5 show all sampling sites have a highest number of microbes in 0-3cm sediment depth.

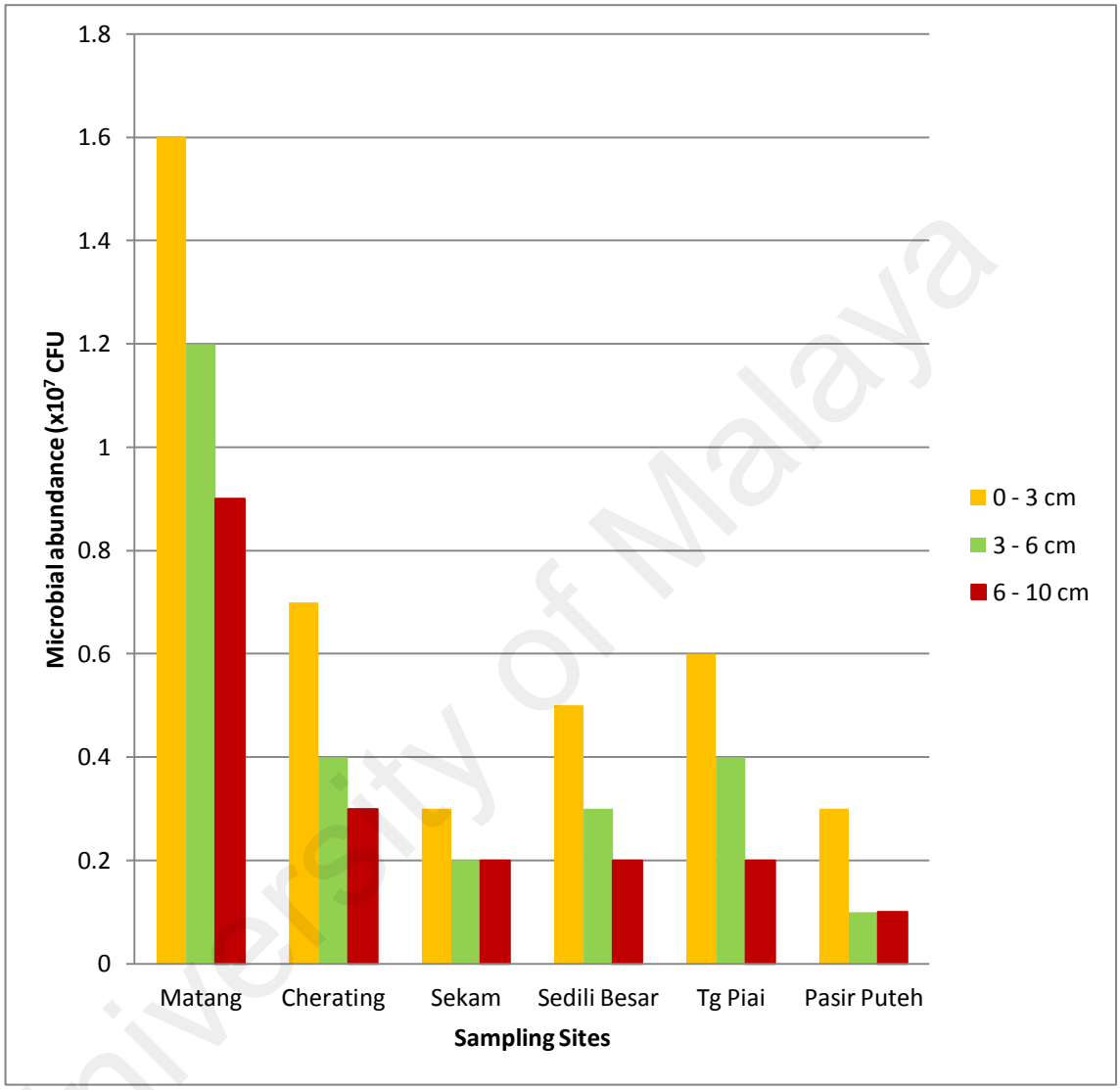


Figure 4.5 : Microbial abundance (based on layer of sampling sites)

From the result obtained, layer 1 has the highest number of microbes (Figure 4.6). The abundance of oxygen is high in the upper part of sediment, since most of the bacteria isolated in this study are aerobic bacteria. It was supported by previous study done by Cole (2011), which stated that aerobic microbial number are higher on top of the sediment as compared to the layer below due to the reduced avaibility of oxygen.

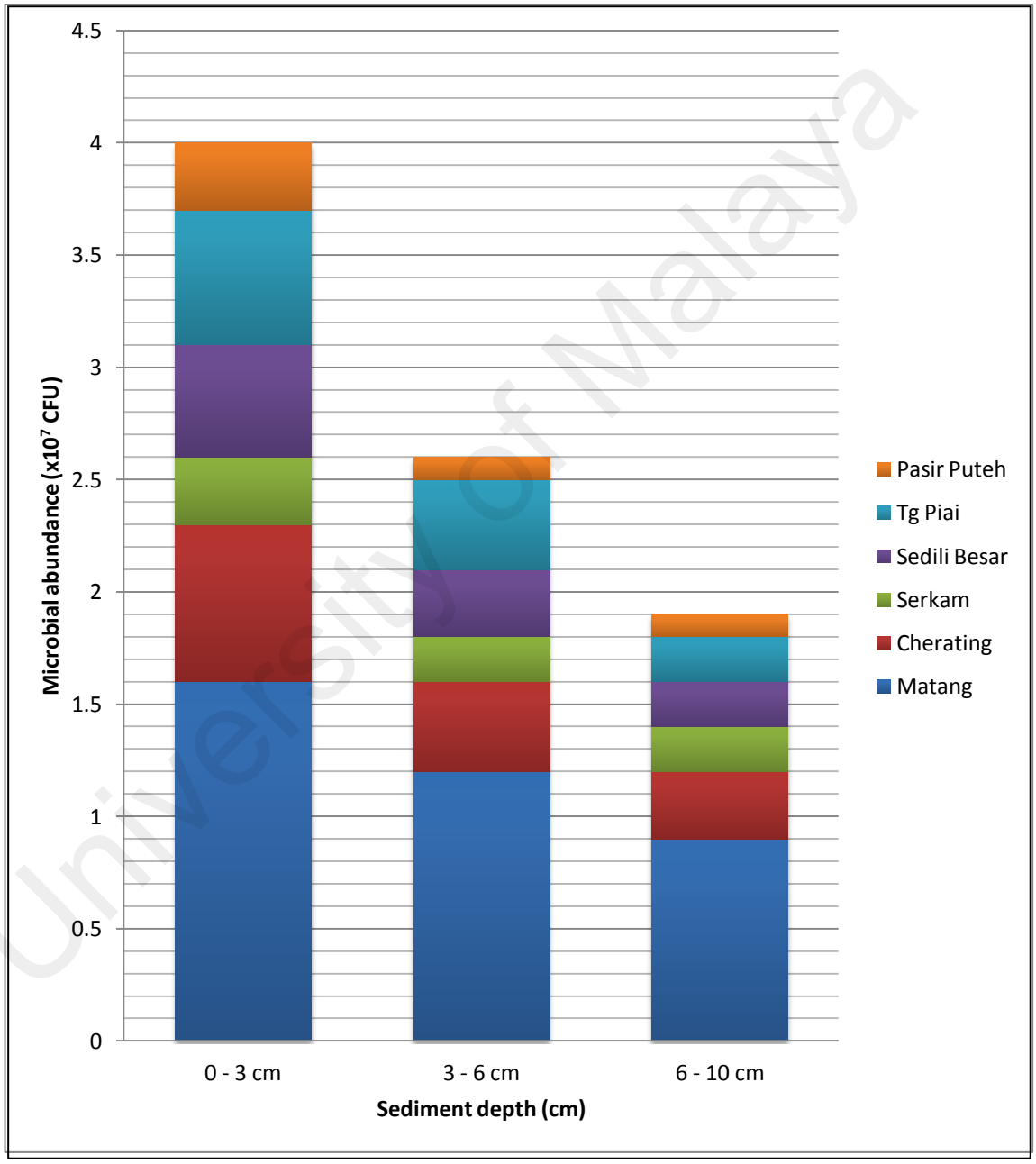


Figure 4.6 : Microbial abundance by layer

4.3 Isolation and identification of bacteria

A total of 38 morphologically different bacteria were isolated from the six selected mangrove sites. The isolated bacterium was identified using BIOLOG GEN III Microbial Identification system and 16S rRNA sequence. The result is shown in Table 4.16.

Table 4.16: Identification of bacteria

Isolate	Identification	Isolate	Identification
M1	*Bacillus cereus*	M20	*Bacillus thurigiensis*
M2	*Bacillus aquimaris*	M21	*Bacillus thurigiensis*
M3	*Bacillus sonorensis*	M22	*Exigluobacterium* sp.
M4	*Bacillus thuringiensis*	M23	*Bacillus cerius*
M5	*Bacillus vietnamensis*	M24	*Bacillus aquimaris*
M6	*Bacillus ruris*	M25	*Bacillus thuringiensis*
M7	*Sporosarcina* sp.	M26	*Bacillus toyonensis*
M8	*Bacillus thuringiensis*	M27	*Bacillus toyonensis*
M9	*Bacillus cibi*	M28	*Bacillus flexus*
M10	*Acinetobactor* sp .	M29	*Bacillus toyonensis*
M11	*Enterococcus* sp.	M30	*Pseudomonas strutzel*
M12	*Bacillus cerius*	M31	*Bacillus toyonensis*
M13	*Bacillus gothell*	M32	*Bacillus thuringiesis*
M14	*Strenothropomonas* sp.	M33	*Alcaligenes* sp.
M15	*Bacillus pseudomycoides*	M34	*Bacillus toyonensis*
M16	*Bacillus stratophericus*	M35	*Alcaligenes* sp.
M17	*Bacillus pumilus*	M36	*Rhodococcus* sp.
M18	*Alcaligenes* sp.	M37	*Alcaligenes* sp.
M19	*Bacillus pumilus*	M38	*Bacillus gotheir*

The result obtained shows that nine bacterial genera namely *Bacillus* sp., *Alcaligenes* sp., *Rhodococcus* sp, *Pseudomonas* sp. *Exiguobacterium* sp., *Strepnothropomonas* sp., *Enterococcus* sp., *Acinetobactor* sp., S*porosarana* sp. were identified from the six mangrove sediment. All microbes are grown in standard disposable petri dish with size 100 mm diameter by 15mm height.

4.3.1 *Bacillus* sp.

In total, there are 13 types of *Bacillus* sp. found in this studies. There are *Bacillus cereus, Bacillus aquimaris, Bacillus sonorensis, Bacillus thuringiensis, Bacillus vietnamensis, Bacillus ruris, Bacillus cibi, Bacillus gothell, Bacillus pseumycoides, Bacillus stratophericus, Bacillus pumilus, Bacillus toyonensis* and *Bacillus flexus.*

4.3.1.1 *Bacillus cereus*

There are three isolates which share same species identification of *Bacilllus cereus* namely M1, M12 and M23. However they appeared to be morphologically different on agar plates. All *Bacilllus cereus* isolates are Gram positive and have rod shape. They differ in colour and opacity, while being isolated from the different layer of the mud sediment.

In agar plates, *Bacillus cereus* M1 shows white-yellowish colony with a wavy margins (Plate 4.1).

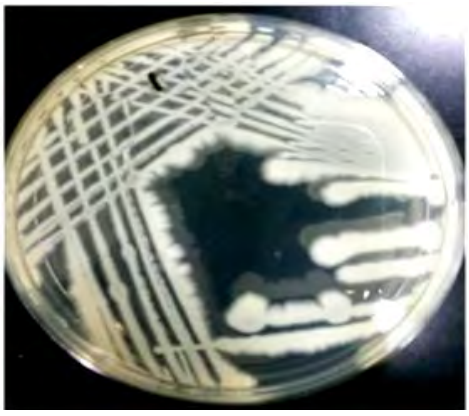


Plate 4.1: *Bacillus cereus* (M1) on nutrient agar

Unlike *Bacillus cereus* M1, the different of this isolates from M1 is its surface of isolates is moist and smooth and colouring which is more yellowish (Plate 4.2). *Bacillus cereus* M12 is more favourable found in mangroves area as it was found in Matang, Cherating, Sekam and Sedili Besar sediment samples. It was found at 0-10 cm of sediment depth which indicates the survival of this types of isolates in various condition.

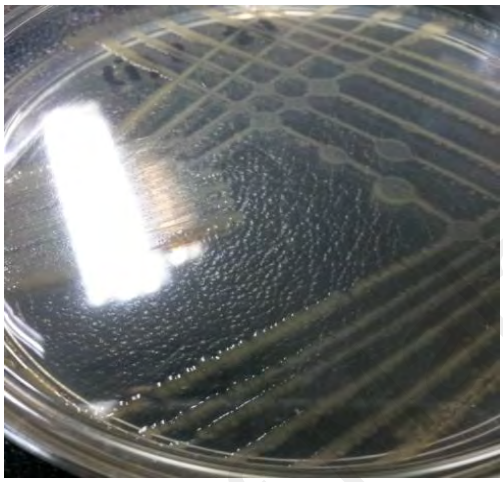


Plate 4.2 : *Bacillus cereus* (M12) on nutrient agar

Comparing to above isolates, *Bacillus cereus* M23 was quite similar to *Bacillus cereus* M1 isolates, but have is more whitish colour and the surface is filamentous (Plate 4.3). *Bacillus cereus* M23 was found in Matang and Pasir Puteh sediment samples. It is found at 0 - 3 cm of the sediment samples which indicates that the species needs oxygen for respiration.

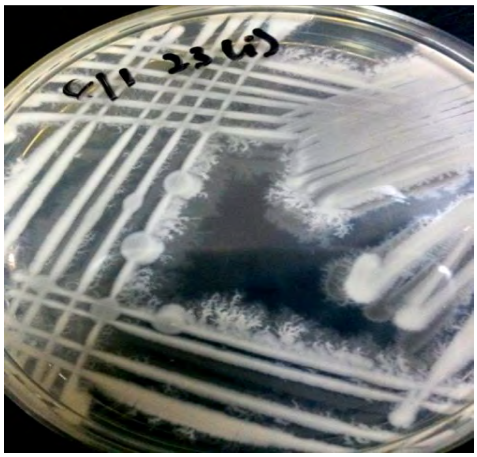


Plate 4.3 : *Bacillus cereus* (M23) on nutrient agar

4.3.1.2 *Bacillus aquimaris*

Isolates M2 and M24 were identify as *Bacillus aquimaris*. Both are Gram positive bacteria and has circular shape. *Bacillus aquimaris* M2 was found in Cherating and Tanjung Piai at layer 3 - 6 cm deep in the mud. It is orange in colour and have smooth surface (Plate 4.4).

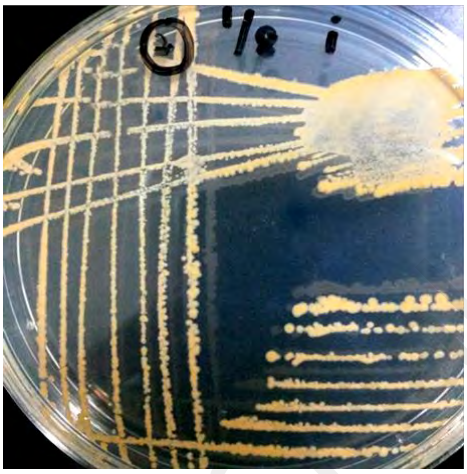


Plate 4.4 : *Bacillus aquimaris* (M2) on nutrient agar

Apart of that, *Bacillus aquimaris* M24 are different from *Bacillus aquimaris* M2 as it is pinkish in colour (Plate 4.5). The colony have a smooth surface and margins. This type of isolates are more favourable in mangroves as it was found in all sampling sites except for Pasir Puteh. It was isolated from various layer of sediment.

Plate 4.5: *Bacillus aquimaris* (M24) on nutrient agar plate

4.3.1.3 *Bacillus sonorensis*

Bacillus sonorensis (M3) is Gram positive and has rod shape. The colour of the colony is cream yellow and was rather transparent looking with smooth surface (Plate 4.6). It was isolated from Matang mangroves from 0 – 3 cm deep.

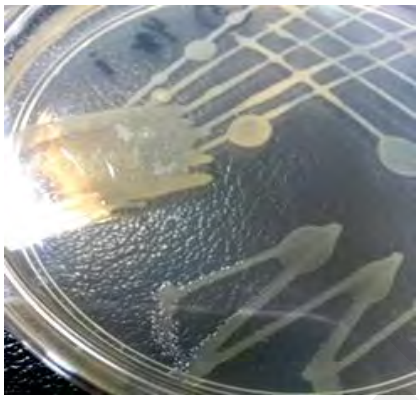


Plate 4.6: *Bacillus sonorensis* (M3) on nutrient agar plate

4.3.1.4 *Bacillus thuringiensis*

Six isolates were identified as *Bacilllus thuringiensis* namely M4, M8, M20, M21, M25 and M32. However they have different morphological appearance on agar plates. All *Bacilllus thuringiensis* isolates are Gram positive and have irregular shapes. They are different in colour, opacity, origin and level of sediment isolated. *Bacilllus thuringiensis* (M4) is white in colour, irregular shape (Plate 4.7). It was isolated only from from 0 – 3 cm deep sediment.

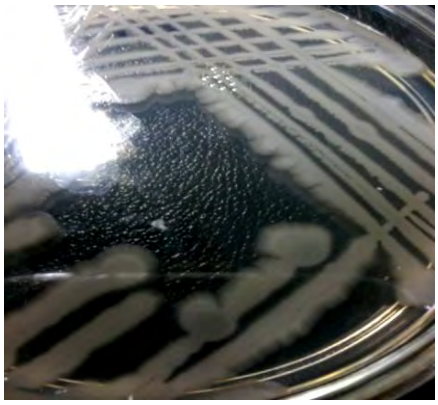


Plate 4.7: *Bacilllus thuringiensis* (M4) on nutrient agar plate

M8 are different from other *Bacilllus thuringiensis* isolates as it is yellow in colour (Plate 4.8). The colony has rough surface. It was isolated from a depth of 6 – 10 cm mangrove sediment.

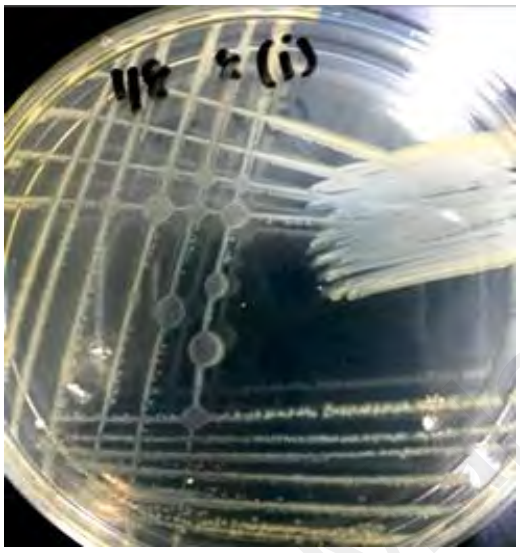


Plate 4.8: *Bacilllus thuringiensis* (M8) on nutrient agar plate

Bacilllus thuringiensis M20 was white colonies with filamentous margins (Plate 4.9). *Bacilllus thuringiensis* M20 was isolated from 0 – 3 cm deep of in Cherating mangrove sediment. In agar plates,

Plate 4.9: *Bacilllus thuringiensis* (M20) on nutrient agar plate

Bacilllus thuringiensis M21 has a white colonies with rough surface (Plate 4.10). *Bacilllus thuringiensis* M21 was isolated from Matang and Cherating mangroves, from 0 – 10 cm deep of mangroves sediment.

Plate 4.10: *Bacilllus thuringiensis* (M21) on nutrient agar plate

Bacilllus thuringiensis (M25) is cream in colour, smooth surface, irregular shape and translucent opacity (Plate 4.11). It was isolated from 0-6 cm deep in Matang and Pasir Puteh mangroves.

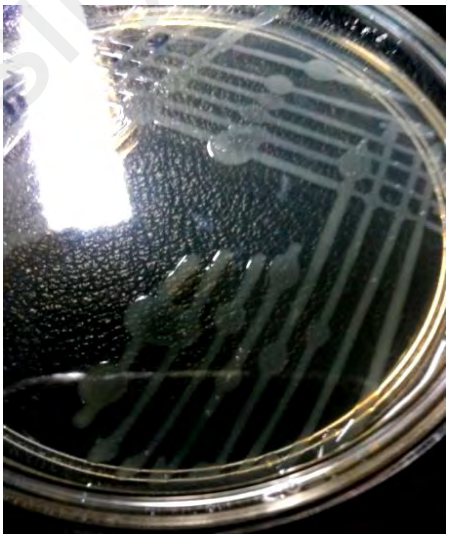


Plate 4.11: *Bacilllus thuringiensis* (M25) on nutrient agar plate

Bacilllus thuringiensis (M32) grows into white and rough surface colony on nutrient agar (Plate 4.12). It was found at 0 – 3 cm deep of Matang.

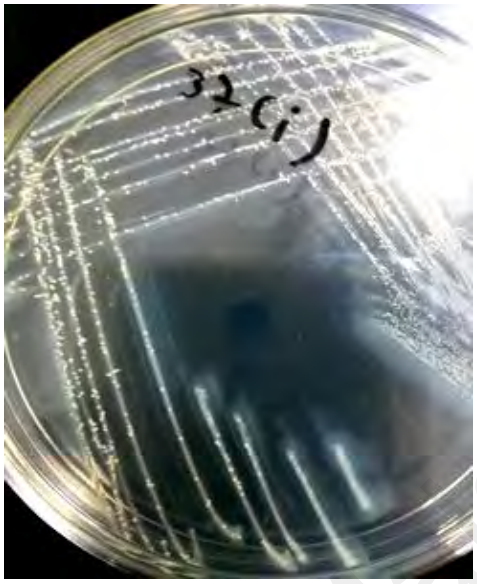


Plate 4.12: *Bacilllus thuringiensis* (M32) on nutrient agar plate

4.3.1.5 *Bacillus vietnamensis*

Bacillus vietnamensis (M5) is Gram positive with irregular shapes. The colour of the colony is orange and surface is smooth (Plate 4.13). It was found at 0 - 6 cm deep at Matang and Serkam mangrove sediment.

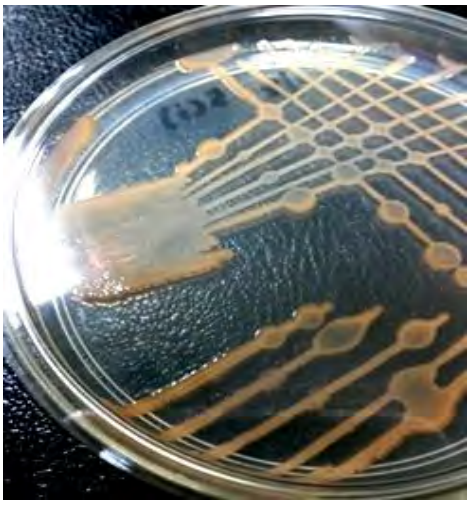


Plate 4.13: *Bacilllus vietnamensis* (M5) on nutrient agar plate

4.3.1.6 *Bacillus ruris*

M6 was identify as *Bacillus ruris,* Gram positive bacteria. It is yellowish with filamentous margins and flat colony on nutrient agar (Plate 4.14). Isolated from Cherating and Pasir Puteh mangroves the depth of 0 – 3 cm of mangroves sediment indicating its requirement of oxygen for survival

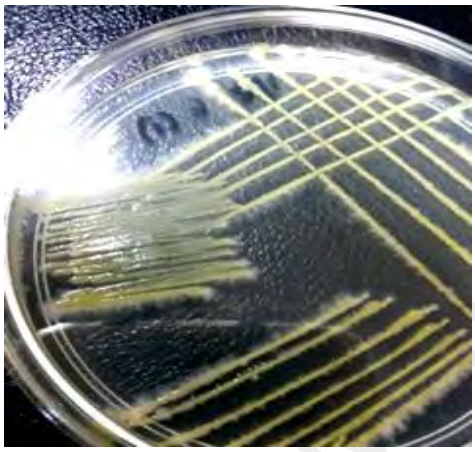


Plate 4.14: *Bacilllus ruris* (M6) on nutrient agar plate

4.3.1.7 *Bacillus cibi*

Bacillus cibi is a Gram positive bacteria with yellowish colony and smooth surfaces (Plate 4.15). *Bacillus cibi* (M9) was found at Matang, Cherating and Pasir Puteh mangroves from0 – 10 cm deep. This indicates that this types of isolates trives in various conditions.

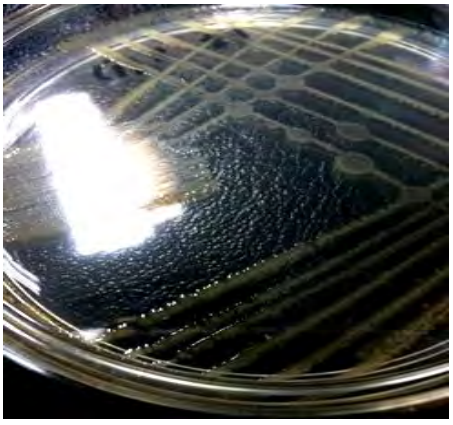


Plate 4.15: *Bacillus cibi* (M9) on nutrient agar plate

4.3.1.8 *Bacillus gotheill*

There are two isolates identified as *Bacilllus gotheill* namely M13 and M38. Both have different morphological appearance on agar plates. *Bacilllus gotheill* isolates are Gram positive and rod shaped.

On nutrient agar *Bacilllus gotheill* (M13) was slightly yellow and smooth (Plate 4.16). *Bacilllus gotheill* (M13) was found at 0 – 3 cm deep of Matang and Cherating sediment. While *Bacilllus gotheill* (M38) show white and filamentous colony appearances (Plate 4.17). It was found at Cherating, Sekam and Sedili Besar.

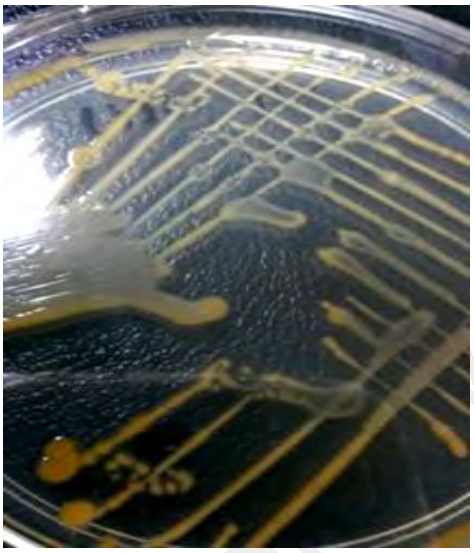


Plate 4.16: *Bacillus gotheill* (M13) on nutrient agar plate

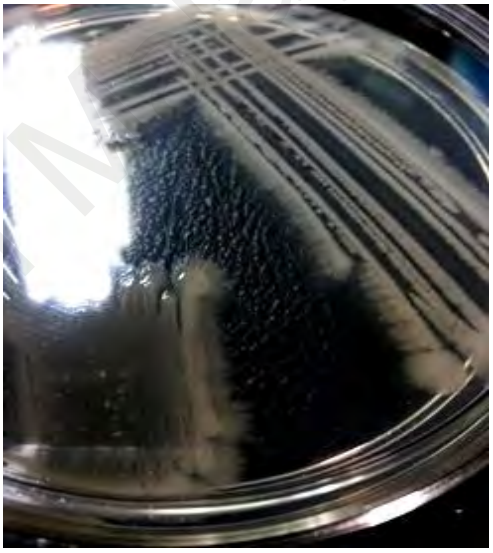


Plate 4.17: *Bacillus gotheill* (M38) on nutrient agar plate

4.3.1.9 *Bacillus psudomycoides*

Bacillus psedomycoides (M15) is a Gram positive bacteria, with white colours and opaque rhizoid (Plate 4.18). It was favourable in mangroves as it was found at all sampling area except for Tg. Piai. It *Bacillus psedomycoides* (M15) was isolated from surface to 10 cm deep of sediment collected and this indicate that indicate that the bacteria is facultative anaerobic.

Plate 4.18: *Bacillus psedomycoides* (M15) on nutrient agar plate

4.3.1.10 *Bacillus stratophericus*

Bacillus stratophericus (M16) is Gram positive and has distinct pigmented yellowish colony, flat surface and smooth edges (Plate 4.19). itwas only found at 0 – 3 cm deep of Matang mangrove sediment.

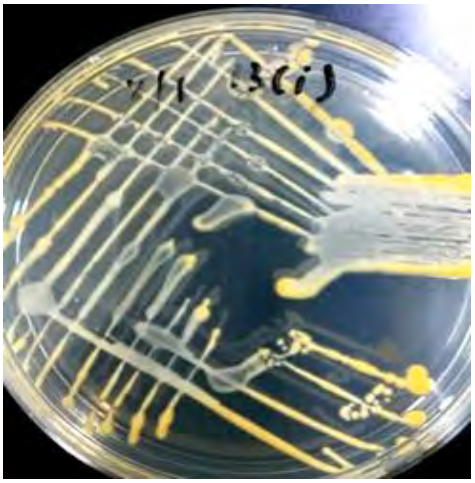


Plate 4.19: *Bacillus stratophericus* (M16) on nutrient agar plate

4.3.1.11 *Bacillus pumilus*

M17 and M19 was identified as *Bacillus pumilus. Bacilllus pumilus* isolates are Gram positive with motile rod shape with flagella.

Bacilllus pumilus is a facultative anaerobe as it was isolated from different depth of mangrove sediment. On nutrient agar, *Bacilllus pumilus* (M17) has slightly yellowish (Plate 4.20) while *Bacilllus pumilus* (M19) was whitecolony with filamentous edge (Plate 4.21). *Bacilllus pumilus* (M17) was found at Pasir Puteh and *Bacilllus pumilus* (M19) Matang and Cherating.

Plate 4.20: *Bacilllus pumilus* (M17) on nutrient agar plate

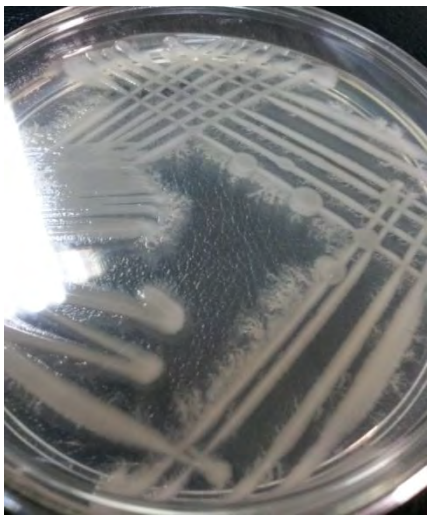


Plate 4.21: *Bacilllus pumilus* (M19) on nutrient agar plate

4.3.1.12 *Bacillus toyonensis*

There are five isolates that share similar identification aws *Bacilllus toyonensis* namely M26, M27, M29,M31 and M34. All *Bacilllus toyonensis* isolates are Gram positive and have rod shape. However, they are different in terms of colour and places of isolation.

On Agar plates, *Bacillus toyonensis* (M26) shows a white tiny colony with a flat structure (Plate 4.22). It was found in first layer of mangroves sediment sample. *Bacillus toyonensis* (M26) was only found in Matang mangroves sediment.

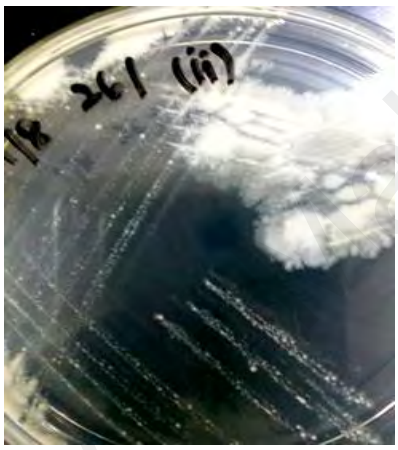


Plate 4.22: *Bacillus toyonensis* (M26) on nutrient agar plate

Bacillus toyonensis (M27) form white colonies with wavy edges. It form white colonies with wavy edges (Plate 4.23). *Bacillus toyonensis* was found in Matang and Cherating mangrove from 0 – 6 cm of the sediment layer

Plate 4.23: *Bacillus toyonensis* (M27) on nutrient agar plate

Bacillus toyonensis shows circular white tiny colonies, with smooth surface and white in colour (Plate 4.24). (M29) was found in Matang, Cherating, Serkam and Pasir Puteh. It was found from 0 – 10 cm of the sediment samples which indicates the survival of this types of isolates in various condition

Plate 4.24: *Bacillus toyonensis* (M29) on nutrient agar plate

Bacilllus toyonensis (M31) forms white, tiny and flat surface colonies (Plate 4.25). It was only found in Cherating mangrove from the surface 0 – 3 cm.

Plate 4.25: *Bacillus toyonensis* (M31) on nutrient agar plate

Different from other *Bacilllus toyonensis,* isolates M34 has yellowish and smooth surface colony (Plate 4.26). It was isolated from Matang, Cherating, Sekam and Sedili Besar mangroves.

Plate 4.26: *Bacillus toyonensis* (M34) on nutrient agar plate

4.3.1.13 *Bacillus flexus*

M28 was identify as *Bacillus flexus* and a Gram positive bacteria. It form colonies that are circular and smooth (Plate 4.27). It was isolated from 0 - 3 cm deep of the sediment and isolates from Pasir Puteh mangroves. This indicates that this bacteria require oxygen for its survival.

Plate 4.27: *Bacillus flexus* (M28) on nutrient agar plate

4.3.2 *Sporosarcina* sp.

Sporosarcina sp. is a Gram positive bacteria with spherical, white, and smooth colonies (Plate 4.28). *Sporosarcina* sp. (M7) was isolated from Matang, Cherating and Pasir Puteh mangroves. It was isolated from layer 0 – 3 cm depth of the mangrove sediment.

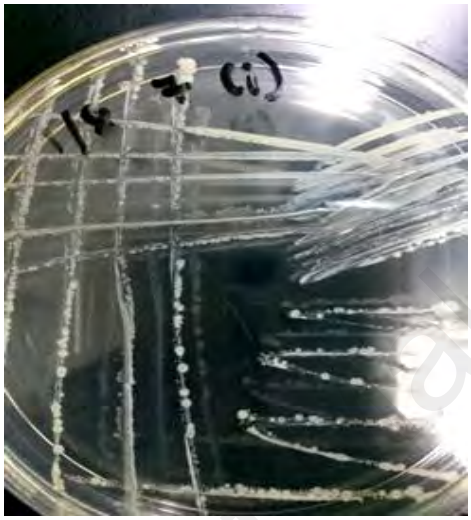


Plate 4.28: *Sporosarcina* sp (M7) on nutrient agar plate

4.3.3 *Acinetobactor* sp.

Acinetobactor sp. is a Gram negative bacteria and has a circular, convex, and smooth colonies (Plate 4.29). *Acinetobactor* sp. (M10) was found only in Cherating from the 0 – 3 cm layer of sediment.

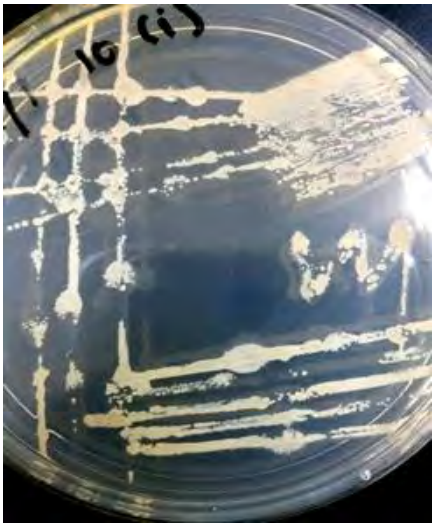


Plate 4.29: *Acinetobactor* sp. (M10) on nutrient agar plate

4.3.4 *Enterococcus* sp.

Isolates M11 was identified as *Enterococcus sp.*, gram positive isolates form yellow, circular and smooth colonies (Plate 4.30). It was isolated from 3 – 6 cm layer Cherating magroves.

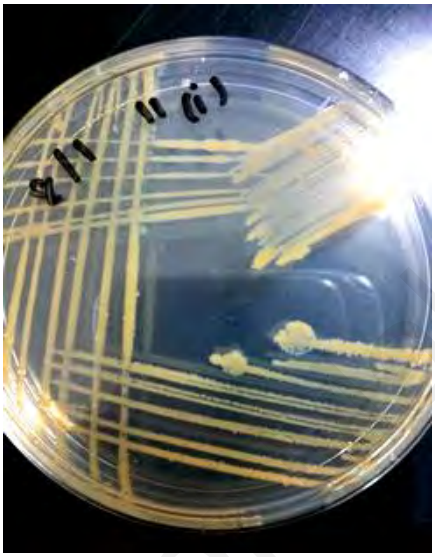


Plate 4.30: *Enterococcus* sp. (M11) on nutrient agar plate

4.3.5 *Strenothropomonas* sp.

*Strenothropomonas s*p. is Gram negative bacteria, that has white and smooth colonies (Plate 4.31). *Strenothropomonas s*p. (M14) was found in Matang and Cherating from the top 3 cm layer.

Plate 4.31: *Strenothropomonas* sp. (M14) on nutrient agar plate

4.3.6 *Alcaligenes* sp.

There are four isolates that share similar species identification for *Alcaligenes* sp., namely M18, M33, M35, and M37. All *Alcaligenes* sp isolates are Gram negative, obligate aerobics as all are isolated from the top 3 cm layer of the sediment.

Alcaligenes sp. (M18) were yellow colonies with filamentous and smooth surface (Plate 4.32). isolated from Matang and Cherating mangrove

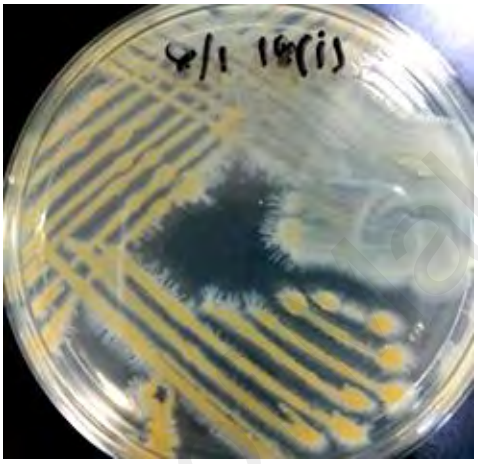


Plate 4.32: *Alcaligenes* sp. (M18) on nutrient agar plate

Alcaligenes sp. (M33) colonies are circular and smooth (Plate 4.33). It was found in all mangroves area except Pasir Puteh mangrove

Plate 4.33: *Alcaligenes* sp. (M33) on nutrient agar plate

On Nutrient agar, *Alcaligenes* sp. (M35) form filamentous colonies (Plate 4.34). On the other hand, *Alcaligenes* sp. (M37) found in Matang and Cherating sampling sites, forms white, smooth, and circular coloies (Plate 4.35). (M35) was found only in Sekam sampling sites.

Plate 4.34: *Alcaligenes* sp. (M35) on nutrient agar plate

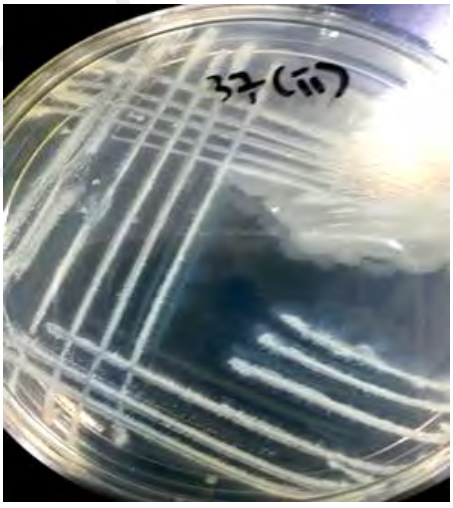


Plate 4.35: *Alcaligenes* sp. (M37) on nutrient agar plate

4.3.7 *Exiguobacterium* sp.

Exiguobacterium sp is Gram positive bacteria, with yellow, flat and smooth colonies (Plate 4.36). *Exiguobacterium* sp. (M22) was found in Matang, Sedili Besar, Tanjung Piai, and Pasir Puteh mangroves.

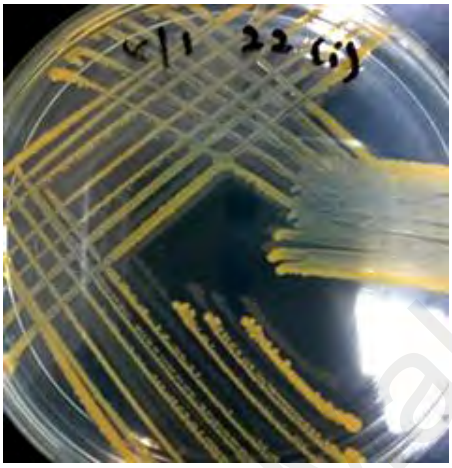


Plate 4.36: *Exiguobacterium* sp. (M22) on nutrient agar plate

4.3.8 *Pseudomonas strutzeri*

Isolate M30 was identified as *Pseudomonas strutzeri* which is a Gram negative bacteria. It forms smooth colonies (Plate 4.37). *Pseudomonas strutzeri* was found in Matang, Cherating, Tg Piai and Pasir Puteh mangroves. It is strictly aerobic as it was isolated from the top 3 cm of the mangrove sediment.

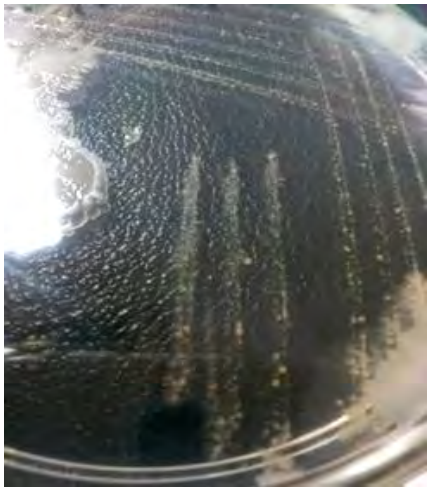


Plate 4.37: *Pseudomonas strutzeri* (M30) on nutrient agar plate

4.3.9 *Rhodococcus* sp.

*Rhodococcus s*p. is a Gram positive bacteria, white in colour and has irregular shape with smooth surfaces (Plate 4.38). *Rhodococcus s*p. (M36) was isolated in Serkam from 0 – 6 cm deep of the sediment.

Plate 4.38: *Rhodococcus sp* (M36) on nutrient agar plate

This study reveals that *Bacillus* sp. and gram positive bacteria are the dominant genera in mangrove sediments. There are 31 Gram positive bacteria and seven Gram negative bacteria. These results are comparable with research done by Devendran (1987), which obtained a high percentage of Gram-positive bacteria from the mangrove sediment in Pichavaram, India.

According to Prescott *et al*. (1996), *Bacillus* sp and other Gram positive bacteria are important components of microbial community of soil . This could be due to the spore forming nature of allows its wide distribution in terrestrial habitats. As soil conditions are often extremely diverse, endospores have an apparent advantage in surviving periods of nutrient deprivation or droughts.

Table 4.17 and Figure 4.7 shows the distribution of different isolated bacteria based on sampling sites.

Table 4.17 : Distribution of different isolated bacteria

Isolated bacteria	Matang	Cherating	Serkam	Sedili Besar	Tg Piai	Pasir Puteh
M1	✓					
M2		✓			✓	
M3	✓					
M4		✓				
M5	✓		✓			
M6		✓				✓
M7	✓	✓				
M8						✓
M9	✓	✓			✓	
M10		✓				
M11		✓				
M12	✓	✓	✓	✓		
M13	✓	✓				
M14	✓	✓				
M15	✓	✓	✓	✓		✓
M16	✓					
M17						✓
M18	✓	✓				
M19	✓	✓				
M20		✓				
M21	✓	✓				
M22	✓			✓	✓	✓
M23	✓					✓
M24	✓	✓	✓	✓	✓	
M25	✓					✓
M26		✓				
M27	✓	✓				
M28						✓
M29	✓	✓	✓			
M30	✓	✓			✓	✓
M31		✓				
M32	✓					
M33	✓	✓	✓	✓	✓	
M34	✓	✓	✓	✓		
M35			✓			
M36			✓			
M37	✓	✓				
M38		✓	✓	✓		
Total	24	25	10	7	6	9

✓ Indicate the present of bacteria at the respective sampling sites

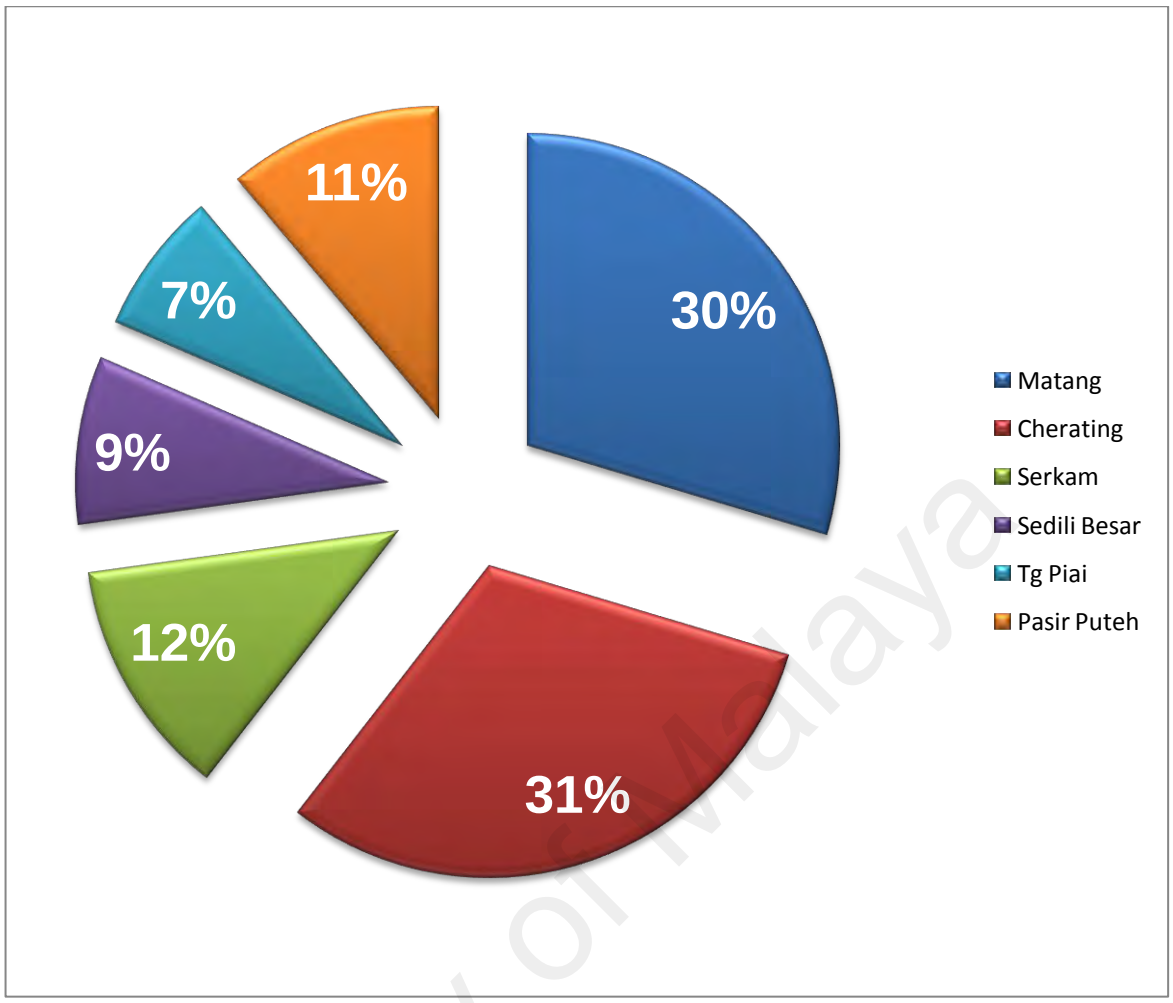


Figure 4.7 : Percentage of different bacteria found in each sampling sites

Sediments collected from Matang and Cherating mangrove had the most variety of bacteria in which out of 38, 24 types of bacteria are present. This is due to the availability of an optimum environmental condition, making it favourable for bacteria to grow. The sediment with the least variety is from Tg Piai Mangrove which has six types of bacteria. This may indicate that the area is less favourable for bacterial growth.

Out of 38 types of bacteria, the common bacteria found on several sites are M15 (*Bacillus pseudomycoides*), M24 (*Bacillus aquimaris*), and M30 (*Pseudomonas strutzeri*), which are present in five out of the six selected mangroves as shown in Figure 4.8.

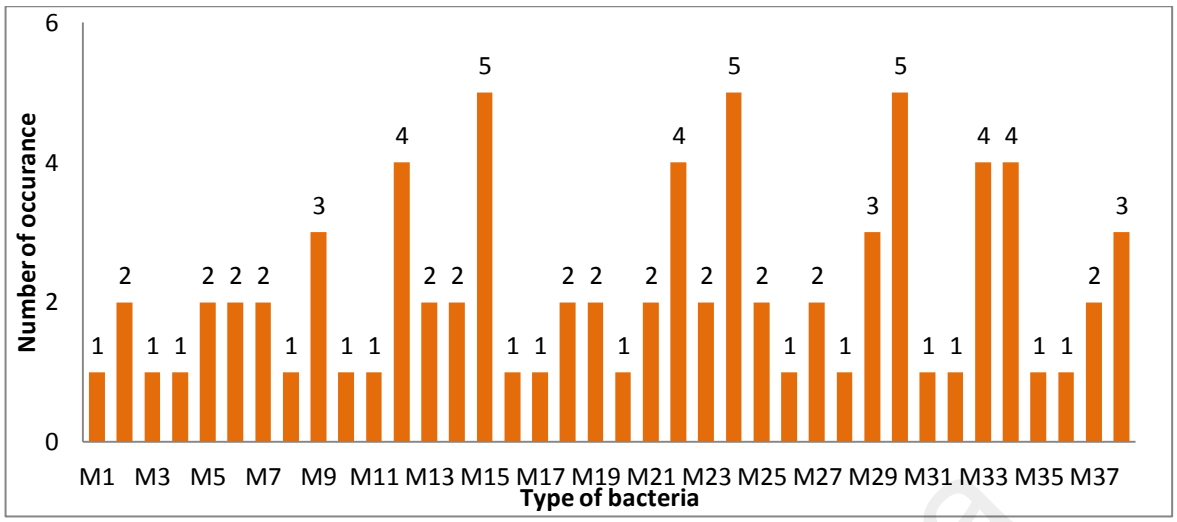


Figure 4.8 : Microbial distribution based on number of occurrence in six selected mangrove area.

Subsequently, all 38 isolated microorganisms were further screen in the laboratory condition to identify their potential of degrading microplastic.

4.4 Screening of Potential Degrading Microplastic Bacteria Using Bushnell's Haas Agar

Out of 38 isolated, only 19 isolates shows the capability to growth in the selective media. There are 17 isolates that growth in polystyrene, 15 isolates in PET, 10 isolates in polypropylene and 13 isolates in polyethylene agar. Table 4.18 summarize the capability of isolate to growth in *Bushnell-Hass* agar.

According to Amal *et al.* (2015), bacteria which can growth in this media have higher ability to utilize plastic as carbon sources and degrade the plastics. Therefore, it can be the good candidates for bioremediation studies. These isolates were further screen to identify their potential to degrade plastics.

Table 4.18: Capability of isolate to growth in Bushnell-Hass agar

Bacteria	Polystyrene	PET	Polypropylene	Polyethylene
M1	-	-	-	-
M2	++	-	-	-
M3	-	-	-	-
M4	++	++	++	++
M5	++	++	+	++
M6	-	-	-	-
M7	-	-	-	-
M8	++	++	+	++
M9	++	+	++	++
M10	-	-	-	-
M11	-	-	-	-
M12	++	++	+	+
M13	-	-	-	-
M14	-	-	-	-
M15	+	-	-	-
M16	++	++	-	+
M17	++	++	++	++
M18	++	+	+	+
M19	++	++	-	-
M20	++	++	++	++
M21	-	-	-	-
M22	-	-	-	-
M23	-	-	-	-
M24	-	-	-	-
M25	-	-	-	-
M26	-	-	-	-
M27	++	++	+	+
M28	+	+	-	-
M29	-	-	-	-
M30	-	-	-	-
M31	-	-	-	-
M32	-	-	-	-
M33	-	-	-	-
M34	-	-	-	+
M35	-	-	-	-
M36	++	++	++	++
M37	+	+	+	-
M38	++	++	++	++
TOTAL	17	15	10	13

** **++ : Very positive growth of isolate**
+ : Positive growth of isolate
- : No growth

Plate 4.39 to Plate 4.42 show some of the positive growth of the isolates in the Bushnell-Haas agar supplemented with polypropylene, polyethylene, polystyrene and PET.

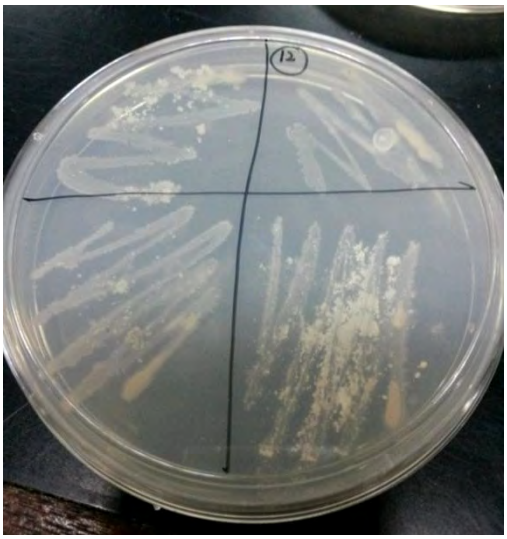


Plate 4.39: *Bacillus cerius* shows positive growth for all type of plastic in selective agar

Plate 4.40: *Alcaligenes* sp.shows positive growth for all type of plastic in selective agar

Plate 4.41: *Bacillus toyonensis* shows positive growth for all type of plastic in selective agar

Plate 4.42: *Rhodococcus* sp. shows positive growth for all type of plastic in selective agar

Plate 4.43 and Plate 4.44 show no growth on Bushnell-Haas agar supplemented with polypropylene, polyethylene, polystyrene and PET.

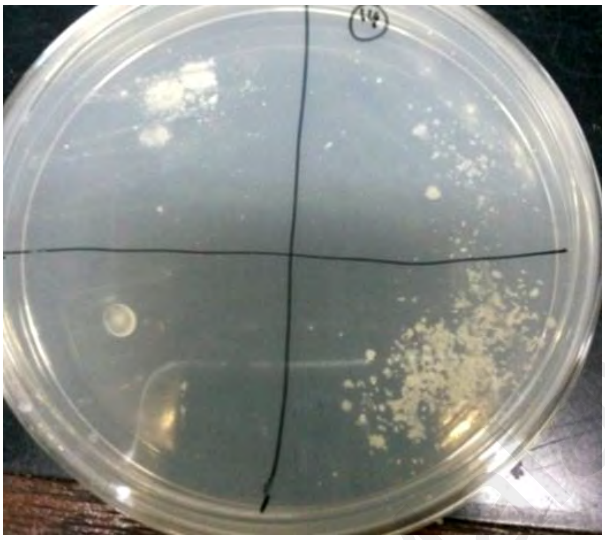


Plate 4.43: *Strenothropomonas* sp. shows no growth

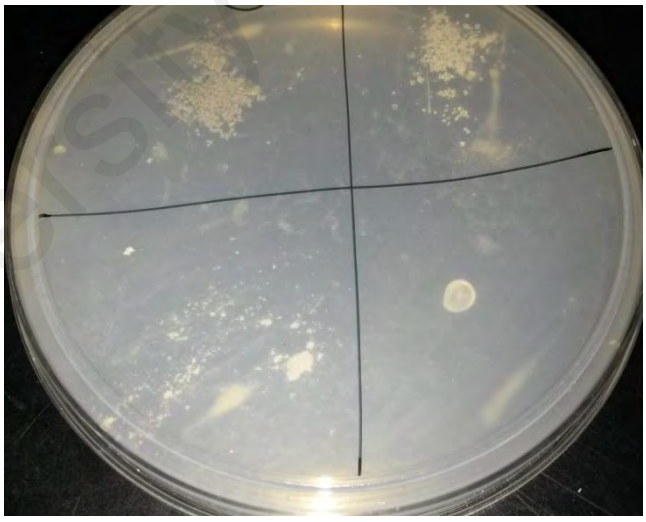


Plate 4.44: *Acinetobactor* sp. shows no growth

4.5 Microbial formulation for consortium

Many researchers agreed that a higher degree of biodegradation and mineralisation can be expected when co-metabolic activities exist within a microbial community that complement a consortium (Tripathi *et al.,* 2001). In this condition the organisms can act synergistically to degrade a contaminant. In this study, four consortia of microbial cocktails were developed based on the ability of the bacteria to degrade each types of plastics (Table 4.19). The culture was prepared at 1.3 ABS at 600nm to be used in bioremediation set up (Emenike *et al.,* 2016).

Table 4.19: Microbial cocktail formulation

Plastic Content	Polystyrene	Polypropylene	Polyethylene	Polyethyelene Terephtalate
Microbial Consortium	M2 M4 M5 M8 M9 M12 M15 M16 M17 M18 M19 M20 M27 M28 M36 M37 M38	M4 M5 M8 M9 M12 M17 M18 M20 M27 M36 M37 M38	M4 M5 M8 M9 M12 M16 M17 M18 M20 M27 M34 M36 M38	M4 M5 M8 M9 M12 M16 M17 M18 M19 M20 M27 M28 M36 M37 M38

Plate 4.45 to Plate 4.48 shows some of the clear zones produced by isolates in the Bushnell-Haas agar supplemented with polypropylene, polyethylene, polystyrene and PET.

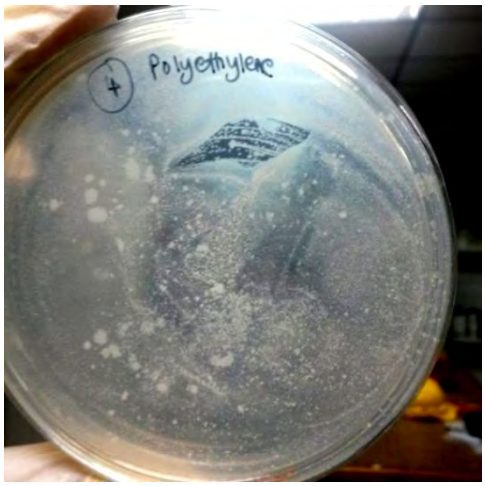


Plate 4.45: Clear zone produced by microbial cocktail with polyethylene

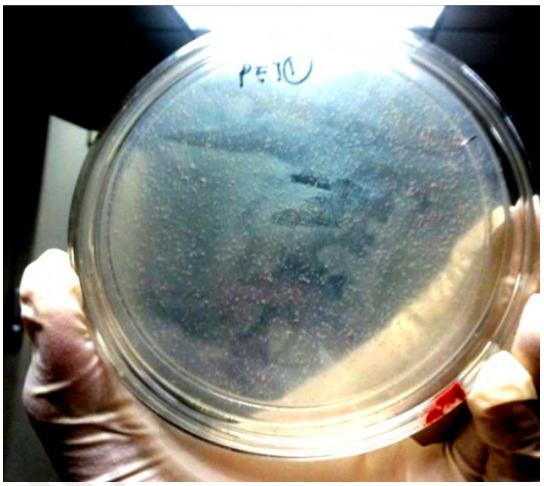


Plate 4.46: Clear zone produced by microbial cocktail with PET

Plate 4.47: Clear zone produced microbial cocktail with polypropylene

Plate 4.48: Clear zone produce by microbial cocktail with polystyrene

4.6 Microplastic bioremediation analysis in Bushnell's Haas broth

Figure 4.9 illustrates the bacterial isolates growth profile during the biodegradation assay. From the result obtained, it can be seen that the bacteria grew better and survived longer when plastics is present in the medium. Microbes in polyethylene plates show the highest growth rate in which indicate successful use of polyethylene in their biochemical reaction. The microbes utilize the polymer and use it as the carbon sources. The control flasks containing non-inoculated supplemented show low growth.

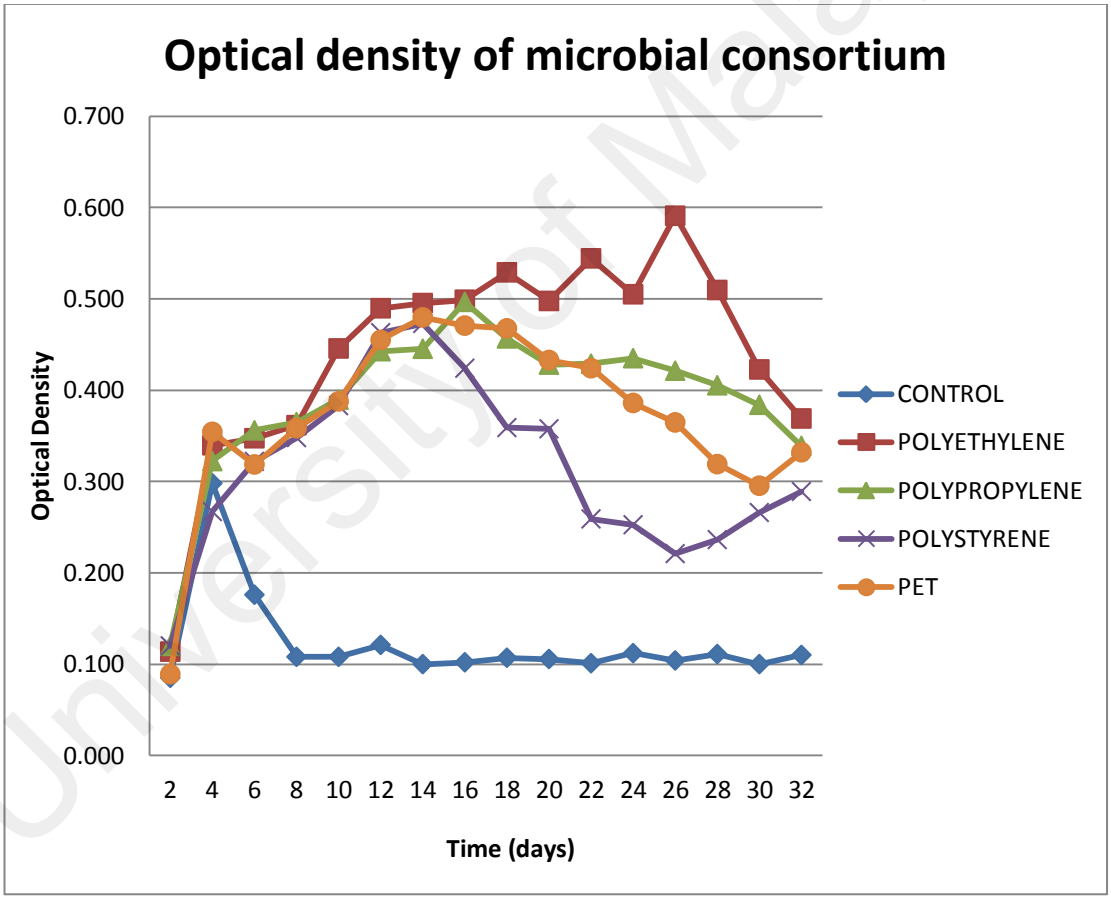


Figure 4.9: Optical density of microbial consortium with plastics

The percentage of weight reduction was recorded after the 30 days incubation period. It was observed that degradation has taken place as reflected by the reduction in the mean weight for all four classes of plastics,. Grima *et al.* (2000), reported that biological hydrolysis and biological oxidation are typically the mechanisms for this degradation. The percentage of polymer weight loss reflects the biodegradation (Figure 4.10).

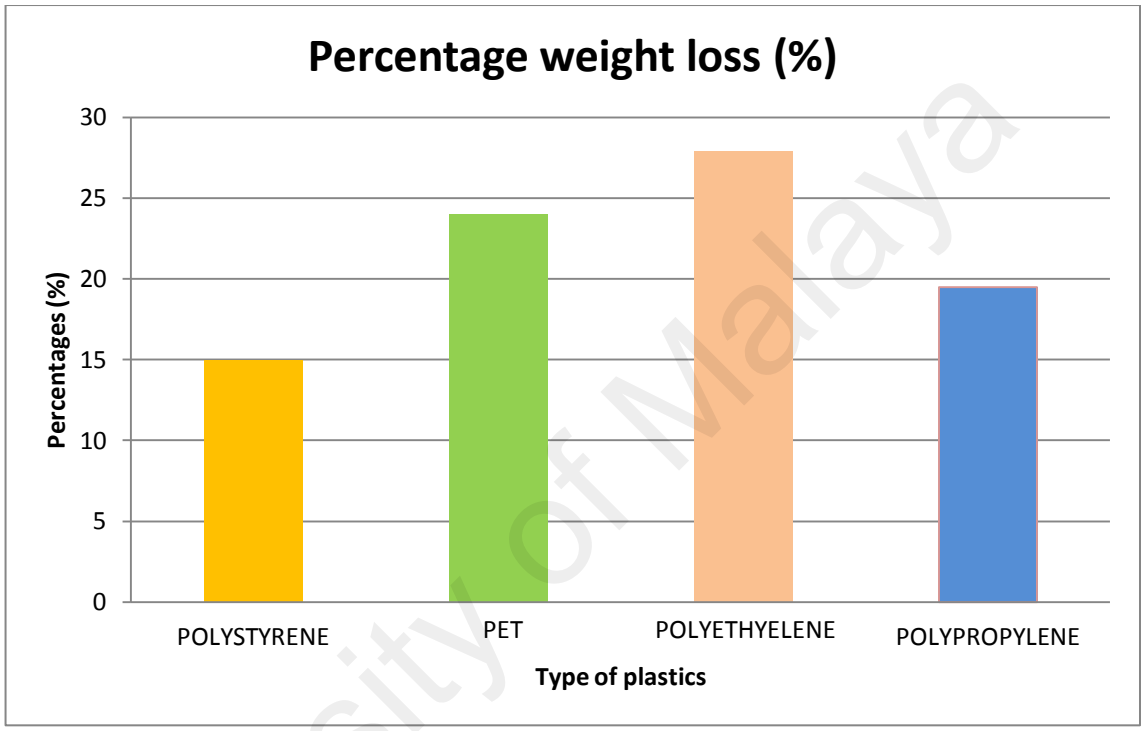


Figure 4.10: Weight loss of polymer after 30 days incubation with microbial consortium.

Polyethyelene recorded the highest weight loss which is 27.9%, follow by PET 24%, polypropylene 19.5%, and polystyrene 15%. The percentage of weight loss in this study is higher than past research degradation. Percentage of polyethylene weight loss is higher than that obtained by Kathiresan and Bingham (2001), which reported that bacteria caused the biodegradation ranging from 2.19 to 20.54%.

This result might due the use of microbial consortium in this study instead of single colony microorganism. Polystyrene shows the lowest weight loss and this result are supported by Berit *et al.* (2015), who stated that polystyrene is considered to be the most durable thermoplastic polymer to undergo biodegradation as compared to other polymers. This is probably due to its complex structure that makes it unsusceptible for microbial degradation.

4.7 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The changes in the structure of polypropylene, polyethylene terephtalate, polystyrene, and polyethylene with subsequent bacterial inoculation were analyzed by Fourier Transform Infrared Spectroscopy (Shimadzu) in the frequency range of 0 – 4000 cm^{-1} (Figure 4.11-4.14). Greater peak intensity means that there are more types of bond.

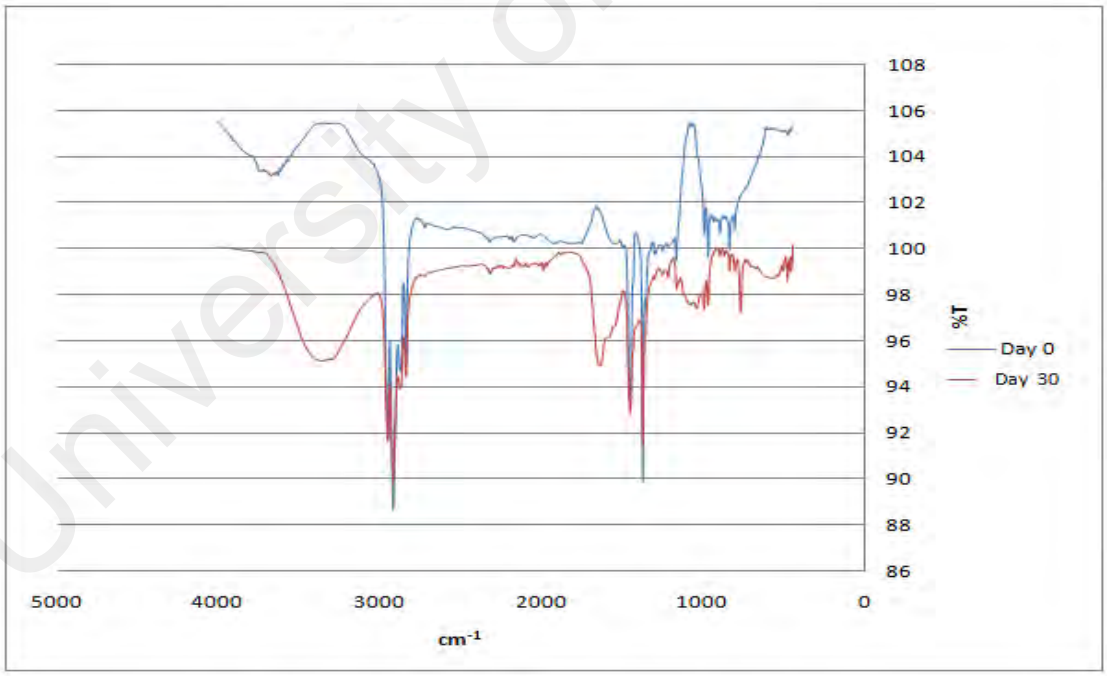


Figure 4.11 : FTIR spectra of polypropylene

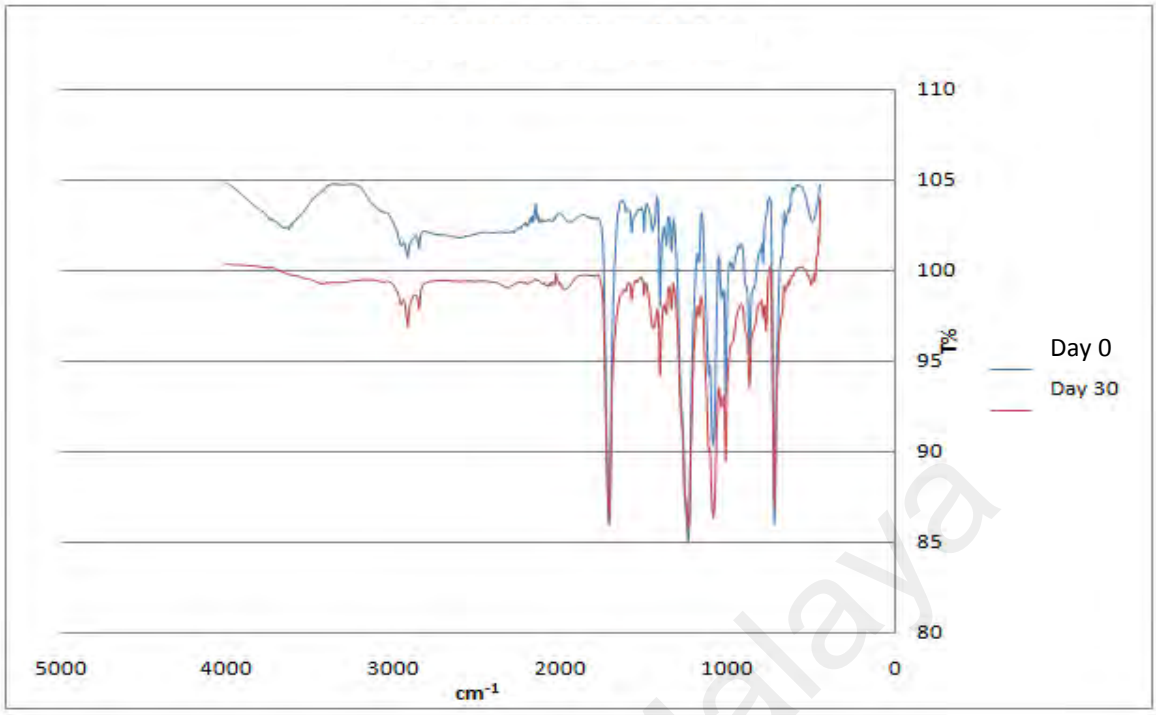


Figure 4.12 : FTIR spectra of polyethylene terephtalate

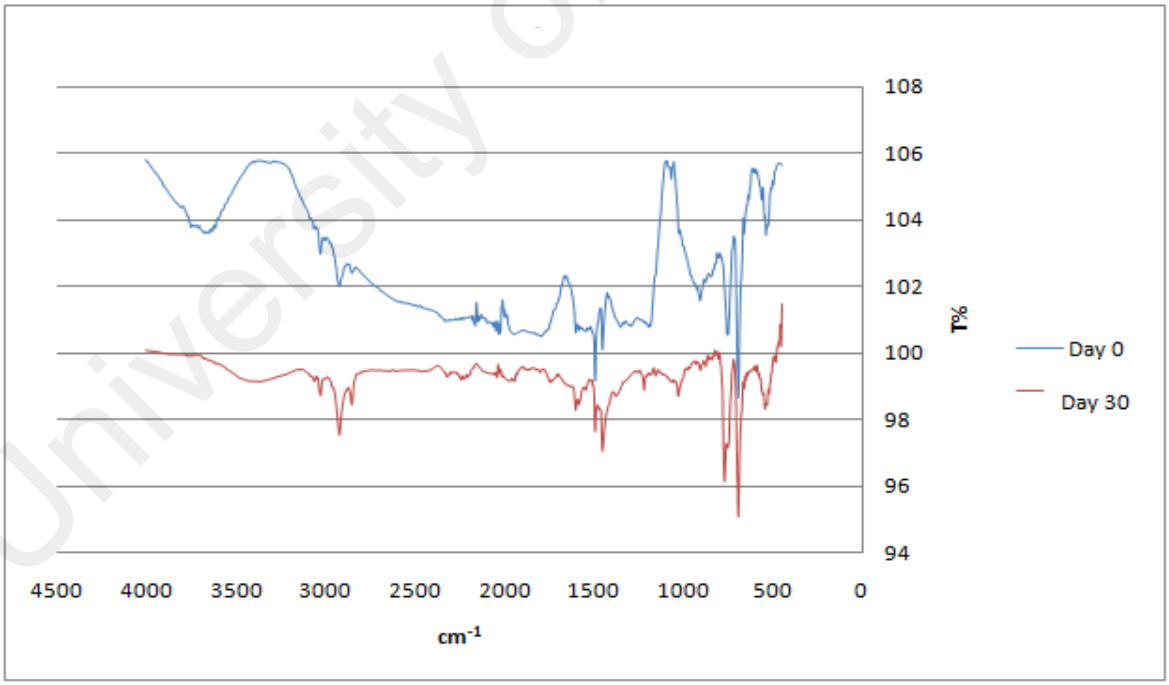


Figure 4.13 : FTIR spectra of polystyrene

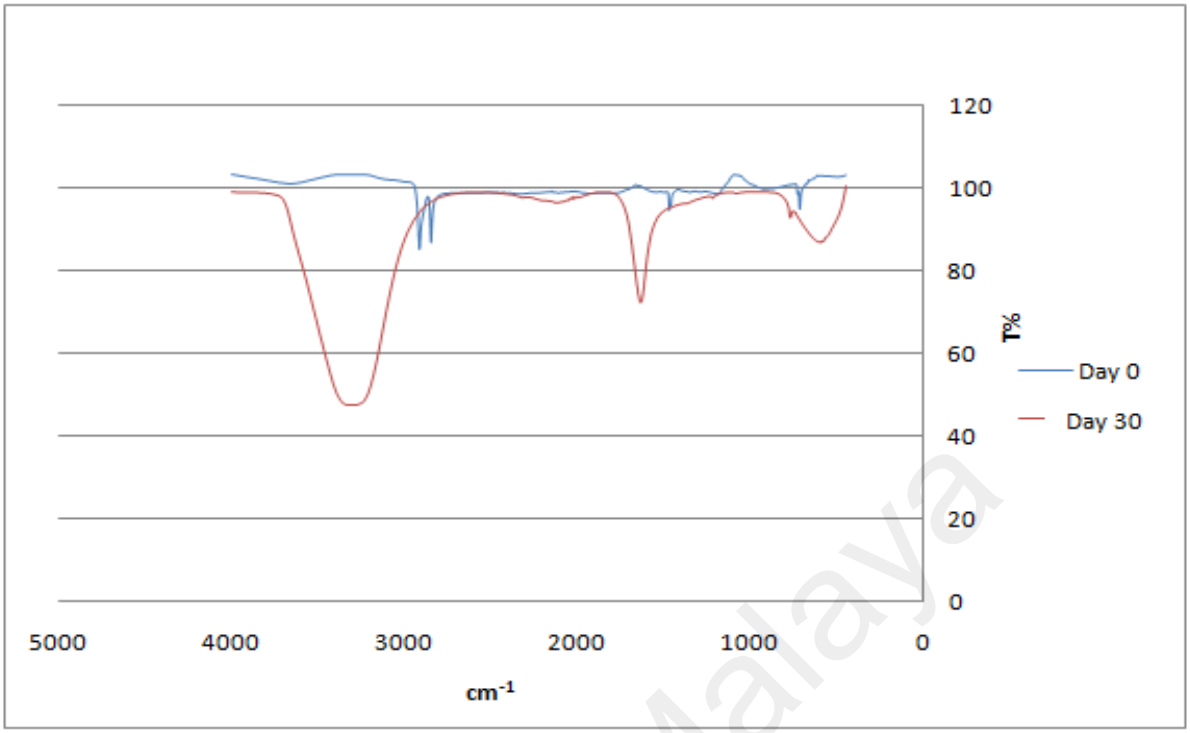


Figure 4.14 : FTIR spectra of polyethylene

The main band of 2920-2851 cm^{-1} reflects the C-H stretch. Furthermore, based on the IR spectroscopy , the bacterial degradation led to a substantial increase in the C-H stretch band of the polyethylene at 2920-2851 cm^{-1}.

The degradation potential of these bacteria is confirmed through the change in the peak of FTIR result. Chandrakant and Shwetha (2011) claimed that the degradation occurred due to the microbes secretion of extracellular enzymes that break the plastics complex molecular structure. Any changes, in forms of new peak formation, peak disappearance or change in the peak range, are considered as the monitoring parameter and seen as the changes that occurred on the polymer surface as a result of bacterial isolate actions. Finally, FTIR results indicate the formation of carboxylic acids, aldehyde, ketone, and alcohols after the biodegradation process.

CHAPTER FIVE: CONCLUSION

In general, most of the water quality parameters are below the acceptable limit except for COD which will influence the microbial population in the area. It was found that, microbial abundance at Matang Mangrove, Perak recorded the highest number of bacteria with 3.7 x 10^7 CFU/ml, indicating that there is high nutrient flow into the area to flourish the bacterial colonies. The microbial abundance was significantly correlated with water dissolved oxygen and BOD_5 values. This study established the potential of bacteria isolated from mangrove sediments to degrade microplastics. The usage of microbial cocktail enhance the biodegradation process of the plastics in which the percentage of degradation is range from 15% to 27.9%. Hence, when properly optimized and applied on polluted sites, the degradation effect will reduce the environmental impact of plastic polymers in the environment.

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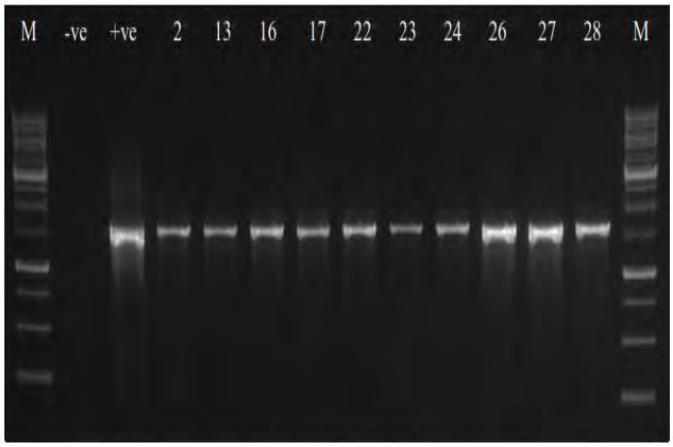
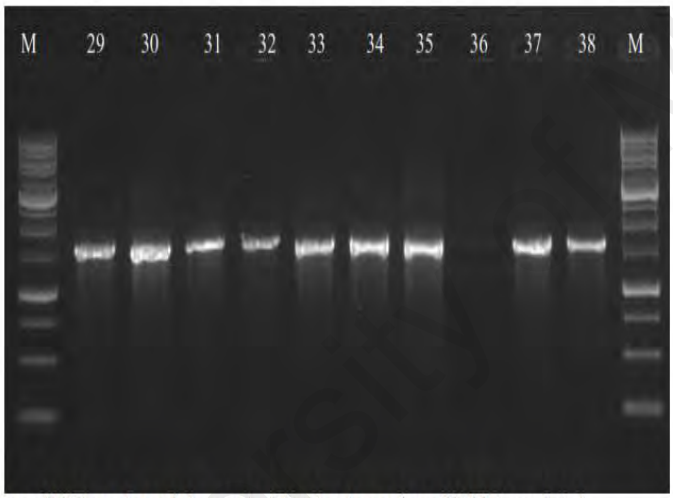
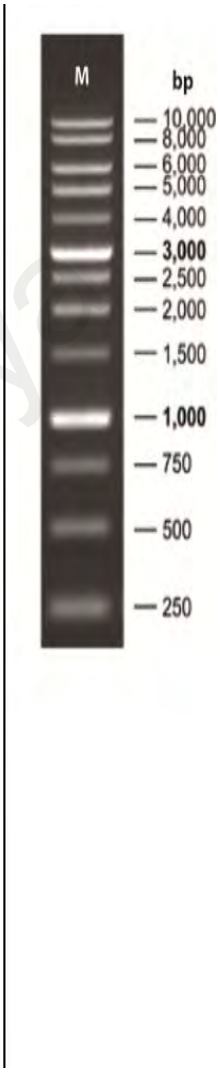
APPENDIX

Appendix 1: Gel Electrophoresis results for some of the isolate sample





-ve: PCR no-template control (water to replace DNA template)
+ve: Positive control (DNA extracted from *E.coli* is used as template)



Appendix 2: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M2)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus aquimaris strain TF-12 16S ribosomal RNA gene, partial sequence	2300	2300	99%	0.0	99%	NR_025241.1
Bacillus marisflavi strain TF-11 16S ribosomal RNA gene, partial sequence	2291	2291	99%	0.0	99%	NR_118437.1
Bacillus marisflavi strain TF-11 16S ribosomal RNA gene, partial sequence	2291	2291	99%	0.0	99%	NR_025240.1
Bacillus vietnamensis strain NBRC 101237 16S ribosomal RNA gene, partial sequence	2266	2266	99%	0.0	99%	NR_113995.1
Bacillus acidicola strain 105-2 16S ribosomal RNA gene, complete sequence	2228	2228	99%	0.0	98%	NR_041942.1
Bacillus shackletonii strain LMG 18435 16S ribosomal RNA gene, partial sequence	2205	2205	99%	0.0	98%	NR_025373.1
Bacillus kochii strain WCC 4582 16S ribosomal RNA gene, partial sequence	2192	2192	99%	0.0	98%	NR_117050.1
Bacillus purgationiresistens strain DS22 16S ribosomal RNA gene, partial sequence	2186	2186	99%	0.0	98%	NR_108492.1
Bacillus isabeliae strain CVS-8 16S ribosomal RNA gene, complete sequence	2176	2176	99%	0.0	98%	NR_042619.1
Bacillus coahuilensis strain m4-4 16S ribosomal RNA gene, partial sequence	2170	2170	97%	0.0	98%	NR_115933.1

Appendix 3: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M13)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus gottheilii strain WCC 4585 16S ribosomal RNA gene, partial sequence	2509	2509	100%	0.0	98%	NR_108491.1
Bacillus eiseniae strain A1-2 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	97%	NR_108906.1
Bacillus pocheonensis strain Gsoil 420 16S ribosomal RNA gene, partial sequence	2480	2480	99%	0.0	97%	NR_041377.1
Bacillus niacini strain IFO15566 16S ribosomal RNA gene, partial sequence	2471	2471	100%	0.0	97%	NR_024695.1
Bacillus kochii strain WCC 4582 16S ribosomal RNA gene, partial sequence	2464	2464	100%	0.0	97%	NR_117050.1
Bacillus koreensis strain BR030 16S ribosomal RNA gene, partial sequence	2452	2484	100%	0.0	97%	NR_116851.1
Bacillus bataviensis strain IDA1115 16S ribosomal RNA gene, partial sequence	2450	2450	99%	0.0	97%	NR_036766.1
Bacillus niacini strain NBRC 15566 16S ribosomal RNA gene, partial sequence	2446	2446	99%	0.0	97%	NR_113777.1
Bacillus foraminis strain CV53 16S ribosomal RNA gene, complete sequence	2446	2446	100%	0.0	97%	NR_042274.1
Bacillus novalis strain IDA3307 16S ribosomal RNA gene, partial sequence	2444	2444	99%	0.0	97%	NR_042168.1

Appendix 4: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M16)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus stratosphericus strain 41KF2a 16S ribosomal RNA gene, partial sequence	2668	2668	99%	0.0	99%	NR_042336.1
Bacillus altitudinis strain 41KF2b 16S ribosomal RNA gene, partial sequence	2666	2666	99%	0.0	99%	NR_042337.1
Bacillus pumilus SAFR-032 strain SAFR-032 16S ribosomal RNA, complete sequence	2634	2634	99%	0.0	99%	NR_074977.1
Bacillus aerius strain 24K 16S ribosomal RNA gene, partial sequence	2630	2630	98%	0.0	99%	NR_118439.1
Bacillus safensis strain NBRC 100820 16S ribosomal RNA gene, partial sequence	2625	2625	98%	0.0	99%	NR_113945.1
Bacillus pumilus strain NBRC 12092 16S ribosomal RNA gene, partial sequence	2625	2625	98%	0.0	99%	NR_112637.1
Bacillus stratosphericus strain 41KF2a 16S ribosomal RNA gene, partial sequence	2621	2621	97%	0.0	99%	NR_118441.1
Bacillus safensis strain FO-36b 16S ribosomal RNA gene, partial sequence	2547	2547	96%	0.0	99%	NR_041794.1
Bacillus pumilus strain ATCC 7061 16S ribosomal RNA gene, partial sequence	2547	2547	96%	0.0	99%	NR_043242.1
Bacillus atrophaeus 1942 strain 1942 16S ribosomal RNA, complete sequence	2509	2509	99%	0.0	98%	NR_075016.1

Appendix 5: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M22)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Exiguobacterium sp. AT1b strain AT1b 16S ribosomal RNA, complete sequence	2599	2599	99%	0.0	99%	NR_074970.1
Exiguobacterium profundum strain 10C 16S ribosomal RNA gene, partial sequence	2590	2590	99%	0.0	99%	NR_043204.1
Exiguobacterium aestuarii strain TF-16 16S ribosomal RNA gene, partial sequence	2581	2581	99%	0.0	99%	NR_043005.1
Exiguobacterium marinum strain TF-80 16S ribosomal RNA gene, partial sequence	2556	2556	99%	0.0	99%	NR_043006.1
Exiguobacterium aurantiacum strain DSM 6208 16S ribosomal RNA gene, partial sequence	2486	2486	99%	0.0	98%	NR_043478.1
Exiguobacterium aurantiacum strain NBRC 14763 16S ribosomal RNA gene, partial sequence	2477	2477	99%	0.0	98%	NR_113666.1
Exiguobacterium alkaliphilum strain 12/1 16S ribosomal RNA gene, partial sequence	2473	2473	99%	0.0	98%	NR_116296.1
Exiguobacterium himgiriensis strain K22-26 16S ribosomal RNA gene, partial sequence	2444	2444	97%	0.0	98%	NR_118534.1
Exiguobacterium aquaticum strain IMTB-3094 16S ribosomal RNA gene, partial sequence	2443	2443	97%	0.0	98%	NR_109413.1
Exiguobacterium mexicanum strain 8N 16S ribosomal RNA gene, complete sequence	2322	2322	92%	0.0	98%	NR_042424.1

Appendix 6: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M23)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	2598	2598	99%	0.0	99%	NR_074540.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence	2598	2598	99%	0.0	99%	NR_115714.1
Bacillus cereus strain ATCC 14579 16S ribosomal RNA gene, partial sequence	2598	2598	99%	0.0	99%	NR_114582.1
Bacillus anthracis str. Ames strain Ames 16S ribosomal RNA, complete sequence	2583	2583	99%	0.0	99%	NR_074453.1
Bacillus cereus strain JCM 2152 16S ribosomal RNA gene, partial sequence	2583	2583	99%	0.0	99%	NR_113266.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA gene, partial sequence	2583	2583	99%	0.0	99%	NR_115526.1
Bacillus cereus strain NBRC 15305 16S ribosomal RNA gene, partial sequence	2581	2581	99%	0.0	99%	NR_112630.1
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	2580	2580	100%	0.0	99%	NR_121761.1
Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA gene, partial sequence	2580	2580	99%	0.0	99%	NR_114581.1
Bacillus thuringiensis Bt407 16S ribosomal RNA, complete sequence	2576	2576	99%	0.0	99%	NR_102506.1

Appendix 7: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M24)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus aquimaris strain TF-12 16S ribosomal RNA gene, partial sequence	2484	2484	99%	0.0	98%	NR_025241.1
Bacillus marisflavi strain TF-11 16S ribosomal RNA gene, partial sequence	2457	2457	99%	0.0	98%	NR_025240.1
Bacillus marisflavi strain TF-11 16S ribosomal RNA gene, partial sequence	2423	2423	98%	0.0	98%	NR_118437.1
Bacillus vietnamensis strain 15-1 16S ribosomal RNA gene, partial sequence	2381	2381	96%	0.0	98%	NR_024808.1
Bacillus acidicola strain 105-2 16S ribosomal RNA gene, complete sequence	2367	2367	100%	0.0	97%	NR_041942.1
Bacillus shackletonii strain LMG 18435 16S ribosomal RNA gene, partial sequence	2347	2347	99%	0.0	96%	NR_025373.1
Bacillus licheniformis strain DSM 13 16S ribosomal RNA gene, complete sequence	2334	2334	100%	0.0	96%	NR_118996.1
Bacillus licheniformis strain ATCC 14580 16S ribosomal RNA gene, complete sequence	2325	2325	100%	0.0	96%	NR_074923.1
Bacillus licheniformis strain BCRC 11702 16S ribosomal RNA gene, partial sequence	2320	2320	99%	0.0	96%	NR_116023.1
Bacillus licheniformis strain NBRC 12200 16S ribosomal RNA gene, partial sequence	2316	2316	99%	0.0	96%	NR_113588.1

Appendix 8: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M26)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	2444	2444	100%	0.0	100%	NR_121761.1
Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA gene, partial sequence	2444	2444	100%	0.0	100%	NR_114581.1
Bacillus thuringiensis Bt407 16S ribosomal RNA, complete sequence	2439	2439	100%	0.0	99%	NR_102506.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA gene, partial sequence	2432	2432	99%	0.0	100%	NR_043403.1
Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	2428	2428	99%	0.0	99%	NR_112780.1
Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	2426	2426	100%	0.0	99%	NR_074540.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence	2426	2426	100%	0.0	99%	NR_115714.1
Bacillus cereus strain ATCC 14579 16S ribosomal RNA gene, partial sequence	2426	2426	100%	0.0	99%	NR_114582.1
Bacillus cereus strain JCM 2152 16S ribosomal RNA gene, partial sequence	2414	2414	99%	0.0	99%	NR_113266.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA gene, partial sequence	2414	2414	99%	0.0	99%	NR_115526.1

Appendix 9: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M27)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	2675	2675	99%	0.0	99%	NR_121761.1
Bacillus thuringiensis Bt407 16S ribosomal RNA, complete sequence	2675	2675	99%	0.0	99%	NR_102506.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA gene, partial sequence	2666	2666	99%	0.0	99%	NR_043403.1
Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	2661	2661	99%	0.0	99%	NR_074540.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence	2661	2661	99%	0.0	99%	NR_115714.1
Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA gene, partial sequence	2661	2661	99%	0.0	99%	NR_114581.1
Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	2654	2654	98%	0.0	99%	NR_112780.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA gene, partial sequence	2648	2648	99%	0.0	99%	NR_115526.1
Bacillus cereus strain ATCC 14579 16S ribosomal RNA gene, partial sequence	2643	2643	99%	0.0	99%	NR_114582.1
Bacillus anthracis str. Ames strain Ames 16S ribosomal RNA, complete sequence	2639	2639	99%	0.0	99%	NR_074453.1

Appendix 10: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M28)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus flexus strain IFO15715 16S ribosomal RNA gene, partial sequence	2592	2592	99%	0.0	99%	NR_024691.1
Bacillus flexus strain NBRC 15715 16S ribosomal RNA gene, partial sequence	2578	2578	99%	0.0	99%	NR_113800.1
Bacillus megaterium strain ATCC 14581 16S ribosomal RNA gene, partial sequence	2526	2526	99%	0.0	99%	NR_117473.1
Bacillus aryabhattai strain B8W22 16S ribosomal RNA gene, partial sequence	2518	2518	99%	0.0	99%	NR_115953.1
Bacillus megaterium strain NBRC 15308 16S ribosomal RNA gene, partial sequence	2513	2513	98%	0.0	99%	NR_112636.1
Bacillus megaterium strain IAM 13418 16S ribosomal RNA gene, partial sequence	2504	2504	98%	0.0	99%	NR_043401.1
Bacillus megaterium QM B1551 strain QM B1551 16S ribosomal RNA, complete sequence	2495	2495	98%	0.0	99%	NR_074290.1
Bacillus megaterium strain ATCC 14581 16S ribosomal RNA gene, partial sequence	2450	2450	96%	0.0	99%	NR_116873.1
Bacillus flexus strain SBMP3 16S ribosomal RNA gene, partial sequence	2419	2419	97%	0.0	98%	NR_118382.1
Bacillus simplex strain DSM 1321 16S ribosomal RNA gene, partial sequence	2414	2414	98%	0.0	98%	NR_115603.1

Appendix 11: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M29)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	2659	2659	100%	0.0	99%	NR_121761.1
Bacillus thuringiensis Bt407 16S ribosomal RNA, complete sequence	2659	2659	100%	0.0	99%	NR_102506.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA gene, partial sequence	2652	2652	99%	0.0	99%	NR_043403.1
Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA gene, partial sequence	2648	2648	99%	0.0	99%	NR_114581.1
Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	2646	2646	100%	0.0	99%	NR_074540.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence	2646	2646	100%	0.0	99%	NR_115714.1
Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	2641	2641	99%	0.0	99%	NR_112780.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA gene, partial sequence	2634	2634	99%	0.0	99%	NR_115526.1
Bacillus cereus strain ATCC 14579 16S ribosomal RNA gene, partial sequence	2630	2630	99%	0.0	99%	NR_114582.1
Bacillus anthracis str. Ames strain Ames 16S ribosomal RNA, complete sequence	2625	2625	99%	0.0	99%	NR_074453.1

Appendix 12: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M30)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas stutzeri A1501 16S ribosomal RNA, complete sequence	2654	2654	100%	0.0	100%	NR_074829.1
Pseudomonas stutzeri strain ATCC 17588 16S ribosomal RNA gene, complete sequence	2648	2648	100%	0.0	99%	NR_103934.1
Pseudomonas stutzeri strain VKM B-975 16S ribosomal RNA gene, partial sequence	2639	2639	100%	0.0	99%	NR_116489.1
Pseudomonas stutzeri strain NBRC 14165 16S ribosomal RNA gene, partial sequence	2634	2634	99%	0.0	99%	NR_113652.1
Pseudomonas stutzeri strain ATCC 17588 16S ribosomal RNA gene, partial sequence	2628	2628	99%	0.0	100%	NR_041715.1
Pseudomonas stutzeri strain CCUG 11256 16S ribosomal RNA gene, complete sequence	2626	2626	98%	0.0	100%	NR_118798.1
Pseudomonas chloritidismutans strain AW-1 16S ribosomal RNA gene, partial sequence	2545	2545	100%	0.0	98%	NR_115115.1
Pseudomonas kunmingensis strain HL22-2 16S ribosomal RNA, partial sequence	2531	2531	100%	0.0	98%	NR_133828.1
Pseudomonas otitidis strain MCC10330 16S ribosomal RNA gene, complete sequence	2518	2518	100%	0.0	98%	NR_043289.1
Pseudomonas guariconensis strain PCAVU11 16S ribosomal RNA, partial sequence	2516	2516	100%	0.0	98%	NR_135703.1

Appendix 13: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M31)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	2668	2668	100%	0.0	99%	NR_121761.1
Bacillus thuringiensis Bt407 16S ribosomal RNA, complete sequence	2668	2668	100%	0.0	99%	NR_102506.1
Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA gene, partial sequence	2661	2661	99%	0.0	99%	NR_114581.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA gene, partial sequence	2659	2659	99%	0.0	99%	NR_043403.1
Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	2654	2654	100%	0.0	99%	NR_074540.1
Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	2654	2654	99%	0.0	99%	NR_112780.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence	2654	2654	100%	0.0	99%	NR_115714.1
Bacillus cereus strain ATCC 14579 16S ribosomal RNA gene, partial sequence	2643	2643	99%	0.0	99%	NR_114582.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA gene, partial sequence	2641	2641	99%	0.0	99%	NR_115526.1
Bacillus cereus strain NBRC 15305 16S ribosomal RNA gene, partial sequence	2637	2637	99%	0.0	99%	NR_112630.1

Appendix 14: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M33)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Alcaligenes faecalis strain NBRC 13111 16S ribosomal RNA gene, partial sequence	2621	2621	99%	0.0	99%	NR_113606.1
Alcaligenes aquatilis strain LMG 22996 16S ribosomal RNA gene, partial sequence	2583	2583	99%	0.0	99%	NR_104977.1
Alcaligenes faecalis strain IAM 12369 16S ribosomal RNA gene, complete sequence	2567	2567	98%	0.0	99%	NR_043445.1
Alcaligenes faecalis subsp. parafaecalis strain G 16S ribosomal RNA gene, partial sequence	2473	2473	95%	0.0	99%	NR_025357.1
Alcaligenes faecalis subsp. phenolicus strain J 16S ribosomal RNA gene, partial sequence	2437	2437	98%	0.0	97%	NR_042830.1
Pusillimonas noertemannii strain BN9 16S ribosomal RNA gene, complete sequence	2343	2343	99%	0.0	95%	NR_043129.1
Pusillimonas ginsengisoli strain DCY25 16S ribosomal RNA gene, partial sequence	2333	2333	99%	0.0	95%	NR_116103.1
Paenalcaligenes suwonensis strain ABC02-12 16S ribosomal RNA, partial sequence	2329	2329	97%	0.0	96%	NR_133804.1
Candidimonas bauzanensis strain BZ59 16S ribosomal RNA gene, partial sequence	2327	2327	99%	0.0	95%	NR_108569.1
Parapusillimonas granuli strain Ch07 16S ribosomal RNA gene, partial sequence	2324	2324	98%	0.0	95%	NR_115804.1

Appendix 15: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M34)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	2679	2679	99%	0.0	99%	NR_121761.1
Bacillus thuringiensis Bt407 16S ribosomal RNA, complete sequence	2670	2670	99%	0.0	99%	NR_102506.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA gene, partial sequence	2663	2663	99%	0.0	99%	NR_043403.1
Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	2657	2657	99%	0.0	99%	NR_074540.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence	2657	2657	99%	0.0	99%	NR_115714.1
Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA gene, partial sequence	2657	2657	99%	0.0	99%	NR_114581.1
Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	2650	2650	98%	0.0	99%	NR_112780.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA gene, partial sequence	2645	2645	99%	0.0	99%	NR_115526.1
Bacillus cereus strain ATCC 14579 16S ribosomal RNA gene, partial sequence	2639	2639	99%	0.0	99%	NR_114582.1
Bacillus weihenstephanensis strain DSM 11821 16S ribosomal RNA gene, partial sequence	2639	2639	99%	0.0	99%	NR_024697.1

Appendix 16: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M35)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Alcaligenes faecalis strain NBRC 13111 16S ribosomal RNA gene, partial sequence	2621	2621	99%	0.0	99%	NR_113606.1
Alcaligenes aquatilis strain LMG 22996 16S ribosomal RNA gene, partial sequence	2587	2587	99%	0.0	99%	NR_104977.1
Alcaligenes faecalis strain IAM 12369 16S ribosomal RNA gene, complete sequence	2571	2571	99%	0.0	99%	NR_043445.1
Alcaligenes faecalis subsp. parafaecalis strain G 16S ribosomal RNA gene, partial sequence	2473	2473	95%	0.0	99%	NR_025357.1
Alcaligenes faecalis subsp. phenolicus strain J 16S ribosomal RNA gene, partial sequence	2441	2441	99%	0.0	97%	NR_042830.1
Pusillimonas noertemannii strain BN9 16S ribosomal RNA gene, complete sequence	2347	2347	100%	0.0	95%	NR_043129.1
Pusillimonas ginsengisoli strain DCY25 16S ribosomal RNA gene, partial sequence	2336	2336	100%	0.0	95%	NR_116103.1
Candidimonas bauzanensis strain BZ59 16S ribosomal RNA gene, partial sequence	2331	2331	100%	0.0	95%	NR_108569.1
Paenalcaligenes suwonensis strain ABC02-12 16S ribosomal RNA, partial sequence	2329	2329	97%	0.0	96%	NR_133804.1
Parapusillimonas granuli strain Ch07 16S ribosomal RNA gene, partial sequence	2324	2324	98%	0.0	95%	NR_115804.1

Appendix 17: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M36)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhodococcus ruber strain DSM 43338 16S ribosomal RNA gene, complete sequence	2639	2639	100%	0.0	99%	NR_026185.1
Rhodococcus phenolicus strain DSM 44812 16S ribosomal RNA gene, partial sequence	2513	2513	100%	0.0	98%	NR_115082.1
Rhodococcus zopfii strain DSM 44108 16S ribosomal RNA gene, partial sequence	2493	2493	99%	0.0	98%	NR_041775.1
Rhodococcus pyridinivorans 16S ribosomal RNA, complete sequence	2473	2473	100%	0.0	97%	NR_121768.1
Rhodococcus ruber strain DSM 43338 16S ribosomal RNA gene, partial sequence	2471	2471	93%	0.0	99%	NR_118602.1
Rhodococcus rhodochrous strain 372 16S ribosomal RNA gene, partial sequence	2464	2464	100%	0.0	97%	NR_037023.1
Rhodococcus pyridinivorans strain PDB9 16S ribosomal RNA gene, partial sequence	2462	2462	99%	0.0	97%	NR_025033.1
Rhodococcus biphenylivorans strain TG9 16S ribosomal RNA, partial sequence	2446	2446	100%	0.0	97%	NR_134798.1
Rhodococcus coprophilus strain CUB 687 16S ribosomal RNA gene, partial sequence	2444	2444	100%	0.0	97%	NR_029206.1
Rhodococcus aetherivorans strain DSM 44752 16S ribosomal RNA gene, partial sequence	2434	2434	93%	0.0	99%	NR_118619.1

Appendix 18: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M37)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Alcaligenes faecalis strain NBRC 13111 16S ribosomal RNA gene, partial sequence	2621	2621	99%	0.0	99%	NR_113606.1
Alcaligenes aquatilis strain LMG 22996 16S ribosomal RNA gene, partial sequence	2583	2583	99%	0.0	99%	NR_104977.1
Alcaligenes faecalis strain IAM 12369 16S ribosomal RNA gene, complete sequence	2567	2567	99%	0.0	99%	NR_043445.1
Alcaligenes faecalis subsp. parafaecalis strain G 16S ribosomal RNA gene, partial sequence	2473	2473	95%	0.0	99%	NR_025357.1
Alcaligenes faecalis subsp. phenolicus strain J 16S ribosomal RNA gene, partial sequence	2437	2437	99%	0.0	97%	NR_042830.1
Pusillimonas noertemannii strain BN9 16S ribosomal RNA gene, complete sequence	2343	2343	99%	0.0	95%	NR_043129.1
Pusillimonas ginsengisoli strain DCY25 16S ribosomal RNA gene, partial sequence	2333	2333	99%	0.0	95%	NR_116103.1
Paenalcaligenes suwonensis strain ABC02-12 16S ribosomal RNA, partial sequence	2329	2329	97%	0.0	96%	NR_133804.1
Candidimonas bauzanensis strain BZ59 16S ribosomal RNA gene, partial sequence	2327	2327	99%	0.0	95%	NR_108569.1
Parapusillimonas granuli strain Ch07 16S ribosomal RNA gene, partial sequence	2324	2324	98%	0.0	95%	NR_115804.1

Appendix 19: Blast results against NCBI 16S ribosomal RNA sequences (Isolate M38)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus gottheilii strain WCC 4585 16S ribosomal RNA gene, partial sequence	2228	2228	100%	0.0	99%	NR_108491.1
Bacillus kochii strain WCC 4582 16S ribosomal RNA gene, partial sequence	2206	2206	100%	0.0	98%	NR_117050.1
Bacillus firmus strain NBRC 15306 16S ribosomal RNA gene, partial sequence	2199	2199	100%	0.0	98%	NR_112635.1
Bacillus firmus strain IAM 12464 16S ribosomal RNA gene, partial sequence	2197	2197	100%	0.0	98%	NR_025842.1
Bacillus niacini strain NBRC 15566 16S ribosomal RNA gene, partial sequence	2194	2194	99%	0.0	98%	NR_113777.1
Bacillus niacini strain IFO15566 16S ribosomal RNA gene, partial sequence	2194	2194	99%	0.0	98%	NR_024695.1
Bacillus eiseniae strain A1-2 16S ribosomal RNA gene, partial sequence	2188	2188	100%	0.0	98%	NR_108906.1
Bacillus pocheonensis strain Gsoil 420 16S ribosomal RNA gene, partial sequence	2188	2188	100%	0.0	98%	NR_041377.1
Bacillus cibi strain JG-30 16S ribosomal RNA gene, partial sequence	2188	2188	100%	0.0	98%	NR_042974.1
Bacillus kyonggiensis strain NB22 16S ribosomal RNA, partial sequence	2181	2181	100%	0.0	98%	NR_132682.1

Appendix 20: BIOLOG Identification (Isolate M1)

Result: Species ID: Bacillus cereus/thuringiensis
Comment
Notice

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.569	0.569	6.330	GP-Rod-SB	Bacillus cereus/thuringiensis
2	0.088	0.088	8.037	GP-Rod-SB	Bacillus thuringiensis/cereus
3	0.043	0.043	8.923	GP-Rod-SB	Bacillus weihenstephanensis/cereus
4	0.042	0.042	8.941	GP-Rod-SB	Bacillus pumilus/safensis

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	< 190	< 382	< 450	< 283	{ 114	< 402	-5	{ 138 -	< 1666	< 1776	< 1214
B	10	-22	-18	< 309	{ 88	< 446	16	-8	11	< 1784	< 749	< 486
C	< 350	{ 48	< 410	-74	-54	-61	30	-32	{ 51 +	< 893	96	< 1739
D	22	{ 131	-69	26	{ 147	< 195	< 205	-26	{ 120	{ 118	< 1683	{ 112 +
E	< 254	{ 117	{ 80	{ 128	< 192	< 181	{ 101	-14	{ 125	{ 139	< 2061	102
F	< 198	{ 45	-40	{ 170	-29	-64	-13	-51	-34	{ 123	< 498 -	{ 299 -
G	-13	{ 136	-40	{ 178	{ 101	35	{ 62	< 269	{ 91	{ 133	< 1238	< 1642
H	< 251	< 251 -	{ 88	{ 149	{ 74	{ 90	{ 99	{ 118	{ 104	< 1123	< 1144	< 472

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Appendix 21: BIOLOG Identification (Isolate M3)

Result: Species ID: Bacillus sonorensis
Comment
Notice

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.509	0.509	7.294	GP-Rod-SB	Bacillus sonorensis
2	0.136	0.136	7.403	GP-Rod-SB	Bacillus vallismortis/subtilis
3	0.114	0.114	7.619	GP-Rod-SB	Bacillus pumilus/safensis
4	0.100	0.100	7.782	GP-Rod-SB	Bacillus marisflavi

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	159	< 258	< 283	< 269	< 271	< 271	< 271	< 270	153	< 276	< 280	102
B	{ 203	{ 190	< 272	< 259	< 278	{ 225	{ 212	{ 195	158	< 283	< 262	< 264
C	< 253	< 284	< 275	< 257	{ 172	{ 197	{ 183	< 281	< 268	< 285	78	78
D	{ 187	< 278	118	{ 187 +	< 268	{ 223	< 246	< 268	69	75	89	91
E	< 245	< 273	< 262	< 260	< 266	< 273	137	{ 238	{ 227	95	87	87
F	< 243	{ 218	{ 227 -	< 263	{ 225	{ 193	{ 216	< 251 -	< 247	80	{ 142	90
G	86	< 275	{ 226	< 261	< 286	{ 181	166 +	< 269	< 262	< 280	< 263	< 289
H	< 253	{ 216	< 271 -	< 269 -	{ 212	166 +	< 261	< 264	117	< 310	< 246	123

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Appendix 22: BIOLOG Identification (Isolate M4)

Result: Species ID: Bacillus thuringiensis/cereus
Comment
Notice

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.689	0.689	4.465	GP-Rod-SB	Bacillus thuringiensis/cereus
2	0.187	0.187	4.990	GP-Rod-SB	Bacillus cereus/thuringiensis
3	0.030	0.030	6.086	GP-Rod-SB	Bacillus weihenstephanensis/cereus
4	0.006	0.006	7.071	GP-Rod-SB	Bacillus pseudomycoides/cereus

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	138	< 200	< 198	< 211	< 197	145	148	{ 159	135	< 255	< 252	64
B	133	145	145	< 197 -	{ 183 -	< 215	{ 155	146	147	< 246	< 253	< 234
C	< 201	{ 184	< 204	143	146	{ 155	149	{ 150	< 187	< 263	68	< 249
D	134	136	133	{ 170	< 191	< 222	< 234	124	{ 179	63	< 258 -	73
E	< 237	{ 160	{ 160	{ 171	{ 174	{ 186	{ 182	{ 159	< 216	79	< 233	69
F	{ 152	{ 156	{ 160	< 203	{ 164	{ 177	{ 150	140	{ 159	64	119	82
G	115	< 201	142	{ 181	147	{ 165	146	< 224	{ 181	{ 124	< 255	< 245
H	{ 153	138	127	139	108	{ 176	127	{ 163	< 204 -	< 245	< 250	{ 174

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Appendix 23: BIOLOG Identification (Isolate M5)

Result: Species ID: Bacillus vietnamensis
Comment
Notice

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.660	0.660	4.876	GP-Rod-SB	Bacillus vietnamensis
2	0.156	0.156	5.548	GP-Rod-SB	Bacillus cibi
3	0.009	0.009	7.280	GP-Rod-SB	Bacillus firmus
4	0.003	0.003	7.838	GP-Rod-SB	Bacillus idriensis

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 wel

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	165	< 225	{ 188	< 206	145	{ 172	{ 193	{ 170	{ 160	< 255	< 249	71
B	147	149	156	{ 165	{ 161	< 230	{ 179	{ 163	{ 167	< 243	< 247	< 251
C	{ 198	75	< 224	127	123	{ 158	155	136	< 211 -	< 244	72	{ 137
D	150	{ 194	131	{ 158	{ 194	{ 179	< 210 -	{ 174	72	70	85	82
E	< 270	< 211	{ 170	< 212	< 207	< 202	< 229	{ 201 -	{ 172	91	79	81
F	< 217	{ 185	153	< 230	{ 188 -	{ 178	{ 176	{ 183	{ 169	72	{ 138	84
G	113	{ 189	{ 178	{ 189 -	{ 160	{ 164	{ 173	< 233	{ 159	108	{ 212	< 223
H	{ 178	114	131	< 228	104	{ 192	118	< 223	148	< 252	{ 178	109

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Appendix 24: BIOLOG Identification (Isolate M6)

Result: Species ID: Bacillus ruris
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.549	0.549	6.619	GP-Rod-SB	Bacillus ruris
2	0.155	0.155	6.897	GP-Coccus	Micrococcus yunnanensis
3	0.101	0.101	7.422	GP-Rod-SB	Sporolactobacillus terrae
4	0.078	0.078	7.744	GP-Coccus	Micrococcus lylae C

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	{ 30	< 285	< 214	{ 40	-5	< 230	< 298 -	2	< 366	< 302	{ 123
B	{ 35	24	8	-8	-18	{ 34	{ 61	{ 38	-46	< 320	< 562	< 363
C	< 336	< 147	{ 54 +	-10	-50	{ 37	11	18	{ 120 -	{ 237	{ 120	< 411
D	{ 69	-5 +	{ 30	18	16	-42	24	-10	16	{ 114	{ 139	{ 130
E	{ 74	{ 91 -	{ 42	0	-26	3	-70	22	{ 34	{ 130	{ 130	{ 109
F	< 310	16	{ 35	{ 120 -	-8	11	-29	{ 70	{ 94	{ 118	< 408 -	{ 229
G	-75	26	6	{ 66	-53	27	{ 93	14	{ 88	< 285	< 334	< 366
H	-77	{ 74	{ 50	{ 80	-46	{ 98	{ 67	{ 90	-54	< 283	< 387	< 454

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Appendix 25: BIOLOG Identification (Isolate M7)

Result: Species ID: Sporosarcina globispora (26C)
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.575	0.575	6.205	GP-Rod-SB	Sporosarcina globispora (26C)
2	0.145	0.145	6.518	GP-Rod-SB	Brevibacillus panacihumi
3	0.111	0.111	6.858	GP-Rod-SB	Viridibacillus arvi/neidei
4	0.110	0.110	6.868	GP-Rod	Nocardia araoensis

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	52	< 124	{ 94	{ 93	{ 84	70	{ 95	{ 107	57	< 261	< 219	65
B	55	54	67	52	57	59	68	55	75	< 249	87	63
C	{ 104	51	{ 104	65	64	{ 83	73	75	67	< 203	70	< 206 -
D	57	53	56	61	{ 117	< 140	< 181 -	61	68	68	< 203 -	78
E	63	52	61	59	57	73	57	73	62	< 229 -	< 217 -	79
F	{ 113	73	66	75	{ 98	{ 117 -	73	66	75	75	{ 132	85
G	51	16	52	62	26	{ 77	61	{ 92	57	< 258	{ 133	< 255
H	< 125	48	55	57	66	63	54	45	59	{ 170	75	{ 103

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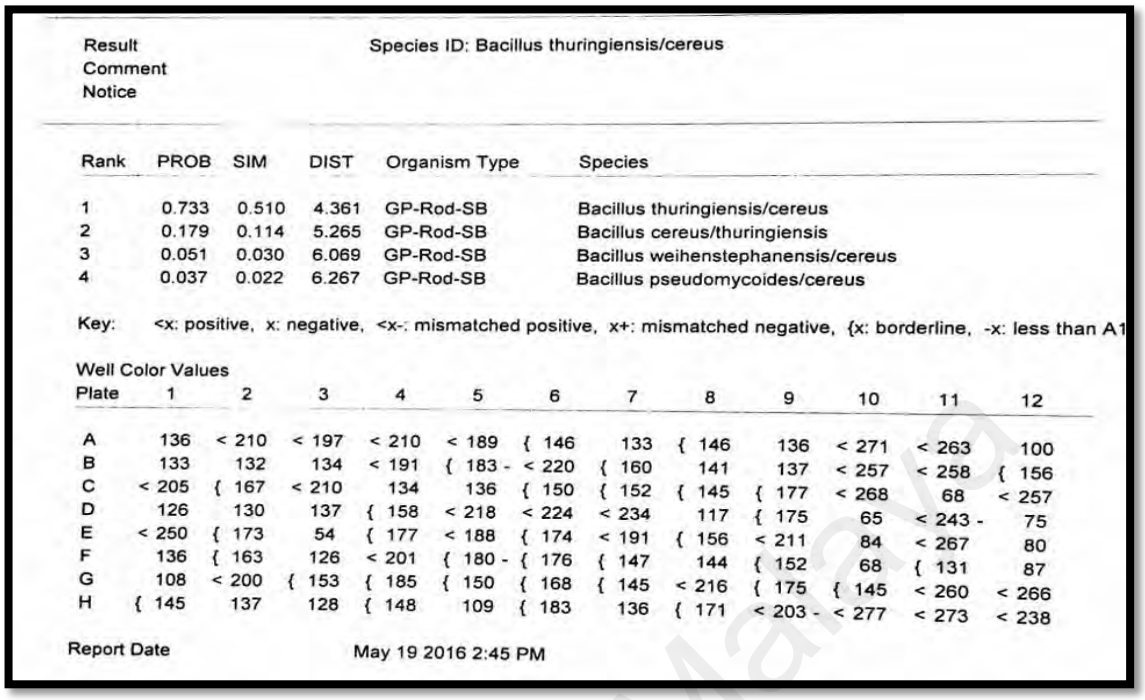
Appendix 26: BIOLOG Identification (Isolate M8)



Result
Comment
Notice

Species ID: Bacillus thuringiensis/cereus

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.733	0.510	4.361	GP-Rod-SB	Bacillus thuringiensis/cereus
2	0.179	0.114	5.265	GP-Rod-SB	Bacillus cereus/thuringiensis
3	0.051	0.030	6.069	GP-Rod-SB	Bacillus weihenstephanensis/cereus
4	0.037	0.022	6.267	GP-Rod-SB	Bacillus pseudomycoides/cereus

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	136	< 210	< 197	< 210	< 189	{ 146	133	{ 146	136	< 271	< 263	100
B	133	132	134	< 191	{ 183 -	< 220	{ 160	141	137	< 257	< 258	{ 156
C	< 205	{ 167	< 210	134	136	{ 150	{ 152	{ 145	{ 177	< 268	68	< 257
D	126	130	137	{ 158	< 218	< 224	< 234	117	{ 175	65	< 243 -	75
E	< 250	{ 173	54	{ 177	< 188	{ 174	< 191	{ 156	< 211	84	< 267	80
F	136	{ 163	126	< 201	{ 180 -	{ 176	{ 147	144	{ 152	68	{ 131	87
G	108	< 200	{ 153	{ 185	{ 150	{ 168	{ 145	< 216	{ 175	{ 145	< 260	< 266
H	{ 145	137	128	{ 148	109	{ 183	136	{ 171	< 203 -	< 277	< 273	< 238

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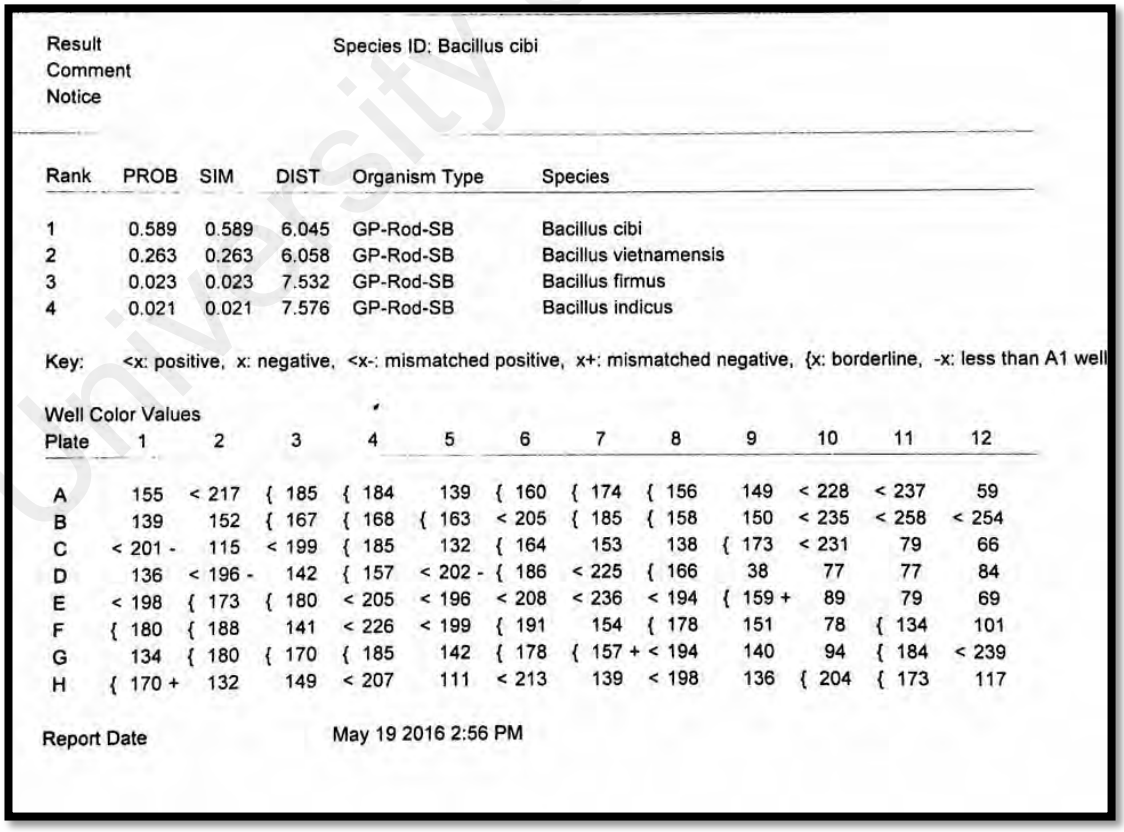
Appendix 27: BIOLOG Identification (Isolate M9)



Result
Comment
Notice

Species ID: Bacillus cibi

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.589	0.589	6.045	GP-Rod-SB	Bacillus cibi
2	0.263	0.263	6.058	GP-Rod-SB	Bacillus vietnamensis
3	0.023	0.023	7.532	GP-Rod-SB	Bacillus firmus
4	0.021	0.021	7.576	GP-Rod-SB	Bacillus indicus

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	155	< 217	{ 185	{ 184	139	{ 160	{ 174	{ 156	149	< 228	< 237	59
B	139	152	{ 167	{ 168	{ 163	< 205	{ 185	{ 158	150	< 235	< 258	< 254
C	< 201 -	115	< 199	{ 185	132	{ 164	153	138	{ 173	< 231	79	66
D	136	< 196 -	142	{ 157	< 202 -	{ 186	< 225	{ 166	38	77	77	84
E	< 198	{ 173	{ 180	< 205	< 196	< 208	< 236	< 194	{ 159 +	89	79	69
F	{ 180	{ 188	141	< 226	< 199	{ 191	154	{ 178	151	78	{ 134	101
G	134	{ 180	{ 170	{ 185	142	{ 178	{ 157 +	< 194	140	94	{ 184	< 239
H	{ 170 +	132	149	< 207	111	< 213	139	< 198	136	{ 204	{ 173	117

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Appendix 28: BIOLOG Identification (Isolate M10)

Result: Species ID: Acinetobacter schindleri
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.596	0.596	5.870	GN-Nent	Acinetobacter schindleri
2	0.147	0.147	6.253	GN-Nent	Serpens flexibilis
3	0.125	0.125	6.445	GN-Nent	Acinetobacter towneri
4	0.104	0.104	6.681	GN-Nent	Acinetobacter genomospecies 15TU

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	-37	-10	22	-5	-22	-6	3	-2	< 278	< 280	{ 122
B	3	-11	2	-11	-22	-8	-10	-2	-21	< 291	< 451 -	< 440 -
C	{ 34	{ 42	-14	-34	-35	-34	-27	-11	0	< 450	{ 141	{ 224
D	18	-6	-5	-26	-16	-3	-13	14	0	{ 130	{ 160	{ 155
E	22	11	{ 34 +	10	-21	-13	-13	-18	3	{ 158	< 259	{ 110 +
F	-10	{ 50	18	< 155 -	-18	-19	-34	-14	{ 43	< 301	< 488	< 262
G	-67	< 221	{ 42	< 149	-45	{ 58	24	{ 69	{ 66	{ 203	{ 221	{ 210
H	< 227	{ 67	-2	{ 82	-11	{ 42	-11	{ 96	-43	{ 235	{ 218	{ 243 -

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Appendix 29: BIOLOG Identification (Isolate M11)

Result: Species ID: Enterococcus faecium
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.596	0.596	5.922	GP-Coccus	Enterococcus faecium
2	0.124	0.124	6.465	GP-Rod	Cellulomonas hominis
3	0.114	0.114	6.533	GP-Rod	Listeria innocua/welshimeri/seeligeri
4	0.102	0.102	6.631	GP-Rod	Carnobacterium inhibens

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	< 299	< 232	< 138	< 208	< 221	< 171	-6	16	< 486	< 323	{ 141
B	24	{ 43 +	{ 86	< 230	< 259	< 238	< 227	32	{ 35	< 411	< 301	< 349 -
C	< 174	-14 +	< 227	{ 64 +	24	19	{ 43	0	{ 107	< 277	{ 118	{ 112
D	-30	< 216	-6	30	< 134 -	22	21	{ 35	-59	{ 104	{ 149	{ 125
E	{ 131 -	{ 64	{ 51	{ 98	{ 35	{ 66	{ 58	18	{ 54	{ 120	< 282	{ 162
F	{ 38	16	27	{ 110	22	19	6	10	18	{ 138	{ 258	69
G	-51	29	13	22	-16	5	-30	{ 48	6	< 453	< 421	< 464
H	10	18	19	13	6	{ 70	11	{ 43	8	{ 258	< 325	{ 227

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Appendix 30: BIOLOG Identification (Isolate M12)

Result: Species ID: Bacillus thuringiensis/cereus
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.653	0.653	4.995	GP-Rod-SB	Bacillus thuringiensis/cereus
2	0.194	0.194	5.172	GP-Rod-SB	Bacillus weihenstephanensis/cereus
3	0.109	0.109	5.518	GP-Rod-SB	Bacillus cereus/thuringiensis
4	0.072	0.072	5.771	GP-Rod-SB	Bacillus pseudomycoides/cereus

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	127	< 224	< 228	< 215	< 192	{ 150	120	140	{ 144	< 262	< 252	90
B	133	{ 144	134	< 201	< 193 -	< 232	{ 149	138	133	< 251	< 261	91 +
C	< 203	{ 172	< 225	127	128	142	{ 144	140	< 195	< 263	75	< 250
D	126	127	128	130	< 237	{ 152 +	< 200	118	{ 176	71	< 243 -	78
E	< 249	{ 160	{ 171	{ 177	{ 186	{ 176	< 201	{ 145	< 230	85	< 250	76
F	129	{ 168	122	< 206	{ 182	{ 174	137	139	136	71	{ 138	101
G	116	< 234	{ 149	< 196	{ 154	{ 168	142	< 202	{ 163	{ 200	< 256	< 256
H	{ 153	141	{ 147	{ 155	{ 145	< 205	{ 157	{ 184	< 213 -	< 256	< 265	{ 219

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Appendix 31: BIOLOG Identification (Isolate M14)

Result: Species ID: Stenotrophomonas maltophilia
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.854	0.666	3.154	GN-Nent	Stenotrophomonas maltophilia
2	0.146	0.101	4.288	GN-Nent	Stenotrophomonas rhizophila
3	0.000	0.000	8.699	GN-Nent	Stenotrophomonas acidaminiphila
4	0.000	0.000	9.432	GN-Nent	Vibrio harveyi

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	58	< 244	< 268	54	58 +	{ 86	50	60	53	< 336	< 317	{ 223
B	57	58	{ 94	65	65	< 271	{ 93	< 269	62	< 327	{ 217	65
C	< 188	< 255	{ 177	{ 126	{ 117	{ 126	{ 130	{ 108	69	< 324	< 268	< 266
D	59	60	67	53	66	{ 101	{ 164	63	77	< 329	< 335	80
E	< 307	< 265	< 248	68	< 201	{ 153	< 212	70	< 243	< 330	< 279	{ 223
F	59	{ 141	{ 143 -	75	{ 151	{ 140	80	62	68	< 314	< 365	< 392
G	53	< 247	{ 158	< 251	< 283	< 266	65	< 289	< 270	{ 229	< 275	77
H	< 263	57	< 204	75	{ 84 +	{ 147	< 257	< 276	52	< 330	{ 255	54

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Appendix 32: BIOLOG Identification (Isolate M15)

Result: Species ID: Bacillus pseudomycoides/cereus
Comment
Notice

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.549	0.549	6.623	GP-Rod-SB	Bacillus pseudomycoides/cereus
2	0.108	0.108	8.123	GP-Rod-SB	Bacillus thuringiensis/cereus
3	0.032	0.032	9.606	GP-Rod-SB	Bacillus weihenstephanensis/cereus
4	0.009	0.009	11.155	GP-Rod-SB	Lysinibacillus fusiformis

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	96	< 177	< 182	< 175	89	89	89	107	104	< 250	< 216	78
B	92	91	87	92	{ 145	< 195	< 171 -	114	102	< 204	65	67
C	{ 127	55	{ 128 +	{ 118	79	{ 143	{ 141	{ 118	< 199	{ 143	{ 106	< 200
D	85	89	95	{ 116	110	< 189	< 219	97	101	74	92	99
E	< 224	110	{ 123	< 200 -	{ 134	{ 153	{ 137	{ 121	{ 137	{ 104	< 217	90
F	101	{ 158 -	< 165 -	109	< 184 -	{ 156	108	107	{ 116	79	{ 138	{ 117
G	104	< 192	101	{ 151	{ 121	111	94	< 179	{ 145	99	81	< 195
H	{ 115	104	{ 132	104	{ 135	107	{ 124	{ 132	{ 122 +	< 219	80	94

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Appendix 33: BIOLOG Identification (Isolate M17)

Result: Species ID: Bacillus pumilus/safensis
Comment
Notice

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.624	0.624	5.406	GP-Rod-SB	Bacillus pumilus/safensis
2	0.238	0.238	5.674	GP-Rod-SB	Bacillus safensis/pumilus
3	0.004	0.004	8.158	GP-Rod-SB	Bacillus muralis
4	0.004	0.004	8.178	GP-Rod-SB	Bacillus amyloliquefaciens ss amyloliquefaciens

Key: <x. positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	< 288 -	-16	< 438	< 478	< 466	< 462	27	{ 171	< 1114	< 539	{ 141 +
B	< 258	{ 77	{ 155	< 539	< 490	< 446	{ 75	14	6	< 1014	< 371	< 373
C	< 286	< 250	< 270	{ 166	22	{ 45	22	5	< 221	< 907	{ 88	{ 101 +
D	18	< 261	29	27	< 261	{ 74	{ 64	< 245	-32	{ 106	{ 141	{ 86
E	< 229	{ 115	{ 150	< 373	< 250	< 344	{ 48	{ 110	{ 141	{ 114	< 558	{ 104
F	{ 114	26	{ 77	{ 186	{ 88	37	{ 48	< 363	8	{ 93	{ 312 -	< 747 -
G	37	{ 96	21	{ 80	< 299	{ 74	26	{ 190	{ 99	{ 192	< 475	< 667
H	{ 53	< 227	30	26	26	{ 106	{ 136	{ 88	32	< 368	< 346	{ 158

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Appendix 34: BIOLOG Identification (Isolate M18)

Result: Species ID: Alcaligenes faecalis ss faecalis
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.736	0.608	2.463	GN-Nent	Alcaligenes faecalis ss faecalis
2	0.194	0.142	3.747	GN-Fas	Bordetella trematum
3	0.037	0.023	5.337	GN-Nent	Achromobacter denitrificans/ruhlandii
4	0.034	0.021	5.434	GN-Nent	Achromobacter insolitus

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	51	65	48	48	47	56	47	49	48	< 319	< 313	{ 127
B	49	53	59	57	57	57	59	57	56	< 314	< 267	108
C	49	56	61	{ 81	{ 75	{ 88	{ 93	68	54	< 336	< 304	{ 245
D	49	55	62	56	63	62	{ 122 -	65	{ 98	< 275	< 323	< 295
E	51	60	< 212	59	{ 139	< 256	< 197	{ 148	50	< 311	< 285	< 307
F	{ 83	{ 88	{ 86	62	{ 94	{ 110	65	59	59	< 316	< 349	< 351
G	< 203	< 239	66	< 271	< 276	< 189	65	< 288	< 173 -	106	< 293	66
H	{ 84	58	{ 131	< 200	{ 140	71	< 234	< 163	{ 126	< 261	< 309	60

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Appendix 35: BIOLOG Identification (Isolate M19)

Result: Species ID: Bacillus pumilus/safensis
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.624	0.624	5.406	GP-Rod-SB	Bacillus pumilus/safensis
2	0.238	0.238	5.674	GP-Rod-SB	Bacillus safensis/pumilus
3	0.004	0.004	8.158	GP-Rod-SB	Bacillus muralis
4	0.004	0.004	8.178	GP-Rod-SB	Bacillus amyloliquefaciens ss amyloliquefaciens

Key: <x. positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	< 288 -	-16	< 438	< 478	< 466	< 462	27	{ 171	< 1114	< 539	{ 141 +
B	< 258	{ 77	{ 155	< 539	< 490	< 446	{ 75	14	6	< 1014	< 371	< 373
C	< 286	< 250	< 270	{ 166	22	{ 45	22	5	< 221	< 907	{ 88	{ 101 +
D	18	< 261	29	27	< 261	{ 74	{ 64	< 245	-32	{ 106	{ 141	{ 86
E	< 229	{ 115	{ 150	< 373	< 250	< 344	{ 48	{ 110	{ 141	{ 114	< 558	{ 104
F	{ 114	26	{ 77	{ 186	{ 88	37	{ 48	< 363	8	{ 93	{ 312 -	< 747 -
G	37	{ 96	21	{ 80	< 299	{ 74	26	{ 190	{ 99	{ 192	< 475	< 667
H	{ 53	< 227	30	26	26	{ 106	{ 136	{ 88	32	< 368	< 346	{ 158

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Appendix 36: BIOLOG Identification (Isolate M20)

Result Species ID: Bacillus thuringiensis/cereus
Comment
Notice

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.555	0.555	6.553	GP-Rod-SB	Bacillus thuringiensis/cereus
2	0.121	0.121	7.515	GP-Rod-SB	Bacillus vietnamensis
3	0.065	0.065	8.290	GP-Rod	Nocardia thailandica
4	0.062	0.062	8.341	GP-Rod-SB	Bacillus siralis

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	{ 99	{ 102	< 152	{ 88	{ 106 -	-24	16	-8	< 586	< 634	{ 138
B	-2	-34	-61	< 158 -	{ 54	< 344	2	-27	-37	< 507	< 339	{ 307
C	< 224	14	{ 112	-91	-74	-83	-77	-90	{ 123	< 709	{ 86	{ 299
D	{ 29	21	-13	-51	< 149	{ 53 +	{ 94	-26	-11	{ 101	< 618 -	{ 107
E	< 594	< 195 -	{ 34	{ 38	{ 69	-24	{ 35	-67	{ 64 +	{ 168	< 862	{ 98
F	-24	{ 70	-26	< 296	-35	-6	{ 38	-38	-10	{ 106	{ 118	67
G	-45	{ 67	-8	< 150	-3	-2	18	{ 78	24	{ 197	< 363	< 754
H	{ 107	{ 50	14	{ 94 -	0	{ 70	{ 54	{ 29	{ 29	< 659	< 592	{ 226

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Appendix 37: BIOLOG Identification (Isolate M21)

Result Species ID: Bacillus thuringiensis/cereus
Comment
Notice

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.589	0.589	6.027	GP-Rod-SB	Bacillus thuringiensis/cereus
2	0.101	0.101	7.384	GP-Rod-SB	Bacillus pseudomycoides/cereus
3	0.072	0.072	7.795	GP-Rod-SB	Bacillus oleronius
4	0.071	0.071	7.816	GP-Rod-SB	Lysinibacillus odysseyi

Key <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	< 120	< 243	< 144	-14	-26	{ 35	-29	-54	< 722	< 1770	{ 162
B	-40	-64	-88	{ 114 -	5	< 368	-19	5	{ 30	< 1213	< 974	< 418
C	< 128	10	{ 35	-123	-75	-69	-53	-35	{ 93	< 614	86	< 1523
D	-46	-64	-93	-21	10	{ 46 +	{ 112	5	{ 69	{ 99	< 765 -	{ 101
E	< 322	-29	-72	-46	-13	-35	-43	-54	< 251	{ 102	< 1114	{ 114
F	-42	-83 +	-74	-21	-83	-75	-40	-59	{ 29	93	{ 339 -	{ 168
G	-78	{ 43	-90	-13	-8	-3	-27	{ 74	{ 54	{ 178	< 707	< 1502
H	{ 69	-14	-72	13	-35	11 +	-8	-5	-26	< 598	< 427	< 883

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Appendix 38: BIOLOG Identification (Isolate M25)

Result: Species ID: Bacillus thuringiensis/cereus
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.516	0.516	7.216	GP-Rod-SB	Bacillus thuringiensis/cereus
2	0.120	0.120	7.898	GP-Rod-SB	Paenibacillus anaericanus
3	0.084	0.084	8.342	GP-Rod-SB	Bacillus oleronius
4	0.070	0.070	8.567	GP-Rod-SB	Lysinibacillus odysseyi

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	< 149	{ 61 +	< 142	5	-6	-74	-14	-27	< 565	< 610	{ 163
B	-16	-62	-74	{ 115 -	{ 22	< 355	-32	5	{ 29	< 602	< 454	< 554
C	< 157	10	{ 50	-94	-58	-72	-43	-14	< 147	< 824	{ 187	< 526
D	-30	-67	-77	-51	13	{ 66	{ 77	-6	{ 58	{ 173	< 690 -	{ 186
E	< 450	{ 67	13	3	6	-22	-6	-22	< 176	{ 205	< 944	{ 234
F	-96	-45 +	-75	{ 24	-64	-62	-27	-26	{ 61	{ 197	< 358 -	{ 326 -
G	-32	11	-35	{ 50	-3	-2	-27	{ 54	{ 27	{ 162	{ 192	< 506
H	{ 74	{ 22	-24	{ 58	-14	{ 38 +	{ 38	{ 34	13	< 467	< 509	{ 325

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Appendix 39: BIOLOG Identification (Isolate M32)

Result: Species ID: Bacillus thuringiensis/cereus
Comment:
Notice:

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.653	0.653	4.995	GP-Rod-SB	Bacillus thuringiensis/cereus
2	0.194	0.194	5.172	GP-Rod-SB	Bacillus weihenstephanensis/cereus
3	0.109	0.109	5.518	GP-Rod-SB	Bacillus cereus/thuringiensis
4	0.072	0.072	5.771	GP-Rod-SB	Bacillus pseudomycoides/cereus

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	127	< 224	< 228	< 215	< 192	{ 150	120	140	{ 144	< 262	< 252	90
B	133	{ 144	134	< 201	< 193 -	< 232	{ 149	138	133	< 251	< 261	91 +
C	< 203	{ 172	< 225	127	128	142	{ 144	140	< 195	< 263	75	< 250
D	126	127	128	130	< 237	{ 152 +	< 200	118	{ 176	71	< 243 -	78
E	< 249	{ 160	{ 171	{ 177	{ 186	{ 176	< 201	{ 145	< 230	85	< 250	76
F	129	{ 168	122	< 206	{ 182	{ 174	137	139	136	71	{ 138	101
G	116	< 234	{ 149	< 196	{ 154	{ 168	142	< 202	{ 163	{ 200	< 256	< 256
H	{ 153	141	{ 147	{ 155	{ 145	< 205	{ 157	{ 184	< 213 -	< 256	< 265	{ 219

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