METAGENOMICS OF MICROBIAL DIVERSITY IN BOTH ACTIVE AND CLOSED LANDFILLS AND THEIR TOLERANCE TOWARDS SELECTED HEAVY METALS

MOHAMAD YUSOF BIN ZAINUN

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

METAGENOMICS OF MICROBIAL DIVERSITY IN BOTH ACTIVE AND CLOSED LANDFILLS AND THEIR TOLERANCE TOWARDS SELECTED HEAVY METALS

MOHAMAD YUSOF BIN ZAINUN

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **MOHAMAD YUSOF BIN ZAINUN**

Matric No: **SGR160012**

Name of Degree: **MASTER OF SCIENCE**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

METAGENOMICS OF MICROBIAL DIVERSITY IN BOTH ACTIVE AND CLOSED LANDFILLS AND THEIR TOLERANCE TOWARDS SELECTED HEAVY METALS

Field of Study: **MICROBIOLOGY**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

METAGENOMICS OF MICROBIAL DIVERSITY IN BOTH ACTIVE AND CLOSED LANDFILLS AND THEIR TOLERANCE TOWARDS SELECTED HEAVY METALS

ABSTRACT

The municipal landfill is an example of human-made environment that contain high level of heavy metals contamination and harbors a complex diversity of microorganisms. To evaluate the landfill complexity, this study aims to assess the structures of bacterial communities in active and closed landfills with culture independent metagenomics approaches. At the same time, the potential of indigenous landfill bacteria to treat heavy metals and their succession in bioaugmentation process were analysed. Several points of soil samples were collected from 0 to 20 cm depth and were subjected to physicochemical test. The bacterial enumeration was examined while the microbial soil DNA was extracted prior to sequence the 16S rRNA gene for bioinformatics analyses. As a result, the higher bacterial operational taxonomic units (OTUs) sequenced was recorded in closed landfills compared to active landfill i.e. 6625 and 4552 OTUs respectively. The data from both landfills showed that the predominant phyla belonged to Proteobacteria (55.7 %). Bacteroidetes was the second highest phylum followed by Firmicutes for the active landfill. While the phyla for communities in closed landfill were dominated by phyla from Acidobacteria and Actinobacteria. These composition of bacterial communities shows some variances between the bacterial communities found in active and closed landfills. On the other hand, twenty nine heavy metal resistant bacteria were isolated from both landfills displayed different degree of metal ions tolerance. The STB7 strain with identification as *Delftia tsuruhatensis* shown the most potential isolate toward the test. The result in preliminary tests of the isolated microbes suggests their suitability for enhanced bioremediation of heavy metal polluted environment. Furthermore, the succession of inoculated bacteria that potentially to treat heavy metal in landfill soil were

assessed via denaturing gradient gel electrophoresis (DGGE) approach. The result shown the inoculated bacteria treatment of bacteria into soil sample did survive at the beginning of treatment before gradually disappear when the time passes, especially when reaching 100 days incubation.

Keywords: Bacteria community structure, 16S rRNA gene, contaminated soil, molecular technique, DGGE.

METAGENOMIK KEPELBAGAIAN MIKROB DALAM TAPAK PELUPUSAN AKTIF DAN TERTUTUP SERTA TOLERANSINYA TERHADAP LOGAM BERAT TERPILIH

ABSTRAK

Tapak pelupusan sampah merupakan contoh bagi persekitaran ciptaan manusia yang mengandungi aras pencemaran logam berat yang tinggi serta mempunyai pelbagai diversiti mikroorganisma yang kompleks. Bagi menilai hal tersebut, kajian ini dijalankan bertujuan untuk mengetahui struktur komuniti bakteria bagi tapak pelupusan yang aktif dan tertutup dengan menggunakan pendekatan tanpa kultur metagenomik. Pada waktu yang sama, potensi bagi bakteria tempatan untuk tujuan pemulihan logam berat and kemampanan meraka untuk proses bioaugmentasi turut di analisa. Beberapa titik lokasi sampel tanah di ambil merangkumi kedalaman dari 0 hingga 20 cm dan seterusnya diperiksa keadaan fisikokimianya. Perhitungan jumlah bakteria juga diperiksa dan DNA bakteria tanah di ekstrak pada rantaian 16S rRNA bagi tujuan bioinformasi analisis. Hasil keputusan urutan operasi unit bakteria (OTUs) dicatitkan lebih tinggi bagi tapak pelupusan tertutup berbanding aktif dengan masing-masing berjumlah 6625 and 4552 OTUs. Data bagi kedua-dua tapak pelupusan menunjukkan bahawa phyla yang dominan dimiliki oleh Proteobacteria (55.7 %). Bacteroidetes merupakan phylum kedua tertinggi di ikuti Firmicutes bagi tapak pelupusan aktif. Manakala, bagi tapak pelupusan tertutup di dominasi oleh phyla daripada Acidobacteria dan Actinobacteria. Oleh yang demikian, komposisi stuktur bacteria ini memperlihatkan sedikit variasi yang wujud di antara bakteria komuniti bagi tapak pelupusan aktif dan tertutup. Pada waktu yang sama, dua puluh sembilan bacteria yang tahan terhadap logam berat berjaya di isolasi daripada kedua-dua tapak pelupusan dengan menunjukkan berbagai peringkat toleransi logam berat. Strain STB7 dengan pengenalan sebagai *Delftia tsuruhatensis* menunjukkan bacteria yang paling berpotensi terhadap ujian yang telah dijalankan. Tindak balas yang

ditunjukkan daripada mikrob-mikrob ini menunjukkan kesesuaian mereka bagi tujuan bioremediasi terhadap logam berat yang terdapat pada alam sekitar. Sebagai tambahan, kemampanan bioaugmentasi inokulasi bakteria yang berpotensi untuk memulih logam berat di nilai melalui pendekatan gradiasi penyahaslian gel elektroforesis (DGGE). Hasil menunjukkan rawatan bakteria ke dalam tanah kekal hidup pada permulaan inokulasi dan membantu untuk memulih logam berat sebelum secara perlahan-lahan menghilang apabila logam berat berkurangan seiring masa berlalu terutamanya menghampiri detik 100 hari pengeraman.

Kata kunci: Struktur komuniti bakteria, 16S rRNA gen, tanah tercemar, teknik molecular, DGGE.

ACKNOWLEDGEMENTS

In the name of God, the Most Gracious and the Most Merciful. Alhamdulillah, praise to Allah SWT at last I had successfully finished my thesis writing for my degree of Master of Science. In this opportunity I want to dedicate my appreciation firstly to my supervisor Dr Khanom Simarani because for her willingness to guide me throughout the years and without her guidance, this dissertation would not have been possible. I gained a lot of new knowledge and learned so much during handling this project.

Besides that, my special thanks go to University of Malaya and the Institute of Biological Sciences for granting financial support for this research project.

Last but not least, I wish to express my deepest appreciation to my beloved parents and family who always give me a support in term of moral and financial based. Additionally, I'm thanks a lot towards all the staffs working in Microbiology Division, postgraduates and friends from Biotechnology and Applied Microbiology Laboratory which already help me so much during conducting the project. I'm really appreciated all of their efforts.

TABLE OF CONTENTS

Abstract…… iii-iv

Abstrak……..... v-vi

Acknowledgements……... vii

Table of Contents…….. viii-xi

List of Figures……... xii

List of Tables…… xiii

List of Symbols and Abbreviations…….. xiv-xvi

List of Appendices…… xvii

CHAPTER 1: INTRODUCTION..... **1**

1.1 Background……. 1

1.2 Objectives……... 4

CHAPTER 2: LITERATURE REVIEW……. **5**

2.1 Introduction……. 5

2.2 Metal contamination and their interaction in soil……... 6

2.3 Techniques in microbial diversity studies…….. 7

2.4 Metal remediation techniques from soil……. 9

2.4.1 Physico-chemical techniques……. 9

2.4.2 Biological techniques……..... 11

2.4.2.1 Interaction of microorganisms with heavy metals……. 13

2.5 Bioaugmentation……. 15

2.6 Denaturing gradient gel electrophoresis (DGGE)…...…….. 17

CHAPTER 3: MATERIALS AND METHODS…….. **19**

3.1 Site and soil sampling……. 19

3.2 Soil analysis and physicochemical determination……..……... 22

3.3 Enumeration and isolation of bacteria populations……. 23

3.4 Study on metagenomics profiling for assessing microbial diversity in both active and closed landfills……..……..... 23

3.4.1 Microbial soil DNA extraction and purification…… 23

3.4.2 The 16S rRNA amplicon Illumina sequencing soil bacteria……. 24

3.4.3 Statistical and bioinformatics analyses……..…… 25

3.5 Study on the potential of landfill bacterial strains for heavy metal remediation……..... 25

3.5.1 Bacteria isolation and purification……. 25

3.5.2 Metal tolerance screening…….. 26

3.5.3 Determination of Minimum Inhibitory Concentration (MIC)…….... 27

3.5.4 Antibiotic sensitivity test……... 26

3.5.5 Bacterial identification of six selected isolates…….. 28

3.5.5.1 Identification using MALDI-TOF……. 28

3.5.5.2 16S rRNA analysis for bacteria identification……... 29

3.6 Study on the microbial succession in bioaugmentation process of contaminated soil via PCR-DGEE approach……... 31

3.6.1 Microbial inoculation preparation……. 31

3.6.2 Bacteria and microbial soil DNA preparation …….. 32

3.6.3 PCR-DGGE analysis..……..... 33

CHAPTER 4: RESULTS……... **34**

4.1 Soil physicochemical properties……. 34

4.2 Metagenomics profiling for assessing microbial diversity in both active and closed landfills…….. 37

4.2.1 Analysis of sequencing and data depth……. 39

4.2.2 Diversity index analysis on microbial community…… 40

4.2.3 The analysis of microbial community structure and dominant phyla……... 40

4.2.4 Clustering pattern of microbial community composition from active and closed landfills……. 42

4.3 Potential of landfill bacterial strains for heavy metal remediation……. 44

4.3.1 Microbial isolated from landfills soil…… 44

4.3.2 Metal tolerance screening…….. 48

4.3.3 Determination of Minimum Inhibitory Concentration (MIC)…….... 50

4.3.4 Antibiotic sensitivity test……... 51

4.3.5 Bacteria identification of six selected strains..... 52

4.4 The microbial succession in bioaugmentation process of contaminated soil via PCR-DGEE approach……. 54

4.4.1 Analysis of DGGE on different inoculated bacteria as referral indicator .....……..... 54

4.4.2 Analysis of microbial succession…….. 57

CHAPTER 5: DISCUSSION……. **61**

5.1 Metagenomics profiling for assessing microbial diversity in both active and closed landfills…….. 61

5.2 Potential of landfill bacterial strains for heavy metal remediation……. 66

5.3 The microbial succession in bioaugmentation process of contaminated soil via PCR-DGEE approach……. 71

CHAPTER 6: CONCLUSIONS…….……...…… **74**

References……..... 75

List of Publication and Papers Presented…….. 89

Appendices...……. 91

LIST OF FIGURES

Figure 3.1 : The location of both landfills and their sampling points, a) Bukit Beruntung and b) Taman Beringin……..... 21

Figure 4.1 : Venn Graph (a) and the rarefraction Curve (b) based on 97 % similarity……..…….. 38

Figure 4.2 : The Choa1 index (a) and Shannon's diversity index curve (b) based on 97 % similarity……...……. 39

Figure 4.3 : Taxonomic summary of 16S rRNA gene sequences from both active and closed landfills for each different sampling point at the phylum level……. 40

Figure 4.4 : Principal component analysis of the seven samples from both Group B-active landfill and Group T-closed landfill……... 42

Figure 4.5 : Hierarchical clustering of microbiomes form active and closed landfills from difference sampling points locations…. 43

Figure 4.6 : Phylogenetic analysis STB7 using Mega 7. Phylogenetic reconstruction was performed by using neighbour-joining and test Bootstrap method of 1000 replicates…..... 53

Figure 4.7 : DGGE analyses of 16S rRNA fragment of each inoculated bacteria obtained after PCR amplification…..... 55

Figure 4.8 : DGGE profile of 16S rRNA gene amplification of soil samples on day 0 (a), day 60 (b) and day 100 (c) for different bacterial treatments……. 57

LIST OF TABLES

Table 3.1	:	General condition of the landfills……	20
Table 3.2	:	Description of sampling sites……..	20
Table 3.3	:	PCR Master Mix Preparation……..	30
Table 3.4	:	Conditions for PCR amplification…….....	30
Table 3.5	:	List of bacteria in the treatments set up……	32
Table 4.1	:	Physicochemical properties of landfills soil and microbial population in both Bukit Beruntung and Taman Beringin landfills……...	35
Table 4.2	:	Composition of soil heavy metal in both landfills…….....	35
Table 4.3	:	Standard of heavy metal limit in soil (mg/L) in Malaysia……	36
Table 4.4	:	Summary of 16S rRNA gene sequencing of both group B (active landfill) and group T (closed landfill) for bacterial diversity analysis…….	37
Table 4.5	:	Colony morphological characteristic of isolated bacteria from Bukit Beruntung and Taman Beringin……	44
Table 4.6	:	Cell morphological characteristic and biochemical test of isolated bacteria from Bukit Beruntung and Taman Beringin	47
Table 4.7	:	The susceptibility of 29 isolated bacterial strains toward 10 selected metal ions……..	49
Table 4.8	:	Tolerance patterns of 10 heavy metals ions in 29 soil microbial strains……..	49
Table 4.9	:	MIC of 10 metal ions against the selected bacteria strain isolated from both landfills soil…….....	50
Table 4.10	:	Antibiotic sensitivity profile of selected bacterial isolate……	51
Table 4.11	:	Isolated bacterial species from the landfill soils……..	52
Table 4.12	:	The indicator represents of mixed bacteria……..	55

LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS

ºC	:	Degree celcius
cm	:	Centimeter
g	:	Gram
g/L	:	Gram per liter
h	:	Hours
min	:	Minutes
mg/L	:	Milligram per liter
mL	:	Milliliter
mm	:	Millimeter
mM	:	Millimolar
ppm	:	Parts per million
rpm	:	Revolutions per minutes
v/v	:	Volume per volume
µl	:	Microliter
%	:	Percent

ABBREVIATIONS

Ag	:	Silver
Al	:	Aluminium
As	:	Arsenic
BBL	:	Bukit Beruntung landfill
Cd	:	Cadmium
$CdCl_3$	:	Deuterated chloroform
Co	:	Cobalt
Cr	:	Chromium
Cu	:	Copper
DGGE	:	Denaturing gradient gel electrophoresis
Fe	:	Iron
Hg	:	Mercury
HNO_3	:	Nitric acid
ICPMS	:	Inductively Coupled Plasma-Mass Spectrometry
MCA	:	MacConkey agar
MIC	:	Minimum inhibitory concentration
Mg	:	Magnesium
Mn	:	Manganese
MSA	:	Mannitol salt agar
MSW	:	Municipal solid waste
NA	:	Nutrient agar
Ni	:	Nickel
OTUs	:	Operational taxonomic units
Pb	:	Lead

PCR-DGGE	:	Polymerase chain reaction - denaturing gradient gel electrophoresis
TBL	:	Taman Beringin landfill
Zn	:	Zinc
ZnCl	:	Zinc chloride

LIST OF APPENDICES

Appendix A : Taxonomic summary of 16S rRNA gene sequences from the active and closed landfill of each difference sampling points at the class level……... 91

Appendix B : Taxonomic affiliation of 16s rRNA metagenomics fragments of Bukit Beruntung and Taman Beringin sample soil …….. 92

Appendix C : Preparation of culture medium……. 96

Appendix D : Gram staining technique……..... 97

Appendix E : Biochemical test of oxidase and catalase……..... 98

Appendix F : Picture of colony and cell morphology of six selected bacteria…… 99

CHAPTER 1: INTRODUCTION

1.1 Background

Municipal solid waste (MSW) landfills have turned into a habitual spot to dump solid wastes in many countries, including Malaysia. As such, the case of leachate has emerged as a primary concern that has yet to be addressed due to this landfills waste disposal practice (Oller *et al.*, 2011) for the increasing rate of deleterious soil and groundwater pollutions, as a consequence of discharged leachate, is rather alarming (Han *et al.*, 2016). Furthermore, leachate that consists of contaminants like heavy metals, organic matter, as well as chlorinated organic and inorganic salts (Dao *et al.*, 2016) that are hazardous to the surrounding environment, are also affecting the public health. In addition, this contamination is slow in its degradation process and its harmful residue can last for more than three decades (Perez-Leblic *et al.*, 2012).

The microbial communities, especially in leachate and soil landfill has a potential to transform most pollutants and organic elements into less toxic compounds (Staley *et al.*, 2015). The study of microbial communities in contaminated landfills can reflects the level of contamination, whereby this precise knowledge can be applied as a measurement to predict and monitor their rates of natural degradation (Jain *et al.*, 2005; Tavares *et al.*, 2016). Although several studies have tapped into the basic microbial reaction at a lab scale, along with a pilot study concerning landfill bioreactor (Sang *et al.*, 2008); the aspects of structural and functional in microorganism communities in the actual landfill have yet to be discovered.

In general, researches concerning bacteria in landfills have looked into the application of conventional methods, such as culture dependent and culture independent techniques. The latter method of genetic molecular tools, for instance, denaturing gradient

gel electrophoresis (DGGE) (Nayak *et al.*, 2009), fluorescence *insitu* hybridization (Burrell *et al.*, 2004), and PCR cloning (Huang *et al.*, 2005), have been employed to characterize microbial communities without undergoing the cultivation process.

At the same time, the existence of high concentration of heavy metal in leachate and landfill soil has cause a number of environmental problems. Zinc, magnesium, chromium and iron are some of heavy metals that been reported to be dominant in soil landfill (Jayanthi *et al.*, 2017). Most heavy metal are toxic to animals, plants and humans. Metal such as arsenic, manganese, nickel, led, copper and cadmium are easily to accumulate in vital organ and therefore can threaten human health. As for example, history showed us the itai-itai and minamata disease had strike in Japan caused by the cadmium poisoning due to mining activity and mercury contamination in the water stream (Hema *et al.*, 2014). Therefore, heavy metal pollution in soil is a growing environmental problem, which requires immediate attention.

There are some report showed that the indigenous microbial communities are capable in running extensive bioremediation activities (Staley *et al.*, 2015). Bacteria bioremediation with the utilising of bacteria is one of the effective method to remove contamination in the environment (Sheng *et al.*, 2008). Mixed population of bacteria are undoubtedly play a significant role in the degradation process of toxic substances like heavy metals especially in complex landfill soil condition. These bacteria can transform, absorb, reduce the mobility and bioavailability of heavy metals in order to remove the contaminants as reviewed by Wu *et al.* (2010).

One of the methods to apply soil bioremediation is via the biological augmentation process. This technique functioning by adding a specific of bacterial cultures that required to speed up the rate of contamination degradation. Normally, microorganisms that originated from contaminated areas may possess an ability to break down wastes, but

perhaps in slow rate and inefficient (Al-Mailem *et al.*, 2017). Therefore, studying the indigenous microbial varieties present in the modification ecosystem is important to determine if biostimulation is possible to stimulate the microbial population that capable of bioremediation. The PCR-DGGE is one of the advanced methods which can be used to determine the present of existing bacterial population in a particular sample. The PCR could amplified the common region of internal DNA simultaneously within the same reaction tube to reduce the variability (Pintado *et al.*, 2003). It may become a good option to quantify bioaugmentation of microbial mixed cultures.

Therefore, this study aimed to investigate bacteria communities through the use of novel and high-throughput sequencing approaches that offer more readable sequences for analyses that enabling a more complete picture pertaining to landfill microbial communities. Two non-sanitary landfills represent operational and non-operational landfill soil samples were used for HiSeq-based 16S rRNA gene sequence analysis in order to carry out an in-depth genetic survey, as well as to gain better taxonomic resolution. The physicochemical test and ICPMS were also conducted to quantify the relative level of heavy metals in the soil samples. At the same time, the heavy metal tolerance bacteria isolated from both landfills were screened as an early exploration in their bioremediation capabilities. These selected bacterial were further assess for their present in landfill soil via the PCR-DGGE approaches. This method allowed the investigation on the succession of inoculated bacteria into the microcosms landfill soil as an artificial, simplified ecosystems that are used to stimulate and predict the behaviour of natural ecosystems under controlled conditions after 100 days of bioaugmentation.

1.2 Objectives

- To identify bacteria diversity of closed and active landfill using 16s rRNA metagenomic analysis.
- To screen the most potential bacteria for heavy metal degradation.
- To determine the microbial succession in bioagumentation process of contaminated soil via PCR-DGEE approach.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The modern industrialization, urbanization and rapid development driven by the exponential growth rate of world population has directly influence the balance of ecosystems and the surrounding environment. According to Feng *et al.* (2017), the consequential of poor waste management and contaminations from these activities has become a major risk generally toward all life cycle of ecosystem. One of the worldwide environmental problems is concerning the production of waste which is come from the anthropogenic activities (Wu *et al.*, 2015). Land filling is one of the major parts of municipal solid waste (MSW) disposal especially in developing countries. Therefore, better understanding on this system is crucial to improve the MSW disposal management and development.

However, very little discovery were made up on landfill condition and their potential of local microbial diversity because of many obstacles involved including the sampling problems and solid wastes itself that are potentially hazardous (Adelopo *et al.*, 2017). Renou *et al.* (2008) mentioned that landfill will produce a leachate through decomposing solid wastes due to the infiltration of rain water and snowmelt. This has resulted in long-term pollution emissions with high concentration of organic and inorganic substances. Furthermore, the formation of leachate from the landfill usually contain contaminants such as organic matter, chlorinated organic, inorganic salts as well as heavy metals (Dao *et al.*, 2016).

Heavy metal is one of the major concern in environmental problem as it is extensive be used, widely distributed in every corner of the world and particularly are very toxic to human beings and the biosphere (Prasad & Freitas, 2003). In Malaysia, the

Department of Environment has set a standard limit for certain heavy metals in soil with maximum perimeter such as 507.2 mg/L of Mg, 14.40 mg/L of Cr, 3.99 mg/L of Mn, 28.90 mg/L of Ni, 19.8 mg/L of Cu, 54.3 mg/L of Zn, 0.09 mg/L of Cd and 0.42 mg/L of Hg (Sakawi *et al.*, 2013). However, some of landfills have been reported to be exceed this permissible limit (Jayanthi *et al.*, 2016). Generally, heavy metal will cause negative effect on human physiology and other biological system. They show a great affinity for other elements such as sulphur disrupting enzyme functions in living cells by forming bond with this group. Cadmium, mercury and lead ions have the ability to bind to cell membranes, interfering with the cell transport processes (Bailey *et al.*, 1999). Heavy metals also not easily degraded and more likely to cause bioconcentration in which the condition of heavy metals accumulated in the tissues living organisms (Kobya *et al.*, 2005). In addition, their slow degradation process make these harmful contamination residue to be last more than thirty years in the environment (Perez-Leblic *et al.*, 2012; Su, 2014).

2.2 Metal contamination and their interaction in soil

Landfill soil is compost of a complex mixture of mineral, clay, organic such as humic substances, water and gaseous constituents. It is a firm system with variations of pH, moisture content, ionic strength and redox potential conditions. These factors will contribute and affected the availability of metals in soil (Calli *et al.*, 2005).

Generally, the absorption of heavy metal in soil will increases with increasing pH. The metal ions particularly become mobile under acidic environment while the increasing in pH will reduce and restricted the availability of the ions (Giller *et al.*, 1998). Cationic metals are becoming free ionic species or soluble organo-metals under the acidic condition. A report from Sandrin and Maier (2002) demonstrated that the pH play an important role in order to determine the solubility of cadmium. The ionic cadmium (Cd^{2+})

was measured with high concentration at pH 4 (44mg/l) while at pH 7 the value decreased (4mg/l), creating insoluble cadmium phosphate $Cd_3(PO_4)_2$.

2.3 Techniques in microbial diversity studies

One of the most important component in the soil ecosystem is the soil microbes. They play a key role in the soil health by regulating the material cycles and balance the energy flow in soil (Chen *et al.*, 2011). With the advance molecular based studies, there are estimated about 6000 species of microorganism in a gram of soil (Curtis *et al.*, 2002). A report from Kochling *et al.* (2015) find that landfill soil harbors complex microbial communities in which members of the bacterial Firmicutes, Proteobacteria, and Bacteroidetes phyla are the most abundant taxonomic groups, whereas archaeal populations typically consist of methanogenic species.

However, the understanding of prokaryotes are still remain incomplete and controversial even with all the technological advances. In order to study the microbial diversity within a microbial community, several characteristics such as microbial phylogenetic diversity and microbial functionality diversity are essential to have more details on the microbial community (Schloss & Handelsman, 2004).

Unfortunately, the conventional method of culture dependent technique is only be able to identified identify small part of the total microorganisms in the sample even with the application of enriched medium provided (Chien *et al.*, 2008). At the beginning of modern microbiology, there are several tools and methods that microbiologist was rely to, including the microscope, staining methods and pure cultures (Brehm-Stecher & Johnson, 2004). However, once the microorganism appearance is overlapping and similar with each other such as coccus or rod in shape, there is impossible to differentiate between the organisms using the morphology characteristics. There is also another common

method known as biochemical test and metabolic activities properties usually been used for this purposed. However, these methods are not really robust to be used for microbial classification as the microorganisms can adapt and change their properties and give difference outcome according to the environment conditions. Therefore, molecular based method has been identified as an essential alternative method to categorized microorganisms and microbial communities.

The advancement of molecular techniques has allowed the researcher to study and discover the microorganisms in variety of environments (Carini *et al.*, 2017). Pace *et al.* (1986) were become the pioneer that introduce the usage of culture independent method to study the microbial populations. This discovery including the analysis of 16S rRNA or 5S gene sequences that been directly extracted from the environmental samples. The progression in Polymerase Chain Reaction (PCR) was also facilitate the performance of this analysis. In recent year, there are a lot of methods has been implemented including PCR amplification of 16S rRNA, cloning of amplicons and next generation gene sequencing that comparing the obtained data with existing sequences information to allow the assessment of phylogenetic group microorganisms within a phylogenetic tree. Metagenomic analysis should make it easier to decipher taxonomic and functional assemblages of indigenous communities in natural environments, determine their potential roles in the biological functioning of ecosystems, and identify the associated services. As a result from these advance techniques has make the number of recognized phyla to increase significantly from 11 phyla in 1987 (Hugenholtz *et al.*, 1998) to 53 phyla as reported by Handelsman (2004).

The researcher could recognize and identify the active microorganism in the landfill environments that are potentially for bioremediation (Akob *et al.*, 2007). This is due to their domination and abundance that obviously play a key role in biochemical

processes such as organic matter and nutrients cycle, nitrogen fixation, microbial interaction with plants and bioremediation of harmful substances (Doney *et al.*, 2004).

2.4 Metal remediation techniques from soil

One of the serious environmental problems is concerning the emergence of heavy metal contamination. This hazard has affected the worldwide ecosystem and given a negative impact toward living organisms including the human beings. Heavy metal can be found in the environment especially in contaminated soil and water as free cations, as complexes form (e.g. ZnCl, $CdCl_3$) when combine with other organic or inorganic materials and always bind together with soil colloids (Wang *et al.*, 2010). The excess amount of these metals in the environment can lead toward the accumulation of it into the biological system and food web through various mechanisms (Giller *et al.*, 1998).

There are a lot of methods that been used to contain and/or remove heavy metal contamination from the environment. However, the selection of technique for the treatment is depend on the contaminated site characteristic, regulatory requirement, cost and time constraints.

2.4.1 Physico-chemical techniques

(i) Mechanical separation

This method was applied to separate the large particles from the smaller polluted particles. Usually this technique was used in mine ore processing and recently implemented in remediation of heavily contaminated soil (Mulligan *et al.*, 2004).

(ii) Isolation and containment

Isolation and containment techniques can be used in order to prevent the movement of heavy metals contaminations. The large physical barrier made up of different materials were usually been used for capping contaminants vertically and/or horizontally containment. This application has showed a good result to reduce water mobility thus lowering the metals permeability and availability (Mulligan *et al.*, 2004).

(iii) Chemical treatment

The mobility of heavy metal also can be reduce with chemical reactions like oxidation and reduction process. The contaminated water is one of the example condition that usually applying this kind of method to treat contamination. Some chemical such as potassium permanganate, chlorine gas or hydrogen peroxide were widely been used and added into the contaminated sites. Unfortunately, this method has certain drawback as it can create a new source of contamination (Mulligan *et al.*, 2004).

(iv) Soil washing

The contamination of heavy metal in soils can be removed by adding several type of chemicals into it. These chemicals are included the usage of chelating agent known as ethylenediaminetetraacetic acid (EDTA) organic, inorganic and organic acids such as acetic acid and sulphuric acid. Usually the process of cleaning soil was conducted in a reactor and returned to the original location after the process completed. However, the effectiveness of this method is depend on the soil condition and characteristics (Mulligan *et al.*, 2004).

(v) Electrokinetics

With this technique, the low amount of currents was passed between two different electrode of cathode and anode that been submerged in the contaminated soil. The electromigratian and electrophoresis movement were generated when the electric current was applied. The metals will be collected and removed through this process of electroplating, precipitation and recovering that separated them from the remaining soils. Europe has implemented this technique in their metal recovery processed (Mulligan *et al.*, 2004).

(vi) Ion exchange

This ion exchange is one of the common techniques that been used to remove the metals contamination. This process required an insoluble exchange materials that targeting a specific metal species to be displaced. Chelating resins, zeolites, plant materials and microorganism are example of exchange material that can be used in this method. However, the process is very sensitive toward pH conditions and very costly (Mulligan *et al.*, 2004).

2.4.2 Biological techniques

Some of these contaminated soils and sediments contain a potential microorganism including the prokaryotes and eukaryotes that can dealing with the contaminants (Zettler *et al.*, 2002). Some microorganisms that have displayed the potential to degrade these pollutants are fungi, protozoa and bacteria (Fang *et al.*, 2014). For instance, *Bacillus* spp., *Aspergillus* spp., *Staphylococcus* spp., and *Streptomyces* spp., (Sineriz *et al.*, 2009) have been described to be able to tolerate with high concentration of various heavy metal (Hema *et al.*, 2014). A diverse array of bacteria individually or

cooperatively play such a key role in degrading the organic and inorganic matter over time (Krishnamurthi & Chakrabarti, 2013).

The microorganisms are one of the main actor that play an important role in recycling nutrients and heavy metals in the stressed environment (Moffett *et al.*, 2003). Some processes such as metal homeostasis, detoxification, metabolic exploitation, solubilisation and precipitation of heavy metal were carried by these organisms by modified the physicochemical conditions of the contaminated surrounding (Bruneel *et al.*, 2006). Several number of studies also suggested the role of soil/sediment microbial community in heavy metal remediation (Collins *et al.*, 2004). These finding highlighted the importance of metal microbes interaction and the biological process involved including the oxidation/reduction and the sorption of metals on the cell surfaces in order to determine the fate of heavy metals in environment.

Bioremediation is a process to degrade the contaminants from soil or any medium by microorganism under optimum condition. The successful of microorganisms to survive and carry out bioremediation activities were strongly depends on several key factor such as the biochemical properties, physiological and morphology of the organisms, genetic adaptation as well as environmental modification of metal speciation (Abou-Shanab *et al.*, 2007). Several studies carried out by Perez-de-Mora *et al.* (2006) showed that the exposed of microorganism toward the heavy metals for a long period of time has led to the adaptation/selection of microbial community to be survived in the contaminated soil. The ability of the microorganism to tolerate and transforming the heavy metal into less toxic compound enable them to live in the contaminated site and potentially useful in bioremediation purposed.

2.4.2.1 Interaction of microorganisms with heavy metals

Certain heavy metals such as zinc, nickel, copper and cobalt are essential for metabolic activity for bacterial cellular process. However only low concentration of these metals are needed for the activities such as enzymatic function and growth processes. In high concentration, the metal ions become toxic to cells. On the other hand, other metals such as Hg, Pb, Cr and Cd are very harmful toward the cells and contain no known effect in cellular activities (Abou-Shanab *et al.*, 2007; Chen *et al.*, 2005).

It is known that microbial activity plays an important role in the metal speciation and transport in the environment (Rajkumar *et al.*, 2012). The microorganism may have certain specific mechanism in order to enable them to do so. Some microorganisms have been recognised to exhibit tolerance toward various heavy metals by immobilizing them on their cell surfaces or transforming the metals into less harmful substances.

Bacterial surface structure is one of the important material that working and interacting with metal ions from surrounding environment. Basically the bacteria can be categorised into Gram negative and Gram positive bacteria depends on the structure that made up their outer layer of cells. Gram positive bacteria is a cell with thick peptidoglycan that made up as much as 90 % of the cell wall together with small percentage of teichoic acid (Guine *et al.*, 2007). On the other hand, the Gram negative cell contain multi-layered structure with an outer layer cell consist of lipopolysaccharides, phospholipid and small amount of peptidoglycan constituent. These structures are forming a negatively charged toward the outer layer of cells and interacting with surrounding metal ions (Guine *et al.*, 2007).

Bioaccumulation is example of mechanisms on how microbial action reduce the concentration of metal ions in contaminated soil. This process is a substrate specific

mechanism that driven by adenosine triphosphate (ATP) and required active transport for heavy metal uptake. This active transport is one of three mechanism apart from passive and facilitated transport that responsible to allow any substances to be in or out of the cells. Usually, the active transport required selective metal transport system and only specific transporter is able to carry the specific metal. However there are some exceptions on it as more discovery revealed that Cd ions also can be transported by the same protein transporter as Zn (Ansari & Malik, 2007).

On the other hand, biosorption is refer to other process of metals reduction that required no ATP but controlled by the physico-chemical factor such as the chemiosmotic gradient of the cell. This passive metal uptake system may take place in either the living or dead biomass (Chen *et al.*, 2005). The negative charge of cell wall from both Gram positive and negative are very important in metals cation sorption (Krishnamurthi & Chakrabarti, 2013). Errasquin and Vazquez (2003) reported that the biosorption has high tendency to remove heavy metal contaminants in the environment especially from wastewater. However, the cost for this treatment is quite expensive and play an important factor before been implemented. Therefore, low-cost biomass is always been take into consideration for practical application in biosorption (Chen *et al.*, 2005).

The other mechanisms that lead toward bacterial resistance included the metal complexation, reduction, active efflux and sequestration that transform the metal ions into less toxic substances (Nies, 2003). Most of these methods are driven by the special sequences of gene in plasmid that can contributes and transferred from cell to cell (Valls & de Lorenzo, 2002), and encoded in chromosome resistance gene that belong to certain bacterial species (Abou-Shanab *et al.*, 2007). From the metabolic point of view, a special group of metal-chelating protein that known as metallothioneins plays are an important

role in metal resistance. These small cysteine-rich polypetides functioning by binding the metal ions from the cell and remove it (Valls & de Lorenzo, 2002).

In recent years, the importance of bacteria as heavy metals remediation agents has increased especially with the technology advancement that allow discovery of potential microorganisms to remove metal contaminations (Valls & de Lorenzo, 2002). Errasquin and Vazquez (2003) stated that bacteria is one of a good biosorbents that can become a promising and a good alternative for metals removal from the environment in the near future. Several studies included the isolated metal resistance bacteria are been tested for bioremediation processes. *Cupriavidus metallidurans* strain CH34 has shown a potential in heavy metal remediation in polluted soil and water. This strain of bacteria able to accumulate the metals included Gold (Au), Selenium (Se) and volatilize Hg via reactive processes (Guine *et al.*, 2007). Meanwhile, studied on *Pseudomonas stutzeri* isolated from foundry soil showed a capability to tolerate with chromium up to 1 mM (Tsai *et al.*, 2005).

2.5 Bioaugmentation

The remediation technology known as bioaugmentation has started since the late 70's. Bioaugmentation is refer to the process of addition of cultured microorganisms into the subsurface for promoting specific biodegradation (Ellis *et al.*, 2000). Normally it been used to transform the contaminants in soil and groundwater into less harmfull substances. The prominent of bioaugmentation begin when the first large scale bioaugmentation was applied to clean up British beaches that been contaminated by the Torrey Canyon spill in 1970's. Since that time, the environmental agencies in US had gain support on the effort toward bioremediation activities and success in Exxon Valdez oil spill in 1989 (Rothmel *et al.*, 1998).

Cornu *et al.* (2017) had suggested the list of sites that suitable for bioaugmentation with the following conditions:

- Site that contain low or non-identifiable of contaminant degrading microorganism.
- Site with contaminations that require a long time to be clean up.
- Site that involve in time constraint to be clean up that may not enough if using biostimulation alone.
- Site that encompass with compound that needed multi-process remediation.
- Site with small scale area by which the cost for bioaugmentation is lower compare to cost for extensive testing.

After determining the possibility for the successful bioaugmentation on particular contaminated site, an appropriate microbes or suitable group of microorganism must be chosen. There is some commercial available product that contain microbial strain with capability to degrade contaminants or selected microbial strain can be obtained through research and screening. Usually the growth of selected microbes are tested under the restricted environment to enhance their capability to degrade the contaminants. Bioaugmentation can be more successful when the selective cultures microbes are already adapted toward the target contaminants and the condition in site location (Mrozik & Piotrowska-Seget, 2010).

However there is also several limiting step in bioaugmentation should be considered in order to make this application to be more effective on field trial such as the seed microorganisms must be possess an ability to degrade most of the target contaminants, able to survive and proliferate in new and hostile environment, maintaining the genetic stability and viability during storage, effectively compete with local microorganisms, have direct contact with the pollutant and can pass through the pores of

sediment to the target area of contaminants location. The complex and unpredictable condition of the subsurface in full scale experiment has created an option to run up a bioaugmentation test in the laboratory. This small scale experiment usually required a microcosm and column studies that represent the system dynamic of the real situation with controlled condition applied.

2.6 Denaturing gradient gel electrophoresis (DGGE)

Microbial ecology in soil are very prone to the changes of their surrounding environment, temporal changes or the response toward specific experimental treatments. In order to address the structural differences among the communities, a technique that can address the structural difference among the whole communities should be implemented (Valaskova & Baldrian, 2009). Bhakta *et al.* (2017) mentioned that in last twenty years, the method used to describe the diversity and availability of bacterial populations in soils have undergone major changes as the cultivation based approaches were switched toward a new and comprehensive culture independent methods. This is very crucial and important shift as the old cultivation dependent methods can only analysed minor fraction of a soil microbial community. In contrast, the most recent molecular methods are able to analyse the vast genetic materials from environmental samples. Among the methods currently used to compare microbial communities based on nucleic acid sequences, the techniques based on differences in the melting properties of double stranded molecules, denaturing gradient gel electrophoresis (DGGE) are the most widely used (Valaskova & Baldrian, 2009).

DGGE was developed in the 1980s for the identification of point mutations and was first used for the analysis of microbial communities in the early 1990s (Muyzer *et al.*, 1993). This method is based on the separation of the same length but difference in G-C base pair on bacterial 16S rRNA by electrophoresis in a gradient of a denaturant.

Muyzer *et al.* (1993) have adopted this application to extend the knowledge on microbial ecological in various environmental samples. DGGE has been used to separate the same length of targeted fragment but with different sequences in an acrylamide gel with a gradient of denaturing chemical (Aydin *et al.*, 2015). This technique will allow the sequence variation of bacterial hypervariable region in 16S rRNA gene to be determined and thus can be used to study the microbial diversity and relative abundance in natural ecosystem (Muyzer & Smalla, 1998; Nubel *et al.*, 1999).

The outcome from DGGE analysis will provide an image composed an array of bands with difference intensities. The band intensities were reflecting the frequency of each PCR products in the reaction mixture (Valaskova & Baldrian, 2009). Therefore, this molecular application provided a rapid, simultaneous and reproducible analysis of sample even though with limited resolution (Kowalchuk *et al.*, 2004).

CHAPTER 3: MATERIALS AND METHODS

3.1. Site and soil sampling

Two types of non-sanitary landfills had been selected for this study; the active landfill situated at Bukit Beruntung (BBL) and the closed landfill located at Taman Beringin (TBL), Selangor, Malaysia (Figure 3.1). These landfills have and had served as domestic and industrial waste dumping sites; known as MSW landfill.

Table 3.1 presents the general condition of the landfills. Soil samples with a depth of 0-20 cm were collected from several selected points at each landfill from areas contaminated with leachate by using a one-piece auger in adherence to 2014 ASTME – 1197 standard guideline in performing terrestrial soil-core microcosm test (Sprocati *et al.*, 2012). As for the Taman Beringin landfill site, four various sampling points were opted, while three samplings points for Bukit Beruntung landfill, as described in Table 3.2. Besides, for each point, several small sub-points were gathered and mixed well to obtain the final homogenised soil. After that, some portion of the composite soil samples were kept in a sampling bag with ice pack for transportation purpose before stored at -20 ˚C for further analysis.

Table 3.1: General condition of the landfills.

Landfills	Bukit Beruntung	Taman Beringin
Status	Active (operational)	Closed (non-operational)
Landfill type	Non sanitary	Non sanitary
Location	3 32.14N, 101 25.80E	3 13.78N, 101 39.72E
Classification	Mature	Stabilized
Landfilling period	2001-to date	1995-2005
Waste type	Household, commercial, industrial	Household, commercial, industrial and others
Fate of landfill gas generated	No facility	No facility

Source: Adapted from Jayanthi *et al.* (2016)

Table 3.2: Description of sampling sites.

Location landfill	Sampling points	Latitude	Longitude	Detail
Bukit Beruntung (Group B)	BB	3°42’49.32N	101°54’45.34E	Top side and fresh wastes deposited
	BC	3°42’24.44N	101°54’38.21E	Middle side of landfill
	BD	3°36’33.46N	101°47’31.33E	Lower end of landfill
Taman Beringin (Group T)	TA	3° 7’ 26.34N	101°39’13.65E	Near collected area of leachate
	TB	3°13’37.88N	101°39’51.37E	Top side of landfill
	TC	3°13’25.95N	101°39’52.38E	Middle side of landfill
	TD	3°13’14.69N	101°39’45.45E	Lower slope of landfill

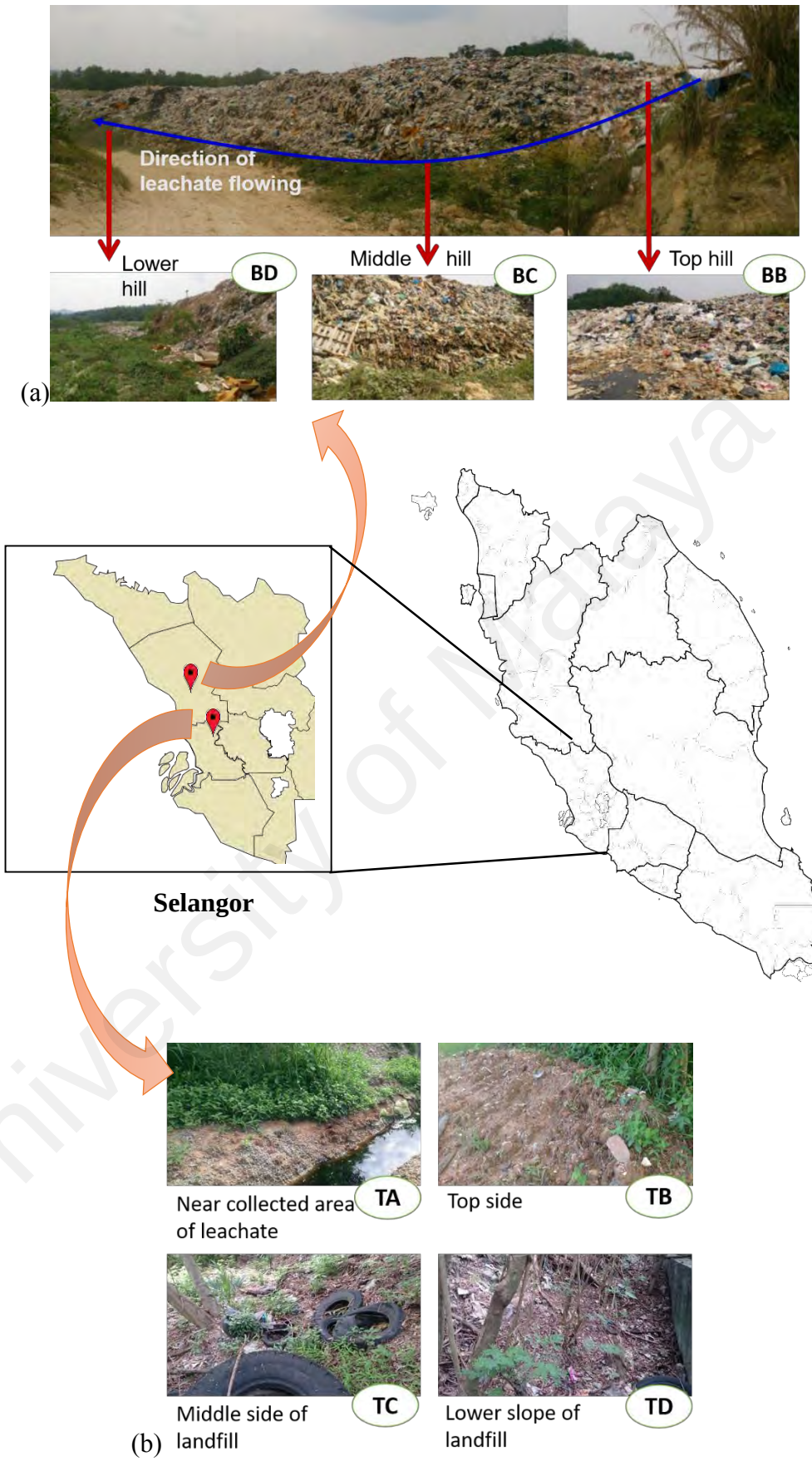


Figure 3.1: The location of both landfills and their sampling points, a) Bukit Beruntung and b) Taman Beringin.

3.2. Soil analysis and physicochemical determination

The sample soil suspensions were prepared by mixing 1 gram of sieved composite soil sample with 2.5 ml sterile distilled water (1:2.5 ratio) before measuring its pH by dipping it in PB-11 pH probe (Sartorius, USA) (Azlan Halmi *et al.*, 2018). The moisture content was determined based on the dry mass of the sample soil that had been oven dried at 105 °C for overnight (Estefan *et al.*, 2013) .

In addition, the composition of heavy metals concentration had been analysed via USEPA 3050B method by using the Agilent 7500 Series Inductively Coupled Plasma-Mass Spectrometry (ICP-MS ChemStation G1834B) (Agilent Technologies, Japan). The most common method to determine the concentration of metals contaminants in soil is via total elemental analysis (USEPA Method 3050). In this study, an initial mass of 1g of soil sample was put into a flask. A 10 ml of HNO_3 was added into the flask with covered vapour recovery device and placed on a hot plate to be heated. The sample was heated at 90˚C ± 5˚C and refluxed for 10-15 minutes without boiling according to USEPA 3050 (total-recovered). The sample was cooled before been added with another 5 ml of concentrated HNO_3 and further refluxed for another 30 minutes. This step was repeated again and again until there is no more brown fumes generated which indicated the complete reaction with HNO_3 was done. Finally, the solution was heated at 90˚C ± 5˚C without boiling for two hours. The end product of the digestion was cold down before be filtered using Whatman No. 41 filter paper and brought to a total volume of 50 mL with deionised water in a volumetric flask. The total heavy metal concentration was measured using ICP-MS.

3.3. Enumeration and isolation of bacteria populations

As for isolation of bacteria, 1g of soil sample was transferred into a tube that contained 9 ml of saline water (0.9 % NaCl) and was homogenised via vortex followed by serial dilution to produce the next dilution factor solutions. Next, 100 µl of sample diluted soil was pipetted on agar media and spread using a hockey stick. Three types of media were used in this research, i.e. nutrient agar (NA), MacConkey agar (MCA) (Nigam *et al.*, 2010), and mannitol salt agar (MSA) (Adekanle & Akindele, 2017), which were prepared based on the instructions provided by the manufacturer (Appendix C). After that, all inoculated plates were incubated at 30 ºC for two days. The growth of bacteria colony was observed daily and the colony forming unit (CFU) was determined (Breza-Boruta, 2016). The colony with different morphology was collected and purified on the fresh NA plate prior to maintain in the slant agar and stored at 4 ºC for future use.

3.4 Study on metagenomics profiling for assessing microbial diversity in both active and closed landfills

3.4.1 Microbial soil DNA extraction and purification

The DNA of landfill soil samples were extracted directly by using the Powersoil® DNA Isolation Kit by adhering to the instructions given by the manufacturer (MO BIO, USA). The purity of the harvested DNA was measured by using Nanodrops 2000 UV-Vis spectrophotometer (Thermo Scientific, USA) in order to check if there is any contaminants in the samples and followed by 1 % (v/v) agarose gel electrophoresis to quantify the amount of DNA (Masek *et al.*, 2005).

3.4.2 The 16S rRNA amplicon Illumina sequencing soil bacteria

The recovered DNA samples were further analysed for sequencing at Novogene Bioinformatic Technology Co., Ltd (Beijing, China). Briefly, the 16S protocol was designed to amplify prokaryotes (bacteria and archaea) using paired-end 16S community sequencing on the Illumina platform. The Polymerase Chain Reaction (PCR) amplification was conducted using primers with barcode 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) which target the V4 region of the 16S rRNA. The 30 µl PCR reaction mixture contained 15 µl of Phusion® High Fidelity PCR Master Mix (New England Biolabs): 0.2µM of forward and reverse primers, and about 10ng templates DNA. The PCR was performed using standard procedure: initial denaturation at 98 °C for 1 min followed by 30 cycles of denaturation (98 °C for 10 s) , annealing (50 °C for 30 s), and elongation (72 °C for 60 s) with a final extension at 72 °C for 5 min. The PCR products were mixed with 1X loading buffer containing SYB green with the ratio of 1:1 and analysed on electrophoresis using 2 % (v/v) agarose gel. Later, the samples with the bright and sharp band between 400 and 450 bp were selected for further sequencing analysis.

The selected PCR products were mixed in equidensity ratios and purified with the Qiagen Gel Extraction kit (Qiagen, Germany). After that, sequencing libraries were generated by using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) by adhering to the recommendations given by the manufacturer, where index coded had been added. The quality of the library was assessed using Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Lastly, the library was sequenced on IlluminaHiSeq2500 platform, where 250 bp paired-end reads were generated.

3.4.3 Statistical and bioinformatics analyses

After eliminating the barcode and primer sequences, paired-end reads from the original DNA fragments were merged by using FLASH (Magoc & Salzberg, 2011). The paired-end reads were assigned to each sample based on the unique barcode. After that, the tags were compared with the reference database (Gold database) using UCHIME algorithm to detect the chimera sequences before removal to obtain the final Effective Tags

Sequence analysis was carried out using the UPARSE software program (Edgar, 2013). As such, a sequence with ≥ 97 % similarity was assigned to similar OTUs. A representative sequence for each OTU (Green Gene Database) based on RDP classifier was employed to annotate taxonomic information. In order to compute Alpha Diversity (within the sample) , the complexity of species diversity was analysed via several indices, including Chao 1, Shannon index, and Observed-species, which were computed using QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). In addition, the Beta Diversity (among samples) was analysed on both weighted and unweighted unifrac calculated with QIIME (Version 1.7.0). Cluster analysis was preceded by principal component analysis (PCA) while the graphical representation of the relative abundance of bacterial diversity from phylum to species was visualized by using the Krona chart.

3.5 Study on the potential of landfill bacterial strains for heavy metal remediation

3.5.1 Bacteria isolation and purification

The isolation of bacteria was performed by mixed 1 g of soil samples into a tube contained 9 ml of normal saline water (0.9 % NaCl) and well homogenized by using vortex before followed by 20 times serial dilution (Jayanthi *et al.*, 2017). 100 µl of sample diluted soil was pipetted on nutrient agar media and spread using a hockey stick. Then,

the inoculated plates were incubated at 30 ºC for 24 h. Developed colony were subsequently sub-cultured to obtain the pure colony bacteria.

The characteristic of the isolates bacteria were recorded based on their morphology such as colony size, colour, margin, elevation and transparency (Brenner *et al.*, 2015). Each of the isolates then further characterized by using Gram staining and biochemical test such as catalase and oxidase test (Appendix D and E).

3.5.2 Metal tolerance screening

Metal tolerance screening was performed by using twenty nine bacteria cultures that previously isolated from the landfill soil against 10 type of heavy metals. These heavy metal salts (Cu^{2+}, Cr^{3+}, Fe^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+}, Zn^{2+}, Al^{3+} and Hg^{2+}) were used for the screening of heavy metal tolerance. Metal solutions were prepared in phosphate buffer saline with pH 6.8 to maintain the pH metal solutions. For each of the heavy metal, there are six different concentration were prepared ranging from 0.1, 1, 5, 10, 15 and 20 mM except for Hg which was 0.005, 0.01, 0.05, 0.1 and 1 mM (Abou-Shanab *et al.*, 2007).

Before conducting the test, all the glasswares were leached with strong acid HNO_3 and rinse with distilled water to remove any unwanted foreign substances and ensure there was no metal residue contamination on the container. The rapid test metal tolerance was performed based on direct agar diffusion method with some modification from Chandy (1999).

The nutrient agar (NA) plate was used as a growth media that been prepared according to the manufacturer before been sterilized at 121 °C for 15 min. A loop full of fresh pure colony of bacterial was picked and transferred into nutrient broth (NB) and incubated at 30 °C, 100 rpm for 24 hours. The suspension of bacterial was diluted, mixed and compared with 0.5 % MacFarland solution before been lawn onto the growth medium

by using sterile cotton swab. The plates were kept in the room temperature for at least 30 min to let the bacteria suspension dried up before been used.

The surface of lawn bacterial plate were divided into six partitions that represent for each concentration of the heavy metal. 10µL of each heavy metal concentration was carefully pipetted onto the partition of test plate. All plates were incubated at 30 °C for 24 hours and the present of clear zone were observed. The diameter of inhibition zone (mm) at the spotted area were measured. The halozone that appear on the plates indicate that the bacteria was susceptible toward selected heavy metal, while the absence of halozone represent the metal tolerance capability of that bacterial strains toward defining metal (Hema *et al.*, 2014).

3.5.3 Determination of Minimum Inhibitory Concentration (MIC)

Different types of heavy metal concentration were tested to determine the MIC value for each selected strain (six bacteria). These six bacteria were chosen based on their capability to tolerance with previous heavy metal screening test. The MIC values was measured using various concentrations of heavy metals with 20 mM, 15 mM, 10 mM, 5 mM, 1 mM and 0.1 mM were assayed against the tested bacteria. Each of the well in 96 well plates were filled with 100 µl of nutrient broth together with metal ions salt and 10 µl of tested bacteria. At another column of the plates, the well was filled with serial of heavy metal concentration without been added with bacteria inoculums that act as a negative control. The plates were incubated at 30 ºC for 24 h. The minimum inhibitory concentration was defined as the lowest concentration that able to inhibit any visible bacteria growth (Wiegand *et al.*, 2008).

3.5.4 Antibiotic sensitivity test

The antibiotic sensitivity test of the bacterial isolates were determined using disc plate method (Rajkumar *et al.*, 2012). Each bacteria was lawn on the entire fresh nutrient agar prior the placing of antibiotic disc such as Ampicillin (25 mcg), Chloramphenicol (30 mcg), Erythromycin (15 mcg), Neomycin (30 mcg), Streptomycin (10 mcg) and Vancomycin (30 mcg). All the plates were incubated at 30 °C for 24 hours. The result of inhibition zones diameter were quantified to the nearest mm and categorized as susceptible (S), intermediate (I) or resistance (R) classification according to Tomova *et al.* (2015).

3.5.5 Bacterial identification of six selected isolates

3.5.5.1 Identification using MALDI-TOF

Six bacteria were chosen based on their capability to tolerance with previous heavy metal screening test. All of these selected strains of bacteria were undergo identification process using MALDI-TOF to know their bacterial species. Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has emerged as one of the method to perform a microbial identification. The preparation of sample was depend on the constituent of the bacterial cell Gram staining. Some studies reported that the Gram positive bacteria were necessary to undergo preparatory extraction step before been analysis by MALDI-TOF MS while the Gram negative bacteria were not (Alatoom *et al.*, 2012).

The direct cell profiling method that been identified by MS was used by taken up a colony of cell bacteria using the sterilized toothpick and directly spotted on to the sample metal plate. The prepared matrix solution (consist of saturated HCCA powder, α-Cyano-4-hydroxycinnamic acid in a solution of 50 % acetonitrile, 47.5 % ionized water

and 2.5 % trifluoroacetic acid) was then dropped on the same spot to overlay the bacteria smeared. The MALDI sample metal plate was set to be air-dried before the spotted samples were analysed by mass spectrometry (Jeong *et al.*, 2014).

3.5.5.2 16S rRNA analysis for bacterial identification

Those selected bacteria also were tested on 16S rRNA sequencing analysis for their confirmation identification. The extraction of genomic bacterial DNA was performed by using the Genomic DNA Extraction Mini Kit according to standard procedure specified by the manufacturer (Yeastern Biotech Co., Ltd). The purity of final DNA aliquots were analysed by using Nanodrop 2000/2000c with optimum DNA range of 1.8-2.0.

For PCR purposed, a set of primer was used; 27F (forward primer): 5'-CACGGAGAGTTTGATCCTGGCTCAG-3' and 1492R (reverse primer): 5'-GGTTACCTTGTTACGACTT-3' to amplify the 16S rRNA gene of bacterial DNA (Pisol *et al.*, 2015). The PCR Master Mix was prepared as in Table 3.3. The PCR process was performed in Veriti 96 Well Thermal Cycler (Applied Biosystems) under condition as describe in Table 3.4. Agarose gel electrophoresis (1.0 %, v/v) were prepared in order to run the amplified DNA templates. The UV transluminator (Clever Scientific, UK) was used to observe the DNA amplified products before send for sequencing at Firstbase, Malaysia.

The generated sequences of 16S rRNA gene data were analysed using software Sequence Scanner Version 1.0 (Applied Biosystems). The partial sequence of the data was identify to determine the identification of isolates using EzTaxon-e server on the basis of partial 16S rRNA gene sequence database. Then the phylogenetic analysis was

conducted by using MEGA (Molecular Evolutionary Genetics Analysis) version 5 with neighbor joining of bootstrap analysis 1000 replications (Tamura *et al.*, 2011).

Table 3.3: PCR Master Mix Preparation.

Component	Stock concentration	Final concentration	Volume/ reaction (µl)
GoTaq® Green Flexi Buffer	5X	1X	5.0
dNTP mix	10 mM	0.2 mM	0.5
$MgCl_2$	25 mM	1.5 mM	1.5
GoTaq® DNA polymerase	500 U	0.02 U/ µl	0.125
Primer 27F	10 µM	0.2 µM	0.5
Primer 1492R	10 µM	0.2 µM	0.5
DNA template		~50 ng	2.0
Sterile dH_2O			14.875
Total			25 µL

Table 3.4: Conditions for PCR amplification.

Step	Temperature (°C)	Time	Number of cycle
Initial denaturation	95	2 min	1
Denaturation	95	30 s	35
Annealing	53	30 s	35
Extension	72	1 min 30 s	35
Final extension	72	10 min	1

3.6 Study on the microbial succession in bioaugmentation process of contaminated soil via PCR-DGEE approach

3.6.1. Microbial inoculation preparation

There were eighteen strains of potential bacteria were used to perform the heavy metal remediation in landfill soil bioaugmentation pilot study (Table 3.5). These selected bacteria were previously isolated from the above landfills and confirmed by using molecular method by Jayanthi *et al.* (2016). Each strain was grown as a pure culture in a nutrient agar (NA) plate for two days at 30 °C before being transferred into the nutrient broth (NB) and further incubated for another day at 30 °C with 150 rpm rotating shaker.

Two set of soil microcosm contamination (Soil Bukit Beruntung and Taman Beringin landfills) were prepared according to the ASTM guidelines that consist of 2.0 kg soil sample microcosm stored at room temperature. For each set, three types bioaugmentation treatments in triplicates were evenly dispersed with 20 % (w/v) of following bacteria cocktails; (A) mixture of all bacteria stated, (F) proteobacteria and (G) non proteobacteria (Table 3.5). Soil microcosm without any bacteria was used as a control. Regular watering was conducted by adding approximately 20 ml distilled water to ensure the moisture content in the soil were maintained. However, the excess watering must be avoided as it can create leachate which is not required in this experiment in order to prevent the wash out of metal contents.

The bioaugmentation process was maintained for 100 days under controlled environment at room temperature. A portion (5g) of soil samples were taken during 0, 60 and 100 days to determine the presence of inoculated bacteria in the microcosm system by using DGGE approaches (Hassanshahian *et al.*, 2016).

Table 3.5: List of bacteria in the treatments set up.

Label	Bacteria	Control	A	F	G
1	*Bacillus cereus*	-	+	-	+
2	*Aeromonas caviae*	-	+	+	-
3	*Delftia tsuruhatensis*	-	+	+	-
4	*Pseudomonas alcaligenes*	-	+	+	-
5	*Chryseobacterium gleum*	-	+	-	+
6	*Pseudomonas mendocina*	-	+	+	-
7	*Serratia marcescens*	-	+	+	-
8	*Ochrobacterium intermedium*	-	+	+	-
9	*Burkholderia vietnamiensis*	-	+	+	-
10	*Stenotrophomonas acidaminiphilia*	-	+	+	-
11	*Acidovorax ebreus*	-	+	+	-
12	*Brevundimonas diminuta*	-	+	+	-
13	*Cloacibacterium*	-	+	-	+
14	*Rhodococcus rubber*	-	+	-	+
15	*Bacillus aryabhattai*	-	+	-	+
16	*Bacillus Pumilus*	-	+	-	+
17	*Bacillus kochii*	-	+	-	+
18	*Janibacter hoylei*	-	+	-	+

Note for treatments: A: All microbes, F: Proteobacteria, G: Nonproteobacteria while Control treatment where none of the strain were inoculated.

3.6.2 Bacteria and microbial soil DNA preparation

Each bacterial genomic DNA of eighteen selected bacteria (Table 3.5) were obtained by using the Bacteria DNA Extraction kit as mentioned in 3.5.5.2. Meanwhile, the total microbial soil DNA samples were extracted as previously explained in 3.4.2.

3.6.3 PCR-DGGE analysis

For PCR amplification of 16S rRNA, both bacteria DNA (that later act as indicator) and microbial soil extracted DNA were amplified with bacterial primers F357GC:5"CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCGACGGGGGCTCCT ACGGGAGGCAGCAG- 3" and 518R: 5"-ATT ACC GCG GCT GCT GG-3" (Webster *et al.*, 2004). The PCR master mix was consist of 100 ng of DNA template, 2 µl 10 mM primers, 25 µl Taq DNA polymerase (MyTaq Red Mix) in a total of volume 50 µl. The condition for the PCR amplification was setup at 94 °C for 3 min, 30 cycle of denaturation at 94 °C for 1 min; annealing at 65 °C for 1 min; extension at 72 °C for 1 min; and the final extension was at 72 °C for 10 min . This PCR amplification of V3-region of 16S rRNA was conducted in a Veriti 96 Well Thermo Cycler (Applied Biosystems). The quality of the final products were observed under 1 % (w/v) agarose gel electrophoresis with pre-stained Flourosafe before been viewed under the UV light (Hassanshahian *et al.*, 2016).

For DGGE analysis, 8 % (v/v) of 1 mm thick polyacrylamide gel (acrylamide-bisacrylamide 37.5:1, Bio-Rad) were prepared and loaded with the samples before been electrophoresed. The DGGE condition were setup from 30 – 60 % urea gradient, for 5 hrs at 200 V. The gels were then stained with SYB green for 30 min before been photographed with UV transluminator (BIO-RAD GEL DOC XR System). The observation of DGGE emerged bands on treatments sample were compared with indicator to determine the presence of inoculated bacteria throughout 100 days.

CHAPTER 4: RESULTS

4.1 Soil physicochemical properties

The physicochemical characteristics of both active and closed landfills are summarised in Table 4.1. The pH values for both landfills had been recorded between 7.59 and 8.91, whereas the temperature and the moisture content varied between the sample locations at the following ranges; 26.6 - 30.3 °C and 10.63 - 37.42 %, respectively.

Investigation on culture dependant microorganisms using agar-based plate revealed more bacteria growth on the NA plate from closed landfill, when compared to active landfill. This indicated that more culturable bacteria populated in older landfill compared to the other one. A similar pattern was noted in culturing the bacteria using MSA plates as they allowed most of the Gram positive to be successfully isolated from the closed landfill. Nonetheless, the bacteria that grew on MCA plates had been found to be more abundant in active landfill, in comparison to closed landfill. In general, the MCA plate is a selective and differential medium that supports the Gram negative rod bacteria and inhibits most of the Gram positive bacteria due to the presence of bile salts and crystal violet (Ortiz, 2015).

The composition of soil for heavy metal measured by ICPMS is tabulated in Table 4.2. As a result, both landfills displayed some metal concentrations that exceeded the standard limit listed by local (Table 4.3) and international level (Aweng *et al.*, 2011; Sakawi *et al.*, 2013). The higher metal concentration was observed at the Bukit Beruntung landfill (BBL) compared to that in the Taman Beringin landfill (TBL) probably because the BBL is an active landfill that receives waste deposits on a daily basis, hence producing more leachate that contains metal that leaches into the surrounding area. In contrast, the closed landfill of TBL exhibited less metal concentration due to its inactivity. Similar

observations were also reported by many other researchers; thus proving that the concentration of metals in active landfills is indeed higher compared to that in non-active landfills (Calli *et al.*, 2005; Yusof *et al.*, 2009).

Table 4.1: Physicochemical properties of landfills soil and microbial population in both Bukit Beruntung and Taman Beringin landfills.

Location		pH	Moisture content (% soil)	Temp (°C)	cfu/g of soil (X10^6) on different media plates		
					NA	MSA	MCA
Bukit Berun-tung	BB	8.61±0.03	17.92±0.21	30.3±0.4	8.67	1.27	7.34
	BC	8.91±0.07	29.70±0.54	29.8±0.2	10.33	2.51	10.37
	BD	7.79±0.11	10.63±0.23	29.5±0.1	26.00	10.73	30.20
Taman Beringin	TA	8.12±0.08	21.27±0.67	28.2±0.6	29.33	14.82	4.43
	TB	8.77±0.13	37.42±0.22	27.8±0.1	155.67	6.24	8.41
	TC	7.59±0.07	24.14±0.19	26.5±0.8	100.00	4.17	2.23
	TD	7.62±0.04	20.49±0.46	26.6±0.8	81.33	17.30	3.39

Table 4.2: Composition of soil heavy metal in both landfills.

Location	Active Landfill			Closed Landfill			
Heavy metal (mg/L)	BB	BC	BD	TA	TB	TC	TD
Mg	46.94	74.26	27.92	39.66	139.70	64.78	171.10
Al	1112.00	909.10	875.40	699.60	926.80	567.10	696.00
Si	93.61	85.07	99.67	73.72	62.63	64.55	83.41
Cr	26.35	16.47	18.77	6.61	38.84	10.10	13.99
Mn	197.00	242.60	81.71	16.03	111.80	52.49	80.16
Co	3.88	2.09	0.82	0.19	1.07	0.67	1.61
Ni	19.95	2.62	10.01	3.50	8.95	5.51	3.01

Table 4.2, continued.

Location	Active Landfill			Closed Landfill			
Heavy metal (mg/L)	BB	BC	BD	TA	TB	TC	TD
Cu	46.03	144.6	26.74	0.00	38.92	72.05	0.00
Zn	24.11	218.70	53.00	2.26	36.43	17.80	29.18
As	0.49	0.57	0.53	0.10	0.07	0.06	0.08
Ag	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Cd	0.77	4.22	0.21	0.00	0.53	0.00	0.43
Hg	0.04	0.09	0.00	0.15	0.21	0.00	0.07
Pb	5.45	9.85	5.16	2.64	3.68	3.09	14.13
U	0.04	0.06	0.03	0.03	0.02	0.02	0.03

Table 4.3: Standard of heavy metal limit in soil (mg/L) in Malaysia.

Heavy metal	Maximum limit (mg/L)	Minimum limit (mg/L)	Mean (mg/L)
Mg	507.2	0.9	141.4
Al	53900	33500	42567
Si	-	-	-
Cr	14.40	0.02	6.00
Mn	3.99	3.95	3.97
Co	11.90	3.90	7.90
Ni	28.90	0.70	5.77
Cu	19.8	4.0	13.8
Zn	54.3	6.9	21.9
As	43.0	1.1	15.6
Ag	<0.5	<0.5	NA
Cd	0.09	11.9	14.40
Hg	0.42	0.02	0.12
Pb	36.00	0.18	10.37
U	-	-	-

Source: Adapted from Department of Environment 2009

4.2 Metagenomics profiling for assessing microbial diversity in both active and closed landfills

4.2.1 Analysis of sequencing and data depth

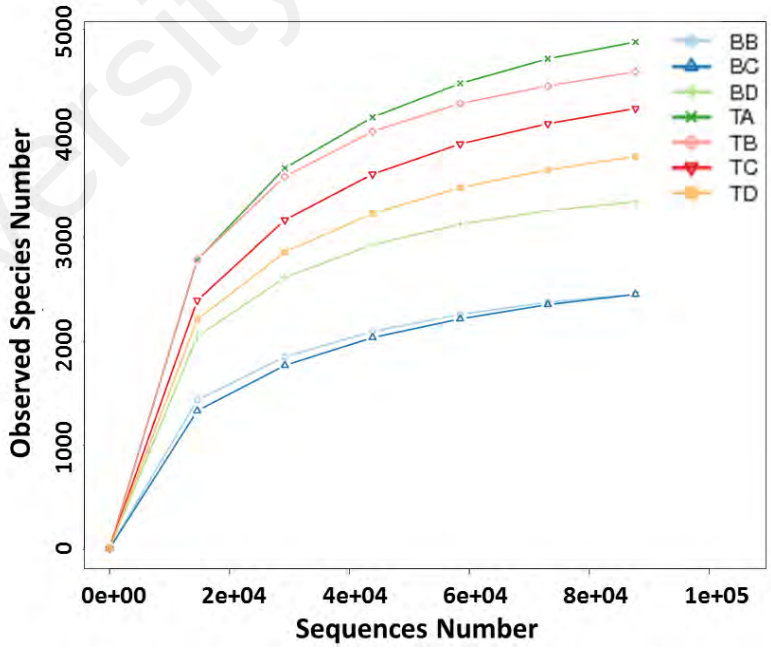
Table 4.4: Summary of 16S rRNA gene sequencing of both group B (active landfill) and group T (closed landfill) for bacterial diversity analysis.

Sample name	sample group	Total reads	Combined reads	OTU num	chao1	Observed species	shannon
BB	group B	132,756	125,562	2735	2721	2453	7.941
BC	group B	120,424	114,734	2751	2764	2451	6.276
BD	group B	120,058	113,387	3613	3550	3341	9.261
TB	group T	131,350	125,683	5348	5364	4879	9.832
TD	group T	131,369	125,608	5010	5147	4593	10.304
TA	group T	139,868	132,935	4696	4611	4239	9.301
TC	group T	135,720	128,460	4187	4173	3777	9.681
Total		911,545	866,369				

The general analysis of Illumina sequencing data shows a total of 911 545 reading retrieved from seven samples (Table 4.4). After removing short and low-quality reads, replicates, chimeras, and singletons; some 866 369 sequences that ranged from 113 387 to 132 935 per sample were successfully gathered for 16S rRNA. With that, a total of 8 812 unique operational taxonomic units (OTUs) were successfully identified from all seven landfill soil samples (Figure 4.1a). From these findings, (91 to 99.9) % of sequence were classified as bacteria, while the remaining ones (0.1 to 9) % were classified as Archaea.

Additionally, rarefaction curves were generated to determine if the depth of sequencing was indeed acceptable to reflect a complete microbial diversity from the samples. The rarefaction analysis (Figure 4.1b) exemplified that the number of OTUs for 16S rRNA had the tendency to go plateau at a similar level with 97 % similarity; indicating that the sequencing result could relatively reflect most of the microbial diversity and the richness of these total samples in an accurate manner.

(a)

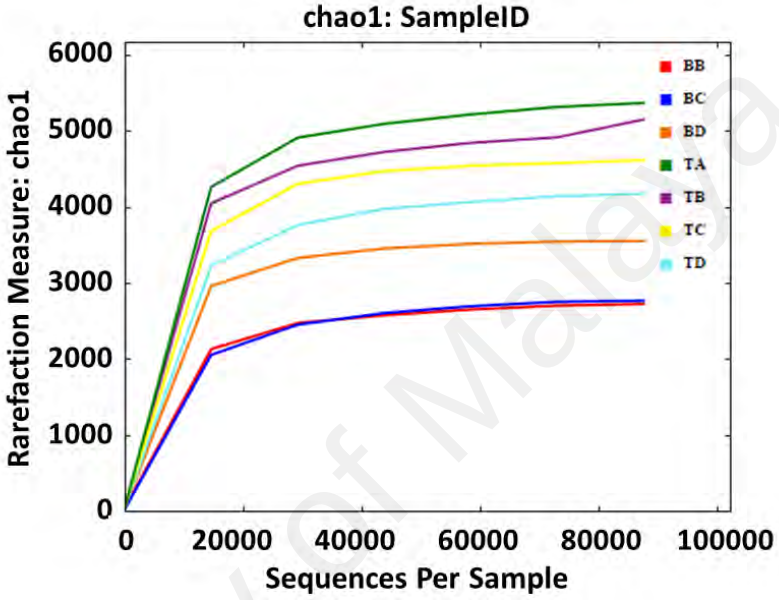


(b)

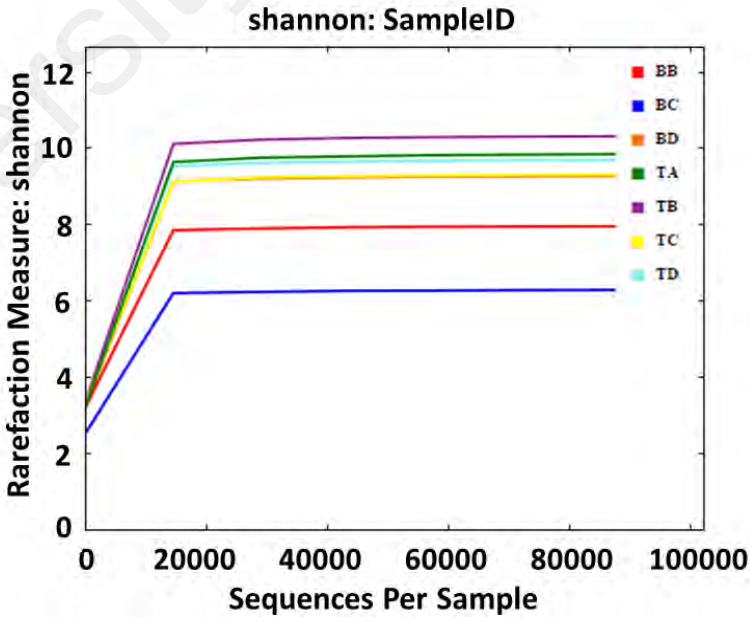
Figure 4.1: Venn Graph (a) and the rarefraction Curve (b) based on 97 % similarity. Venn diagrams showing the unique and shared OTUs in the different communities between Group B and Group T.

4.2.2 Diversity index analysis on microbial community

The Choa1 and Shannon index (Figure 4.2) shows that the abundance and the diversity of the bacterial communities in each closed landfill sample had been higher than those from active landfill.



(a)



(b)

Figure 4.2: The Choa1 index (a) and Shannon's diversity index curve (b) based on 97 % similarity. Group B-active landfill (BB, BC and BD) and Group T-closed landfill (TA, TB, TC and TD).

4.2.3 The analysis of microbial community structure and dominant phyla

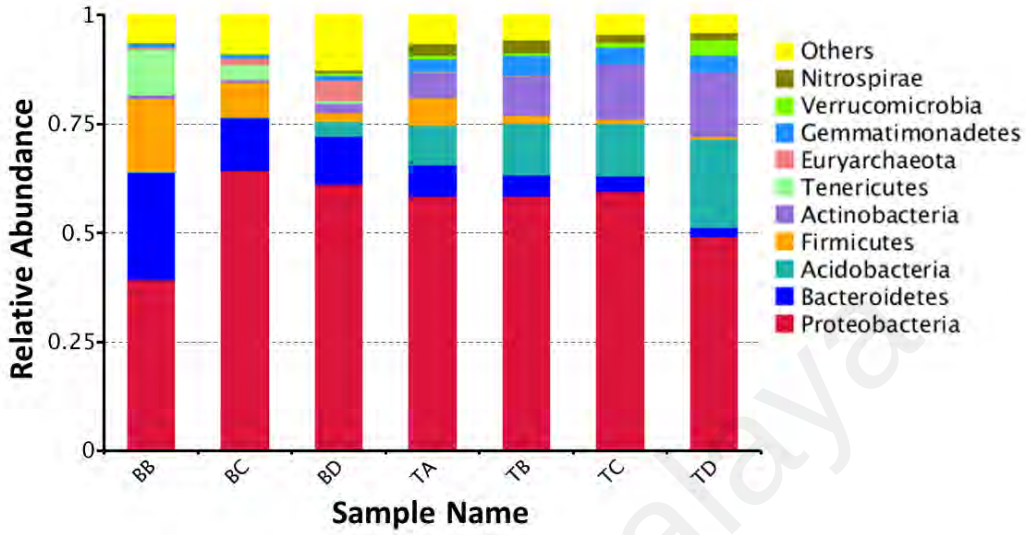


Figure 4.3: Taxonomic summary of 16S rRNA gene sequences from both active and closed landfills for each different sampling point at the phylum level.

The high-throughput sequencing illustrates the diversity of the microbial community in varied samples at the phylum level (Figure 4.3) and Appendix B. The most commonly classified reads were linked with the phylum Proteobacteria as the composition of this phylum remained high across the samples for both active and closed landfills. This result increased in relative abundance from 39 % in BB to 64 % in BC, which then, remained high throughout samples BD, TA, TB, and TC, before observing a slight decrease in TD at 49 %.

Furthermore, various other dominant phyla were discovered for the active landfill, including Bacteroidetes, Firmicutes, Tenericutes, and Euryarchaeota (Archaea), whereas Acidobacteria, Actinobacteria, Gemmatimonadetes, Nitrospirae, and Verrucomicrobia at the closed landfill. In fact, the reading for these prominent phyla accounted for up to 95 % of the entire bacterial sequences; thus giving out only a tiny portion of the bacterial richness from the soil sample to the other remaining phyla.

Apart from the relative dominance of Proteobacteria, in active landfill the other taxonomic group from phyla Bacteroidetes, Firmicutes, and Tenericutes displayed a sharp drop from BB (25 %, 17 %, and 11 %, respectively) to BD (11 %, 2 %, and 0.5 %, respectively), and later remained in the smaller percentage of closed landfill soil samples TA, TB, TC, and TD. Other than that, phyla Euryarchaeota increased slightly from the BB sampling point (0.5 %) to BD (5 %), which later declined drastically at less than 0.06 % when entering the closed landfill sample point TA to TD. This occurrence could be associated with the function of Methano sp. bacteria that gradually inhabited the fresh and new landfill soil to convert waste materials into methane and other by-products. However, their role showed a decrease in closed landfill and remained low in number when the environment failed to support their growth.

Nonetheless, from a dissimilar perspective, phyla Acidobacteria and Actinobacteria turned dominant in closed landfill, but only a small fraction of the bacteria population had been discovered in active landfill. Besides, in closed landfill, the phyla Acidobacteria populated at about 9 – 21 % from each soil sample, while phylum Actinobacteria occupied between 6 and 15 % of the total bacterial composition. As for active landfill, the results showed less than 3 % and 2 % of phyla Acidobacteria and Actinobacteria, respectively. While phyla from Gemmatimonadetes, Nitrospirae, and Verrucomicrobia emerged as minority groups in closed landfill with less than 4 %, 3 %, and 3 %, respectively.

The above findings suggest significant variance for the relative abundance of bacterial phyla between active and closed landfills. The diversity and the abundance of bacterial composition also leaned more towards closed landfill compared to the other one.

4.2.4 Clustering pattern of microbial community composition from active and closed landfills

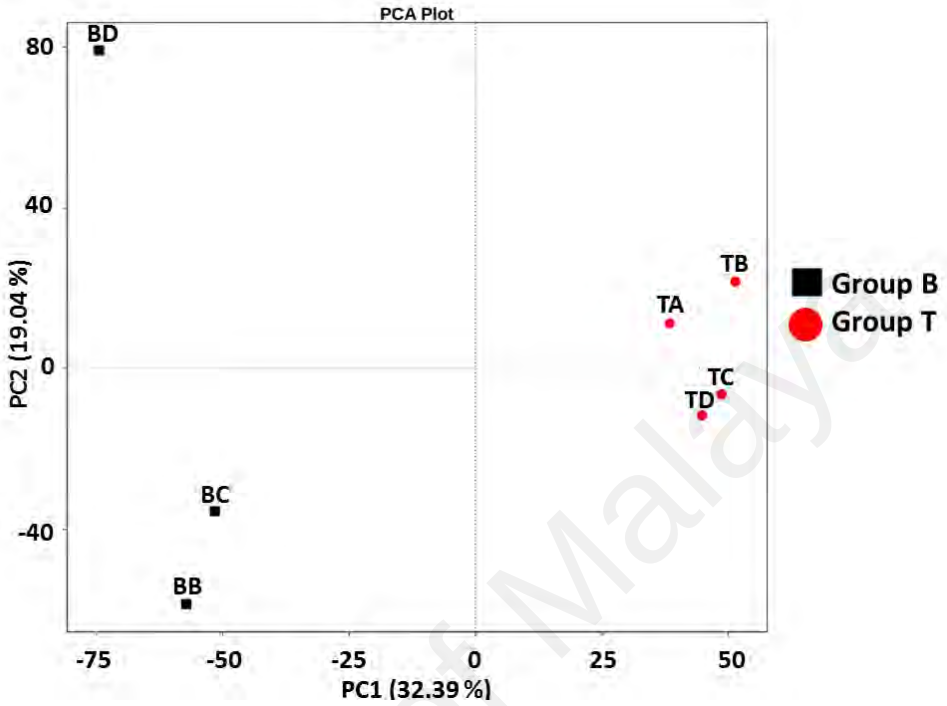
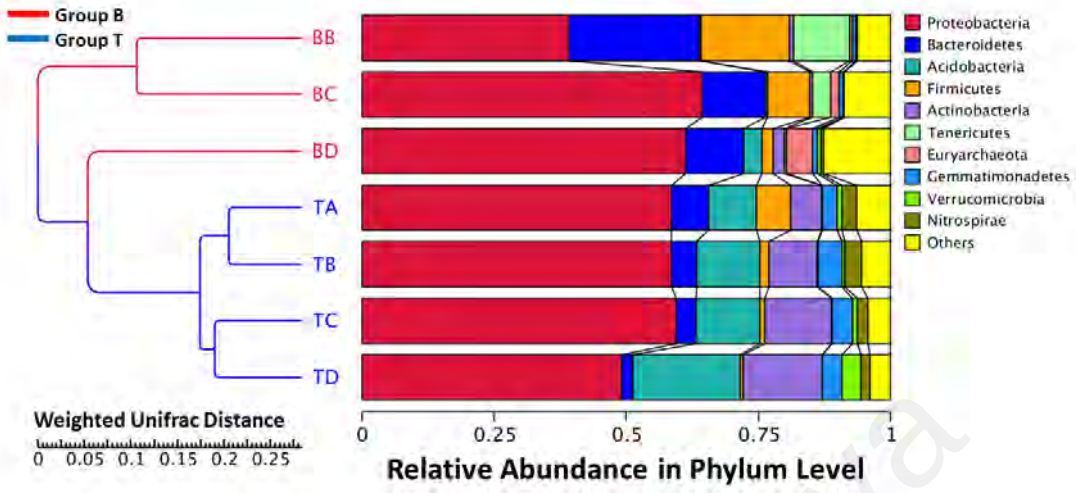
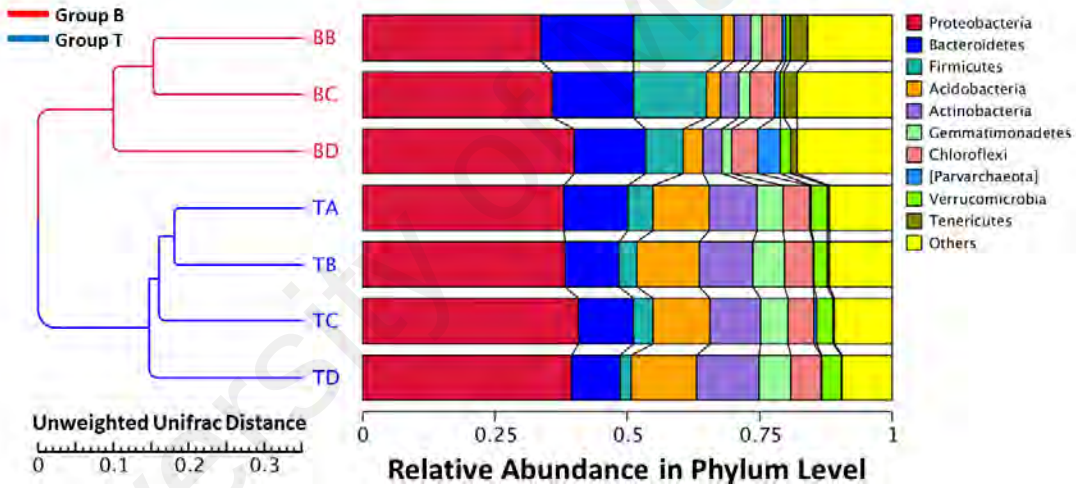


Figure 4.4: Principal component analysis of the seven samples from both Group B-active landfill and Group T-closed landfill.

As illustrated in Figure 4.4, the PCA plot indicated that the microbial community did display a significant difference between Group B (active landfill sample) and Group T (closed landfill sample). In fact, active waste deposition activities and the difference in ages between these two landfills could influence the microbial diversity and structures in the landfill soil samples. Therefore, when the activity of waste deposition is halted and as the landfill becomes older, a shift could have taken place in the microbial structure, which could eventually increase microbial diversity in landfill soil. The dissimilarities in microbial communities were further confirmed by making weighted and unweighted UniFrac calculations (Stephens *et al.*, 2016).



(a)



(b)

Figure 4.5: Hierarchical clustering of microbiomes form active and closed landfills from difference sampling points locations. Distance matrices were created with (a) weighted and (b) unweighted UniFrac calculations.

The weighted UniFrac (Figure 4.5) was applied to observe the variances in the microbial communities from the light of both the occurrence and the abundance of OTUs. Meanwhile, the unweighted UniFrac calculations, basically, determined the occurrence of OTUs to identify the variables between these communities.

4.3 **Potential of landfill bacterial strains for heavy metal remediation**

The additional waste materials, especially from industrial waste, add up to the accumulating heavy metal in both leachate and soil landfill which affected the diversity of the local microorganisms. Metals like calcium (Ca), chromium (Cr), copper (Cu), magnesium (Mg), manganese (Mn), nickel (Ni), sodium (Na), and zinc (Zn) were benefited and exhibited a biological function towards the microorganisms as micronutrients for metabolic activities. On the other hand, several other metals offer no biological role, such as aluminium (Al), cadmium (Cd), silver (Ag), and mercury (Hg). In fact, these metals can sometimes function as toxic towards microorganism.

4.3.1 Microbial isolated from landfills soil

Twenty nine isolates strains were successfully isolated from both Bukit Beruntung and Taman Beringin landfills using culture dependent method. Microscopic observation and cell characteristic (Brenner *et al.*, 2015) on each of bacteria were recorded in Table 4.5 and Table 4.6.

Table 4.5: Colony morphological characteristic of isolated bacteria from Bukit Beruntung and Taman Beringin.

Bacteria	Size (mm)	Colour	Form / Texture	Margin	Elevation	Transparency
BB 1	2.0	Creamy whitish	Circular	Entire	Convex	Opaque
BB 2	2.0	Creamy yellowish	Irregular	Entire	Convex	Opaque
BB 3	2.0	Creamy yellowish	Circular	Entire	Convex	Opaque
BB 4	3.0	Slightly yellowish	Irregular	Undulate	Flat	Transparent

Table 4.5, continued.

Bacteria	Size (mm)	Colour	Form / Texture	Margin	Elevation	Transparency
BB 5	2.0	Creamy yellowish	Circular	Entire	Convex	Opaque
BB 6	1.5	Creamy yellowish	Circular	Entire	Convex	Opaque
BB 7	1.0	Whitish	Irregular	Entire	Convex	Opaque
BB 8	2.0	Creamy yellowish	Irregular	Entire	Flat	Opaque
BB 9	<1.0	Light peach	Circular	Entire	Convex	Opaque
BB 10	1.5	Creamy yellowish	Circular	Entire	Raised	Opaque
BB 11	1.5	Creamy yellowish	Circular	Entire	Convex	Opaque
BB 12	1.0	Dried whitish	Irregular	Entire	Convex	Opaque
BB 13	<1.0	Creamy yellowish	Circular	Entire	Convex	Opaque
BB 14	1.5	Creamy orange	Circular	Entire	Convex	Opaque
TB 1	1.0	Creamy yellowish	Circular	Entire	Convex	Opaque
TB 2	3.0	Creamy whitish	Irregular	Crenated	Convex	Opaque
TB 3	1.5	Creamy yellowish	Circular	Entire	Convex	Opaque
TB 4	< 1.0	Creamy yellowish	Circular	Entire	Convex	Opaque
TB 5	1.0	Creamy yellow	Circular	Entire	Convex	Opaque
TB 6	1.0	Creamy yellowish	Circular	Entire	Convex	Opaque

Table 4.5, continued.

Bacteria	Size (mm)	Colour	Form / Texture	Margin	Elevation	Transparency
STB 7	1.0	Creamy yellowish	Circular	Entire	Convex	Opaque
STB 8	2.0	Creamy yellowish	Circular	Entire	Convex	Opaque
STB 9	2.5	Creamy orange	Circular	Entire	Convex	Opaque
TB 10	1.0	Creamy yellowish	Circular	Entire	Convex	Opaque
TB 11	1.0	Creamy yellowish	Circular	Entire	Convex	Opaque
TB 12	1.0	Creamy yellowish	Circular	Entire	Convex	Opaque
TB 13	2.0	Creamy orange	Irregular	Undulate	Raised	Opaque
TB 14	1.5	Creamy orange	Circular	Entire	Convex	Opaque
TB 15	4.0	Creamy yellowish	Irregular	Entire	Convex	Opaque

Table 4.6: Cell morphological characteristic and biochemical test of isolated bacteria from Bukit Beruntung and Taman Beringin.

Bacteria	Gram-positive/negative	Shape	Arrangement	Catalase	Oxidase
BB 1	Negative (-)	Bacillus	Single	Positive	Positive
BB 2	Positive (+)	Coccus	Clump	Positive	Negative
BB 3	Negative (-)	Bacillus	Single	Positive	Negative
BB 4	Negative (-)	Bacillus	Single	Positive	Negative
BB 5	Negative (-)	Bacillus	Single	Positive	Negative
BB 6	Negative (-)	Bacillus (short rod)	Single	Positive	Positive
BB 7	Negative (-)	Bacillus	Single	Positive	Negative
BB 8	Positive (+)	Coccus	Clump	Positive	Negative
BB 9	Positive (+)	Cocco-Bacillus	Single	Negative	Negative
BB 10	Negative (-)	Bacillus	Single	Positive	Negative
BB 11	Positive (+)	Coccus	Clump	Positive	Negative
BB 12	Positive (+)	Bacillus	Single	Positive	Negative
BB 13	Positive (+)	Coccus	Clump	Negative	Negative
BB 14	Negative (-)	Bacillus	Clump	Positive	Negative
TB 1	Positive (+)	Staphylo coccus	Clump	Positive	Negative
TB 2	Positive (+)	Bacillus	Clump	Positive	Negative
TB 3	Negative (-)	Bacillus (short rod)	Single	Positive	Positive
TB 4	Positive (+)	Bacillus (short rod)	Single	Positive	Negative
TB 5	Positive (+)	Staphylo coccus	Clump	Positive	Negative

Table 4.6, continued.

Bacteria	Gram-positive/negative	Shape	Arrangement	Catalase	Oxidase
TB 6	Negative (-)	Bacillus (short rod)	Single	Positive	Negative
STB 7	Negative (-)	Bacillus	Single	Negative	Positive
STB 8	Negative (-)	Bacillus	Single	Positive	Negative
STB 9	Positive (+)	Coccus	Clump	Positive	Negative
TB 10	Negative (-)	Bacillus	Single	Positive	Negative
TB 11	Positive (+)	Coccus	Diplode & Tetrade	Positive	Negative
TB 12	Negative (-)	Bacillus (short rod)	Single	Positive	Negative
TB 13	Positive (+)	Coccus	Single	Positive	Negative
TB 14	Positive (+)	Staphylo coccus	Clump	Positive	Negative
TB 15	Negative (-)	Bacillus	Single	Positive	Negative

4.3.2. Metal tolerance screening

All isolates (29 bacteria) were tested against 10 type of heavy metals. The result for percentage of susceptible isolates against various concentration of these heavy metal ions were shown in Table 4.7. Although there is no standard regulation for distinguish the concentration of metal ions between metal resistant and metal sensitive bacteria, the strain that be able to grow at and above 1.0 mM of metal ions except Hg which is 0.1 mM were considered as resistant (Abou-Shanab *et al.*, 2007). The frequencies of resistance toward the metal ions of the isolates bacteria were shown as follows: Hg, 13.8 %; Cd, 86.2 %; Pb, 93.1 %; Cr, 93.1 %; Co, 96.6 %; and 100 % toward Fe, Cu, Ni, Al and Zn. The outcome indicated that the mercury was the most toxic, inhibiting 10.3 % of the isolates at 0.005 mM. In this study, the metal toxicity can be found as Hg > Cd > Cr > Pb > Co >

Zn > Al> Ni > Cu > Fe. In overall, the toxic effect of metal ions were increased when the concentration increase.

Table 4.7: The susceptibility of 29 isolated bacterial strains toward 10 selected metal ions.

Metal ion	Cumulative % of strains susceptible to the metal ion concentration (mM)								
	0.005	0.01	0.05	0.1	1	5	10	15	20
Fe	-	-	-	0	0	0	3.4	3.4	3.8
Cu	-	-	-	0	0	0	0	3.4	24.1
Ni	-	-	-	0	0	3.4	3.4	6.9	24.1
Al	-	-	-	0	0	6.9	20.7	31	34.5
Zn	-	-	-	0	0	10.3	24.1	44.8	58.6
Co	-	-	-	0	3.4	17.2	20.7	34.5	51.7
Pb	-	-	-	0	6.9	17.2	20.7	31	37.9
Cr	-	-	-	0	6.9	17.2	24.1	37.9	41.4
Cd	-	-	-	6.9	13.8	69.0	89.7	96.6	100.0
Hg	10.3	20.7	65.5	86.2	100.0	-	-	-	-

Table 4.8: Tolerance patterns of 10 heavy metals ions in 29 soil microbial strains.

No. of different tolerance	**Type of tolerance**	**Percentage of tolerance strains (%)**
10	Fe, Cu, Ni, Al, Zn, Co, Pb, Cr, Cd, Hg	10.3
9	Fe, Cu, Ni, Al, Zn, Co, Pb, Cr, Cd	65.5
	Fe, Cu, Ni, Al, Zn, Co, Pb, Cr, Hg	3.4
8	Fe, Cu, Ni, Al, Zn, Co, Pb, Cd	10.3
	Fe, Cu, Ni, Al, Zn, Co, Cr, Cd,	3.4
7	Fe, Cu, Ni, Al, Zn, Pb, Cd	3.4
	Fe, Cu, Ni, Al, Zn, Co, Cd	3.4

The overall result from Table 4.7 indicated that there were high level of resistance and extensive tolerance bacteria was establish among the isolates that been tested. All the tested microorganisms were showed multiple tolerant toward the metal ions. However,

the pattern of tolerance among these 29 microbes varied (Table 4.8). Most of the cultures can be found be tolerant up to nine and ten type of heavy metals which is 68.97 % and 10.34 % of the total isolates respectively.

4.3.3 Determination of Minimum Inhibitory Concentration (MIC)

Table 4.9: MIC of 10 metal ions against the selected bacteria strain isolated from both landfills soil.

Bacteria	MIC (mM)									
	Fe	Cu	Ni	Al	Pb	Cr	Co	Zn	Cd	Hg
TB2	>20	>20	>20	>20	>20	15	>20	10	5	0.1
TB3	20	>20	>20	>20	15	>20	>20	20	5	0.1
STB7	>20	>20	>20	>20	>20	5	>20	>20	15	0.05
BB1	>20	>20	>20	15	1	>20	20	20	10	0.05
BB3	>20	>20	>20	>20	20	>20	15	15	5	0.1
BB14	>20	>20	>20	>20	>20	10	20	>20	10	0.05

The MIC value of six selected strains against ten type of heavy metal ions was shown in Table 4.9. These six bacteria were chosen based on their capability to tolerance with previous heavy metal screening test. The MIC is the lowest concentration of particular substances that be able to inhibit any visible bacteria growth (Wiegand *et al.*, 2008). The lowest concentration of heavy metal that prevented growth was taken as the MIC value. The strains coded STB7, TB2 and BB14 show more tolerance compare to the rest of strains.

4.3.4 Antibiotic sensitivity test

Table 4.10: Antibiotic sensitivity profile of selected bacterial isolates.

Antibiotic disc (conc.)	Diameter of inhibition zone (mm)					
	TB2	TB3	STB7	BB1	BB3	BB14
Ampicillin (25 mcg)	7 (R)	30 (S)	- (R)	29 (S)	28 (S)	7 (R)
Chloramphenicol (30 mcg)	22 (S)	27 (S)	- (R)	32 (S)	20 (I)	19 (I)
Erythromycin (15 mcg)	21 (S)	15 (R)	- (R)	17 (I)	27 (S)	14 (R)
Neomycin (30 mcg)	10 (R)	14 (R)	- (R)	15 (R)	15 (R)	14 (R)
Streptomycin (10 mcg)	16 (I)	16 (I)	- (R)	17 (I)	16 (I)	16 (I)
Vancomycin (30 mcg)	18 (I)	16 (I)	21 (S)	19 (I)	18 (I)	- (R)

The Letter in parentheses specify sensitivity: S susceptible (≥ 21 mm), I intermediate (16 – 20 mm), R resistant (≤ 15 mm) (Tomova *et al.*, 2015).

Six of the selected bacteria strain were also tested for their sensitivity toward six different antibiotics. The resistance patterns of those selected bacteria against the antibiotics were determined and the results were shown in Table 4.10. Isolate STB7 was resistant to as many as five antibiotics but showed susceptible response to the Vancomycin. The BB14 were resistant to four antibiotics followed by both TB2 and TB3 showed two antibiotics were resistant while the remained only resistant to Neomycin antibiotics tested.

4.3.5 Bacteria identification of six selected strains

Table 4.11: Isolated bacterial species from the landfill soils.

Bacteria	Identification with MALDI-TOF MS	Confirmation with 16S rRNA sequencing
TB2	*Bacillus cereus* 4080 LBK 1.409	*Bacillus cereus* (AE016877)
TB3	*Aeromonas caviae* CECT 838T DSM 2.081	*Aeromonas punctata subsp. caviae* (CDBK01000019)
STB7	*Delftia acidovorans CCM 2410 CCM 1.958*	*Delftia tsuruhatensis (BCTO01000107)*
BB1	*Ochrobactrum intermedium LMG 3301T HAM 2.226*	*Orchrobacterium intermedium* (ACQA01000003)
BB3	-	*Stenotrophomonas acidaminiphila (LDJO01000053)*
BB14	*Serratia marcescens 13103_1 CHB 2.405*	*Serratia marcencens subsp. sakuensis* (CLG_48654)

The selected of six bacteria strains were identified using MALDI-TOF and confirmed with 16S rRNA analysis as showed in Table 4.11. The nucleotide sequences that obtained from FirstBase Laboratory Sdn. Bhd. were identified using EzTaxon e-server.

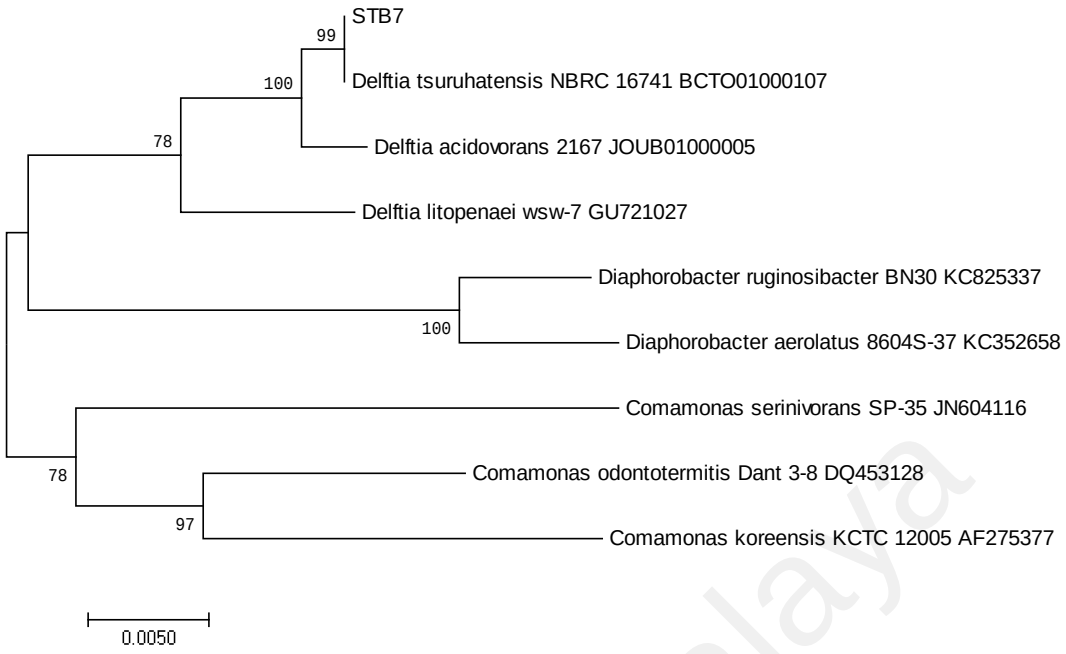


Figure 4.6: Phylogenetic analysis STB7 using Mega 7. Phylogenetic reconstruction was performed by using neighbour-joining and test Bootstrap method of 1000 replicates. Bootstrap values indicated at branch point were 50 % or more.

In the current study, The STB7 with identification as *Delftia tsuruhatensis* shown the most potential isolate toward the metal screening, MIC and antibiotic test. The response of the isolated microbes suggests the suitability for it in heavy metal bioremediation activities. Some of the previous studies also has revealed that microorganisms isolated from the landfill have the potential to treat heavy metal elements in the environment (Jayanthi *et al.*, 2016).

4.4 **The microbial succession in bioaugmentation process of contaminated soil via PCR-DGEE approach**

The selective bacteria that potentially to carry out heavy metal remediation were chosen to undergo the biaugmentation process in laboratory. The microbial bioaugmentation in this study carry out by adding a group of potential heavy metal resistant bacteria into the soil landfill samples. These introduction of potential bacteria often involves in the immobilizing and to stabilize the metal movement in the soil.

However, bioaugmentation is challenging processes as the introduced microbial population are not necessarily establish themselves among the indigenous populations (Singer *et al.*, 2005). Therefore, in this study, the succession of inoculated bacterial treatment that potentially to remediate heavy metal were evaluated through the DGGE approaches.

4.4.1 Analysis of DGGE on different inoculated bacteria as referral indicator

Eighteen selected bacteria were obtained from the previous isolation of culture collection that potentially to remediate heavy metal were chosen for DGGE analysis. The PCR products of each bacteria were loaded individually or as a mixed bacteria that act as a reference marker or indicator bands into the parallel wells denaturing gradient gel. In this analysis, V3 region of 16S rRNA was selected as a target region for the DGGE study. This region has been widely used in analysis of bacteria communities and bacteria identification purpose (Ercolini *et al.*, 2003; Ogier *et al.*, 2002).

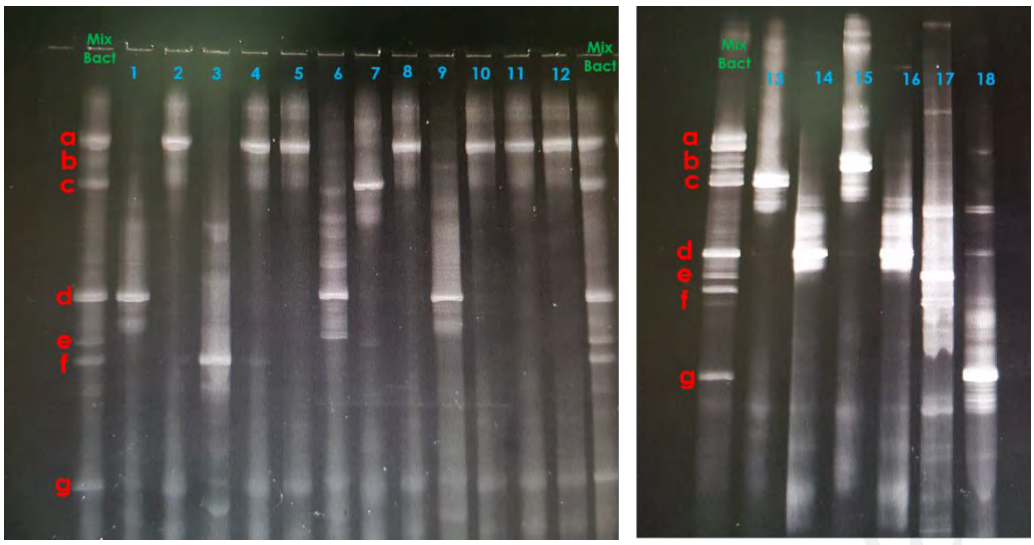


Figure 4.7: DGGE analyses of 16S rRNA fragment of each inoculated bacteria obtained after PCR amplification. Both photomicrograph showed the position of each bacteria band (labelled as 1 – 18). The most left of the gel is the mixed bacteria with indicator made up of mix bacteria (a: 2,4,5,8, 10,11,12 b: 15 c: 7,13 d: 1,6,9,14,16 e: 17 f: 3 g: 18).

Table 4.12: The indicator represents of mixed bacteria.

Indicator	Consist of bacteria	Bacterial species
a	2,4,5,8, 10,11,12	2: *Aeromonas caviae* 4: *Pseudomonas alcaligenes* 5: *Chryseobacterium gleum* 8: *Ochrobacterium intermedium* 10: *Stenotrophomonas acidaminiphilia* 11: *Acidovorax ebreus* 12: *Brevundimonas diminuta*
b	15	15: *Bacillus aryabhattai*
c	7,13	7: *Serratia marcescens* 13: *Cloacibacterium*

Table 4.12, continued.

Indicator	Consist of bacteria	Bacterial species
d	1,6,9,14,16	1: *Bacillus cereus* 6: *Pseudomonas mendocina* 9: *Burkholderia vietnamiensis* 14: *Rhodococcus rubber* 16: *Bacillus Pumilus*
e	17	17: *Bacillus kochii*
f	3	3: *Delftia tsuruhatensis*
g	18	18: *Janibacter hoylei*

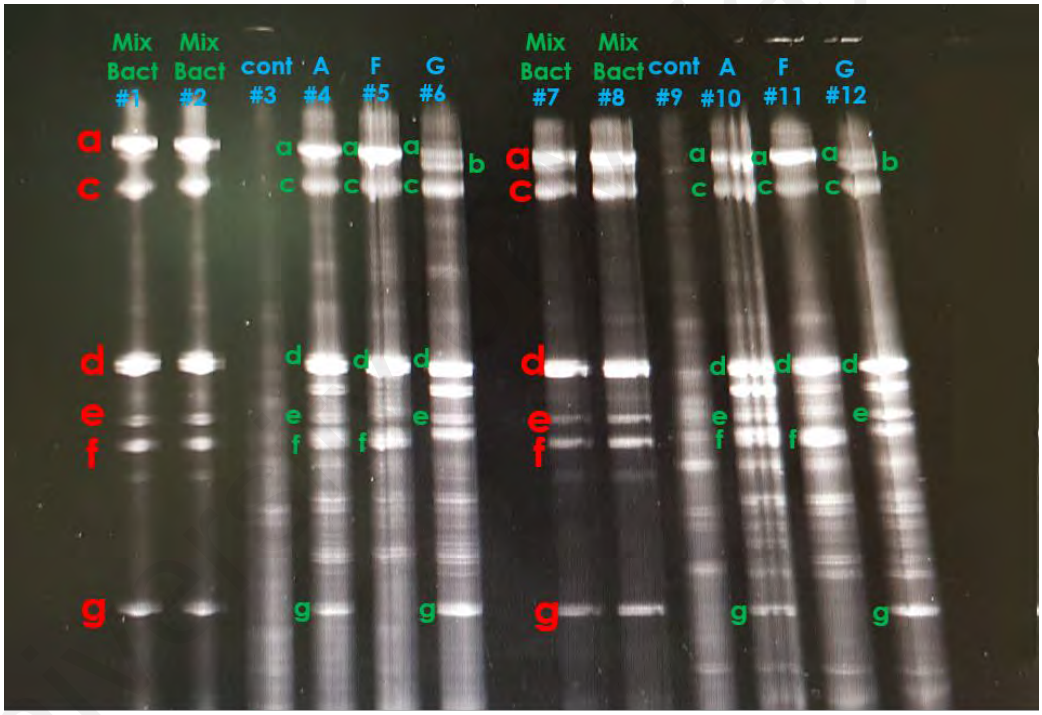
As shown in Fig 1, Lane 1 to 18 represented the individual pattern of the bacteria on the DGGE analysis. At the same time, mix bacteria that act as an indicator bands were developed containing mixed of known eighteen bacteria was used in the analysis (Table 4.12). The indicator bacteria were loaded in order to determine the position of each bacteria. These indicator bands also were later be used as a comparison in treatment soil samples in investigating the sustainability of these bacteria community from day 0 to 100.

The majority of the bands obtained were sequenced without interfere, suggesting that each band represent one microorganism. However, lane number 13 to 18 were contained smear band which make it harder to determine the exact band position of each strain (Figure 4.7). The result showed only seven out of eighteen expected bands were successfully emerged on the mixed bacteria indicator. This limitation happened as some of the microorganism might have only slightly different in their targeted DNA GC content sequences which lead the difficulty for DGGE to separate them completely into different band. Additionally, the preparation of denaturing gel condition and chemicals used during

preparing the gradient gel also can result the significant gel-to-gel variations that make this large samples set comparison become more difficult (Valaskova & Baldrian, 2009).

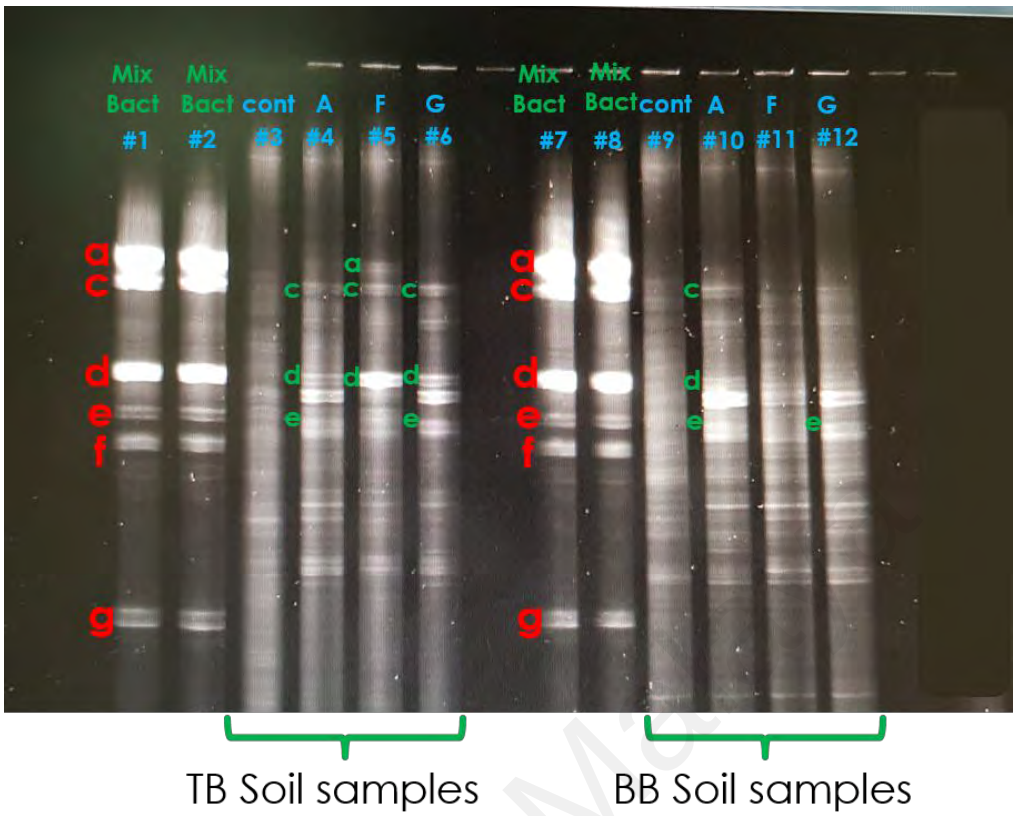
4.4.2 Analysis of microbial succession

The PCR-DGGE approaches was used in order to investigate the succession of inoculated bacteria treatment into the landfill soil for 100 days. The bacterial community analysis structure was done based on the bacterial 16S rDNA gene sequences according to Muyzer *et al.* (1993).

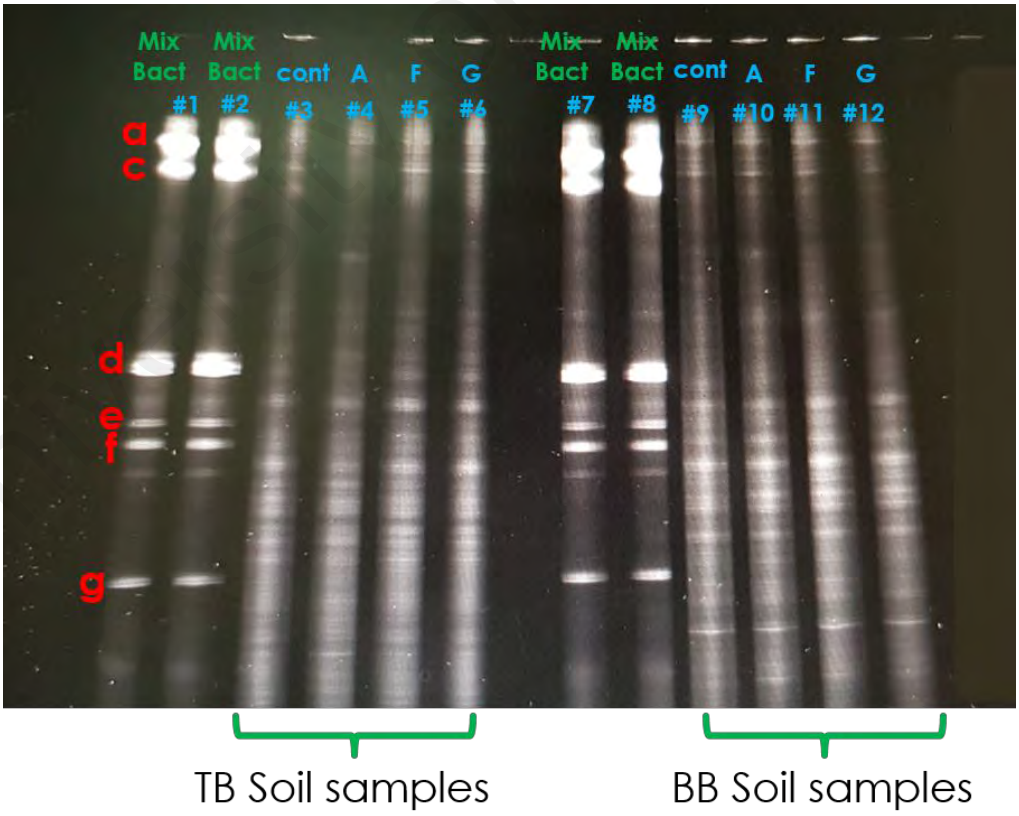


(a)

Figure 4.8: DGGE profile of 16S rDNA gene amplification of soil samples on day 0 (a), day 60 (b) and day 100 (c) for different bacterial treatments. Control (non-bacteria inoculated), treatment A (all 18 bacteria), F (proteobacteria), G (non-proteobacteria) and mix bacteria (as indicator). TB (Taman Beringin) soil sample and BB (Bukit Beruntung) soil sample.



(b)



(c)

Figure 4.8, continued.

The DGGE profile displayed in Figure 4.8 (a) showed the result for day 0 analysis from the sample treatments. The results treatment from lane #4, #5, #6, #10, #11 and #12 demonstrated the presence of many distinguishable bands in the separation pattern indicated many different bacterial species were established in these population. It was also revealed that most of the inoculated bacteria treatment soil were survive with bright emerged bands from both Taman Beringin and Bukit Beruntung soil samples; in treatment A, F and G compared with the control ones (non-bacteria inoculated).

The DGGE pattern from lane #3 and #9 are represent control sample (un-inoculated bacteria) of two different landfills. The results show the bands were not clearly visible and weak as the intensity of targeted DNA were absence or very few in those control landfills soil. It also suggesting that bacterial functional diversity was quite low in heavy metal contaminated landfill soils. However, the sample with treatment A (line #4 and #10) showed the highest number emerged bands as they contain all the inoculated bacteria in the treatment. While the treatment F and G (line #5, #11, and #6, #12 respectively) for both groups of treatment showed different pattern compare to each other as a different bacteria were added within the treatments.

It is normal to obtain a molecular fingerprints result with various communities and very complex patterns. Previous study on soil microbial diversity showed that in a gram of soil contain approximately ten thousand of bacteria species with variation of community evenness (Roesch *et al.*, 2007). Therefore, the result such as DGGE output may appear as many equally intense bands and sometime even by a smear with no clear resolution of individual bands (Valaskova & Baldrian, 2009).

On the other hand, for day 60 PCR-DGGE pattern Figure 4.8 (b), it shows there were still several bands emerged for all type of treatments. However there were noticed some changes in band number that been treated compare to previous day 0 band pattern.

The relative intensities of the bands among the soils under the treatments were quite different, indicating the differences in their bacterial densities too. Therefore it was indicated that treatment of some inoculated bacteria were still survive while some may not base on the bands appearances especially during 60 day incubation.

In Figure 4.8 (c) the number of bands had been decreased in all samples treatments (lane #4, #5, #6, #10, #11 and #12) and their intensity become fading indicating the overall treatments bacteria were decline and shows as similar band pattern with control treatment soil. The treatment in TB soil lane #4, #5 and #6 were shows a similar band with their control sample in lane #3 while treatment in BB soil of lane #10, #11 and #12 were similar with control treatment lane #9. Therefore, we can assume that the additional of bacterial population through different treatments in the bioaugmentation process had survived in the first place before gradually disappeared when approaching 100 days of incubation.

CHAPTER 5: DISCUSSION

5.1 Metagenomics profiling for assessing microbial diversity in both active and closed landfills

Landfills are the most common spots that harbour various types of microorganisms, including bacteria and archaea, which are capable in running extensive bioremediation activities (Azari *et al.*, 2017; Gomez *et al.*, 2011; Lu *et al.*, 2012). These microbes have been hardly analysed mainly because the condition of these landfills is mixed with high physical and chemical heterogeneity. In fact, several prior studies have made the attempt to address this heterogeneity by conducting several analyses on samples collected from a single landfill (Stamps *et al.*, 2016) or making sampling within a sole location of landfill, primarily to comprehend the stratification of waste and landfill soil (Suflita *et al.*, 1992). These methods are expensive, time-consuming, and only reflect a minor portion of the wide spectrum microorganism distribution. Hence, via cutting-edge technology available to date, this study investigated several points from active and closed landfill soil with high throughput sequencing of 16S rRNA gene libraries, especially to gain a better picture of bacterial distribution.

The microbial taxonomic distributions in this study displayed the presence of phyla Proteobacteria, Firmicutes, Bacteroidetes, Tenericutes, Euryarchaeota, Acidobacteria, Actinobacteria, Gemmatimonadetes, Nitrospirae, and Verrucomicrobia. It has been reported that phyla Proteobacteria, Firmicutes, and Bacteroidetes were also found in anaerobic ecosystem, for example aquifer sediment (Wan *et al.*, 2012), river sediment (Wang *et al.*, 2015), and wastewater bioreactor (Qiu *et al.*, 2013), along with landfills (Song *et al.*, 2015).

This study discovered that phylum Proteobacteria was dominant in both landfills. From the bacterial metagenomics sequencing results, it is clearly shown that the Proteobacteria percentage for both active landfill samples (BB, BC, and BD) and closed landfill samples (TA, TB, TC, and TD) were dominant and remained as high throughout among the samples. This indicated that Proteobacteria phylum may have the main role in degrading both organic and inorganic substances, including heavy metal contaminants in both leachate and landfill soil. Therefore, it is believed that the more prominent bacterial species function as the most important microorganisms in the leachate ecosystem (Kochling *et al.*, 2015).

On top of that, the class of Gammaproteobacteria appeared as the most abundant within the Proteobacterial group for active landfill, but lower count in closed landfill (TBL). On the contrary, the Alphaproteobacteria clearly dominated the closed landfill samples (Appendix A, Figure S1). Gammaproteobacteria and Alphaproteobacteria, hence, were extensively studied as a functional group microorganism in landfill for the occurrence of methanotrophs (Semrau *et al.*, 2010). It was also reported that the large diversity of methanotrophs, associated with both Gammaproteobacteria and Alphaproteobacteria, had been discovered in landfills from various parts of the world, such as the United States, Ireland, Canada, Germany, and England (Uz *et al.*, 2003). The high amount of Gammaproteobacteria in the BBL samples showed a similar finding reported from several landfills, such as Japanese landfill (Sawamura *et al.*, 2010) and in aged refuse located at Shanghai landfills (Xie *et al.*, 2012). Besides, the type order of Pseudomonadales from Gammaproteobacteria had been widely found in various environments, which could be responsible for the processes of degradation and denitrification of organic matter (Guo *et al.*, 2013; Lalucat *et al.*, 2006). For instance, (Xie *et al.*, 2012) also found that Pseudomonas was an important species in aged landfill

for leachate treatment, which further indicates that these bacteria may have a substantial role in organic matter decomposition.

Meanwhile, in active landfill (BBL), the dominating phyla were Bacteroidetes and Firmicutes. Bacteroidetes is known for its well-established hydrolytic capacities (Song *et al.*, 2015) that depends on the amount of oxygen available to survive (Janssen, 2006). On the other hand, phylum Firmicutes is believed to be part of cellulose decomposition activities in landfills. It has been recently reported that the member of Firmicutes could contribute to an important proportion of microbial taxonomy in landfill ecosystem (Kochling *et al.*, 2015). Besides, the type order of Clostridiales emerged as a major fraction in phylum Firmicutes, which is responsible in a wide spectrum of fermentation reactions for anaerobic treatment systems (Huang *et al.*, 2005).

In contrast, two phyla of Tenericutes and Euryarchaeota displayed a minor abundancy in active landfill sample, but almost completely absent in the closed landfill soil samples. As for Tenericutes, less than 11 % of this group of bacteria were found to contaminate the soil in the BBL. It has been reported that this community inhabits inside the gut of red palm weevil, as these intestinal microbes ingest tender interior fibrous tissues of date palm trunks (Jia *et al.*, 2013).The Tenericutes also has the potential to perform cellulolytic fermentation (Song *et al.*, 2015) although only a handful of studies have looked into this phylum. At the same time, the phylum Euryarchaeota also was found in a small portion in the bacterial communities for active landfill with a higher percentage of methanogenic archaea, when compared to that for closed landfill soil samples. This result is in agreement with the findings retrieved by (Nayak *et al.*, 2009; Uz *et al.*, 2003) on two types of wastes: (1) waste of different ages in situ, and (2) samples from bioreactors filled with municipal solid waste over some period of time that involved this bacteria group. Their results showed that “young” waste demonstrated greater

methanogenic diversity, in comparison to "older" waste. In addition, this study also found that at the early stage of active landfill (BBL), sample BB exhibited a lower abundance of archaea compared to other older site of the same landfill for sample BD. This phenomenon can be explained as (Kochling *et al.*, 2015) mentioned that in a new spot of landfill, the methanogenic population in leachate, probably, is present at the preliminary stage of expansion, which resulted in a low number of archaea. Hence, the landfill has to adapt to the high amount of organic and inorganic materials from the waste deposited. Nonetheless, it could be too young to offer a good environmental condition towards archaea communities to grow optimally.

From a different standpoint, phyla Acidobacteria and Actinobacteria turned dominant in closed landfill. Phylum Acidobacteria is one of the five phyla, including Gemmatimonadetes, Verrucomicrobia, Chloroflexi, and Planctomycetes, which have been proven to be hardly cultivated and poorly characterized (Bergmann *et al.*, 2011; Janssen, 2006) due to their slow-growth properties that lead towards dormancy (Janssen *et al.*, 2002; Stott *et al.*, 2008). This scenario reflects the results obtained in this study because the closed landfill could contain less organic matter when deposition of waste materials had been stopped. Commonly, the rich and fast-growing species could probably utilise the energy sources within the ecosystem to grow competitively, while slower-growing species use anabiosis-type strategies to keep out competitors and stay dormant, especially during starvation periods (Jones & Lennon, 2010; Rappe & Giovannoni, 2003). Hence, the oligotrophic condition permits these slow-growing phyla to become dominant.

Other than that, Acidobacteria is a versatile phylum that is capable of inhabiting at various environments like neutral soil (Dunbar *et al.*, 1999), wastewater treatment bioreactor (LaPara *et al.*, 2000), mine drainage (Kishimoto *et al.*, 1991), sewage sludge (Layton *et al.*, 2000), and even at the surface of paleolithic painting cave (Zimmermann

et al., 2005). As such, based on the abundance and phylogenetic complexity, it could have an essential function in the soil ecosystem. Moreover, (Radajewski *et al.*, 2002) asserted that some Acidobacteria might promote methanol assimilation.

For Actinobacteria, some studies have reported that the richness found in this phylum depends on the quantity and the quality of the organic matter, as well as other abiotic factors (Eichorst *et al.*, 2007; Fierer *et al.*, 2007; Ward *et al.*, 2009). The results further revealed that the adaptation of this group of bacteria towards low substrate concentration in soil and gave a negative response toward the increases in pH value and carbon availability. Hence, the oligotrophic environment of closed landfill may contribute to the richness of such phyla for dominance.

Several minority bacteria group populations were recorded in closed landfill, such as Gemmatimonadetes, Verrucomicrobia, and Nitrospirae. Phylum Gemmatimonadetes contains only one representative described bacteria that is known as *Gemmatimonas aurantiacus,* a Gram negative aerobic heterotroph isolated from sewage treatment plant (Zhang *et al.*, 2003). Based on the previous reports the highest relative abundance of Gemmatimonadetes had been discovered near the neutral pH (Lauber *et al.*, 2009; Vishnivetskaya *et al.*, 2011). However the diversity of general description of this group has remained to be discovered. Next, the second phylum that contributed towards the minority taxonomy structure of closed landfill is Verrucomicrobia. This group of bacteria is particularly abundant and could be found widespread in the nature (Zhang & Xu, 2008). Various locations like freshwater lakes, acid rock drainage, rice paddies, sewage sludge, landfill leachate, guts of several animals, and human intestine have been successfully retrieved from this group of bacteria with 16S rRNA sequencing (Op den Camp *et al.*, 2009). However, the pure culture of these bacteria is still limited to only 12 described genera been recorded (Janssen, 2006; Op den Camp *et al.*, 2009).

Other than that, phylum Nitrospirae was also detected in this closed landfill soil samples. This phylum Nitrospirae 16S rRNA was found under vegetated soil, such as uninterrupted and unfertilized grassland soil (Kowalchuk *et al.*, 2000; Webster *et al.*, 2002) or rhizosphere soil near leachate irrigation (Sundberg *et al.*, 2007). This microorganism was clearly found in abundance at the closed landfill, which was surrounded with trees and vegetative plants. The rhizosphere of the root plant could serve as the best condition for Nitrospirae bacteria to propagate. However, very small percentage from this phylum in active landfill had been discovered as it has limited trees that could support the Nitrospirae population to further grow.

5.2 Potential of landfill bacterial strains for heavy metal remediation

The heavy metal contamination that introduced into the environment has lead toward the environmental problem and affected the structure of microbial communities and their activities (Hema *et al.*, 2014). As the heavy metal will caused inhibitory action on living cells including the microorganism by blocking the essential functional proteins, modifying the active sites of biological molecules or replacing the essential molecule with metal ions (Doelman *et al.*, 1994; Li & Tan, 1994). However, the response of these microorganisms are varied and depend on the level of heavy metal contamination and availability of the metal ions in the polluted environment. At low concentration, certain transition metals such as zinc, nickel and cobalt are necessary for many cellular process in bacteria as they help as a co-factor for metallo-proteins and enzymes functioning. However, the metals will cause cytotoxic effect toward the microorganisms if the concentration become too high. Other heavy metals such as lead, mercury, cadmium and chromium have no beneficial effects to the bacteria cells at all and can be toxic even with the small concentration (Doelman *et al.*, 1994).

The metal tolerance screening test in this study indicated that there were high level of resistance and extensive tolerance bacteria was established among the isolates that been tested. This is due to the condition of the high metal content in their natural environment of both Bukit Beruntung and Taman Beringin landfills. The bacteria was already adapted to this condition that exposed them toward the high concentration of heavy metal since metal ions were available either in aqueous solution or absorbed into soil particle (Giller *et al.*, 1998). The metal toxicity of the isolated bacteria can be found as Hg > Cd > Cr > Pb > Co > Zn > Al> Ni > Cu > Fe.

While the MIC test showed the isolate of STB7, TB2 and BB14 were more tolerance compare to the rest of strains. According to Ahmed *et al.* (2005) bacteria that exposed to the high concentration of heavy metal in the environment have adapted to metal stress and develops numerous resistance mechanism. The variation in responses by tested bacteria toward heavy metals resistance might be due to the differences in bacterial cell wall structure and composition (Tomova *et al.*, 2015). A report from Jayanthi *et al.* (2017) mentioned that the binding ability to the metal, ionic interaction or complex formation, and precipitation ability of the isolate are the factors toward the bacterial heavy metal tolerance and inhibition. The tolerance of soil bacteria to heavy metals has been comprised as a bio-indicator of heavy metal toxicity (Hassen *et al.*, 1998).

At the same time, the environment complex-polluted by those heavy metals and antibiotics in landfill soil leachate has lead toward a collaborative and cross-resistance in the local microbial communities. The antibiotic test in this study showed that isolate STB7 was resistant to five out of six antibiotics and at the same time also gave a good result in heavy metal MIC test. A reported from Zhang *et al.* (2012) suggested that there was a positive correlation between the capability of bacteria resistance toward the antibiotic when there are resistance on heavy metals. The capability of the

microorganisms and their resistance mechanisms can be potentially be utilized in bioremediation approaches (Filali *et al.*, 2000; Malik, 2004).

The resistance mechanisms toward the heavy metal and antibiotics has been discovered for several decades. The ability of resistance bacteria to the heavy metals were usually associated with some mobile elements such as plasmid which also encode the resistance gene to antibiotics, although there are still unclear a direct correlation between these two factors (Tomova *et al.*, 2015). However, it is well known that the specific antibiotic resistance mechanisms can be gain by mutation of encoded gene in bacterial genome or by acquired additional sequences from other strains (Herreros *et al.*, 2005). This is a normal phenomenon with clustered resistance gene that always been simultaneously transferred to other bacteria in order to keep them survival in harsh environment (Filali *et al.*, 2000). The antibiotic resistance gene that located at plasmid sequences are easily to be horizontally transferred among the communities of bacteria population, thus leading toward the widespread of antibiotic resistance in the environment (Herreros *et al.*, 2005). Therefore, with the complexes of contaminants that harbor the landfill environment such as heavy metals and antibiotic has created multi stresses condition toward the local microorganisms. Thus it would be more advantage for the microbial survival ship to have resistance on both stresses (De Souza *et al.*, 2006; Miller *et al.*, 2009).

Based on the complete sequence genome of *Cupriavidus* sp. strain BIS7 and BLAST, there are several identified protein that play a role in heavy metal resistance mechanism has been acknowledged including the ZntA (P-type ATPase involved in Pb^{2+}, Cd^{2+} and Zn^{2+} resistance) and CzcE (involved Co^{3+}, Zn^{2+} and Cd^{2+} resistance) (Hong *et al.*, 2012). It is also believe that both heavy metals and antibiotics could activated some enzyme for antioxidant including peroxide (POD), superoxide dismutase (SOD) and

catalase (CAT), which help the bacteria to overcome the oxidative stress and let it to live under pressure (Liu *et al.*, 2012; Weihe *et al.*, 2010). This statement had been further explained by Abskharon *et al.* (2010) that found a total protein content in *E.coli* ASU3 was decreased and the induction of these enzymes increased with the increasing of copper concentration. That it is why some antibiotic resistance can be strengthen by the present of certain concentration of heavy metals. In addition, the cooperative resistance also can come from some unknown chemical reaction that occur between heavy metals and antibiotic or it decomposed residues; by which the heavy metal might alter the target site of action to its affinity with antibiotics. This reaction will create a complexion between heavy metals and antibiotic that in overall decreasing the toxicity toward the bacteria and form a co-resistance phenomenon (Zhang *et al.*, 2012).

In the current study, the isolate of STB7 showed the most promising strain that be able to tolerate with various concentration of heavy metal and resistance toward many antibiotic tested. This bacteria Gram negative short bacillus was revealed with MALDI-TOF as *Delftia* sp. and this outcome was further confirmed by the sequencing of 16S rRNA identified as *Delftia tsuruhatensis*. This strain contains a similarity up to 100 % to *Delftia tsuruhatensis* (Accession number BCTO01000107).

Delftia tsuruhatensis was firstly isolated by Shigematsu *et al.* (2003) from activated sludge in Tsuruhata, Kumamoto Prefecture, Japan. This isolate showed colony morphology with irregular and cream-coloured colonies. This strain is a Gram negative bacteria with slightly curved in rod shape (0.7 – 1.2 × 2.4 – 4.0 μm). The cell usually can be found individually or in pair under the microscope. It growth optimally at 35 °C with pH 7.0. *Delftia tsuruhatensis* is non-fermentative bacteria, does not hydrolyse starch and not possess denitrification ability, although nitrate reduction is found. However, this

bacteria showed a positive result toward catalase, arginine dihydrolase, lipase (Tween 80 hydrolysis) and urease activities (Shigematsu *et al.*, 2003).

The assessment of MIC test in this study indicated that isolate STB7 (*Delftia tsuruhatensis*) has developed potential in tolerance toward most of the concentration of heavy metals tested-including zinc and lead. The main result of this study is demonstrated the strength of this bacteria to survive with various degree of heavy metal concentration and could possibly removing the metal ions from the environment. This assumption is supported by report from (Bautista-Hernández *et al.*, 2012) that showed a potential of *Delftia tsuruhatensis* as it obtained maximum biosorption of 0.216 and 0.207 mmol/g for Pb and Zn respectively.

This strain also has showed positive result toward the antibiotic test with several antibiotic resistance be determined. The wide resistance of strain STB7 to antibiotic may indicate that it hold rich mobile genes that carry simultaneous resistance to antibiotics and metals, as it is thought the bacterial resistance of heavy metals is associated with the capability of antibiotic resistance (Zhang *et al.*, 2012). Therefore, this results finding showed some promising for the isolated heavy metal tolerant bacteria strain *Delftia tsuruhatensis* (STB7) to be utilised in heavy metal bioremediation treatment. Further studies on this metals resistance strain bacteria need to be enhanced and the genome sequence investigation are required to discover the heavy metal tolerance genes capabilities.

5.3 The microbial succession in bioaugmentation process of contaminated soil via PCR-DGEE approach

In this study, eighteen selected resistance heavy metal bacteria were undergo bioaugmentation processes according to their treatments group to remediate heavy metal. Bioaugmentation has been found to be successful in the many tested of small scale trial (Emenike *et al.*, 2017; Sinha *et al.*, 2012). However, there are still several issues need to take into consideration to ensure the optimal condition for bioaugmentation had been achieved before it can be applied into the mainstream.

There are several factors that may contribute toward the culture viability in the field. Many researchers have conducted an experiment to study on the competitive characteristic of local microorganisms community in the present of foreign microbes (Mars *et al.*, 1998). The new population is need to compete for nutrients such as N, H, P and etc in the new habitat (Fennell *et al.*, 1997). Probably the missing information in bioaugmentation is what happens to the cultures after been inserted into the macrocosm samples. Therefore, in this study, the use of PCR-DGGE method has been applied to monitor the succession of inoculated cultures throughout 100 days of bioaugmentation periods.

Based on the data obtained, it is showed that bioaugmentation is not a simple process and required further research to evaluate the condition of overall process in more details. However, the PCR-DGGE method that been conducted revealed the landfills soil sample consist of a complex bacterial community. At the beginning of inoculation treatment, the DGGE analysis demonstrated variation in the DNA bands intensity for all treatment samples from both Bukit Beruntung and Taman Beringin landfill soil (Figure 4.8 (a)). This PCR-DGGE band intensity indicated that the different group of bacteria for the treatments did survive at the first place in bioaugmentation period with variant of

inoculated bacteria populations. On the other hand, the control samples from both landfills showed weak intensity bands which reflecting less diverse of indigenous bacteria populating the landfill soil samples. The heavy metals constrain from the local landfill soil may contribute toward this phenomenon to occur. A study carried out by Yao *et al.* (2017) on bacterial community by using DGGE revealed that heavy metal contamination in marine sediments changes the bacterial community structure, while study from Altimira *et al.* (2012) showed agricultural soils close to copper and zinc smelters may provoke changes in the composition of soil bacterial community and a decrease of the bacterial diversity. However, changes in the soil bacterial community exposed to heavy metals may vary depending of soil properties, heavy metal bioavailability and the indigenous microbial groups in soil (Ranjard *et al.*, 2006).

The other important observations of this study is the substantial change produced in bioaugmentation treatments on day 60. The community profiles DGGE bands of treatment A, F and G from both landfill were decreased as the intensity and number of bands emerged were depleted (Figure 4.8 (b)). Some of the bands were no longer on the DGGE pattern compared to the previous day 0 observation. This result suggest that only certain group of bacteria were capable to stay viable inside the samples until that period compare to several other bacteria that were already diminished. At the same time, the number of emerged bands for Taman Beringin (TB) treatments were higher compare to those bands treatments from Bukit Beruntung (BB) samples. This phenomenon may happen because of the different contamination heavy metal concentration level in the landfill soil samples itself. As mentioned by Zainun and Simarani (2018) the concentration of metals in active landfill (BB) is indeed higher compared to that in non-active landfill (TB). In most case, the higher metals levels causes more stress on bacterial population and lead toward lower community diversity (Sobolev & Begonia, 2008).

In overall, this result show that the diversity of the bacteria varied little over time. Highly diverse ecosystems, such as soils, sediments and activated sludge sample will usually produce DGGE banding patterns that are very complex to interpret (Boon *et al.*, 2002). The lost and emerged new bands on the gel may indicate the absence and presence of particular bacteria in the sample. However, the number of bands generated by DGGE may not accurately reflect the number of difference sequences present in a given mixture. Indeed, DNA of bacteria that are present in relatively low numbers in the environment might escape amplification by PCR (Cébron *et al.*, 2004). Meanwhile, several emerged bands were detected on day 60 band pattern which indicating some indigenous bacteria were discovered during bioaugmentation process. These bacteria were emerged as the soil condition start support and become favourable for them to start survived.

Nevertheless, at the end of experimental period on day 100, PCR-DGGE patterns in all treatments sample were showed similarity in bands degradation. All the samples treatment from both landfills were shows similar band pattern with the control landfill soil samples in lane #3 and #9 (Figure 4.8 (c)). This is a good sign for bioaugmentation process as the inoculated treatment bacteria should not altering the indigenous bacterial community for a long period of time. These responsible bacteria were available when they utilised the contaminants as their nutrient sources and then disappeared gradually after some period of time.

CHAPTER 6: CONCLUSION

This metagenomics study that employed the HiSeq methodology suggests a new possibility to determine the microorganism diversity within landfill soil in a detailed manner. The study outcome exemplified the variances found in microbial richness and diversity between active and closed landfills. Both landfills were dominated by Proteobacteria phylum with Bacteroidetes and Firmicutes phyla more dominance in the active landfill, whereas Acidobacteria and Actinobacteria groups seemed dominant in the closed landfill. The diversity and richness of these microorganisms were believed to promote the remediation activities that occurs in landfills.

On the other hand, the bacterial isolates from both landfills have showed some potential toward heavy metal tolerance characteristic included copper, iron, nickel, lead, chromium, cobalt, zinc, cadmium and mercury. The selected of six isolates from the soil landfills also displayed the variance in metals MIC test and their association with antibiotic test. *Delftia tsuruhatensis* strain was able to tolerate with multiple metal ions tested and features more resistance toward antibiotics. These responsible metal tolerant microorganisms obtained in the current study could be explored for future used in heavy metal remediation activity.

The application of PCR-DGGE to evaluate the presence of inoculated bacteria in bioaugmentation provide a better interpretation in the process. The study might be concluded that PCR-DGGE pattern varied with different treatments and the survival of inoculated bacteria could be monitored as the pattern changed from day 0 to day 100.

REFERENCES

Abou-Shanab, R. A. I., van Berkum, P., & Angle, J. S. (2007). Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*. *Chemosphere, 68*(2), 360-367.

Abskharon, R. N. N., Hassan, S. H. A., Kabir, M. H., Qadir, S. A., El-Rab, S., & Wang, M. H. (2010). The role of antioxidants enzymes of *E. coli* ASU3, a tolerant strain to heavy metals toxicity, in combating oxidative stress of copper. *World Journal of Microbiology & Biotechnology, 26*(2), 241-247.

Adekanle, M., & Akindele, A. (2017). Bacteria isolated from waste-dump sites soil at Osogbo, Osun State. *Journal of Microbiology and Biotechnology Research, 4*(3), 66-69.

Adelopo, A. O., Haris, P. I., Alo, B., Huddersman, K., & Jenkins, R. O. (2017). Comparative characterisation of closed and active landfill composites using EDX, FTIR and proximate techniques. *Waste and Biomass Valorization, 8*(4), 1313-1323.

Ahmed, N., Nawaz, A., & Badar, U. (2005). Screening of copper tolerant bacterial strains and their potential to remove copper from the environment. *Bulletin of Environmental Contamination and Toxicology, 74*(2), 219-226.

Akob, D. M., Mills, H. J., & Kostka, J. E. (2007). Metabolically active microbial communities in uranium-contaminated subsurface sediments. *Fems Microbiology Ecology, 59*(1), 95-107.

Al-Mailem, D. M., Al-Deieg, M., Eliyas, M., & Radwan, S. S. (2017). Biostimulation of indigenous microorganisms for bioremediation of oily hypersaline microcosms from the Arabian Gulf Kuwaiti coasts. *Journal of Environmental Management, 193*, 576-583.

Alatoom, A. A., Cazanave, C. J., Cunningham, S. A., Ihde, S. M., & Patel, R. (2012). Identification of non-*diphtheriae Corynebacterium* by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology, 50*(1), 160-163.

Altimira, F., Yáñez, C., Bravo, G., González, M., Rojas, L. A., & Seeger, M. (2012). Characterization of copper-resistant bacteria and bacterial communities from copper-polluted agricultural soils of central Chile. *BMC Microbiology, 12*(1), 193.

Ansari, M. I., & Malik, A. (2007). Biosorption of nickel and cadmium by metal resistant bacterial isolates from agricultural soil irrigated with industrial wastewater. *Bioresource Technology, 98*(16), 3149-3153.

Aweng, E., Karimah, M., & Suhaimi, O. (2011). Heavy metals concentration of irrigation water, soils and fruit vegetables in Kota Bharu area, Kelantan, Malaysia. *Journal of Applied Sciences in Environmental Sanitation, 6*(4), 463-470.

Aydin, S., Shahi, A., Ozbayram, E. G., Ince, B., & Ince, O. (2015). Use of PCR-DGGE based molecular methods to assessment of microbial diversity during anaerobic treatment of antibiotic combinations. *Bioresource Technology, 192*, 735-740.

Azari, M., Walter, U., Rekers, V., Gu, J. D., & Denecke, M. (2017). More than a decade of experience of landfill leachate treatment with a full-scale anammox plant combining activated sludge and activated carbon biofilm. *Chemosphere, 174*, 117-126.

Azlan Halmi, M., Hasenan, S., Simarani, K., & Abdullah, R. (2018). Linking soil microbial properties with plant performance in acidic tropical soil amended with biochar. *Agronomy, 8*(11), 255.

Bailey, S. E., Olin, T. J., Bricka, R. M., & Adrian, D. D. (1999). A review of potentially low-cost sorbents for heavy metals. *Water Research, 33*(11), 2469-2479.

Bautista-Hernández, D. A., Ramírez-Burgos, L. I., Duran-Páramo, E., & Fernández-Linares, L. (2012). Zinc and lead biosorption by *Delftia tsuruhatensis*: A bacterial strain resistant to metals isolated from mine tailings. *Journal of Water Resource and Protection, 4*(04), 207.

Bergmann, G. T., Bates, S. T., Eilers, K. G., Lauber, C. L., Caporaso, J. G., Walters, W. A., . . . Fierer, N. (2011). The under-recognized dominance of Verrucomicrobia in soil bacterial communities. *Soil Biology & Biochemistry, 43*(7), 1450-1455.

Bhakta, J. N., Lahiri, S., Bhuiyna, F. A., Rokunuzzaaman, M., Ohonishi, K., Iwasaki, K., & Jana, B. B. (2017). Profiling of heavy metal (loid)-resistant bacterial community structure by metagenomic-DNA fingerprinting using PCR–DGGE for monitoring and bioremediation of contaminated environment. *Energy, Ecology and Environment*, 1-8.

Boon, N., De Windt, W., Verstraete, W., & Top, E. M. (2002). Evaluation of nested PCR–DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiology Ecology, 39*(2), 101-112.

Brehm-Stecher, B. F., & Johnson, E. A. (2004). Single-cell microbiology: Tools, technologies, and applications. *Microbiology and Molecular Biology Reviews, 68*(3), 538.

Brenner, D. J., Krieg, N. R., & Staley, J. T. (2015). Bergey's Manual of Systematic Bacteriology (2nd ed.). USA: Springer.

Breza-Boruta, B. (2016). The assessment of airborne bacterial and fungal contamination emitted by a municipal landfill site in Northern Poland. *Atmospheric Pollution Research, 7*(6), 1043-1052.

Bruneel, O., Duran, R., Casiot, C., Elbaz-Poulichet, F., & Personne, J. C. (2006). Diversity of microorganisms in Fe-As-rich acid mine drainage waters of Carnoules, France. *Applied and Environmental Microbiology, 72*(1), 551-556.

Burrell, P. C., O'Sullivan, C., Song, H., Clarke, W. P., & Blackall, L. L. (2004). Identification, detection, and spatial resolution of *Clostridium* populations responsible for cellulose degradation in a methanogenic landfill leachate bioreactor. *Applied and Environmental Microbiology, 70*(4), 2414-2419.

Calli, B., Mertoglu, B., & Inanc, B. (2005). Landfill leachate management in Istanbul: Applications and alternatives. *Chemosphere, 59*(6), 819-829.

Carini, P., Marsden, P. J., Leff, J. W., Morgan, E. E., Strickland, M. S., & Fierer, N. (2017). Relic DNA is abundant in soil and obscures estimates of soil microbial diversity. *Nature Microbiology, 2*(3), 16242.

Cébron, A., Coci, M., Garnier, J., & Laanbroek, H. J. (2004). Denaturing gradient gel electrophoretic analysis of ammonia-oxidizing bacterial community structure in the lower Seine River: Impact of Paris wastewater effluents. *Applied and Environmental Microbiology, 70*(11), 6726-6737.

Chandy, J. P. (1999). Heavy metal tolerance in chromogenic and non-chromogenic marine bacteria from Arabian Gulf. *Environmental Monitoring and Assessment, 59*(3), 321-330.

Chen, B., Liu, J.-n., Wang, Z., Dong, L., Fan, J.-h., & Qu, J.-j. (2011). Remediation of Pb-resistant bacteria to Pb polluted soil. *Journal of Environmental Protection, 2*(2), 130.

Chen, J. Z., Tao, X. C., Xu, J., Zhang, T., & Liu, Z. L. (2005). Biosorption of lead, cadmium and mercury by immobilized *Microcystis aeruginosa* in a column. *Process Biochemistry, 40*(12), 3675-3679.

Chien, C. C., Kuo, Y. M., Chen, C. C., Hung, C. W., Yeh, C. W., & Yeh, W. J. (2008). Microbial diversity of soil bacteria in agricultural field contaminated with heavy metals. *Journal of Environmental Sciences, 20*(3), 359-363.

Collins, B., McArthur, J. V., & Sharitz, R. R. (2004). Plant effects on microbial assemblages and remediation of acidic coal pile runoff in mesocosm treatment wetlands. *Ecological Engineering, 23*(2), 107-115.

Cornu, J.-Y., Huguenot, D., Jézéquel, K., Lollier, M., & Lebeau, T. (2017). Bioremediation of copper-contaminated soils by bacteria. *World Journal of Microbiology and Biotechnology, 33*(2), 26.

Curtis, T. P., Sloan, W. T., & Scannell, J. W. (2002). Estimating prokaryotic diversity and its limits. *Proceedings of the National Academy of Sciences of the United States of America, 99*(16), 10494-10499.

Dao, H. T. N., Kuroda, K., Nakahara, N., Danshita, T., Hatamoto, M., & Yamaguchi, T. (2016). 16S rRNA gene-based comprehensive analysis of microbial community compositions in a full-scale leachate treatment system. *Journal of Bioscience and Bioengineering, 122*(6), 708-715.

De Souza, M. J., Nair, S., Bharathi, P. A. L., & Chandramohan, D. (2006). Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic Marine waters. *Ecotoxicology, 15*(4), 379-384.

Doelman, P., Jansen, E., Michels, M., & Vantil, M. (1994). Effects of heavy-metals in soil on microbial diversity and activity as shown by the sensitivity-resistance index, an ecologically relevant parameter. *Biology and Fertility of Soils, 17*(3), 177-184.

Doney, S. C., Abbott, M. R., Cullen, J. J., Karl, D. M., & Rothstein, L. (2004). From genes to ecosystems: The ocean's new frontier. *Frontiers in Ecology and the Environment, 2*(9), 457-466.

Dunbar, J., Takala, S., Barns, S. M., Davis, J. A., & Kuske, C. R. (1999). Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology, 65*(4), 1662-1669.

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nature Methods, 10*(10), 996.

Eichorst, S. A., Breznak, J. A., & Schmidt, T. M. (2007). Isolation and characterization of soil bacteria that define *Teniglobus* gen. nov., in the phylum Acidobacteria. *Applied and Environmental Microbiology, 73*(8), 2708-2717.

Ellis, D. E., Lutz, E. J., Odom, J. M., Buchanan, R. J., Bartlett, C. L., Lee, M. D., . . . Deweerd, K. A. (2000). Bioaugmentation for accelerated in situ anaerobic bioremediation. *Environmental Science & Technology, 34*(11), 2254-2260.

Emenike, C. U., Liew, W., Fahmi, M. G., Jalil, K. N., Pariathamby, A., & Hamid, F. S. (2017). Optimal removal of heavy metals from leachate contaminated soil using bioaugmentation process. *CLEAN–Soil, Air, Water, 45*(2), 1500802.

Ercolini, D., Hill, P. J., & Dodd, C. E. (2003). Bacterial community structure and location in Stilton cheese. *Applied and Environmental Microbiology, 69*(6), 3540-3548.

Errasquin, E. L., & Vazquez, C. (2003). Tolerance and uptake of heavy metals by *Trichoderma atroviride* isolated from sludge. *Chemosphere, 50*(1), 137-143.

Estefan, G., Sommer, R., & Ryan, J. (2013). Methods of soil, plant, and water analysis. *International Biodeterioration & Biodegradation, 113*, 170-176.

Fang, H., Cai, L., Yang, Y., Ju, F., Li, X. D., Yu, Y. L., & Zhang, T. (2014). Metagenomic analysis reveals potential biodegradation pathways of persistent pesticides in freshwater and marine sediments. *Science of the Total Environment, 470*, 983-992.

Feng, S. J., Gao, K. W., Chen, Y. X., Li, Y., Zhang, L. M., & Chen, H. X. (2017). Geotechnical properties of municipal solid waste at Laogang Landfill, China. *Waste Management, 63*, 354-365.

Fennell, D. E., Gossett, J. M., & Zinder, S. H. (1997). Comparison of butyric kid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. *Environmental Science & Technology, 31*(3), 918-926.

Fierer, N., Bradford, M. A., & Jackson, R. B. (2007). Toward an ecological classification of soil bacteria. *Ecology, 88*(6), 1354-1364.

Filali, B. K., Taoufik, J., Zeroual, Y., Dzairi, F. Z., Talbi, M., & Blaghen, M. (2000). Waste water bacterial isolates resistant to heavy metals and antibiotics. *Current Microbiology, 41*(3), 151-156.

Giller, K. E., Witter, E., & McGrath, S. P. (1998). Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. *Soil Biology & Biochemistry, 30*(10-11), 1389-1414.

Gomez, A. M., Yannarell, A. C., Sims, G. K., Cadavid-Restrepo, G., & Herrera, C. X. M. (2011). Characterization of bacterial diversity at different depths in the Moravia Hill landfill site at Medellin, Colombia. *Soil Biology & Biochemistry, 43*(6), 1275-1284.

Guine, V., Martins, J. M. F., Causse, B., Durand, A., Gaudet, J. P., & Spadini, L. (2007). Effect of cultivation and experimental conditions on the surface reactivity of the metal-resistant bacteria *Cupriavidus metallidurans* CH34 to protons, cadmium and zinc. *Chemical Geology, 236*(3-4), 266-280.

Guo, H. L., Chen, C., Lee, D. J., Wang, A. J., & Ren, N. Q. (2013). Sulfur-nitrogen-carbon removal of *Pseudomonas* sp C27 under sulfide stress. *Enzyme and Microbial Technology, 53*(1), 6-12.

Han, Z. Y., Ma, H. N., Shi, G. Z., He, L., Wei, L. Y., & Shi, Q. Q. (2016). A review of groundwater contamination near municipal solid waste landfill sites in China. *Science of the Total Environment, 569*, 1255-1264.

Handelsman, J. (2004). Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews, 68*(4), 669.

Hassanshahian, M., Bayat, Z., Cappello, S., Smedile, F., & Yakimov, M. (2016). Comparison the effects of bioaugmentation versus biostimulation on marine microbial community by PCR–DGGE: A mesocosm scale. *Journal of Environmental Sciences, 43*, 136-146.

Hassen, A., Saidi, N., Cherif, M., & Boudabous, A. (1998). Resistance of environmental bacteria to heavy metals. *Bioresource Technology, 64*(1), 7-15.

Hema, T. G., Getha, K., Tan, G. Y. A., Sahira, H. L., Syamil, A. M., & Fairuz, M. Y. N. (2014). Actinobacteria isolates from tin tailings and forest soil for bioremediation of heavy metals. *Journal of Tropical Forest Science, 26*(1), 153-162.

Herreros, M., Sandoval, H., González, L., Castro, J., Fresno, J., & Tornadijo, M. (2005). Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). *Food Microbiology, 22*(5), 455-459.

Hong, K. W., Thinagaran, D. A. L., Gan, H. M., Yin, W. F., & Chan, K. G. (2012). Whole-genome squence of *Cupriavidus* sp strain BIS7, a heavy-metal-resistant bacterium. *Journal of Bacteriology, 194*(22), 6324-6324.

Huang, L. N., Zhu, S., Zhou, H., & Qu, L. H. (2005). Molecular phylogenetic diversity of bacteria associated with the leachate of a closed municipal solid waste landfill. *Fems Microbiology Letters, 242*(2), 297-303.

Hugenholtz, P., Goebel, B. M., & Pace, N. R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology, 180*(18), 4765-4774.

Jain, R. K., Kapur, M., Labana, S., Lal, B., Sarma, P. M., Bhattacharya, D., & Thakur, I. S. (2005). Microbial diversity: Application of microorganisms for the biodegradation of xenobiotics. *Current Science, 89*(1), 101-112.

Janssen, P. H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology, 72*(3), 1719-1728.

Janssen, P. H., Yates, P. S., Grinton, B. E., Taylor, P. M., & Sait, M. (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Applied and Environmental Microbiology, 68*(5), 2391-2396.

Jayanthi, B., Emenike, C. U., Agamuthu, P., Simarani, K., Mohamad, S., & Fauziah, S. H. (2016). Selected microbial diversity of contaminated landfill soil of Peninsular Malaysia and the behavior towards heavy metal exposure. *Catena, 147*, 25-31.

Jayanthi, B., Emenike, C. U., Auta, S. H., Agamuthu, P., & Fauziah, S. H. (2017). Characterization of induced metal responses of bacteria isolates from active non-sanitary landfill in Malaysia. *International Biodeterioration & Biodegradation, 119*, 467-475.

Jeong, Y. S., Choi, S., Chong, E., Kim, J. H., & Kim, S. J. (2014). Rapid detection of Bacillus spore aerosol particles by direct in situ analysis using MALDI-TOF mass spectrometry. *Letters in Applied Microbiology, 59*(2), 177-183.

Jia, S. G., Zhang, X. W., Zhang, G. Y., Yin, A., Zhang, S., Li, F. S., . . . Yu, J. (2013). Seasonally variable intestinal metagenomes of the red palm weevil (*Rhynchophorus ferrugineus*). *Environmental Microbiology, 15*(11), 3020-3029.

Jones, S. E., & Lennon, J. T. (2010). Dormancy contributes to the maintenance of microbial diversity. *Proceedings of the National Academy of Sciences of the United States of America, 107*(13), 5881-5886.

Kishimoto, N., Kosako, Y., & Tano, T. (1991). *Acidobacterium capsulatum* gen. nov., sp. nov.: An acidophilic chemoorganotrophic bacterium containing menaquinone from acidic mineral environment. *Current Microbiology, 22*(1), 1-7.

Kobya, M., Demirbas, E., Senturk, E., & Ince, M. (2005). Adsorption of heavy metal ions from aqueous solutions by activated carbon prepared from apricot stone. *Bioresource Technology, 96*(13), 1518-1521.

Kochling, T., Sanz, J. L., Gavazza, S., & Florencio, L. (2015). Analysis of microbial community structure and composition in leachates from a young landfill by 454 pyrosequencing. *Applied Microbiology and Biotechnology, 99*(13), 5657-5668.

Kowalchuk, G., Smit, E., de Bruijn, F., Head, I., Akkermans, A., & van Elsas, J. (2004). Community analysis using PCR-denaturing gradient gel electrophoresis (DGGE). *Molecular Microbial Ecology Manual*, 771-788.

Kowalchuk, G. A., Stienstra, A. W., Heilig, G. H. J., Stephen, J. R., & Woldendorp, J. W. (2000). Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcareous grasslands. *Environmental Microbiology, 2*(1), 99-110.

Krishnamurthi, S., & Chakrabarti, T. (2013). Diversity of bacteria and archaea from a landfill in Chandigarh, India as revealed by culture-dependent and culture-independent molecular approaches. *Systematic and Applied Microbiology, 36*(1), 56-68.

Lalucat, J., Bennasar, A., Bosch, R., Garcia-Valdes, E., & Palleroni, N. J. (2006). Biology of *Pseudomonas stutzeri*. *Microbiology and Molecular Biology Reviews, 70*(2), 510.

LaPara, T. M., Nakatsu, C. H., Pantea, L., & Alleman, J. E. (2000). Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. *Applied and Environmental Microbiology, 66*(9), 3951-3959.

Lauber, C. L., Hamady, M., Knight, R., & Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology, 75*(15), 5111-5120.

Layton, A. C., Karanth, P. N., Lajoie, C. A., Meyers, A. J., Gregory, I. R., Stapleton, R. D., . . . Sayler, G. S. (2000). Quantification of *Hyphomicrobium* populations in activated sludge from an industrial wastewater treatment system as determined by 16S rRNA analysis. *Applied and Environmental Microbiology, 66*(11), 5106.

Li, F., & Tan, T. C. (1994). Effect of heavy-metal ions on the efficacy of a mixed bacilli bod sensor. *Biosensors & Bioelectronics, 9*(4-5), 315-324.

Liu, Y., Guan, Y. T., Gao, B. Y., & Yue, Q. Y. (2012). Antioxidant responses and degradation of two antibiotic contaminants in *Microcystis aeruginosa*. *Ecotoxicology and Environmental Safety, 86*, 23-30.

Lu, Z. M., He, Z. L., Parisi, V. A., Kang, S., Deng, Y., Van Nostrand, J. D., . . . Zhou, J. Z. (2012). Geochip-based analysis of microbial functional gene diversity in a landfill leachate-contaminated aquifer. *Environmental Science & Technology, 46*(11), 5824-5833.

Magoc, T., & Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*(21), 2957-2963.

Malik, A. (2004). Metal bioremediation through growing cells. *Environment International, 30*(2), 261-278.

Mars, A. E., Prins, G. T., Wietzes, P., de Koning, W., & Janssen, D. B. (1998). Effect of trichloroethylene on the competitive behavior of toluene-degrading bacteria. *Applied and Environmental Microbiology, 64*(1), 208-215.

Masek, T., Vopalensky, V., Suchomelova, P., & Pospisek, M. (2005). Denaturing RNA electrophoresis in TAE agarose gels. *Analytical Biochemistry, 336*(1), 46-50.

Miller, R. V., Gammon, K., & Day, M. J. (2009). Antibiotic resistance among bacteria isolated from seawater and penguin fecal samples collected near Palmer Station, Antarctica. *Canadian Journal of Microbiology, 55*(1), 37-45.

Moffett, B. F., Nicholson, F. A., Uwakwe, N. C., Chambers, B. J., Harris, J. A., & Hill, T. C. J. (2003). Zinc contamination decreases the bacterial diversity of agricultural soil. *Fems Microbiology Ecology, 43*(1), 13-19.

Mrozik, A., & Piotrowska-Seget, Z. (2010). Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. *Microbiological Research, 165*(5), 363-375.

Mulligan, C. N., Kamali, M., & Gibbs, B. F. (2004). Bioleaching of heavy metals from a low-grade mining ore using *Aspergillus niger*. *Journal of Hazardous Materials, 110*(1-3), 77-84.

Muyzer, G., Dewaal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S rRNA. *Applied and Environmental Microbiology, 59*(3), 695-700.

Muyzer, G., & Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 73*(1), 127-141.

Nayak, B. S., Levine, A. D., Cardoso, A., & Harwood, V. J. (2009). Microbial population dynamics in laboratory-scale solid waste bioreactors in the presence or absence of biosolids. *Journal of Applied Microbiology, 107*(4), 1330-1339.

Nies, D. H. (2003). Efflux-mediated heavy metal resistance in prokaryotes. *Fems Microbiology Reviews, 27*(2-3), 313-339.

Nigam, A., Jit, S., & Lal, R. (2010). *Sphingomonas histidinilytica* sp. nov., isolated from a hexachlorocyclohexane dump site. *International Journal of Systematic and Evolutionary Microbiology, 60*(5), 1038-1043.

Nubel, U., Garcia-Pichel, F., Kuhl, M., & Muyzer, G. (1999). Quantifying microbial diversity: Morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. *Applied and Environmental Microbiology, 65*(2), 422-430.

Ogier, J.-C., Son, O., Gruss, A., Tailliez, P., & Delacroix-Buchet, A. (2002). Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Applied and Environmental Microbiology, 68*(8), 3691-3701.

Oller, I., Malato, S., & Sanchez-Perez, J. A. (2011). Combination of advanced oxidation processes and biological treatments for wastewater decontamination. *Science of the Total Environment, 409*(20), 4141-4166.

Op den Camp, H. J. M., Islam, T., Stott, M. B., Harhangi, H. R., Hynes, A., Schouten, S., . . . Dunfield, P. F. (2009). Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. *Environmental Microbiology Reports, 1*(5), 293-306.

Ortiz, M. (2015). Antimicrobial activity of onion and ginger against two food borne pathogens *Escherichia Coli* and *Staphylococcus Aureus*. *MOJ Food Processing & Technology, 1*(4), 1-9.

Pace, N. R., Stahl, D. A., Lane, D. J., & Olsen, G. J. (1986). The analysis of natural microbial-populations by ribosomal-RNA sequences. *Advances in Microbial Ecology, 9*, 1-55.

Perez-de-Mora, A., Burgos, P., Madejon, E., Cabrera, F., Jaeckel, P., & Schloter, M. (2006). Microbial community structure and function in a soil contaminated by heavy metals: Effects of plant growth and different amendments. *Soil Biology & Biochemistry, 38*(2), 327-341.

Perez-Leblic, M. I., Turmero, A., Hernandez, M., Hernandez, A. J., Pastor, J., Ball, A. S., . . . Arias, M. E. (2012). Influence of xenobiotic contaminants on landfill soil microbial activity and diversity. *Journal of Environmental Management, 95*, S285-S290.

Pintado, J., Guyot, J. P., & Ampe, F. (2003). Multiple competitive PCR-DGGE as a tool for quantifying and profiling defined mixed cultures of lactic acid bacteria during production of probiotics from complex polysaccharides. *Journal of Applied Microbiology, 95*(5), 921-933.

Pisol, B., Abdullah, N., Khalil, K. A., & Nuraida, L. (2015). Isolation and identification of lactic acid bacteria from different stages of traditional Malaysian tempeh production. *Malaysian Journal of Microbiology, 11*(4), 358-364.

Prasad, M. N. V., & Freitas, H. M. D. (2003). Metal hyperaccumulation in plants - Biodiversity prospecting for phytoremediation technology. *Electronic Journal of Biotechnology, 6*(3), 285-321.

Qiu, G. L., Song, Y. H., Zeng, P., Duan, L., & Xiao, S. H. (2013). Characterization of bacterial communities in hybrid upflow anaerobic sludge blanket (UASB)-membrane bioreactor (MBR) process for berberine antibiotic wastewater treatment. *Bioresource Technology, 142*, 52-62.

Radajewski, S., Webster, G., Reay, D. S., Morris, S. A., Ineson, P., Nedwell, D. B., . . . Murrell, J. C. (2002). Identification of active methylotroph populations in an acidic forest soil by stableisotope probing. *Microbiology-Sgm, 148*, 2331-2342.

Rajkumar, B., Sharma, G. D., & Paul, A. K. (2012). Isolation and characterization of heavy metal resistant bacteria from Barak River contaminated with pulp paper mill effluent, South Assam. *Bulletin of Environmental Contamination and Toxicology, 89*(2), 263-268.

Ranjard, L., Echairi, A., Nowak, V., Lejon, D. P., Nouaïm, R., & Chaussod, R. (2006). Field and microcosm experiments to evaluate the effects of agricultural Cu treatment on the density and genetic structure of microbial communities in two different soils. *FEMS Microbiology Ecology, 58*(2), 303-315.

Rappe, M. S., & Giovannoni, S. J. (2003). The uncultured microbial majority. *Annual Review of Microbiology, 57*, 369-394.

Renou, S., Givaudan, J. G., Poulain, S., Dirassouyan, F., & Moulin, P. (2008). Landfill leachate treatment: Review and opportunity. *Journal of Hazardous Materials, 150*(3), 468-493.

Roesch, L. F., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K. M., Kent, A. D., . . . Triplett, E. W. (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. *Isme Journal, 1*(4), 283-290.

Rothmel, R. K., Peters, R. W., St Martin, E., & Deflaun, M. F. (1998). Surfactant foam bioaugmentation technology for in situ treatment of TCE-DNAPLs. *Environmental Science & Technology, 32*(11), 1667-1675.

Sakawi, Z., Ariffin, M. R., Mastura, S. S., & Jali, M. F. M. (2013). The analysis of heavy metal concentration per distance and depth around the vicinity of open landfill. *Research Journal of Applied Sciences, Engineering and Technology, 5*(24), 8619-8625.

Sandrin, T. R., & Maier, R. M. (2002). Effect of pH on cadmium toxicity, speciation, and accumulation during naphthalene biodegradation. *Environmental Toxicology and Chemistry, 21*(10), 2075-2079.

Sang, N. N., Soda, S., Sei, K., & Ike, M. (2008). Effect of aeration on stabilization of organic solid waste and microbial population dynamics in lab-scale landfill bioreactors. *Journal of Bioscience and Bioengineering, 106*(5), 425-432.

Sawamura, H., Yamada, M., Endo, K., Soda, S., Ishigaki, T., & Ike, M. (2010). Characterization of microorganisms at different landfill depths using carbon-utilization patterns and 16S rRNA gene based T-RFLP. *Journal of Bioscience and Bioengineering, 109*(2), 130-137.

Schloss, P. D., & Handelsman, J. (2004). Status of the microbial census. *Microbiology and Molecular Biology Reviews, 68*(4), 686.

Semrau, J. D., DiSpirito, A. A., & Yoon, S. (2010). Methanotrophs and copper. *Fems Microbiology Reviews, 34*(4), 496-531.

Sheng, X. F., Xia, J. J., Jiang, C. Y., He, L. Y., & Qian, M. (2008). Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. *Environmental Pollution, 156*(3), 1164-1170.

Shigematsu, T., Yumihara, K., Ueda, Y., Numaguchi, M., Morimura, S., & Kida, K. (2003). *Delftia tsuruhatensis* sp nov., a terephthalate-assimilating bacterium isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology, 53*, 1479-1483.

Sineriz, M. L., Kothe, E., & Abate, C. M. (2009). Cadmium biosorption by *Streptomyces* sp. F4 isolated from former uranium mine. *Journal of Basic Microbiology, 49*, S55-S62.

Singer, A. C., van der Gast, C. J., & Thompson, I. P. (2005). Perspectives and vision for strain selection in bioaugmentation. *TRENDS in Biotechnology, 23*(2), 74-77.

Sinha, A., Pant, K. K., & Khare, S. K. (2012). Studies on mercury bioremediation by alginate immobilized mercury tolerant *Bacillus cereus* cells. *International Biodeterioration & Biodegradation, 71*, 1-8.

Sobolev, D., & Begonia, M. (2008). Effects of heavy metal contamination upon soil microbes: lead-induced changes in general and denitrifying microbial communities as evidenced by molecular markers. *International Journal of Environmental Research and Public Health, 5*(5), 450-456.

Song, L. Y., Wang, Y. Q., Tang, W., & Lei, Y. (2015). Bacterial community diversity in municipal waste landfill sites. *Applied Microbiology and Biotechnology, 99*(18), 7745-7756.

Sprocati, A. R., Alisi, C., Tasso, F., Marconi, P., Sciullo, A., Pinto, V., . . . Cremisini, C. (2012). Effectiveness of a microbial formula, as a bioaugmentation agent, tailored for bioremediation of diesel oil and heavy metal co-contaminated soil. *Process Biochemistry, 47*(11), 1649-1655.

Staley, C., Johnson, D., Gould, T. J., Wang, P., Phillips, J., Cotner, J. B., & Sadowsky, M. J. (2015). Frequencies of heavy metal resistance are associated with land cover type in the Upper Mississippi River. *Science of the Total Environment, 511*, 461-468.

Stamps, B. W., Lyles, C. N., Suflita, J. M., Masoner, J. R., Cozzarelli, I. M., Kolpin, D. W., & Stevenson, B. S. (2016). Municipal solid waste landfills harbor distinct microbiomes. *Frontiers in Microbiology, 7*, 11.

Stephens, W. Z., Burns, A. R., Stagaman, K., Wong, S., Rawls, J. F., Guillemin, K., & Bohannan, B. J. (2016). The composition of the zebrafish intestinal microbial community varies across development. *The ISME Journal, 10*(3), 644.

Stott, M. B., Crowe, M. A., Mountain, B. W., Smirnova, A. V., Hou, S. B., Alam, M., & Dunfield, P. F. (2008). Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand. *Environmental Microbiology, 10*(8), 2030-2041.

Su, C. (2014). A review on heavy metal contamination in the soil worldwide: Situation, impact and remediation techniques. *Environmental Skeptics and Critics, 3*(2), 24.

Suflita, J. M., Gerba, C. P., Ham, R. K., Palmisano, A. C., Rathje, W. L., & Robinson, J. A. (1992). The worlds largest landfill - A multidisciplinary investigation. *Environmental Science & Technology, 26*(8), 1486-1495.

Sundberg, C., Stendahl, J. S. K., Tonderski, K., & Lindgren, P. E. (2007). Overland flow systems for treatment of landfill leachates - Potential nitrification and structure of the ammonia-oxidising bacterial community during a growing season. *Soil Biology & Biochemistry, 39*(1), 127-138.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*(10), 2731-2739.

Tavares, T. C. L., Normando, L. R. O., de Vasconcelos, A. T. R., Gerber, A. L., Agnez-Lima, L. F., & Melo, V. M. M. (2016). Metagenomic analysis of sediments under seaports influence in the Equatorial Atlantic Ocean. *Science of the Total Environment, 557*, 888-900.

Tomova, I., Stoilova-Disheva, M., Lazarkevich, I., & Vasileva-Tonkova, E. (2015). Antimicrobial activity and resistance to heavy metals and antibiotics of heterotrophic bacteria isolated from sediment and soil samples collected from two Antarctic islands. *Frontiers in Life Science, 8*(4), 348-357.

Tsai, Y. P., You, S. J., Pai, T. Y., & Chen, K. W. (2005). Effect of cadmium on composition and diversity of bacterial communities in activated sludges. *International Biodeterioration & Biodegradation, 55*(4), 285-291.

Uz, I., Rasche, M. E., Townsend, T., Ogram, A. V., & Lindner, A. S. (2003). Characterization of methanogenic and methanotrophic assemblages in landfill samples. *Proceedings of the Royal Society B-Biological Sciences, 270*, S202-S205.

Valaskova, V., & Baldrian, P. (2009). Denaturing gradient gel electrophoresis as a fingerprinting method for the analysis of soil microbial communities. *Plant Soil and Environment, 55*(10), 413-423.

Valls, M., & de Lorenzo, V. (2002). Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. *Fems Microbiology Reviews, 26*(4), 327-338.

Vishnivetskaya, T. A., Mosher, J. J., Palumbo, A. V., Yang, Z. K., Podar, M., Brown, S. D., . . . Elias, D. A. (2011). Mercury and other heavy metals influence bacterial community structure in contaminated Tennessee streams. *Applied and Environmental Microbiology, 77*(1), 302-311.

Wan, R., Zhang, S. Y., & Xie, S. G. (2012). Microbial community changes in aquifer sediment microcosm for anaerobic anthracene biodegradation under methanogenic condition. *Journal of Environmental Sciences, 24*(8), 1498-1503.

Wang, F., Yao, J., Si, Y., Chen, H. L., Russel, M., Chen, K., . . . Bramanti, E. (2010). Short-time effect of heavy metals upon microbial community activity. *Journal of Hazardous Materials, 173*(1-3), 510-516.

Wang, Z., Yang, Y. Y., Dai, Y., & Xie, S. G. (2015). Anaerobic biodegradation of nonylphenol in river sediment under nitrate- or sulfate-reducing conditions and associated bacterial community. *Journal of Hazardous Materials, 286*, 306-314.

Ward, N. L., Challacombe, J. F., Janssen, P. H., Henrissat, B., Coutinho, P. M., Wu, M., . . . Kuske, C. R. (2009). Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils. *Applied and Environmental Microbiology, 75*(7), 2046-2056.

Webster, G., Embley, T. M., & Prosser, J. I. (2002). Grassland management regimens reduce small-scale heterogeneity and species diversity of beta-proteobacterial ammonia oxidizer populations. *Applied and Environmental Microbiology, 68*(1), 20-30.

Webster, G., Parkes, R. J., Fry, J. C., & Weightman, A. J. (2004). Widespread occurrence of a novel division of bacteria identified by 16S rRNA gene sequences originally found in deep marine Sediments. *Applied and Environmental Microbiology, 70*(9), 5708-5713.

Weihe, E., Kriews, M., & Abele, D. (2010). Differences in heavy metal concentrations and in the response of the antioxidant system to hypoxia and air exposure in the Antarctic limpet *Nacella concinna*. *Marine Environmental Research, 69*(3), 127-135.

Wiegand, I., Hilpert, K., & Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols, 3*(2), 163.

Wu, G., Kang, H. B., Zhang, X. Y., Shao, H. B., Chu, L. Y., & Ruan, C. J. (2010). A critical review on the bio-removal of hazardous heavy metals from contaminated soils: Issues, progress, eco-environmental concerns and opportunities. *Journal of Hazardous Materials, 174*(1-3), 1-8.

Wu, Q., Leung, J. Y., Geng, X., Chen, S., Huang, X., Li, H., . . . Lu, Y. (2015). Heavy metal contamination of soil and water in the vicinity of an abandoned e-waste recycling site: Implications for dissemination of heavy metals. *Science of the Total Environment, 506*, 217-225.

Xie, B., Xiong, S. Z., Liang, S. B., Hu, C., Zhang, X. J., & Lu, J. (2012). Performance and bacterial compositions of aged refuse reactors treating mature landfill leachate. *Bioresource Technology, 103*(1), 71-77.

Yao, X.-f., Zhang, J.-m., Tian, L., & Guo, J.-h. (2017). The effect of heavy metal contamination on the bacterial community structure at Jiaozhou Bay, China. *Brazilian Journal of Microbiology, 48*(1), 71-78.

Yusof, N., Haraguchi, A., Hassan, M. A., Othman, M. R., Wakisaka, M., & Shirai, Y. (2009). Measuring organic carbon, nutrients and heavy metals in rivers receiving leachate from controlled and uncontrolled municipal solid waste (MSW) landfills. *Waste Management, 29*(10), 2666-2680.

Zainun, M. Y., & Simarani, K. (2018). Metagenomics profiling for assessing microbial diversity in both active and closed landfills. *Science of the Total Environment, 616*, 269-278.

Zettler, L. A. A., Gómez, F., Zettler, E., Keenan, B. G., Amils, R., & Sogin, M. L. (2002). Microbiology: Eukaryotic diversity in Spain's river of fire. *Nature, 417*(6885), 137.

Zhang, H., Sekiguchi, Y., Hanada, S., Hugenholtz, P., Kim, H., Kamagata, Y., & Nakamura, K. (2003). *Gemmatimonas aurantiaca* gen. nov., sp nov., a gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum *Gemmatimonadetes* phyl. nov. *International Journal of Systematic and Evolutionary Microbiology, 53*, 1155-1163.

Zhang, L., & Xu, Z. H. (2008). Assessing bacterial diversity in soil. *Journal of Soils and Sediments, 8*(6), 379-388.

Zhang, Y., Cai, X. Y., Lang, X. M., Qiao, X. L., Li, X. H., & Chen, J. W. (2012). Insights into aquatic toxicities of the antibiotics oxytetracycline and ciprofloxacin in the presence of metal: Complexation versus mixture. *Environmental Pollution, 166*, 48-56.

Zimmermann, J., Gonzalez, J. M., Saiz-Jimenez, C., & Ludwig, W. (2005). Detection and phylogenetic relationships of highly diverse uncultured acidobacterial communities in altamira cave using 23S rRNA sequence analyses. *Geomicrobiology Journal, 22*(7-8), 379-388.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

Zainun, M. Y., & Simarani, K. (2018). Metagenomics profiling for assessing microbial diversity in both active and closed landfills. *Science of the Total Environment, 616*, 269-278.