BIODEGRADATION OF SECONDARY MICROPLASTICS BY SELECTED BACTERIAL CONSORTIUM IN MANGROVE SEDIMENTS UNDER LABORATORY CONDITIONS

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ABSTRACT

Universal abundance of microplastics poses a grave threat to the welfare of human beings and other living organisms. Hence, biodegradation is often suggested for remediation of microplastic pollution in the environment. Thus, the objectives of this research were to investigate the effect of daily input of bacterial inoculum on biodegradation, to study the effect of different concentrations of inoculum on biodegradation, and to explore the effect of different size of microplastics on their biodegradation. Research experiments were carried out in homogenized mangrove soil that was collected from 0 – 5 cm depth from six mangrove sites in Peninsular Malaysia. Three types of microplastic namely, High-Density Polyethylene (HDPE), Polypropylene (PP) and Polystyrene (PS) were chosen to study the impact of daily application of inoculum, while PP and PS were selected to further investigate the effect of concentration of inoculum and microplastic size due to their higher resistance to biodegradation. A consortium of nine bacteria was used as inoculum. Three methods were selected to determine biodegradation of microplastics namely weight loss, Fourier-transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). When microplastics were treated with inoculum on daily basis, 1.26% of weight loss was observed for HDPE, 1.15% for PP, and 0.5% for PS. Whereas, the lowest concentration of inoculum resulted in higher degradation of PS as weight loss recorded was 0.17%. Lastly, 1–4 mm^2 PP showed the most weight reduction of 0.5%. No significant differences between weight loss values of treated and control microplastics was found except for PP treated 1% inoculum concentration. Despite the low weight loss values, FTIR spectra of treated microplastics have shown evidence of biodegradation with the changes in peak intensities and absence of typical peaks. The

formation of new peaks indicated disappearance of typical functional groups and appearance of new functional groups due to polymer oxidation. Similarly, SEM analysis of treated PP and PS of 1-4 mm^2 with 0.25% inoculum concentration, showed formation of pits and surface erosion suggesting structural damages to microplastics due to assimilation of polymers by microbes. Selected bacterial consortium has shown the capabilities of biodegradation of HDPE, PP and PS. The rate constant of microplastics treated with daily application of inoculum suggested higher rate of biodegradation for HDPE, PP and PS which was 0.002 day^{-1}, 0.00018 day^{-1} and 0.00008 day^{-1}, respectively. Similarly, among other sizes of microplastics, 1-4 mm^2 sized PP and 25 mm^2 sized PS have shown higher biodegradation rate of 0.000056 day^{-1} and 0.000011 day^{-1}, respectively as well.

Keywords: Secondary microplastic; Biodegradation; Bacterial consortium; Mangrove soil; Microplastic film

BIOPENGURAIAN MICROPLASTIK SEKUNDER OLEH KONSORTIUM BAKTERIA TERPILIH DARI SEDIMEN PAYA BAKAU DI DALAM PERSEKITARAN MAKMAL

ABSTRAK

Pencemaran mikroplastik di seluruh dunia menimbulkan ancaman yang serius terhadap kesejahteraan manusia dan organisma hidup yang lain. Oleh itu, biodegradasi sering dicadangkan sebagai langkah pemulihan untuk masalah pencemaran plastik dalam persekitaran. Objektif kajian ini adalah untuk mengkaji kesan input inokulum harian terhadap biodegradasi, mengenal pasti kesan kepekatan inokulum yang berlainan terhadap biodegradasi dan menguji kesan saiz mikroplastik yang berbeza terhadap biodegradasi mikroplastik tersebut. Penyelidikan dijalankan menggunakan tanah paya bakau homogen yang diambil daripada kedalaman 0-5 cm di enam kawasan paya bakau di Semenanjung Malaysia. Tiga jenis mikroplastik iaitu High-Density Polyethylene (HDPE), Polypropylene (PP) dan Polystyrene (PS) telah digunakan untuk mengkaji kesan input inokulum harian terhadap degradasi manakala PP dan PS digunakan untuk menguji kesan kepekatan inokulum dan saiz mikroplastik disebabkan daya ketahanan yang lebih tinggi terhadap biodegradasi. Suatu konsortium bakteria yang mengandungi sembilan jenis bakteria telah dipilih sebagai inokulum. Tiga kaedah untuk menentukan biodegradasi mikroplastik adalah penyusutan berat, Fourier-transform Infrared Spectroscopy (FTIR) dan Scanning Electron Microscopy (SEM). Apabila mikroplastik dirawat dengan inokulum secara harian, penyusutan berat sebanyak 1.26% telah diperhatikan untuk HDPE, 1.15% untuk PP dan 0.5% untuk PS. Sebaliknya, kepekatan inokulum yang paling rendah menyebabkan degradasi yang tinggi untuk PS disebabkan penyususstan berat yang dicatatkan sebanyak 0.17%. Akhir sekali, PP bersaiz 1-4 mm^2 menunjukkan penyusutan berat yang paling tinggi sebanyak 0.5%. Tiada perbezaan yang signifikan di antara nilai-nilai penyusutan berat mikroplastik yang dirawat dengan

mikroplastik kontrol kecuali dalam PP apabila dirawat dengan 1% inokulum. Walaupun penyusutan nilai berat adalah rendah, spektra FTIR mikroplastik yang dirawat menunjukkan bukti pengoksidaan polimer dalam bentuk perubahan puncak-puncak keamatan dan ketiadaan puncak-puncak tipikal. Pembentukan puncak-puncak baru menunjukkan kehilangan kumpulan berfungsi tipikal dan kemunculan kumpulan berfungsi baru disebabkan oleh pengoksidaan polimer. Seumpamanya, analisis SEM pada PP berukuran 1-4 mm^2 yang dirawat dan pada PS yang dirawat dengan 0.25% kepekatan inokulum menunjukkan formasi lubang-lubang dan retakan pada permukaan, menandakan kemusnahan struktur pada mikroplastik disebabkan oleh tindakan mikrob. Konsortium bakteria tertentu telah menunjukkan keupayaan biodegradasi HDPE, PP dan PS. Kadar degradasi mikroplastik yang dirawat dengan aplikasi inokulum harian adalah lebih tinggi bagi HDPE pada 0.002 $hari^{-1}$, PP pada 0.00018 $hari^{-1}$ dan PS pada 0.00008 $hari^{-1}$. Seumpamanya, PP bersaiz 1-4 mm^2 dan PS bersaiz 25 mm^2 telah menunjukkan kadar biodegradasi yang lebih tinggi sebanyak 0.000056 $hari^{-1}$ untuk PP dan 0.000011 $hari^{-1}$ untuk PS, berbanding dengan lain-lain saiz PP dan PS.

Kata kunci: mikroplastik sekunder; biodegradasi, konsortium bakteria, media tanah

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LIST OF SYMBOLS AND ABBREVIATIONS

A_n	:	Absorbance
C	:	Carbon
CH_4	:	Methane
CO_2	:	Carbon Dioxide
E	:	East
g/mol	:	Molar Mass
H_2O	:	Water
H_2O_2	:	Hydrogen Peroxide
HCl	:	Hydrochloric Acid
K^{-1}	:	Rate Constant
KOH	:	Potassium Hydroxide
N	:	North
NaOH	:	Sodium Hydroxide
O	:	Oxygen
^{O}C	:	Celsius
sp.	:	Species
T%	:	Transmittance Percentage
µm	:	Micrometer
cm	:	Centimeter
g	:	gram
kg	:	Kilogram
L	:	Litre
m	:	Meter
ml	:	Millilitre

mm	:	Millimetre
mM	:	Millimolar
Mn	:	Manganese
nm	:	Nanometre
rpm	:	Revolution Per Minute
2D	:	2-Dimensional
3D	:	3-Dimensional
ABS	:	Acrylonitrile Butadiene Styrene
ABS	:	Absorbance
ABTS	:	2-2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
ATR-FTIR	:	Attenuated total reflectance – Fourier transform infrared spectroscopy
BSA	:	Bovine Serum Albumin
CFU	:	Colony Forming Units
CI	:	Carbonyl Index
CV	:	Crystal Violet
EPS	:	Expanded Polystyrene
EU	:	European Union
FTIR	:	Fourier Transmittance Infrared Spectroscopy
GDP	:	Gross Domestic Product
HDPE	:	High-Density-Polyethylene
HIPS	:	High-Impact-Polystyrene
HT-GPC	:	High-temperature gel-permeation chromatography
LDPE	:	Low-Density-Polyethylene
LLDPE	:	Linear-Low-Density-Polyethylene
LMWPE	:	Low-Molecular-Weight-Polyethylene

MARELITT	:	Marine Litter Program
MEG	:	Monoethylene Glycol
MSW	:	Municipal Solid Waste
PAH	:	Polycyclic Aromatic Hydrocarbons
PBDE	:	Polybrominated diphenyl Ethers
PBS	:	Phosphate Buffer Solution
PCA	:	Plate Count Agar
PCB	:	Polychlorinated biphenyls
PE	:	Polyethylene
PES	:	Polysulfone
PET	:	Polyethylene Terephthalate
POP	:	Persistent Organic Pollutants
PP	:	Polypropylene
PP&A	:	Polyamide and Acrylic
PS	:	Polystyrene
PTA	:	Terephthalic Acid
PUR	:	Polyurethane
PVA	:	Polyvinyl Alcohol
PVC	:	Polyvinyl Chloride
SDGs	:	Sustainable Development Goals
SDS	:	Sodium Dodecyl Sulphate
SEM	:	Scanning Electron Microscopy
SWCorp	:	Solid Waste Management and Public Cleansing Corporation
TN	:	Total Nitrogen
TOC	:	Total Organic Carbon
TP	:	Total Phosphorous

UNEP		United Nations Environment Programme
US	:	United States
UV	:	Ultraviolet

CHAPTER 1: INTRODUCTION

1.1 What is Microplastic Pollution?

The conundrum of microplastic seems to be getting more complicated with time. Just when scientific community and general public were trying to grasp the findings of microplastics in drinking water (Pivokonsky *et al*., 2018; Schymanski *et al*., 2018; Oßmann *et al*., 2018), new research findings have highlighted that human beings might be breathing microplastics from the air as well (Gasperi *et al*., 2018; Dris *et al*., 2017). Hitherto microplastics were reported to have been, intentionally or unintentionally, ingested by marine and terrestrial fauna (Fauziah *et al*., 2018) only. While at that time, there were concerns of unintentional intake of microplastics by human beings through consumption of infected seafood (Van Cauwenberghe *et al*., 2014), potential human exposure to microplastics through drinking water and air should be the straw that broke camel's back. Human beings, including terrestrial and marine fauna, should not be ingesting microplastics. Plastic material regardless of size, is non-degradable, that living organisms naturally do not possess the capabilities of assimilating plastic material for carbon source and energy.

Microplastics are plastic particles that are smaller than 5 mm (Anderson *et al*., 2016) and are pervasively present in the environment. They have been found in beach sediments, in deep sea sediments, in surface water of oceans, rivers and lakes and, as well as, in ice cores (Fauziah *et al*., 2018). Microplastics can either originate from fragmentation of plastic debris (secondary microplastics) or can be produced intentionally for intended purposed (primary microplastics) (Auta *et al*., 2017). The abundance of plastic litter in aquatic and terrestrial environment cannot be neglected due to strong positive correlation found between the abundance of plastic litter and the abundance of microplastics (Fok *et al*., 2015). Nearly 5.25 trillion particles of plastic litter (including microplastics) are reported to have been floating on the surface of world oceans from 2007 to 2013 (Eriksen,

et al., 2014). Due to the great abundance of plastic litter, the quantity of microplastics is expected to increase with continuous degradation of plastic litter in the aquatic environment (Eriksen, *et al*., 2014). In 2014, an estimation of global inventory of microplastics suggested approximately 51 trillion particles of microplastics floating on the surface of world oceans (van Sebille *et al*., 2015). Whereas, 32 billion particles of primary microplastics i.e. microbeads were reported in Hong Kong (Cheung & Fok, 2016). Similarly, 20,000 items/km^2 to 170,000 items/km^2 of microplastics can be found in cyclonic and anticyclonic eddies in North Atlantic Ocean (Brach *et al*., 2018).

Owing to universal abundance of microplastics in nature, both terrestrial and marine organisms have come in contact with microplastics directly or indirectly. Microplastics on average have similar size range as that of benthos and planktons; within the range of 5 mm to 1 µm. Due to this similarity of size with benthos and planktons, microplastics can be mistaken as food by many organisms and hence can be ingested (Hidalgo-Ruz *et al*., 2012). Qualitative findings show ingestion of microplastics by several organisms such as freshwater ducks (Holland *et al*., 2016;), stripped red mullet fish (Alomar *et al*., 2017), Nile perch (Biginagwa *et al*., 2016), mussels (Vandermeersch *et al*., 2015), crustacean (Murray & Cowie, 2011), and blood worms (Nel & Froneman, 2018). Table 1.1 highlights the concentration of microplastics found in the infected fauna.

Table 1.1: Quantitative Findings of Microplastic Ingestion by Fauna

Fauna	Quantity of Microplastic	Reference
Gular pouch	8.99 items/gular pouch	Amelineau *et al*., 2016
Sand trout	1.83 ± 1.34/fish	Peters *et al*., 2017
Atlantic spadefish	2.96 ± 5.21/fish	Peters *et al*., 2017
Fish (P. indicus)	1.850 ± 0.455 (fish muscle)	Akhbarizadeh *et al*., 2018
Copepods	103.49 items/m^3	Sun *et al*., 2017
Mussels	0.9–4.6/g	Li *et al*. 2016
Mussels	9.2 items/g	Kolandhasamy *et al*., 2018

Several studies have also shown the presence of microplastics in seafood (Li *et al*., 2018; Karami *et al*., 2018) intended for human consumption. Additionally, microplastics have been reported in other human consumables such as salts (Gündoğdu, 2018), in honey and sugar (Liebezeit *et al*., 2014), and in beer (Kosuth *et al*., 2018), thereby increasing the chances of human ingestion of microplastics. While the impacts of microplastic ingestion in human beings are still unknown, several negative impacts of microplastic ingestion are seen in marine fauna (Katsnelson, 2015). These impacts include clogged digestive organs that result in reduced appetite (Wright *et al*., 2013), increased mortality (Browne *et al*., 2013), inflammation, liver stress, and endocrine disruption (Rochman *et al*., 2014). Nanoplastics, plastic particles smaller than 100 nm (Koelmans *et al*., 2015), pose an even greater threat as they can be transmitted to the next generation once ingested (Agamuthu, 2018).

Microplastics act as a sponge, absorbing toxins from the aquatic environment due to characteristics like crystallinity, large surface area, strong Van der Waal's force of attraction and hydrophobicity (Fauziah *et al*., 2018). Toxins such as dioxin, Polychlorinated biphenyls (PCBs) and Dichlorodiphenyltrichloroethane had shown high affinity for adherence on microplastic pellets where pellets were carrying one million times more concentration than that of the sea water (Takada, 2013). A number of toxins are listed in Table 1.2, that were found adhered to the surface of microplastics. Once these toxins loaded microplastics are ingested by organisms, toxins can be transferred into the body of organisms (Tanaka *et al*., 2013). However, there are more knowledge gaps in understanding the impacts of toxins loaded microplastics ingestion than what we know at this point (Katsnelson, 2013).

Table 1.2: Some Toxins found adhered on microplastics

Toxins	Reference
Dioxin	Takada, 2013
Polychlorinated biphenyls (PCBs)	Takada, 2013; Rochman *et al*., 2013; Hirai *et al*., 2011
Dichlorodiphenyltrichloroethane	Takada, 2013
Polycyclic Aromatic Hydrocarbons	Rochman *et al*., 2013
Polybrominated diphenyl Ethers (PBDEs)	Tanaka *et al.,* 2013; Rochman *et al*., 2014; Hirai *et al*., 2011
Nonylphenol	Browne *et al*., 2013
Phenanthrene	Browne *et al*., 2013

1.2 Management of Microplastic Pollution

The publishing of these staggering numbers of microplastics in global oceans and ample reports on microplastic ingestion in marine biota has caught the attention of national governments and international authorities, therefore several initiatives have been taken globally over the years. As recent as in 2016, United Nations Environmental Assembly, passed a stand-alone resolution on marine litter without any opposing and motioned countries to put marine litter issue high on their national environmental agenda (United Nations Environment Assembly, 2016). Other initiatives such as formulation of Honolulu Strategy (The Honolulu Strategy, 2018), Global Partnership on Marine Litter (Global Partnership on Marine Litter, n.d.), and G7 summit (Ruiz, 2018), all focus on prevention, reduction and mitigation of marine litter, especially marine plastic litter and microplastics by formulating framework, strategies and charter, respectively. Additionally, legislative and market-based instruments have also been deployed for tackling plastic (and microplastic) debris issues. For instance, ban on using microbeads in cosmetic products will reduce the generation of primary microplastics litter by prevention. Whereas, levies or partial bans on using single-use plastic bags will help reduce the generation of secondary microplastics by reducing the generation of plastics waste in the environment (Agamuthu *et al*., 2018). European Union (EU) has legislation

on recycling and reusing packaging waste, Directive 94/62/EC, that includes plastics (EUR-Lex, 2015) and more recently EU passed a legislation, Directive 2015/720, on reduction in consumption of lightweight plastic bag (EUR-Lex, 2015).

Four Sustainable Development Goals (SDG) have targets that encourage indirect prevention and reduction in the generation of primary and secondary microplastics (Sustainable development goals, 2015). SDG 11 (Sustainable cities and communities), SDG 12 (Responsible consumption and production), and SDG 14 (Life Below Water) aim to reduce waste generation. By reducing waste generation, especially plastic waste generation, the generation of secondary microplastics can be decreased. Whereas, SDG 6 (Clean water and sanitation) has target on halving the proportion of untreated wastewater. Hence, in conjunction with the ban on microbeads, SDG 6 will reduce the generation of microbeads in the aquatic environment. However, the best possible way of curbing microplastic pollution is prevention at source, therefore adopting ideal waste hierarchy and moving towards circular economy are the only sustainable and long-term solutions of tackling microplastics issue. Lastly, changing people's behaviour of throwaway mind-set and habit of littering will also go a long way in tackling this issue (Lohr *et al*., 2017).

In addition to global and national initiatives for prevention and reduction of microplastic and marine debris issue, manual removal of marine debris and beach clean-ups have also been executed in several locations worldwide (Schneider *et al*., 2018). EU funded MARELITT program and Global Ghost Gear Initiatives are the examples of regional initiatives taken to manually remove derelict fishing equipment, fishing nets and other components of marine debris (Agamuthu *et al*., 2018). In order to reduce the generation of secondary microplastics in the environment, manual removal of existing marine debris is vital as many studies have suggested fragmentation of plastic marine debris that leads to formation of secondary microplastics (Kim *et al*., 2015; Fok &

Cheung, 2015). However, the efficiency of marine litter collection depends on the method deployed as there are a variety of collection methods (Schneider *et al*., 2018). It is often feared that microplastics smaller than the mesh size of the net (i.e. 0.33 mm mesh) cannot be trapped in the net (Eriksen *et al*., 2014) and hence they stay in the aquatic system. Moreover, other processes such as ingestion by organisms, decreased buoyancy due to biofouling, and entrainment in settling detritus may lead to microplastics' escape from manual removal from the aquatic ecosystem (Eriksen *et al*., 2014). However, the major challenge of manual removal of microplastics from the aquatic environment is the low density of microplastics, as well as, the overlapped size of microplastics with that of benthos and planktons (Hidalgo-Ruz *et al*., 2012). So, it is feared that by trapping microplastics from the oceans, benthos and planktons might also be removed from the ocean surfaces. On the other hand, microplastics in sediments have similar size range of sand and silt; 5 mm to 500 µm for sand, and 500 µm to 1 µm for silt (Hidalgo-Ruz *et al*., 2012), which also makes manual removal of microplastics from terrestrial sediments practically impossible. Hence, management/mitigation of existing microplastics in the environment is also important in addition to prevention and reduction at source.

1.3 Is bioremediation the solution?

Generally, environmental pollution is remediated by using microbes, such as in the case of heavy metal pollution (Jacob *et al*., 2018), petroleum hydrocarbons pollution (Varjani, 2017), and persistent organic pollutants pollution (Guar *et al*., 2018). The phenomenon of utilizing microbes for remediation is called bioremediation (Dezionek *et al*., 2016). Plastic material used to be considered as resistant to microbial attack due to hydrophobicity characteristic. But several studies have manifested the capabilities of microbes (bacteria and fungus) to excrete extracellular enzymes that break down complex, hydrophobic polymers into monomers and/or dimers (Muthukumar *et al*., 2015). These simpler chains of plastic material are then degraded into carbon dioxide and

water (Tokiwa *et al*., 2009) and the process is called biodegradation. Therefore, biodegradation of mesoplastics (greater than 5 mm in size) have been studied in many experiments, where deployed microorganisms have utilized plastic polymers as source of carbon and energy (Ariba Begum *et al*., 2015; Gnanavel *et al*., 2012; Kale *et al*., 2015). Similarly, bioremediation of microplastics have also been thought as the means of tackling microplastic pollution (Auta *et al*., 2017; Paco *et al*., 2017). Utilizing microbes for potentially eliminating microplastics from the environment will provide a low-cost and environment friendly option.

1.4 Problem Statement and Objectives

Mangrove forests are one of the diverse habitats on the planet. Located between marine and terrestrial environment, these habitats are well known for distinctive plant species that can tolerate salinity, oxygen and pH variations and are also well known for common ground for marine and terrestrial food web (Alongi, 2014). Mangrove soil is rich in organic matter and salts with a texture between silt and clay (Hossain *et al*., 2016). Mangrove forests in Malaysia cover about 2% of total area of the country (Abd Shukor, 2004). However, in race to become a developed country, mangrove forests have been reclaimed for several development projects such as for aquaculture pond, tourism, commercial industrial complex and extension of commercial ports (Mazlan *et al*., 2005). Due to development and anthropogenic activities, plastic debris and microplastics have been found in mangrove forests such as in Singapore (Mohamed Nor & Obbard, 2014) and Peninsular Malaysia (Norkhairah, 2018; Jayanthi *et al*., 2014). With increasing accumulation of plastic waste in the environment and the struggle to achieve efficient plastic waste management, especially in developing countries, there is even a greater need for research on the biodegradation of microplastic. For plastic littering is a serious issue which leads to deposition of plastic on or in soil sediments through sedimentation (Claessens *et al*., 2011). While manual removal of macroplastic may be possible, removal

of microplastics from environment is practically impossible at the moment. Moreover, collection of microplastics from the environment and their management for recycling or other disposal method is also not cost-effective (Andrady, 2017).

While biodegradation is regarded as the solution, there are still several gaps in the biodegradation studies of microplastics. This research attempts at finding the answers to four of those gaps. Since the majority of studies have been performed in shake-flask method (Auta *et al*., 2018; Auta *et al*., 2017; Sowmya *et al*., 2017; Mohan *et al*., 2016), there is uncertainty on the biodegradation capabilities of same microbes in an experimental set-up that is closely related to natural environment, i.e. in mangrove soil. As environmental factors such as humidity, temperature, pH, salinity, and aerobic or anaerobic conditions will vary in shake-flask or in soil set-up. Another gap realized in the biodegradation studies of microplastics was lack of scientific data on the effect of microplastics size on biodegradation since microplastic can be found in highly variable sizes in the environment (Fok *et al*., 2017; Zhao *et al*., 2014) and biodegradation is a surface reaction (Chinaglia *et al*., 2018). Lastly, the effect of different concentrations of bacterial inoculum on biodegradation of microplastics in soil medium and the effect of increased input of inoculum into treatment are also unknown. Baring these gaps in the biodegradation studies of microplastic, it is imperative to acquire comprehensive knowledge on biodegradation of microplastic as biodegradation can provide a viable option for tackling the crises of microplastic pollution in the environment.

The objectives of this research are as follow:

1. To explore the biodegradation rate of microplastics treated with increased frequency of input of selected bacterial consortium.
2. To determine the biodegradation rate of microplastics treated with different inoculum concentrations.
3. To investigate the effect of different size of microplastics on biodegradation.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to Plastic

Manufacturing of plastic was a revolutionary invention. Just like all other major inventions of mankind, plastic or the very first synthetic polymer was invented due to the increasing demand and necessity. Back in 1869, the necessity for the substitute of ivory was increasing as ivory was acquired from hunting wild elephants (Sherman, 2018; The history & future of plastics, n.d.). Billiards was gaining substantial popularity and ivory was required for making billiards balls (Sherman, 2018). John Wesley Hyatt, treated cellulous from cotton fibre with camphor and discovered a material that could be shaped in any desired shape (Bellis, 2018; Sherman, 2018; The history & future of plastics, n.d.). Interestingly though, at the time of discovery, this plastic material was thought to have saved environment by reducing the demand for ivory from elephant and tortoise (The history & future of plastics, n.d.). However, billiard balls made from synthetic polymer were not strong enough and the discovery of this plastic material did not succeed (Bellis, 2018; Sherman, 2018).

The first fully synthetic polymer, Bakelite, was created in 1907 by Leo Baekeland (Knight, 2014). Bakelite was durable, heat resistant, could be moulded into any shape and was suited for mechanical mass production (Bellis, 2017; The history & future of plastics, n.d.) Major chemical companies started investing in research and development of new synthetic polymers after seeing the success of Hyatt and Baekeland (The history & future of plastics, n.d.).However, World War II acted as the catalyst in expansion of plastic industry in United States & United Kingdom (History of Plastics, n.d.; Knight, 2014; PHS *et al*., 2014). Nylon, invented by Wallace Carothers in 1935 as synthetic silk was used for manufacturing of parachutes, ropes, body armour and more (The history & future of plastics, n.d.). During World War II, plastic production increased by 300% (The history & future of plastics, n.d.). Hence, after the war plastic manufacturing industry turned

towards consumer products as they wanted to continue their boom (History of Plastics, n.d.; Knight, 2014).

2.2 Plastic Production

Since World War II, plastic manufacturing has been a great success worldwide, contributing greatly to national GDPs. The turnover related to plastic industry has been immense throughout the world as well. Table 2.1 highlights the turnover of plastic industry of three big plastic producers in the world and of Malaysia. Additionally, plastic industry also provides plenteous jobs to people globally. For instance, it created over 1.5 million jobs in Europe (PlasticsEurope, 2018) and about 1.75 million jobs (including suppliers) in US (Plastics, 2017) alone. Plastic is an organic polymer with high molecular weight that can be moulded into any desired shape on application of heat or pressure (Cowie, 1973). Due to this versatile characteristic of plastic, the demand has been very high for plastic in market sectors such as packaging, building and construction, automotive etc. Moreover, characteristics like durability, low cost, stability and lightweight make plastic material appealing for manufacturing a variety of commodities (Muthukumar & Veerappapillai, 2015). Due to positive impacts of plastic manufacturing industry i.e. turnovers, job creation and enticing characteristics, plastic production has been increasing steadily over the years.

Table 2.1: Turnover of plastic industries of three major producers and of Malaysia

Country/Region	Year	Turnover (US Dollars)	Reference
European Union	2016	396 billion	PlasticsEurope, 2018
United States of America	2016	418 billion	Plastics, 2017
China	2015	330 billion	Statista, 2017
Malaysia	2016	6 billion	Malaysia Petrochemical Country Report, 2017

2.2.1 Global Plastic Production

Based on available data, plastic production in 1950 was 1.5 million tonnes (Beckman, 2018). Since then the production of plastic has been exponentially increasing over the years as shown in Figure 2.1. According to one estimate, approximately 8,3000 million tonnes of virgin plastic materials namely, resins, additives, polyester fibre, polyamide fibre and acrylic fibre (PP&A fibres) have been produced until 2015 (Geyer *et al*., 2017). Following 2015, global plastic production has increased by approximately 4% in one year as 335 million tonnes of plastic was manufactured in 2016 compared to 322 million tonnes of plastic material manufactured in 2015 (PlasticsEurope, 2018). The plastic materials manufactured in 2016 included thermoplastics, polyurethanes, thermostats, adhesives, coatings, and sealants (PlasticsEurope, 2017). Thermoplastics cover a wide range of plastic material such as polypropylene (PP), polyethylene (PE), polystyrene (PS) etc. Whereas, in report by PlasticsEurope (2018) the amount of plastic fibres produced was not included in the total amount of plastic material manufactured globally. Hence the total production of synthetic polymers would be even higher. Asia manufactured half the plastic materials produced globally in 2016 (PlasticsEurope, 2017), where China was the biggest producer of plastic material, manufacturing approximately 97 million tonnes (Statista, 2018). European Union (EU), on the other hand, had produced 60 million tonnes of plastic in 2016 (PlasticsEurope, 2017).

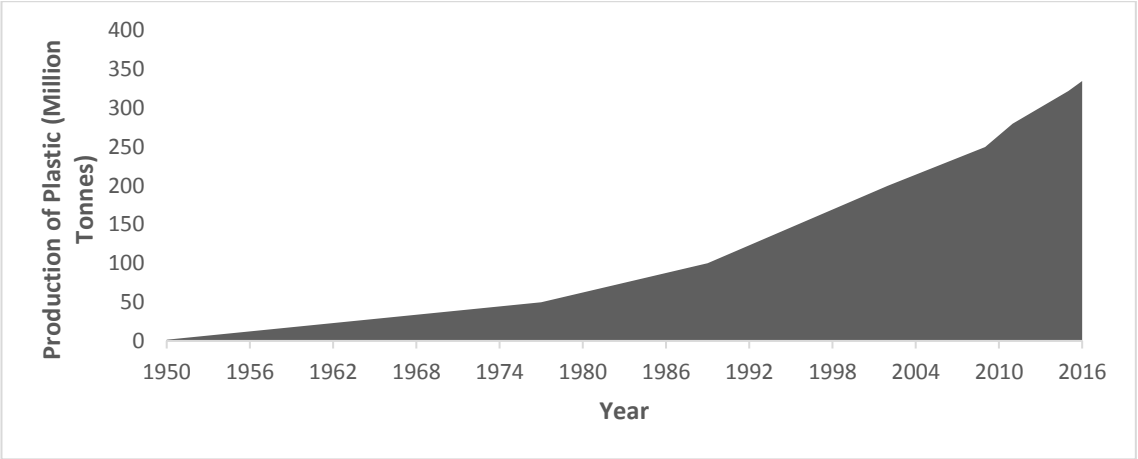


Figure 2.1: World production of plastics from 1950 – 2016 (Adapted from Beckman, 2018; PlasticsEurope, 2018)

When it comes to production of plastic for industries, packaging industry accounts for the most plastic produced for, followed by building and construction industry (Geyer *et al*., 2017). Figure 2.2 shows the percentage of plastic demand in different industries.

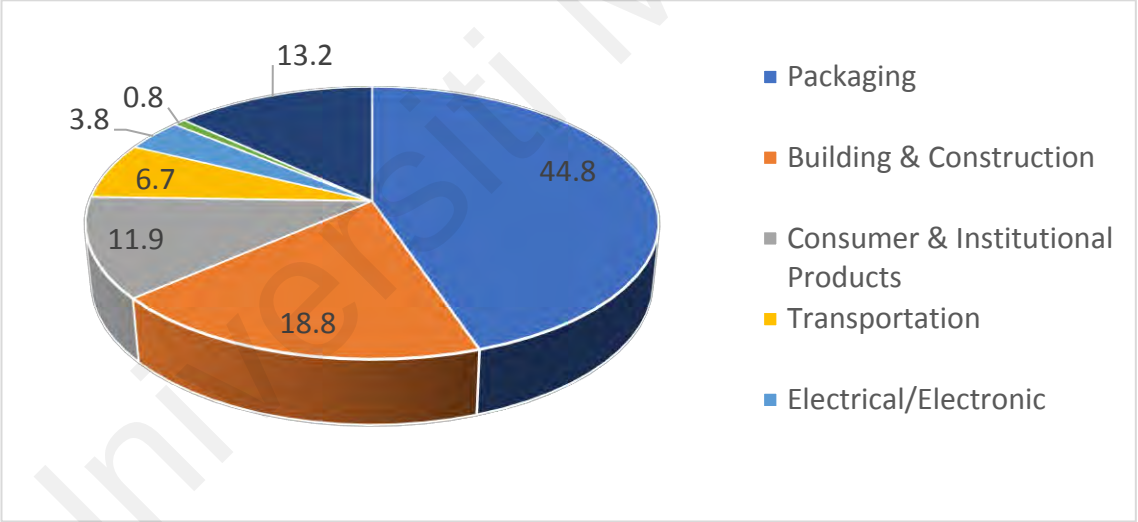


Figure 2.2: Total plastic production according to industries in Europe, US, China, and India from 2002 – 2014 (Adapted from Geyer, 2017)

2.2.2 Plastic Production in Malaysia

Malaysia produced a variety of plastic material, such as PP, PE and ethylene polyethylene terephthalate (PET) etc. as listed in Table 2.2. In 2016, Malaysia manufactured 7.7 million tonnes of plastic materials (Malaysia Petrochemical Country Report, 2017). However, Malaysia also imported 3.2 million tonnes of plastic material in 2016 (Malaysia Petrochemical Country Report, 2017). Plastic industry is expected to

grow in coming years due to the expansion of manufacturing operations by current manufacturing companies, as well as due to the establishment of new plastic manufacturing companies in the country (Malaysia Petrochemical Country Report, 2017).

Table 2.2: Quantity of each type of plastic material produced in Malaysia in 2016

Type of Plastic Material	Quantity (tonnes)
ABS	350,000
Ethylene	1,723,000
HDPE	525,000
LDPE	485,000
LLDPE	60,000
Monoethylene Glycol (MEG)	380,000
PET	666,000
Polyethylene (PE)	1,070,000
PP	373,000
Propylene, Total	1,077,000
PS	110,000
PVC	110,000
Styrene	240,000
Terephthalic Acid (PTA)	600,000
TOTAL	7,769,000

Source: Malaysia Petrochemical Country Report, 2017

2.3 Plastic Composition

Plastics are the synthetic polymers that are composed of covalently bonded long chains of repeating units of monomers (McKeen, 2013). Monomers are composed mainly of carbon and hydrogen but may also contain oxygen, nitrogen and other inorganic or organic compounds and/or elements. Thermoplastics and thermosets are two different categories of plastics which ae differentiated by their response to heat. Former softens and melts upon heating and hardens on cooling whereas later can melt upon heating but once it takes shape (after setting reaction), it can withstand heat for longer period (McKeen, 2013; Speight, 2010; Agamuthu & Omar, 2009). Both types of plastics are manufactured through a process called polymerization. Polymerization is a process in which monomer building blocks are chemically bonded to form long chains of polymers

(Chanda, 2017). In this process of polymerization, the affinity of adding more monomers is enhanced due to the presence of double bonds or active function group in a monomer. For instance, by creating favourable conditions such as optimum heat, light and/or catalyst, the chain reaction of self-addition is instigated in ethylene monomer, which results in formation of higher molecular polymer called polyethylene. The process of polymerization of polyethylene is shown in Figure 2.3. There are several methods of polymerization but two of the most commonly deployed methods are addition polymerization and condensation polymerization (McKeen, 2013). In the process of addition polymerization, new monomer units are added successively through double or triple bonds in a chain reaction (Chanda, 2017; McKeen 2013). Whereas, condensation polymerization takes place stepwise by reacting the monomer units with growing polymer chain end group and as a by-product, small molecule i.e. water is released (McKeen, 2013).

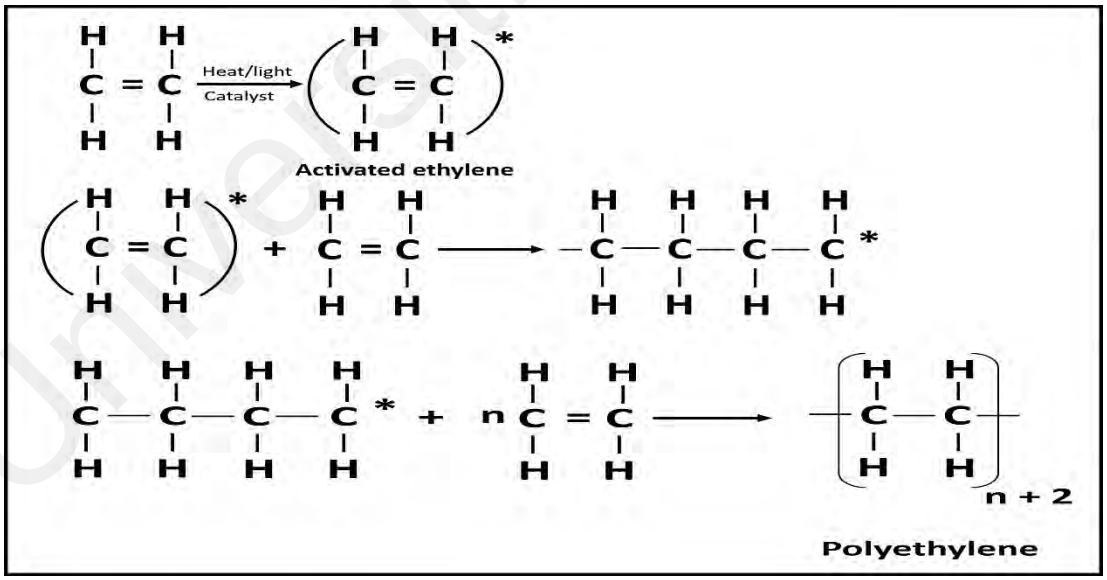


Figure 2.3: Polymerization process of polyethylene (Adapted from Chanda, 2017)

There are several important characteristics of these synthetic polymers. Permeability is one of the significant characteristics. The greater the polarity of polymers, the higher the permeability to water (McKeen, 2013). Figure 2.4 shows the polarity of common synthetic polymers.

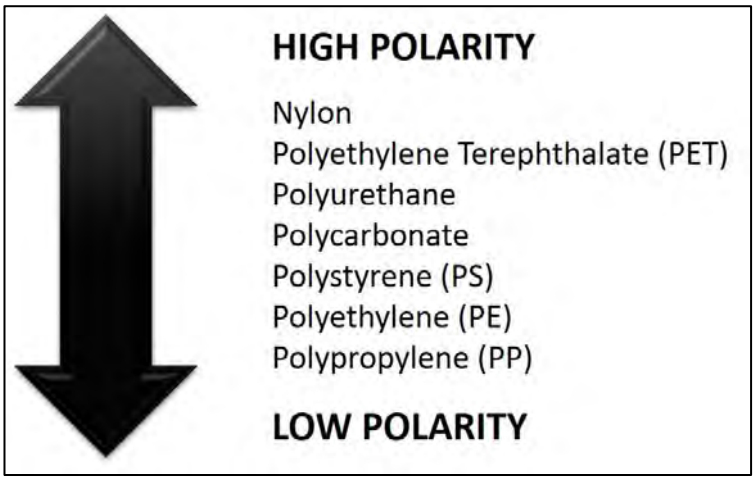


Figure 2.4: Polarity of selected plastics (Adapted from Mckeen, 2013)

The classification of plastic polymers is based on properties such as molecular weight, thermosets or thermoplastics and crystalline or amorphous (McKeen, 2013). The strengths of crystalline and amorphous resins are different, while the former has superior chemical resistance, greater stability at high temperatures and better creep resistance, later has better impact strength, less mould shrinkage and less final part warping (McKeen, 2013). The amorphous characteristics of synthetic polymers are more susceptible to biodegradation than synthetic polymers with crystalline properties (Wilkes & Aristilde, 2017). Thus, crystallinity, absence of favourable functional groups and higher molecular weight limits the biodegradation of plastic material (Devi *et al*., 2016). The higher crystalline nature (in addition to higher tensile strength and melting point) of plastic polymers are due to hydrogen bond between polymer chains (McKeen, 2013). Whereas, molecular weights of synthetic polymers can be as low as 20,000 and as high as hundreds of thousands (Chanda, 2017). Hence, molecular weight is another important property of plastics.

On the other hand, neat polymers, essentially pure compounds of monomers, rarely exist in the world as they are not suitable for production and end use (McKeen, 2013). Therefore, two or more polymers are blended together as they offer more physical properties than neat polymers (McKeen, 2013) and hence are called copolymers. Majority

of the commercial polymers are manufactured from two different polymers with addition of compatibilizing polymer also known as block of graft copolymer (McKeen, 2013).

Additives are also added to the synthetic polymers to enhance its properties, which leads to better processing and performance (Agamuthu & Omar, 2009). The type and choice of additives depend on the required properties of final product of synthetic polymers. Carbon black as an additive offers excellent colour strength, UV performance and cost-effectiveness which makes it utilization widespread in the production of thermoplastics. Carbon black has smaller particle size and higher oil absorption as compared to other commercially available pigments (McKeen, 2013). Table 2.3 enlists different additives used in the production of synthetic polymers. Addition of these additives can retard the process of biodegradation (Kolvenbach *et al*., 2014) and can be toxic to microorganisms (Arutchelvi *et al*., 2008). For instance, Dibutyl tin dilaurate, an additive present in polyurethane containing synthetic polymer can be harmful to microbes (Cregut *et al*., 2013).

Table 2.3: Types of additives and the benefits of their use in synthetic polymers

Type of Additives	Examples of Additives	Effects on Polymers
Reinforcement filler	Glass or carbon fibres	To enhance creep resistance, stiffness
Non – fibrous filler	Glass spheres, Mineral powders, Mica, Talc, Clays	Increase stiffness
Extender (Particulates or Pigments)	Carbon black, China Clay, Kaolin, Titanium dioxide	Reduce overall costs by relative less use of expansive resin or improve physical properties such as brightness, opacity, metallic appearance
Platelet		Impart colour, lustre, metallic appearance, pearlescent effect
Impact Modifiers / Tougheners	ethylene propylene diene monomer rubber (EPDM rubber), Ionomers	Improve impact resistance and flexibility
Plasticizers	Phthalates	Maintain flexibility of plastics

Source: McKeen, 2013

Lastly, there is a wide range of plastic material being produced nowadays and some are assigned with resin codes. Table 2.4 shows the polymer composition and resin code assigned to it.

Table 2.4: Resin codes assigned to different composition of plastic

Polymer Name	Resin Code
Polyethylene terephthalate (PET)	1
High Density Polyethylene (HDPE)	2
Polyvinyl Chloride (PVC)	3
Low Density Polyethylene (LDPE)	4
Polypropylene (PP)	5
Polystyrene (PS)	6

Therefore, it is highlighted that plastic composition and the structural arrangement play a significant role in biodegradation of synthetic polymers (Wilkes & Aristilde, 2017; Tribedi *et al*., 2015). Consequently, it is also important to understand the structures and composition of synthetic polymers, namely High-density-polyethylene (HDPE), PP and PS to understand their biodegradability potentials.

2.3.1 High-Density-Polyethylene

HDPE is a type of polyethylene that has less or no branches of ethylene. The molecules of HDPE are stacked and thus the intermolecular forces are stronger (Wilkes & Aristilde, 2017). The structure of HDPE is manifested in Figure 2.5. HDPE is produced through addition polymerization process (McKeen, 2013) and it is highly hydrophobic (Wilkes & Aristilde, 2017) and therefore it is usually considered inert. However, Satlewal *et al.* (2008) proposed that due to compact nature of HDPE and consequently greater cross-linking and higher carbon content offers enhanced sites to microbes to attack to utilize HDPE as source of carbon and energy.

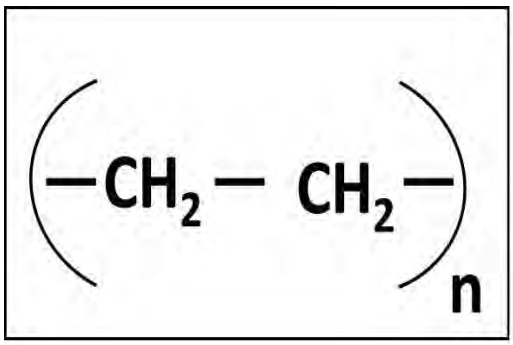


Figure 2.5: Structure of High-Density-Polyethylene

2.3.2 Polypropylene

PP is composed of repetitive methylene units without an active functional group (Arkatkar *et al*., 2009). This attribute, in addition to higher molecular weight and subsequent high hydrophobicity make PP recalcitrant to biodegradation and chemical abrasion (Arkatkar *et al*., 2009; Shah *et al*., 2008). This inertness of PP is associated with the absence of "weak zones" of polymers where microbes can attack; carbon – oxygen bonds (C=O, C–OR, C–OH) (Motta *et al*., 2009). The structure of PP is shown in Figure 2.6.

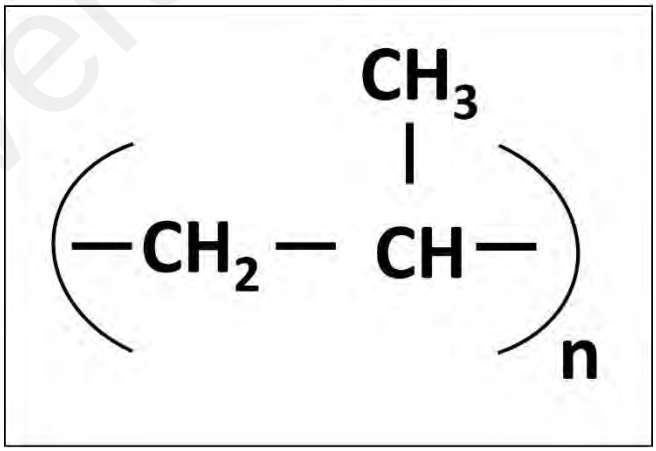


Figure 2.6: Structure of Polypropylene

2.3.3 Polystyrene

The distinctive feature of PS is the presence of aromatic ring/phenyl group in form of pendants (Wilkes & Aristilde, 2017; Atiq *et al*., 2010). The structure of PS is demonstrated in Figure 2.7. PS is manufactured through addition polymerization process

(McKeen, 2013). The properties that make the use of PS common are thermal insulation, stiffness and lightweight. Upon chemical or thermal degradation of PS, toluene, benzene, styrene and acrolein are released as by-products (Muthukumar &Veerappapillai, 2015).

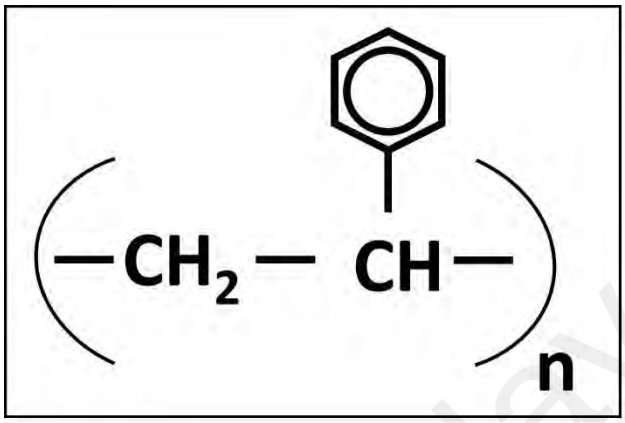


Figure 2.7: Structure of Polystyrene

2.4 Municipal Solid Waste Management

After the end of intended use of plastic commodities, they are discarded as waste and such plastic waste is a major component of Municipal Solid Waste (MSW). Hence, generation and subsequent management of MSW is discussed here

2.4.1 Global Municipal Solid Waste Management

Globally 0.74 kg of MSW per capita per day was generated in a world populated with approximately 7.4 billion people (World Bank, 2018), which led to total generation of 2.01 billion tonnes of MSW in 2016 (Kaza & Yao, 2018). The factors related to MSW generation are mainly population growth, affluence and urbanization (Kaza *et al*., 2018). Due to growing population, national economies and urbanization, the amount of MSW and plastic waste is also expected to increase.

The composition of globally generated MSW (2.01 billion tonnes in 2016) is mainly constituted by food and green waste which is 44%. Organic fraction of MSW is followed by paper and cardboard waste which is 17%. While this 61% of MSW is biodegradable and is recyclable, 12% of MSW is composed of plastic waste which is notoriously known

due to its recalcitrant characteristic. Table 2.5 lists the quantity of various waste streams of MSW. The composition of MSW varies with income level. High organic fraction is found in MSW generated by low-income and lower-middle-income countries (Kaza & Yao, 2018; Agamuthu, 2001). Food and green waste percentage decreases as income level increases whereas, in higher income level countries the number of dry recyclables also increases. The generation of greater amount of plastic and paper waste is linked with increase in affluence (Kamran *et al*., 2015). Therefore, countries striving towards economic boost tend to generate more plastic waste such as Malaysia (Latifah *et al*. 2009).

Table 2.5: Composition of MSW (Adapted from Kaza *et al*., 2018)

Type of Waste	Quantity (Million Tonnes)	Percentage
Food and green	884	44
Paper and cardboard	342	17
Other	281	14
Plastic	242	12
Glass	100	5
Metal	80	4
Rubber and leather	40	2
Wood	40	2
Total	2010	100

Throughout the world, 37% of MSW is disposed of at landfills, where 8% are sanitary landfills with landfill gas collection system, 33% of MSW is disposed of in open dumps, 19% of waste is recycled and composted and remaining 11% is incinerated (Kaza *et al*., 2018). While a little more than half of MSW is disposed of at landfills or open dumps globally, landfilling or open dumping is almost completely preferred over other waste management techniques in low-income countries (Kaza *et al*., 2018). In addition to high landfilling and open dumping rates, collection is a significant issue in developing countries. According to an estimate, solid waste is not collected from approximately 2 billion people globally (Modak *et al*., 2015). Similarly, Jambeck *et al.* (2015) evaluated mismanagement of 68% of generated MSW in developing countries. Figure 2.8 shows the global collection rates of MSW. This mismanagement of MSW results in

mismanagement of plastic waste as well. Thus, plastic waste escapes into the environment especially lacustrine, fluvial and marine environment, contributing to the generation of marine debris (Jambeck *et al*., 2015).

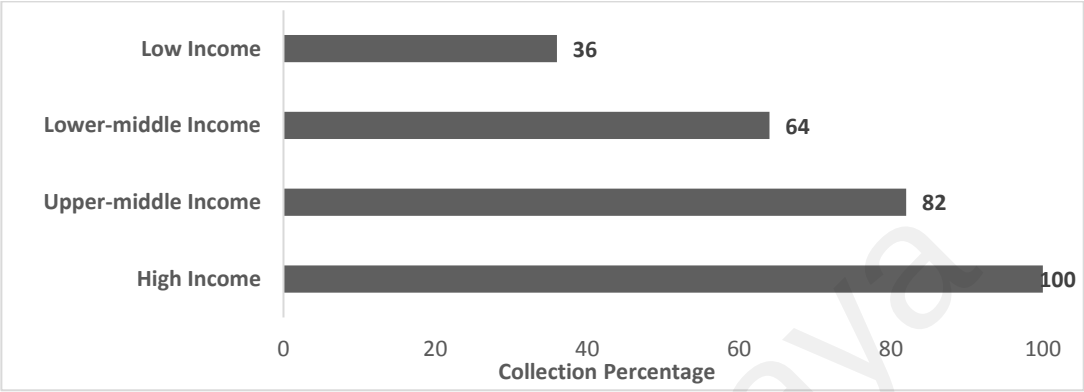


Figure 2.8: Collection rate of MSW income wise (Adapted from Kaza *et al*., 2018)

2.4.2 Municipal Solid Waste Management in Malaysia

Malaysia generated approximately 13 million tonnes (12,982,685 million tonnes) of MSW in 2014 (Agamuthu, 2017; Kaza *et al*.,2018), where per capita MSW generation was 1.21 kg/capita/day (Kaza *et al*., 2018). Moreover, landfilling is the most deployed technology in Malaysia as MSW sent to landfills accounts for 81.5% whereas only 17.5% is recycled (Agamuthu, 2017).

On the other hand, the composition of MSW generated in Malaysia is shown in Figure 2.9. Approximately 44.5% of food waste is mixed with 25% of dry recyclables (plastic, paper, glass) which makes it economically infeasible for recycling, especially recycling of plastic waste. Moreover, composting is not feasible as food waste and organic waste gets contaminated with 1.3% household hazardous waste such as e-waste. (Agamuthu, 2017). This comingling of MSW is one of the reasons of high landfilling rate in Malaysia.

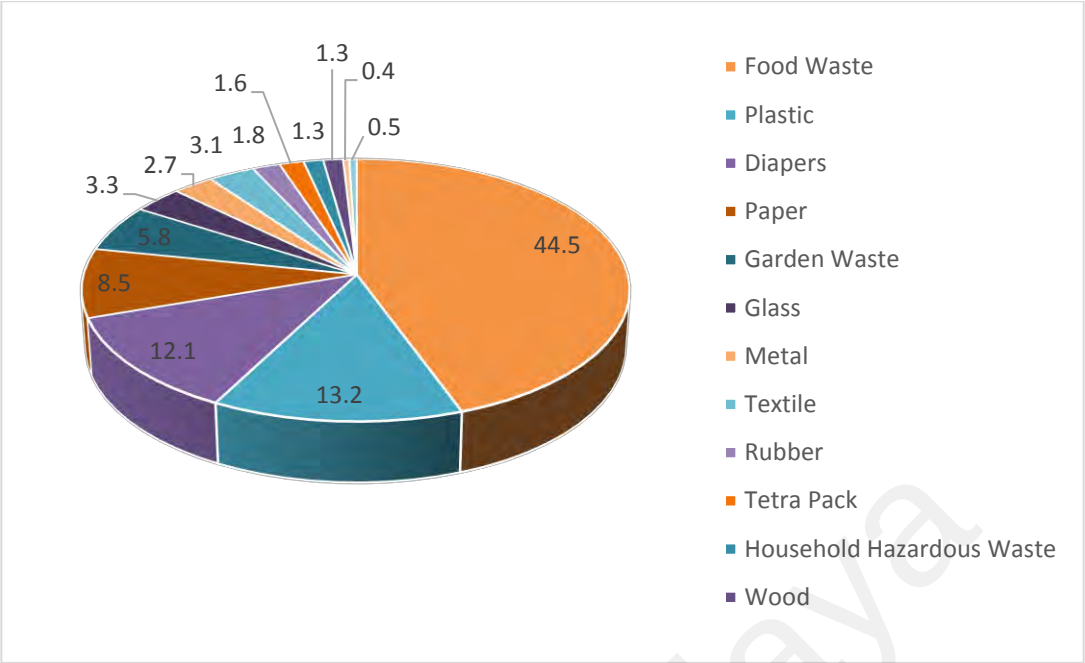


Figure 2.9: Composition of MSW in Malaysia (Adapted from Agamuthu, 2017)

Now that it has been established that plastic waste is one of the major components of MSW, the current practices of plastic waste management are discussed in more detail in following section.

2.5 Plastic Waste Management

2.5.1 Global Plastic Waste Management

The amount of plastic waste generated worldwide is astoundingly high and UNEP (2018) elucidated in their report that plastic waste generation has been increasing for last 60 years. The persistence of throwaway culture in majority of the countries is one reason of continuous increase in generation of plastic waste (UNEP, 2018). Geyer *et al.* (2017) estimated that since 1950 approximately 6,300 million tonnes of plastic waste had been generated globally and global plastic waste generation in 2015 was 300 million tonnes. When it comes to composition of plastic waste, approximately half of total plastic waste generated globally is plastic packaging waste (UNEP, 2018). It should not come as surprise since the largest sector of plastic manufacturing is packaging industry (30%) (UNEP, 2018). In EU, approximately 60% of plastic waste is packaging waste

(PlasticsEurope, 2018). Figure 2.10 displays the increasing plastic waste generation by sectors.

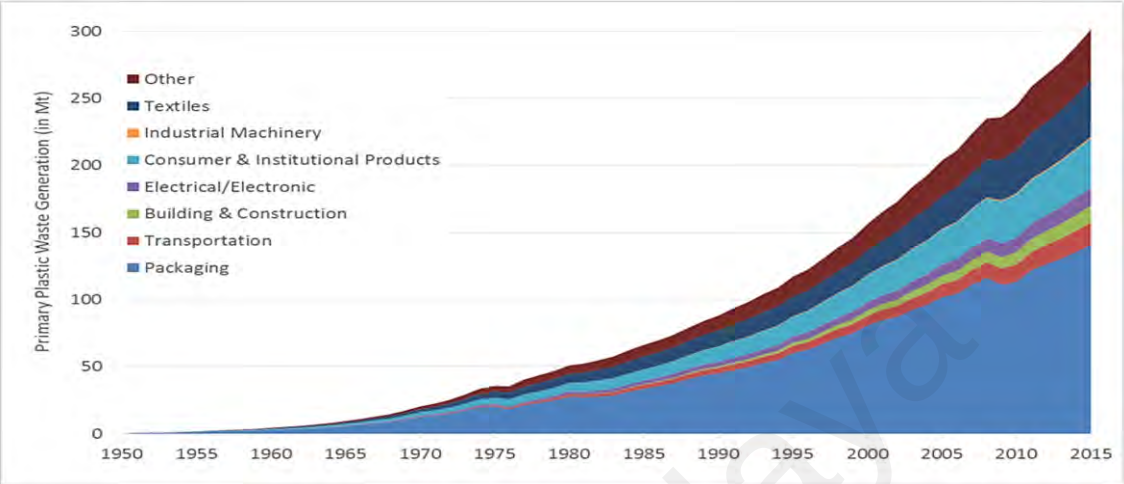


Figure 2.10: Generation of plastic waste from industries (Geyer *et al*., 2017)

Plastic waste generated in EU is either recycled or sent for energy recovery through incineration. For the first time in approximately 10 years, the rate of landfilling has fallen below recycling (PlasticsEurope, 2018). Approximately a total of 27.1 million tonnes of plastic waste was collected in 2016 from EU member states, Switzerland and Norway, of which 27.3% was sent to landfills for final disposal, 31.1% was recycled and 41.6% was incinerated for energy recovery (PlasticsEurope, 2018). In total, EU, Switzerland and Norway diverted approximately 73% of plastic waste from landfills. This scenario is in stark contrast to developing countries, where plastic waste mixed with other fractions of MSW such as organic waste, paper waste, metal, E-waste etc is mainly disposed of in landfills. While, EU plastic waste management on average looks sustainable, the actual practice is far from it. Even in EU, landfilling rate of plastic waste is uneven and many countries prefer landfilling as the final destination for plastic waste (PlasticsEurope, 2018). However, the factor that strikes out in determining management of plastic waste in the EU is the landfill restrictions for recyclables. Out of 28 member states of EU, Switzerland and Norway, only 10 countries have landfill restrictions. Consequently, recycling and energy recovery rate of plastic waste in these countries is 90 – 100%.

Among these 10 countries, Switzerland, Austria, Germany, Netherlands and Sweden have approximately 100% of recycling and energy recovery rate from plastic waste (PlasticsEurope, 2018).

In contrast to above mentioned scenario, according to Geyer *et al.* (2017), only 9% of plastic waste has been recycled globally until 2015. It shows that management of plastic waste is ambiguous to an extent. For instance, majority of developed countries exported plastic waste to China up until 2018 instead of managing it in the country of generation (Parker, 2018). Similarly, approximately half of plastic waste collected (29.7%) in EU for recycling was exported to China and Hong Kong in 2014, whereas, United States exported 2.1 million tonnes of plastic waste to China in 2013 (QDB, 2017).

On the other hand, developing countries mainly dispose plastic waste along with other waste streams of MSW in landfills and/or open dumps. However, informal sector contributes to national recycling rates as they segregate and collect plastic waste from dumps or landfills for their livelihood. For instance, in India recycling rate of PET bottles is 70% due to informal sector (Aryan *et al*., 2019). Whereas, informal sector in Beijing, China, collect more than 50% of plastic bottles and other dry recyclables (Liu *et al*., 2015). Management of plastic waste in Africa is poor as plastic is usually not collected and if plastic waste is collected, it is disposed of in open dumps (Oyake-Ombis *et al*., 2015).

2.5.2 Plastic Waste Management in Malaysia

Plastic waste generation in Malaysia was approximately 1.8 million tonnes in 2016 (Pauze, 2016). According to Bedi (2018), only 15% of plastic waste was recycled in 2016. The plastic waste that reached recycling industries in Malaysia, approximately 90% of it was recycled into plastic resins. Recycled plastic resins were then sold to plastic commodity manufacturing industries such as stadium seats, office furniture, motor cycle

parts, fruit baskets etc. (Bedi, 2018). Malaysian recycling industry has a turnover of RM 4.5 billion and it provides jobs to 13,000 people in the country (Bedi, 2018).

Malaysians, predominately generate highly mixed MSW. While Solid Waste Management and Cleansing Act 2007 (Act 627) requires Malaysian citizens to segregate their MSW, it fails to induce a significant change throughout the country due to its implementation in only six states and even then, it is only applicable to landed houses. The number of high-rise residential buildings are increasing in comparison to landed houses and yet source separation has not been implemented on high-rise residential buildings. Due to unsegregated MSW, plastic waste becomes highly contaminated which reduces its economic value as it would require more labour, energy and time to recycle it. Moreover, low volume and low quantity of plastic waste generated in Malaysia also make it economically infeasible for plastic recyclers to collect plastic waste frequently.

Therefore, 80 – 90% recycling industries depend on imported plastic waste (Bedi, 2018). Since China banned the import of plastic waste at the start of 2018, world's biggest exporters of plastic waste have started sending their plastic waste to Southeast Asian countries like Malaysia, Thailand, and Indonesia (Parker, 2018). Approximately 19 countries including US, Japan, Britain, Germany, Belgium, France, Spain, and Estonia have been exporting their plastic waste to Malaysia (Chu, 2018). Just within seven months, January to July 2018, 754,000 tonnes of plastic waste were imported to Malaysia from these countries (Greenpeace International, 2018). While Malaysian plastic recycling industries did not have the capacity to recycle this huge amount of imported plastic, plastic waste was then passed on to illegal recyclers (Greenpeace International, 2018). Illegal recyclers are those recycling plants that do not have required permit by the Government of Malaysia. These illegal recyclers have been reported to burn plastic waste openly or dump it in abandoned buildings, fishing ponds or in open ground (The Straits

Times, 2018; Greenpeace International, 2018). Greenpeace International, a Malaysian NGO, published a report on plastic management in Malaysia that showed images of inefficient management of plastic waste i.e. open dumping (Figure 2.11) and open burning (Figure 2.12).

Figure 2.11: Open dumping of plastic waste in aquaculture pond (Greenpeace, 2018)

Figure 2.12: Open burning of plastic waste in Malaysia (Greenpeace, 2018)

In addition to open dumping of plastic waste by illegal recyclers, littering is also another issue in Malaysia (Malaysia Today, 2018). Plate 2.1 depicts the plastic littering

observed in Matang mangrove in Malaysia. Moreover, it is estimated that Malaysia releases approximately 30,000 tonnes of plastic waste into oceans yearly (The Star, 2018).

Plate 2.1: Plastic litter observed in Matang mangrove, Pahang during sampling

This opening dumping and littering of plastic waste into environment lead to generation of marine plastic debris. Therefore, marine plastic debris is elucidated in the following section.

2.6 Marine Plastic Debris

Marine debris or Marine litter is any type of waste that is generated on land or at sea by human activities but accumulates in the aquatic environment such as in lakes, rivers, seas and oceans (Lohr *et al*., 2017). Generally, marine debris consists of varying sizes such as macro (> 25 mm), micro (5 mm – 0.01 mm) and nano (>0.01 mm) size (Agamthu, 2018). However, this section discusses marine debris of sizes greater than 5 mm.

Plastic is the major composition of marine debris (UNEP, 2016; Galgani *et al*., 2015) and usually is referred as marine plastic debris. Other constituents of marine debris are

glass, wood, metal and paper (Agamuthu *et al*., 2018). Figure 2.12 shows the composition of marine debris found in European seas. Plastic is the main component of marine debris as even majority of derelict fishing gear i.e. nylon nets, fishing lines etc., are made up of plastic material. Thus, in conclusion the percentage of plastic in marine debris can be more than 70%.

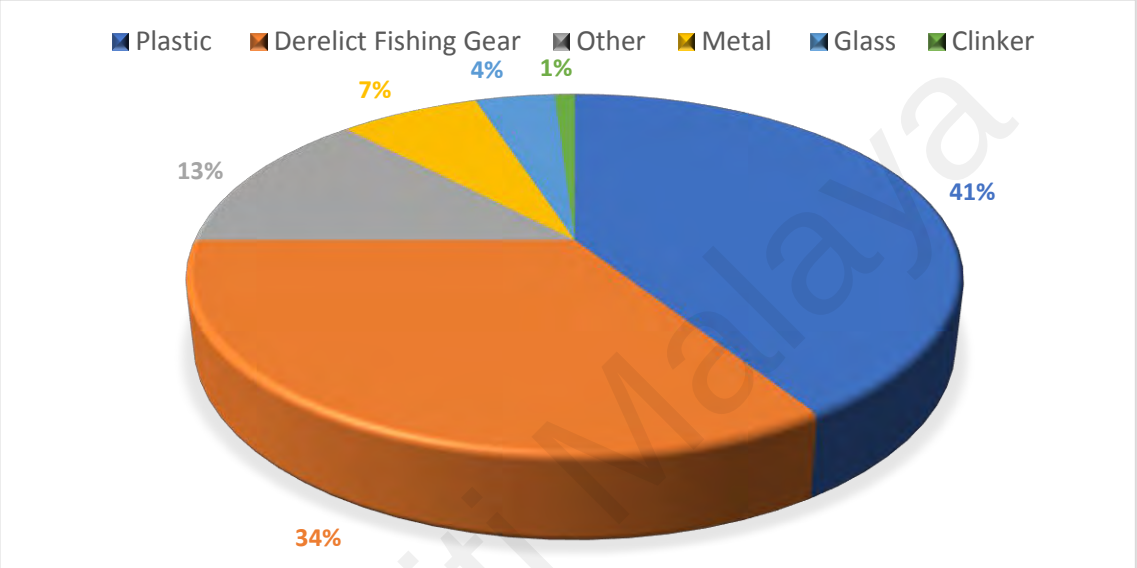


Figure 2.13: Composition of marine debris (Adapted from Pham *et al*., 2014)

It is estimated that 80% of marine debris comes from land whereas 20% of marine debris originates from sea (Agamuthu, 2018). Since plastic is also part of marine litter it is safe to assume that majority of plastic waste also originates from land-based source (Galgani *et al*., 2015). Majority of this plastic ends up in oceans due to mismanagement (Jambeck *et al*., 2015; Galgani *et al*., 2015). Jambeck *et al*. (2015) estimated that 31.9 out of 99.5 million tonnes of plastic waste was mismanaged in coastal areas of the world and approximately 4.8 to 12.7 million tonnes of mismanaged plastic waste ended up as marine plastic litter in 2010 alone. Another source of marine plastic debris is ghost or abandoned fishing gear. UNEP assessment revealed that 640,000 tonnes of abandoned fishing equipment enter the world oceans every year (UNEP, 2016).

For example, buoys made up of expanded polystyrene (EPS) are extensively used in South Korea and it is common practice in South Korea to abandon these buoys once their intended use is over (Kim *et al*., 2015). Marine plastic litter breaks down into smaller fragments due to mechanical abrasion, photodegradation and oxidation (Barnes *et al*., 2009). Hence, continued fragmentation of marine plastic litter leads to production of microplastics. The abundance of microplastics will be discussed in detail in the following sections.

2.7 Abundance of Microplastics

Microplastics are plastic particles that are less than 5 mm in size (Anderson *et al*., 2016). They are introduced into the environment either directly; in form of microbeads (Rochman *et al*., 2015), and industrial abrasives (Cole *et al*., 2011) or indirectly; by accidental spillage of virgin plastic pellets (Veerasingam *et al*., 2016), and by degradation of littered or discarded plastic debris (Andrady, 2011). Microbeads, industrial abrasives and pellets are the microplastics that are produced for intended purposes and hence are called primary microplastics (Auta *et al*., 2017). For instance, microbeads are present in cosmetics (as exfoliants), in toiletries and in detergents that are intended for domestic usage (Napper *et al*., 2015; Rochman *et al*., 2015). Pellets are utilized as raw material in manufacturing plastic commodities (Veerasingam *et al*., 2016). On the other hand, abrasives are primary microplastics that are used in industries for blast cleaning or paint removal (Auta *et al*., 2017; Cole *et al*., 2011). Chemical or physical degradation due to photo-oxidation and/or wave action in the environment (Barnes *et al*., 2009) breaks down plastic litter into particles that are smaller than 5 mm in size and these are referred as secondary microplastics (Andrady, 2011).

The source of generation of microplastics in the environment is mostly associated with anthropogenic activities (Fauziah *et al*., 2018). Table 2.6 enlists the type of human activities that result in the generation of microplastics. Additionally, unintentional or

accidental release of primary microplastics, microbeads or pellets, from wastewater treatment plants or ships transporting pellets can also contribute to microplastic pollution (Gallagher *et al*., 2016; Veerasingam *et al*., 2016). Washing of synthetic fabrics also releases fibres that escape the wastewater treatment plants and enter the oceans (Browne *et al*., 2011).

Table 2.6: Summary of activities that generate microplastics

Type of Activity	Plastic Litter	Source
Tourism	Food wrappers, plastic bottles, and plastic detergent containers,	Mohamed Nor & Obbard, 2014 Stolte *et al*., 2015 Kim *et al.,* 2015
Fishery Activities	Fishing nets, Buoys, Fishing lines	
Wastewater Treatment Plant	Primary Microplastics (microbeads)	Lima *et al*., 2015 Cincinelli *et al*., 2017
Maritime Transport	Primary Microplastics (pellets)	Gallagher *et al*., 2016 Veerasingam *et al.,* 2016
Road Surface Markings	Thermoplastic composite paints	Horton *et al.*, 2017

2.7.1 Global Abundance

It has been established that microplastics mainly exist in the environment due to the mismanagement of plastic waste. Fragmentation of plastic waste can also occur on land, leading to abundance of microplastics in sediments (Mohamed Nor & Obbard, 2014). In addition to the source, the circulation and subsequent abundance of microplastics is governed by environmental factors (Imhof *et al*., 2017; Kim *et al*., 2015; Veerasingam *et al*., 2016). Wave currents, tides, cyclones, wind directions and river hydrodynamics, define the distribution and redistribution of microplastics in the marine environment (Besseling *et al*., 2017; Liubartseva *et al*., 2016; Kim *et al*., 2015; Sadri & Thompson, 2014; Thiel *et al*., 2013; Kukulka *et al*., 2012; Browne *et al*., 2010). Tables 2.7 and 2.8 enlist the concentrations of microplastics found in sediments and in aquatic environment, respectively. It must be noted that there may be multiple research findings for microplastic concentrations in one city or in one country (Fauziah *et al*., 2018), or the reporting units and methodology may vary (Hidalgo-Ruz *et al*., 2012) but elucidating

such details is beyond the scope of this thesis. Therefore, the listed concentrations of microplastics is to emphasize the universal abundance of microplastics.

Table 2.7: Microplastics concentration in sediments from different countries around the world

Location	Concentration	Source
Nakdong River Estuary, South Korea	27,606 items/m^2	Lee *et al*. 2013
Goa, India	3000 pellets	Veerasingam *et al*., 2016
Singapore	12.0–62.7 items/kg	Mohamed Nor & Obbard 2014
Beijing, China	544 ± 107 items/kg	Wang *et al*. 2017
Coral Island, Maldives	647 ± 720 items/m^2	Imhof *et al*. 2017
Pearl River, Hong Kong	5595 items/m^2	Fok & Cheung, 2015
Waimushan Beach, Taiwan	508 items/0.0125 m^3	Kunz *et al*. 2016
Bostanu, Iran	1258 ± 291 items/kg	Naji *et al*. 2017
Lido di Dante, Italy	1512 ± 187 items/kg	Lots *et al*. 2017
Barcelona, Spain	148 ± 23 items/kg	Lots *et al*. 2017
Cassis, France	124 ± 36 items/kg	Lots *et al*. 2017
Dikili, Turkey	248 ± 47 items/kg	Lots *et al*. 2017
Pilion, Greece	232 ± 93 items/kg	Lots *et al*. 2017
Vik, Iceland	792 ± 128 items/kg	Lots *et al*. 2017
Porto, Portugal	140 ± 26 items/kg	Lots *et al*. 2017
Rhine river, Germany	228–3763 items/kg	Klein *et al*., 2015
River Thames Basin, United Kingdom	660 items/kg	Horton *et al*. 2017
Hawai'i, Untied States of America	1774 items/m^2	Young & Elliot, 2016
South Africa	340.7–4757 items/m^2	Nel & Froneman, 2015
Ontario Lake, Canada	980 items/kg	Ballent *et al*. 2016
Boa Viagem, Brazil	310 items/kg	Costa *et al*. 2010

Table 2.8: Microplastics concentration in water surface from different countries around the world

Location	Concentration	Source
Nakdong River Estuary, South Korea	210 to 15,560 items/m^3	Kang *et al*. 2015
Guangdong Province, China	6701.375 items/m^3	Fok *et al*. 2017
Japanese Sea, Japan	1.72 million items/km^2	Isobe *et al*. 2015
Bay of Bengal, India	Few hundreds to 20,000	Eriksen *et al*. 2017
Hong Kong	51–27,909 items/100 m^3	Tsang *et al*. 2016
Mongolia	20,264 items/km^2	Free *et al*. 2014
Mediterranean Sea	130,000 items/km^2	Faure *et al*. 2015
Algarve, Portugal	6980 items/m^3	Frias *et al*. 2016
Southampton water, United Kingdom	960 items/m^3	Gallagher *et al*.2016
Lake Bolsena, Italy	0.82 to 4.42 items/m^3	Fischer *et al.* 2016
Danube River, Austria	0.3168 ± 4.6646 items/m^3	Lechner *et al*. 2014
Ukaleqarteq, Greenland	2.38 items/m^3	Amelineau *et al*. 2016
Canada	8–9200 items/m^3	Desforges *et al*. 2014
Illinois, United States of America	6,698,264 items/m^2	McCormick *et al*. 2014
Arctic Ocean	38–234 items/m^3	Obbard *et al*. 2014
Antarctica	0.17 ± 0.34 items/m^3	Cincinelli *et al*., 2017
South Africa	204.5 to 1491.7 items/m^3	Nel & Froneman, 2015

2.7.2 Microplastics Abundance in Malaysia

The number of research studies on abundance of microplastics in Malaysia is increasing. These findings provide baseline data on concentration of microplastics (Khalik *et al*., 2018) at the moment. Thus, highlighting the need for more research. Table 2.9 highlights the concentration of microplastics reported in sediments and in aquatic environment. Ingestion of microplastics by bivalves *Scapharca cornea* (Ibrahim *et al*., 2016) and fish *Lates calcarifer* (Ibrahim *et al*., 2017) have been reported, further highlighting the existence of microplastics in Malaysia. In another study it was estimated that 0.19 trillion microbeads are released every year, due to usage of personal care products with exfoliants, in Malaysia (Praveena *et al*., 2018). The abundance of marine

plastic litter has been reported in various research findings which can only worsen the situation of microplastics pollution in Malaysia (Khairunnisa *et al*., 2012; Agamuthu *et al*., 2012) due to subsequent fragmentation.

Table 2.9: Concentration of microplastics in Malaysia

Location	Concentration	Source
Port Dickson, Negeri Sembilan **Kuala Terengganu, Terengganu** **Kota Kinabalu, Sabah**	687 pieces	Fauziah *et al*., 2015
Matang Mangrove, Perak	65 particles/kg	Norkhairah, 2018
Kukup Mangrove, Johor	38 particles/kg	Norkhairah, 2018
Serkam Mangrove, Melacca	43 particles/kg	Norkhairah, 2018
Sedili Besar Mangrove, Johor	53 particles/kg	Norkhairah, 2018
Cherating Mangrove, Pahang	26 particles/kg	Norkhairah, 2018
Semerak Mangrove, Kelantan	30 particles/kg	Norkhairah, 2018
Kuala Nerus, Terengganu	0.13 – 0.69 pieces/litre	Khalik *et al*., 2018
Kuantan Port, Pahang	0.14 – 0.15 pieces/litre	Khalik *et al*., 2018
Sepetang River, Perak	101.39 particles/kg (sediments) $6.2x10^{-3}$ particles/m^3 (surface water)	Norkhairiyah, 2018
Serkam River, Melacca	31.88 particles/kg (sediments) $2.8x10^{-3}$ particles/m^3 (surface water)	Norkhairiyah, 2018
Ayer Masin River, Johor	42.92 particles/kg (sediments) $1.0x10^{-2}$ particles/m^3 (surface water)	Norkhairiyah, 2018
Sedili Besar River, Johor	32.36 particles/kg (sediments) $3.2x10^{-3}$ particles/m^3 (surface water)	Norkhairiyah, 2018
Cherating River, Pahang	32.15 particles/kg (sediments) $3.8x10^{-3}$ particles/m^3 (surface water)	Norkhairiyah, 2018
Semerak River, Kelantan	22.64 particles/kg (sediments) $9.7x10^{-3}$ particles/m^3 (surface water)	Norkhairiyah, 2018
Semanta mangrove, Klang	418 particles/m^2 (sediments)	Jayanthi *et al*., 2014

2.7.3 Microplastics in the Environment

Microplastics behave differently depending on the type of residing environment. Microplastics in aquatic environment commonly float on the surface of water due to low

density. However, biofouling can increase their density which could result in partial sinking or settlement at the bottom of sea (Andrady, 2017). Moreover, microplastics can also be ingested by marine mammals (Lusher *et al*., 2015), seabirds (Tanaka *et al*., 2013), zooplanktons (Ferreira *et al*., 2016) and invertebrates (Goldstein & Goodwin, 2013). On the other hand, microplastics or plastic litter also experience photooxidative and thermal degradation. However, photooxidative and thermal degradation is higher on land (Andrady, 2011) than that in aquatic environment (Halle *et al*., 2016), which leads to the generation to more microplastics.

Table 2.10 summarizes the behaviour of microplastics in the environment. In addition to density that determines the buoyancy of microplastics, crystallinity also impacts buoyancy. It must be noted that higher crystallinity leads to higher density in microplastics (Andrady, 2017).

Table 2.10: Summary of environmental impacts on characteristics of microplastics

Characteristics	Related Properties	Description
Buoyancy	Density	Determines the buoyancy and hence where microplastics will end up within water column or in deep sea sediments
Weathering	Partial Crystallinity	Determine the oxidation and further degradation of microplastics; lower crystallinity will be weathered faster
Biofouling	Surface Properties	Surface energy determines the rate of fouling; higher the surface area, faster the biofouling
Toxins Sorption	Crystallinity, Surface properties	Lower crystallinity often leads to higher loading of POPs

Source Andrady, 2017: Fazey & Ryan, 2016; Andrady, 2011

2.8 Degradation of Plastic / Microplastic

The term degradation is often tossed for changes in physical and/or chemical properties of synthetic polymers due to abiotic factors (Kale *et al*., 2015; Shah *et al*., 2008). The abiotic factor can include light, heat, moisture, and chemical conditions (Kale *et al*., 2015). On the other hand, when microbes consume plastic material for energy and carbon

source, it is referred as biodegradation (Kale *et al*., 2015; Muthukumar & Veerappapillai, 2015). However, some authors use the term degradation for both abiotic and biotic factors that alter the physical and/or chemical characteristics of plastics. Hence, the term biodegradation and degradation can be interchangeable. Nevertheless, in this dissertation, "degradation" is referred to changes in plastic properties due to abiotic factors only whereas, "biodegradation" is referred to changes in properties of plastic due to microbes (biotic factors). Muthukumar and Veerappapillai (2015) described the types of degradation that macro-plastic can undergo which include chemical, thermal and photo degradation.

The decomposition of plastic takes longer than the decomposition of other waste material such as organic waste, paper and cardboard etc. and that is why plastic is persistent in the natural environment. It can take as long as 1000 years for plastic bags to be decomposed completely (Kale *et al*., 2015). Without degradation of plastic polymers, the decomposition can take even longer (Wilkes & Aristilde, 2017). Thus, highlighting the importance of degradation. Polyolefins are commonly recalcitrant to the acidic and basic treatment. But polyolefins were reported to be oxidized by concentrated sulphuric acid (Neu, 1996). Similarly, other highly concentrated acids such as sulphuric, chromic and nitric acids have also shown capabilities of oxidization of PP (Arkatkar *et al*., 2010). Albertsson *et al.* (1987) reported that synergistic interaction exists between degradation (photo-oxidation) and biodegradation of polyethylene. Thus, pre-treatment strategies before subjecting the polymer to biodegradation can be effective. Other factors that may affect the biodegradation or degradation of synthetic polymers are molecular composition, physical form i.e. films, powder, fibres and pellets and structure of synthetic polymers such as branching or linearity, type of bonds etc. (Kale *et al*., 2015).

Degradation of plastics results in cracking, erosion, crazing, discoloration, delamination or phase separation (Shah *et al*., 2008) as well as, bond scission, formation of new functional groups and chemical transformation (Pospisil & Nespurek, 1997). Additionally, degradation of plastics can reduce the molecular weight. But still extremely weathered plastic can have relatively higher average molecular weight (tens of thousands g/mol) (Andrady, 2011). There are several types of degradation depending on the active agent.

2.8.1 Photodegradation

Photodegradation occurs when plastic material is exposed to ultraviolet (UV) radiation in the form of sunlight. The absorption of UV-A (~315 – 400 nm) and UV-B (~295 – 315 nm) radiation by synthetic polymers instigates its degradation, which is also known as photolysis (Shah *et al*., 2008). Moreover, degradation is further increased due to the heat of sunlight (Resmeriță *et al*., 2018). Therefore, plastic material present in sandy beaches or on land experiences faster photo-oxidation than those plastic in the ocean or aquatic ecosystem where temperatures are relatively cooler (Andrady, 2015). The most damage is caused by UV-A region between 320 – 340 nm which usually leads to yellowing (Andrady, 2015). Additionally, cracking can occur by the induction of mechanical stress from the existence of heated and non-heated surfaces/patches within a plastic material (Resmeriță *et al*., 2018). Photodegradation is often preceded by oxo-degradation.

Figure 2.14 shows the photooxidation of PE and PP where absorption of UV radiation leads to yellowing of plastic, oxidation, chain scission and chain relinking. During photooxidation, amorphous part of polymer is degraded first which leads to increase in relative crystallinity as only crystalline part of polymer is left, after degradation of amorphous part. (Andrady, 2017). Hence, oxidation occurs at this stage of

degradation/weathering and results in generation of ketones, aldehydes, carboxylic acids, alcohol groups and hydroperoxides

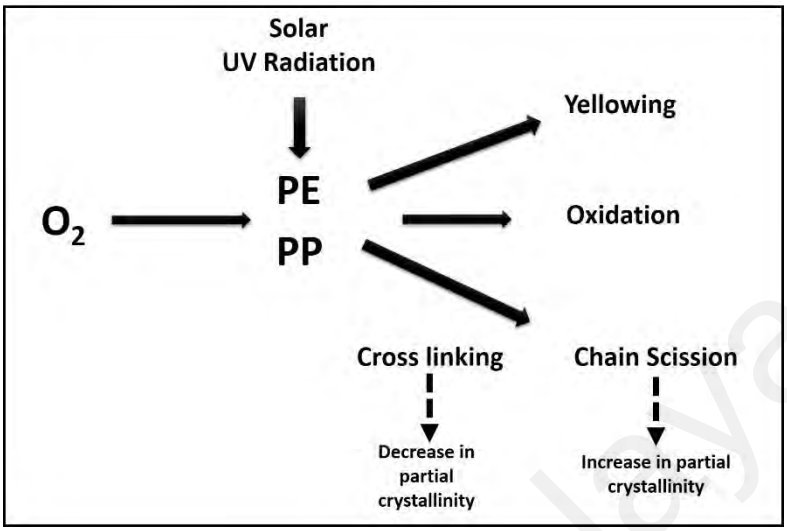


Figure 2.14: Photooxidation of polypropylene and polyethylene in the environment (Adapted from Andrady, 2017)

2.8.2 Oxo-degradation

As mentioned earlier, oxo-degradation starts after or in conjunction with photodegradation and with thermal degradation (Agamuthu & Omar, 2009). It often involves pro-oxidants such as Mn^{2+} or Mn^{3+} that use oxygen from the environment to degrade synthetic polymers (Muthukumar & Veerappapillai, 2015). Hydroperoxides are formed leading to chain scission of synthetic polymers. This process reduces the molecular mass of synthetic polymers by generating products such as alcohols, carboxylic acids, ketones and hydrocarbon (Muthukumar & Veerappapillai, 2015; Shah *et al*., 2008). Due to oxidative degradation, the hydrophobicity of synthetic polymers is decreased, making them more favourable to the attack of microorganisms. The process of photo-oxidation of PP and PE starts with alkyl radicals' generation leading to chain scission and chain branching due to hydroperoxides. This results in alteration of chemical, mechanical and physical properties of synthetic polymers (Resmeriță *et al*., 2018: Andrady, 2015).

2.8.3 Thermal Degradation

Thermal degradation occurs when the temperature increases greatly, resulting in molecular deterioration from overheating (Shah *et al*., 2008). Andrady (2011) stated that thermal degradation is generally not an environmental form of degradation. Whereas, Shah *et al.* (2008) described the effects of thermal degradation such as reduction in ductility, discolouration, embrittlement, changes to molecular changes etc., hinting at environmental degradation (combination of photochemical and thermal degradation). Similarly, photochemical and thermal degradations were considered similar and classified together as oxidative degradation by Singh and Sharma (2008). Agamuthu and Omar (2009) summarized thermal degradation and converged to utilization of thermal degradation to produce feedstock material and by-products. Generally, high temperature often causes molecular scission that leads to change in polymeric properties due to reaction of separated macromolecules (Agamuthu & Omar, 2009; Shah *et al*., 2008).

2.8.4 Hydrolytic Degradation

Hydrolytic degradation occurs when plastic polymers comes in contact with water. For hydrolytic degradation to occur, the existence of hydrolysable groups is important (Agamuthu & Omar, 2009). It includes ether, ester, anhydride or amide groups found in starch, polycarbonates, polyesters, polyamides, polyanhydrides (van der Zee, 2011). The effect of hydrolysis was negligible or absent in the degradation of PP (Resmeriță *et al*., 2018). Similarly, the hydrolysis is understood to be the least important mechanism in marine environment (Andrady, 2011).

It is understood that four different types of plastic degradation, photodegradation, oxo-degradation, thermal degradation and hydrolytic, may occur in combination in the natural world. However, microbial attack may also happen concurrently with physical degradation (Figure 2.15). But it starts with photodegradation, followed by oxo-

degradation along with thermal degradation and often leads to biodegradation as molecular weight along with hydrophobicity of synthetic polymers would have reduced due to physical degradation (Webb *et al*., 2013). Biodegradation of plastic polymers, which is the main theme of this dissertation, is elucidated next.

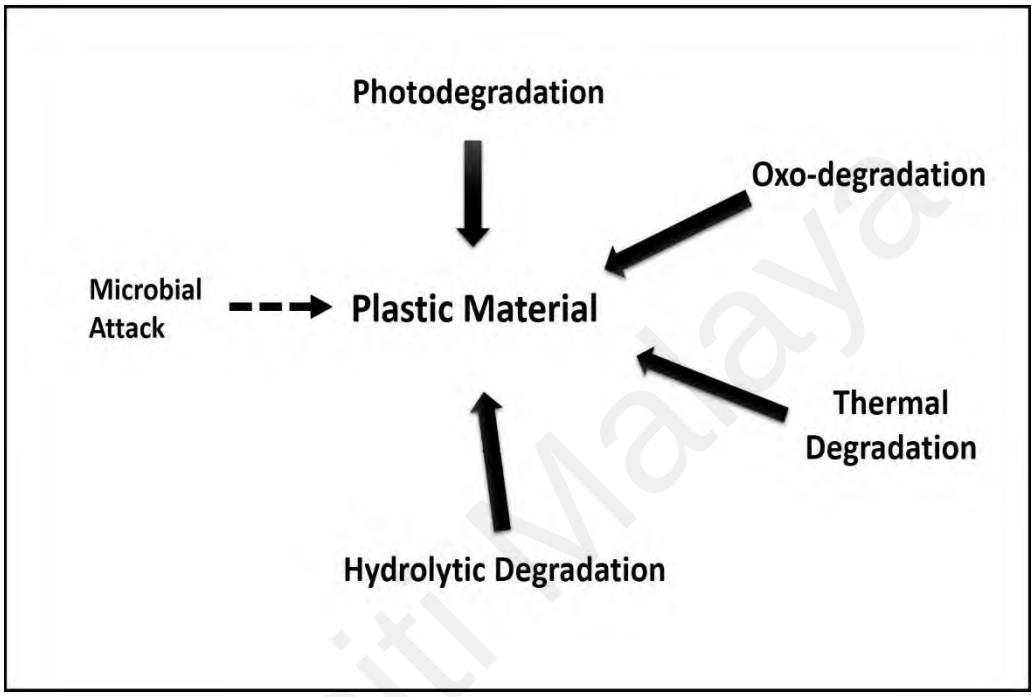


Figure 2.15: Concurrent occurrence of degradation processes (solid lines) along with probable biodegradation (dashed line) on plastic material

2.9 Biodegradation of Plastic / Microplastic

In the environment biodegradation usually takes places along with photo-degradation and hydrolysis (van der Zee, 2011). Since synthetic polymers are composed of thousands of monomers, synthetic polymers cannot be assimilated in microbial cell membranes. Therefore, these polymers are first broken down into dimers, monomers and oligomers (Shah *et al*., 2008) with the help of extracellular enzymes such as depolymerases (Gu *et al*., 2000) and hydrolases (Wilkes & Aristilde, 2017). This process is known as depolymerization or chain cleavage step (van der Zee, 2011; Shah *et al*., 2008) These smaller chains of synthetic polymers are then absorbed in the outer bacterial cell membranes for further metabolism by intercellular depolymerases (Shah *et al*., 2008; Gu *et al*., 2000). Intercellular enzymes such as oxidases and peroxidases reduce respective

polymers into carbonyls, alcohols or aldehyde groups (Atiq *et al*., 2010). Whereas, peroxidases break down dissolved oxygen to peroxide, and laccases convert oxygen to water and oxidize phenolic and non-phenolic into quinones or phenoxy radicals and cation radicals (Moen & Hammel, 1994; Rabinovich *et al*., 2004; Atiq *et al*., 2010). It must be noted that when it comes to intercellular enzymes and metabolism, there is a lot of inferences involved (Wilkes & Aristilde, 2017).

When the final products of polymer biodegradation are carbon dioxide and water, it is known as mineralization (van der Zer, 2011; Gu *et al*., 2000). The end of anaerobic biodegradation process yields carbon dioxide, methane and water, whereas the end of aerobic biodegradation process produces only carbon dioxide and water (Gu *et al*., 2000). Equations, 2.1 and 2.2, give the chemical perspective of aerobic and anaerobic biodegradation of polymer material (van der Zee, 2011).

Aerobic Biodegradation

$$C_{polymer} + O_2 \rightarrow CO_2 + H_2O + C_{residue} + C_{biomass} \quad \text{Eq. (2.1)}$$

Anaerobic Biodegradation

$$C_{polymer} \rightarrow CO_2 + CH_4 + H_2O + C_{residue} + C_{biomass} \quad \text{Eq. (2.2)}$$

In summary, biodegradation of plastic material is a complex process. Hence, there are several methods that are used to study and determine the biodegradation of synthetic polymers. Mostly these methods are deployed in conjunction. The examples of methods are monitoring uptake of oxygen, evolution rate of carbon dioxide, physical and chemical changes in synthetic polymers, growth rate of microbes and others (Kale *et al*., 2015). Hence, a novel approach is taken here to understand and synthesize the findings of biodegradation of plastic material. Instead of describing how different factors affect the

biodegradation of plastic material, biodegradation of plastics is deciphered from the perspective of several techniques that are widely executed in the biodegradation studies.

2.10 Physical Indicators of Biodegradation

2.10.1 Biofilm

The initial stage of biodegradation/biodeterioration of any material is the formation of biofilm on that material (Arkatkar *et al*., 2010; Gu, 2003). The attachment of microbes to the surface of any material in form of colony is called biofilm (Corsterton *et al*., 1995). Hence, the initiation of biodegradation of plastic material is also carried out by the formation of biofilm (Hadad *et al*., 2005). The consensus model explains that formation of microbial biofilm is instigated when planktonic culture proliferates to a cell density level that triggers attachment of cells to the surface by sending quorum sensing signals, which then leads to formation of microcolonies. These microcolonies upon maturation will develop three-dimensional sessile structure (Corsterton *et al*., 1995). The process of biofilm formation either takes place by aggregation of bacteria or in some cases, attachment to substratum by single cells that leads to clonal growth (Tolker-Nielsen *et al*., 2000). Formation of biofilm is reported in all successful biodegradation studies of plastic material. But microbial strain must be hydrophobic in order to form biofilm on plastic material (Sivan *et al*., 2006) as less hydrophobic strains have shown incapability to develop biofilm on plastic material as highlighted in Sivan *et al* (2006) and Gilan (Or) *et al* (2004).

However, due to formation of biofilm, synthetic polymers become more hydrophilic which improves the biodegradation rate (Kyaw *et al*., 2012). The availability of nutrients in the surrounding environment also plays a role in formation of biofilms (Wilkes & Aristilde, 2017). In the absence of sufficient nutrients, biofilm formation on plastic material was higher (Tribedi & Sil, 2013a) than in nutrient rich environment where the

formation of biofilm had reduced (Nauendorf *et al*., 2016). Similarly, carbon starvation has been reported to increase hydrophobicity of bacteria (Sakharovski *et al*., 1999), which could lead to the formation of biofilms on hydrophobic microplastics in carbon starved conditions (Sivan *et al*., 2006; Mor & Sivan, 2008). *Rhodococcus corallinus* in carbon starved conditions, became more hydrophobic and hence it increased their adhesion to the polymers compared to non-starved cells (Sivan *et al*., 2006).

On the other hand, the hydrophobicity of microbes, especially bacteria, also plays a significant role in formation of biofilm on plastic material. A study showed that in addition to hydrophobic nature of bacteria, properties of microbes and substrate, forces of bacterial adherence to surface and type of experimental conditions play equally decisive role (Manijeh *et al*., 2008). *Salmonella* sp., (73% hydrophobic bacterium) can develop biofilms on hydrophilic surface of substrates i.e. glass, stainless steel but could not form biofilm on HDPE as hydrophobicity of bacterium and HDPE repelled each other in this case (Manijeh *et al*., 2008). Bacterial strain either release biosurfactants extracellularly or produce biosurfactants on their cell surface that leads to increase in hydrophobicity of that bacterial strain and hence improves its adherence capabilities. These biosurfactants can be produced by both hydrophilic and hydrophobic bacteria (Arkatkar *et al*., 2010). Generally hydrophobic bacteria prefer hydrophobic surfaces for development of microcolonies whereas hydrophilic bacteria prefer hydrophilic surfaces.

Visual observation is deemed sufficient for qualitative confirmation of biofilm formation on plastic material. However, the quantification of biofilm involves studying of biomass (population density) and protein content. Standard method of estimating population density is based on direct cell counting or plating (Sivan *et al*., 2006). When direct counting is not possible, Crystal Violet (CV) staining method is performed (Mor & Sivan, 2008; Sivan *et al*., 2006). A detailed procedure of Crystal Violet straining method

is delineated in Mor and Sivan, (2008) and Feoktistova *et al.* (2016). To summarize the process, biomass is first stained with crystal straining solution and then removed from plastic material by using ethanol (Mor & Sivan, 2008) or methanol (Feoktistova *et al*., 2016). The biofilm cell density is determined by direct correlation of bacterial biomass and absorbance of intracellular strain. On the other hand, protein content is measured by alkaline hydrolysis technique (Mor & Sivan, 2008). In alkaline hydrolysis method, plastic material with adhering biofilm is washed and boiled for 20 minutes in 4.0 ml of 0.5 N NaOH solution. Then, it is centrifuged to remove biomass and the protein content of this biomass is determined under spectrophotometry at wavelength of 280 nm (Mor & Sivan, 2008). Often microbes are also extracted from biofilms adhered to plastic material for isolation and determination of potentially plastic degrading microbes. These isolated microbes are then deployed in plate assay technique.

2.10.2 Plate Assay

Plate assay is often deployed to determine the biodegradation capabilities of selected microbes where microplastics/plastic material is incubated in agar plates providing the sole carbon source to microbes. After incubation for predetermined period, development of clear hallow zones and formation of biofilm around these hallow zones indicate biodegrading potential of the microbes (Sowmya *et al*., 2014) as it determines if plastic material supports microbial growth (van der Zee, 2011). Deepika and Jaya (2015) chose microorganisms that produced clear zones in PE powder containing agar petri dishes at the end of incubation period of 2 – 4 weeks at 30 – 35oC. However, it is often argued by some authors that in addition to formation of clear zones, mechanical properties of plastic material should also be determined before concluding the potential of microbes for biodegradation (van der Zee, 2011).

2.10.3 Weight Loss

Weight loss is one of most common method deployed in the biodegradation study of plastic material. Breakdown of plastic material by microbes usually results in the reduction in mass (van der Zee, 2011). However, the findings of weight loss are often supported by other techniques. The reason is that loss of weight does not give indications of mineralization or the extent of mineralization (van der Zee, 2011). Regardless, it is economical and simple technique that makes it famous among researchers.

Different species of bacteria may result in varying degree of weight loss. For instance, three bacterial strains, *Pseudomonas aeruginosa*, *Pseudomonas syringae* and *Pseudomonas putida*, resulted in three different weight loss values in shake flask method after 120 days (Kyaw *et al*., 2012). The location of bacteria isolating site may also determine the potential of biodegradation and result in different weight loss values. For instance, *Pseudomonas* species from sewage sludge (29.1%) biodegraded polyethylene more than from textile effluent drainage site (19.6%) and municipal garbage dump (16.3%) (Nanda *et al*., 2010). Plastic biodegradation potential of bacteria or fungi may vary and even different species of bacteria or fungi result in different weight loss values. Moreover, absence or existence of alternative carbon source in the experimental set-up can also have different results. For instance, 3.8% of weight loss was observed after 30 days' incubation in shake flask method whereas introduction of gelatin supplement resulted in weight loss of 12.4% in PS (Sekhar *et al*., 2016).

Weight loss values may also vary depending on the pre-treatment deployed. PE, after autoclave, UV treatment and surface sterilization, resulted in different values of weight loss in a biodegradation experiment with *Bacillus cereus* for three months respectively (Sowmya *et al*., 2014). Shah *et al.* (2008) summarized that composition of polymer, type of organism and type of pre-treatment are the factors that play a significant role in

biodegradation of synthetic polymers. Weight loss values from different biodegradation studies is summarized in Table 2.11.

Table 2.11: Weight loss of plastic material in biodegradation studies of plastic material

Polymer Type	Duration of Experiment	Weight Loss %	Reference
LDPE	120 days	20*, 11.3*, 9*	Kyaw *et al*., 2012
PE	3 months	7.2$^{\alpha}$, 14 $^{\alpha}$, 2.4 $^{\alpha}$	Sowmya *et al*., 2014
PS	8 weeks	0.8	Mor & Sivan, 2008
LDPE	225 days	0.28	Nowal *et al*., 2011
PE	8 weeks	7.5	Sivan *et al*., 2006
PP	12 months	2.5	Arkatkar *et al*., 2010
LDPE	6 months	16 – 46	Deepika & Jaya, 2015
Polythene	30 days	16.2*, 20.1*	Ariba Begum *et al*., 2015
LDPE	30 days	0.13*, 1.29*, 1.31*	Kavitha *et al*., 2014
HIPS	30 days	12.4	Sekhar *et al*., 2016
HDPE	90 days	1.54	Agamuthu & Omar, 2009
Polythene	9 months	6$^{\square}$, 18.1$^{\square}$	Abdullhi & Saidu, 2013
LDPE	60 days	9	Das and Kumar, 2014
Polythene	1 month	20*, 30*	Thakur, 2012
Polythene	3 weeks	40.5*, 37.5*, 33*	Nanda & Sahu, 2010
LDPE	30 days	7.59*, 3.79*	Singh *et al*., 2012
LDPE	2 weeks	36*, 32*, 30*	Singh & Gupta, 2014
LDPE	126 days	29.5$^{\alpha}$, 15.8$^{\alpha}$	Esmaeili, 2013
PP	40 days	6.4*, 4*	Auta *et al*., 2018
PE	40 days	1.6	Auta *et al*., 2017
PET	40 days	6.6	Auta *et al*., 2017
PS	40 days	7.4	Auta *et al*., 2017

* Different species of bacteria/fungi resulted in different weight loss values
α Different pre-treatment of plastic resulted in different weight loss values
□ Different type of soil medium

Lastly, complete biodegradation of plastic material or 100% efficiency of biodegradation is an allusive goal. 100% decrease in mass of plastic material has not been reported till date. For instance, in a study mechanical property such as elongation at break had observed to be reduced by 98%, whereas the reduction in mass of plastic had only

been in the range of 0.03 % - 17% in all experiments (Nowak *et al*., 2011). It brings forth the question, what is 100% efficiency of biodegradation process of synthetic polymer. As authors consider 100% biodegradation of polymer when mechanical property is reduced by 100% (Muthukumar & Veerappapillai, 2015; Shah *et al*., 2008).

2.10.4 Scanning Electron Microscopy

Degradation or biodegradation of plastic material leads to changes in the surface morphology. Scanning Electron Microscopy (SEM) is deployed to observe the surface morphology of plastic material at the end of degradation or biodegradation study. Generally, formation of cavities, puts, cracks etc. manifest microbial attack on the surface of plastic material. The common morphological changes that are reported are summarized in the Table 2.12.

Table 2.12: Summary of SEM findings in biodegradation studies of plastic material

Polymer Type	Duration of Experiment	Results Achieved	Reference
LDPE	120 days	Formation of micro-cracks and surface deformation	Kyaw *et al*., 2012
PP	12 months	Formation of valleys or pits	Arkatkar *et al*., 2010
LDPE	225 days	Surface exfoliation resulting in threads formation at the edges of film	Nowak *et al*., 2011
LDPE	100 days	Formation of pits, surface erosion	Zahra *et al*., 2010
PE	3 months	Formation of holes	Sowmya *et al*., 2014
PE	30 days	Erosion, and formation of cavities	Kavitha *et al*., 2014
HIPS	30 days	Formation of pores and grooves	Sekhar *et al*., 2016
PE	28 days	Enhanced Irregularity	Paco *et al.,* 2017
HDPE	90 days	Formation of aperture and cracks	Agamuthu & Omar, 2009
LDPE	3 months	Formation of fissures	Negi *et al*., 2011
LDPE	60 days	Formation of irregularities and holes	Das and Kumar, 2014

2.10.5 High-Temperature Gel-Permeation Chromatography

High-temperature gel-permeation chromatography (HT-GPC) is used to determine the changes in molecular weight of synthetic polymers. Due to random scission of polymer chains, average molecular weight and/or molecular weight distribution of plastic material

reduces (van der Zee, 2011). While it is true that degradation of plastic compliments biodegradation, yet there is no guarantee that fragmentation of plastic litter from photooxidation degradation will result in biodegradation of microplastic. This is because microplastics still have higher molecular weight and biodegradation of polyethylene was oligomers with molecular weight of 500 g/mol (Andrady, 2011). Zahra *et al.* (2010) reported a decreased in molecular weight of irradiated LDPE at the end of treatment with fungi.

2.11 Chemical Indicators of Biodegradation

The are several techniques that gives indication of biodegradation of plastic material from chemical properties.

2.11.1 Fourier Transmittance Infrared Spectroscopy

Every material whether it is a pure element or a compound has functional groups depending on its composition that vibrate at specific wavelength when infrared beams are bombarded at them. The vibration of these functional groups at specific wavelengths give rise to peaks in FTIR spectrum that can be used to differentiate different polymers. Figure 2.16 displays the FTIR spectrum of LDPE microplastic. The FTIR spectrum of LDPE is simple as LDPE molecule contains polymers of ethylene. FTIR spectrum of LDPE only shows the peaks of methylene groups (Smith, 1998). Methylene groups have peaks due to asymmetrical and symmetrical stretches at 2917 and 2852 cm^{-1}, and scissoring vibration stretches at 1468 cm^{-1}. An umbrella mode appears at 1377 cm^{-1} due to methyl groups that terminate the long chains of LDPE (Smith, 1998). Similarly, FTIR spectrum is also used to determine the degradation of biodegradation of polymers. The absence of typical peaks or formation of new peaks give indication of oxidation of plastic material (Sudhakar *et al*., 2008).

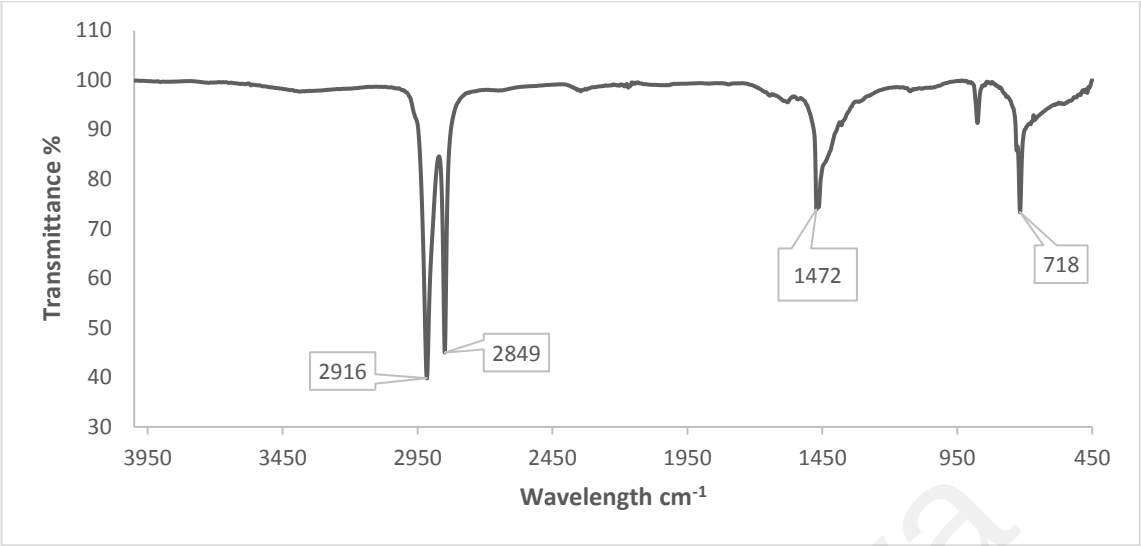


Figure 2.16: FTIR Spectrum of Low-Density-Polyethylene microplastic

Carbonyl group or carbonyl index (CI) is a yardstick to determine the chemical oxidation of polyolefin such as PE, PP etc. Thus, biodegradation of PP can be monitored by focusing on peaks associated with carbonyl group at wavelength between 1700 cm^{-1} and 1800 cm^{-1} (Arkatkar *et al*., 2010). Arkatkar *et al* (2010) monitored the relative changes in peaks of ester group at wavelength of 1748 cm^{-1} and changes in the peaks of ketone group at wavelength of 1715 cm^{-1} or of 1711 cm^{-1} to the peaks of methylene group at wavelength of 1456 cm^{-1}. They deployed Equation 2.3 and 2.4 to determine the ester and ketone group indices.

$$Ester\ carbonyl\ index\ = \frac{A_{1748}}{A_{1456}} \qquad \text{Eq. (2.3)}$$

$$Ketone\ carbonyl\ index = \frac{A_{1715}}{A_{1456}}\ or\ \frac{A_{1711}}{A_{1456}} \qquad \text{Eq. (2.4)}$$

The carbonyl index for PE was used to determine the biodegradation by selected bacteria (Kyaw *et al*., 2012). The value of CI can be obtained by Equation 2.5.

$$Carbonyl\ Index\ (CI) = \frac{Absorption\ at\ 1740\ cm^{-1} (maximum\ of\ carbonyl\ peak)}{Absorption\ at\ 1460\ cm^{-1}\ (maximum\ of\ carbonyl\ peak)} \qquad \text{Eq. (2.5)}$$

Sometimes, the FTIR spectrum of synthetic polymers must be approached with caution. As no chemical changes were detected in FTIR spectrum of expanded

polystyrene (EPS) but on analysis of metabolic activities of selected bacterial strains; *Paenibacillus urinalis*, *Bacillus sp*., and *Pseudomonas aeruginosa*, metabolites were found in the extracellular environment (Atiq *et al*., 2010). The presence of metabolites manifest utilization of carbon from EPS films by microbes. Due to slow process of carbon assimilation, FTIR spectrum did not reveal any chemical changes.

Since microbes cannot attack synthetic polymers due to hydrophobicity property, by executing chemical or biological oxidation, hydrophobicity of synthetic polymers can be decreased. This decrease in hydrophobicity leads to generation of characteristic functional groups i.e. carbonyl groups, alcohol etc. which increase the affinity of synthetic polymers for microbial attachment and subsequent biodegradation (Albertson *et al*., 1995; Lucas *et al*., 2008; Arkatkar *et al*., 2010; Wilkes & Aristilde, 2017). Once carbonyl functional groups are formed along with other characteristic products of degradation, microbial cells can metabolize them via tricarboxylic acid cycle and β-oxidation cycle (Shah *et al*., 2008; Restrepo-Florez *et al*., 2014; Wilkes & Aristilde, 2017). Similarly, as highlighted by Shah *et al.* (2008) after reduction in higher molecular weight of plastics, microbial enzymes can efficient and effectively breakdown smaller molecules of synthetic polymers that can be assimilated through microbial cell membranes. These carbonyl groups are formed due to ultraviolet light or other oxidation agents as mentioned above. As cited by Sowmya *et al.* (2013) in their paper, the formation of these groups plays the decisive role in adherence of microbes on the surface of PE chains, thus microbes can then consume smaller segments of PE chains (Albertsson *et al*., 1987). The formation of these functional groups also increases the hydrophilicity of plastic surface. Table 2.13 enlists some of the results of FTIR analysis performed in the biodegradation study of synthetic polymers.

Table 2.13: Summary of FTIR results in biodegradation studies of plastic material

Polymer Type	Duration of Experiment	Results Achieved	Reference
PP	12 months	Formation and subsequent disappearance of keto carbonyl & ester carbonyl groups at 1715 cm^{-1} & 1748 cm^{-1} respectively	Arkatkar *et al*., 2010
LDPE	225 days	Formation of ketone and aldehyde groups at 1710 – 1750 cm^{-1}, band intensity increased at 1200 – 1300 cm^{-1},	Nowak *et al*., 2011
LDPE	100 days	Bond intensity of C=O at 1700 – 1760 cm^{-1} decreased.	Zahra *et al*., 2010
PE	3 months	Formation of carboxylic acids, aldehydes, alcohols, esters, ethers, aromatics, and alkene, change in peak intensities	Sowmya *et al*., 2014
PE	30 days	Broadening of bands, Formation of C-O, ketone or aldehyde peak at 1710 – 1750 cm^{-1}, peak of hydroxylated compounds at 3800 – 3100 cm^{-1} and peak of carboxylated compounds at 1900 – 1500 cm^{-1}, peak of new vinyl group at 948 cm^{-1}	Kavitha *et al*., 2014
HIPS	30 days	Narrowing of peaks associated with C-O stretch and =C-H bend, decrease in peak associated with C-Br at 690 – 515 cm^{-1}	Sekhar *et al*., 2016
PE	28 days	New peaks formed at 1700 – 1500 cm^{-1} due to carbonyl groups and at 1200 – 950 cm^{-1} due to double bond. Formation of peaks of hydroperoxide and hydroxyl groups at 3700 – 3000 cm^{-1}	Paco *et al*., 2017
PE	120 days	Reduction in carbonyl index	Kyaw *et al*., 2012
HDPE	90 days	Reduction in peak intensities, formation of new peaks between 1200 – 900 cm^{-1}	Agamuthu & Omar, 2009
PUR	6 days	Loss of C(O)-O ester linkage related peak at 1735 cm^{-1}	Russell *et al*., 2011
LDPE	60 days	Increase in peaks linked with C=O and O-H stretch at 1079 cm^{-1} and 2418 cm^{-1} and distortion of peak at 2920 cm^{-1}	Das and Kumar, 2014
LDPE	3 months	Absence of CH_3 bending and CH_2 deformation	Negi *et al*., 2011

2.11.2 Surface Energy

Hydrophobicity of plastic material is one of the characteristics that hinder the biodegradation of plastic material. However, under the influence of degradation and/or biodegradation, plastic material can become hydrophilic. This is where surface energy

can be useful. Surface energy method determines the intermolecular forces of plastic material. Lower intermolecular forces indicate hydrophobicity whereas, higher intermolecular forces manifest hydrophilicity. Arkatkar *et al.* (2010) utilized Easy Drop Contact Angle Measuring System where Milli-Q water and formamide (Polar liquids) and diiodomethane (non-polar liquid) determined the surface energy of plastic material using instrument, which is based on the Fowkes method, with DSA2 software.

2.11.3 Carbon Dioxide Evolution

In aerobic conditions, microorganisms use oxygen and release carbon dioxide by oxidizing carbon of synthetic polymers (Shah *et al*., 2008). Based on this understanding, carbon dioxide evolution is often deployed to study biodegradation of synthetic polymers. Gas is trapped from the biodegradation set-up in base i.e. KOH containing bottle/container and titrated with barium chloride solution. The precipitations formed are then weighed to calculate produced carbon dioxide (Gnanavel *et al*., 2012) or carbon dioxide is monitored using infrared detectors (van der Zee, 2011). For some polymers, often 60% conversion of carbon to carbon dioxide is considered sufficient to declare biodegradation of plastic material as some carbon also gets incorporated in biomass due to microbial growth (van der Zee, 2011).

2.12 Biological Indicators of Biodegradation

Biological indicators mainly highlight the metabolic activity of the cell that manifests changes in synthetic polymers.

2.12.1 Bacterial Viability Test

Bacterial viability test is confined to bacterial biodegradation studies of plastic material. Baclight bacterial viability test kit is often deployed to test the presence of living and dead bacterial cells on the plastic material. SYTO 9 is green coloured stain that adheres to both living and dead bacterial cells whereas, propidium iodide dye is red

coloured stain that adheres to only dead bacterial cells. STYO 9 is applied to the plastic material, followed by propidium iodide dye, then plastic material is incubated for 15 – 20 minutes in the dark and images are taken with the help of fluorescence microscope with a blue filter at an excitation of 475 nm (Arkatkar *et al*., 2010; Arkatkar *et al*., 2009).

2.12.2 Carbohydrate and Protein Content

Another indicator of biodegradation of plastic material is the presence of carbohydrate and protein content of extracellular matter that is excreted in the formation of biofilm (Arkatkar *et al*., 2010). Microbes secrete proteins that adhere to complex substrates to acquire nutrients (Shimpi *et al*., 2012). The carbohydrate and protein content are measured by using Phenol-sulphuric acid (Arkatkar *et al*., 2010; Sadasivam & Manickam, 2005) and Barkford's methods (Arkatkar *et al*., 2010; Bradford, 1976) respectively. Bradford assay was also used by Sekhar *et al* (2016) to determine protein content. Lowry's method involving Bovine Serum Albumin (BSA) was deployed to determine protein estimates by Sowmya *et al.* (2014). On the other hand, Mor and Sivan (2008) extracted protein from biofilm and determined it using spectrophotometer at 280 nm. This protein and carbohydrate content are an important indicator of development of biofilm on any surface (Arkatkar *et al*., 2010). Presence of protein content corresponds to biodegradation of synthetic polymers (Shimpi *et al*., 2012) Often stressful conditions in the experimental set-up can result in significant metabolization of highly stable polymers by some microorganisms through their extracellular and intercellular proteins (Sekhar *et al*., 2016).

2.12.3 Enzyme Activity

Microbes excrete extracellular and intercellular enzymes to biodegradation plastic material. Enzymes biodegrade the plastic material by first adhering to its surface and then catalysing the hydrolytic cleavage (Shah *et al*., 2008). Oxidation is a significant part of

biodegradation process (Arkatkar *et al*., 2010). Enzyme assays involves introduction of a certain enzyme into a buffered or pH-controlled set-up, also containing polymer substrate. For instance, Arkatkar *et al.* (2010) confirmed the presence of laccasse enzyme by preparing a reaction mixture of extracellular culture fluid and 2-2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in the system of 50 mM of glycine – HCl buffer at a pH of 3.0 and at a temperature of 25ºC, where reaction was studied by monitoring the changes in absorbance A_{346} for 5 minutes.

Sowmya *et al.* (2014) used laccase screening medium (LSM) to determine the presence of laccase enzyme. They inoculated selected bacteria in LSM and incubated in dark for 7 days. In order to determine manganese peroxidase enzyme, they added H2O2 in LSM plates. Therefore, presence of enzymes in enzyme assay and/or higher concentration of enzymes is related with biodegradation of synthetic polymers (Sekhar *et al*., 2016; Sowmya *et al*., 2014). Some of the extracellular enzymes linked with biodegradation of synthetic polymers are listed in Table 2.14.

Table 2.14: List of Enzymes reported in biodegradation studies of plastic material

Plastic Type	Enzyme	Source
PE	Hydrolase	Tribedi & Sil, 2013b
Polyester	Esterase	Biffinger *et al*., 2015; Shah *et al*., 2013; Mukherjee *et al*., 2011
LMWPE	Alkane hydroxylase	Yoon *et al*., 2012
Polyester	Lipase	Hung *et al*., 2016; Biffinger *et al*., 2015
PES	Esterase	Tribedi *et al*., 2012
HIPS	Esterase	Mohan *et al*., 2016
PVA	Esterase	Kawai & Hu, 2009
PU	Urease, Protease, Esterase, Laccase	Loredo-Trevino *et al*., 2011
PE	Laccase	Bhardwaj *et al*., 2012
PE	Laccase, Manganese	Sowmya *et al*., 2014

2.13 Mechanical Indicators of Biodegradation

Tensile strength, elongation at break and modulus of the synthetic polymers are studied to observe the changes in these properties of synthetic polymers. Tensile strength

decreases in successful biodegradation experiments and is usually measured in materials testing machine (Kyaw *et al*., 2012). Usually changes in tensile strength is greater in physical degradation of synthetic polymers but when changes in tensile strength are studied along with other parameters, biodegradation of plastic polymers can be inferred (Shah *et al*., 2008). However, biodegradation rate must be high for changes in tensile strength to be significant (Shah *et al*., 2008). Kyaw *et al* (2012) reported 20% reduction in mass of LDPE, and the findings of tensile strength were backed by aforementioned weight loss, FTIR, and SEM analysis. Similarly, changes in percentage elongation is also used to determine biodegradation (Sowmya *et al*., 2014).

2.14 Fungal Biodegradation Vs Bacterial Biodegradation

Fungi is considered as potential biodegrading organisms for two reasons; firstly, they can survive in unfavourable conditions such as in environment of low nutrient and moisture, secondly, they naturally possess rich source of enzymes (Trishul & Doble, 2010). But the mechanism of biodegradation of synthetic polymers remains the same for both bacteria and fungi (Kawai *et al*., 2004). However, in the same study, the biodegradation rate of PE wax was higher for bacteria than fungi (Kawai *et al*., 2004).

The formation of fungal biofilm on plastic material can also lead to physical degradation i.e. swelling and bursting of plastic material which in return enhances biodegradation chances as fungi can penetrate into the substrate (Muthukumar & Veerappapillai, 2015). Nevertheless, fungi have also shown potential for assimilating plastic material for carbon and energy source. *Zalerion maritimum* had shown the potential for biodegradation of PE microplastics in shake flask method (Paco *et al*., 2017). An increase in fungal biomass and subsequent reduction in PE mass was observed. Moreover, PE microplastic pellets were found to have been broken down into threads after the incubation in *Zalerion maritimum* induced medium for 28 days (Paco *et al*.,

2017). Hence, both fungi and bacteria have been shown to biodegrade plastic material but the choice of either microorganism depends on the experimental set-up and the objectives of the research.

2.15 Research Gaps

Following research gaps were noted from reviewing literature:

1. Majority of the studies have been focused on the biodegradation of macro-plastic. Comparatively, lesser researches have examined biodegradation of microplastic.
2. Similarly, most of the biodegradation studies have been performed in shake-flask experiments or in liquid medium. There is a strong need for substantial amount of biodegradation studies carried out in soil medium. Since, shake-flask experiments are usually screening tests, experiments performed in soil medium would resemble environment more closely.
3. Since biodegradation of plastic is a slow process, more experiments are required that examine the biodegradation of microplastic for a long duration such as a year or longer. Comprehensive understanding of interaction of microbes and microplastic for a long period time is lacking. Experiment duration of 40 days or few months are too short for biodegradation of microplastic.
4. The effect of different shapes and sizes of microplastics on its biodegradation are not fully explored yet. There are limited number of studies on this. Hence, more research is required.

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Preparation of Media for Bioremediation Set-ups

3.1.1 Soil Sampling Location

A total of six mangroves sites were selected along the coast of Peninsular Malaysia (Figure 3.1). Table 3.1 enlists the names and coordinates of the mangrove sites.

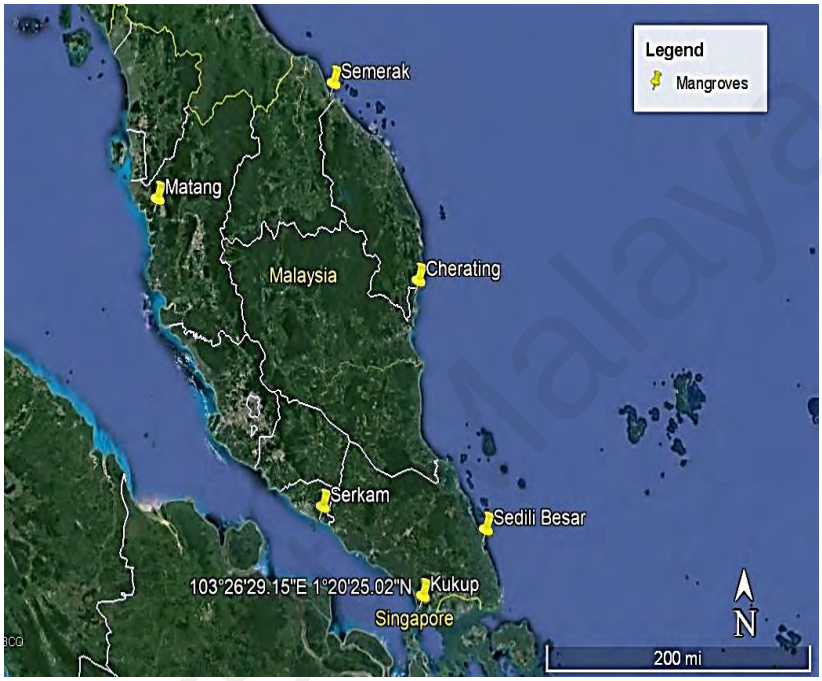


Figure 3.1: Locations of mangrove selected for soil sampling in Peninsular Malaysia (Google Earth)

Table 3.1: Coordinates of Soil Sampling Locations in Peninsular Malaysia

No.	Sampling Locations	Longitude	Latitude	Coast Location
1	Semerak Mangrove, Kelantan	102°29'32.66"E	5°52'4.88"N	East Coast
2	Cherating Mangrove, Pahang	103°23'15.0"E	4°07'32.0"N	
3	Sedili Besar Mangrove, Johor	104° 6'21.34"E	1°55'44.25"N	
4	Matang Mangrove, Perak	100°38'0.65"E	4°50'36.5"N	West Coast
5	Serkam Mangrove, Malacca	102°22'58.9"E	2°08'05.3"N	
6	Kukup Mangrove, Johor	103°26'29.15"E	1°20'25.02"N	

3.1.2 Soil Collection

In each of the six sampling locations, mangrove soil samples were taken randomly. Therefore, the distance between sampling point to the shoreline or to any other significant coastal landmark was not recorded. A big shovel was used to scoop the mangrove soil from the surface to 5 cm depth (0 – 5 cm) (Jayanthi *et al*., 2014) and placed in a 270 × 280 mm plastic bag. Approximately 320 kilograms (kg) of mangrove soil was collected in total for biodegradation experiments. The soil was kept in cold room at 4^{o}C until research experiments were initiated. Plate 3.1 (a – f) show the places where mangrove soil was extracted in all six sampling locations.

(a) Semarak mangrove, Kelantan

(b) Cherating mangrove, Pahang

(c) Sedili Besar mangrove, Johor

(d) Kukup mangrove, Johor

(e) Serkam mangrove, Malacca

(f) Matang mangrove, Perak

Plate 3.1: Soil sampling places in each mangrove location

The rationale behind acquiring soil from six different mangroves in Peninsular Malaysia was to produce a homogenized media to represent a typical mangrove media for the biodegradation study. Mangrove soil collected from the six locations were thoroughly mixed manually to homogenize them.

3.1.3 Soil Analysis

To characterize the soil, six parameters namely, soil pH, moisture content, Total Organic Carbon (TOC), Total Nitrogen (TN) Total Phosphorous (TP) and soil microbial count were selected for soil analysis. Standard protocols were used to determined TOC (US EPA 9060, n.d.), TN (APHA 4500, n.d.) and TP (US EPA 6010B, n.d.) at the start and at the end of biodegradation experiments. This analysis is important to determine the condition of the media. Soil nutrients have been reported to change due to biodegradation of plastic material (Abdullahi & Saidu, 2013). Therefore, soil characterization at the start and end of experiment was performed.

Soil samples for determination of soil pH and microbial count were taken from point where biodegradation occur i.e. the burial of microplastics. where sampling bags were pulled out, then soil samples were weighed and placed in distilled water.

On the other hand, pH of soil samples was measured at the beginning of experiment, at every 15-day interval, and at the end of experiment. pH was measured by potentiometric method at a soil-distilled water ratio of 1:4. A determined weight of soil and determined volume of distilled water (50 g: 200 ml) were mixed. The soil-distilled water solution was stirred thoroughly and pH reading was recorded using YSI multiprobe.

Viable bacterial count in the soil medium was determined by pour plate method (Atlas & Bartha, 1998) at the start of experiment, at 15-day interval and at the end of experiment. One gram of soil was taken from the burial point and mixed with 9 ml of autoclaved

distilled water. Then, serial dilution was performed and aliquots of dilutions of 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} were plated with plate count agar (PCA) and incubated at 35ºC for 24 hours. The number of viable colonies was calculated using Colony Counter (Galaxy 230).

Lastly, standard protocol was followed to determine moisture content (Camuffo, 2018). Moisture content was determined at the initial and final day of the experiment. For moisture content, the difference in weight was calculated after soil samples were oven dried at 110ºC for 24 hours. Porcelain crucible dishes were used for this purpose. The moisture content was calculated by using formula given in Equation 3.1.

$$\text{Moisture Content } \% = \left(\frac{wet\ soil\ (g) - dry\ soil\ (g)}{dry\ soil\ (g)}\right) \times 100 \qquad \text{Eq. 3.1}$$

3.2 Preparation of Samples for Bioremediation Set-ups

3.2.1 Preparation of Bags for Experiment

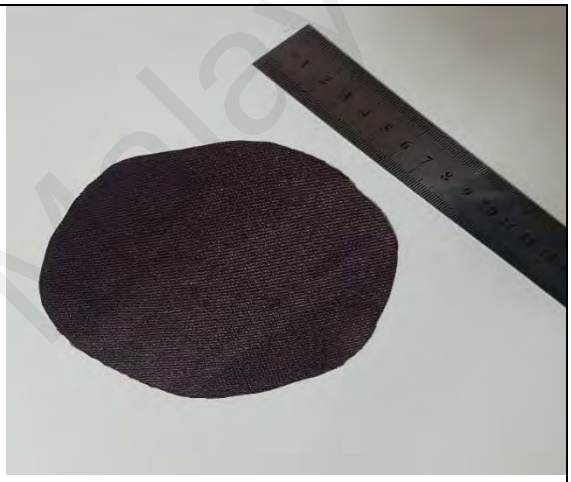
Several paraphernalia have been deployed in biodegradation studies that involved burying (macro or micro) plastics in-situ or in lab set-up. Their main objectives are to hold plastics in one place so that the (macro or micro) plastic samples can be retrieved at the end of experiment and hence there is strictly no loss of plastic material (especially microplastics). The examples of paraphernalia are meshes, metal cages, envelops (Tosin *et al*., 2012), sampling bags (Auta, 2018) or even direct exposure (for macroplastics) in form of burial (Gnanavel *et al*., 2012).

In this study, sampling bags were deemed suitable as paraphernalia for microplastics due to the innate smaller size (< 5 mm) of microplastics. Additionally, cloth was selected as the material for making sampling bags as it would have kept smaller fragments of microplastics from escaping. It is a new approach as netlike sampling bags have been used before (Auta, 2018). For that matter Satin cloth was chosen to make sampling bags. Cloth was cut into a square of 12 × 12 cm (Plate 3.2 a) and then the edges of square were

cut to make it circular (Plate 3.2 b). In order to tie the bags, fancy cord/thread was poked through the bag at 1 cm below the circumference (Plate 3.2 c). Plate 3.2 (d) shows the finalized sampling bag.

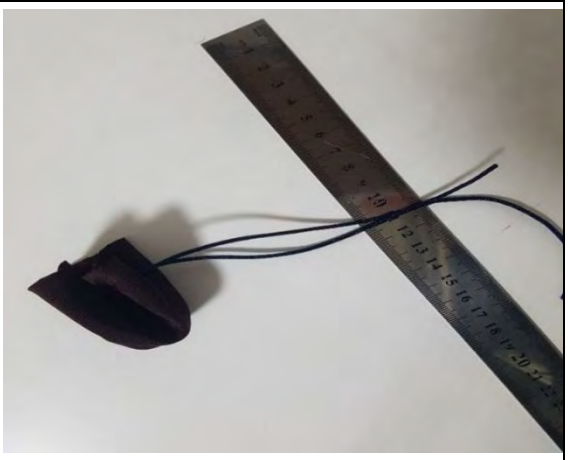
Since microplastics for both control and treatment were placed in satin bags, the interaction of microplastics would be same to their surroundings in both settings. Moreover, biodegradation of satin bags is not the scope of this study. Hence, biodegradation of satin bags was not determined.

(a) 12 X 12 cm square pieces of Satin Cloth



(b) Shape of cloth after cutting the edges of square

(c) Sampling bag after sowing fancy cord

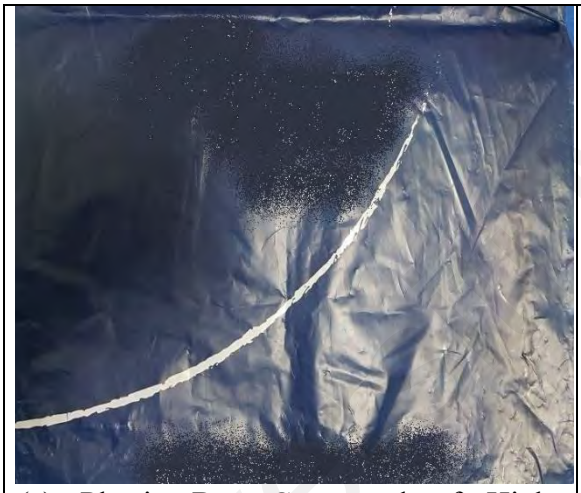


(d) Final shape of sampling bag

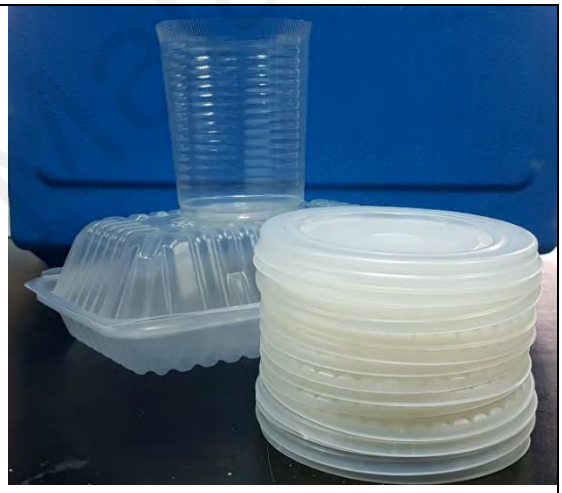
Plate 3.2: Preparation of Sampling Bag

3.2.2 Types of Microplastics

Three polymers were selected for the biodegradation experiments namely, high-density-polyethylene (HDPE), (polypropylene PP) and (polystyrene) PS. Majority of plastic commodities have resin codes marked on them depending on their polymeric composition. i.e. resin codes 5 and 6 are for PP and PS respectively. That is how PP (resin code 5) was attained from takeaway food and drink containers, whereas PS (resin code 6) was acquired from the lids of takeaway (cold) drink and coffee containers. On the other hand, HDPE was obtained from plastic bags. Plate 3.3 (a) shows the HDPE plastic bag used in this study and Plate 3.3 (b) shows the PP and PS used in this research.



(a) Plastic Bag Composed of High-density-polyethylene



(b) Polypropylene and Polystyrene takeaway food and drink containers and lids, respectively

Plate 3.3: Source of Microplastics for the experiments

To acquire secondary microplastics, all three types of plastics were manually cut using scissors into the required size range for biodegradation experiments. Plate 3.4 shows HDPE and PS microplastics, respectively.

(a) High-density-polyethylene microplastics after manual cutting of plastic bag

(b) Polystyrene microplastics after manually cutting takeaway drink container lids

Plate 3.4: Microplastics utilized in the experiments

3.3 Bioremediation Set-up

3.3.1 Containers for Trials

The media for the biodegradation experiments was placed in a 171 L rectangular tub with dimensions of 95 cm (length) × 60 cm (width) × 30 cm (height) (Figure 3.2). The homogenized mangrove soil was placed up to 10 cm height from the base of the tubs. 10 cm layer was chosen to study the effect of top surface layer in the biodegradation of microplastics. Each experiment had separate tubs for the control and for the treatment. Moreover, each experiment had separate tubs as well. For instance, experiments involving treatment with 1% inoculum concentration, treatment with 0.5% inoculum concentration, treatment with 0.25% inoculum concentration, as well as, for experiment involving different sizes of microplastics, and treatment with daily input, all had separate tubs respectively. Such an arrangement was chosen to eliminate any chances of cross contamination between different trials. It must be noted that only one tub was used for treatment of different sizes of microplastics as it received fixed (0.5%) inoculum concentration.

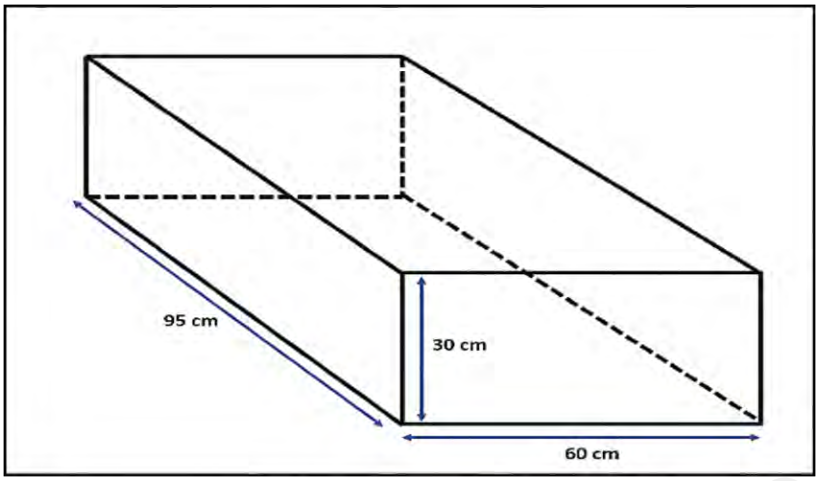


Figure 3.2: 3-Dimensional (3D) illustration of container for experiment

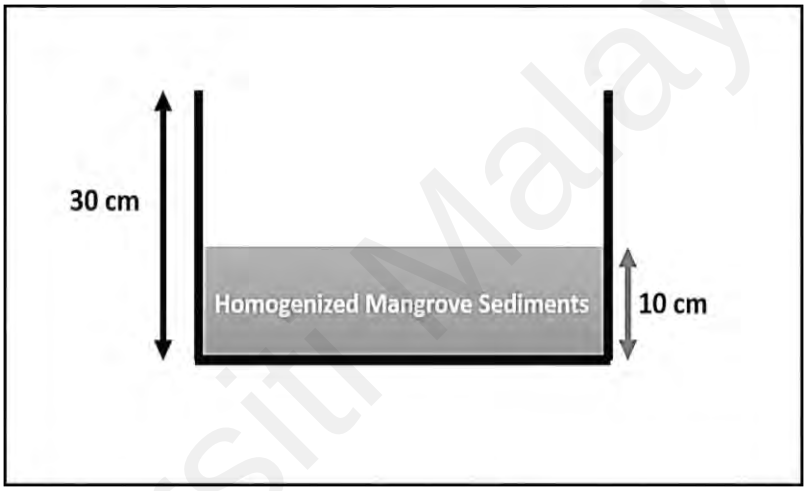


Figure 3.3: 2-Dimensional drawing of soil filled container

3.3.2 Experimental Set up

One gram of microplastics was placed in satin bags. Afterwards the satin bags containing microplastics were irradiated in ultraviolet light for 10 minutes for sterilization purpose. Then these bags were buried (in triplicates) in mangrove sediments in respective tubs at the depth of 5 cm. 5 cm depth was chosen based on the findings of a study on abundance of microplastics in Malaysian mangroves which reported highest concentration of microplastics in the depth range of 3 – 6 cm as compared to depth ranges of 0 – 3 cm and 6 – 9 cm (Norkhairah, 2018). Sampling bags were buried 3 cm away from the bordering walls of tub and also 3 cm away from adjacent sampling bags (Figure 3.4). This setting was randomly selected.

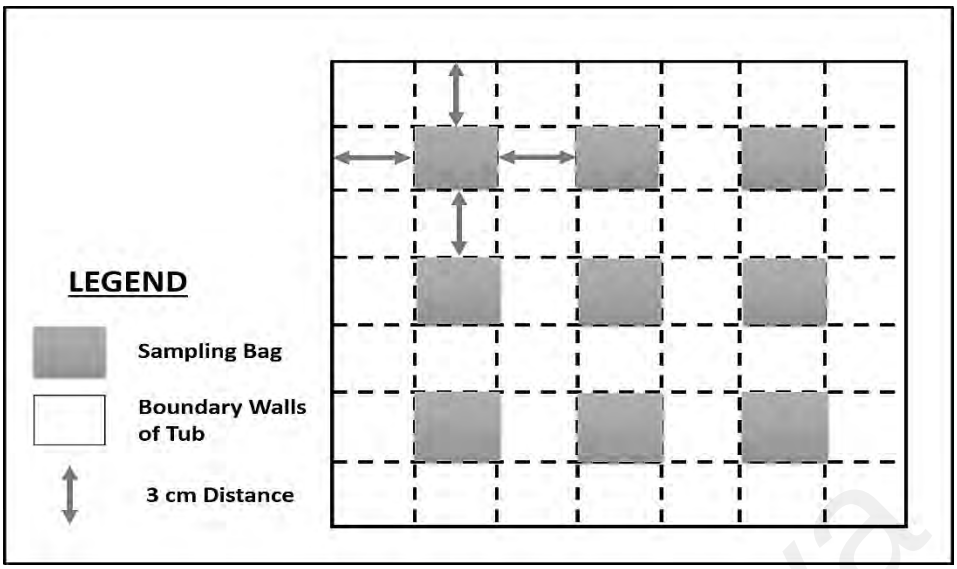


Figure 3.4: Setting of Sampling Bag Placement in Containers for Experiment (Note the diagram is not drawn to scale)

3.4 Inoculum Preparation

3.4.1 Bacteria Used for Trials

A total of nine bacterial samples were selected for this research. These bacteria were isolated from six mangrove sites in Peninsular Malaysia including Matang mangrove Perak, Cherating mangrove Pahang, Tanjung Piai Johor, Sekam mangrove Melaka, Sedili Besar Johor, and Pasir Puteh mangrove Kelantan, (Auta *et al*., 2017). The list of bacteria selected are:

1. *Bacillus cereus*
2. *Bacillus sonorensis*
3. *Bacillus vietnamensis*
4. *Sporosarcina globispora*
5. *Alcaligenes taecalis*
6. *Staphylococcus epidermidis*
7. *Bacillus flexus*
8. *Rhodococcus rubber*
9. *Bacillus gottheilii*

These bacteria have been studied separately (Auta *et al*., 2018; Auta, 2018; Auta *et al*., 2017) and together in combinations of bacterial consortium (Auta, 2018) for determining the biodegradation potential for microplastics. In this study, all nine bacteria were deployed in blended form to examine other aspects of biodegradation study.

3.4.2 Inoculum Preparation in Nutrient Broth

Standard protocol was followed for the preparation of inoculum in nutrient broth. The subculture of all nine bacteria were prepared by growing them separately onto nutrient agar (NA) at 35ºC for 24 hours. These subcultures of nine bacteria were inoculated into nutrient broth separately and were left to grow to stationary phase (1.2 absorbance [ABS] at 600 nm) in a rotating shaker at 29ºC and 150 rpm. Then, these prepared inoculums of individual bacterium were mixed to get an inoculum of bacterial consortium. Fresh inoculum was prepared for every application.

3.4.3 Inoculum Preparation in Phosphate Buffer Solution

Phosphate Buffer Solution (PBS) was prepared by the standard protocol stated in Clescerl *et al*. (1998) using chemical given in Table 3.2. After dissolving these chemicals, pH of the phosphate buffer solution was maintained at pH 7.4.

Table 3.2: Amount of Chemicals Used for Making 1 Litre of PBS Solution

No.	Chemicals	Amount (grams)
1	Potassium Dihydrogen Orthophosphate, KH_2PO_4	8.5
2	di-Potassium Hydrogen Phosphate, K_2HPO_4	21.75
3	Sodium phosphate dibasic heptahydrate, $Na_2HPO_4{\cdot}7H_2O$	33.4
4	Ammonium Chloride, NH_4Cl	1.7

For the purpose of direct inoculum preparation, PBS was used to suspend the bacterial consortium collected from the stationary phase (1.2 absorbance [ABS] at 600 nm). This bacterial consortium was harvested by centrifuging the stock inoculum (in Sorvall ST 16 Centrifuge Series) at 10,000 rpm at 40ºC for 10 minutes. Supernatant was

discarded and pellets were washed twice with PBS. These pellets were then resuspended in PBS buffer to the final bacterial concentration of 2.0×10^8 CFU/ml.

3.5 Biodegradation Trials

3.5.1 Bacterial Biodegradation by Increased Frequency of Inoculum Input

The effect of daily dosage of inoculum on the biodegradation of microplastics was examined, since such an approach for studying biodegradation has not been taken before. The inoculum prepared in nutrient broth was slowly poured over the soil medium in the treatment tub from flask every day for 60 days. The concentration of inoculum introduced into the treatment tub was 1% (403 ml) for the first 15 days and 0.5% (201.5 ml) for the rest of 45 days. For control set-up, non-inoculated nutrient broth was used. The concentration of 1% inoculum was selected for first 15 days in order to provide higher microbial population to the treatment as induced microbes will be adapting to the new environment in the beginning. Due to this higher microbial population, microbes would have higher probability to survive in the new environment. Afterwards, the concentration of inoculum was reduced as metabolites produced by earlier microbial population, in conjunction with newly added inoculum would be attacking microplastics in the treatment. This selected method is the first attempt in biodegradation study of microplastics.

At every 15-day interval, three representative bags of each microplastic, HDPE, PS and PP, were pulled out of treatment and control tub. The justifications of removing microplastic samples on every 15-day interval from the experiment were that Auta (2018) had shown in her experiments that biodegradation of microplastics had initiated by 15^{th} day of the experiment, and more importantly, weight loss data from regular internals was required to determine the biodegradation rate using slope analysis. If microplastic samples were only extracted at the end of experiment, the gradual decrease of mass or the

phenomenon of biodegradation from the perspective of weight loss could not be delineated. The size range of all microplastics was 1 – 25 mm^2. They were soaked in 70% ethanol solution (v/v) overnight to kill all microorganisms. In order to wash microplastics samples, the techniques used by Auta *et at* (2017) and Deepika and Jaya (2015) were modified slightly. Microplastics were washed under the running tap water in sieves (mesh # 0.5 mm and 0.025 mm) for 5 minutes. Then microplastics were soaked in 70% (v/v) solution of ethanol for 15 minutes. Afterwards microplastics were again washed under running tap water in sieves (mesh # 0.5 mm and 0.025 mm) for 5 minutes as done by Ariba Begum *et al.* (2015). Washed microplastics were then dried at 50o C in oven overnight (Sowmya *et al*., 2014). However, this method was not found to be efficient in removing biofilm as residual weight of microplastics was still higher than 1.000 g (initial weight).

3.5.1.1 Weight Loss

The residual weight of microplastic samples was measured using weighing balance (A&D Weighing GF-300 Digital Scale). Weight loss was calculated by subtracting the initial weight of microplastics from final/residual weight of microplastics. Then, the resulted value was divided by final weight (Sekhar *et al*., 2016; Kavitha *et al*., 2014).

$$Weight\ Loss\ \% = \left(\frac{Initial\ Mass - Residual\ Mass}{Initial\ Mass}\right) \times 100 \qquad \text{Eq. (3.2)}$$

3.5.1.2 Rate of Mass Reduction of Microplastics

Mass reduction rate constant of HDPE, PP and PS microplastics was determined using formula involving first-order kinetic model (Alaribe & Agamuthu, 2015). It must be noted that total experiment time (t) is used in this formula. Therefore, the time used in the formula for this experiment, studying daily input of inoculum, was 60 days.

$$K = -\frac{1}{t}\left(\ln\frac{W}{Wo}\right)$$ Eq. (3.3)

Where,
K = first – order rate constant for polymer uptake per day, t = time in days,
W= residual mass of microplastics (g), W_O = initial mass of microplastics (g)

After determining rate constant (K), Half-life ($t\frac{1}{2}$) of microplastics was calculated using the following formula;

$$Half\ Life\ (t\frac{1}{2}) = \frac{\ln(2)}{K}$$ Eq. (34)

3.5.1.3 Fourier Transform Infrared Spectroscopy

Attenuated total reflectance – Fourier transform infrared spectroscopy (ATR-FTIR), using Perkin-Elmer 400 FT-IR/FT-FIR, was performed at the frequency range of 4000 – 450 cm^{-1} on microplastics films recovered at the end of each experiment. FTIR or ATR-FTIR is often used to study the changes in chemical composition of synthetic polymers (Paco *et al*., 2017). FTIR spectra of treated microplastics was compared with FTIR spectra of microplastics from control. Any significant changes such as disappearance of typical peaks or formation of new peaks is associated with biodegradation of synthetic polymer (Sekhar *et al*., 2016).

3.5.2 Bacterial Biodegradation by Different Inoculum Concentration

Biodegradation studies often involve examining the impact on substrate biodegradation with varying concentration of inoculum (Auta, 2018; Dada *et al.,* 2012; Liu *et al*., 2011) and similar approach was taken in this trail. For the purpose of investigating the effect of inoculum concentration on biodegradation of plastic, PP and PS with size range of 1 – 25 mm^2 were selected. The reason only PP and PS were used in this experiment was that these two types of plastic are considered highly resistant to biodegradation (Jeyakumar *et al*., 2013; Mor & Sivan, 2008). Inoculum in PBS with varying concentrations namely 1%, 0.5% and 0.25% was re-applied every 15 days, for 90 days. For control experiment, non-inoculated PBS was used. The duration of experiment

was extended to 90 days for this experiment due to the results obtained from experiment mentioned in section 3.5.1. At the end of previously mentioned experiment (section 3.5.1), it was discovered that microbial interaction with microplastic was still ongoing and more time was required for the biodegradation of microplastics. Plate 3.5 displays experimental tub where 0.25% inoculum was introduced in the tub. The protruding threads show the buried microplastics containing satin bags.

Plate 3.5: Experimental Set-up for 0.25% inoculum concentration

Triplicates of PS and PP were removed from treatment and control at every 15-day interval with the rationale described in section 3.5.1. Recovered microplastic samples were then soaked in 70% ethanol solution (v/v) overnight to kill all microorganisms. Afterwards these microplastics were incubated in 2% (w/v) solution of Sodium Dodecyl Sulphate (SDS) for four hours at 50°C (Kavitha *et al*., 2014; Kyaw *et al*., 2012). Then, they were washed with lukewarm water in the sieves (mesh # 0.5 mm and 0.025 mm) first, followed by washing under running tap water for 5 minutes. Afterwards microplastics were oven dried at 50°C (Sowmya *et al*., 2014). Microplastics were washed with SDS to remove biofilm. It must be highlighted that despite the use of SDS in other studies (Kavitha *et al*., 2014; Kyaw *et al*., 2012; Mor & Sivan, 2008), SDS was not found to be very effective in removing biofilm. Whereas, washing microplastics under running tap water on sieves was more effective to separate fine sand and silt from microplastics.

3.5.2.1 Weight Loss

The weight loss was determined as mentioned in section 3.5.1.1.

3.5.2.2 Rate of Mass Reduction of Microplastics

Rate constant of biodegradation and half-life of PP and PS microplastics were determined as elucidated in section 3.5.1.2.

3.5.2.3 Fourier Transform Infrared Spectroscopy

FTIR spectroscopy analysis was performed as previously mentioned in section 3.5.1.2.

3.5.2.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM), using Leica EM SCD005, Austria, was carried out at magnification of 1000x, 8000x and 10,000x to study the morphological changes on the surface of microplastics films. Samples were obtained at the end of respective experiments. The selection of samples for SEM was based on weight loss and FTIR Spectra; the sample with highest weight loss and major changes in FTIR spectrum were chosen for SEM.

3.5.3 Different Size of Microplastics for Bacterial Biodegradation

Studying the effect of different sizes of microplastics on the biodegradation is a new approach as majority of the studies have been focused on screening capabilities of microorganisms (Chinaglia *et al*., 2018; Paco *et al*., 2017). Therefore, the effect of three different sizes of PP and PS microplastics at; 1 – 4 mm^2, 9 mm^2 and 25 mm^2, on the biodegradation was also examined for 90 days. PP and PS were selected for this study as well and the length of the experiment was 90 days because they are highly resistant to biodegradation (Jeyakumar *et al*., 2013; Mor & Sivan, 2008) than HDPE and LDPE, thus they required more time for biodegradation. 0.5% of inoculum in PBS and 0.5% of non-

inoculated PBS was applied into the treatment and control, respectively at every 15-day interval.

Triplicate samples of PP and PS were removed at every 15-day interval from treatment and control on the basis of justification described in section 3.5.1. These microplastics were then soaked in 70% ethanol solution (v/v) to kill all microbes. In order to remove biofilm formed on the microplastics films, SDS was used as mentioned in Section 3.5.2.1.

3.5.3.1 Weight Loss

The weight loss values were determined as previously explained in section 3.5.1.1.

3.5.3.2 Rate of Mass Reduction of Microplastics

Rate constant of biodegradation and half-life of PP and PS microplastics were calculated as described in section 3.5.1.2.

3.5.3.3 Fourier Transform Infrared Spectroscopy

The FTIR analysis was performed on all samples as elucidated in section 3.5.1.3.

3.5.3.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed on only one sample as mentioned in section 3.5.1.4. The selection of samples for SEM was based on highest weight loss value and the most changes observed in FTIR Spectra.

3.5.3.5 3.6 Statistical Analysis

For statistical analysis, t-test was performed in Excel 2016 software to determine statistically significant differences between residual mass of microplastics from control and treatment. Additionally, for experiments focused on different concentration of applied inoculum and different sizes of microplastics, Analysis of Variance (ANOVA) was performed in Excel 2016 software on residual mass of microplastics from treatment

to determine the statistically significant difference between different inoculum concentrations applied and different sizes of microplastics, respectively.

Lastly, in addition to calculation of rate of mass reduction using formula mentioned in section 3.5.3.2, trend-line analysis was also carried out in Excel 2016 software of treated microplastics from all three biodegradation experiments to determine biodegradation rate. Average residual values were plotted on the y-axis and sampling intervals were plotted on the x-axis for trend-line analysis.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Soil Analysis

4.1.1 Bacterial Biodegradation by Increased Frequency of Inoculum Input

The changes in pH of treatment and control soil are displayed in Figure 4.1.

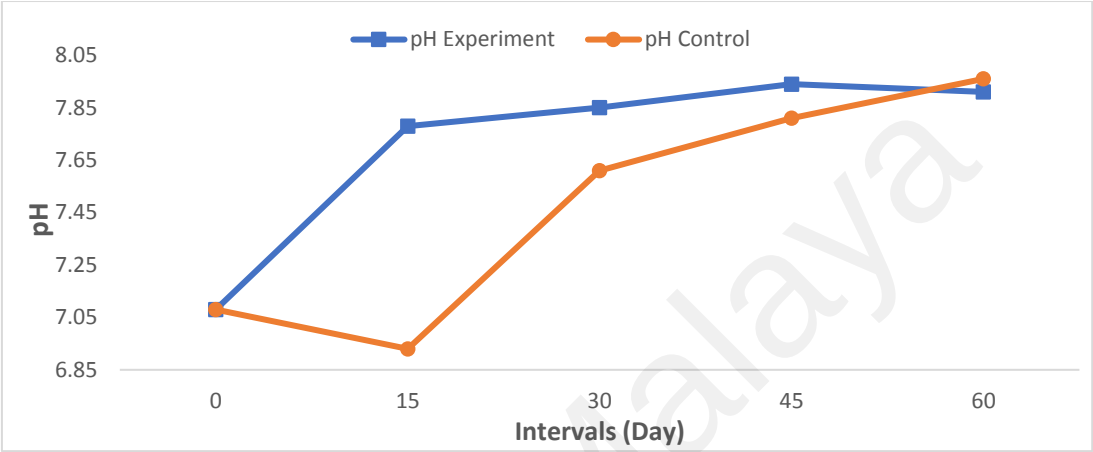


Figure 4.1: pH changes observed in treatment and control soil

The final pH of treatment soil increased by 11.4% at the end of experiment (90 days). It was neutral at the start of the experiment and then became slightly basic. This increase in soil pH could be due to ammonification of nitrogen as a result of microbial activities (Esmaeili *et al*., 2013; Zahra *et al*., 2010).

There was sharp increase in pH of treatment soil from 0-day (pH 7.08) to 15-day (pH 7.78). Then a gradual increase was observed for next two intervals and followed by a decrease in pH from pH 7.94 to pH 7.91 at the end of experiment. This slight decrease in pH of the treatment set-up from 45th day to 60th day of trial could be due to enzymatic activities of bacterial consortium applied. Enzymatic activities would have led to production of organic acids as pH 6 – 6.5 were reported in biodegradation of HDPE (Orhan *et al*., 2004).

Even though, pH of control set-up increased from the beginning of the experiment to the end, but the trend was different for the treatment set-up. Control soil became slightly

acidic from neutral at 1st interval (day 15). Then the pH of control soil increased by 12.4% at the end of experiment, pH 7.96. However, no statistically significant difference was found between pH of control pH 7.48 ± 0.45 and treatment pH 7.71 ± 0.36 soil, $t(8) = 0.91$, $p = 0.39$.

Table 4.1 enlists the values of TOC, TN and TP along with other characteristics of soil such as pH and moisture content. Generally, plant assimilation and mineralization by microbes may cause the changes in the nutrients in the environment (Alongi, 1996). Since there were no plants in the experimental set-ups, any changes in soil nutrients may have been due to microbial activities.

Table 4.1: Physico-chemical characterization of treatment and control soil at 0 Day and 60 Day

Parameters	**Treatment**			**Control**		
	0 Day	**60 Day**	**% Change**	**0 Day**	**60 Day**	**% Change**
pH	7.08	7.91	+ 11.7 %	7.08	7.96	+ 12.4 %
Moisture Content %	49.5	45.0	- 9 %	49.5	45.8	- 7.48 %
TN mg/kg	3220	1580	- 50.9 %	3220	1352	- 58 %
TP mg/kg	320	620	+ 93.6 %	320	590	+ 84 %
TOC %	1.9	2.9	+ 52.6 %	1.9	3.6	+ 89.5 %

TN decreased at the end of the trial in both control and treatment soil. This reduction of 51 and 58% of TN in treatment and control soil, respectively might be due to ammonification of nitrogen as aforementioned (supported by increase in soil pH over time as well).

On the contrary, an increase of 84% and 94% in TP and increment of 89% and 53% in TOC was observed for control and treatment soil, respectively at the end of experiment. However, TOC value was higher in control soil as compared to treatment soil, whereas TN and TP values were higher in treatment soil in contrast to control soil. TOC could have increased due to the daily input of nutrient broth in control and inoculum (bacterial consortium was grown in nutrient broth) in treatment. The amount of TOC in the control

set-up was higher probably because a smaller number of microbes were using the nutrients. Whereas, bacteria in the treatment inoculum would have depleted the nutrients and hence the lower amount of TOC in the treatment soil. On the other hand, the increase in TP could be due to increase in biomass in the soil (Oje *et al*., 2015). Relatively higher value of TP in treatment than control would be due to input of inoculum in treatment, since inoculum contained higher population of bacteria. The moisture content recorded ranged at 45 – 49%.

Microbial count of both treatment and control soil was determined at every 15th day of the experiment. Figure 4.2 shows the microbial count of treatment and control with respect to sampling intervals. The number of bacterial colonies in both treatment and control soil decreased with time.

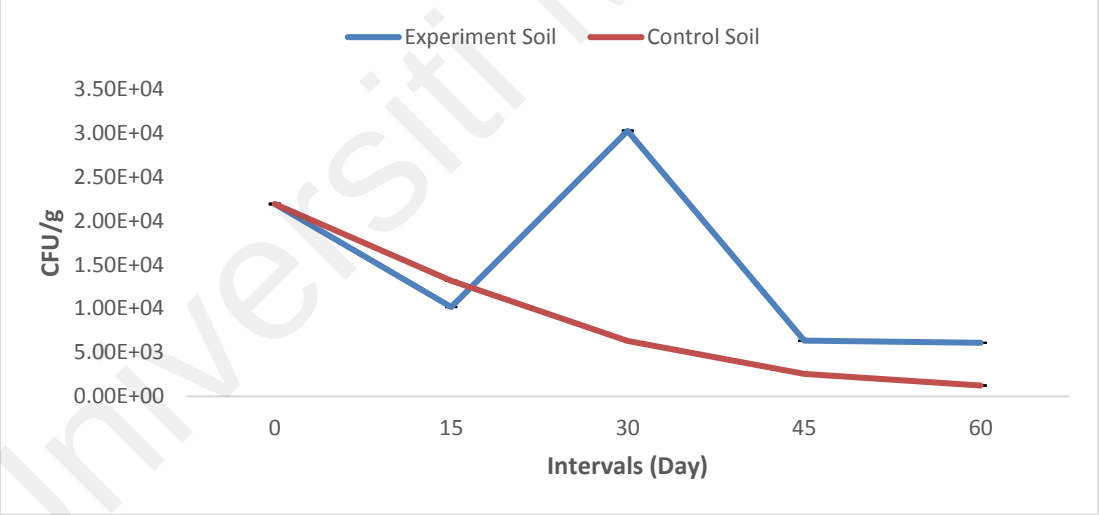


Figure 4.2: Bacterial population profile in control and treatment soil (n = 3)

Overall, microbial count in the treatment soil was higher than in control soil throughout the experiment except at 15-day interval. Relatively higher concentration of viable bacterial count in the treatment soil could be due to utilization of carbon and energy from provided microplastics (Kyaw *et al*., 2012). The period from day 0 to day 15 can be associated with lag phase of bacterial growth. Afterwards, microbes induced into the treatment tub reached exponential phase from 15th day of experiment to the 30th day of

experiment and immediately followed by death phase at 45th day of experiment, where number of bacterial colonies decreased substantially. Perhaps a short stationary phase would have occurred between 30th and 45th day of experiment. On the other hand, number of bacterial colonies in the control soil kept decreasing with time until the end of the trial.

4.1.2 Bacterial Biodegradation at Different Inoculum Concentration

At the beginning of the study, pH was slightly acidic (pH 6.8) for all three experimental set-ups; 1% inoculum, 0.5% inoculum and 0.25% inoculum set-up. Then, pH began to increase over the period of 90 days, becoming more alkaline from the acidic state. Moreover, in all three set-ups, pH in control soil was higher than pH in treatment soil. Figures 4.3, 4.4 and 4.5 display the pH changes in 1%, 0.5% and 0.25% inoculum set-up over six intervals.

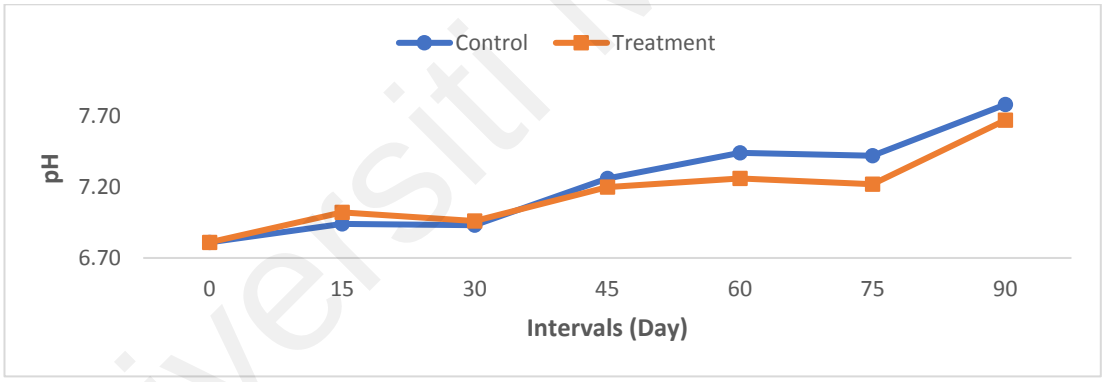


Figure 4.3: pH changes observed in treatment and control soil with 1% inoculum concentration

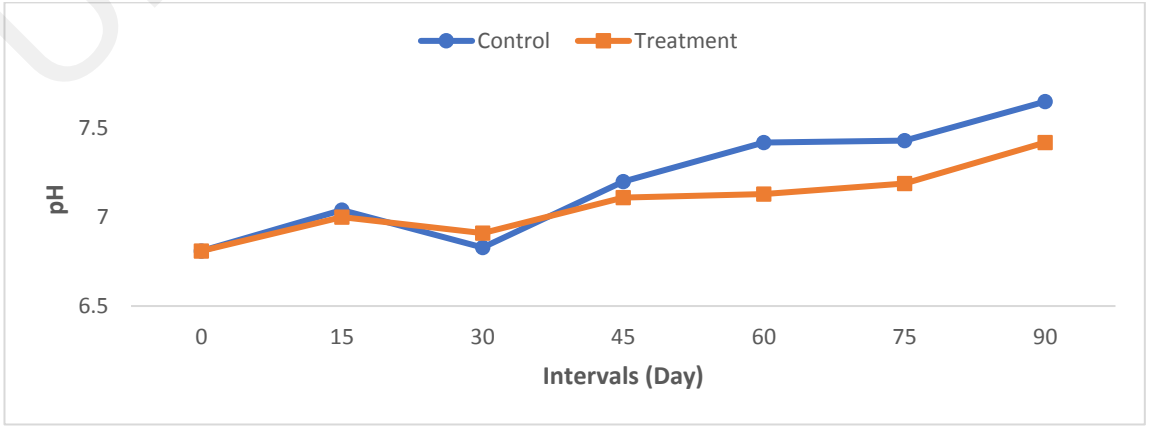


Figure 4.4: pH changes observed in treatment and control soil with 0.5% inoculum concentration

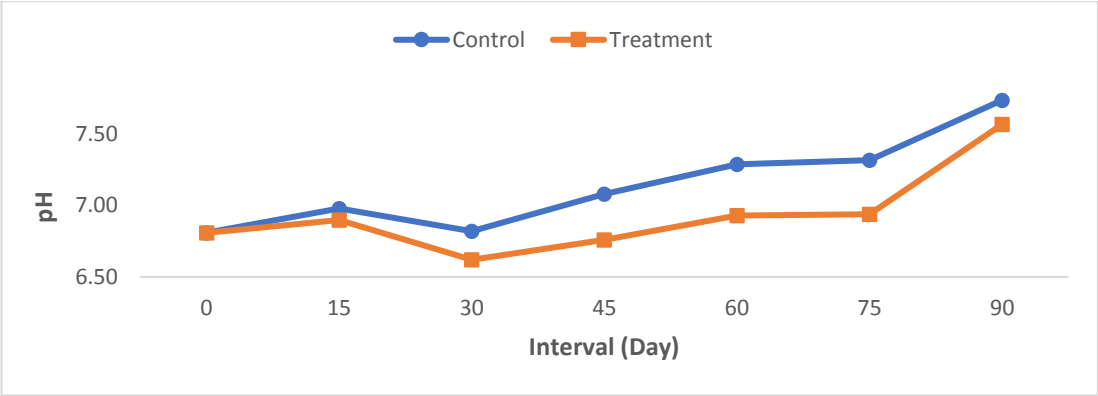


Figure 4.5: pH changes observed in treatment and control soil with 0.25% inoculum concentration

The values of soil pH are higher than those reported in most biodegradation studies of synthetic polymers. Generally, microbial activities pertaining to biodegradation of synthetic polymers result in generation in organic acids which lowers the pH of medium, either liquid or soil, (Ghorpade *et al*., 2001). However, this could be the reason of relatively lower pH values of treatment soil. A t-test performed on pH values of treatment and control soil revealed no statistically significant differences between pH of control and treatment soils. The values of selected soil parameters of treatment and control of 1%, 0.5% and 0.25% inoculum concentration set-up are given in Tables 4.2, 4.3 and 4.4, respectively.

Table 4.2: Physico-chemical characterization of treatment and control soil with 1% bacterial inoculum

Parameters	Treatment			Control		
	0 Day	90 Day	% Change	0 Day	90 Day	% Change
pH	6.81	7.67	+12.6 %	6.81	7.78	+14.2 %
Moisture Content %	48.73	44.09	-9.5 %	48.73	44.32	-9.05 %
TOC %	2	2.4	+20 %	2	2	0
TN mg/kg	1260	1100	-12.7 %	1260	1460	+15.8 %
TP mg/kg	340	1440	+323.5 %	340	2710	+697 %

Table 4.3: Physico-chemical characterization of treatment and control soil with 0.5% bacterial inoculum

Parameters	Treatment			Control		
	0 Day	**90 Day**	**% Change**	**0 Day**	**90 Day**	**% Change**
pH	6.81	7.42	+ 8.9 %	6.81	7.65	+12.3 %
Moisture Content %	48.73	42.35	- 13.1 %	48.73	41.52	-14.8 %
TOC %	2	2.4	+ 20 %	2	2	0
TN mg/kg	1260	1610	+ 27.8 %	1260	2020	+60.3 %
TP mg/kg	340	3600	+ 958.8 %	340	4460	+1211.7 %

Table 4.4: Physico-chemical characterization of treatment and control soil with 0.25% bacterial inoculum

Parameters	Treatment			Control		
	0 Day	**90 Day**	**% Change**	**0 Day**	**90 Day**	**% Change**
pH	6.81	7.57	+11.2 %	6.81	7.74	+13.6 %
Moisture Content %	48.73	44.02	-9.6 %	48.73	44.97	-7.7 %
TOC %	2	2.7	+35 %	2	2.2	+10 %
TN mg/kg	1260	1500	+19.05 %	1260	1850	+46.8 %
TP mg/kg	340	2240	+558.8 %	340	1940	+470.6 %

In treatment soil of 1% inoculum TOC, and TP increased at the end of experiment, while, TN decreased. Similar result was reported in the biodegradation study of polythene in soil medium where treatment soil had shown a decrease in TN whereas an increase in TP was recorded (Abdullahi & Saidu, 2013), which was associated with microbial activities on plastics. Thus, lower TN and higher TP values might indicate the increase of microbial activities to biodegrade the microplastics.

The values of primary nutrients, TOC, TN and TP, in soil with 0.5% and 0.25% inoculum increased at the end of experiment as well. However, the amount of TN, TP observed in control soil had shown greater increase. The percentage of TOC in the treatment and control soil is lower than those reported in other mangrove soils' suggesting that organic content had decomposed to greater extent (Barreto *et al*., 2016). Moreover, applied bacterial consortium in treatment would have consumed nitrates and phosphates in soil, which would have resulted in relatively lower values of TP, and TN in treatment soil as described by Odokuma and Okara (2005). However, it must be noted that

phosphate buffer solution (PBS) was introduced into the treatment (inoculated PBS) and control soil (pure PBS) at every interval. The composition of PBS is various phosphate salts and ammonium chloride. This may have led to extremely high TN and TP values in soil.

The viable bacterial count was higher in treatment soil as compared to control soil. While the introduction of bacterial consortium in the treatment would have resulted in higher total bacterial count than in control, the increase in bacterial growth in treatment soil over the span of trial, suggests that bacterial consortium had accustomed to the soil medium and their interaction with provided microplastics had been positive. Figures 4.6, 4.7 and 4.8 demonstrate the bacterial growth profile in 1%, 0.5% and 0.25% inoculum, respectively.

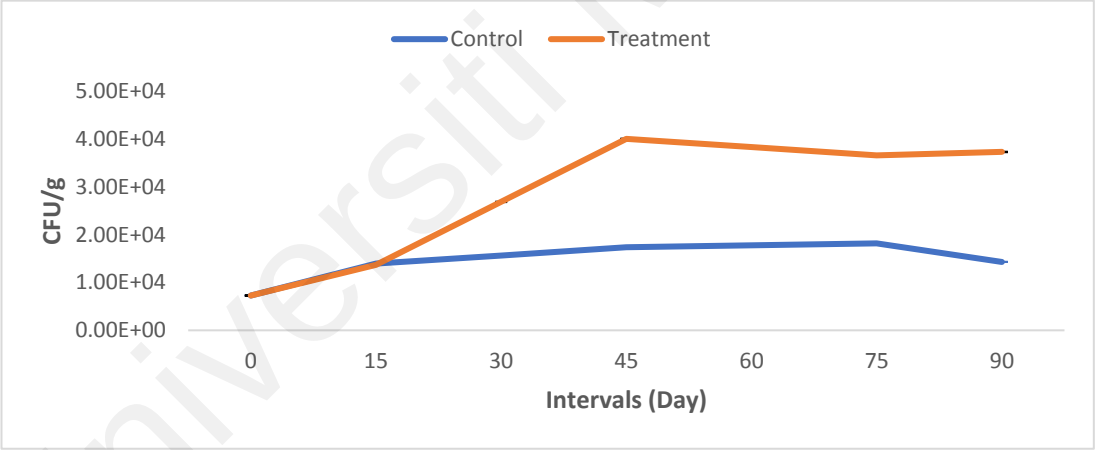


Figure 4.6: Bacterial population profile in control and treatment soil with 1% inoculum (n = 3)

In soil with 1% inoculum, the lag phase was observed within the first 15 days of experiment, where the bacterial population increased sharply from 15th day to 45th day highlighting the exponential phase of bacterial growth. From 45th day onwards until the end of experiment (90th day), the viable count of bacterial colonies remained steady, hence suggesting stationary phase of bacterial growth. Similar microbial growth profile was also reported by Mor and Sivan, (2008) in biodegradation study of plastic material.

On the other hand, microbial growth in the control soil remained lower than treatment soil and it stayed steady until it began to decrease from day 75th to 90th.

The bacterial growth profile of soil with 0.5% inoculum (Figure 4.7) shows continuous increase in bacterial colonies in contrast to 1% inoculum set-up. The bacterial colony forming unit (CFU) at the beginning of the experiment was 7.3×10^3 CFU/g and lag phase was observed from day 0 to 15 day, followed by gradual increase from day 15 until day 75. From day 75 to day 90, there was a sharp increase in the total bacterial count. At the end of the experiment, total bacterial count was 4.54×10^4 CFU/g. Thus, from day 15th until day 90, it can be inferred that exponential phase was observed. Similar logarithmic phase was also observed in biodegradation study of plastic (Kavitha *et al*., 2014). There was a steady increase in concentration of microbial colonies in control soil as well, despite the lower values of colony forming unit.

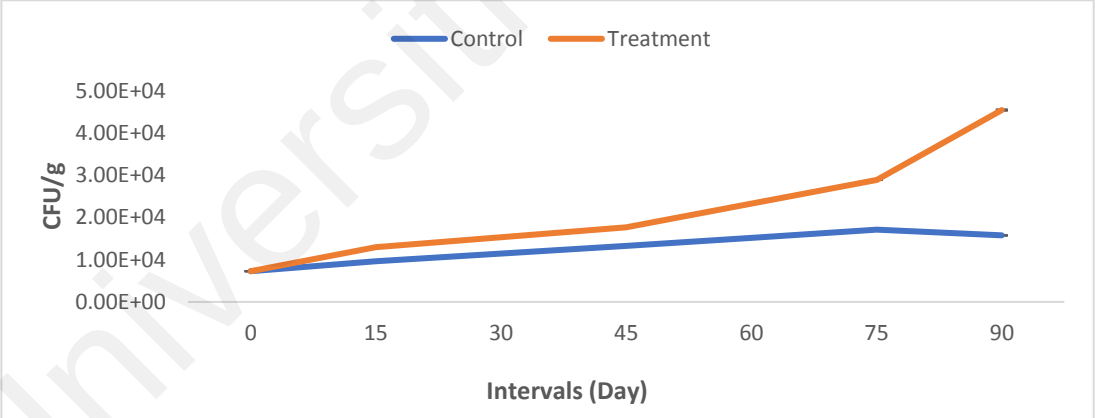


Figure 4.7: Bacterial population profile in control and treatment soil with 0.5% inoculum (n = 3)

In soil with 0.25% inoculum, bacterial growth did not increase as substantially as seen in 1% inoculum and 0.5% inoculum concentration (Figure 4.8). A decrease in bacterial growth was recorded from Day 15 to Day 45 but began to increase significantly after the Day 45. Thus, from the start of experiment until day 45, the lag phase was observed whereas, the second half could be associated with exponential phase.

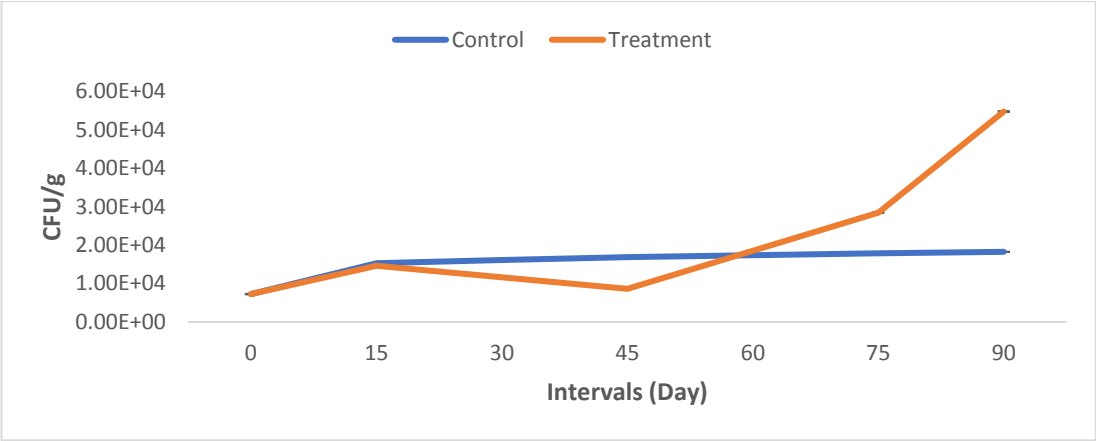


Figure 4.8: Bacterial population profile in control and treatment soil with 0.25% inoculum (n = 3)

The pattern that must be noted here is that with the increase in concentration of inoculum, bacterial growth had proliferated faster as three phases of bacterial growth were observed i.e. lag phase, exponential phase and stationary phase in soil with 1% inoculum. On the other hand, with decreasing concentration of inoculum, the growth of bacterial consortium was also hindered as lesser phases of bacterial growth were observed in 0.5% and 0.25% inoculum set-up. However, the CFU of 0.5% (4.5×104 CFU/g) and 0.25% (5.5×104 CFU/g) inoculum set-up were higher at 90th day than that of 1% (3.7×104 CFU/g) inoculum.

4.1.3 Different Size of Microplastics for Biodegradation

Gradual increase was observed in soil pH of control and treatment of experiment with 1 – 4 mm^2 microplastics. Both control and treatment soil were acidic at the beginning of the start of experiment (pH 6.81) and became slight basic at the end of experiment (> pH 7.5). However, unlike other experimental set-ups, the pH of treatment soil was relatively higher than control soil throughout the trial period (Figure 4.9). At the end of the trial, pH of treatment soil was pH 7.53 and while the control soil was pH 7.35.

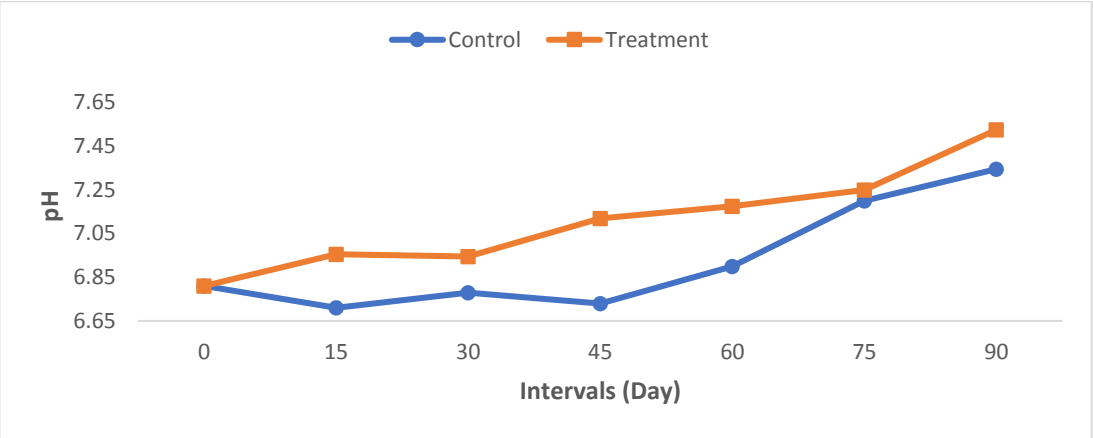


Figure 4.9: pH changes observed in treatment and control soil with 1 – 4 mm^2 microplastics

On the contrary, for the experiment with 9 mm^2 microplastics, pH of the control soil was higher than the treatment soil (Figure 4.10). Nevertheless, similar pattern was observed in terms of changes in soil pH where soil started with acidic nature and became basic as the experiment continued for 90 days. At the end of the experiment, pH of control soil was pH 7.65 and pH 7.42 for the treatment soil.

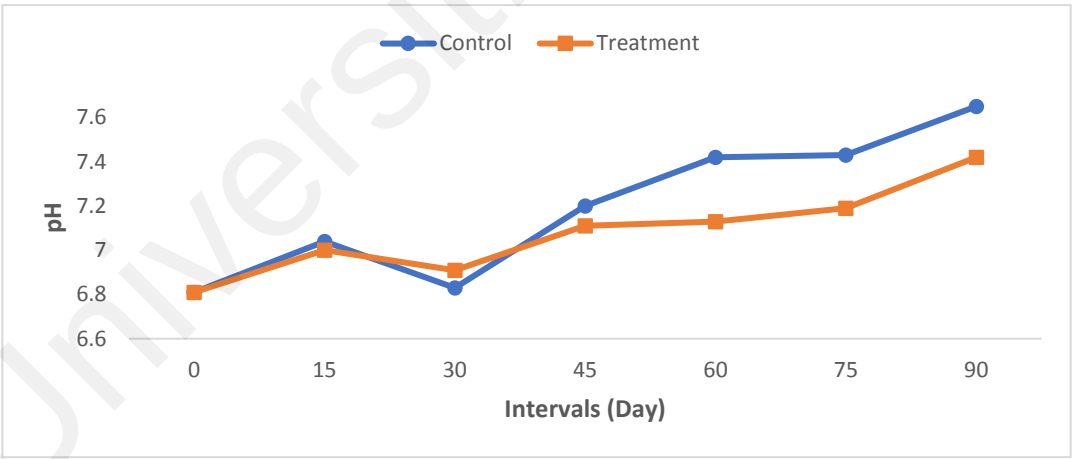


Figure 4.10: pH changes observed in treatment and control soil with 9 mm^2 microplastics

Experimental set-up with 25 mm^2 microplastics also showed the similar pattern of increase in pH as the biodegradation experiment went on (Figure 4.11). Control soil had higher pH at the end of experiment (pH 7.65), as compared to treatment soil (pH 7.42). In this experiment, the overall pattern of pH changes over time is similar to throughout

the study. The pH of soil becomes basic which could have been due to ammonification of nitrogen (Zahra *et al*., 2010).

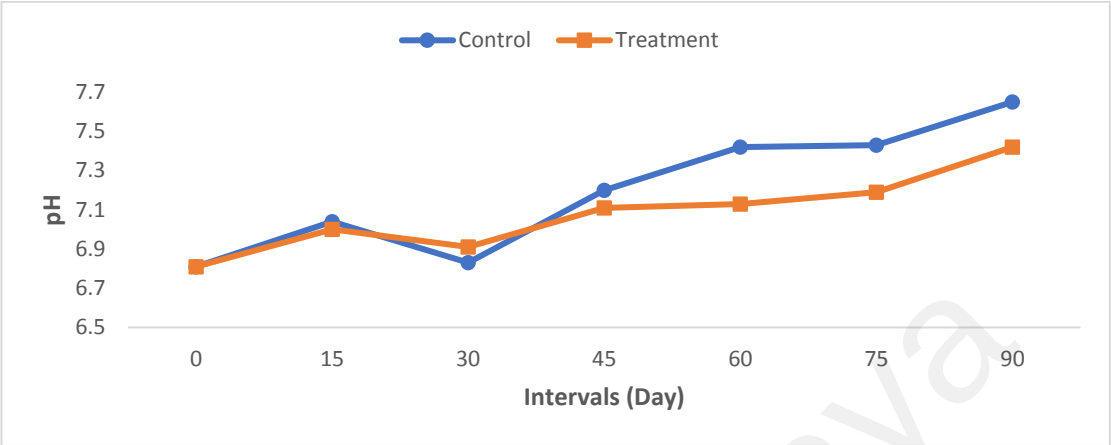


Figure 4.11: pH changes observed in treatment and control soil with 25 mm^2 microplastics

Based on the results obtained from t-test there was no statistically significant difference between pH of control and treatment soil of all three experiments with different concentrations; 1%, 0.5% and 0.25%.

The values of pH, MC, TOC, TN, and TP of each experimental set-up of 1 – 4 mm^2, 9 mm^2 and 25 mm^2 sized microplastics are given in Tables 4.5, 4.6 and 4.7, respectively. The values of TOC in all three experimental set-ups are lower than the typical values of TOC in mangrove soils (Adame *et al*., 2016). TN of both control and treatment soil increased at the end of the experiment. However, the value of TN for control was higher than that of treatment soil with different plastic. This increase in TN values indicates the mineralization of nitrogen and the favourable conditions for applied microbial consortium. Lower TN values suggest lower microbial count in the soil due to which lesser mineralization of nitrogen occurs (Abdullahi & Saidu, 2013). On the contrary, TP values are expected to decrease due to microbial activity, as microbes utilize phosphorous in the form of phosphates along with sulphates (Odokuma & Okara, 2005) which was not observed in the TP values of treatment and control soil. Nevertheless, the extremely high

values of both TP and TN could be due to the introduction of phosphate saline buffer at every interval of the experiment which among others was composed of phosphate salts and ammonium chloride.

Table 4.5: Physico-chemical characterization of treatment and control soil with 1 – 4 mm^2 microplastics

Parameters	Treatment			Control		
	0 Day	90 Day	% Change	0 Day	90 Day	% Change
pH	6.81	7.53	+ 10.6 %	6.81	7.35	+ 7.9 %
Moisture Content %	48.73	42.35	- 13.1 %	48.73	41.52	- 14.8 %
TOC %	2.0	2.3	+ 15 %	2.0	2.5	+ 25 %
TN mg/kg	1260	1600	+ 26.9 %	1260	1815	+ 44 %
TP mg/kg	340	3730	+ 997 %	340	3320	+ 876.5 %

Table 4.6: Physico-chemical characterization of treatment and control soil with 9 mm^2 microplastics

Parameters	Treatment			Control		
	0 Day	90 Day	% Change	0 Day	90 Day	% Change
pH	6.81	7.42	+ 8.9 %	6.81	7.65	+12.3 %
Moisture Content %	48.73	42.35	-13.1 %	48.73	41.52	-14.8 %
TOC %	2.0	2.4	+20 %	2.0	2.0	0
TN mg/kg	1260	1610	+27.8 %	1260	2020	+60.3 %
TP mg/kg	340	3600	+958.8	340	4460	+1211.7 %

Table 4.7: Physico-chemical characterization of treatment and control soil with 25 mm^2 microplastics

Parameters	Treatment			Control		
	0 Day	90 Day	% Change	0 Day	90 Day	% Change
pH	6.81	7.42	+9.9 %	6.81	7.65	+12.3 %
Moisture Content %	48.73	42.35	-13.1 %	48.73	41.52	-14.8 %
TOC %	2.0	2.4	+20 %	2.0	2.0	0
TN mg/kg	1260	1610	+27.8 %	1260	2020.0	+60.3 %
TP mg/kg	340	3600	+958.8	340	4460.0	+1211.7 %

Figures 4.12, 4.13 and 4.14 display the bacterial growth in control and treatment soil of experimental set-up with 1 – 4 mm^2, 9 mm^2 and 25 mm^2, respectively. The patterns of bacterial growth are similar in all experimental set-up. The treatment soil depicted gradual increase of bacterial growth over the period of 90 days. Exponential

phase started from 60-day onwards. On the contrary, the microbial growth of control soil showed relative decrease over time.

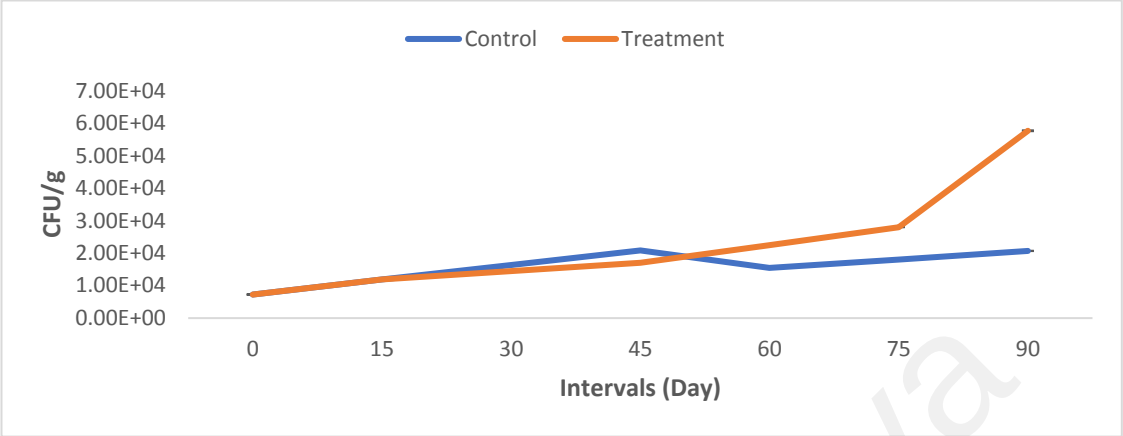


Figure 4.12: Bacterial population profile of control and treatment soil with 1 – 4 mm^2 microplastics experiment (n = 3)

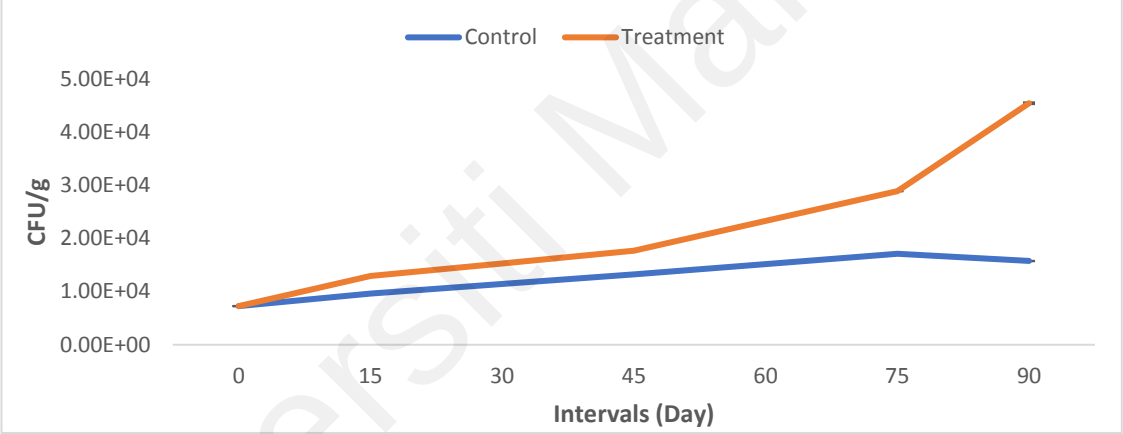


Figure 4.13: Bacterial population profile of control and treatment soil with 9 mm^2 microplastics experiment (n = 3)

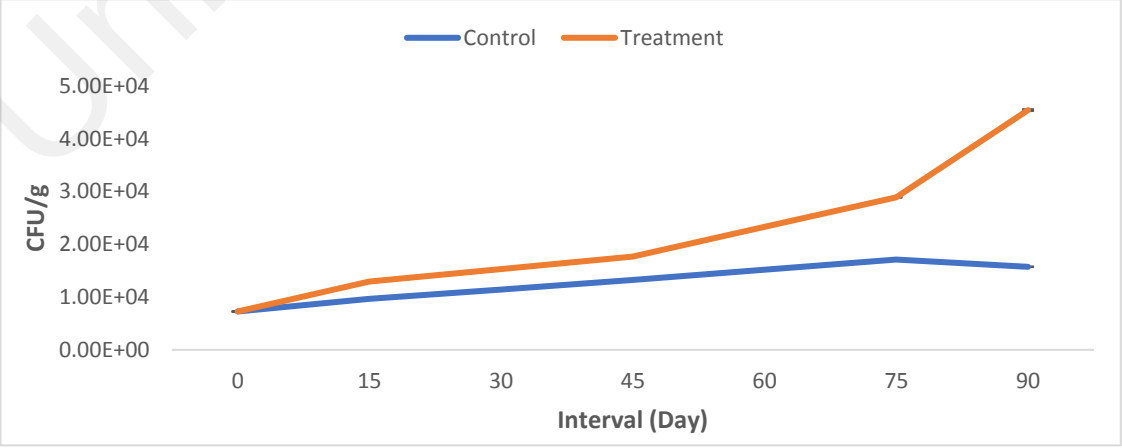


Figure 4.14: Bacterial population profile of control and treatment soil with 25 mm^2 microplastics experiment (n = 3)

4.2 Bacterial Biodegradation by Increased Frequency of Inoculum Input

4.2.1 High-Density-Polypropylene

The selected bacterial consortium was able to reduce the weight of High-Density-Polyethylene (HDPE) microplastic after 60 days' exposure. Figure 4.15 displays the weight loss percentage of HDPE. Among the three types of microplastics used, the highest reduction in weight was observed in HDPE, 1.26%. HDPE microplastics from control set-up also showed reduction in weight, albeit lesser weight loss than the weight loss of microplastic treated with selected bacterial consortium. Microbes in mangrove soil have shown capabilities to biodegrade synthetic polymers (Auta *et al*., 2018). Therefore, 0.57% of weight loss in HDPE could have been due to the presence of microbes in the mangrove soil used for experiment. However, as viable bacterial count of control soil continued to reduce throughout the experiment, this weight loss in microplastics from control cannot be associated with biodegradation until seen in conjunction with FTIR analysis.

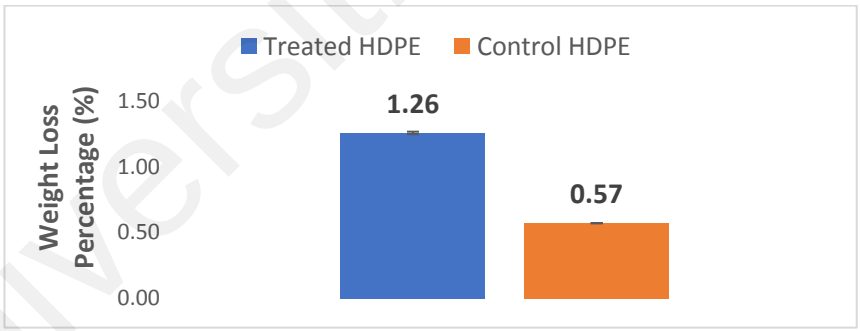


Figure 4.15: Weight loss of high-density-polyethylene (n=3)

The trend in weight loss of HDPE microplastic over 90 days in both treatment and control tub is similar (Figure 4.16). Weight of HDPE increased at the first interval and then, gradually decreased towards the end of the experiment. The weight of HDPE from treatment tub within 15 days increased to 1.004 g ± 0.008, then it reduced to 1.003 g ± 0.001, followed by 0.999 g ± 0.003 and 0.988 g ± 0.009. In the experiment of plastic biodegradation, selected microbes form colonies on the surface of plastic material will lead to biofilm formation (Usha *et al*., 2011). Hence, the slight increase in the weight

as observed in this experiment could be due to formation of biofilm on the surface of plastic material. Similar increase in weight of plastic was reported by Deepika and Jaya (2015) due to the formation of biofilm on plastic surface. The weight of HDPE microplastics from control tub was increased to 1.003 g ± 0.002 at interval 1 as well and then reduced to 1.001 ± 0.001, 0.999 g ± 0.002 and 0.994 g ± 0.001 at intervals 2, 3, and 4, respectively.

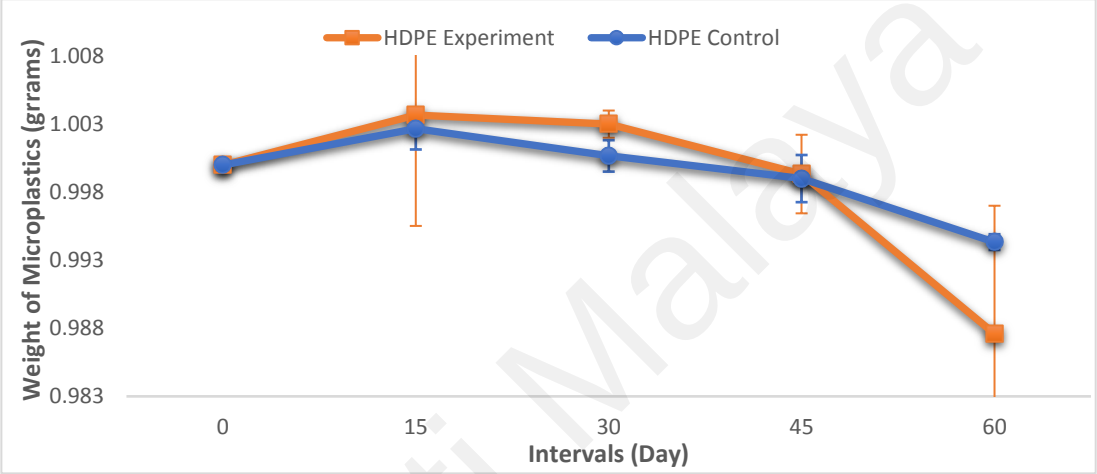


Figure 4.16: Trend of high-density-polyethylene microplastics weight over four intervals (n = 3) Bars represent standard deviation.

Yet from statistical analysis using t-test, there was no significant difference ($p > 0.05$) between treated microplastics, 0.999 g ± 0.004 and untreated microplastics 0.998 g ± 0.007 [$t(4) = -0.18$, $p = 0.86$]. This shows that statistically, introduction of bacterial consortium did not have any significant effect on the weight loss of microplastics.

Figure 4.17 displays the linear trend-line analysis of treated HDPE. It was performed to determine the biodegradation rate of treated microplastics. The R^2 value of 0.5 shows that data was moderately scattered. The slope of linear equation given in Figure 4.17, is the biodegradation rate as it predicts the changes in y-value (weight) over x-value (time). Hence, biodegradation rate of HDPE treated with daily input of bacterial consortium was calculated to be 1.9×10^{-4} g/day.

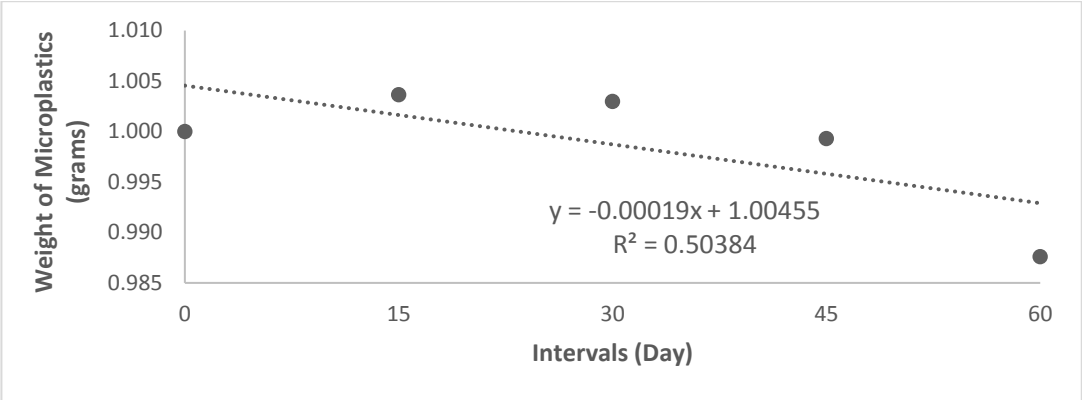


Figure 4.17: Linear Trend-line analysis of high-density-polyethylene microplastics treated with daily input of bacterial consortium

Since reduction in weight was observed in HDPE microplastics from both treatment and control, FTIR-ATR analysis was performed on raw microplastics, in addition to treated and untreated microplastics, so that any changes in FTIR spectra of control HDPE and treated HDPE could be examined by comparing the FTIR spectrum of raw HDPE. The FTIR-ATR spectra of raw, control and treated HDPE microplastic showed typical peaks at wavelength 2915 cm^{-1} and 2848 cm^{-1} which represented the asymmetrical and symmetrical stretch between C-H bond, respectively (Figure 4.18). Due to the closely packed methylene chains, methylene rocking vibration is split into two peaks manifested at wavelength of 731 cm^{-1} and 718 cm^{-1}. The broadening of bands between 1385 cm^{-1} and 1476 cm^{-1} and the formation of new peak at 876 cm^{-1} in FTIR spectra of control and treated HDPE shows biodegradation of HDPE. However, higher broadening of bands and formation of another peak at 1638 cm^{-1} in FTIR spectrum of treated HDPE represents greater level of biodegradation compared to control HDPE. Formation of new peaks at 876 cm^{-1} and 1638 cm^{-1}, as observed in this study as well, have been attributed to oxidation of polyethylene (Kavitha *et al*., 2014).

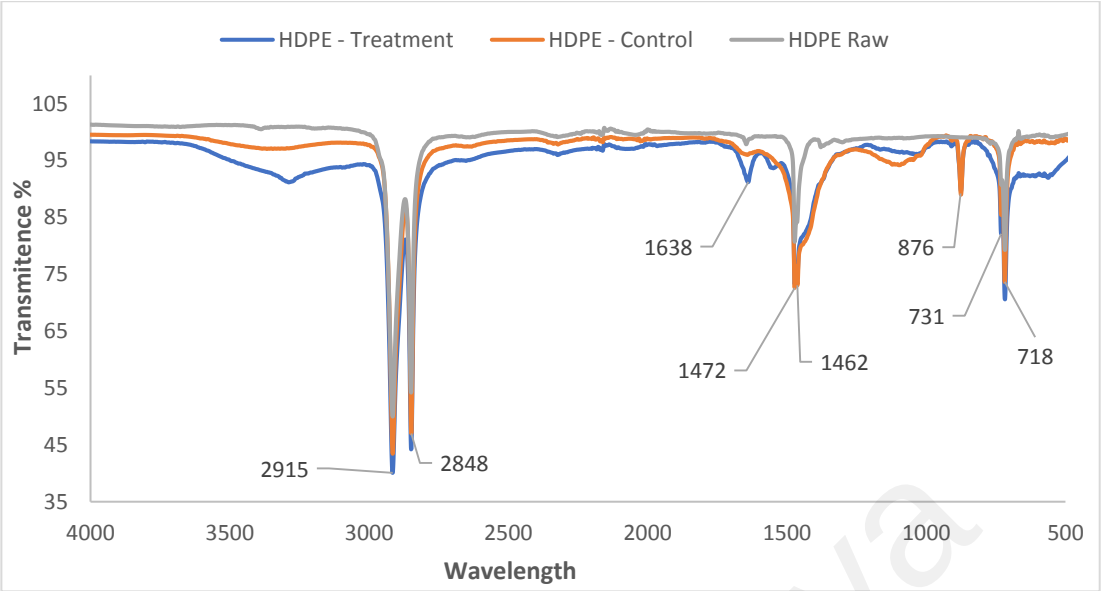


Figure 4.18: FTIR spectra of raw, control and treated high-density-polyethylene

The rate constant and half-life for treated HDPE was 0.0002 day^{-1} and 3448 days respectively. The highest rate constant was recorded for treated HDPE as higher weight loss was observed for treated HDPE. Consequently, half-life of treated HDPE is the shortest. Since reduction in weight and changes in FTIR spectrum of HDPE from control were also recorded, the rate constant and half-life values of control HDPE was determined. The rate constant of HDPE from control was 0.0001 day^{-1} and the half-life was 6931.5 days. The rate constant of control HDPE is slower, and the half-life is longer than the rate constant and half-life of treated HDPE. It suggests that daily input of bacterial consortium speeded up the process of biodegradation of HDPE in soil medium.

From this study, the increase in the incubation period of HDPE would have encouraged more biodegradation. This has also been proposed by Sivan (2011). But longer incubation time does not guarantee faster biodegradation rate as LDPE was the least biodegraded plastic at the end of the experiment (Sivan, 2011). Similar observation has also been reported for LDPE degradation in a six months' incubation (Chielinli *et al*., 2006). But, HDPE from experiment had shown affinity towards biodegradability due to introduction of selected bacterial consortium. While 1.26% of weight loss in two months may appear

to be meagre biodegradation capabilities of bacterial consortium, the weight loss findings are congruent with other biodegradation studies on HDPE. The biodegradation experiment of HDPE in liquid medium resulted in weight loss of 0.92% in two months, however after six months of incubation of HDPE, 7.8% of weight loss was reported by Ingavale and Raut (2018). It highlights the complexity and slow nature of biodegradation process of synthetic polymers as observed and recorded in this study. Similarly, only 3.86% of weight loss was recorded for HDPE in twelve months long experiment (Vijaya & Reddy, 2008) where 0% weight loss was recorded for HDPE after two months of incubation. 1.3% of weight loss was observed after nine months' incubation of HDPE in another experiment (Orhan *et al*. 2004).

There was no pretreatment of HDPE microplastics in this experiment as HDPE was only exposed to UV light for 15 minutes which was intended to disinfect microplastics (and sampling bag). Perhaps lack of pretreatment resulted in slow biodegradation of HDPE microplastics by bacterial consortium as biodegradation rate of HDPE calculated was 1.9×10^{-4} g/day. In contrast, thermal pretreatment of HDPE enhanced the biodegradation as 18.4% of weight loss was observed after incubation with *Klebsiella pneumoniae* CH001 in 60 days (Awasthi *et al*., 2017).

Nevertheless, in comparison with other studies, weight loss values along with changes in FTIR spectrum of treated HDPE hint towards increasing affinity to biodegradation by selected bacterial consortium.

4.2.2 Polypropylene

The selected bacterial consortium had also shown ability to reduce Polypropylene (PP) microplastic weight after 60 days of experiment. Figure 4.19 exhibits the weight loss percentage of PP. The weight of PP was reduced by 1.15% after 60 days of incubation.

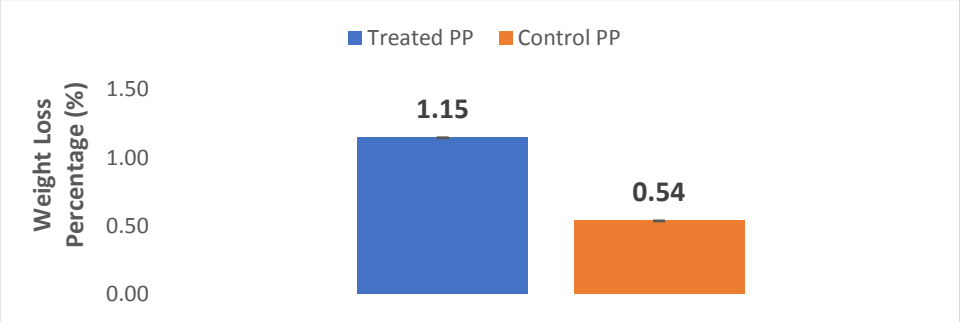


Figure 4.19: Weight loss of polypropylene (n=3)

However, PP microplastics from control also showed reduction in weight, as 0.54% of weight loss was recorded in control PP. Auta (2018) reported 16.4% and 19% weight loss for PET and PS microplastics buried in in situ setting in mangrove soil, respectively. Since, bacteria in selected bacterial consortium were also isolated from Malaysian mangroves, weight loss of 0.54% in control PP mass would have been the effect of native microbes. But as suggested earlier, the FITR spectra of control PP must reflect the changes associated with biodegradation and only weight loss may not be a true indication of biodegradation. As weight loss of 0.54% of control PP microplastics could have been due to loss of volatiles or soluble impurities as pointed out by Ho *et al.* (2017).

The trend in weight loss for treated PP is different from control PP over four intervals' time, as shown in Figure 4.20. A non-liner weight loss pattern was observed in treated PP recovered at every 15th day of experiment. Although, residual weight of treated PP was less than the initial weight at all four intervals which shows the advanced biodegradation capabilities of selected bacterial consortium. The weight of PP from treatment tub was recorded as 0.997 g ± 0.005, 0.990 g ± 0.013, 0.997 g ± 0.003 and 0.989 g ± 0.003 in intervals 1, 2, 3, and 4, respectively.

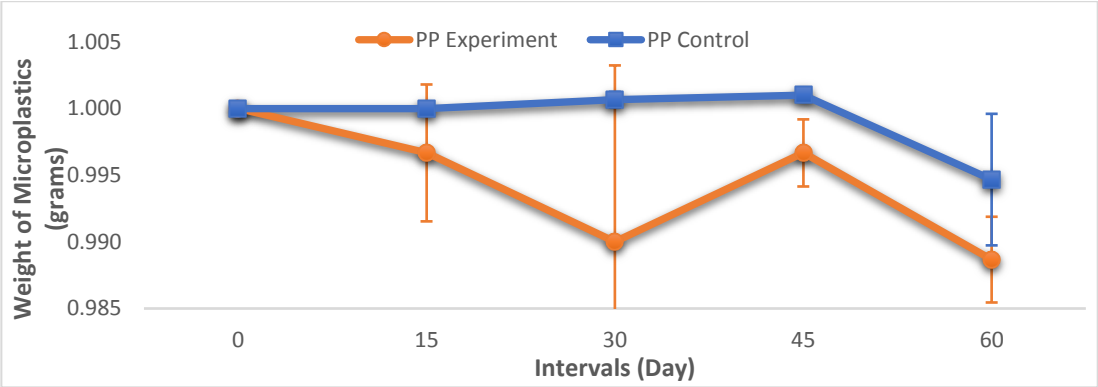


Figure 4.20: Trend of polypropylene microplastics weight over four intervals (n = 3) Bars represent standard deviation

Upon visual examination of PP microplastics, greater amount of biofilm formation was observed in treated PP since interval one as compared to other types of microplastics. The first step of biodegradation of plastic material is adherence of microbes on the surface of substrate upon contact, before consuming it for carbon and energy. Plate 4.1 shows the PP samples from trial (daily input of inoculum) pulled out at the end of experiment.

Plate 4.1: Biofilm formed on the treated polypropylene microplastics

This could have resulted in relatively faster weight loss of PP after the instigation of experiment in comparison to HDPE and PS. The recorded non-linear weight loss of PP microplastics in respective intervals in not uncommon, as it was also observed by Ingavale and Raut, (2018). The mass of PP from control tub seemed to increase slightly in the second and third intervals, then reduction in weight was observed in fourth interval.

No change was observed in weight of control PP at interval one. Slight increase in weight, 1.001 g ± 0.001, was observed at intervals 2 and 3, and followed by the decrease in weight, 0.995 g ± 0.005, at interval four. The loss of weight in control microplastics also highlights the biodegradation potential of native microbes in mangrove soil. Yet there is no significant difference between the two values ($p > 0.05$).

The values of residual weight of PP over time was moderately scattered as R^2 value was 0.55. It was due to non-linear reduction in weight of treated PP. Figure 4.21 displays the linear trend-line analysis of treated PP. The biodegradation rate of PP treated with daily input of bacterial consortium was calculated to be 1.5×10^{-4} g/day.

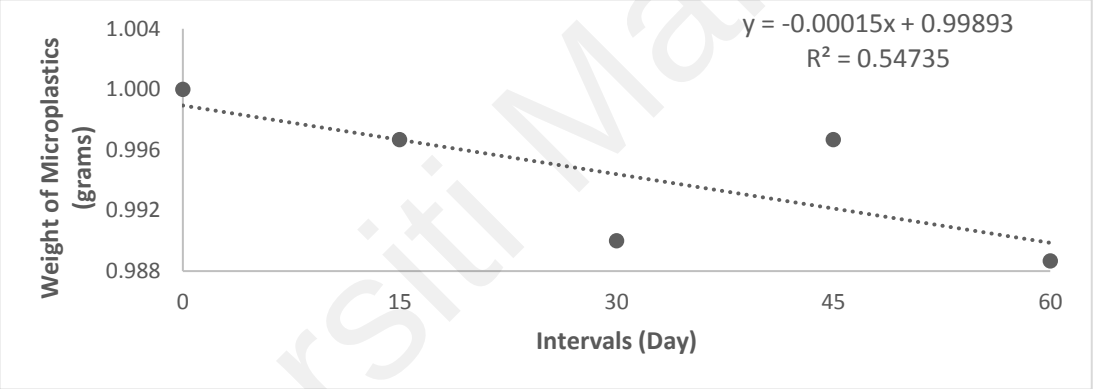


Figure 4.21: Linear Trend-line analysis of polypropylene microplastics treated with daily input of bacterial consortium

FTIR-ATR spectra of raw, control and treated PP showed typical peaks (Figure 4.22). Peaks at 2951 cm^{-1} and 2868 cm^{-1} are associated with asymmetrical and symmetrical vibrations of methyl group, respectively and the umbrella mode at 1375 cm^{-1}. On the other hand, peaks at 2918 cm^{-1} and 2839 cm^{-1} are associated with CH_2 stretching vibrations. Although there were no new peaks observed in the region between wavelength 969 cm^{-1} and 1110 cm^{-1}, reduction in peak intensity was observed among treated, control and raw PP samples. It suggests initiation of some chemical changes in treated PP. The spectrum of control PP is almost identical to spectrum of raw PP which suggests no chemical changes had occurred in control PP.

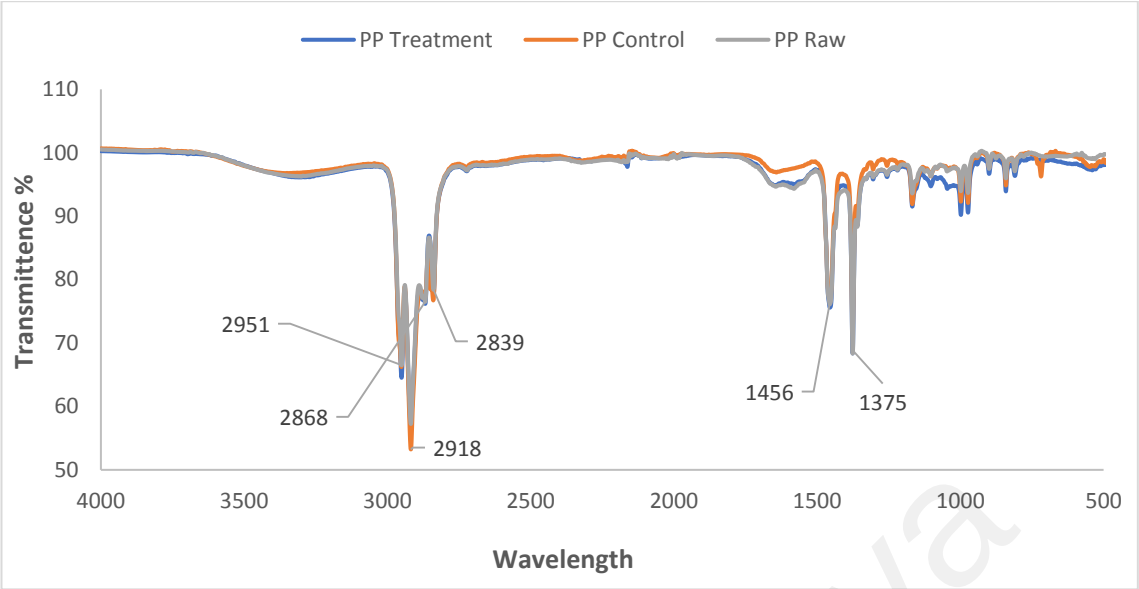


Figure 4.22: FTIR spectra of raw, control and treated polypropylene

The rate constant and half-life determined for treated PP was 0.00018 day^{-1} and 3767 days respectively. No changes in FTIR spectrum of control PP were observed, the rate constant and half-life were determined regardless. The rate constant and half-life of control PP was 0.000083 day^{-1} and 8351 days, respectively.

1.15% reduction in mass of treated PP after 60 days indicates biodegradation of PP microplastics. While less changes were observed for FTIR spectrum of treated PP, reduction in mass since the beginning of experiment indicates towards biodegradation as supported by reduction in pH, and higher bacterial growth in treatment soil. The decrease in TN values and increase in TP values at the end of experiment also supports the activity of bacterial consortium applied into treatment to biodegrade microplastics. Biofilm was also observed in all treated PP samples recovered from experiment.

The determination of protein content in or of biomass of biofilm was not selected for this study. Therefore, the formation of biofilm and subsequent weight loss observed in treated PP microplastics suggest that bacterial consortium had developed hydrophobicity to attach themselves to PP microplastics and utilize it for carbon and energy source. This has been reported in other findings (Arkatkar *et al*., 2010).

In this experiment, PP microplastics (inside sampling bags) were exposed to UV light for 15 minutes only to disinfect them. However, it is often suggested to pre-treat PP under UV light or increased temperature before deploying PP into the biodegradation experiment or addition of metal ions or starch to improve the affinity of PP for biodegradation (Jeyakumar *et al*., 2013). The daily input of bacterial consortium had resulted in weight loss of 1.15% in treated PP microplastics in two months' period of incubation. In comparison, in the abovementioned study, untreated PP had recorded less 5% weight loss in 12 months of incubation. Thus, with respect to commonly practiced prolonged pretreatment of PP with UV light, current study shows that by just increasing input of microbial consortium, the biodegradation of PP can be achieved.

4.2.3 Polystyrene

The least amount of weight loss was observed for Polystyrene (PS) treated with daily input of bacterial consortium for 60 days. Figure 4.23 displays the weight loss percentage of treated and control PS. Only 0.54% percentage of weight reduction was recorded for PS.

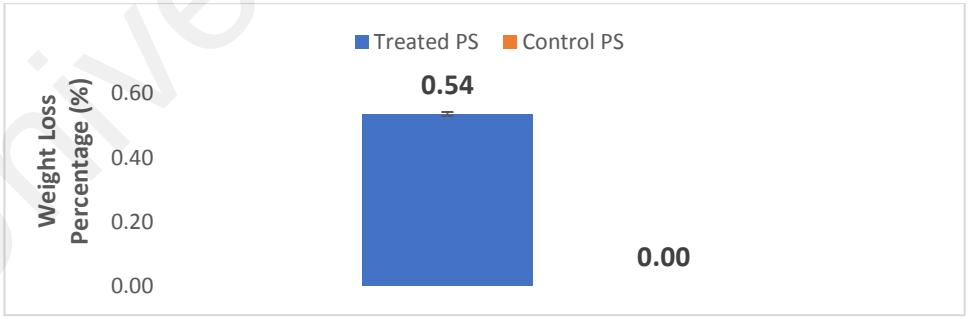


Figure 4.23: Weight loss of polystyrene (n=3)

The trend of residual weight of PS from treatment and control is shown in Figure 4.24. The control PS samples showed no change in weight for first two intervals, then slight increase in weight was observed in samples from third interval, 1.001 g ± 0.001 and no change in weight was observed from fourth interval 1.000 g ± 0.006. However, PS samples from treatment showed non-linear pattern. There was an increase in weight of

PS samples recovered from 1 interval, 1.004 g ± 0.003, that probably suggested formation of biofilm on PS surface. Then, subsequent relative decrease in weight was observed in PS samples pulled from 2nd interval, 1.002 g ± 0.001. But PS samples recovered from 3rd interval recorded an increase in weight, 1.003 g ± 0.002. Whereas, reduction was observed in weight of PS recovered from 4th interval, 0.995 g ± 0.006. This non-linear trend of weight loss has also been reported by Sudhakar *et al*., (2008).

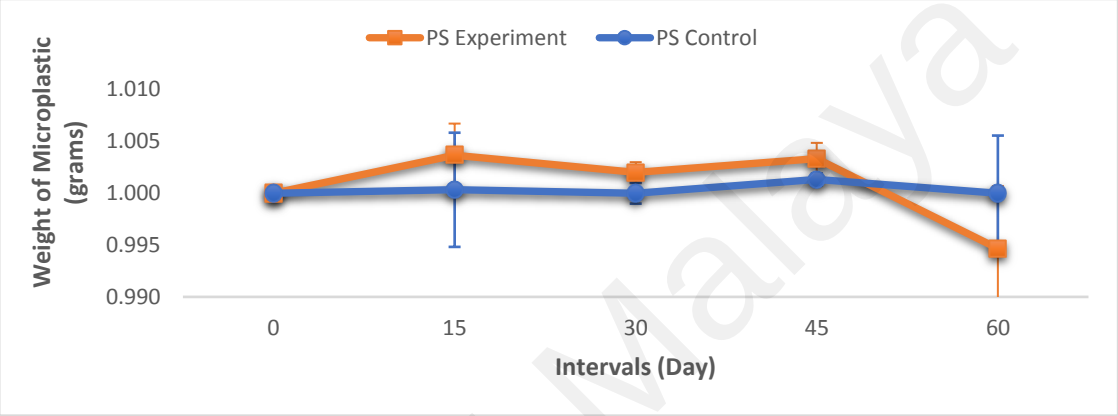


Figure 4.24: Trend of polystyrene microplastics weight over four intervals (n = 3) Bars represent standard deviation.

No statistically significant difference ($p > 0.05$) existed between treated PS microplastics, 1.001 g ± 0.004, and control PS microplastics, 1.000 g ± 0.001 from the t-test. Based on this statistical analysis, treatment of daily input of bacterial consortium had no effect on biodegradation of PS microplastics.

The values of residual weight of PS over four intervals were highly scattered as R^2 value was 0.22. Nevertheless, the biodegradation rate of PS treated with daily input of bacterial consortium was 6.9×10^{-5} g/day. The trend-line analysis of treated PS is presented in Figure 4.25. The lowest biodegradation rate of PS highlights the higher recalcitrant characteristics of PS to biodegradation (Sivan, 2011).

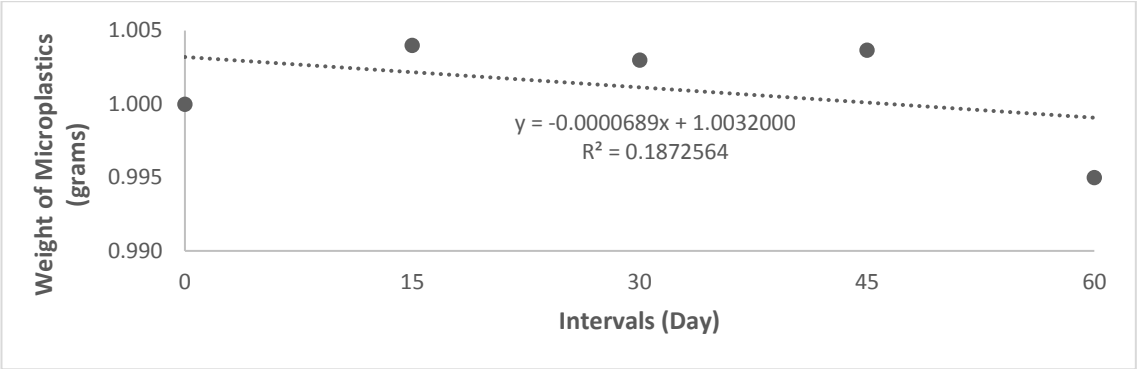


Figure 4.25: Linear Trend-line analysis of polystyrene microplastics treated with daily input of bacterial consortium

The FTIR-ATR spectra of raw, control and treated PS are shown in Figure 4.26. Peaks at wavelength 2922 cm^{-1} and 2854 cm^{-1} are associated with asymmetrical and symmetrical stretches of methylene. Whereas, peaks between 3024 cm^{-1} and 3083 cm^{-1} are attributed to a group of aromatic C-H stretches. Benzene ring modes are observed at peaks 1603 cm^{-1} and 1493 cm^{-1}. Peaks at 752 cm^{-1} and 696 cm^{-1} are associated with out-of-plane C-H bend of aromatic ring hydrogens and ring-bending vibration, respectively. The intensity of bands in treated PS decreased as compared to raw PS and control PS which suggests chemical changes occurring on the surface of PS microplastics. Similar decrease in intensity of treated PS spectrum had been interpreted as biodegradation of PS (Syranidou *et al*., 2017; Mohan *et al*., 2016).

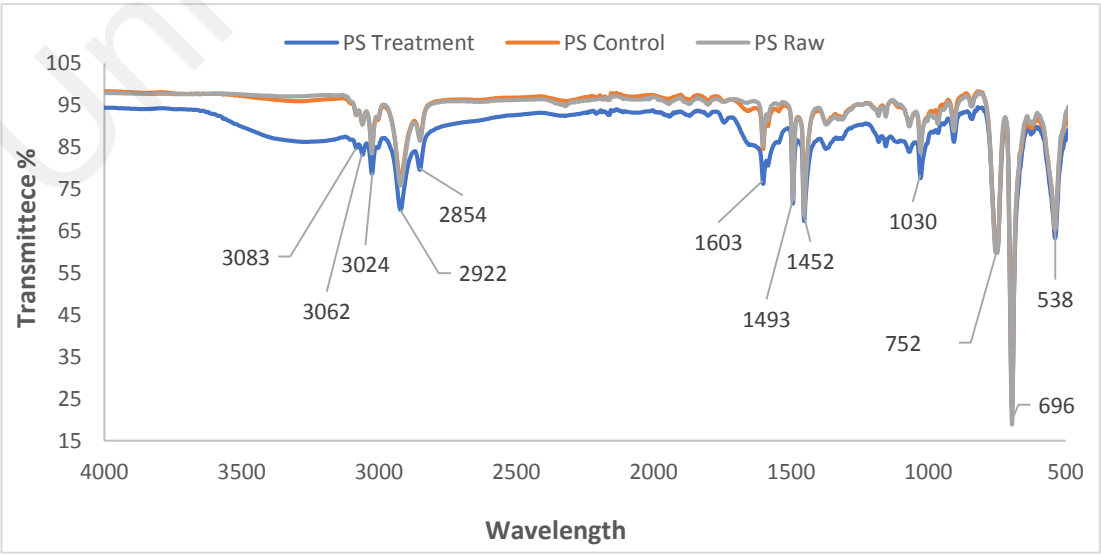


Figure 4.26: FTIR spectra of raw, control and treated polystyrene

The rate constant and half-life were also calculated for treated PS. The rate constant of treated PS was 0.00008 day^{-1} and the half-life based on rate constant was determined to be 8351 days. Since no weight loss was observed for control PS, its rate constant and half-life could not be determined.

From this study, PS was considered to be highly resistant to microbial attack. Therefore, the lower percentage of weight loss (0.54%) was recorded for PS at the end of incubation of 60 days regardless of the daily application of inoculum. Biofilm was also detected in PS as well due to increase weight of microplastics and supported by visual observation. Weight loss of 0.54% and reduced intensity in FTIR spectrum of treated PS present a noteworthy result in comparison to results by Mor & Sivan (2008). It was reported that despite the recalcitrant nature of PS to biodegradability, carbon starved environment induces microbes to adhere to the surface of PS and thus utilize it for carbon and energy source. Carbon starved conditions resulted in decline of planktonic population of C208 but it was able to adhere and sustain biofilm on the surface of PS (Mor & Sivan, 2008). However, in this study bacterial consortium was grown in nutrient broth and it was introduced into experiment. TOC values of treatment soil were 53% higher at the end of experiment.

It suggests that bacteria can adhere to PS surface and utilize it for carbon and energy source even in the presence of carbon source. Sekhar *et al.* (2016) also reported that addition of carbon source (gelatin) improved the biodegradation of PS. Greater weight loss of PS has also been reported by authors which highlights the biodegradability of PS. For instance, 2.5% of weight loss was recorded for PS macroplastic after incubation of four months in mangrove soil (Asmita *et al*., 2015).

4.3 Bacterial Biodegradation by Different Inoculum Concentration

4.3.1 Polypropylene

The percentage weight loss of PP from the application of different concentrations of bacterial inoculum is shown in Figure 4.27. Maximum percentage of weight loss, 0.43%, was observed in PP treated with 0.5% inoculum concentration. Whereas, comparatively minimum percentage of weight loss, 0.23%, was observed in PP treated with both 1% and 0.25% inoculum concentration. These lower weight loss values of treated PP microplastics are possibly due to highly recalcitrant nature of polyolefin (PE and PP) which are also considered non-biodegradable (Sivan, 2011). As aforementioned, either pretreatment with UV light for extended period of time or blending PP with starch, make conditions favourable for microbes to attack. Without the blending of starch, *Pseudomonas aeruginosa* only resulted in 0.08% of weight loss in PP at the end of six months' incubation (Khoramnejadian, 2013), which is even lower value than observed in this experiment.

The residual mass of PP microplastics from control was also found to be less than initial mass of 1 gram. Only 0.03% of weight loss was observed in control of 1% set-up. Whereas, 0.23% of weight loss was recorded for control of 0.5% set-up. Lastly, 0.167% of weight loss was observed in control of 0.25% set-up. However, the weight loss of PP from control must be seen in conjunction with FTIR to delineate probable cause of weight loss.

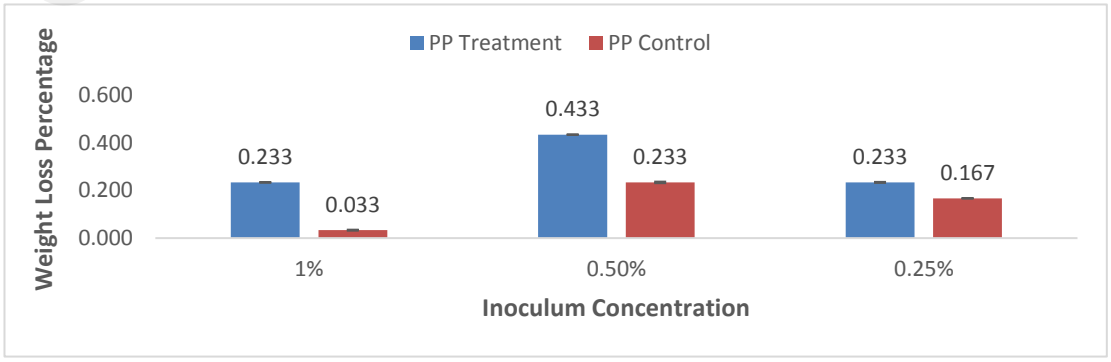


Figure 4.27: Weight loss of polypropylene microplastics treated with 1%, 0.5% and 0.25% inoculum concentration and untreated PP (n = 3)

As triplicates of microplastics were pulled out of experiment at every 15th day, the average of triplicates is plotted against six intervals to study the pattern of weight loss of microplastics. Figure 4.28 displays the residual weight of PP from 1% inoculum concentration set-up. The residual weight of PP microplastics from treatment shows slight reduction since the first interval. However, the reduction is linear with time. Highest weight loss was observed at 60th day, 0.997 g ± 0.002, whereas PP recovered at the end of experiment had residual mass of 0.998 g ± 0.002. PP microplastics recovered from control show maximum weight reduction of 0.999 g ± 0.001 at first interval and as well as at interval third, 0.999 g ± 0.005 and fifth, 0.999 g ± 0.001.

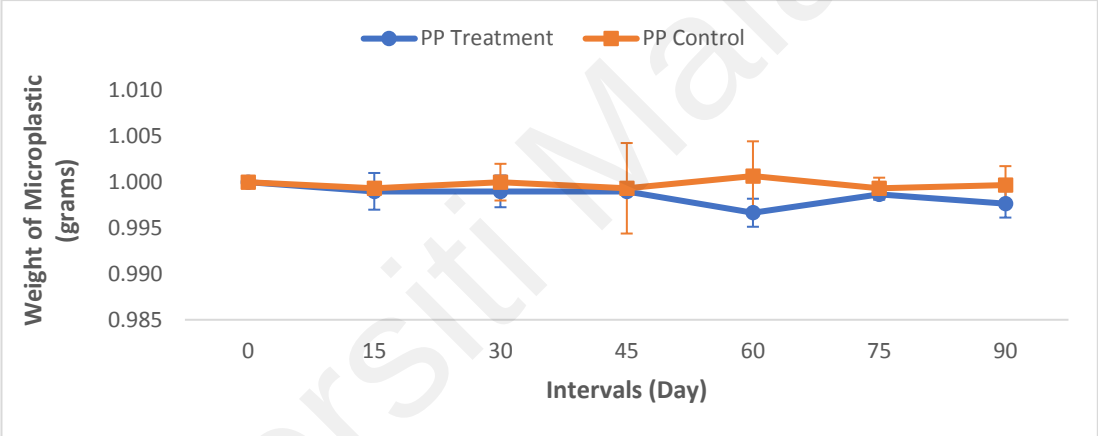


Figure 4.28: Residual weight of treated and control polypropylene from 1% inoculum set-up (n = 3)

For PP microplastics that was treated with 0.5% inoculum concentration, weight loss decreased with time (Figure 4.29). PP recovered at first interval from treatment showed increase in weight, 1.001 g ± 0.001. Afterwards, weight reduction was recorded in PP microplastics recovered from respective intervals, where relatively less weight reduction was observed at 5th interval 0.998 g ± 0.002. PP microplastics recovered from control also showed reduction in weight with time. However, no reduction in weight of microplastics recovered from 3rd and 4th interval was observed as compared to 2nd interval where microplastics recovered had mass of 0.999 g ± 0.001.

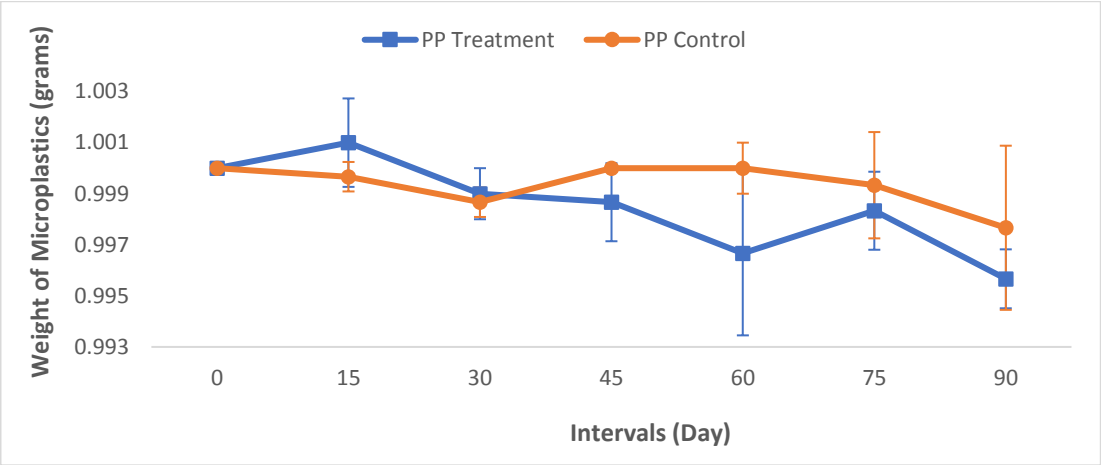


Figure 4.29: Residual weight of treated and control polypropylene from 0.5% inoculum set-up (n = 3)

The weight loss pattern for treated PP recovered from experimental set-up of 0.25% inoculum concentration is more erratic (Figure 4.30). Weight reduction was only observed in PP from 2nd interval (0.998 g ± 0.003), 4th interval (0.999 g ± 0.001), and 6th interval (0.998 g ± 0.002). Whereas, increase in weight was observed in PP from 3rd interval (1.002 g ± 0.002). PP microplastics pulled out from control show less erratic pattern. However, this pattern of non-linear weight loss is suspected to be due to close proximity of microplastics to each other, where microplastics pollution load is higher than inoculum input. This is so as concentration and subsequent activity of microbes are decisive factors in bioremediation (Kunioka *et al*., 2006).

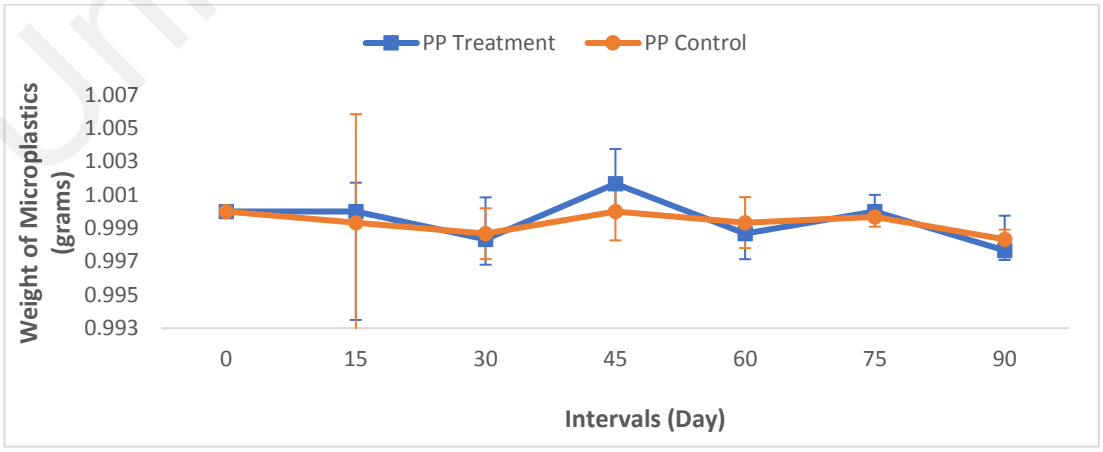


Figure 4.30: Residual weight of treated and control polypropylene from 0.25% inoculum set-up (n = 3)

Results obtained from t-test indicated there is a significant difference ($p < 0.05$) between final weight of untreated PP and treated PP with 1% inoculum treatment. On the other hand, for 0.5% treatment, no statistically significant difference ($p > 0.05$) was observed in residual mass of PP microplastics from control and treatment. Similarly, in 0.25% treatment, no statistically significant difference ($p > 0.05$) was observed in residual weight values of PP microplastics from control and treatment.

One-way ANOVA was also performed to compare the results of weight loss of PP microplastics treated with 1%, 0.5% and 0.25% inoculum. However, no statistically significant difference ($p > 0.05$) was found in biodegradation of PP microplastics when treated with different concentration of inoculum.

The biodegradation rate of PP microplastics treated with 1% inoculum of bacterial consortium was 2.4×10^{-5} g/day. However, R^2 value in Figure 4.31 is 0.5 highlighting that residual weight values of treated microplastics are scattered. It suggests that biodegradation rate of PP treated with 1% inoculum concentration is not straightforward.

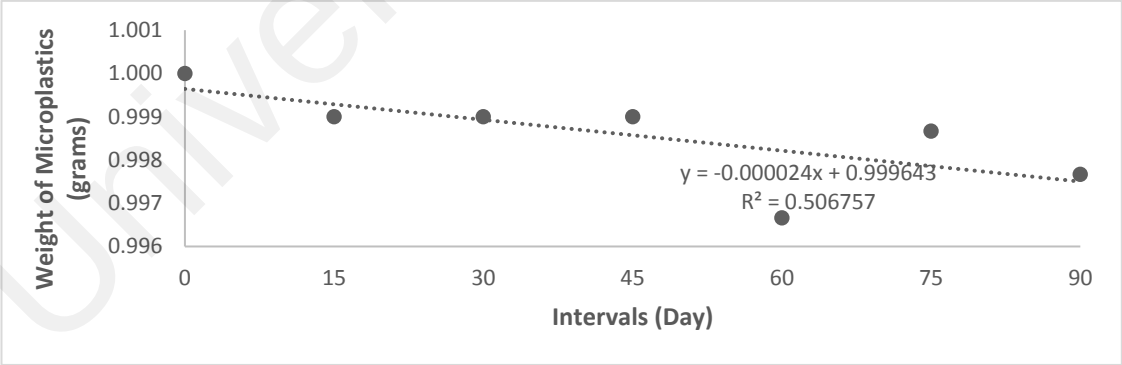


Figure 4.31: Linear trend-line analysis of polypropylene microplastics treated with 1% inoculum concentration

The residual mass values of PP microplastics treated with 0.5% inoculum concentration were relatively more clustered ($R^2 = 0.7$) around best fit line than PP treated with 1% inoculum of bacterial consortium shown above. The biodegradation rate of PP

treated with 1% inoculum concentration, signified by value of slope (m) was 5.0×10^{-4} g/day (Figure 4.32).

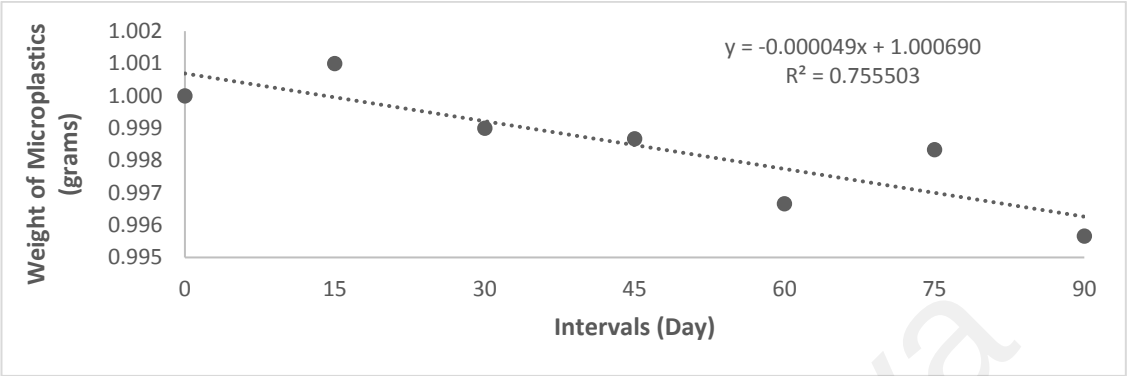


Figure 4.32: Linear trend-line analysis of polypropylene microplastics treated with 0.5% inoculum concentration

Lastly, the values of residual mass of PP treated with 0.25% inoculum concentration of bacterial consortium were highly scattered as shown by R^2 value which was 0.15 (Figure 4.33). The biodegradation rate of PP treated with 0.25% inoculum was 1.6×10^{-5} g/day.

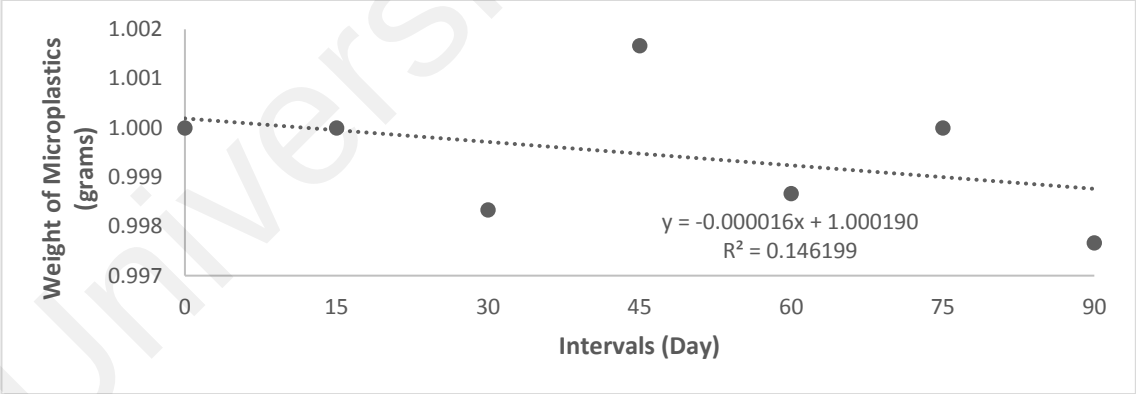


Figure 4.33: Linear trend-line analysis of polypropylene microplastics treated with 0.25% inoculum concentration

Typical peaks were observed in control and treated PP microplastics. The presence of methyl bands at wavelength of 2950 and 2867 cm^{-1} because of asymmetrical and symmetrical stretches and at 1375 cm^{-1} due to umbrella mode of methyl bands is shown in the PP FTIR spectra. On the other hand, asymmetrical and symmetrical stretches of methylene bands at 2919 and 2839 cm^{-1} is due to methylene groups. Figures 4.34, 4.35

and 4.36 display the FTIR spectra of control PP and PP treated with 1%, 0.5% and 0.25% inoculum concentrations, respectively. Both treated PP and control PP has identical FTIR spectra suggesting no changes in chemical structures of PP. Typical peaks of PP are also present in FTIR spectra of treated PP hint at intact methylene and methyl groups. Based on FTIR spectra, bacterial consortium at different concentration have no significant degradation.

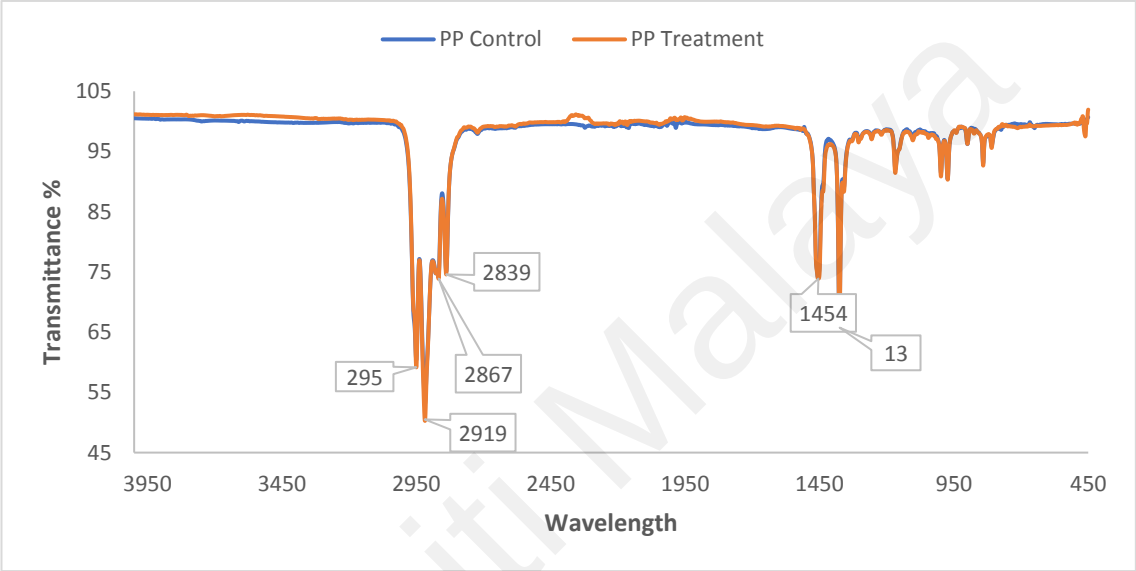


Figure 4.34: FTIR spectra of control and treated polypropylene with 1% concentration of bacterial consortium

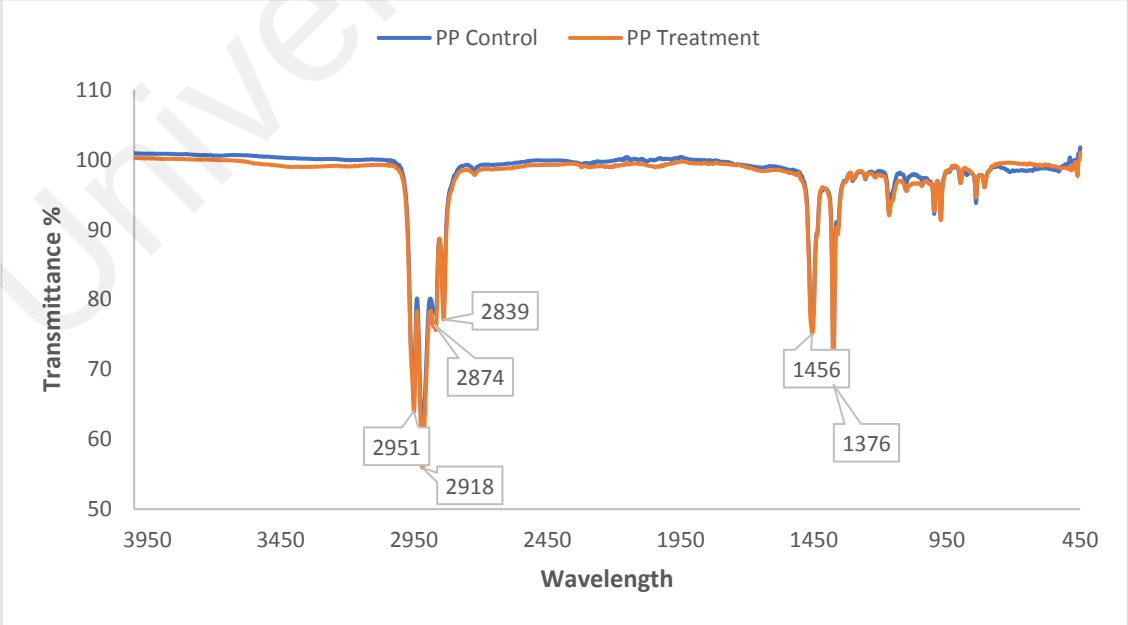


Figure 4.35: FTIR spectra of control and treated polypropylene with 0.5% concentration of bacterial consortium

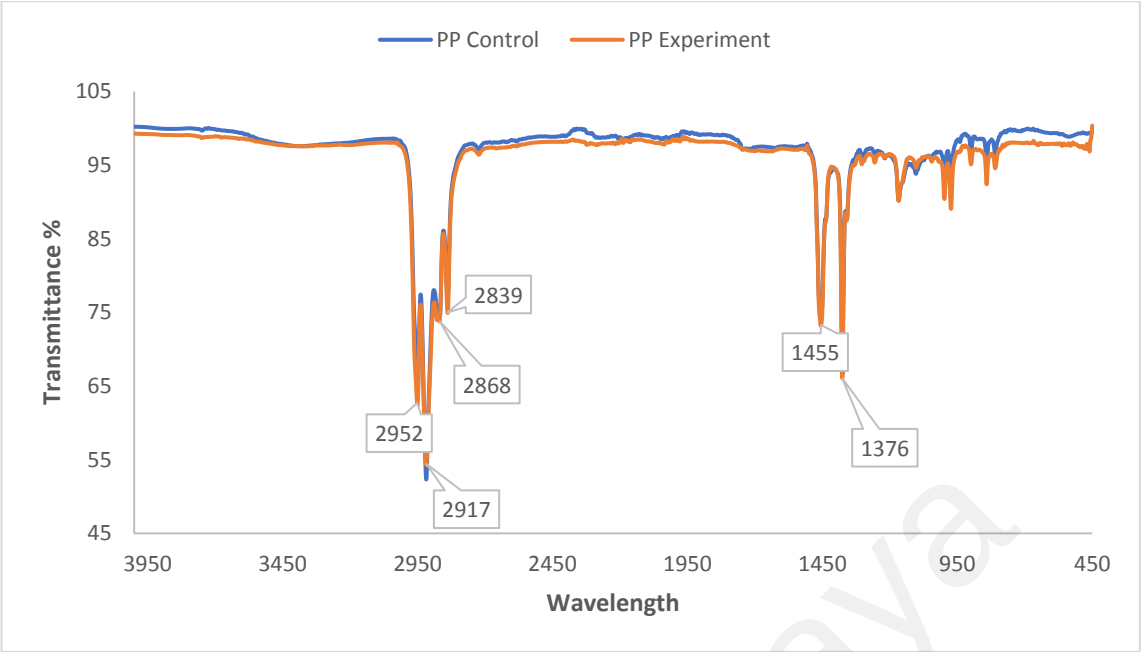


Figure 4.36: FTIR spectra of control and treated polypropylene with 0.25% concentration of bacterial consortium

The rate constant (K) and half-life (H½) of treated PP with all three inoculum concentration was determined. The highest rate constant calculated was 0.000048 day^{-1} for PP treated with 0.5% inoculum concentration, and since weight loss of PP treated with 1% and 0.25% inoculum concentrations was same, their rate constant was same as well i.e. 0.000026 day^{-1}. The shortest half-life was determined to be of PP treated with 0.5% inoculum concentration (14365 days) as compared to PP treated with 1% and 0.25% inoculum concentrations (26704 days).

4.3.2 Polystyrene

As for PS, highest weight reduction, 0.17%, was observed for PS treated with 0.25% concentration of inoculum, followed by PS treated with 1% inoculum (0.13%). Whereas, no weight reduction was found in PS microplastics treated with 0.5% inoculum concentration (Figure 4.37). Similar to PP microplastics from control, PS microplastics from control were also found to have reduced residual mass. Highest weight reduction, 0.13%, was observed in PS from control settings of 1% experiment, followed by 0.7%

weight loss in PS from control of 0.5% experiment. However, residual mass of PS from control of 0.25% experiment had an increased weight (0.03% increase).

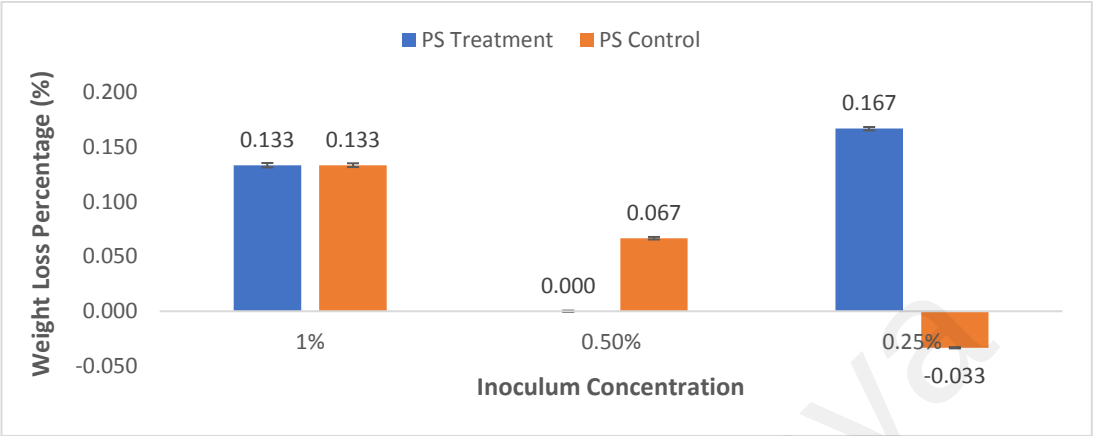


Figure 4.37: Weight loss of polystyrene microplastics treated with 1%, 0.5% and 0.25% inoculum concentration and of PS from control (n = 3)

The residual weight of PS treated with 1% inoculum decreased from Day 45 onwards (Figure 4.38) with a maximum weight reduction of 0.999 g ± 0.002 at Day 90. However, no increase in residual weight was observed in the control.

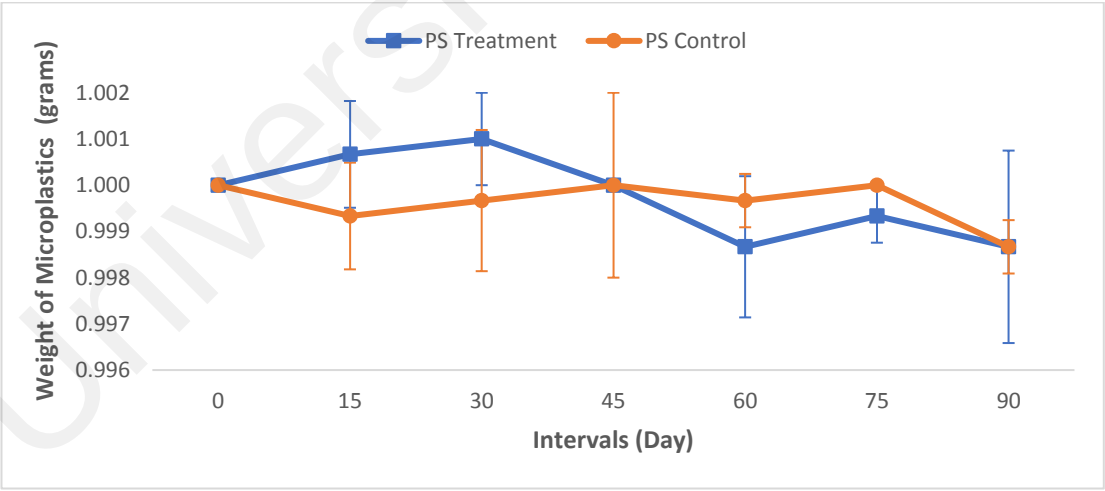


Figure 4.38: Residual weight of treated and control polystyrene from 1% inoculum set-up (n = 3)

Weight loss pattern for PS microplastics treated with 0.5% inoculum concentration is interesting as no weight loss was observed at the end of experiment for treated PS as compared to PS from control which recorded residual weight of 0.999 g ± 0.001. Figure 4.39 displays the residual weight of treated and control PS plotted over six intervals.

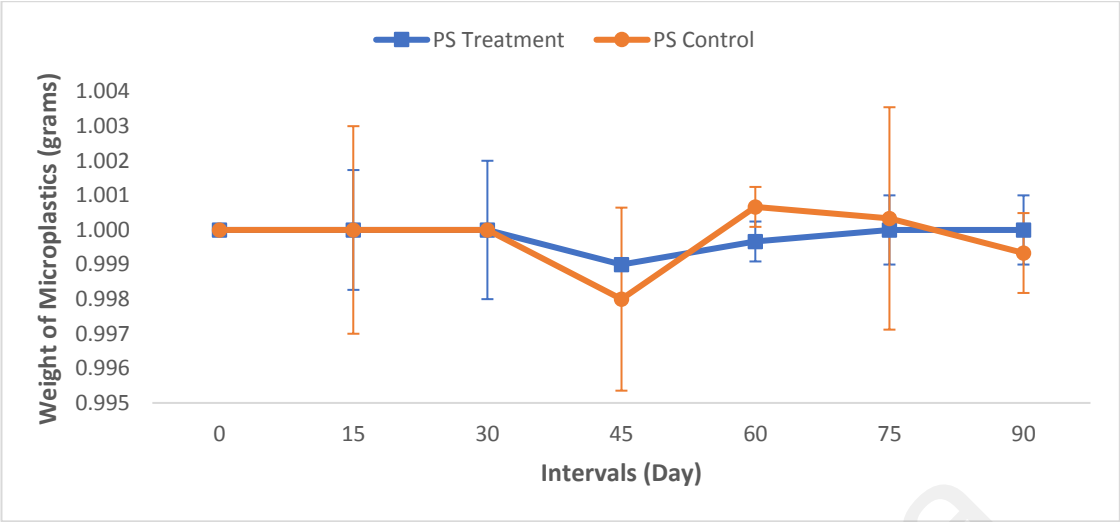


Figure 4.39: Residual weight of treated and control polystyrene from 0.5% inoculum set-up (n = 3)

At 45th day, the weight of treated PS was 0.999 g ± 0. Similar pattern was observed in biodegradation study of HDPE as well where weight loss at the end of experiment was lower than weight loss observed in preceding intervals of experiment (Sudhakar *et al*., 2008). PS from control set-up at 45th day of experiment showed lower residual weight (0.998 g ± 0.003) than treated PS. However, such a non-linear trend hasn't been addressed by respective authors. Additionally, biodegradation studies of plastic polymer generally report final mass of plastic polymer calculated at the end of experiment. The number of researches on reporting weight loss of plastic polymer at decided intervals are not prevalent. However, Auta (2018) reported approximately linear decrease in weight loss of microplastics over different intervals in biodegradation study. However, in that in-situ experiment, microplastic samples that were removed at respective sampling intervals were approximately 30 cm apart. Whereas, in this study, each microplastic sample was 3 cm apart from the next neighbouring microplastic sample. Perhaps, separate microcosm or separate flasks (for liquid media) provides maximum possible concentration of microbes to the synthetic polymer.

The pattern of weight loss of PS microplastics treated with 0.25% concentration of inoculum is crisscross (Figure 4.40). But overall, treated PS showed greater weight loss

as compared to PS from control. Similarly, unlike patterns of above-mentioned experimental set-ups, highest weight reduction was observed in PS microplastics recovered at the end of experiment. The residual mass of treated PS at 90th day was 0.998 g ± 0.003. Moreover, mass of microplastics in the experimental set-up increased in the beginning of experiment, and then it decreased with time. Similar observation was also made for abovementioned weight loss patterns of microplastics.

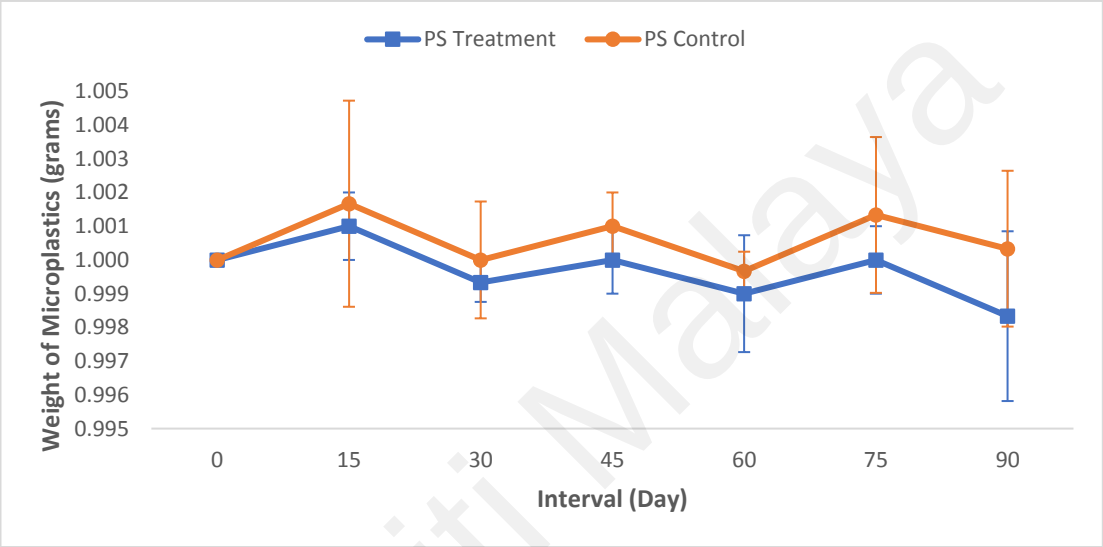


Figure 4.40: Residual weight of treated and control polystyrene from 0.25% inoculum set-up (n = 3)

Result from t-test showed that there was no statistically significant difference ($p > 0.05$) in residual weight values of untreated and treated PS with 1%, 0.5% and 0.25% inoculum concentration. Since the objective was to find out the effect of different concentrations of inoculum on biodegradation of microplastics, one-way ANOVA was performed to compare the weight loss of PS treated with 1%, 0.5% and 0.25% inoculum. However, no statistically significant difference ($p > 0.05$) was found.

When it comes to PS treated with 1% inoculum concentration, the residual weight values were slightly less scattered, as R^2 was 0.57 (Figure 4.41). The biodegradation rate of PS microplastics treated with 1% inoculum concentration of bacterial consortium was 2.0×10^{-4} g/day.

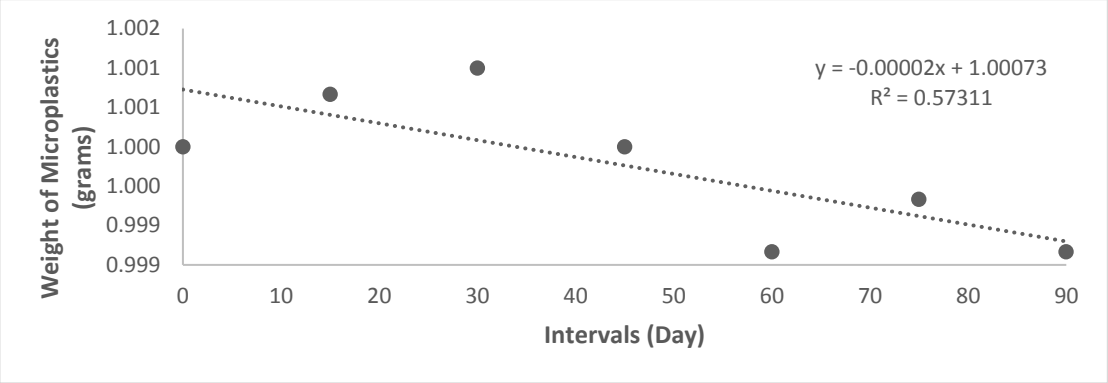


Figure 4.41: Linear trend-line analysis of polystyrene microplastics treated with 1% inoculum concentration

The residual mass of PS microplastics treated with 0.5% inoculum concentration of bacterial consortium was highly scattered (Figure 4.42). The R^2 value was 0.005. Therefore, the biodegradation rate of treated PS microplastics is less reliable which was 1.0×10^{-5} g/day. On the contrary, the biodegradation rate of PS treated with inoculum concentration of 0.25% was 1.7×10^{-5} g/day where R^2 value was 0.4. Thus, suggesting that residual mass of treated PS is moderately scattered (Figure 4.43).

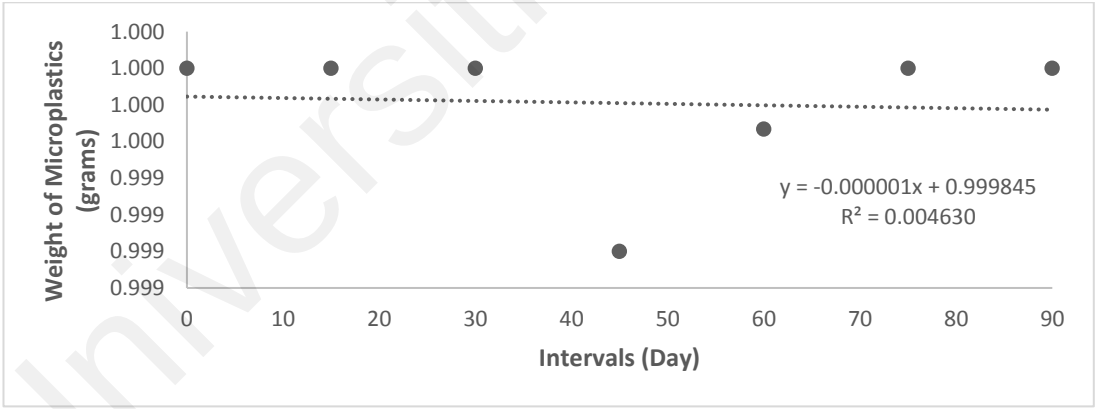


Figure 4.42: Linear trend-line analysis of polystyrene microplastics treated with 0.5% inoculum concentration

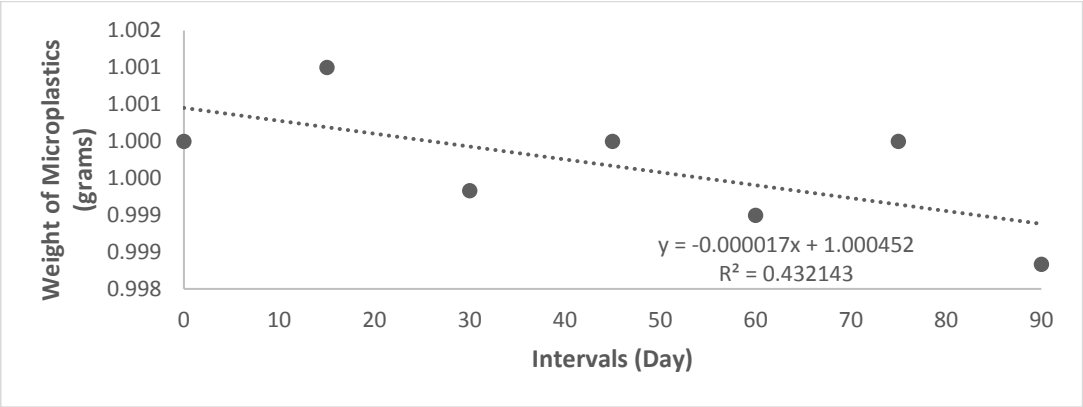


Figure 4.43: Linear trend-line analysis of polystyrene microplastics treated with 0.25% inoculum concentration

The FTIR spectrum of PS shows presence of methylene group due to peaks at 2922 and 2849 cm^{-1} as they appear from asymmetrical and symmetrical stretches. The peaks around 3050 cm^{-1} are from stretches of aromatic C-H, whereas benzene ring gives rise to peaks at 1601 and 1493 cm^{-1} wavelength (Smith, 1998). The peaks seen in FTIR spectra of PS samples around 698 and 750 cm^{-1} are due to ring-bending vibration and out-of-plane C-H bending mode of aromatic ring respectively (Sekhar *et al*., 2016). FTIR spectra of PS treated with 1% and 0.5% inoculum concentration did not show any changes as shown in Figure 4.44 and Figure 4.45 respectively. Similarly, PS from control of 1% and 0.5% inoculum concentration also showed no changes in their FTIR spectra.

However, when it comes to FTIR spectrum of PS treated with 0.25% of inoculum, several typical peaks were either missing or had reduced Intensity (Figure 4.46). For instance, peaks at 1452 and 1370 cm^{-1} associated with methylene group had reduced intensity. Atiq (2011) also reported reduction in peaks in FTIR spectrum of treated PS.

Moreover, peaks linked with stretches of aromatic C-H around 3050 cm^{-1} and as well as, asymmetrical and symmetrical stretches of methylene group at 2923 and 2850 cm^{-1} were also missing in the FTIR spectrum of treated PS with 0.25% inoculum concentration. Similar peaks in FTIR spectrum of treated PS were also found to be reduced (Auta, 2018). The reduction in peaks of FTIR spectrum is due to application of

bacterial consortium as described in other studies (Zahra *et al*., 2010). The formation of new peaks at wavelengths 1716 cm^{-1}, 1409 cm^{-1}, 1372 cm^{-1}, 1239 cm^{-1}, 1089 cm^{-1}, 1016 cm^{-1}, and 875 cm^{-1} were also observed. The peaks at 1016 cm^{-1} and 1089 cm^{-1} of FTIR spectrum of PS treated with 0.25% inoculum concentration are associated with vibrational stretching of C-O bonds of carboxylic acids, esters and alcohols (Motta *et al*., 2009).

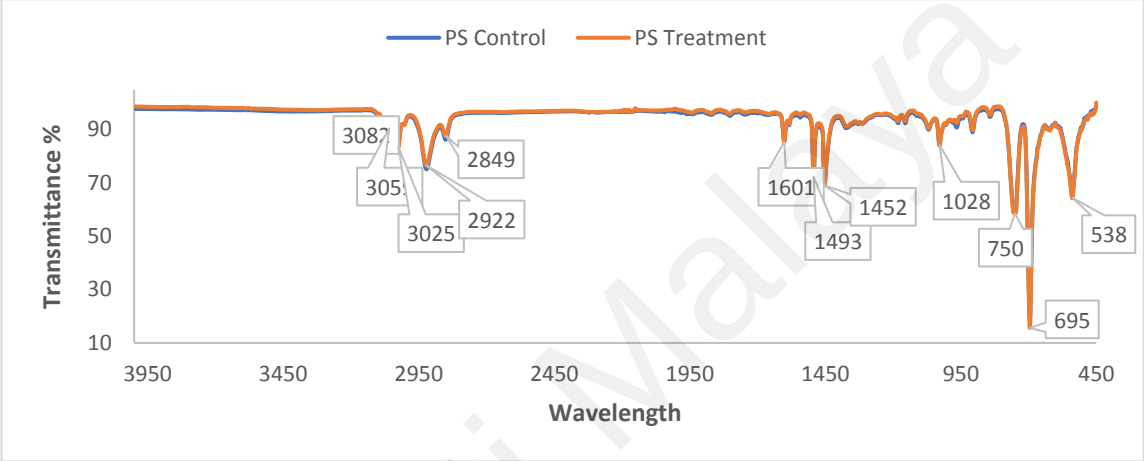


Figure 4.44: FTIR spectra of control and treated polystyrene with 1% concentration of bacterial consortium

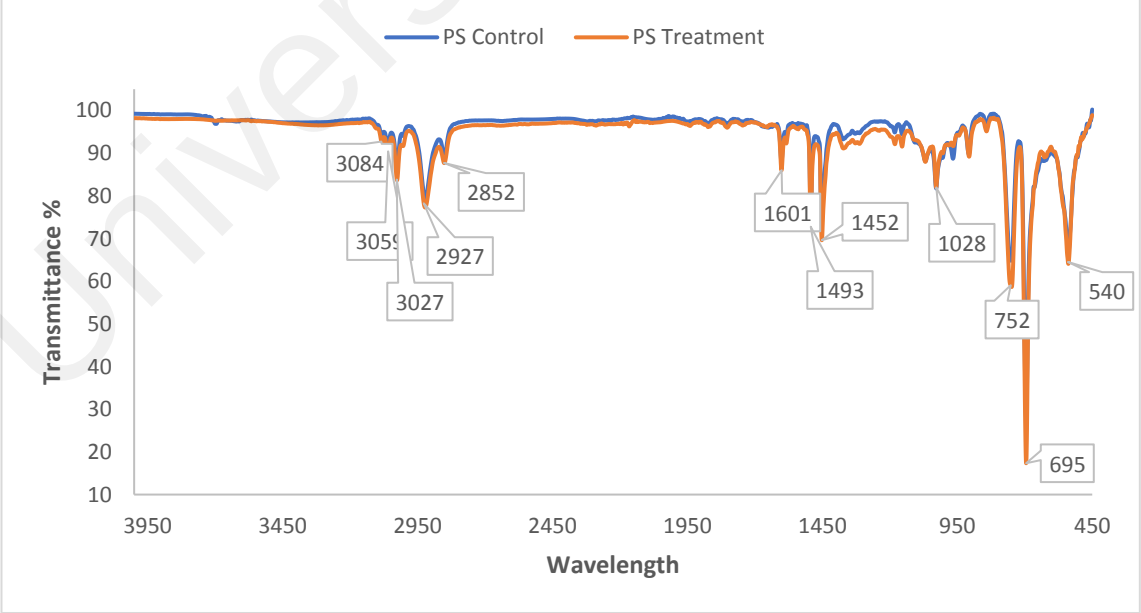


Figure 4.45: FTIR spectra of control and treated polystyrene with 0.5% concentration of bacterial consortium

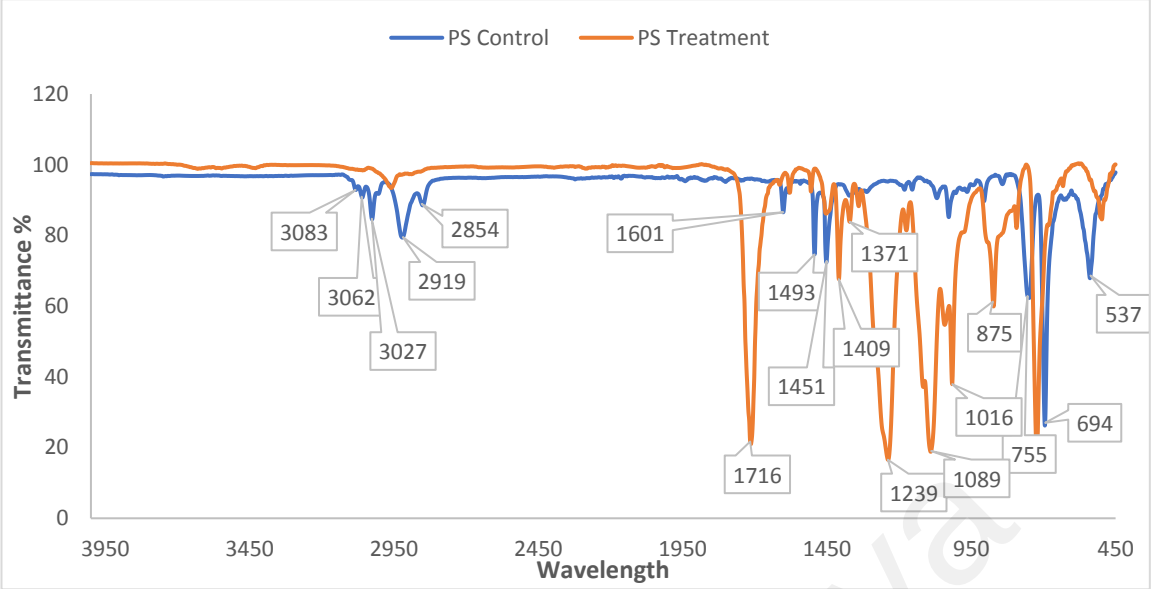
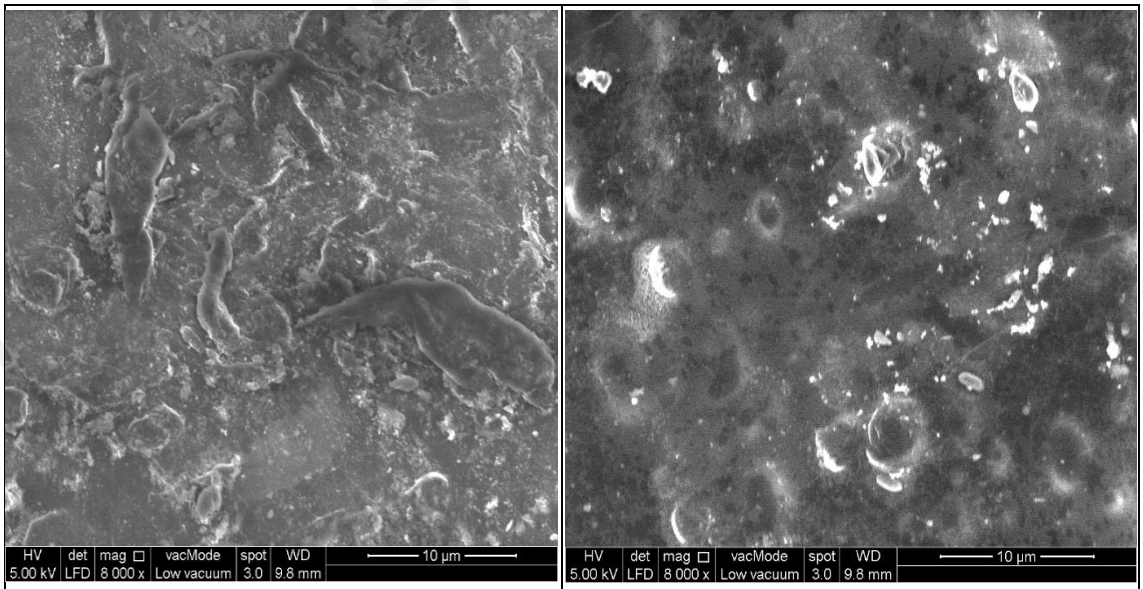


Figure 4.46: FTIR spectra of control and treated polystyrene with 0.25% concentration of bacterial consortium

Scanning Electron Microscopy (SEM) was only performed on PS microplastics samples treated with 0.25% of bacterial consortium and on its control sample. SEM micrograph of PS from control and SEM micrograph of PS treated with 0.25% inoculum concentration of bacterial consortium are shown in Plate 4.2.



SEM micrograph of Control Polystyrene	SEM micrograph of Treated Polystyrene

Plate 4.2: SEM micrograph of Polystyrene treated with 0.25% inoculum concentration

The SEM micrograph of PS from control shows no surface erosion or cracks but only adhered biofilm. Whereas, SEM micrograph of treated PS shows surface erosion and

unevenness. Similar surface erosion and unevenness is linked with microbial attack on the surface of plastic material (Syranidou *et al*., 2017; Tian *et al*., 2017).

The rate constant (K) and half-life (H½) of treated PS with 1%, and 0.25% inoculum concentration was determined to be 0.000015 day^{-1}, 46756 days and 0.000019 day^{-1}, 37399 days, respectively. Since no change was observed in residual value of PS treated with 0.5% inoculum concentration, its rate constant and half-life could not be derived.

4.3.3 General Discussion

Based on the findings of this research, when PS was treated with highest concentration of inoculum (1%) only 0.163% of weight loss was observed. This low value of weight loss for PS is congruent with general understanding of PS, since PS is regarded as the most recalcitrant plastics among other thermoplastic polymers due to poor affinity towards biodegradation (Mor & Sivan, 2008). However, no weight loss was observed in PS at the end of experiment when treated with 0.5% inoculum concentration.

The maximum weight loss (0.17%) was observed when PS microplastic was treated with least inoculum concentration of 0.25%. While no changes were observed in FTIR spectra of PS treated with 1% and 0.5% inoculum concentration, several changes in FTIR spectrum of PS treated with 0.25% concentration were observed. There was formation of new peaks due to carboxylic acids, esters and alcohols at 1016 cm^{-1} and 1089 cm^{-1} and as well as reduction in peak intensities. These findings were also backed up by SEM micrograph of PS treated with 0.25% inoculum concentration as roughening of microplastics surface due to microbial activity was seen.

Application of different concentrations of inoculum in bioremediation studies are not uncommon. Maximum reduction of PS microplastics weight treated with least inoculum concentration is congruent with other studies as well. As in biodegradation study of PS

microplastics, the least concentration (10%) of inoculum had resulted in higher weight loss in PS (31%) in shake-flask experiment for 40 days (Auta, 2018). Similarly, least concentration of inoculum deployed for bioremediation of hydrocarbon contaminated soil also resulted in better results as compared to when higher concentration of inoculum was applied (Liu *et al*., 2011).

On the contrary, when 0.5% of inoculum concentration of bacterial consortium was applied to the PP microplastics, highest weight loss was observed (0.43%). Whereas, similar weight loss, 0.23% was observed in PP microplastics when treated with 1% of inoculum concentration and 0.25% inoculum concentration. Thus, solely based on weight loss data, 0.5% of inoculum concentration was found effective. Similar phenomenon was reported by Dada *et al.* (2012), where increased concentrations of microbes led to improved results, until an optimum concentration (maximum increase). Further increase in concentration resulted in less positive results. Similarly, relatively higher concentrations of microbes are suitable for bioremediation of certain pollutants as Wolski *et al.* (2006) studied the bioremediation of pentachlorophenol and discovered that higher concentration of inoculum of *Pseudomonas* sp. resulted in faster degradation of contaminant. Correspondingly, the bioremediation of benzene was increased with increase in inoculum concentration of mixed microbial culture (Kauselya *et al*., 2015).

4.4 Different Size of Microplastics for Bacterial Biodegradation

4.4.1 Polypropylene

The percentage of weight loss for the three different sizes of PP microplastics after 90 days is presented in Figure 4.47. The highest weight loss was observed in the smallest of treated PP microplastics. The weight loss percentage for PP at 1 – 4 mm^2 was 0.5%. The weight loss percentage of PP decreased with increase in size. Where PP at 9 mm^2 had

lower weight loss, 0.4%, than PP with 1 – 4 mm^2 size. Similarly, the weight loss recorded for 25 mm^2 sized PP was 0.13%, the lowest among the various sizes.

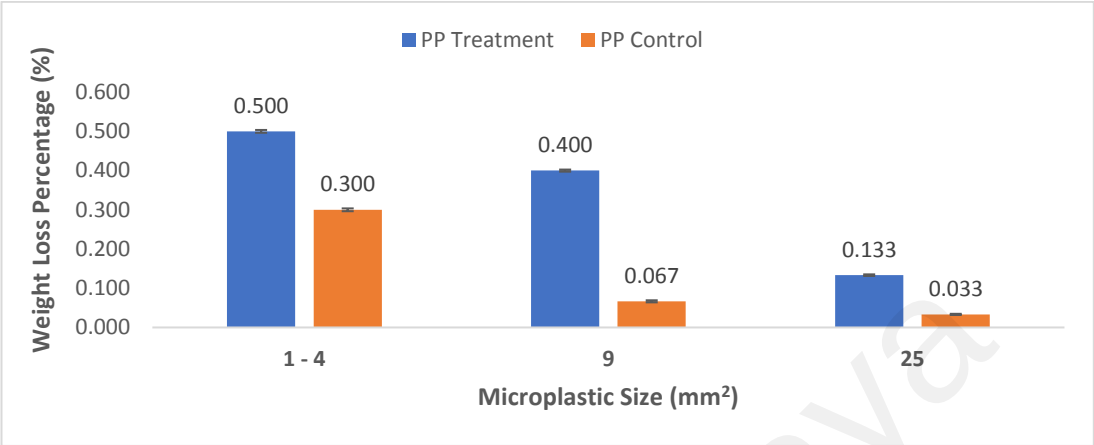


Figure 4.47: Weight loss of control and treated polypropylene microplastics of sizes 1 – 4 mm^2, 9 mm^2 and 25 mm^2 (n = 3)

Weight loss was also observed in the control set-ups. 0.3% of weight loss was recorded for 1 – 4 mm^2 sized PP, followed by 0.07% for 9 mm^2 and 0.03% in 25 mm^2 sized PP.

Mean values of weight of smallest size of PP, 1 – 4 mm^2, has shown decrease in weight since the start of experiment (Figure 4.48). Weight reduction had reached to 0.995 g ± 0.004. Similar pattern was observed for PP microplastics recovered from control as well where reduction in mass was seen from the beginning of experiment. At the end of experiment, the residual weight of PP from control was 0.997 g ± 0.004.

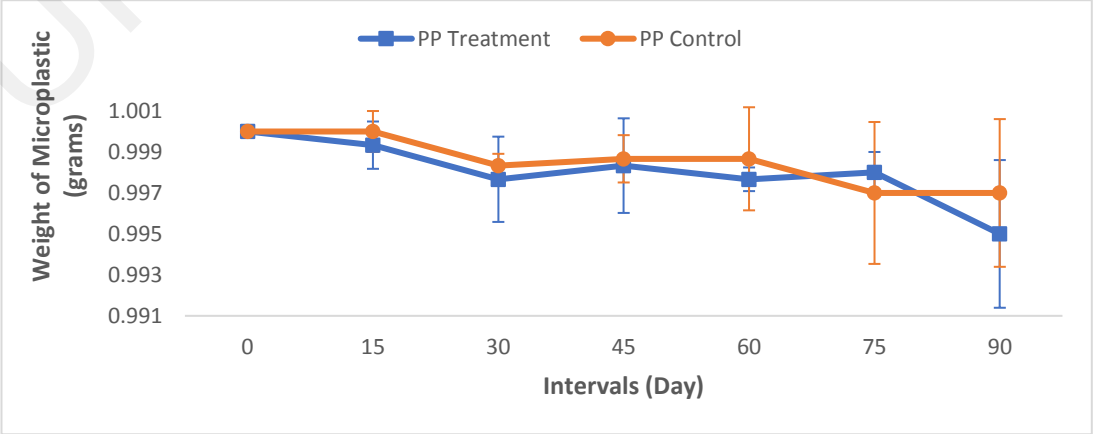


Figure 4.48: Residual weight of treated and control polypropylene sized 1 – 4 mm^2 (n=3)

The weight loss of 9 mm^2 treated PP microplastics over the six intervals roughly shows minimal or no weight loss until at 75th and 90th days (Figure 4.49). The residual weight at 75th day was 0.999 g ± 0.003 and residual weight at the end of experiment (90 day) was 0.996 g ± 0.003. On the other hand, 9 mm^2 sized PP microplastics from control set-ups showed minimal weight loss over the span of 90 days. At the end of experiment (90 day), the residual weight was 0.999 g ± 0.003.

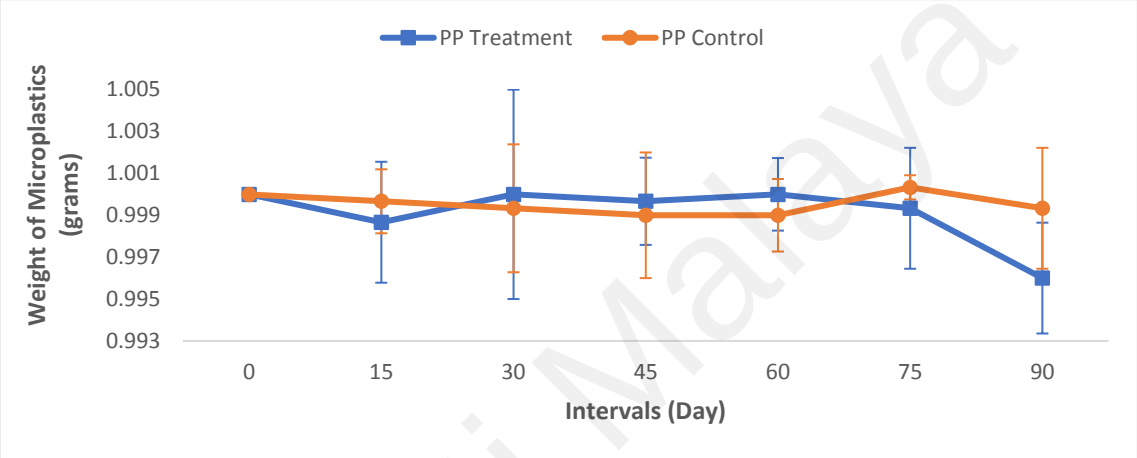


Figure 4.49: Residual weight of treated and control polypropylene sized 9 mm^2 (n = 3)

The residual weight of 25 mm^2 sized PP microplastics is more erratic than 1 – 4 mm^2 and 9 mm^2. The treated 25 mm^2 sized PP did not show any reduction in residual weight until 45 days (Figure 4.50). However, at 60th day residual weight of 25 mm^2 sized treated PP reduced to 0.998 g ± 0.001. Whereas at end of experiment, residual weight loss of 25 mm^2 sized treated PP was only 0.999 g ± 0.003. On the contrary, 25 mm^2 sized PP from control set-ups showed reduction in weight by 0.001 g for samples recovered at interval 2 (30 day), interval 3 (45 day) and interval 5 (day 75).

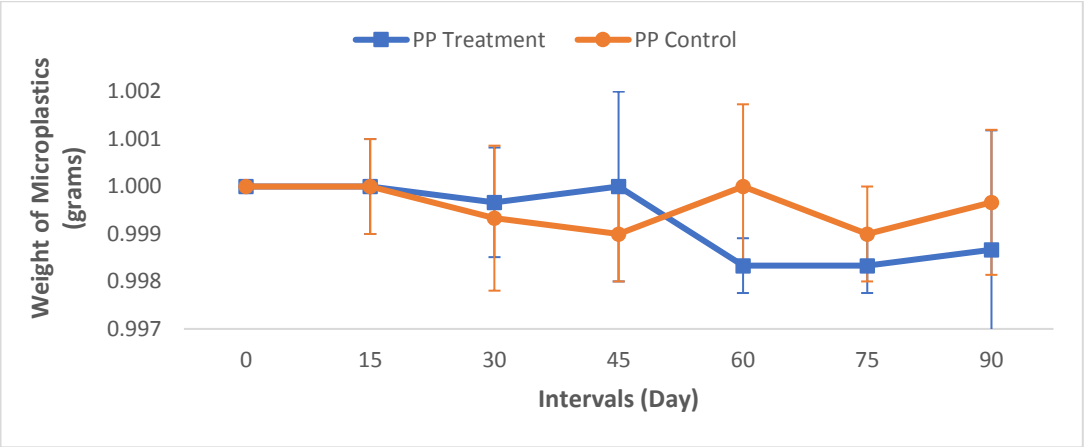


Figure 4.50: Residual weight of treated and control polypropylene sized 25 mm^2 (n = 3)

Results from t-test showed that there was no statistically significant difference ($p > 0.05$) between the residual values of different size of treated and control PP. One-way ANOVA test also showed that there was no significant difference ($p > 0.05$) in the effect of size on biodegradation of PP microplastics.

The mean values of residual weight of 1 – 4 mm^2 sized PP microplastic were moderately clustered as R^2 value was 0.74 (Figure 4.51). Hence, the slope of linear equation gave biodegradation rate 4.0×10^{-4} g/day. On the other hand, average residual weight values of 9 mm^2 sized treated PP were scattered, thus R^2 value was closer to 0, $R^2 = 0.3$. The biodegradation rate of 9 mm^2 sized treated PP was 2.5×10-5 g/day (Figure 4.52). Lastly, the mean values of residual weight of 25 mm^2 sized treated PP microplastics was moderately clustered (Figure 4.53). The R^2 value was 0.7 and thus biodegradation rate of 25 mm^2 sized treated PP microplastics was 2.0×10^{-4} g/day.

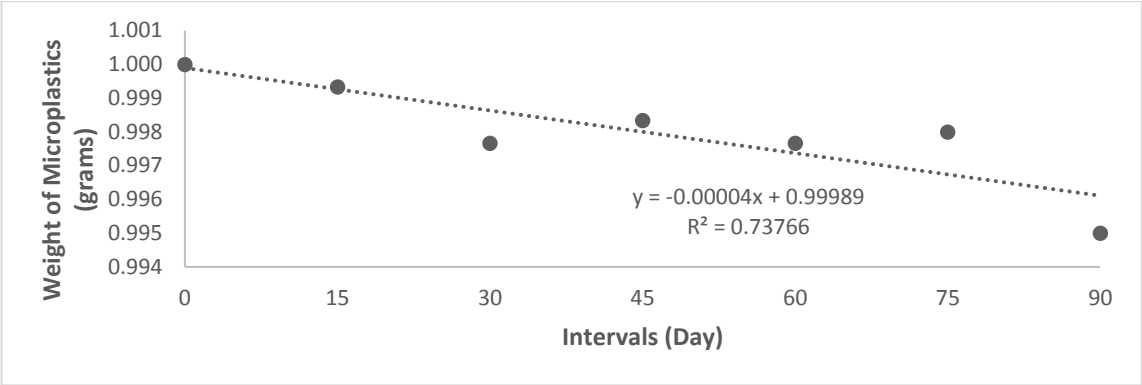


Figure 4.51: Linear trend-line analysis of polypropylene microplastics sized 1 – 4 mm^2

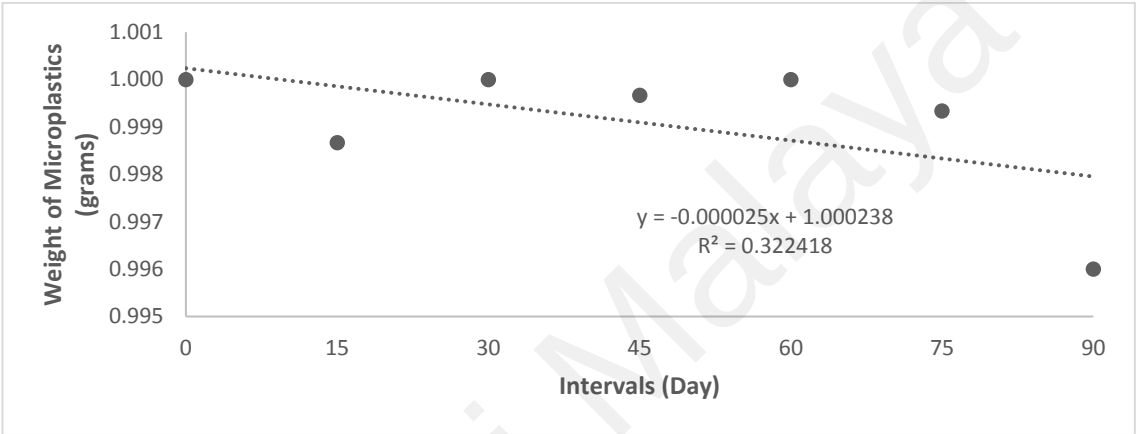


Figure 4.52: Linear trend-line analysis of polypropylene microplastics sized 9 mm^2

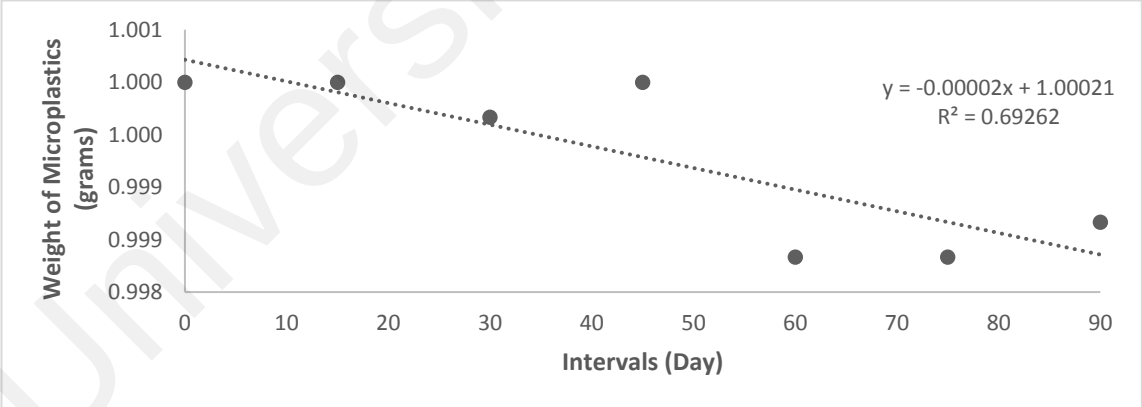


Figure 4.53: Linear trend-line analysis of polypropylene microplastics sized 25 mm^2

Typical peaks were observed in FTIR spectra of 9 mm^2 and 25 mm^2 sized PP from both control and treatment; asymmetrical and symmetrical stretching of methyl group around 2950 and 2870 cm^{-1}, asymmetrical and symmetrical stretches of methylene group around 2920 and 2840 cm^{-1}. Additionally, umbrella mode of methyl band can be seen at 1377 cm^{-1} as well (Smith, 1998).

Among three sizes of PP microplastics, the most notable changes observed were in FTIR spectrum of treated 1 – 4 mm^2 PP (Figure 4.54). In comparison to FTIR spectrum of control 1 – 4 mm^2 sized PP, the peaks associated with asymmetrical and symmetrical stretches of methylene bands around 2920 and 2840 cm^{-1} wavelength, in addition to asymmetrical and symmetrical stretches of methyl group around 2950 and 2870 cm^{-1}, respectively had disappeared in FTIR spectrum of inoculated 1 – 4 mm^2 PP. Moreover, umbrella mode had also reduced in treated 1 – 4 mm^2 sized PP. Formation of new peaks at 1715 cm^{-1}, 1241 cm^{-1}, 1091 cm^{-1}, 1018 cm^{-1}, 874 cm^{-1}, 724 cm^{-1} and 504 cm^{-1} were recorded in FTIR spectrum of 1 – 4 mm^2 sized treated PP. Most reported and discussed peaks in the biodegradation studies of PP are either carbonyl groups or methyl groups. The peak formed at wavelength 1715 cm^{-1} in FTIR spectrum of 1 – 4 mm^2 sized treated PP is linked with process of modification of carbonyl group and it reflects the oxidation of polypropylene (Barbes *et al.*, 2014). Moreover, the peak found at 1377 cm^{-1} wavelength is due to methyl group and the reduction in this peak has also been found due to the treatment of PP with microbes (Jeyakumar *et al.*, 2013). Figure 4.55 displays the segment (1350 – 1400 cm^{-1}) of control and treated 1 – 4 mm^2 PP to highlight the decrease of methyl group peak at 1377 cm^{-1}.

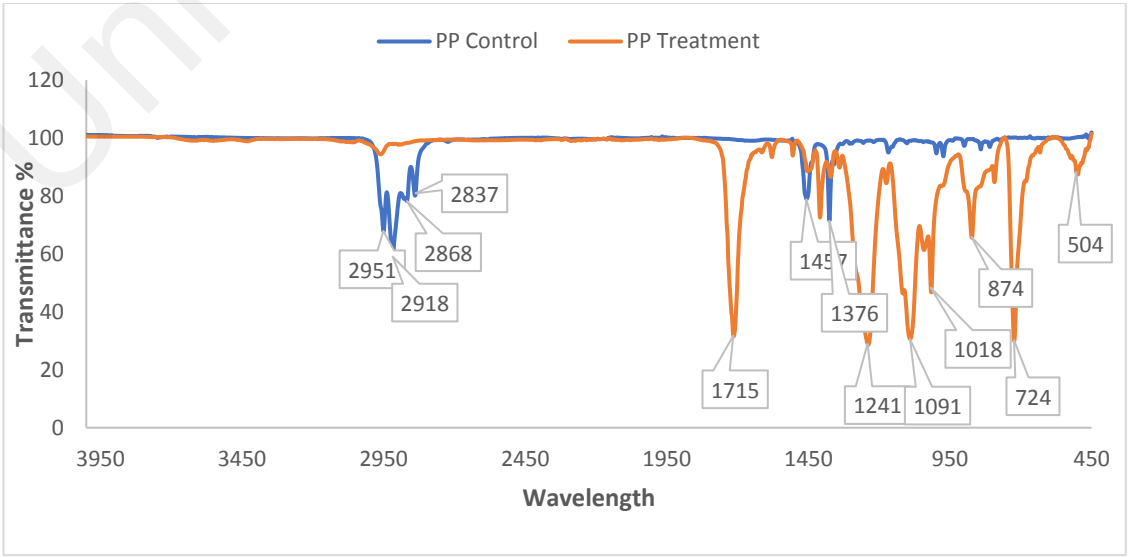


Figure 4.54: FTIR spectra of control and treated polypropylene sized 1 – 4 mm^2

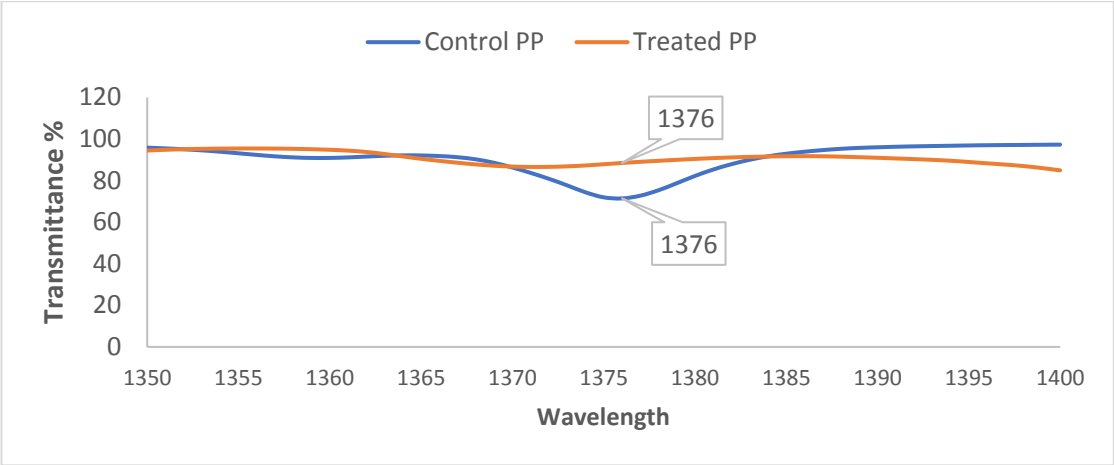


Figure 4.55: FTIR spectra of 1 – 4 cm^2 sized control and treated polypropylene between 1350 – 1400 cm^{-1} wavelength

Since carbonyl groups were found in FTIR spectra of 1 – 4 mm^2 sized treated PP, it is generally understood that carbonyl groups increase when plastics is going through degradation (abiotic forces are active) and they decrease when plastic material is under the influence of biodegradation (biotic forces are active). In a study of LDPE and HDPE, ester and carbonyl groups had increased in the beginning but gradually decreased (Arkatkar *et al*., 2010).

On the other hand, FTIR spectrum of treated 9 mm^2 PP manifested decrease in intensities as compared to FTIR spectrum of untreated 9 mm^2 PP (Figure 4.56). Some form of chemical changes had started to occur in treated 9 mm^2 PP. This decrease in intensities of FTIR spectra is linked with microbial activity on plastic polymers (Ebadi-Dehaghani *et al*., 2016). Lastly, the FTIR spectra of control and treated 25 mm^2 sized PP is identical, suggesting that no chemical changes had occurred in inoculated PP (Figure 4.57).

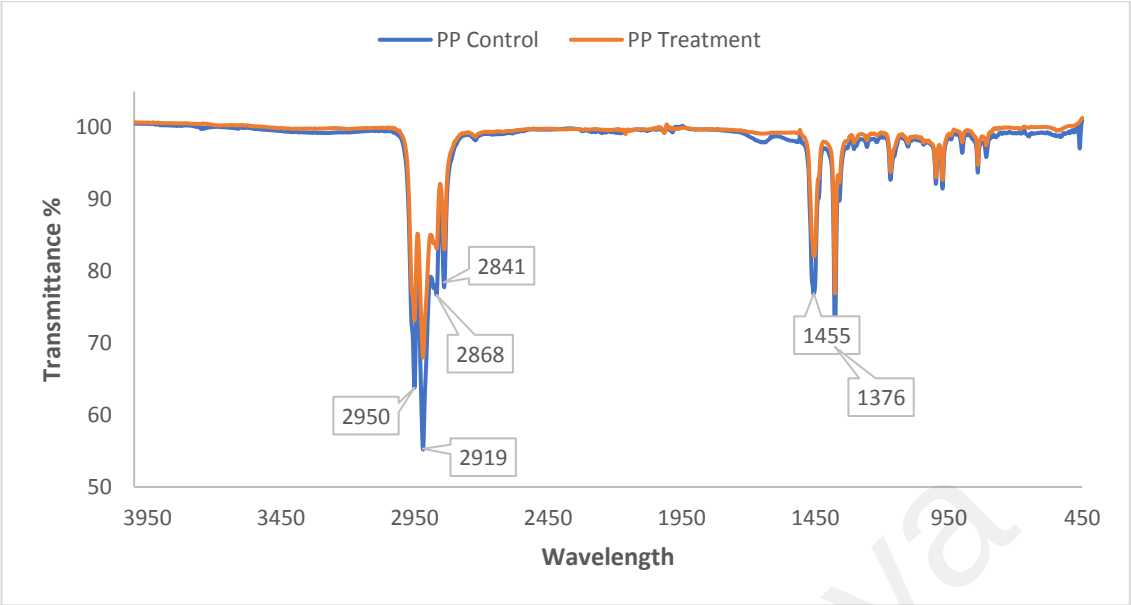


Figure 4.56: FTIR spectra of control and treated polypropylene sized 9 mm^2

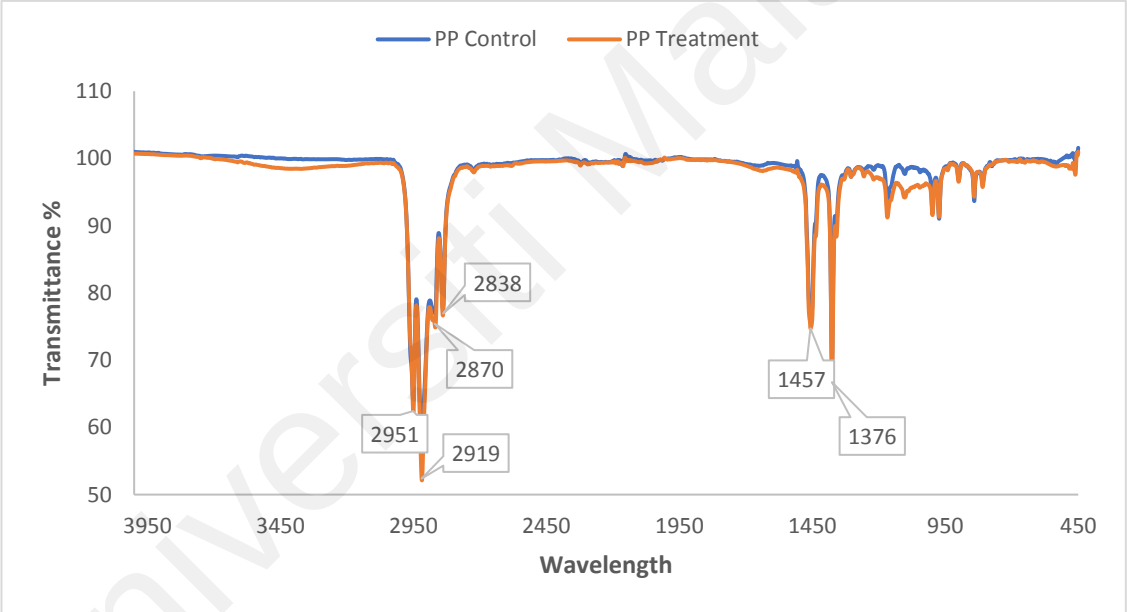
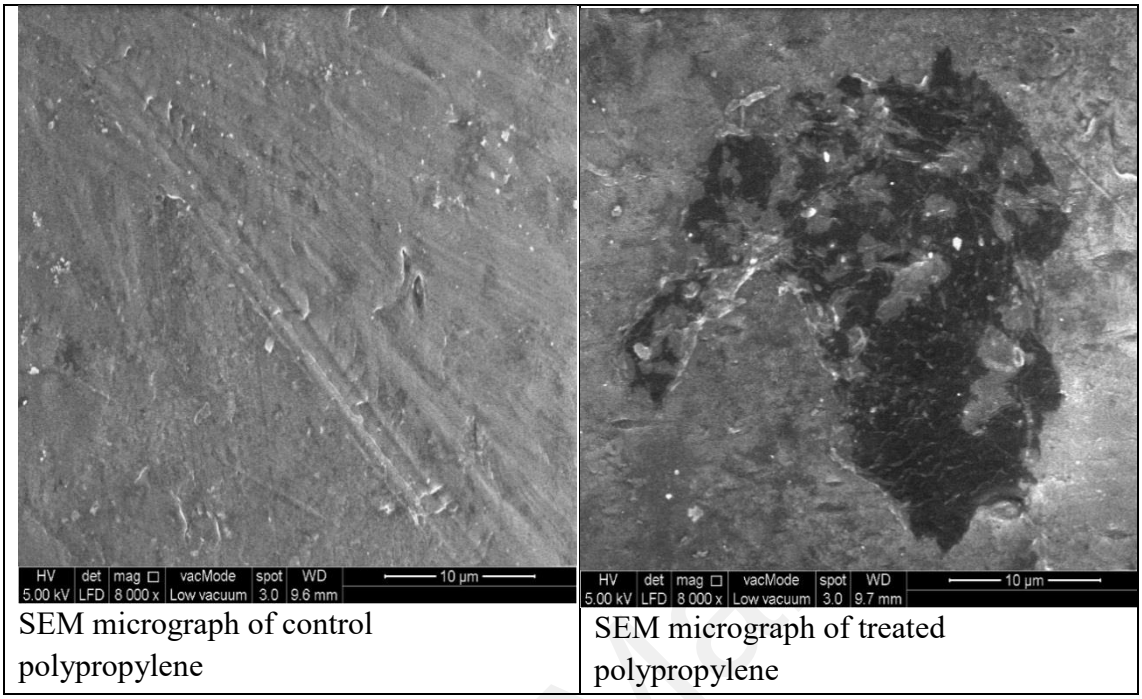


Figure 4.57: FTIR spectra of control and treated polypropylene sized 25 mm^2

SEM micrographs of 1 – 4 mm^2 PP (treatment and control) was taken. Plate 4.3 displays SEM micrograph of 1 – 4 mm^2 PP from control (left side) and treated PP (right side). SEM micrograph of PP from control shows undisturbed surface. On the other hand, treated PS shows massive cavity formed due to microbial attack. Similar deformation of surface of PP plastic have also been reported in other studies (Khoramnejadian, 2013; Arkatkar *et al*., 2010). This type of surface deformation occurs as extracellular enzymes of microbes cannot penetrate into the plastics easily, and usually attack the surface of

plastics, therefore, biodegradation of plastics is often referred as “surface erosion process” (Kale *et al*., 2015).



SEM micrograph of control polypropylene

SEM micrograph of treated polypropylene

Plate 4.3: SEM micrograph of polypropylene sized 1 – 4 mm^2

The highest value of rate constant was 0.000056 day^{-1} and shortest half-life, 12445 days, obtained from 1 – 4 mm^2 treated PP set-ups. The second highest value of rate constant was recorded for 9 mm^2 treated PP, at 0.000045 day^{-1} while the half-life was 15565 days. The lowest values of rate constant and half-life were recorded for 25 mm^2 treated PP, at 0.000015 day^{-1} and 46756 days, respectively.

4.4.2 Polystyrene

At the end of 90 days, equal percentage of weight loss was observed for largest, 25 mm^2, and smallest, 1 – 4 mm^2 treated PS microplastics (Figure 4.58). Only 0.1% of weight loss was recorded for both 1 – 4 mm^2 and 25 mm^2 treated PS at the end of the experiment. However, only marginal weight loss was observed for 9 mm^2 treated PS microplastic. The weight loss percentage value was 0.07% for 9 mm^2 treated PS. On the contrary, no weight loss was recorded for 9 mm^2 and 25 mm^2 PS recovered from control set-ups. Whereas, there was 0.13% increase in the residual weight of 1 – 4 mm^2 PS.

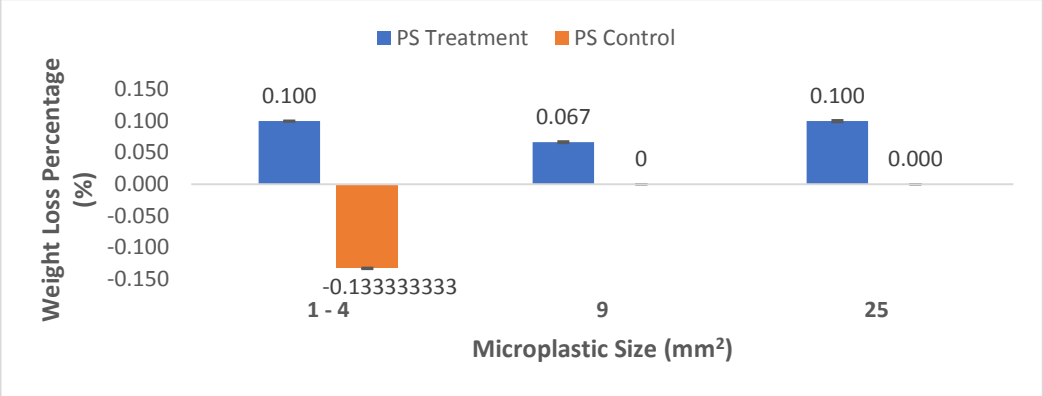


Figure 4.58: Weight loss percentage of control and treated polystyrene microplastics of sizes 1 – 4 mm^2, 9 mm^2 and 25 mm^2 (n = 3)

The residual weight of smallest size of PS microplastic, 1 – 4 mm^2, increased at the beginning of experiment and decreased with time (Figure 4.59). The lowest residual weight of 1 – 4 mm^2 treated PS microplastic was 0.998 g ± 0.001. Whereas, at 90 days the residual weight of PS recorded was 0.999 g ± 0.001. Similar pattern was observed in 1 – 4 mm^2 PS recovered from control set-ups. The increase in weight of untreated microplastics and subsequent reduction in weight over time was observed. The residual weight of 1 – 4 mm^2 PS at the end of experiment was 1.001 g ± 0.001. This increase in initial weight of microplastics is due to the formation of biofilm which is typical feature of plastic biodegradation phenomena (Sivan, 2011).

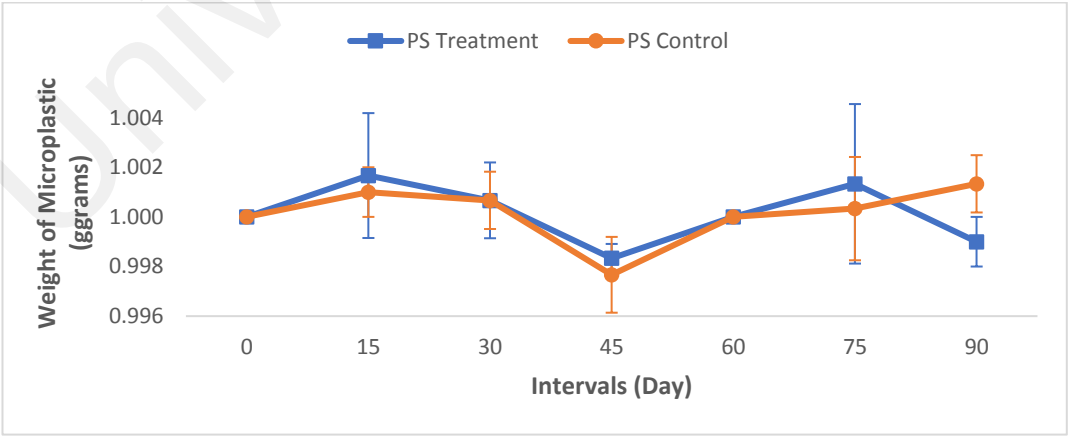


Figure 4.59: Residual weight of treated and control polystyrene sized 1 – 4 mm^2 (n = 3)

Similar pattern was observed in 9 mm^2 treated PS microplastics, where the weight of PS microplastics increased in the beginning and subsequently decreased with time

(Figure 4.60). Maximum increase was recorded on 15th day and the residual weight logged was 1.002 g ± 0.001. On the other hand, maximum weight reduction was recorded on 45th day and at the end of experiment (90 day). The readings of residual weight of both intervals was 0.999 g ± 0.002. On the contrary, 9 mm^2 PS microplastics recovered from control showed no loss in weight throughout the trial except on microplastic samples removed on 45th day. The residual weight of 9 mm^2 PS microplastics from control set-ups was 0.999 g ± 0.001.

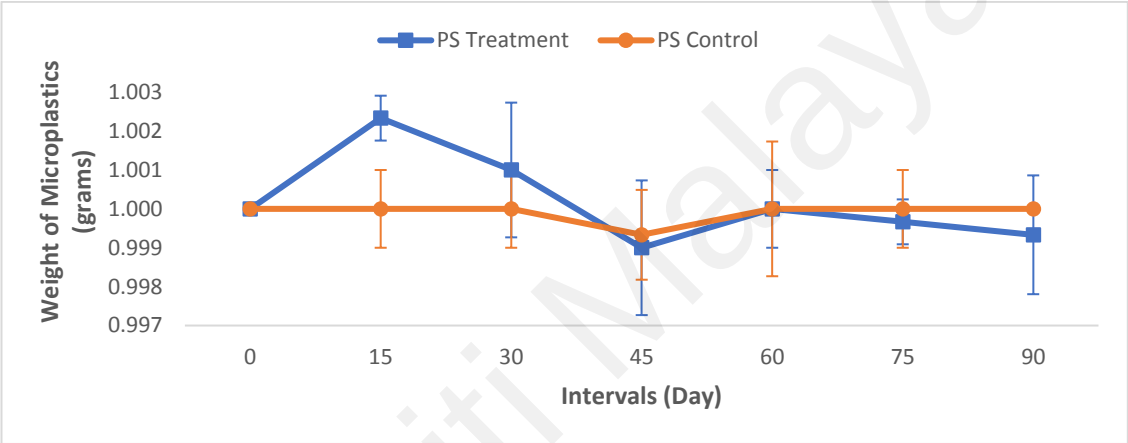


Figure 4.60: Residual weight of treated and control polystyrene sized 9 mm^2 (n = 3)

Irregular pattern was detected for 25 mm^2 treated PS microplastics (Figure 4.61). The weight of 25 mm^2 treated PS increased, 1.003 g ± 0.001, at the beginning of experiment, then decreased to 0.998 g ± 0.001 on 60th day. However, 25 mm^2 treated PS recovered at the end of experiment, logged only 0.999 g ± 0.002 residual weight. In contrast, 25 mm^2 PS recovered from control showed no changes in weight until 45th day where it increased to 1.002 g ± 0.002, and subsequent decrease at 60th day, 0.999 g ± 0.001. No changes in weight were observed in 25 mm^2 PS from control set-ups for the remaining two intervals of the experiment.

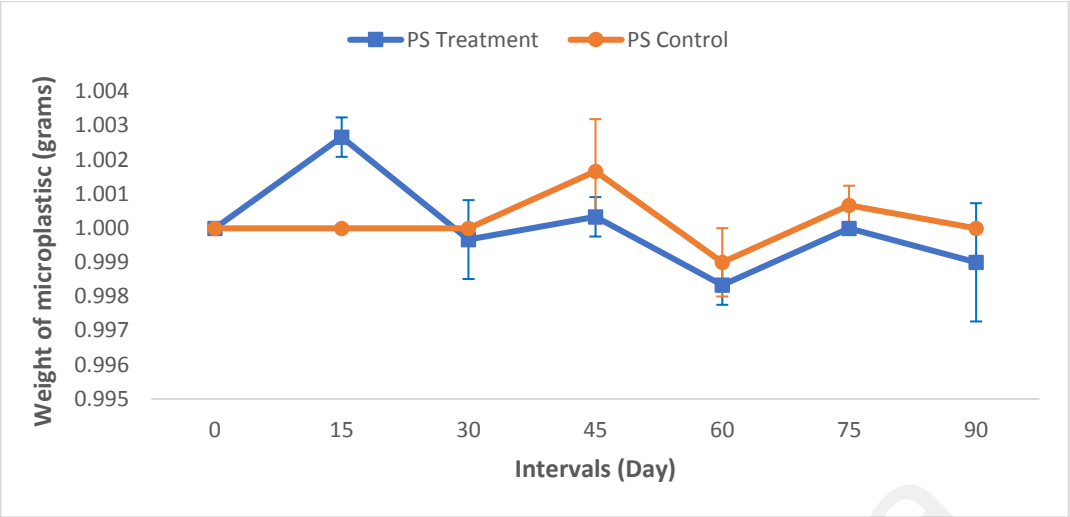


Figure 4.61: Residual weight of treated and control polystyrene sized 25 mm^2 (n = 3)

No statistically significant difference ($p > 0.05$) was recorded in the results of t-test between residual weight of control and treated PS of all sizes. Similarly, no statistically significant difference ($p > 0.05$) was recorded in the results of one-way ANOVA to determine the effect of size on biodegradation of microplastics.

When it comes to 1 – 4 mm^2 treated PS microplastics, the mean residual weight values were highly scattered as R^2 value was 0.07 (Figure 4.62). Thus, the biodegradation rate of treated 1 – 4 mm^2 PS microplastics was 1.0×10^{-4} g/day. Similarly, the mean residual weight values of treated 9 mm^2 PS microplastics was also scattered (Figure 4.63). The R^2 value was 0.3. The biodegradation rate of 9 mm^2 treated PS microplastic was 2.0×10^{-4} g/day. Lastly, the average residual weight values of 25 mm^2 treated PS microplastics was also scattered (Figure 4.64). The R^2 value was 0.3. The biodegradation rate of 25 mm^2 treated PS microplastic was 2.3×10^{-5} g/day.

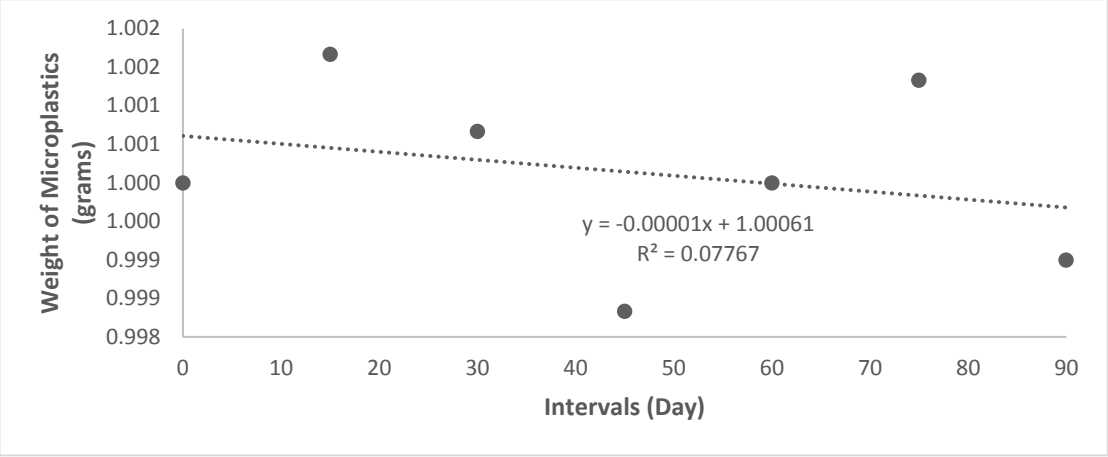


Figure 4.62: Linear trend-line analysis of polystyrene microplastics sized 1 – 4 mm^2

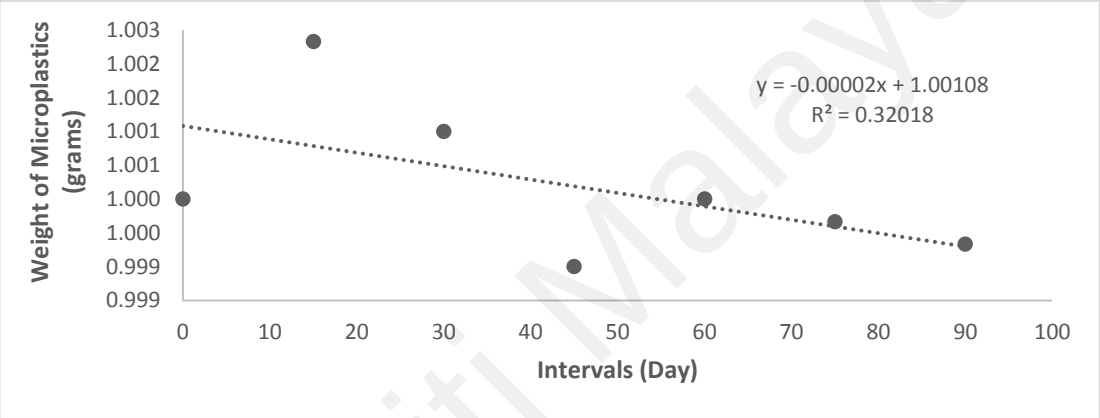


Figure 4.63: Linear trend-line analysis of polystyrene microplastics sized 9 mm^2

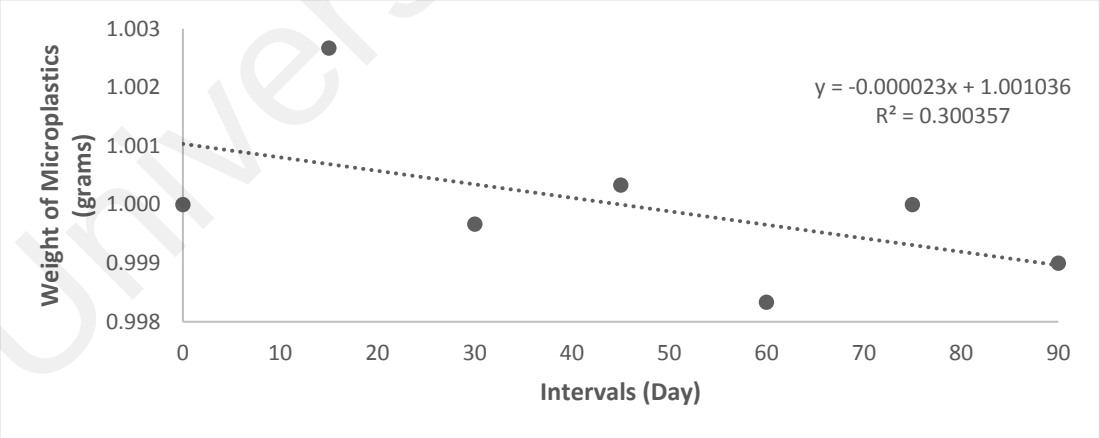


Figure 4.64: Linear trend-line analysis of polystyrene microplastics sized 25 mm^2

The FTIR spectrum of the smallest size of PS microplastics, 1 – 4 mm^2, after treatment with bacterial consortium did not show any chemical changes (Figure 4.65). Moreover, the characteristic peaks found in unaffected PS were observed in both treated and control 1 – 4 mm^2 PS. The peaks associated with asymmetrical and symmetrical stretches of

methylene group were present at wavelength 2922 and 2849 cm^{-1}. The stretches of aromatic C-H were also observed at wavelength 3050 cm^{-1}. Lastly, the peaks linked with benzene rings were also seen at 1601 and 1493 cm^{-1} wavelength.

While characteristic peaks were also observed in 9 mm^2 control and inoculated PS, the band intensities of inoculated PS had reduced as shown in Figure 4.66. Similar type of changes in band intensity such as reduction, reflects some form of chemical changes in the plastic material as also highlighted by Mohan *et al.* (2016).

The largest sized PS, 25 mm^2, after the treatment with bacterial consortium had shown the most changes (Figure 4.67). The peaks at 538 cm^{-1}, 695 cm^{-1}, 752 cm^{-1}, 1603 cm^{-1} had disappeared. The peaks at 695 and 752 cm^{-1} were associated with ring-bending vibration and out-of-plane C-H bending mode of aromatic ring respectively. The disappearance of these peaks has also been reported by Sekhar *et al.* (2016).

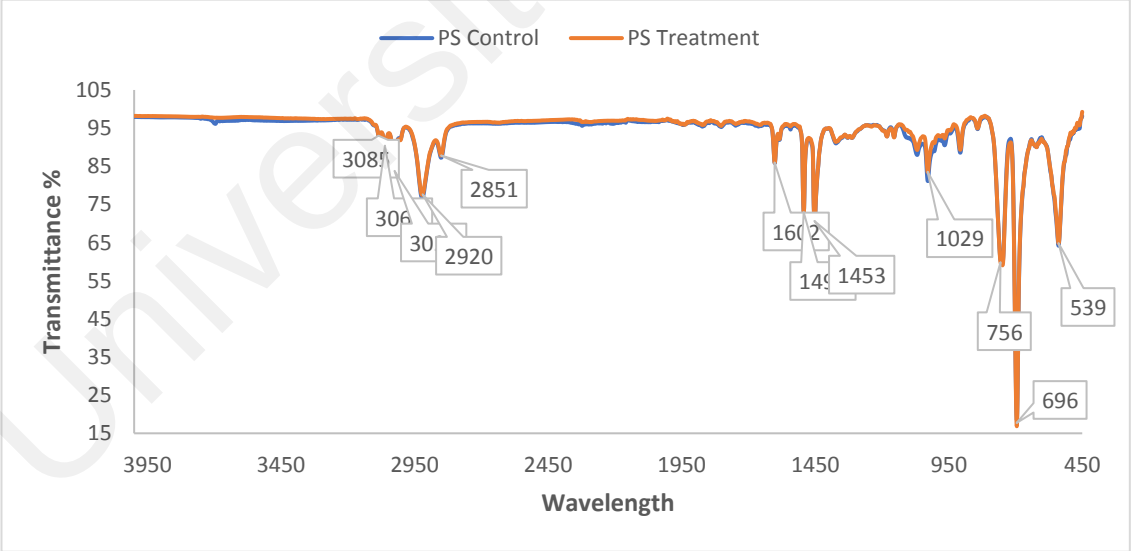


Figure 4.65: FTIR spectra of control and treated polystyrene sized 1 – 4 mm^2

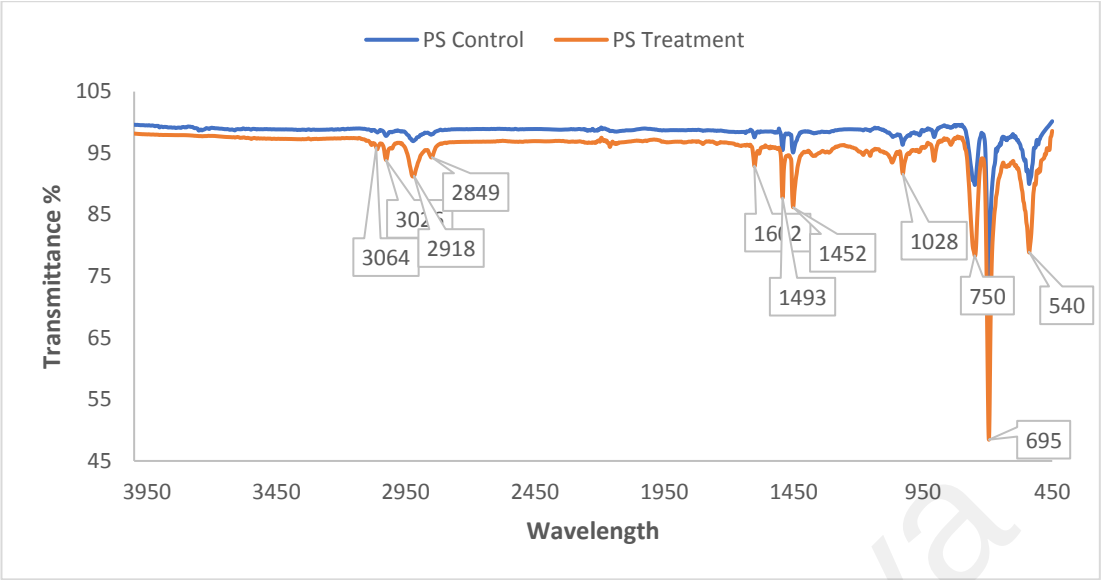


Figure 4.66: FTIR spectra of control and treated polystyrene sized 9 mm^2

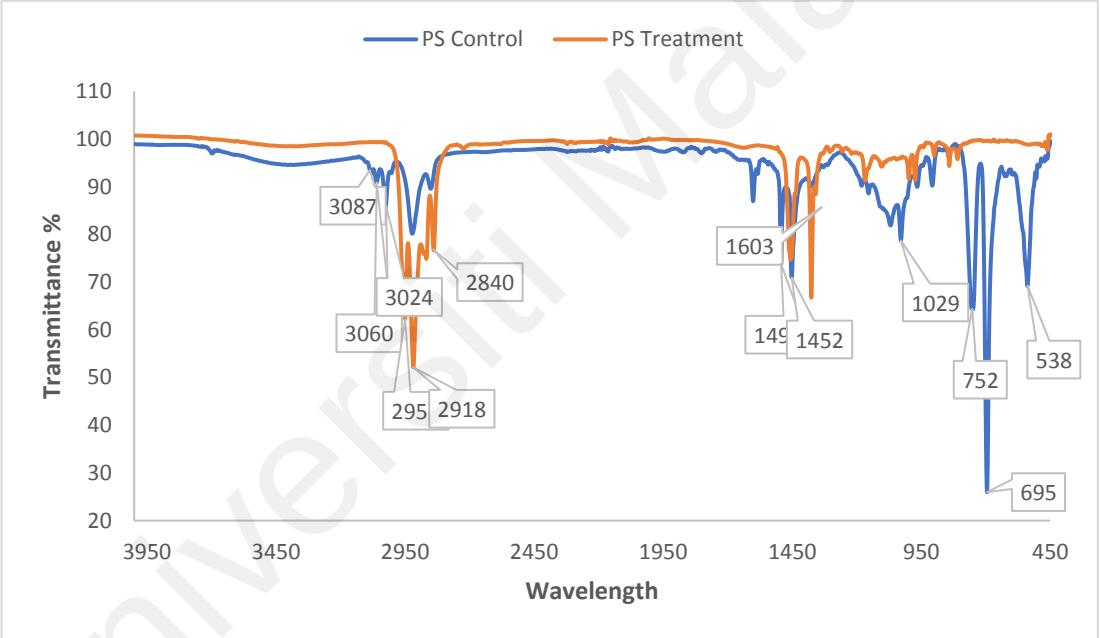


Figure 4.67: FTIR spectra of control and treated polystyrene sized 25 mm^2

The rate constant (K) of treated PS of all three sizes, 1 – 4 mm^2, 9 mm^2, 25 mm^2 were calculated to be 0.000011 day^{-1}, 0.000007 day^{-1}, and 0.000011 day^{-1}, respectively. The half-life (H ½) of 1 – 4 mm^2, 9 mm^2, 25 mm^2 sized treated PS were 62352 days, 93544 days and 62352 days, respectively.

4.4.3 General Discussion

Among the three sizes of PP, the smallest size of PP (1 – 4 mm^2) had shown greater affinity for biodegradation as higher weight loss of 0.5% was recorded. Additionally, changes in FTIR spectrum of treated PP in form of disappearance of methylene and methyl group peaks between wavelength of 2840 – 2950 cm^{-1}, reduction in peak intensity of methyl group at 1377 cm^{-1} and formation of new peak at 1715 cm^{-1}, specifically suggests the oxidation of PP. These findings were also supported by formation of cavities on the surface of 1 – 4 mm^2 treated as seen in SEM micrograph. In biodegradation study of poly(lactic acid) (PLA) powder, powder of particles' size of 0 – 0.125 mm were biodegraded faster than 0.125 mm PLA powder (Kunioka *et al*., 2006). The smallest particle sizes of Polycaprolactone (PCL) were also biodegraded faster (Funabashi *et al*., 2007). Similarly, in a biodegradation study of polyethylene wax, smaller molecules of plastics were consumed faster by microbes than larger molecules (Kawai *et al*., 2004).

On the other hand, 9 mm^2 PP had shown 0.4% weight loss and reduction in peak intensities of FTIR spectrum of treated PP. However, the changes in FTIR spectrum were comparatively less significant than FTIR spectrum of smallest sized PP. The largest size of PP had shown the least reduction in weight at 0.13% and no changes in FITR spectrum. However, it does not suggest that larger size of microplastic or plastic material lessen the affinity of synthetic polymer to biodegrade, since successful biodegradation of PP macroplastics (> 5 mm) have been reported (Jeon & Kim, 2016; Ebadi-Dehaghani *et al*., 2016).

Studying the biodegradation of microplastics films of different sizes is a novel approach as mostly microplastics (regardless of size) are simply acquired to perform biodegradation study. Yet, it is very important facet of biodegradation study as biodegradation of plastic material is a surface reaction (Chinaglia *et al*., 2018) since microbial attack occurs on the surface of plastic. It must be noted that complete

mechanism of biodegradation of microplastic has not been published yet. A few studies have been conducted by exposing microplastics of varying sizes in the same experimental conditions to study the impact of microplastic sizes on its biodegradation (Chinaglia *et al*., 2018; César *et al*., 2009; Funabashi *et al*., 2007; Kunioka *et al*., 2006; Yang *et al*., 2005; Modelli *et al*., 1999). When microbes come in contact with microplastics, they attach themselves to the outer surface and assimilate that layer. The inner part of microplastics stays intact or unaffected (Chinaglia *et al*., 2018).

Majority of biodegradation of microplastics have been conducted on the lower size range (< 1 mm). Microplastics, polyethylene (PE) in composition, with size range of 0.25 – 1 mm were treated with *Zalerion maritimum* fungus in shake-flask experiment for 28 days, which had resulted in more than 65% reduction in weight of microplastics along with increase in growth of fungus and as well as, accompanied by changes in FTIR of microplastics (Paco *et al*., 2017). Auta *et al* (2017) used microplastics, PE, PP, PS and polyethylene terephthalate (PET), less than 0.25 mm in size, in shake-flask experiment for 40 days and it resulted in weight losses from 1.6 – 7.4% of respective composition of microplastics. On the other hand, 0.1 – 0.6 mm sized PE microplastics were used for biodegradation by *Bacillus* sp. and *Paenibacillus* sp. that had also resulted in 14.6% weight reduction and supported by changes observed in FTIR spectra and SEM micrographs (Park & Kim, 2019). The biodegradation of 0.01 mm sized PET microplastics were examined in alkali conditions in combination with engineered strain by Gong *et al.* (2018). It would not be farfetched to conclude that biodegradation studies of microplastics have been performed on smaller sizes of microplastics (< 1 mm). While these studies may suggest that smaller particles of microplastics can be biodegraded, they do not reveal if microplastics of larger size range will show lesser or greater affinity towards biodegradation in similar experiment conditions. But when three sizes of PP microplastics were subject to same experimental set-up, same bacterial consortium and

for the same incubation period, (as is the case of this experiment), higher affinity to biodegradation of each size of PP microplastics was in this order; 1 – 4 mm^2 >> 9 mm^2 >> 25 mm^2 sized PP.

In contrast to PP, the largest size of PS, 25 mm^2, had shown higher affinity to biodegradation. At the end of experiment, 0.1% of weight loss was observed in 25 mm^2 treated PS, that was supported by FTIR spectrum of treated PS where the disappearance of peaks at 695 and 752 cm^{-1} associated with ring-bending vibration and out-of-plane C-H bending mode of aromatic ring. Higher biodegradation for largest size of PS microplastics could have been subjected to styrene as microbial (both bacterial and fungal) degradation of fundamental monomer of polystyrene (Styrene) is well documented (Mooney *et al*., 2006). The medium sized PP, 9 mm^2 recorded the second most affinity to biodegradation among the three sizes as reduction in peak intensities was observed despite lower weight loss of 0.7%. While 1 – 4 mm^2 PS showed relatively higher weight loss of 0.1%, no chemical changes were recorded in FTIR spectrum of treated PS. Therefore, the higher affinity to biodegradation of each sizes of PS microplastics was recorded to be in this order; 25 mm^2 >> 9 mm^2 >> 1 – 4 mm^2.

Lastly, the low values of weight loss of microplastics observed in all three biodegradation experiments do not necessarily mean that biodegradation potential of bacterial consortium is poor. *Bacillus* had shown the capability of biodegrading the synthetic polymer in another study where there was no weight loss or partial weight loss was recorded for LDPE (Nowak *et al*., 2011). Also, it is suggested that weight loss cannot be the determinant of biodegradation of polymer. As they reported that reduction in molecular weight of polymer had occurred and it was also supported by account of reduction in mechanical properties by 98% (Nowak *et al*., 2011). In another study, despite formation of biofilm on polystyrene microplastics, partial biodegradation, 0.8% weight loss, was observed in the experiment (Mor & Sivan, 2008). Therefore, biodegradation of

synthetic polymers can be determined by weight loss in conjunction with addition of functional groups observed in biodegradation studies but not solely on weight loss.

4.5 Overview of Biodegradation with Respect to Results

Increase input of bacterial consortium into the experiment is a novel approach as well. Mostly biodegradation studies involve introduction of microbes at the start of experiment only (Lwanga *et al*., 2018; Negi *et al*., 2011) or simple burial in modified soil (Abdullahi & Saidu, 2013) or in original soil (Nowak *et al*., 2011; Vijaya & Reddy, 2008). However, it must be put forth that Auta (2018) had introduced bacterial consortium at decided intervals (at every 15 days) throughout the experiment. Therefore, the results of daily input of inoculum presents the baseline research findings of the effect of increased input of bacterial consortium.

As microplastics showed irregular pattern of weight loss throughout the span of 90 days of experiment where microplastics recovered from later intervals had shown no or minimal change in weight as compared to microplastics recovered in earlier intervals. This lack of linear decrease in weight of microplastics with time can be linked with the experimental set-up. Note that microplastics were buried within a distance of 3 cm to each other and bacterial inoculum was poured onto the surface of soil medium. Therefore, the microplastics concentration (pollution load) could have been extremely high (Abatenh *et al*., 2017) with respect to inoculum concentration being poured. Additionally, other factors such as percolation of inoculum, availability of alternative nutrients in soil may have influenced as well (Abatenh *et al*., 2017).

As bacterial consortium of nine bacteria was deployed in all three trials, bacteria from the consortium have shown the capabilities of degrading microplastic individually. *Bacillus cereus* and *Rhodococcus rubber* were reported to reduce PP mass by 6.4% and 4.0% in 40 days (Auta *et al*., 2018). In another study *Bacillus cereus* reduced the mass of

microplastic PE and PS by 1.6% and 7.4% in 4 weeks, respectively (Auta *et al*., 2017). Whereas, *Bacillus gottheilii* reduced the mass of microplastic PE, PP and PS by 6.2%, 3.6% and 5.8% in 4 weeks respectively (Auta *et al*., 2017). 14% reduction was reported in UV treated PE plastic by *Bacillus cereus* in 3 months (Sowmya *et al*., 2014). Therefore, the usage of bacterial consortium was selected for biodegradation of selected microplastics. The usage of bacterial consortium over individual strains has also been suggested by some other authors due to synergistic activities of microbes (Wikles & Aristilde, 2017; Negi *et al*., 2011). For instance, biodegradation of polyester PU had increased by consortia of two biodegrading bacteria (Shah *et al*. 2016). Whereas, *P. putida* VM15A and *Pseudomonas* sp. VM15C could not biodegrade PVA individually but were able to form biofilm on PVA symbiotically (Shimao 2001), unlike selected bacterial consortium with known biodegrading bacteria. While the results of this research report relatively less weight loss of microplastics as compared to weight loss values reported for individual bacterium of consortium, it must be noted that those studies of biodegradation were conducted in shake-flask experiments where synthetic polymer was added as single carbon source for microbes in liquid medium i.e. mineral salt medium etc. In such experiments, microbes did not have variety of carbon sources available unlike in soil medium or in the real environment (Krueger *et al*., 2015).

Thus, importance of biodegradation studies in soil medium cannot be ignored which the results of this research indicate. There have been a few biodegradation studies where plastics have been buried in soil medium to study biodegradation (Lwanga *et al*., 2018). Another benefit of studying biodegradation in soil medium is that burial of plastics in soil medium closely resembles natural environmental conditions (Eubeler *et al*., 2009). Similarly, Atiq *et al.* (2010) highlighted that soil burial of synthetic polymer for biodegradation must be deployed to study the phenomena of biodegradation in an experimental environment that is in close resemblance to natural environment.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

4.6 Conclusion

Homogenized mangrove soil used as the media for this biodegradation study was having pH range of 6.81 – 7.08 and TOC at 2%. However, total Nitrogen (TN) and total Phosphorous (TP) were within the range of 1260 – 3220 mg/kg and 320 – 340 mg/kg, respectively. After the experiment, pH, TOC and TP was recorded within the range of 7.42 – 7.91, 2.0 – 3.6 mg/kg, and 590 – 2710 mg/kg, respectively, whereas, value of TN was recorded in range of 1352 – 2020 mg/kg. In the experiment studying the effect of daily input of inoculum, only the value of TN decreased by 51 – 68% at the end of the experiment, whereas, in experiments studying the biodegradation of microplastics by different concentration of inoculum and on different sizes microplastics, all parameters namely pH, TOC, TP and TN had increased at the end of the experiment.

The selected bacterial consortium had shown biodegradation on the various plastic types and sizes.

The effect of microplastic sizes on biodegradation varied between polypropylene (PP) and polystyrene (PS). The biodegradation of smallest size of PP, 1 – 4 mm^2, was higher as 0.5% weight loss was recorded. It was also supported by disappearance of methylene and methyl group peaks in FTIR spectra and as well as, formation of new peak heralding the oxidation of PP. Additionally, SEM micrograph also showed formed cavities. The rate constant determined of 1 – 4 mm^2 PP was 0.000056 day^{-1} and the half-life 12445 days. On the contrary, largest size of PS, 25 mm^2, had shown higher affinity towards biodegradation. The weight loss of 25 mm^2 treated PS was 0.1% recorded. The FTIR spectrum of 25 mm^2 PS revealed disappearance of peaks associated with aromatic rings and shifting of typical peaks of methylene group and stretches of aromatic C-H which

inferred biodegradation of PS. The rate constant and half-life of 25 mm^2 treated PS was 0.000011 day^{-1} and 62352 days, respectively.

The biodegradation rate of PS increased with decrease in concentration of inoculum as maximum weight loss of 0.23% was observed in PS treated with 0.25% (v/w) inoculum concentration. The rate constant of PS treated with 0.25% inoculum concentration was 0.000019 day^{-1}, whereas the half-life was 37399 days. The FTIR spectrum of PS showed addition carboxylic acids, esters and alcohols peaks and disappearance of peaks associated with stretches of aromatic C-H. Moreover, it was also supported by surface erosion detected in SEM micrograph of treated PS.

Lastly, daily application of bacterial inoculum had resulted in highest weight reduction of all types of microplastics. 1.26% of weight loss was recorded for HDPE, 1.15% of weight loss in PP, and 0.5% of weight loss in PS. Weight loss observed in treated HDPE was supported by the addition of new peaks at 876 cm^{-1} and 1638 cm^{-1} in the FTIR spectrum. These peaks are associated with oxidation of PE. On the other hand, weight loss of PP and PS was supported by reduction in peak intensities of FTIR spectra of treated PP and PS, respectively. Thereby, rate constant of treated HDPE, PP and PS was 0.002 day^{-1}, 0.00018 day^{-1} and 0.00008 day^{-1}, respectively. Whereas, the half-life of treated HDPE, PP and PS was 3333 days, 3649 days and 7777 days, respectively.

4.7 Recommendations

Following are the recommendations for further study of microplastic biodegradation:

1. Performing biodegradation studies in a setting such that microplastics are directly buried in the soil samples, instead of placing microplastics in sampling bags, and interaction of selected bacteria occurs directly with microplastics. At the end of the experiment, microplastics could be separated from soil using density separation method.

2. Instead of using a selected weight of microplastics (as 1 g was used in this study), the effect of concentration of microplastics (number of microplastics), in a given area, on the biodegradation capabilities of microbes can be examined. Generally, microplastics in the environment are reported as particles per weight of soil sample or volume of water sample, or particles per meter or kilometre square or cube. Therefore, to imitate the real conditions in laboratory, average particles of microplastics (in the local area) can be used to study the biodegradation capabilities of selected microbes. In this way, results may closely resemble the scenario of bioremediation.

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