EXPLORING THE POTENTIAL OF NATIVE MICROBIAL CONSORTIUM FOR BIODEGRADATION OF PLASTIC WASTES IN COMPOST

by

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DEDICATION

This project work is dedicated to God the Almighty source of divine wisdom and to all the indigent students all over the world.

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ABSTRACT

One of the biggest challenges to the compost industries is plastic contamination. Microorganisms have been reported to have the potential to decompose plastics. A research using next generation amplicon sequencing was conducted to study the microbial diversity and structure of compost piles ranging in age between 2 and 10 years from four compost facilities within Nova Scotia. Five different compost with partially decomposed plastics were randomly collected from compost piles at each location. Additionally, bulk compost samples within 10-cm radius around the sampled partially decomposed plastics were also collected. The bacterial phyla: *Proteobacteria, Bacteroidetes, Actinobacteria* and fungi *Ascomycota* were abundant across all facilities. Our result indicated significant differences in compost microbiomes within compost facilities, which might be related to compost chemical parameters, age of piles and feedstock. However, the presence of plastics in compost had no significant effect on the structure of microbial community the emphasizing inert nature of plastic.

LIST OF ABBREVIATION USED

ADONIS - Analysis of Variance Using Distance Matrices

Balefill - Colchester Composting Facility, Kemptown

DNA - Deoxyribonucleic acid

Fundy - Fundy Compost Inc., Brookfield

Guysborough - Guysborough Composting Facility, Boylston

HDPE - High-density polyethylene

HTS - High throughput sequencing

ITS - Internal transcribed spacer

LDPE - Low-density polyethylene

MDPE - Medium-density polyethylene

MT - Metric tonnes

NGS - Next-generation sequencing

Northridge - Valley – Northridge Farms, Aylesford

OTU – Operational taxonomic unit

PBSA - Polybutylene succinate-co-adipate

PE - Polyethylene

PP - Polypropylene

PS – Polystyrene

PU - Polyurethanes

PVC - Polyvinyl chloride

RRFB - Resource Recovery Fund Board

rRNA – Ribosomal ribonucleic acid

SPI - Society of Plastics Industry

STAMP - Statistical Analysis of Taxonomic and Functional Profiles

QIIME - Quantitative Insights into Microbial Ecology

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CHAPTER 1 INTRODUCTION

1.1 Thesis Overview

Plastic material and its utilization have found wide application in virtually all aspects of human life in both domestic and commercial settings. Thus, hardly anything will one do without encountering plastics or its product daily. The global use of polyethylene and plastic product is approximately 12% per annum, and this continues to rise (Raziyafathima et al., 2016). The high consumer demand drove global production to approximately 140 million tons of synthetic polymers, which has increased by 1.74-fold over the past 15 years to about 243 million tons (Sharma et al., 2015). The rise in production and use of synthetic polymers has increased the amount of global plastic wastes with numerous adverse effects on the environment; a concern expressed by the public including environmental advocates, growers and researchers (Worm et al., 2017).

One of the major environmental threats posed by these plastics is their inability to breakdown or their low rate of breakdown, which thereby, lead to environmental pollution, blockage of water ways causing death to marine and fresh water flora and fauna (Law, 2017; Bläsing and Amelung, 2018). In addition, plastics negatively affect soil ecosystem by releasing and absorbing toxic substances, inhibiting soil dwelling organisms (e. g. soil microbes) (Teuten et al., 2009). Plastic-associated toxic compounds can enter the food chain and affect human health (Hauser and Calafa, 2005). Another possibility is that plastic contamination in the soil can affect seed germination, plant establishment, root penetration, and impede nutrient and water uptake (Kołodziejek, 2017). Interestingly, there are few published scientific literatures to establish these facts.

The main problem facing the compost industry and users in Nova Scotia is contamination from plastic wastes (Figure 1.1). Previous study carried out in Nova Scotia, Canada revealed that approximately 14% of plastic wastes generated in the province in 2008 were diverted from landfill and sold to plastics recyclers (RRFB, 2008). Also, in 2011 and 2012, Divert Nova Scotia (Formally Resource Recovery Fund Board - RRFB) conducted another waste audit to know the amount of plastic sent to landfills in those years. The result showed that 58,550 and 60,600 tons of plastics were generated in 2011 and 2012, respectively. This represented about 20% of all type(s) of plastics that were used domestically, which were sent to landfills across the province during the year under review (RRFB, 2012; Muise, 2016).

Survey of some of the composting facilities in the province revealed that significant amount of revenue is lost annually to contaminations from plastics. For instance, at Fundy Compost Inc., Brookfield, Nova Scotia, an average of 21 metric tons of plastic films was sent back to landfill in 2015 alone at a tipping fee of $160/MT (Personal communication, William Curry, Agronomic Product Sales Manager, June 14, 2016). Also, during screening of compost these plastics may cause damage to equipment; small pieces of plastics from contaminated compost when used on agricultural field or home garden can scattered around which may end up in water bodies or contaminate the environment. Colchester Materials Recovery Facility (Balefill) recently reported approximately 300 to 500 million plastics shopping bags are used by Nova Scotians annually. This amounts to 4,940 lbs (2.24 metric tonnes) and 407,255 plastic bags per day (CWRM, 2018).

Globally, waste management has been geared towards the four important R's in waste management i.e., Reduce, Reuse, Recycle and Recover (Ren, 2003; Waste.net, 2017). Compost is an important

strategic way of organic waste management that helps to accomplish all these four R's. Composting helps to *reduce* the amount of waste sent to landfills; most of the organic matters are *reused* rather than discarded; while larger percentage are *recycled* into useful soil amendment materials. Also, the remaining materials that cannot be recycled are *recovered* into a useful energy content (Waste.net, 2017).

Figure 1.1. Compost pile contaminated with plastics (Picture by Seun Esan 14/06/2016).

Globally, there has been grave public concern and intense advocacy over deterioration in the environment due to indiscriminate disposal of conventional plastic wastes in recent years (Raaman, et al., 2012). These discarded plastics, besides being highly visible are rapidly increasing the percentage of solid waste in landfills. It cost the compost industries in the province much to produce a clean compost that meet the national standards and Compost Council of Canada recommendations. Apart from this, contamination from plastics discourages the users of compost because most of them complain that the small pieces from the plastics contaminate their field, lawn and gardening. However, the possibility of plastics been broken down in solid waste by microorganisms was reported by Yamada-Onodera (2001). Further studies on biodegradation of

various types of plastics, such as high-density polyethylene (HDPE), low density polyethylene (LDPE), polypropylene (PP) and polystyrene (PS) in natural soils revealed that some bacteria and fungi are capable of degrading plastics into carbon and energy.

Yamada-Onodera (2001) also reported that the fungus *Penicillium simplicissimum,* degraded HDPE of a molecular weight of up to 2800 g/mol. Thermophilic bacteria *Brevibaccillus borstelensis* strain 707 and *Rhodococcus ruber* were reported to assimilate LDPE as a carbon source (Gilan et al., 2004; Hadad et al., 2005). Soil inhabiting fungi (e.g., *Aspergillus niger, A. japonicas, A. terreus, A. flavus* and *Mucor* sp.) were also found to have the potential to decompose synthetic plastics; although the results were inconclusive (Raaman et al., 2012). Significant effort has also been done to understand the mechanisms of plastic degradation in various environments (Fotopoulou and Karapanagioti, 2017), as well as the role of microorganisms in this process (Shah, 2008; Sivasankari and Vinotha, 2014).

Many researches in plastic biodegradation has been carried out in soils and raw plastics *in vitro*. However, but there is less work done on plastics decomposition in compost. *Bacillus stearothermophilus* and *B. pumilus* were reported to degrade polybutylene succinate-co– adipate (PBSA) and poly (lactic acid) in soils, compost and activated sludge (Tomita et al., 2000). Native microbial communities can vary with variations in abiotic (e.g., light, temperature) and biotic (e.g., decomposer) factors and from region to region. Thus, there is the need to investigate the potential of native microbial species in any locality that can decompose plastics in compost. However, this study employed next generation amplicon sequencing to assess the diversity and structure of microbial communities from four major compost facilities across Nova Scotia Canada. It was therefore, hypothesized that age of compost and compost pile environmental conditions (in this

case location) and presence of plastics materials can affect the diversity and structure of native microbial community in compost.

1.2. Thesis Objectives

General Objective

To evaluate the potential of microbial communities to degrade plastics in compost.

Specific objectives are to:

- evaluate the diversity and structure of the microbial community in compost contaminated with plastics.
- evaluate the microbial communities associated with plastics degradation from composts obtained from different compost facilities.

1.3. Outline of Thesis and Organization

The thesis is divided into six chapters. Chapter 1 is an introduction to the research and justification for the study, including the present chapter. Chapter 2 is based on review of relevant literatures done in the field of the study, and there is a plan to publish it in a peer-reviewed journal. Chapter 3 focuses on detailed procedures and methods used to carried out various aspect of the research. Chapter 4 and chapter 5 describe the result and discussion of the research respectively. Then, the thesis is concluded with recommendations in Chapter 6, followed by the references and appendix in different sections.

CHAPTER 2. LITERATURE REVIEW

2.1. Compost

Composting is an environmentally friendly method of organic waste management. Historically, composting has been used to recycle agricultural wastes returning the composted organic matter into the soil to maintain fertility and crop productivity with minimum application of synthetic chemical fertilizers (Viaene et al., 2016).

Compost application is an effective way to add nutrient-rich humus to stimulate plant growth and it can also help to restore vitality to depleted soils (Eartheasy, 2014). Compost also serves as means of producing organic soil conditioners while the process itself can stabilize and help to minimize odour generation in the environment from decomposing organic waste in comparison to letting it rot unmanaged. This can provide plants with slow-release nutrients that are made available throughout the growing season (USDA, 2010).

Compost also contains valuable nutrients that can replace or supplement the use of synthetic chemical fertilizers while preventing potential health issues that may arise from organic wastes such as Dengue fever, cholera and malaria (Hoornweg et al., 2000, Chandra et al., 2010). The United States Public Health Service has recently identified 22 human diseases that are directly linked to improper municipal solid waste disposal (Alam and Ahmade, 2013). Compost provides an opportunity to improve overall waste collection and management programs in urban environments (Hoornweg et al., 2000).

Many researchers have proven that composts harbour communities of microorganisms, that are involved in the breakdown of the raw organic material into humus – like materials to release energy, carbon dioxide, water and heat (Jeffries, 2003; Pérez-Piqueres et al., 2006, Antunes et al., 2016). Effective composting helps to stabilize organic carbon (C) and eliminates any potential pathogens within the compost before it is used (Ozores-Hampton et al., 2008).

There are two types of composting processes, namely: aerobic and anaerobic composting. The aerobic process involves naturally occurring microorganisms that depends on the organic wastes for their energy and body proteins. As a result, their activities help to convert the biodegradable organic matter into a humus-like product (Fauziah and Agamuthu, 2009). This process converts nitrogen (N) from unstable ammonia to stable organic forms. Ultimately, the volume, physical structure, chemical and biological properties of the waste are altered. The success of the composting process is influenced by many factors such as C/N ratio, temperature, moisture content, oxygen supply, pH and particle size (Guanzon and Holmer, 2003). Anaerobic composting, or fermentation, is the degradation of organic wastes with very limited oxygen (O_2) to produce methane (CH_4), carbon dioxide (CO_2), ammonia (NH_3) and organic acids. Anaerobic composting is slow as compared to aerobic methods, and it involves microorganisms that do not require oxygen to survive e.g., facultative bacteria. (Guanzon and Holmer, 2003).

2.1.1. Phases of Aerobic Composting

Aerobic composting process has four major phases, namely: mesophilic, thermophilic, cooling down (mesophilic) and maturation and curing (Figure 2.1; Guanzon and Holmer, 2003, Zibilske, 2005).

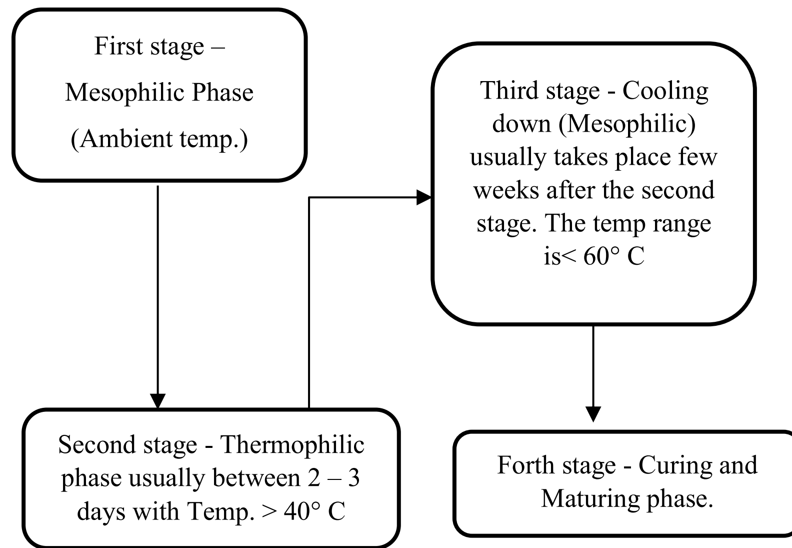


Figure 2.1. Four stages of natural composting process.

2.2. Types and Classification of Plastics

Plastics can be defined as polymers that can be moulded into any form or shapes when heated. (Thakur, 2012). The word plastic comes from the Greek word "plastikos", which means 'able to be molded into different shapes' (Joel, 1995). Plastics are the most versatile synthetic man-made substances created from fossil fuel resources. They may contain resin materials, which made them very stable and not readily degraded under ambient conditions (Raaman et al*.,* 2012). Plastic wastes are inert in nature and they have numerous negative impacts in the environment (Shah et al., 2008). Visible plastic waste in compost can reach 1.2 g per kg (Gajst, 2016; Bläsing and Amelung, 2018).

Common plastics used in recent time are made from inorganic and organic raw materials such as Carbon (C), sulphur (S), hydrogen (H), Nitrogen (N), Oxygen (O) and chlorine (Cl). In addition, plastics are structurally large molecules that are composed of repeated units (called monomers) with carbon as backbones (Thompson et al., 2009). Plastics are divided into two distinct groups,

namely: thermoplastics (mouldable) and thermosets (not mouldable) (Alauddin et al*.,* 1995; Thompson et al., 2009; Ghosh et al., 2013). Plastics can be classified into several groups depending on aim and objective of the classification (Read and Digest, 2018). For instance, Ghosh et al. (2013) classified plastics according to their chemical structure and properties as follows:

1. Classification of plastics according to their thermal properties

Plastic can be grouped into two categories based on their thermal properties namely: Thermoplastics and Thermosetting (Henry, 2014). Thermoplastic polymers are those polymers with linear long chain unlinked polymer molecules (PlasticsEurope, 2018). They cannot be molded into forms or shapes until it has been molten with heat. However, when frozen they become glass – like and can be easily broken (Singh and Sharma, 2008; Ghosh et al*.,* 2013, PlasticsEurope, 2018). They have high molecular weight ranging from 20,000 to 500,000 atomic mass unit (AMU) (Singh and Sharma, 2008; Ghosh et al., 2013). Examples are PE, PP, PS, PVC and polytetrafluoroethylene. Thermosetting or thermoset plastics are synthetic materials that undergo irreversible chemical change when heated and are not recyclable (Singh and Sharma, 2008; PlasticsEurope, 2018). Examples are phenol-formaldehyde and polyurethanes (PU).

2. *Classification of plastics according to their degradability properties*

Due to their chemical properties plastics can be categorized into degradable and non-degradable polymers. Non-biodegradable plastics are the commonly known synthetic plastics made from petrochemicals (Mohan and Srivastava, 2010; Ghosh et al., 2013). Structurally, they have repeated small monomer units and high molecular weights and on example is HDPE. Biodegradable plastics, on the other hand, are made from starch. Therefore, they are not very high in molecular

weight. They break down easily when exposed to biotic and abiotic factors like oxygen, water, micro-organisms, sunlight, enzymes and pH (Mohan and Srivastava, 2010; Ghosh et al., 2013). An example of biodegradable plastic is PBSA. The presence of glycoside linkages and ester groups in the chemical structure of biodegradable plastics represent a point of attack by decomposing enzymes or microorganisms (Reusch, 2013).

3. *Classification of plastics according to Recycling*

This classification is based on numerical code ranging from 1 to 7. It was designed by the Society of Plastics Industry (SPI) in 1988 to allow consumers and recyclers to differentiate types of plastics and for providing a uniform coding system for manufacturers (Earth talk, 2017). The SPI classifications are described below.

Plastic (SPI 1): This is the easiest and common plastics to recycle. They are polyethylene terephthalate (PET) and are accepted in all the recycling centers in Nova Scotia, Canada.

Plastic (SPI 2): Unlike plastics under SPI 1 category, they are not widely accepted in most recycling centers in Nova Scotia, Canada. These groups comprised HDPE plastics e.g., laundry detergents, bleach, milk and shampoo containers.

Plastic (SPI 3): This comprised items made from vinyl. For example, PVC is commonly used in plastic pipes, shower curtains, medical tubing and vinyl dashboards

Plastic (SPI 4): This comprised LDPE plastics used to make thin, flexible plastics like wrapping films, grocery bags, sandwich bags and a variety of soft packaging materials. It is common in all the landfills

Plastic (SPI 5): This comprised PP. Some food containers and most plastic cups are made from this plastic.

Plastic (SPI 6): This comprised PS plastics commonly called Styrofoam. Examples include items such as coffee cups, disposable cutlery and meat trays. PS can be reprocessed into many items, including rigid insulations.

Plastic (SPI 7): Plastics under this category are difficult to recycle, they comprise of others type of plastics or combination of two or more types. Examples are compact discs and medical storage containers.

2.3. Compost-Associated Microorganisms

Compost is a rich reservoir of different microbial communities (Koschinsky et al., 1999; Rawat and Johri, 2013; Neher et al., 2013; Franke-Whittle et al., 2014; Antunes et al., 2016). Identifying the presence of different microbial communities in composts, their co-existence, and the ways by which they interact during the various stages of the biological degradation process can help us to understand the role of these microbial communities during the composting process. Bacteria are the most abundance microbial community found in compost. They account for about 80 – 90% of all the microorganisms in compost (Rawat 2004; Rawat and Johri, 2013). During composting, the diversity and structure of microbial communities, as well as chemical and physical properties of the composting substrates change dramatically (Rawat and Johri, 2013; Franke-Whittle et al., 2014).

In the initial stage, mesophilic microbes form the pioneer community, which rapidly breakdown complex chemical compounds, resulting in the production of heat which raises the temperature of the compost and thus, paves way for thermophilic microbes at above 45°C. The latter forms the climax microbial community in compost (Rawat and Johri, 2013). During the thermophilic phase,

high temperature accelerates the breakdown of proteins, fats and complex carbohydrates like cellulose and hemicellulose. The heat can also affect the physical and chemical structure of plastics in compost. As the supply of these high-energy compounds becomes exhausted, the compost temperature gradually reduces, and mesophilic microorganisms once again take over for the final phase of 'curing' or maturation of the remaining organic matter. The structural divergence and species distribution are probably most significantly affected by temperature distribution. Thermophilic stage shows less structural divergence compared to mesophilic stage (Takaku et al., 2006). Compost is a microcosm of large number and diverse populations of microorganism, which help in the decomposition of organic and some inorganic materials (Koschinsky et al., 1999; Antunes et al., 2016; Friend and Smith, 2017). The diversity and structure of microbial communities can be influenced by abiotic environmental factors, and by biotic microbe-microbe interactions (Fierer, 2017). Furthermore, compost recipes, preparation methods and composting time play a significant role in shaping compost microbiota (Neher et al., 2013); which can also affect the ability of microorganisms to degrade plastics.

2.3.1. Factors Affecting Microbial Abundance and Diversity

Microorganisms play a significant role in the regulation of the ecosystem and are the largest species on Earth (Fierer and Lennon, 2011). The occurrence and biodiversity of microorganisms also varies according to geographical location, environmental factors and ecological variables such as soil, water body, compost, activated sludge, biotic and abiotic factors etc. (Tokiwa et al., 2009; Fierer and Lennon, 2011).

The importance of microbial communities in composting processes cannot be over emphasized (Ryckeboer et al., 2003). Composting involves highly intense microbial process which leads to decomposition of most biodegradable components (Adani et al., 1997; Chandna et al., 2013). Composting is a unique process affected by considerable number of factors (i.e., environmental and biological factors). These factors can influence the way microorganisms interact within compost environment. However, there are other factors that can affect the microbial colonization of finished compost directly or indirectly (Fracchia et al., 2006). These factors are: (a) source and composition of the initial substrates, (b) types of processing conditions, and (c) the quality of the finished compost. Source of feedstocks is an important factor to be considered before composting as different feedstock will produce different amounts of carbon (energy) and nutrients to the compost.

2.4. Importance of Carbon to Nitrogen Ratio

Microorganisms require energy and nutrients including, C, N, phosphorus (P), and potassium (K) in substantial amounts for reproduction, survival, cell wall maintenance, and for effective composting process (USDA, 2010). Typically, C serves the dual purpose of energy source for cellular respiration and as an element in the cell protoplasm (Walter, 2014). Nitrogen on the other hand, is important in the formation of proteins and for reproduction. The adequate C/N ratio range for optimum mixes of compost feedstocks is 25-30:1 (Guanzon and Holmer, 2003). Lower N will reduce the populations of microorganisms and slow down rate of bioconversion of organic wastes. On the other hand, excess N increases ammonia production, which volatilizes with a pungent smell and is lost to the environment. This can reduce microbial activities and the final quality of the compost. Many factors during the composting process determine the dynamics of the microbial

communities. Temperature is the major factor that determines the types of microorganisms, species diversity and the rate of metabolic activities in compost under aerobic conditions (Hassen et al., 2002). A large variety of mesophilic, thermotolerant and thermophilic aerobic microorganisms including bacteria, actinomycetes, yeasts and various other fungi have been extensively reported in composts and other self-heating organic materials (Hassen et al., 2002).

2.5. Identification of Plastic Degrading Microbes

Identification of microorganisms is the first step in establishing the microbial etiology of any disease or presence of any microorganism (Saroch, 2017). The process includes procedures and techniques used to correctly identify microorganism in a medium. As a result, identification of microorganism irrespective of the method requires knowledge of their morphological, biochemical, physiological and genetic characteristics (Saroch, 2017).

Conventional or traditional cultural methods for detecting microorganisms are based on the incorporation of the sample into a nutrient medium in which the microorganisms can multiply. This will also provide visual confirmation of their growth. Conventional methods are simple, easily adaptable, very practical and generally inexpensive (Saroch, 2017). Generally, conventional methods of identification rely on phenotypic identification and biochemical methods (Cloud et al., 2010). However, these methods of identification suffer from two major drawbacks i.e., firstly, they can be used only for organisms that can be cultivated in vitro; and secondly, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species (Saroch, 2017).

2.6. Plastics Degrading Microorganisms

The ability of microorganisms to use polyethylene (plastic) as a carbon and energy source has only been recently studied, and the potential has been confirmed by many researchers (Table 2.1). Generally, the attachment of microorganisms to the surface of the plastics, followed by the colonization of the exposed surface, is the first stage of colonization leading to degradation (Tokiwa et al., 2009). Degradation of plastics by microorganisms is primarily achieved by hydrolytic enzyme activities where bonds in the polymer are broken releasing monomers and oligomers (Lucas et al., 2008). Bacteria and fungi are involved in the degradation of plastics (Gu et al., 2000). For instance, *Streptomyces* spp. and wood degrading fungi (e.g., *Piptoporus betulinus*, *Irpex lacteus* etc.) can attach to the surface of the polyethylene and produce extracellular enzymes, which lead to degradation of polyethylene (Pometto et al., 1992; Kim et al., 2005). Other studies showed that bacteria, such as *Pseudomonas, Streptococcus, Staphylococcus, Micrococcus, Moraxella, Brevibaccillus*, and *Rhodococcus* (Gilan et al., 2004; Hadad et al., 2005), and fungi, including *Aspergillus niger, A. glaucus, Saccharomonospora* spp. and *Actinomycetes* spp. were associated with plastic degradation (Swift, 1997; Raaman et al., 2012).

Furthermore, studies on biodegradation of several types of plastics such as HDPE, LDPE, PP and PS in soils revealed that some fungi and bacteria species are capable of degrading plastics into carbon and energy for cellular metabolism. For instance, Yamada-Onodera (2001) reported that the fungus, *Penicillium simplicissimum,* degraded HDPE up to molecular weight of 2800 g/mol. Thermophilic bacteria *Brevibaccillus borstelensis* and *Rhodococcus ruber* were also reported to assimilate LDPE as a carbon source (Gilan et al., 2004; Hadad et al., 2005). Soil inhabiting fungi (e.g., *A. niger, A. japonicas, A. terreus, A. flavus* and *Mucor* sp.) were also found to have the

potential to decompose synthetic plastics although the results were inconclusive (Raaman et al., 2012). Also, *Bacillus stearothermophilus* and *B. pumilus* were reported to degrade PBSA and poly (lactic acid) in soils, compost and activated sludge (Tomita et al., 2000).

2.7. Four Steps of Plastic Degradation by Microorganisms

Lucas et al. (2008) stated that there are four steps involved in the biodegradation of plastic:

1) Biodeterioration: this refers to the physical and chemical deterioration that result in superficial degradation of plastics by the activities of microbial communities and other bio – decomposer.

2) Biofragmentation: explained the catalytic activities that breaks down polymeric plastics into smaller units such as oligomers, dimers or monomers by various enzymes secreted by microorganisms e.g., Alkane hydroxylase, Lipases, Esterases, Protease, Laccase etc.

3) Assimilation: refers to the integration or assimilation of molecules transported into the cytoplasm of microbes during metabolism.

4) Mineralization: this is the last phase that explain how microorganisms completely excrete the oxidized metabolites i.e., CO_2, N_2, CH_4 and H_2O.

2.8. Plastic Degradation and Biodegradation Mechanism

Generally, commonly used plastics such as polyethylene do not naturally degrade in landfills, soil or water body due to their stable nature. As such, they tend to accumulate in the environment causing severe pollution (Yamada-Onodera et al., 2001; Tseki et al., 2006; Kim et al., 2007; Ray et al., 2007; Hemashenpagam et al., 2013; Ghosh et al., 2013; Restrepo-Flórez et al., 2014; Skariyachan et al., 2015). This property of plastics is perhaps unexpected as one of the primary

reasons for the popularity and widespread application of many plastic material is their exceptionally high stability and durability.

The degradation of plastics can be characterized by many factors such as abiotic or biotic, the type of plastics, type of organisms etc., (Sen and Raut, 2015). Abiotic degradation is a deterioration brought about by environmental agents such as temperature, pressure, light, ultraviolet irradiation while biotic degradation is caused by biological agents such as microorganisms. The effect of these factors on plastic is evident in the structural modification of the plastics leading to changes in its physical and chemical properties (Hakkarainen and Albertsson, 2004). It is important to note that although the degradation of the plastics is usually attributed to either biotic or abiotic factors in nature, it is typical that both act concurrently (Hakkarainen and Albertsson, 2004).

Plastic degradation can be further classified as follows according to the agent causing:

(a) Biodegradation - this is a natural process by which microorganism (bio – agents) breakdown plastics into small units in order to use the carbon and energy sources for growth and energy (Sharma et al., 2015, Sen and Raut, 2015). This process can occur through hydro-biodegradation and oxo-biodegradation (Bonhomme et al., 2003).

(b) Photo degradation - this is brought about by the action of sunlight (Photo). Photo degradation can result into an alteration in the physical and chemical structure of the plastic i.e., usually as a result of sunlight in outdoor exposure (Yousif and Haddad, 2013).

(c) Thermo-oxidative degradation – this is caused by slow oxidative breakdown at moderate temperatures.

(d) Thermodegradation – this is caused by the action of high temperatures.

(e) Hydrolysis – this is the reaction of plastic with water (Hakkarainen and Albertsson, 2004; Andrady, 2011).

Generally, natural degradation of plastics does not occur in isolation. It is a sequence of events starting with photo degradation to thermo-oxidative degradation. UV - light generated by the sun light also play a key role by providing the necessary energy required to incorporate oxygen atoms into the plastic (Andrady, 2011; Raquez et al., 2011). This cause plastics to become brittle and start to break into smaller unit that can be metabolised by microorganisms (Yamada-Onodera et al., 2001; Andrady, 2011). These microorganisms convert the carbon in the plastic to carbon dioxide (Yamada-Onodera et al., 2001; Andrady, 2011).

2.9. Metagenomics

The arrival of next-generation sequencing (NGS) or high throughput sequencing (HTS) has brought about great revolution into the field of microbial ecology and has taken environmental studies to another level (Oulas et al*.,* 2015). The term metagenomics was first mentioned in 1998 by Handelsman et al. (1998). Metagenomics is the analysis of microbial genomes found within an environmental sample (Riesenfeld et al., 2004; Daniel, 2005; Thomas et al., 2012). Through metagenomics over 95% of microorganisms in the natural environment that cannot be cultured by traditional method have been identified (Bintrim et al., 1997; Rondon et al., 1999).

Metagenome sequencing helps us to understand how complex microbial communities are (i.e., microbial diversity) and how microbes interact within their ecosystem (Hayes et al., 2017; He et al., 2017). There are two type of metagenomics analysis namely: “full shotgun metagenomics”, and “marker gene amplification metagenomics” (i.e.,16S or 18S rRNA genes) or “meta-genetics” (Handelsman, 2009; Hayes et al., 2017). The type of method to be used to analyze a dataset

depends on the goals of the study and the available funds. Marker gene amplification metagenomics is restricted to taxonomic composition of the bacterial/archaeal population of the sample whereas full shotgun metagenomics is more detailed and unrestricted, but it is costly (Hayes et al., 2017).

Amplicon based analysis using 16S rRNA and fungal internal transcribed spacer (ITS) gene sequences are commonly used to investigate complex bacterial and fungal communities in the environment (Turnbaugh et al., 2007). There are nine hypervariable regions (V1 – V9) within the bacterial 16S rRNA genes that define bacterial taxa (Chakravorty et al., 2007). The commonly sequenced regions are: V1 – V2, V1 – V3, V3 – V5, V1 – V9, V6 – V9 and V4. Region V6 – V9 happens to be best because it can distinguish the bacterial species than other sub – regions. Unlike 16S, fungal internal transcribed spacer (ITS) region is by far the most commonly sequenced region for queries of systematics and taxonomy ITS is divided into three sub - regions (the spacers ITS1, ITS2 and the 5.8S gene). The first two regions (ITS1 and ITS2) are species specific and show high rate of evolution (White et al., 1990).

The data generated from amplicon-based analysis sequencing requires the use of bioinformatic tools to be able to effectively interpret or derive a taxonomic overview of the microbial community studied (Plummer et al., 2015). There are various tools available to analyse or extract meaningful information from 16S rRNA and fungal ITS gene sequenced data. These include QIIME (Quantitative Insights into Microbial Ecology) (Caporaso et al., 2010), METAGENassist (Arndt et al., 2012) , mothur (Schloss et al., 2009), MG-RAST (Metagenomics - Rapid Annotation using Subsystems Technology) (Meyer et al., 2008), VAMPS (Huse et al., 2014), Genboree (Riehle et al., 2012), CloVR-16S (Angiuoli et al., 2011), EzTaxon (Kim et al., 2012), Pheonix2 (Soh et al., 2013), MEGAN (Mitra et al., 2011), SnoWMan (Stocker et al., 2010), the RDPipeline (Ribosomal

Database Project Pipeline) (Cole et al., 2014), Vegan (Oksanen et al., 2013), ade4 (Dray and Dufour, 2007), and ape (Paradis et al., 2004). Previous studies have shown that of all the various tools available for analysis of 16S rRNA and fungal ITS metagenomics datasets, QIIME seems to stand out as the "gold standard" (Nilakanta et al., 2014; Oulas et al., 2015).

2.9.1. QIIME - Quantitative Insights into Microbial Ecology

QIIME is a bioinformatics pipeline using the PyCogent toolkit (a software for microbiome) for analysis of microbial communities. It helps to interpret dataset from raw DNA data sequence and it has been used successfully to analyze and interpret sequence data from fungal, viral, bacterial, and archaeal communities (Caporaso et al., 2010; Kuczynski et al., 2012). It's can handle bioinformatics of large datasets, which includes de-multiplexing and quality filtering, operational taxonomic unit (OTU) picking, taxonomic assignment and quality graphics and statistics. An OTU is a cluster of closely related individual organisms grouped by DNA sequence similarity (usually 97% similarity) of a specific taxonomic marker gene, such as 16 rRNA gene or fungal ITS. The output of this pipeline is OTU biome table containing a list of identified OTU's, their taxonomic assignment and number of reads representing these OTU's in each sample. This OTU table can be used for downstream phylogenetic reconstruction, and diversity analyses and visualizations. QIIME has been applied to studies based on billions of sequences from tens of thousands of samples (Caporaso et al., 2010). Using QIIME to analyze data from microbial communities consists of typing a series of commands into a terminal window, and then viewing the graphical and textual output. Comeau et al. (2017) gave a comprehensive easy to use workflows or standard operating procedures (SOPs) for analyzing 16S/18S rRNA and metagenomic data using QIIME.

2.9.2. Measurement of Microbial Diversity

One of the major objectives of microbial ecology is to measure and understand the distribution of diversity across or within a community (Birtel et al., 2015). Microbial communities were used in the past to explain patterns of microbial diversity over a range of community (Birtel et al., 2015). The studies of biodiversity and ecosystem function of microbial communities are further confirmed to be a model system to explore how composition of species are formed (i.e., species richness and functional diversity) and abundance of microbial taxa can affect specific ecosystem functions and services. There are various ways to express species diversity than counting species numbers (Jurasinski et al., 2009). However, the first person to develop a terminology and concept for comparison of vegetation diversity was Whittaker (Whittaker, 1956; 1960). He categorised levels of species diversity as follows: (1) *alpha diversity*, the "richness in species of a particular stand or community;" (2) *beta diversity*, the "extent of change of community composition".

2.9.3. Alpha Diversity (α-diversity)

This is the measurement of species richness or species frequencies (number of taxa) within a signal microbial community (Jurasinski et al., 2008). The diversity within a microbial community can be assessed by using the total number of species (species richness), the relative abundances of the species (species evenness), or the combination of the two (Lozupone and Knight, 2008), Alpha diversity in any community is difficult to estimate from sample data because it is very sensitive to the number of samples collected. (Gotelli and Chaol, 2013). Some commonly used indices to describe alpha diversity include Chao 1, Simpson's index and Shannon index.

i. *Chao 1*: measures the richness of species within a community. It can also be described as an estimator that provide the minimal number of OTUs that are present in a sample

ii. *Simpson's index*: describes species evenness that is how equally abundant species are in an environment.

iii. *Shannon index*: it measures the diversity in the environment and indicates species dominance

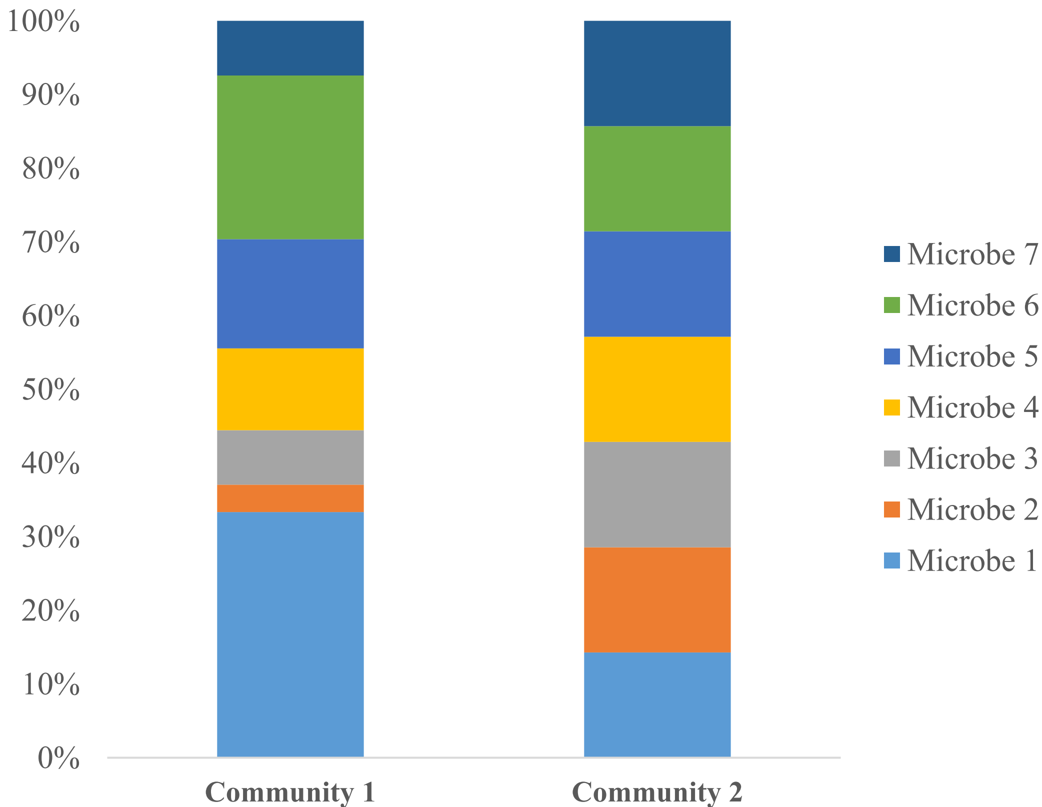


Figure 2.2. Species evenness for community 1 and 2. Each community consist of seven different microbial species.

- Microbes 1 and 5 are over represented in community 1, this represent lower species evenness
- Community 2 have all the seven microbes in equal proportion and this is referred to as higher species evenness.

2.9.4. Beta Diversity (β – diversity)

Beta diversity can be described to be the partitioning of biological diversity among various environments for instance, it can be used to describe number of species shared between two or more environmental locations (Whittaker, 1972; Lozupone, 2007). Beta diversity measures the abundance of species between two or more locations in reference to gain or loss in species. It can also be used to measure the dissimilarity between two communities or two samples.

2.9.5. Distance Metrics

Distance metrics are used for comparison of biological communities**.** They define a distance between each pair of communities in the set (beta-diversity). Many statistical methods have been developed to quantify beta-diversity, including Unique Fraction matrix (UniFrac) and Bray–Curtis distance metrics. UniFrac is a measurement of beta-diversity of any community using phylogenetic lineages (tree) of the taxa in each sample to test if the samples are significantly different (Lozupone et al., 2007; 2011). It can be used for 16S rRNA amplicon sequencing data, because it can produce phylogenetic information to obtain high taxonomic resolution. However, UniFrac cannot be applied to ITS amplicon sequencing data because fungal ITS region is not amenable to alignments across distinct fungal taxa as a matter of fact classification/characterization of fungi in the environment is a major area of concern (Goodrich et al., 2014)

Bray–Curtis diversity calculations are useful for analysis of amplicon sequence data, which cannot produce relabel phytogenic tree, such as fungal ITS amplicon sequencing. Ecologists normally uses Bray-Curtis dissimilarity to measure community assembly processes, species assemblages etc. (Ricotta and Podani, 2017). Bray-Curtis have many advantages which makes it a unique tool in ecological study such as, it is easy to compute, 'the natural analogy to multiple regression for multivariate responses, the nesting of solutions of different dimensionality, the convenient decomposition of variance into additive contributions by ordination dimensions, by cases and by variables, and easy-to-interpret 2 dimensional or 3–dimensional plot graphical displays' (Greenacre, 2017). Both Bray-Curtis and UniFrac matrices can be implemented using many microbial community sequence analysis pipelines such as QIIME (Caporaso et al., 2010) and mother (Schloss et al., 2009). They have a user-friendly web interface that can handle large data sets from high throughput sequencing (HTS).

2.9.6. Analysis of Variance Using Distance Matrices (ADONIS)

Adonis is a statistical test used to analyse and partition sums of squares using dissimilarities metrics such as Bray-Curtis and UniFrac (Anderson, 2001; McArdle and Anderson, 2001; Oksanen et al., 2017). Adonis is a "non-parametric'' statistical method that can be used in microbial community sequence analysis pipelines such as QIIME to determine statistical significance of sample grouping. It can also be used to analysis the size effect (R^2) that is the percentage of variation, as well as a p-value to determine the statistical significance (Caporaso et al., 2010).

2.9.7. Statistical Analysis of Taxonomic and Functional Profiles (STAMP)

STAMP is a statistical software package that gives detailed analysis of taxonomic and functional profiles (Parks et al., 2014). It is also being used to interpret processed data produced by QIIME (Caporaso et al., 2010).

Table 2. 1. Summary of previous research findings on biodegradation of plastics in different environment and their results.

No	Polymer used for the trial	Methods of degradation	Major findings	Microbial source	Method used	Microbes identified	Reference
1.	Low density polyethylene (LDPE)	Weight lost, surface corrosion and tensile strength	After 3 months of regular shaking the polyethylene discs were corroded on the surface and tensile strength decreased and max weight loss of about 12.5% was recorded.	Plastic dumping sites	Morphological keys and biochemical tests	*B. cerues* and *Psedomonas* sp.	Aswale and Ade (2008)
2.	Degradable plastic contained pro-oxidant and 6% starch	Weight loss, changes in tensile strength, percent elongation and molecular weight distribution	50% reduction in tensile strength	Lignocellulose degrading microorganism	The organisms used were known	*S. viridosporus* T7A, *S. badius* 252, *and S. setonii* 75Vi2 (bacteria) and *Phanerochaete chrysosporium* (fungus)	Lee et al., (1991)
3.	Polyethylene bags and plastic cups	Weight loss	After one month of incubation in both bacterial and fungal isolates the maximum degradation by fungi (*Aspergillus niger*) and bacteria (*Streptococcus lactis*) was found as 12.3% and 12.5 %, respectively	1. Medicinal Garden soil 2. Sewage water soil. 3. Energy Park 4. Sludge Area soil. 5. Agricultural soil.	Morphological keys and biochemical tests	B1 (*Pseudomonas*), B2 (*Bacillus subtilis*), B3 (*Staphylococcus aureus*), B4 (*Streptococcus lactis*), B5(*Proteus vulgaris*), B6 (*Micrococcus luteus*), F1 (*Aspergillus niger*), F2 (*Aspergillus nidulance*), F3 (*Aspergillus flavus*), F4 (*Aspergillus glaucus*), F5 (*Penicillium*)	Priyanka and Achana (2011)
4.	Branched low-density (0.92 g cm^{-3}) polyethylene	Gravimetric and molecular weight loss, FTIR	11% (gravimetric) and 30% (molecular) weights loss was reported at 50oC after 30 days.	Soil	Molecular level (using 16S rDNA)	*Brevibaccillus borstelensis* strain 707	Hadad et al. (2005)

No	Polymer used for the trial	Methods of degradation	Major findings	Microbial source	Method used	Microbes identified	Reference
5.	Pure polyethylene (5% starch) and modified polyethylene films (8% starch) and polyethylene with pro-degradant additives (master batch in amount of 20%)	Changes in weight, tensile strength and morphology of polymer	For polyethylene blends in sea water very little microbial degradation was observed in winter. But in summer, the weight loss of polyethylene with the master batch additive after 20 months reached 26%	Baltic Sea water	The incubation of polyethylene samples took 20 months.	Not applicable	Rutkowska et al. (2002)
6.	LPDE in the powdered form	Sturm test where the degradation was attributed to the amount of carbon dioxide evolved and scanning electron microscope (SEM) analysis.	Maximum 4.16 g/L of CO_2 was released after degradation of the polythene	Sea water	Morphological keys	*Aspergillus versicolor* and *Aspergillus* sp.	Pramila and Ramesh (2011)
7.	LDPE films	Weight measurements, tensile strength testing, Fourier Transform infrared spectroscopy – Attenuated Total Reflectance (FTIR) -ATR, Gas Chromatography - Mass Spectrometry (GC-MS) analyses.	The highest level of polythene degradation (weight loss) out of the four bacteria was 20% by *Pseudomonas aeruoginosa* after 120 days		Not applicable	*Pseudomonas aeruginosa* PAO1 (ATCC 15729), *P. aeruginosa* (ATCC 15692), *P. putida* (KT2440 ATCC 47054) and *P. syringae* (DC3000 ATCC 10862)	Kyaw et al. (2012)

No	Polymer used for the trial	Methods of degradation	Major findings	Microbial source	Method used	Microbes identified	Reference
8.	Linear low-density polyethylene torque blended with starch	FTIR spectroscopy, weight loss, SEM, Differential Scanning Calorimetry (DSC), Thermal Gravimetric Analysis (TGA)	The starch content in the blend was found directly proportional to the rate of degradation. Thus, the higher the content of starch, the higher the degree of degradation	Source of the microbes not specified but known cultures were used	Not applicable	*Aspergillus niger, Penicilliurn funiculosum, Chaetomium globosum, Gliocladiurn virens* and *Pullularia pullulans*	Gilan et al. (2004)
9.	LDPE and LLDPE	Chemiluminescence, ATR-FTIR and GC-product analysis	Polythene films 75-85% (containing Fe stearate) and 31-67% (containing Ca stearate) at 45ºC led to reduction in carbonyl index	Polythene films were scattered in agricultural vegetable field and after 30 days were used for the isolation of microbes	Molecular level (16S rRNA gene sequencing)	*Bacillus cereus, B. megaterium, B. subtilis* and *Brevibacillus borstelensis*	Abrusci et al. (2011)
10.	Branched low-density (0.92 g cm^{-3}) polyethylene with an average molecular weight of 191,000 g/mol	Weight loss, SEM analysis and formation of extracellular protein and polysaccharide in biofilm of *Rhodococcus ruber* strain C208 on polyethylene	7.5% of polythene weight loss after eight weeks	Not specified	Not specified	*R. ruber* (C208)	Sivan et al. (2006)
11.	Branched low-density (0.92 g cm^{-3}) polyethylene	Average weight loss, SEM, ATR and FTIR	8% of polyethylene degradation in 4 weeks	Soil with agricultural mulch	Molecular level (16S rDNA sequencing)	*R. ruber* C208	Chandra and Rustgi (1997)
12.	HDPE and LDPE	Mean weight	Nearly 5% of weight loss after a period of eight weeks	Soil	Morphological keys and biochemical tests	*Bacillus, Micrococcus, Listeria* and *Vibrio*	Kumar et al. (2007)

No	Polymer used for the trial	Methods of degradation	Major findings	Microbial source	Method used	Microbes identified	Reference
13.	Polyethylene bags	Weight loss	22.2% of polythene degradation per month was recorded at pH 4, room temperature with regular shaking	Polyethylene dumping site	Morphological keys and biochemical tests	*Serretia marscence*	Aswale and Ade (2009)
14.	Commercially environmentally degradable polythene	Epifluorescence microscopy, SEM and FTIR spectroscopy	After 243 days cross linking, and chain scission was observed at higher temperatures leading to reduction in molecular weight	American type culture collection	Known cultures were used	*Rhodococus rhodocorous* ATCC 29672, *Cladosporium cladosporides* ATCC 20251 and *Nocardia steroids* GK 911	Bonhomme et al. (2003)
15.	Extruded LDPE with 20-micron thickness	SEM and FT-IR	Organism/degrading the polyethylene layer and creating holes in it. Different extracellular enzymes were responsible for the degradation of the shredded polyethylene	Not specified	Known cultures were used	*Staphylococcus epidermis*	Chatterjee et al. (2010)
16.	Commercially available high-density polyethylene (HDPE)	Weight loss, crystallinity and FT-IR spectrum	After 30 days of incubation was nearly 12% (*Arthrobacter* sp.) and 15% (*Pseudomonas* sp.)	Partially decompose polyeyhylene sample with soil sample adhering.	Not specified	*Arthrobacter* and *Pseudomonas* sp.	Balasubramanian et al. (2010)
	Polythene carry bags	Weight loss	25% of weight was observed after 8 months with regular shaking	Landfills	Morphological keys	*Aspergillus niger*	Aswale and Ade (2011)

No	Polymer used for the trial	Methods of degradation	Major findings	Microbial source	Method used	Microbes identified	Reference
17.	Polyethylene carry bags and plastics cups	Weight loss and reduction in tensile strength	In compost culture, highest percentage of weight loss (11.54%) was recorded in LDPE after 12 months while highest percent loss in tensile strength was reported with HDPE in the same time of incubation	Naturally buried polyethylene carry bags and cups in municipal composite	Morphological keys and biochemical tests were used	The predominant bacteria were *Bacillus sp., Staphylococcus sp., Streptococus sp., Diplococcus sp., Micrococcus sp., Pseudomonas sp*. and *Moraxella* sp. The predominant fungi were *Aspergillus niger, A. ornatus, A. nidulans, A. cremeus, A. flavus, A. candidus and A. glaucus*	Reddy, (2008)
18.	LDPE and BPE 10 (10% oxo-biodegradable additive)	Change in tensile strength, percent elongation, FTIR spectroscopy, contact angle and surface energy and SEM analyses	Pre-treated BPE10 after 3 months of incubation with *B. cereus* (C1) changes its tensile strength up to 17% and 17.4^{o} reduction in contact angle.	Municipal compost yard	Morphological keys, biochemical tests and molecular markers	*Bacillus cereus* (C1)	Suresh et al. (2011)
19.	Polyethylene bag wastes (pure water sachets)	Percentage of weight loss	After 8 weeks, only 1.2% weight loss was recorded when treated with 0.5 M HNO_3 followed by slight change in the colour	Landfills	Not specified	*Pseudomonas aeruginosa, Pseudomonas putida, Bacillus subtilis* and *Aspergillus niger*	Nwachukwu et al. (2010)
20	Disposable plastic films	Average weight loss changes in tensile strength and percent elongation	28.5% and 46.5% bacteria and fungi were used in the preliminary report of enzyme(s) responsible for degrading polythene after 10 days of heat treatment.	Nile River Delta	Morphological keys	Eight *Streptomyces* strains and two fungi, *Mucor. rouxii* NRRL 1835 and *Aspergillus flavus*	El-Shafei et al. (1998)

No	Polymer used for the trial	Methods of degradation	Major findings	Microbial source	Method used	Microbes identified	Reference
21.	High-molecular-weight polyethylene	Changes in relative elongation and relative tensile strength (Strograph-R3) and polyethylene molecular weight distribution (Waters model 150-C)	MnP is the key enzyme in polyethylene degradation by lignin-degrading fungi	Not Specified	Not specified	*Phanerochaete chrysosporium* ME-446, *Trametes versicolor* IFO 7043, and IZU-15413	Iiyoshi et al. (1998)
22.	Degradable polyethylene	Percent weight loss and emission of CO_2 gas chromatography (GC)	When *Penicillium frequentans* and *Bacillus mycoides* were used together Weight loss 7.2% (pre-heated at 70°C) and 6.7% (unheated) after 60 days	Soil contaminated with polyethylene bags	Morphological keys and biochemical tests	The most effective fungi and bacteria were *Penicillium frequentans* and *Bacillus mycoides*	Seneviratne et al. (2006)
24.	Low density polyethylene powder	Weight loss	*Actinomycetes* (*Streptomyces* KU8) leads to 46.2% weight loss of the polythene while bacteria (*Pseudomonas spp.*) and fungi (*Aspergillus flavus*) degraded only 37% and 20.6% after six months	Landfills	Morphological keys and biochemical tests	*Streptomyces* KU8, *Streptomyces* KU5, *Streptomyces* KU1, *Streptomyces* KU6, *Pseudomonas sp*., *Bacillus sp*., *Staphylococcus sp*., *Aspergillus nidulans* and *A. flavus*	Usha et al. (2011)
25.	Polythene carry bags	Weight loss, Thin Layer Chromatography (TLC), GC-MS and FTIR analyses	After eight months of regular shaking, 50% weight loss was recorded at room temperature with pH 4 with fungi (*Phanerochaete chrysosporium*) and 35% with bacteria (*Pseudomonas aeruginosa*)	Plastic dumping site	Morphological keys and biochemical tests	*Serratia marcescens* 724, *Bacillus cereus, Pseudomonas aeruginosa, Streptococus aureus* B-324, *Micrococcus lylae* B-429, *Phanerochaete chrysosporiu, Pleurotus ostretus, Aspergillus niger* and *Aspergillus glaucus*	Aswale (2010)

No	Polymer used for the trial	Methods of degradation	Major findings	Microbial source	Method used	Microbes identified	Reference
26.	Natural polyethylene (6% vegetable starch) and synthetic polyethylene	Percentage weight loss	The highest weight loss of natural polythene (46%) and synthetic polythene (29%) was reported with *Pseudomonas* sp. collected from sewage sludge dumping site	Soil	Morphological keys and biochemical tests	*Pseudomonas spp.* (P1, P2 and P3)	Nanda et al. (2010)
27.	Powdered LDPE	Differential scanning calorimetry (DSC), X-ray diffraction (XRD), Fourier Transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM)	After 31 months there were 5% reduction in crystallinity (*Aspergillus niger*), 11% decrease in crystalline thickness (*Pencillium pinophilum*), *P. pinophilum* incubated with and without ethanol showed a higher thermo – oxidized LDPE biodegradation efficiency than *A. niger*. Mineralization was also higher for *P. pinophilum* with the addition of ethanol	Not Specified	Not specified	*Penicillium pinophilum* and *Aspergillus niger*	Volke–Sepulveda et al. (2002)
28.	Polyethylene bags and plastic cups	weight loss	20.5% (*Psedumonas* sp.) 28.8% (*Aspergillus glaucus*) weight loss per month in shaker culture	Mangroves rhizosphere soil	Morphological keys were used	*Streptococcus, Staphylococcus, Micrococcus* (*Gram +ve), Moraxella, and Pseudomonas* (*Gram –ve*) and two species of fungi (*Aspergillus glaucus* and *A. niger*)	Kathiresan, (2003)

Source: Sangale et al., (2012) (Modified)

CHAPTER 3. MATERIALS AND METHODS

3.1. Site Description

Samples were collected in August and September 2016 from four different composting facilities in Nova Scotia, Canada. namely: 1) Colchester Composting Facility (Balefill), Kemptown; 2) Valley – Northridge Farms, Aylesford; 3) Fundy Compost Inc., Brookfield and 4) Guysborough Composting Facility, Boylston. The description of the sites with various sources of feedstock and types of processing at each compost facilities are shown in the table below (Table 3.1).

Generally, the sample piles in all the facilities are mixture of different substances such as rocks, plastics of several types, municipal wastes etc.

Table 3. 1**.** Description of compost facilities.

Name of facility	Coordinate	Age of pile, years	Source of feedstock	Bulking material
Balefill, Colchester Composting Facility, Kemptown	45°27'24.6"N 63°06'20.1"W	2	Colchester county	Wood chips during winter season
Fundy, Fundy Compost Inc., Brookfield	45°15'01.5"N 63°20'46.9"W	10	Halifax, East Hants regional municipality	Wood shaving/woodchips and woods (major source)
Northridge, Valley – Northridge Farms, Aylesford	45°03'20.9"N 64°50'27.6"W	2	Kings, Queens and Annapolis counties	Hays and straw during winter season
Guysborough, Guysborough Composting Facility, Boylston	45°29'33.7"N 61°32'15.2"W	3	Antigonish, Port Hawksbury and Guysborough counties	Hays and straw during winter season

3.2. Collection of compost and plastic samples

Five (5) different compost associated with partially decomposed LDPE were randomly collected from compost piles per location (n = 20). In addition, 500 g of bulk compost samples within 10-cm radius around the sampled partially decomposed plastic films were also collected at each location using sterile hand auger. The plastic and compost samples were kept in labeled sterile plastic bags and immediately placed in a cooling box with icepacks before transporting them to the laboratory. The samples were then processed within 24 hrs.

3.3. Preparation of Compost Samples

Approximately 10 g of the compost samples were sieved using a 2-mm sieve and kept at -80°C for further analysis at the Faculty of Agriculture's Molecular Microbiology laboratory, Dalhousie University. DNA was isolated from 0.25 g of the sieved compost.

3.4. Preparation of Plastic Samples

Five (5) g of compost associated with partially decomposed plastics was placed into a conical flask and 150 ml of sterile 10% glycerol was added before placing on a shaker for 15 min. The mixture was sonicated for 15 min before removing the plastics, which was again placed in another flask, and the process was repeated. The solution from two cleaning steps was combined and centrifuged at 4000 rpm for 30 min and the supernatant was decanted. The plastic pellets formed were transferred into 1.5 ml Eppendorf tubes and centrifuged again at 8,000 rpm for 10 mins. The supernatant was discarded, and the sample was stored at -80°C until processing for DNA isolation.

3.5. Compost Analysis

Compost samples were stored in a cooler during transportation from the sample site and they were stored at -20°C in the laboratory before analysis. The compost nutrients analysis was done at the Nova Scotia Agricultural Laboratory Services (Harlow institute) Truro for standard bulk compost analysis including Mehlich III mineral nutrient concentrations (Mehlich, 1984). Mineral nutrients concentrations were determined using Mehlich III solution (0.2 M acetic acid (CH_3COOH) + 0.25 M ammonium nitrate (NH_4NO_3) + 0.015 M ammonium fluoride (NH_4F) + 0.013 M nitric acid (HNO_3) + 0.001 M ethylene diamine tetra - acetic acid (EDTA) according to Mehlich (1984). Air dried bulk compost samples (10 g) were weighed into 50 mL test tubes and 25 mL of Mehlich III extracting solution added and samples shaken for 5 min using a reciprocating shaker. The solutions were filtered through Whatman #42 filter paper and the resulting filtrate used to determine the mineral nutrients concentration. The percentage carbon, nitrogen, and C/N were analyzed using VarioMAX CN Elementar Americas Inc. New York, 11779, USA.

3.6. DNA Extraction and Sequencing

DNA extraction was carried out using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA quality and concentration were measured using a BioTekSynergy H1 microplate reader (BioTek instrument, Inc, Vermont, USA). Five microlitres of each isolated DNA sample were sent to the Dalhousie University CGEB-IMR (http://cge.b-imr.ca/) for V6-V8 16S rRNA gene and fungal ITS gene library preparation and sequencing. Samples were multiplexed using a dual-indexing approach and sequenced using an Illumina MiSeq with paired-end 300+300 bp reads. Polymerase chain reaction (PCR), primers and Illumina sequencing details were as described by Comeau et al. (2017).

3.7. Sequencing Data Processing

Microbiome Helper Standard operating procedure as described by Comeau et al. (2017) was used to process and analyse the sequencing data. Overlapping paired-end reads were stitched together using PEAR (v0.9.6; (Zhang et al., 2014)). The 16S and fungal ITS reads were successfully stitched at 94.8% and 68.9%, respectively. FASTX-Toolkit (v0.0.14; Gordon, 2009) was later ran to filter out reads that did not have at least 90% of nucleotides (nt) with a quality score greater than 30. In addition, reads shorter than 400 bp that did not contain matching 3'and 5' sequences were filtered out to the appropriate forward and reverse primers using BBMap v35.85 (Bushnell, 2014). Lastly, we ran USEARCH v6.1 (Edgar et al., 2011)) to screen out chimeric reads using the options mindiv=1.5 and minh=0.2.

3.8. OTU Picking and Statistical Analyses

Following the filtering steps in section 3.7 above, we ran open-reference OTU picking using QIIME wrapper scripts (Caporaso et al., 2010). Specifically, SortMeRNA (v2.0-dev; (Kopylova et al., 2012)) was used for the reference OTU picking steps with sortmerna_coverage=0.8 and sumaclust v1.0.00 (Mercier et al., 2013) for the *de novo* OTU picking steps, with 10% of the failures sub-sampled. OTUs that contained fewer than 0.1% of the total sequences were filtered out in order to compensate for MiSeq run-to-run bleed-through (Comeau et al., 2017). Alpha-diversity (Chao1 and richness) and beta diversity i.e., weighted Unifrac distance (Lozupone et al., 2011) and Bray-Curtis matrices (Bray and Cutis, 1957) using QIIME were then generated (Caporaso et al., 2010). Spearman and Tukey's pairwise tests were carried out using Past3 package (Hammer et al., 2001). Adonis tests (999 permutations) were run in QIIME to calculate how sample grouping by sample types or location were related to microbial community structure. These

tests are a measure of how much variation in community structure is explained by the variable of interest (Comeau et al., 2017). Analyses of taxonomic profiles were performed using the STAMP software package (Parks et al*.,* 2014), while analysis of statistical significance (α-level = 0.05) of sample grouping for the samples was done using QIIME (Caporaso et al*.,* 2010). Spearman's rank correlation analysis for alpha-diversity and mean compost nutrient properties were carried out using Minitab v. 18.1 software (Minitab Inc., PA, USA).

3.9. Preparation of LDPE Powder for Media

The procedure described by Pramila and Ramesh (2011) was used to prepare LDPE powder for the test media but it was not successful as it was not possible to get desirable result following the procedures outlined by them. Hence, an already prepared medium density polyethylene (MDPE) was purchased from Aldrich Canada.

3.10. Media Modification for Plastic Degrading Microbes

A preliminary study to develop media to grow plastic degrading microorganisms was carried out but it was not concluded due to several challenges among which are: lack of MDPE powder in large quantity and time constraints. Meanwhile the following media was developed for plastic degrading microorganisms:

a) Minimal media (MDPE) for fungi (1 L): 10.0 g of sucrose, 0.5 g of NH_4Cl, 15.0 g of agar, 970 ml of water, 1.0 ml of thiamine (2 mg/ml), 1.0 ml of biotin (0.2 mg/min 100% EtOH).
b) Minimal media (special fungi) sucrose (1 L): 200 mg of MDPE powder, 15 g of agar, 200 mg of sucrose, 1.0 g of $(NH_4)_2SO_4$, 0.1 g of NaCl, 970 ml of water.

c) Minimal media (special bacterial) (1L): 10 g of MDPE powder, 0.5 g of NH_4Cl, 15.0 g of Agar, 970 ml of water, 1.0 ml of Thiamine (2 mg/ml), 1.0 ml of Biotin (0.2 mg/min 100% EtOH).

d) Minimal sucrose salts I: 20 g of K_2HPO_4, 160 g of K_2HPO_4, 20 g of $MgSO_4$, then autoclave.

All the mixture was autoclaved for 126 mins before cooling to 55°C then 10.0 ml of salt I and II were added. Minimal sucrose salts I: 2 g of $CaCl_22H_2O$, 1 g of $FeSO_4.7H_2O$. Minimal sucrose salts II: 5 g of $Na_2M_oO_4.2H_2O$, 5 g of $Na_2WO_42H_2O$, 5 g of $MnSO_4$.

CHAPTER 4. RESULTS

4.1. DNA Isolation and Analysis

DNA was isolated, as previously described and sequenced to generate qualitative and quantitative data. After the initial analysis (sequencing) seven samples (Balefill plastic 1, Balefill plastic 3, Fundy compost 1, Fundy compost 2, Fundy compost 4, Fundy compost 5, and Guysborough compost 1) had low number of reads and therefore, their DNA were re-isolated and re-sequenced. The quantification and qualification of DNA isolated from Bulk compost and plastic-associated with compost samples were measured by BioTek Synergy H1 microplate reader (BioTek instruments, Inc. Vermont, USA) the results are presented in Appendices 3 and 4.

4.2. Composition of Microbial Communities Across the Four Locations

The data analysis was conducted as stated in Chapter 3 using the standard operating procedure of the CGEB-IMR as outlined in the Microbiome Helper package (QIIME) as described by Comeau et al. (2017). The detailed protocol for sequence data analysis is presented in Appendix 1 (16S rRNA analysis) and Appendix 2 (ITS analysis). The second round of sequence data for the seven re-sequenced samples were combined with data from the first sequencing run using the underlined command to combine the old data with the new data. The blue colour represents each of the old files i.e., BP1, BP3, FC5, FC1, GC1, FC4 and FC2 and the red colour means Redo files. The green colour represents the new raw data

```
cat raw_data/file.fastq.gz EsanRedosITS/file.fastq.gz > raw_data_new/merged_.fastq.gz
```

4.3. Bacterial Communities

A total of 838,769 high-quality 16S rRNA sequences were obtained across the 40 samples of compost. The samples were normalized to a depth of 5,545 reads (i.e., the depth of the smallest reads). After sample normalization, these reads were distributed among 4,391 OTUs at 97% identity and it was later repressed to 593 taxa at the taxonomic class level. The predominant phyla were *Proteobacteria, Bacteroidetes* and *Actinobacteria,* which ranged from 7 to 17% of all the samples. The top 15 most abundant bacterial taxa found from this study at the class level included *Alphaproteobacteria* (17%), *Gammaproteobacteria* (16%), *Cytophagia* (12%), *Saprospirae* (10%), *Actinobacteria* (7%), *Flavobacteriia* (7%), *Deltaproteobacteria* (5%), *Betaproteobacteria* (5%), *Sphingobacteriia* (4%), *Bacilli* (2%), *Anaerolineae* (1%), *Acidobacteria-6* (1%), *Chloracidobacteria* (1%), *Gemmatimonadetes* (0.90%) and *Gemm-5* (0.70%) (Figure 4.1). The sum of the sequences belonging to these classes accounted for more than 90% of bacterial classes identified in the present study (Figure 4.1).

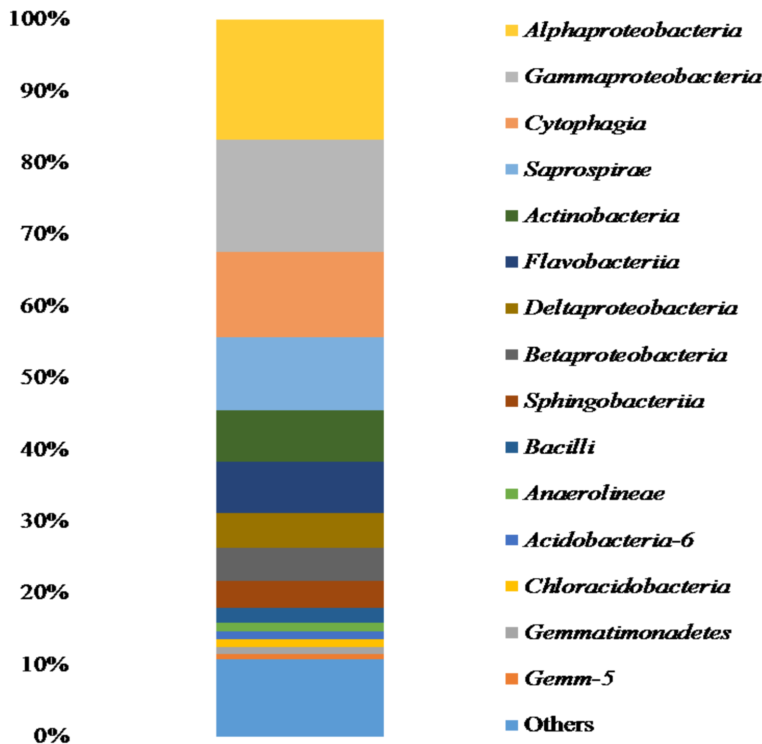


Figure 4.1. Relative abundances of major bacterial16S rRNA microbial taxa identified in bulk compost samples from the four different locations.

4.4. Fungi Community

A total of 825,446 high-quality ITS reads were obtained from 40 samples (data not presented due to large size). The reads were normalized to the depth of 776 reads (i.e., the depth of the smallest reads). After normalization, these reads were distributed among 653 fungal OTUs at 97%. These OTUs comprised of 198 fungal taxa grouped at the class level. The most relatively abundant fungal phylum is *Ascomycota*, representing 99% of all the identified fungal OTUs. The top 15 most abundant fungal taxa at class level representing approximately 91% of all the identified fungi were *Sordariomycetes* (23%), *Agaricomycetes* (22%), *Ascomycota*_unclassified (16%), *Pezizomycetes* (13%), *Dothideomycetes* (5%), *Eurotiomycetes* (5%), *Leotiomycetes* (4%), *Microbotryomycetes* (2%) while *Lecanoromycetes*, *Orbiliomycetes*, *Pezizomycotina_cls_Incertae_sedis*,

Saccharomycetes, *Taphrinomycetes*, *Cystobasidiomycetes*, *Exobasidiomycetes* represented >1% *(*Figure 4.2).

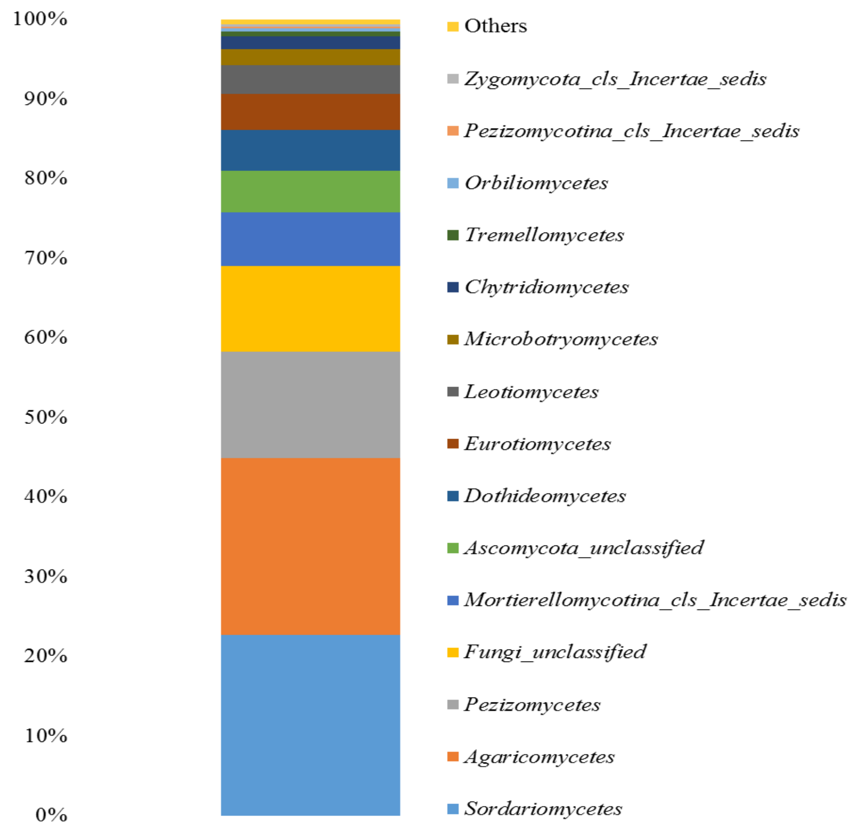


Figure 4.2. Relative abundances of major fungal ITS microbial taxa identified in bulk compost samples from the four different locations.

4.5. Beta-Diversity of Soil Microbial Communities

Principal Coordinate Analysis (PCoA) was used to visualize the diversity of the microbial communities. Figures 4.3a and 4.3b show the visual variations in microbial populations across different compost facilities (locations – Guysborough, Northridge, Balefill and Fundy; Figure 4.3a and 4.3b). Figure 4.3a show the clear visual separation as the colored ring indicates different location while figure 4.3b reveal the visual variation from the three principal coordinate point.

Also, Figures 4.4a and 4.4b represent visual variations due to age of the compost piles (i.e., 2, 3 and 10 years; Figures 4.4a and 4.4b).

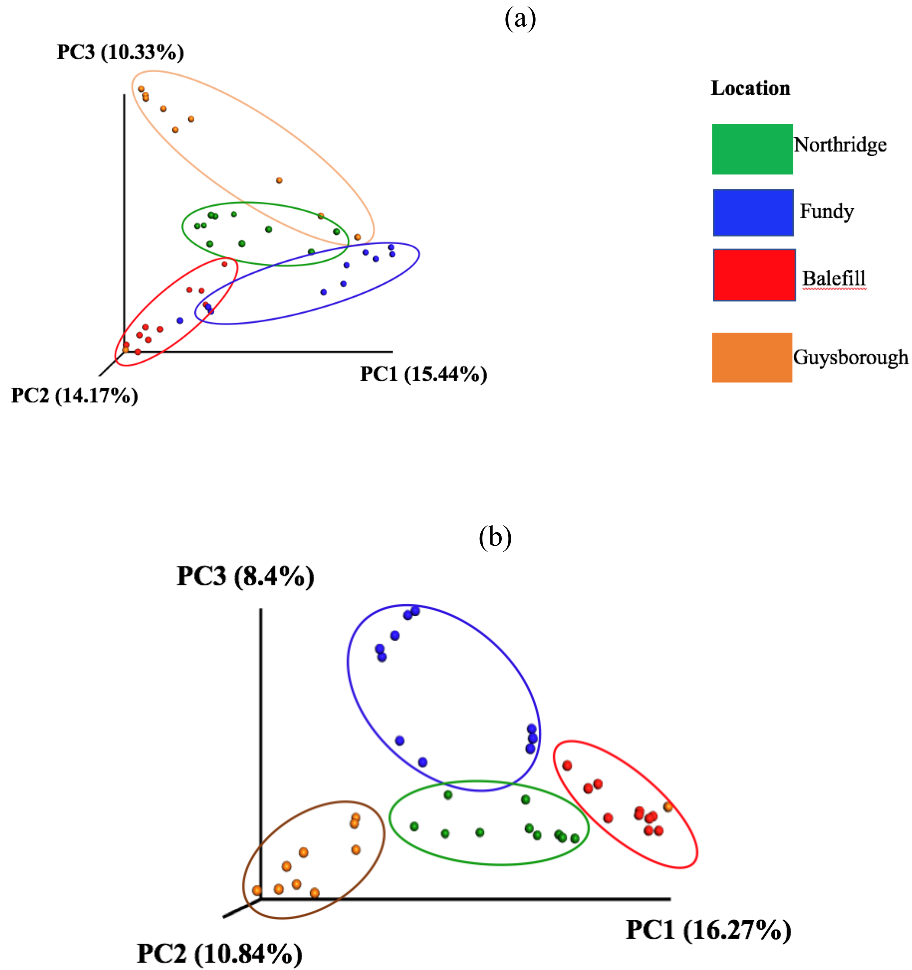


Figure 4.3a. Principal coordinate analysis (PCoA) of microbial communities: a) bacterial and b) fungi based on Bray-Curtis dissimilarity ecological distances for location. Each point represents different sample per location where the colored ring indicates different location.

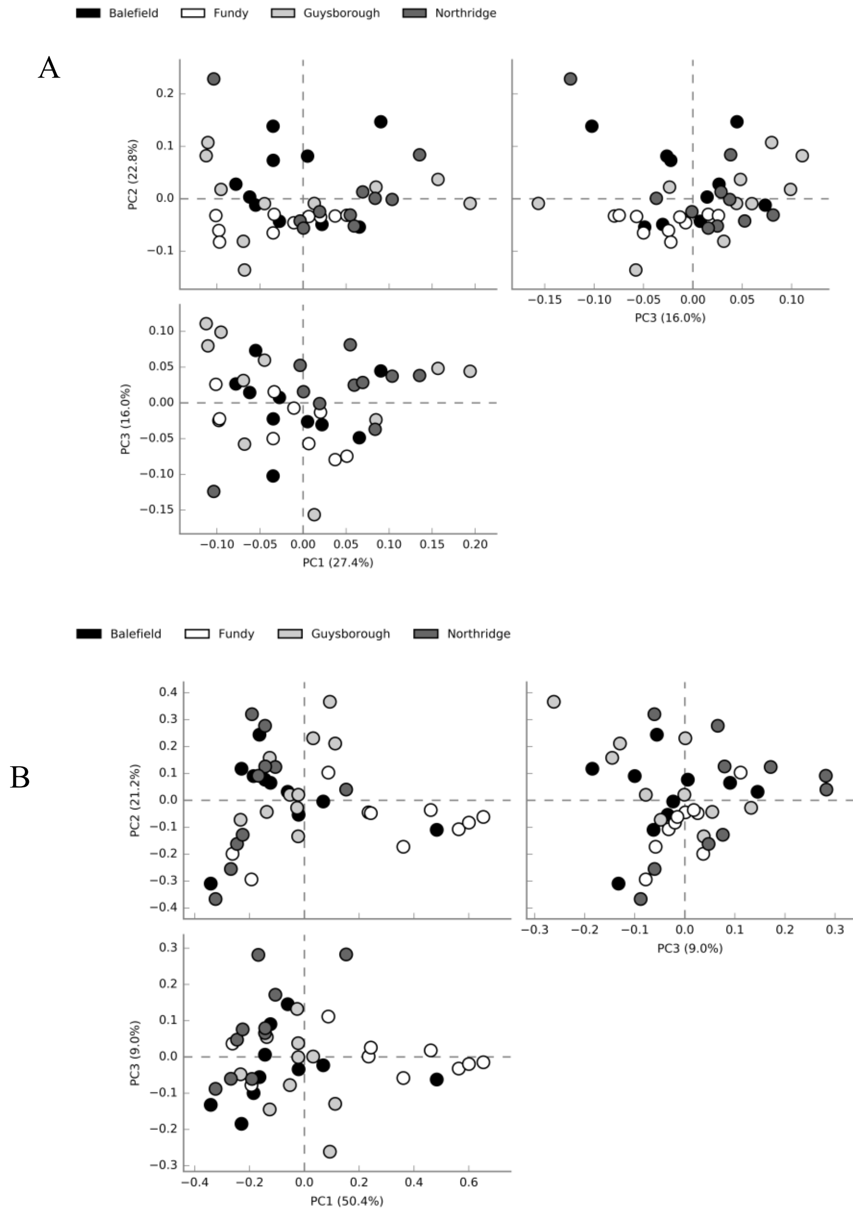


Figure 4.3b. Principal coordinate analysis (PCoA) of microbial communities: a) bacterial and b) fungi based on Bray-Curtis dissimilarity ecological distances for location. Each point represents different sample per location.

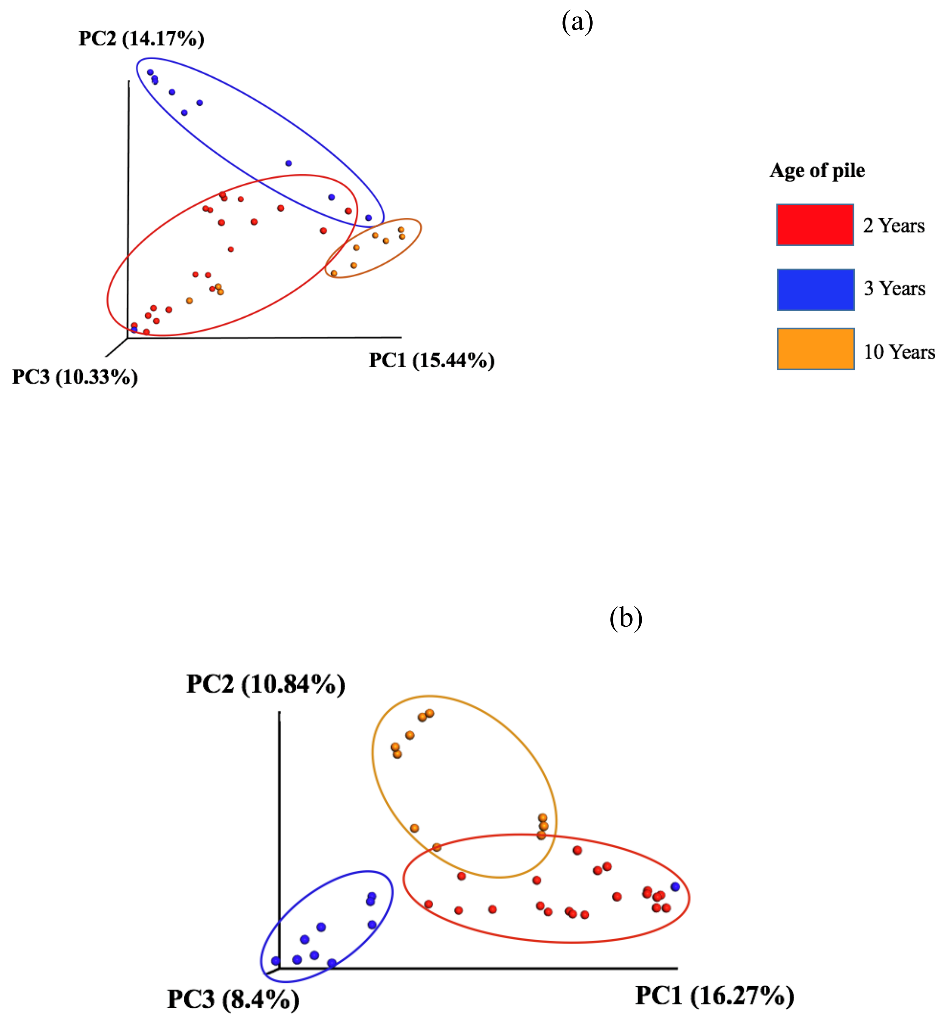


Figure 4.4a. Principal Coordinate Analysis (PCoA) of microbial communities based on a) fungal ITS and b) bacterial 16S rRNA Bray-Curtis dissimilarity ecological distances. Each point represents different age of piles and the colors indicate the different sampling niches.

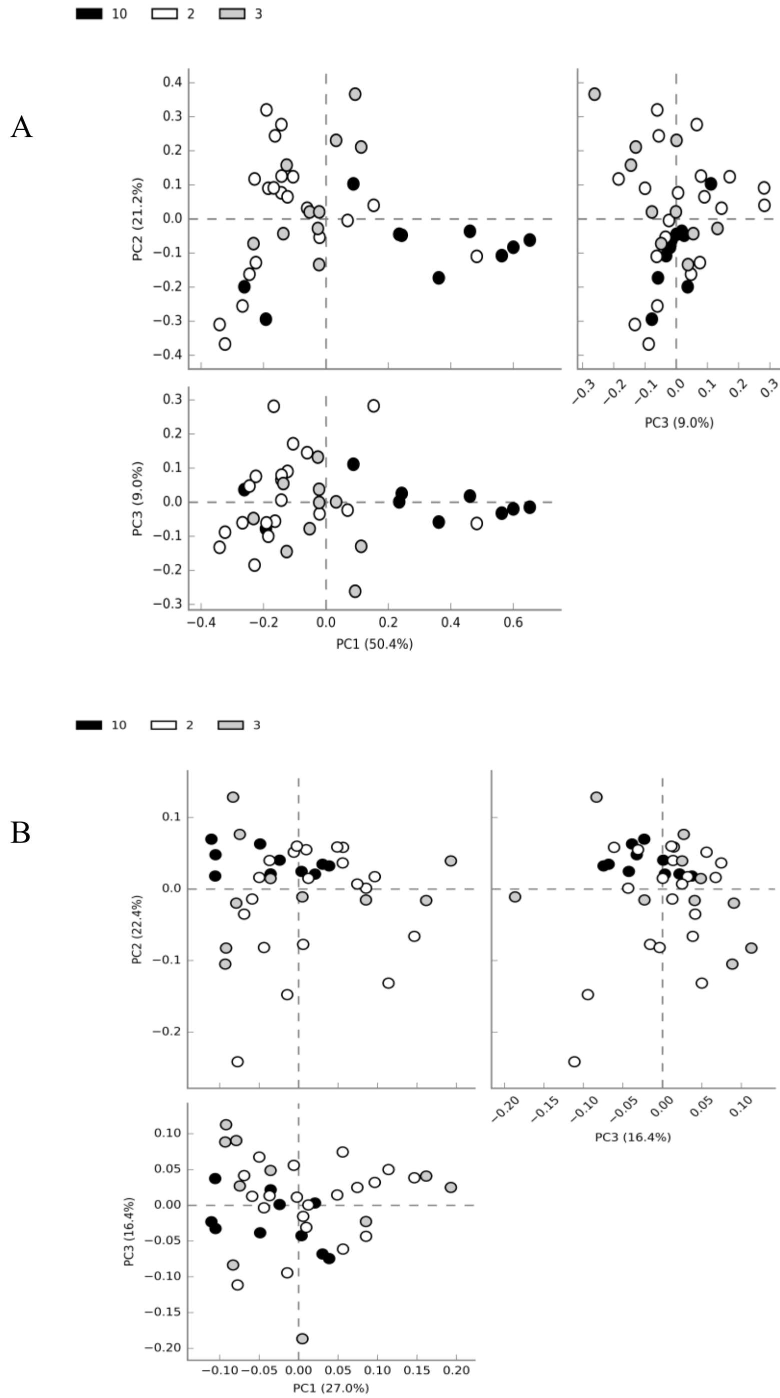


Figure 4.5b. Principal coordinate analysis (PCoA) of microbial communities: a) bacterial and b) fungi based on Bray-Curtis dissimilarity ecological distances for location. Each point represents different age of piles and the colors indicate the different sampling niches.

The data points in the PCoA plot represent the different replicated samples per location (Figure 4.3a, 4.3b, 4.4a and 4.4b). The differences within compost facilities (locations) were determined by the distance between data set clusters on the PCoA plot (Figure 4.3a and 4.3b) while the differences in age of compost pile were determined by distance between data set clusters on the PCoA plot (Figure 4.4a and 4.4b). However, Figure 4.3a and 4.4a revealed some distinct visual variations compare to figure 4.3b and 4.4b as each color and ring indicate the different sampling niches. The ITS sequencing data analysis could not produce a phytogenic tree file, a tool that is available in UniFrac dissimilarity analysis for 16S rRNA data. Therefore, the ecological dissimilarity analysis was carried out using Bray-Curtis ecological distance for both 16S rRNA and ITS analysis. This allowed a direct comparison of ecological distances between bacterial and fungal communities. The command (Appendix 5) was used to build Bray-Curtis dissimilarity ecological distance based on diversity analysis for 16S rRNA and ITS sequencing data. The Bray-Curtis ecological distance analysis revealed that variations in microbial (bacteria and fungi) populations were influenced by locations (i.e., different compost facilities) (Figure 4.3a and 4.3b) and age of the pile (Figure 4.4a and 4.4b) not by the association with plastics (sample type i.e., bulk compost and partially decompose plastic). There were no visual variations observed in both bacterial and fungal communities across sample types (bulk versus plastic associated compost, data not shown).

The analysis of strength and statistical significance of sample groupings referred to as Adonis test (Table 4.1) was carried out as described in Appendices 6 and 7. Adonis test based on Bray-Curtis beta-diversity distances indicated that grouping by location was significant for bacterial ($R^2 = 0.26$ $P < 0.001$) and fungal ($R^2 = 0.27$, $P < 0.001$) microbial communities (Table 4.1). Grouping by age of the piles also showed a significant effect for bacterial ($R^2 = 0.11$, $P < 0.001$) and fungal ($R^2 =$

0.09, $P < 0.001$; Figure 4.3 and 4.4; Table 4.1) communities. However, grouping by sample type was not significant for bacterial ($R^2 = 0.03$, $P > 0.05$) and fungal ($R^2 = 0.03$, $P > 0.05$) populations (Table 4.1). Also, a significant ($P < 0.05$) effect was observed in grouping of bacterial communities by location and age of pile, but not by sample type. Similar effect of grouping by age and location was detected by Adonis test based on Weighted UniFrac beta-diversity distances (Table 4.1).

Table 4. 1 Variations in sample groupings as explained by weighted UniFrac and Bray-Curtis beta-diversity.

	16S rRNA		Fungal ITS
Grouping (subset) [a]	Weighted UniFrac (R^2)	Bray–Curtis (R^2)	Bray–Curtis (R^2)
Location	0.26***	0.26***	0.28***
Age of pile	0.086**	0.11***	0.09***
Sample type [b]	0.03, P > 0.19	0.03, P > 0.41	0.03, P > 0.30
Sample type (Guysborough) [c]	0.03, P > 0.18	0.02, P > 0.39	0.03, P > 0.30
Sample type (Northridge)	0.03, P > 0.19	0.03, P > 0.41	0.03, P > 0.31
Sample type (Balefill)	0.03, P > 0.17	0.03, P > 0.43	0.03, P > 0.19
Sample type (Fundy)	0.03, P > 0.18	0.03, P > 0.43	0.03, P > 0.33

[a] Weighted UniFrac and Bray-Curtis beta-diversity distances were calculated for each subset of samples. Adonis tests were used to assess whether beta-diversity is related to sample groupings, 999 permutations, R^2, *P < 0.05; **P < 0.01, and ***P < 0.001.

[b] Bulk compost vs. plastic-associated microbial communities from all locations combined.

[c] Bulk compost vs. plastic-associated microbial communities from individual locations.

Adonis test was also performed to check whether there was a significant difference between the samples type with each location. The command lines for this analysis it shown in Appendix 8 (Table 4.1). The data showed that grouping by sample type was not significant (P>0.05).

4.6. Differences in Microbial Structure Across Compost Facilities (Locations)

There were visual differences in relative abundances of several bacterial and fungal taxa detected within different compost locations (Figures 4.5a and 4.5b).

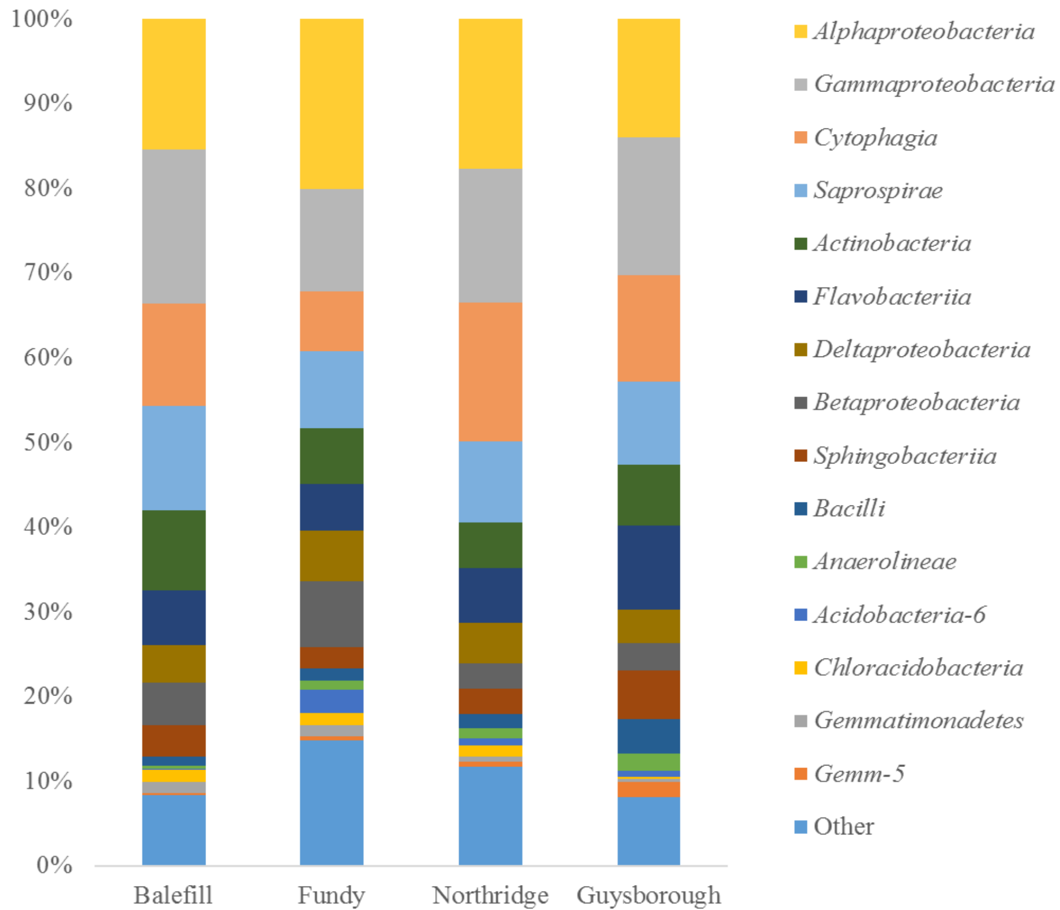


Figure 4.5a. Relative abundances of bacteria taxa identified in the study following analysis of bulk compost samples from the four different locations.

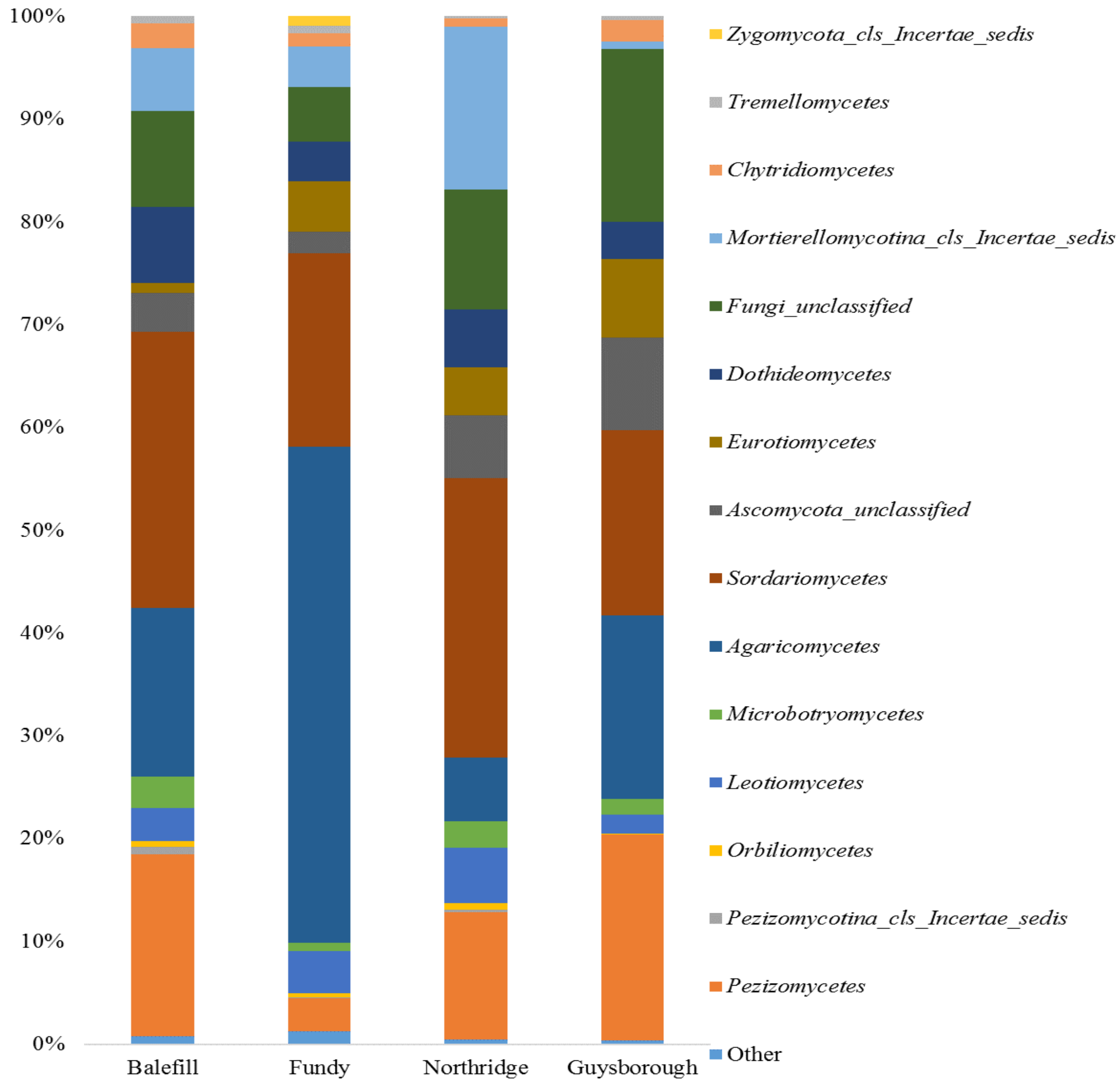


Figure 4.5b. Relative abundances of fungi taxa identified in the study following analysis of bulk compost samples from the four different locations.

The differences between the structures of microbial communities from each location were further analysed using statistical analysis of metagenomics profiles (STAMP) (Figures 4.6 and 4.7). The results showed that 6 out of 15 of the most abundant bacterial classes i.e., *Alpha- and Beta-proteobacteeria, Chloracidobacteria, Cytophagia, Gemmatimonadetes* and *Spingobacteria,* were significantly ($P<0.01$) different in their relative abundances between some of the compost facilities

(Figure 4.6). A higher abundance of *Betaproteobacteria* was detected in Fundy compost facility compared to Northridge, Guysborough and Balefill compost facilities, and relatively less *Chloracidobacteria* was detected in composts from the Guysborough facility compared to the Fundy, Northridge and Balefill compost facilities (Figure 4.6). The abundance of *Alphaproteobacteria* was also higher in Fundy compost facility compared to Guysborough and Balefill compost facilities. Additionally, the relative abundance of *Gemmatimonadetes* was higher in Fundy and Balefill compost facilities, compared to Northridge and Guysborough compost facilities (Figure 4.6). Also, the abundance of *Sphingobacteria* was higher in Guysborough composting facility compare to Fundy and Northridge facilities (Figure 4.6). However, the Northridge composting facility had relative abundance of *Cytophagia* compared to the Fundy composting site (Figure 4.6).

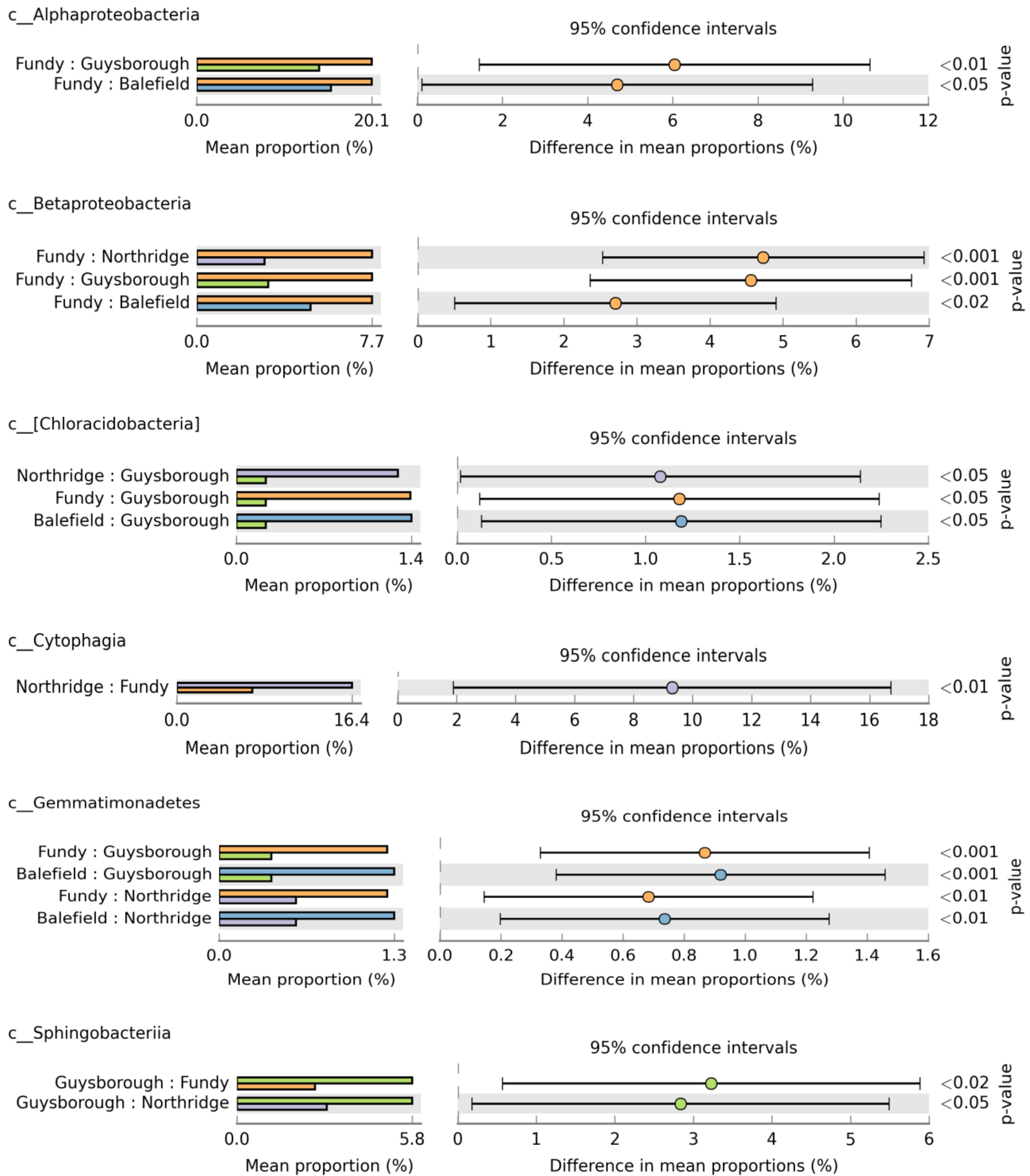


Figure 4.6. Bacterial taxa that were significantly overrepresented in comparison between composting facilities. Only the 15 most abundant bacterial groups were considered. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's *t*-test) *q* value <0.01 were considered significant and were thus retained.

On the other hand, the relative abundances of only 2 out of 15 abundant fungal classes i.e., *Agaricomycetes* and *Mortierellomycotina cls Incertae sedi* differed between compost facilities (Figure 4.7). The relative abundance of *Agaricomycetes* was significantly (P<0.01) higher in the compost collected from Fundy compost facility compared to the other locations (compost facilities), while the Northridge compost facility had relatively more *Mortierellomycotina cls Incertae sedi* compared to Guysborough and Balefill (Figure 4.7).

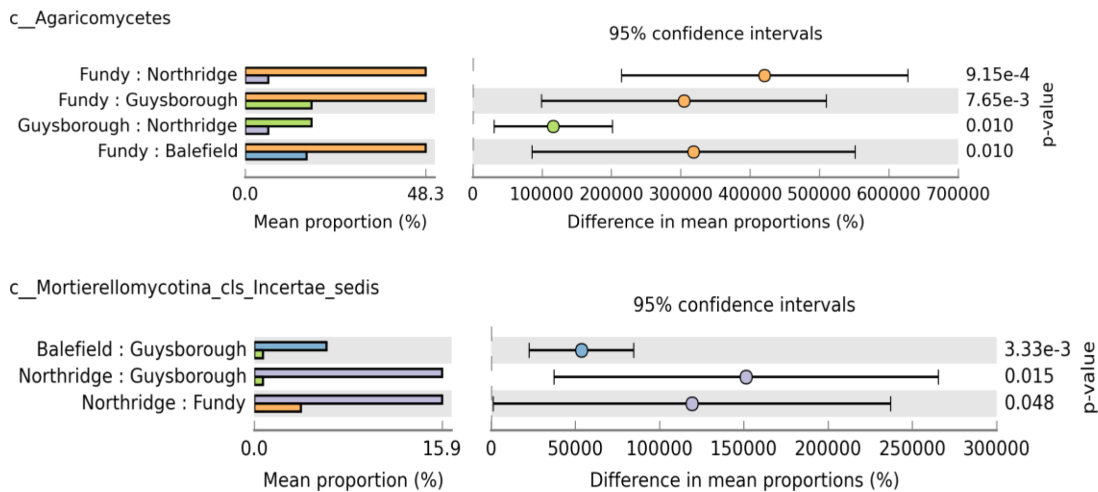
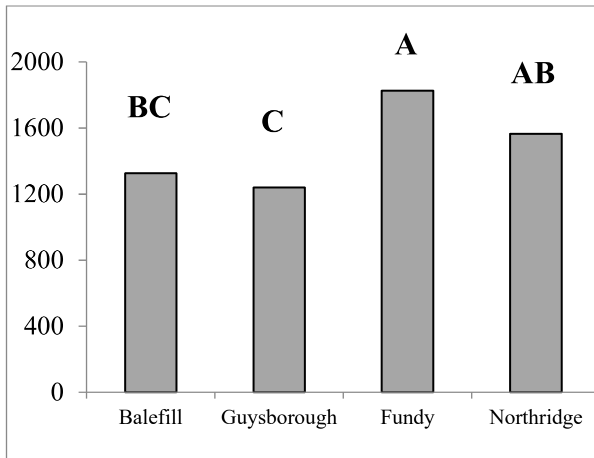


Figure 4.7. Fungal taxa that were significantly overrepresented in comparison between composting facilities. Only the 15 most abundant fungi groups were considered. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's *t*-test) *q* value <0.01 were considered significant and were thus retained.

4.7. Microbial Alpha–Diversity

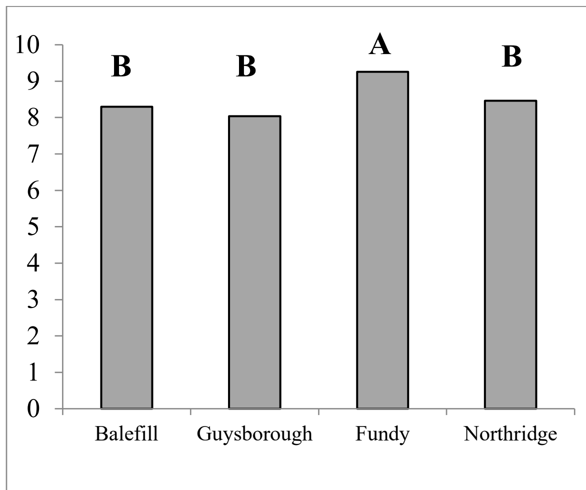
Bacterial alpha-diversity was also affected by location. Bacterial Chao1 richness, Shannon diversity and Simpson evenness were significantly (P<0.05) higher at the Fundy compost site compared to the Northridge, Balefill and Guysborough (Figure 4.8; panels on left column). On the other hand, there were no significant (P>0.05) differences in fungal communities' Chao1 richness,

Shannon diversity and Simpson evenness across all the locations (Figure 4.8; panels on right column). However, there was a consistent trend of small reductions in fungal communities from Balefill > Fundy > Northridge > Guysborough.

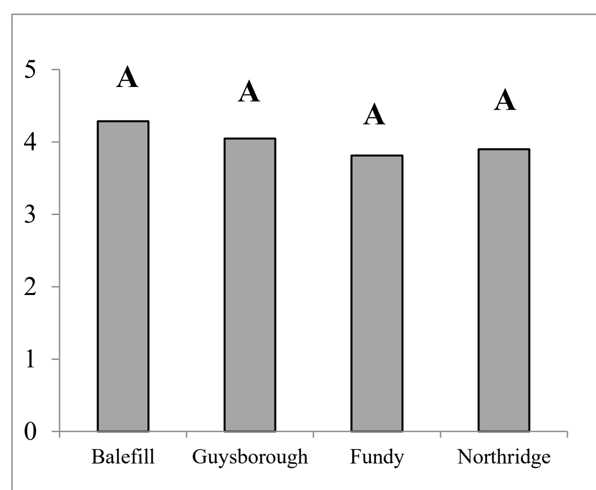


Chao1 richness, 16S rRNA

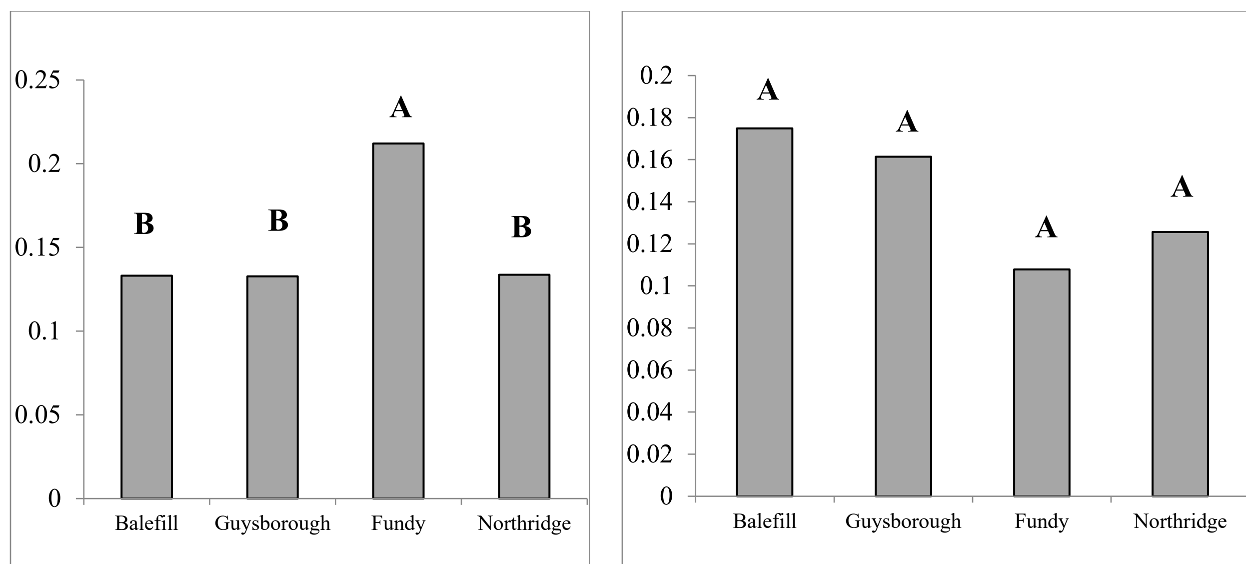
Chao1 richness, Fungal ITS



Shannon diversity, 16SrRNA



Shannon diversity, fungal ITS



Simpson evenness, 16SrRNA **Simpson evenness, Fungal, ITS**

Figure 4.8. Estimated total species richness (Chao1), relative abundance (Shannon) and evenness (Simpson evenness). For each variable, data followed by different letters are significantly ($P < 0.05$) different according to Turkey's pairwise test. N = 40

4.8. Compost Chemical Characteristics

Compost chemical analyses were conducted to provide information about the chemical properties of the various materials used in this study and how these chemical characteristics might affect the microbial structure and diversity. Data from mineral nutrients analyses indicated that bacterial and fungi communities differed in their structures within facilities, which might be linked to differences in compost chemical properties (Table 4.2). Balefill and Fundy facilities were significantly ($P<0.05$) lower in pH compared to Northridge and Guysborough. Also, significantly higher nitrogen and percentage carbon was recorded at Guysborough compare to the other compost facilities. Northridge, Guysborough and Balefill had significantly higher amount of phosphorus compared to Fundy compost. There was no significant difference in C/N in compost from all the

four compost facilities, but Fundy had the highest value. However, Northridge had higher amount of phosphorus. Aluminium and iron were significantly higher in Fundy compost facility compared to the other facilities. Cation exchange capacity (CEC) was significantly higher in Northridge compared to the other compost facilities (Table 4.2).

Table 4. 2. Mean compost chemical contents from the four compost facilities.

Soil parameters	Compost facilities			
	Northridge	Guysborough	Balefill	Fundy
*Nitrogen (%)	1.78^{B}	3.46^{A}	1.52^{B}	1.64^{B}
pH (pH Units)	7.68^{A}	7.42^{AB}	6.94^{B}	7.13B^{B}
*Carbon (%)	18.02^{B}	26.20^{A}	15.87^{B}	19.66^{B}
*C/N	8.53^{A}	7.62^{A}	8.95^{A}	9.59^{A}
P_2O_5 (kg/ha)	3050^{A}	2557^{AB}	3231^{A}	2143^{B}
K_2O (kg/ha)	2831^{AB}	4439^{A}	2378^{AB}	2519^{B}
Calcium (kg/ha)	16431^{A}	11179^{B}	10884^{B}	8180^{C}
Magnesium (kg/ha)	1408.5^{A}	1111.2^{BC}	1386^{AB}	936.4^{C}
Sodium (kg/ha)	1238^{A}	1848^{A}	479^{A}	851^{A}
Sulfur (kg/ha)	143^{B}	176.2^{B}	420.3^{A}	165.4^{B}
Aluminum (ppm)	66.8^{B}	82.2^{B}	109^{B}	222.0^{A}
Boron (ppm)	3.38^{A}	3.05^{AB}	2.29^{B}	2.59^{AB}
Copper (ppm)	2.49^{A}	1.68^{A}	2.23^{A}	2.49^{A}
Iron (ppm)	173.5^{BC}	150.0^{C}	229.7^{AB}	253.6^{A}
Manganese (ppm)	59.5^{B}	47.6^{B}	155^{A}	44.2^{B}
Zinc (ppm)	24.73^{A}	36.84^{A}	41.21^{A}	27.36^{A}
CEC[a] (meq/100 g)	52.7^{A}	41.1^{B}	33.4^{BC}	29.42^{C}

Mean in columns followed by the same letter are not significantly different according to Tukey's test ($P < 0.05$). Uppercase letters indicate significant differences between each parameter.

[a] Cation exchange capacity

* were analyzed using VarioMAX CN Elementar Americas. N = 40

4.9. Correlation between Bacterial and Fungal Alpha-Diversity and Chemical Compost Factors

Spearman's rank correlation analysis was used to explore the relationship between compost chemical factors and microbial alpha-diversity. Fungi richness was negatively correlated with nitrogen and aluminium, while diversity was correlated only with carbon. However, bacteria richness was strongly and positively correlated with aluminium, while it was moderately and positively correlated with iron. Bacterial diversity was negatively correlated with phosphorus, calcium and cation exchange capacity while it was strongly and positively correlated with aluminium and iron. Bacterial evenness was negatively correlated with phosphorus, calcium, magnesium and manganese. In addition, bacteria evenness was moderately and positively correlated with C/N (Tables 4.3).

Table 4.3. Spearman's rank correlation analysis showing the association between soil and chemical factors, bacterial and fungal alpha-diversity.

	Chao1 richness		Shannon diversity		Simpson evenness	
Microbial type	16S	ITS	16S	ITS	16S	ITS
pH	-0.23	-0.18	-0.30	0.11	0.14	0.08
Nitrogen	-0.39	**-0.50***	-0.32	-0.80	-0.32	-0.4
[a]Carbon	-0.20	-0.2	-0.20	**-0.48***	0.60	-0.20
C/N	0.80	0.80	0.80	-0.60	**0.51***	0.00
P_2O_5	-0.186	-0.07	**-0.473***	-0.11	**-0.745*****	-0.01
K_2O	-0.54	-0.50	-0.33	-0.37	0.12	0.10
Calcium	-0.22	-0.04	**-0.53***	-0.19	**-0.63****	-0.03
Magnesium	-0.10	0.16	-0.35	0.16	**-0.61****	0.06
Sodium	-0.44	-0.36	-0.31	-0.30	0.14	0.04
Sulfur	-0.44	**-0.47***	-0.33	-0.20	-0.38	0.10
Aluminum	**0.76*****	**0.51***	**0.77*****	0.38	0.36	-0.12
Boron	-0.20	-0.10	0.16	-0.17	0.05	0.05
Copper	0.37	0.42	0.12	0.35	-0.18	0.03
Iron	**0.55****	0.14	**0.63****	0.07	0.16	-0.17
Manganese	-0.32	0.05	-0.43	0.41	**-0.53***	0.27
Zinc	0.17	0.26	0.00	0.49	-0.37	0.27
[b]CEC	-0.26	-0.22	**-0.52***	-0.34	-0.45	-0.16

Significance levels are shown at ***p<0.05, **p<0.01, and ***p<0.001**. [a]Carbon-nitrogen ratio [b]Cation-exchange capacity. N = 40

CHAPTER 5. DISCUSSION

5.1. Compost Microbial Community

Compost is well-known to be a microcosm of large diverse populations of microorganisms most of which help in the decomposition of organic and inorganic materials (Koschinsky et al., 1999; Antunes et al., 2016; Friend and Smith, 2017). These microorganisms, their co-existence and the ways they interact during various stages of organic matter degradation helps to understand the roles played by different microorganisms during the composting process (Ryckeboer et al., 2003).

This study was carried out to assess the diversity and structure of microbial (bacterial and fungal) communities in municipally derived organics composts contaminated with plastics. We evaluated the effect of the presence of plastics on the structure of microbial community. Our initial hypothesis was that the presence of plastics within the compost samples would have a significant effect on the diversity and structure of microbial communities across four compost facilities in Nova Scotia, Canada. Also, it has been previously reported that environmental niches such as biochars or plant rhizosphere exert selective pressure and species sorting effects that influence microbial community structure and the enrichment of individual microbial taxa (Morrison-Whittle and Goddard 2015; Noyce et al. 2016; Yurgel et al*.,* 2017; Yurgel et al*.,* 2018). However, the results of the present study showed that there were no significant effects on the microbial diversity and structure in compost contaminated with plastics across all the compost facilities. The microbial abundance reported in the present study is only relative and not absolute, which is a major setback for most 16S and fungal 18S/ITS microbiome studies (Yurgel et al., 2017; 2018).

5.2. Bacterial and Fungal Classes Identified in the Study

Previous studies revealed that bacterial communities are the most abundant microbial population in compost (Rawat, 2004; Rawat and Johri, 2013; Antunes et al., 2016). Bacteria are present throughout the composting process and account for approximately 90% of all the microorganisms in compost. Further study (Gupta, 2000; Pereira et al., 2006) confirmed that phylum *Proteobacteria* was the dominant and the most diverse phylum among prokaryotes found in forest soils and compost. It is not a surprise that *Proteobacteria* are reported to be abundant throughout the composting process phases, with various classes being enhanced at each composting stage. This include *Gammaproteobacteria* in the initial stage (mesophilic phase), *Betaproteobacteria* at the thermophilic phase, and *Alphaproteobacteria* during maturation (Partanen et al., 2010; Neher et al., 2013; Huhu et al., 2017). Our study also revealed that *Proteobacteria* was the most abundant phylum recorded at all the four separate compost facilities. This is consistent with the findings reported in the literature where *Proteobacteria* was reported to be abundant in the deep terrestrial biosphere Itavaara et al. (2016) and in forest soil Pereira et al. (2006).

In addition, we found that two other bacterial phyla, *Bacteroidetes* and *Actinobacteria,* were dominant across all four composting facilities evaluated in the present study. These bacterial taxa were previously reported to be dominant in a number of environmental niches including soils (Yamamoto et al., 2009; Yurgel et al., 2017), plant rhizosphere (Fierer et al*.,* 2009; Shi et al*.,* 2015; Foulon et al*.,* 2016), various stages of composting (Neher et al., 2013; Blomström et al., 2016), and vermicompost (Bulgarelli et al*.,* 2013). Also, *Actinobacteria* are among the most important litter decomposers in soils, and this have been confirmed in a wide variety of environments as well (Niva et al., 2006; Kopecky et al., 2011).

In addition to bacteria, fungi are an important part of the soil microbial communities. Using several molecular techniques and conventional methods, it was shown that fungi are the most dominant group of the eukaryotes in several ecosystems (Abed et al., 2013; Peay et al., 2013; Acosta-Martínez et al., 2014; Al-Sadi et al., 2015; Van Geel et al., 2015; Kazeeroni and Al-Sadi, 2016). *Ascomycota* and *Basidiomycota* were reported to be the dominant phyla in soils (Lim et al., 2010; Orgiazzi et al., 2012; Wubet et al., 2012). These phyla were found to be dominant in compost obtained from all the compost facilities evaluated in this study. Similar to the present findings, several researchers also reported that *Ascomycota* was the largest and widespread phylum of fungi in compost including compost obtained from cattle manure (Neher et al., 2013; Kazeeroni and Al-Sadi, 2016; Huhe et al., 2017) as well as in soils (Noyce et al., 2016; Zhou et al., 2017; Yurgel et al., 2017).

5.3. Link between Compost Facilities (Location) and Microbial Communities Structure

In the present study, we used Adonis test, PCoA, alpha-, and beta- diversity and STAMP analyses to show that there were differences in the microbial diversity and structure across all the compost facilities. These differences in microbial communities' structure can be influenced by a number of abiotic and biotic environmental factors. For example, the diversity and structure of microbial communities in compost can be affected by compost pile age, compost recipe (feedstock), composting methods and environmental factors (Neher et al., 2014; Tarlera et al., 2008).

5.4. Effect of Feedstock on Microbial Communities' Structure

Compost recipes or feedstock is an important ingredient that helps to organize and influence microbial communities in compost (Neher et al., 2013). Feedstock varies from one compost facility

to the other. They are mainly sources of carbon and nitrogen for microorganism's energy and growth. For example, carbon sources include straw, paper, woodchips and tree bark, while nitrogen sources include manure, sewage and municipal solid waste. Usually the types of microbial communities found in compost were determined by the feedstock or source material (Neher et al., 2013). For instance, the phylum *Ascomycota* (classes - *Sordariomycetes* and *Agaricomycetes*) that is known for wood decomposition was reported to be commonly associated with hardwood in compost (Neher et al., 2013). Thus, compost with wood chips or straw as the bulking and carbon source may have high content of *Ascomycota* (Rubino and McCarthy, 2003; Ma et al., 2013; Kahl et al., 2015; Kameroni et al., 2017; Yuan et al., 2017). Previous studies confirmed that the bacteria *Proteobacteria, Bacteroidetes* and *Actinobacteria* comprise a number of classes believed to be responsible for various biodegradation activities (e.g., wood chips and shaving decomposition) in compost for instance bacteria from forest topsoil - *Actinobacteria* (Lopez-Mondejar et al., 2016), *Actinobacteria* and Proteobacteria (Zhou et al., 2017).

Generally, forests are known to be global carbon pool because large amount of this global carbon are stored by trees and in the forest floor. The microorganisms within forest zones are responsible for the decomposition of the leaves and plant deposit in the forest floor and thereby, help to achieve soil carbon balance (Austrian Science Fund FWF, 2017). *Ascomycota* and *Basidiomycota* are considered to be very important decomposers of hays, straws and wood in the forest floors and during composting processes (Rubino and McCarthy, 2003; Ma et al., 2013; Kahl et al., 2015; Kameroni et al., 2017; Yuan et al., 2017). These microorganisms were observed within our sampled sites causing similar activities, particularly, in compost from Fundy compost facility, as their abundance were recorded at this site. Moreover, *Proteobacteria, Bacteroidetes* and

Actinobacteria have been previously reported within several forest floors aiding the decomposition of leaves and plant materials (Lopez-Mondejar et al., 2016; Zhou et al.; 2017).

The findings confirmed that *Basidiomycota Agaricomycetes* and *Ascomycota Sordariomycetes* were the dominant classes of fungi across all the four compost facilities studied. This observation was in accordance with De Gannes et al. (2013) who reported similar finding where *Sordariomycetes* was a major class observed at various composting phases during decomposition of different organic materials. Also, Neher et al. (2013) reported that *Sordariomycetes* and *Agaricomycetes* were associated with hardwood degradation in compost. They have the potential to degrade lignin and cellulose in wood and other plant parts during natural decomposition (Zhang et al., 2006; Santiago-Rodriguez *et al*., 2013; Yuan et al., 2013; 2017; Huhe et al., 2017). These findings may be the reasons for the abundance of these classes of fungi across all the compost facilities studied.

One customary practice by all compost facilities evaluated in this study was the addition of plant sources such as wood chip, hay and straw, especially, during the winter season. These materials are used as carbon source during winter to enhance the decomposition rate and to balance the C/N. Of the four composting facilities studied, Fundy compost facility tends to be the only facility that have a high quantity of wood chips and wood shavings in their piles all year-round. The use of wood chips by Fundy compost facility as one of their major sources of feedstock can help to explain the probable reason(s) why higher numbers of the fungi class *Agaricomycetes* were observed in their compost compared to composts from the other facilities.

Interestingly, the compost from Fundy compost facility are different from that of the compost in other facilities. This might be because of the high amount of wood chips used as one of their major feedstocks. This may be linked to the diversity observed in the compost mix from Fundy facility. By all the ecological indices measured, the bacterial community composition, diversity and richness was significantly higher in compost from Fundy compost facility than composts from the other facilities. This further support the findings that bacteria account for approximately 80 – 90% of all the microorganisms in compost (Rawat, 2004; Rawat and Johri, 2013; Antunes et al., 2016).

5.5. Compost Chemical Composition

The chemical characteristics of bulk compost were analyzed to determine how different chemicals and soil factors can influence microbial richness, diversity and evenness at each compost facility. The comparison allowed us to evaluate the relationship between alpha-diversity and bulk compost chemical composition. Correlation analysis between alpha-diversity and chemical compost factors revealed a correlation with some compost chemical parameters i.e., (aluminium and iron) on bacterial composition and structure compared to those of fungal communities (Table 4.4). These data agree with previous works. For example, Lentendu et al. (2014) reported changes in soil chemical composition caused a shift in the composition of eukaryotic microbial communities in the soil. Liu et al. (2014) also reported that the relative abundances of all of the major phyla of bacterial (e.g., Actinobacteria) were influenced by pH and Fe^{3+} concentrations at this tailing site. On the other hand, compost associated microorganisms are actively involved in the breakdown of some and cellulose within forest ecozones, which helps to maintain a C/N ratio of 25-30:1 in compost piles (Lopez-Mondejar et al., 2016; Huhe et al., 2017). C/N ratio is an important compost feedstock and finished compost quality indicator. Although there was no significant difference in

composts C/N from all the facilities. compost from the Fundy facility was higher in C/N compared to the other facilities. This may be due to the amount of wood chips (feedstock), which helps to maintain C/N within acceptable limit in the compost pile. Any change in the available carbon or volatilization of ammonia (the major source of nitrogen) during the composting process can adversely alter the diversity and structure of the microbial community (Boulter et al., 2000).

5.6. Effect of Time on Microbial Communities' Structure

Age of compost piles is a key factor that can significantly affect compost microbial communities (Tarlera et al., 2008). The younger the compost pile, the lower the microbial population; and the older the compost pile, the more abundant the microbial population (Li et al., 2018). This was obvious in the present study where we recorded high relative abundance of microorganisms at Fundy compost facility, which happened to be the oldest site sampled (i.e., between 8 and 10 years). A similar finding was reported for soil by Tarlera et al. (2008) and compost by Li et al. (2018).

5.7. Effect of Plastics on Microbial Diversity and Structure

Our analyses identified in these samples that the presence of plastics in compost did not have any effect on microbial composition and structure in compost from all the studied facilities (Figures 4.3 & 4.4). It was initially hypothesized that the presence of plastics within the compost samples could have a significant effect on the microbial communities. However, after the analysis it was confirmed that there was no significant difference between the microbial communities associated with bulked compost and compost contaminated with plastics (Table 4.2). Therefore, the presence of plastics in the compost pile may serve as an alternative source of carbon needed by

microorganisms to maintain the required C/N ratio balance within the compost mix in the absence of other carbon sources, such as wood chips/shavings. As a matter of fact, the ability of microorganisms to use plastics as a carbon source has been established (Russell et al., 2011; Odusanya et al., 2013). Furthermore, it has been established that during composting the diversity and structure of microbial communities changed dramatically (Yamamoto et al., 2009). This may encourage microorganisms to be able to utilize plastics as a substrate (Rawat and Johri, 2013; Franke-Whittle et al., 2014). This form part of the reasons for the superior abundance of microorganisms (bacterial and fungi) in compost sampled from the Fundy compost facility.

CHAPTER 6. CONCLUSION AND RECOMMENDATIONS

6.1. Overview of Problem and Research Objectives

Plastics contamination in compost is a major problem for both the compost industries and users of compost in Nova Scotia and in Canada; and most places in the world. Previous researches on biodegradation of plastics reported that microorganisms have the potential to degrade plastics. However, native microbial communities can vary in both abiotic (e.g., light, temperature) and biotic (e.g., decomposer) factors and from region to region. This research sought to investigate the microbial community and structure in municipal organic composts from four compost facilities in Nova Scotia using next generation sequencing (NGS). This will be the first research work using NGS to explore native microbial community that have potential to decompose plastics in municipal solid waste compost. We hypothesized that age of compost and pile environmental conditions (i.e., location) and presence of plastic can affect the diversity and structure of native microbial community in compost. The presence of plastics in compost was confirmed to have no significant effect on the microbial community and structure among compost facilities.

Also, the software package used for analyzes of taxonomic or metabolic profiles (STAMP) identified microorganisms up to class level but none was identified below class level. However, the classes of bacterial and fungi identified from this study was in accordance with the previous findings in the area of biodegradation of plastics (Table 2.1). Thus, the research met the objectives of the project. However, it can be concluded that the microbial variations recorded in this study may be due to the source of feedstock, age of pile, and method of processing.

6.2 Recommendations for Future Research

The study provided insights on microbial structure and diversity found within compost contaminated with plastics, which may have the capacity to degrade plastics in compost. However, there are several underlining limitations, which can be looked into for future research. These include the following:

1. The need to isolate the identified bacteria and fungi in the laboratory with plastics modified media.
2. The need to investigate the type of plastics and the phase of composting at which the identified microorganisms degrade plastics.
3. Experiment to investigate the reason(s) for the variation in the microbial community.

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APPENDIX 1

Microbiome Helper Standard Operating procedure for 16S bacteria and archaea **pipeline v1** as described by Comeau et a*l*., 2017 used for the study with little modifications.

1. Run FastQC to allow manual inspection of the quality of sequences

```
mkdir fastqc_out
fastqc -t 4 raw_data_new/* -o fastqc_out/
```

2. Stich paired end reads together (summary of stitching results are written to "pear_summary_log.txt")

```
run_pear.pl -p 4 -o stitched_reads 16S_raw_data/*
```

3. To filter stitched reads by quality score, length and ensure forward and reverse primers match each read (summary written to "readFilter_log.txt" by default). (Note: 255 samples will run for approximately 2 hours but less samples will spend less time)

```
read_filter.pl -q 30 -p 90 -l 400 --thread 20 stitched_reads/*.assembled. *
```

4. Convert FASTQ stitched files to FASTA AND remove any sequences that have an 'N' in them.

```
run_fastq_to_fasta.pl -p 20 -o fasta_files filtered_reads/*fastq
```

5. Remove chimeric sequences with UCHIME (summary written to "chimeraFilter_log.txt" by default). (Note: 255 samples will run for approximately 2 hours but less samples will spend less time)

```
chimera_filter.pl -type 1 -db /home/shared/rRNA_db/Bacteria_RDP_trainset15_092015.fa fasta_files/*
```

6. Create a QIIME "map.txt" file with the first column containing the sample names and another column called "FileInput" containing the filenames. This is a tab-delimited file and there must be columns named "BarcodeSequence" and "LinkerPrimerSequence" that are empty. This file can then contain other columns to group samples which will be used when Fig.ures are created later.

```
create_qiime_map.pl non_chimeras/* > map.txt
```

7. Combine files into single QIIME "seqs.fna" file (This step will take approximately 5 minutes to run).

```
add_qiime_labels.py -i non_chimeras/ -m map.txt -c FileInput -o combined_fasta
```

8. Create OTU picking parameter file.

```
echo "pick_otus:threads 4" >> clustering_params.txt
echo "pick_otus:sortmerna_coverage 0.8" >> clustering_params.txt
```

9. Run the entire qiime open reference picking pipeline with the new sortmerna (for reference picking) and sumaclust (for de novo OTU picking). This does reference picking first, then subsamples failure sequences, de-novo OTU picks failures, ref picks against de novo OTUs, and de-novo picks again any left-over failures. Note: You may want to change the subsampling percentage to a higher amount from the default -s 0.001 to -s 0.01 (e.g 1% of the failures) or -s 0.1 (e.g., 10% of the failures).

   Note: this step can take approximately 24 hours, to save time and multi-task, you can type these lines to run your jobs:

   screen "job" _screen -d (where "job" is whatever command you were using before and -d option means "detach" you can re-attach when you want to check on your job with "screen -r" This will run in the background. To check if it is running type "jobs" or "top -cu (name of your cu)5")

   *Note: Also, you can change the number of threads to 20 at a step within the pipeline where it is more than 20 it will run a lot faste*r

```
pick_open_reference_otus.py -i $PWD/combined_fasta/combined_seqs.fna -o $PWD/clustering/ -p
$PWD/clustering_params.txt -m sortmerna_sumaclust -s 0.1 -v --min_otu_size 1
```

10. Filter OTU table to remove singletons as well as low-confidence OTUs that are likely due to MiSeq bleed-through between runs (reported by Illumina to be 0.1% of reads).

```
remove_low_confidence_otus.py -i $PWD/clustering/otu_table_mc1_w_tax_no_pynast_failures.biom -o
$PWD/clustering/otu_table_high_conf.biom
```

11. Summarize OTU table to determine number of sequences per sample.

```
biom summarize-table -i clustering/otu_table_high_conf.biom -o clustering/otu_table_high_conf_summary.txt
```

12. Normalize OTU table to same sample depth (the minimum sample counts from biom table) - For example, if the lowest depth gotten from the summary table from step (11) is 5545 then change "X''to 5545 in the below command to match the read count of the sample with the lowest (acceptable) number of reads. Note: Don't like the idea of throwing away all that data? You may want to consider trying different normalization methods such as DESeq2 (see below).

```
mkdir final_otu_tables
single_rarefaction.py -i clustering/otu_table_high_conf.biom -o final_otu_tables/otu_table.biom -d X
```

13. Manually add column(s) to map.txt that contain information to group your samples (e.g., healthy vs disease).

14. Create UniFrac beta diversity plots.

```
beta_diversity_through_plots.py -m map_edited.txt -t clustering/rep_set.tre -i final_otu_tables/otu_table.biom -o
plots/bdiv_otu
```

15. Create alpha diversity rarefaction plot (values min and max rare depth as well as number of steps should be based on the number of sequences within your OTU table).

```
alpha_rarefaction.py -i final_otu_tables/otu_table.biom -o plots/alpha_rarefaction_plot -t clustering/rep_set.tre -m map_edited.txt --min_rare_depth 500 --max_rare_depth 5545 --num_steps 10
```

16. Convert BIOM OTU table to tab-separated file to be opened/explored in text editors or Excel, etc.

```
biom convert -i final_otu_tables/otu_table.biom -o final_otu_tables/otu_table_w_tax.txt --to-tsv --header-key taxonomy
```

17. Convert BIOM OTU table to STAMP.

```
biom_to_stamp.py -m taxonomy final_otu_tables/otu_table.biom >final_otu_tables/otu_table.spf
```

18. The following command can be used to creates stacked bar charts at all taxonomic levels (i.e., kingdom, phylum, class, order, family, genius and species) for each sample individually. You can open the file either with ".html" files within plots/taxa_summary/taxa_summary_plots to see the results. or load it as excel file "*.txt" files in "plots/taxa_summary". To load the output as excel file the command is as follows:

```
summarize_taxa_through_plots.py -i final_otu_tables/otu_table.biom -o plots/taxa_summary
```

19. If you want the stacked bar charts to represent collapsed groupings of samples then you can use the following command and you would change the " Discription_level_3" to whatever the column name is in your map file that you want to collapse by. ***NOTE:*** *The command can be used to creates stacked bar charts at all taxonomic levels for each category of data or samples, for instance we can create a stacked bar for Samples (e.g Fundy 1, Fundy 2 etc.), Location (Fundy, Northridge, Balefill and Guysborouboro), Location_Sample_type (Fundy_compost, Fundy_plastics, Northridge_compost, Northridge_compost etc.)*

```
summarize_taxa_through_plots.py -i final_otu_tables/otu_table.biom -o plots/taxa_summary_group -m map_edited.txt -c Discription_level_3
```

For this study grouping by Sample_Type, Location and Location_Sample_Type was used the following code was used:

Groupin by Sample_Type

```
summarize_taxa_through_plots.py -i final_otu_tables/otu_table_5545.biom -o plots/taxa_summary_Location -m map_edited.txt -c Sample_Type
```

Groupin by by Location

```
summarize_taxa_through_plots.py -i final_otu_tables/otu_table_5545.biom -o plots/taxa_summary_Location -m
map_edited.txt -c Location
```

Groupin by Location_Sample_Type

```
summarize_taxa_through_plots.py -i final_otu_tables/otu_table_5545.biom -o
plots/taxa_summary_Location_Sample_Type -m map_edited.txt -c
```

For grouping all samples together, a created a column with name “All” and bou the same value in each row.

```
summarize_taxa_through_plots.py -i final_otu_tables/otu_table_5545.biom -o plots/taxa_summary_all -m
map_edited.txt -c All
```

20. if you want the actual final numbers of OTUs and other diversity metrics (such as Shannon, Simpson, etc.) in each sample, you should be running the "alpha_diversity.py" script on your final OTU table - this will give you a text file with all the values for the different samples (as seen at: http://qiime.org/scripts/alpha_diversity.html).

```
alpha_diversity.py -i final_otu_tables/otu_table.biom -o final_alpha_diversity -m
PD_whole_tree,chao1,observed_otus,shannon,simpson_e -t clustering/rep_set.tre
```

Options for --m: invalid choice: (choose from 'ace, berger_parker_d, brillouin_d, chao1 - richness,' hao1_ci, dominance, doubles, enspie, equitability, esty_ci, fisher_alpha, gini_index, goods_coverage, heip_e, kempton_taylor_q, margalef, mcintosh_d, mcintosh_e, menhinick, michaelis_menten_fit, observed_otus, observed_species, osd, simpson_reciprocal, robbins, shannon, simpson, simpson_e - Simpson’s evenness measure E, singles, strong, PD_whole_tree - Faith's _Phylogenetic Diversity

If you are planning to compare 16S and ITS data you will need to use the same dissimilarity matric for both, and since you can not build Unifrac for ITS we have to build Bray-Curtis for 16S data To build bruy_curtis for 16S. You will use the same function for ITS analysis

```
core_diversity_analyses.py -i final_otu_tables/otu_table_5545.biom -o cdout/ -m map_edited.txt -e 5545 -
-nonphylogenetic_diversity
```

How to build Adonis using Bray-Curtis dissimilarity matric for sample and location

```
biom subset-table -i final_otu_tables/otu_table_5545.biom -a sample -s map_sampleID.txt -o
final_otu_tables/otu_table_5545_map_Samplefundy.biom
```

```
biom subset-table -i final_otu_tables/otu_table_5545.biom -a sample -s map_sampleID.txt -o
final_otu_tables/otu_table_5545_map_SampleBale.biom

biom subset-table -i final_otu_tables/otu_table_5545.biom -a sample -s map_sampleID.txt -o
final_otu_tables/otu_table_5545_map_SampleGuysbo.biom

biom subset-table -i final_otu_tables/otu_table_5545.biom -a sample -s map_sampleID.txt -o
final_otu_tables/otu_table_5545_map_SampleNorth.biom
```

To build Adonis_Sampletype with Weighted_unifrac for 16S

```
compare_categories.py -i final_otu_tables/otu_table_5545_map.biom --method adonis -i
plots/bdiv_otu/weighted_unifrac_dm.txt -m map_edited_Plasticcompost1.txt -c Description -o adonis_Group3 -n
999
```

To build Adonis_Location_Sample_Type_Weighted_unifrac for 16S

```
compare_categories.py -i final_otu_tables/otu_table_5545_map_SampleNorth.biom --method adonis -i
plots/bdiv_otu/weighted_unifrac_dm.txt -m map_edited_1.txt -c Location_Sample_Type -o
adonis_Location_Sample_Type -n 999

compare_categories.py -i final_otu_tables/otu_table_5545_map_SampleBale.biom --method adonis -i
plots/bdiv_otu/weighted_unifrac_dm.txt -m map_edited_1.txt -c Location_Sample_Type -o
adonis_Location_Sample_Type -n 999

compare_categories.py -i final_otu_tables/otu_table_5545_map_SampleGuysbo.biom --method adonis -i
plots/bdiv_otu/weighted_unifrac_dm.txt -m map_edited_1.txt -c Location_Sample_Type -o
adonis_Location_Sample_Type -n 999

compare_categories.py -i final_otu_tables/otu_table_5545_map_SampleFundy.biom --method adonis -i
plots/bdiv_otu/weighted_unifrac_dm.txt -m map_edited_1.txt -c Location_Sample_Type -o
adonis_Location_Sample_Type -n 999
```

To build Adonis_Time_BC for 16S (BRAY _CURTIS)

```
compare_categories.py --method adonis -i cdout/bdiv_even5545/bray_curtis_dm.txt -m map_edited.txt -c Time -o
Adonis_Time_BC/Time -n 999
```

To build Adonis_Time_Weighted_unifrac for 16S

```
compare_categories.py --method adonis -i plots/ taxa_summary_Location/weighted_unifrac_emperor_pcoa_plot.txt
-m map_edited.txt -c Time -o Adonis/Time -n 999
```

To build Adonis_Location_Weighted_unifrac for 16S

```
compare_categories.py -i final_otu_tables/otu_table_5545_map_SampleID.biom --method adonis -i plots/bdiv_otu/weighted_unifrac_dm.txt -m map_edited.txt -c Location -o adonis_Location -n 999
```

APPENDIX 2

Microbiome Helper ITS2 Fungal Standard Operating procedure **for ITS2 pipeline v1** as described by Comeau et al., 2017 used for the study with little modifications

1. Run FastQC to allow manual inspection of the quality of sequences

```
mkdir fastqc_out
fastqc -t 20 raw_data_new/* -o fastqc_out/
```

2. Stitch paired-end reads together (summary of stitching results are written to "pear_summary_log.txt")

```
run_pear.pl -p 20 -o stitched_reads raw_data_new/*
```

3. Filter stitched reads by quality score (at least Q30 over at least 90% of the read), length (at least 200 bp) and ensure forward and reverse primers match 100% each read (summary written to "read_filter_log.txt" by default). If you do not wish to force primer matching, then you must remove the -f/-r/-c options below.

```
read_filter.pl -f GTGAATCATCGAATCTTTGAA -r GCATATCAATAAGCGGAGGA -c both --thread 20 -q 30 -
p 90 -l 200 stitched_reads/*.assembled.*
```

4. Convert FASTQ stitched files to FASTA AND remove any sequences that have an 'N' in them.

```
run_fastq_to_fasta.pl -p -o fasta_files filtered_reads/*fastq
```

5. Remove chimeric sequences with VSEARCH (summary written to "chimera_filter_log.txt" by default).

```
chimera_filter.pl --thread 20 -type 1 -db /home/shared/rRNA_db/UNITE_uchime_ITS2only_01.01.2016.fasta
fasta_files/*
```

6. Create a QIIME "map.txt" file (the map.txt for this study was named map_edited.txt) with the first column containing the sample names and another column called "FileInput" containing the filenames. This is a tab-delimited file and there must be columns named "BarcodeSequence" and "LinkerPrimerSequence" that are empty. This file can then contain other columns to group samples which will be used when Fig.ures are created later.

```
create_qiime_map.pl non_chimeras/* > map.txt
```

7. Combine files into single QIIME "seqs.fna" file (~5 minutes).

```
add_qiime_labels.py -i non_chimeras/ -m map_edited -c FileInput -o combined_fasta
```

8. Create OTU picking parameter file. We chose to cluster the ITS sequences at the 97% identity which is considered the appropriate level for determining species (see Blaalid et al., 2013).

```
echo "pick_otus:threads 4" >> clustering_params.txt
echo "pick_otus:sortmerna_coverage 0.5" >> clustering_params.txt
echo "pick_otus:similarity 0.97" >> clustering_params.txt
echo "assign_taxonomy:id_to_taxonomy_fp
/home/shared/rRNA_db/UNITE_sh_refs_qiime_ver7_dynamic_20.11.2016.goodASCII.txt" >>
clustering_params.txt
echo "assign_taxonomy:reference_seqs_fp
/home/shared/rRNA_db/UNITE_sh_refs_qiime_ver7_dynamic_20.11.2016.fasta" >> clustering_params.txt
echo "assign_taxonomy:assignment_method mothur" >> clustering_params.txt
```

9. Run the entire QIIME open reference picking pipeline with the new sortmerna (for reference picking) and sumaclust (for de novo OTU picking). This does reference picking first, then subsamples failure sequences, de novo OTU picks failures, ref picks against de novo OTUs, and de novo picks again any left over failures. As the ITS2 region is even more variable than 16S/18S, a good reference alignment is not possible for this marker so the alignment and tree-building steps are suppressed. Note: You may want to change the subsampling percentage to a higher amount from the default -s 0.001 to -s 0.01 (e.g 1% of the failures) or -s 0.1 (e.g., 10% of the failures) (~24 hours).

```
pick_open_reference_otus.py -i $PWD/combined_fasta/combined_seqs.fna -o $PWD/clustering/ -p
$PWD/clustering_params.txt -m sortmerna_sumaclust -s 0.1 -v --min_otu_size 1 -r
/home/shared/rRNA_db/UNITE_sh_refs_qiime_ver7_dynamic_20.11.2016.fasta --suppress_align_and_tree
```

10. Filter OTU table to remove singletons as well as low-confidence OTUs that are likely due to MiSeq bleed-through between runs (reported by Illumina to be 0.1% of reads).

```
remove_low_confidence_otus.py -i $PWD/clustering/otu_table_mc1_w_tax.biom -o
$PWD/clustering/otu_table_high_conf.biom
```

11. Summarize OTU table to determine number of sequences per sample.

```
biom summarize-table -i clustering/otu_table_high_conf.biom -o clustering/otu_table_high_conf_summary.txt
```

12. Normalize OTU table to same sample depth - you will need to change the value of (X = 776) shown below to match the read count of the sample with the lowest (acceptable) number of reads. Note: Don't like the idea of throwing away all that data? You may want to consider trying different normalization methods such as DESeq2 (see Additional QIIME Analysis in right panel).

```
mkdir final_otu_tables
single_rarefaction.py -i clustering/otu_table_high_conf.biom -o final_otu_tables/otu_table.biom -d 776
```

13. Manually add column(s) to map.txt that contain information to group your samples (e.g., healthy vs disease).
14. Calculate alpha diversity metrics (you may add/modify the desired metrics with the -m parameter).

```
alpha_diversity.py -i final_otu_tables/otu_table.biom -o final_otu_tables/alpha_diversity.txt -m
chao1,observed_otus,shannon,simpson_e
```

15. Convert BIOM OTU table to tab-separated file to be opened/explored in text editors or Excel, etc.

```
biom convert -i final_otu_tables/otu_table.biom -o final_otu_tables/otu_table_w_tax.txt --to-tsv --header-key
taxonomy
```

16. Convert BIOM OTU table to STAMP:

```
biom_to_stamp.py -m taxonomy final_otu_tables/otu_table.biom > final_otu_tables/otu_table.spf
```

Fix instances of "unidentified" taxa at intermediate taxonomic levels so STAMP can read in table.

```
ITS2_stamp_fix/fix_ITS2_spf.py -i ITS2_stamp_fix/otu_table.spf -o ITS2_stamp_fix/otu_table_fix.spf
```

17. Add sample metadata to BIOM file so that it can be used by other tools like phinch.org and phyloseq.

```
biom add-metadata -i final_otu_tables/otu_table.biom -o final_otu_tables/otu_table_with_metadata.biom -m map.txt
```

All samples

```
summarize_taxa_through_plots.py -i final_otu_tables/otu_table_776.biom -o plots/taxa_summary_all -m
map_edited.txt -c All
```

By Sample

```
summarize_taxa_through_plots.py -i final_otu_tables/otu_table_776.biom -o plots/taxa_summary_by_sample
```

Location_Sample_Type

```
summarize_taxa_through_plots.py -i final_otu_tables/otu_table_776.biom -o
plots/taxa_summary_Location_Sample_Type -m map_edited.txt -c Location_Sample_Type
```

To build Adonis

```
compare_categories.py -i final_otu_tables/otu_table_776.biom --method adonis -i
cdout/bdiv_even776/bray_curtis_dm.txt -m map_edited.txt -c Location -o adonis_Location -n 999
```

Beta diversity for bray_curtis

```
core_diversity_analyses.py -i final_otu_tables/otu_table_776.biom -o cdout/ -m map_edited.txt -e 776 --
nonphylogenetic_diversity
```

```
beta_diversity_through_plots.py -m map_edited.txt -t clustering -i final_otu_tables/otu_table_776.biom -o
plots/bdiv_otu
```

APPENDIX 3

Quantification and qualification of DNA isolated from Bulk compost samples.

Location	260	280	320	260	280	260/280	Concentration, ng/µL
Guysborough 1	0.063	0.056	0.047	0.011	0.006	1.703	10.647
Guysborough 2	0.082	0.073	0.058	0.019	0.012	1.59	19.155
Guysborough 3	0.083	0.074	0.059	0.019	0.012	1.608	19.246
Guysborough 4	0.132	0.117	0.092	0.034	0.022	1.575	34.445
Guysborough 5	0.104	0.091	0.071	0.027	0.017	1.619	27.081
Northridge 1	0.076	0.067	0.053	0.017	0.01	1.667	16.872
Northridge 2	0.090	0.079	0.063	0.022	0.013	1.634	21.896
Northridge 3	0.085	0.077	0.065	0.015	0.009	1.741	14.949
Northridge 4	0.087	0.077	0.06	0.023	0.014	1.647	22.572
Northridge 5	0.063	0.056	0.046	0.012	0.007	1.791	12.082
Balefill 1	0.061	0.055	0.046	0.011	0.006	1.764	10.768
Balefill 2	0.063	0.055	0.044	0.015	0.008	1.757	14.847
Balefill 3	0.068	0.062	0.055	0.009	0.005	1.882	8.838
Balefill 4	0.053	0.048	0.041	0.008	0.004	1.914	7.809
Balefill 5	0.055	0.049	0.041	0.009	0.005	1.8	8.609
Fundy 1	0.064	0.057	0.046	0.013	0.007	1.732	12.942
Fundy 2	0.082	0.073	0.058	0.019	0.012	1.608	18.809
Fundy 3	0.061	0.054	0.043	0.013	0.008	1.768	13.313
Fundy 4	0.06	0.054	0.045	0.011	0.006	1.636	10.533
Fundy 5	0.057	0.051	0.043	0.01	0.006	1.783	10.205

APPENDIX 4

Quantification and qualification of DNA isolated from plastic-associated with compost samples.

Location	260	280	320	260	280	260/280	Concentration, ng/µL
Guysborough 1	0.062	0.055	0.045	0.012	0.007	1.805	11.754
Guysborough 2	0.064	0.059	0.049	0.01	0.007	1.426	9.808
Guysborough 3	0.047	0.043	0.038	0.004	0.002	1.862	4.022
Guysborough 4	0.055	0.05	0.042	0.008	0.005	1.625	8.22
Guysborough 5	0.051	0.047	0.04	0.007	0.004	1.832	6.505
Northridge 1	0.055	0.051	0.043	0.007	0.005	1.468	6.788
Northridge 2	0.05	0.045	0.04	0.005	0.003	1.764	5.282
Northridge 3	0.052	0.047	0.039	0.009	0.005	1.886	8.526
Northridge 4	0.055	0.049	0.043	0.007	0.004	1.843	7.238
Northridge 5	0.051	0.046	0.04	0.006	0.003	1.84	5.924
Balefill 1	0.049	0.045	0.039	0.004	0.003	1.727	4.4
Balefill 2	0.066	0.056	0.042	0.019	0.011	1.713	19.13
Balefill 3	0.046	0.043	0.038	0.003	0.002	1.943	3.468
Balefill 4	0.049	0.044	0.038	0.005	0.003	1.831	5.475
Balefill 5	0.047	0.043	0.038	0.004	0.002	1.954	4.262
Fundy 1	0.048	0.044	0.039	0.004	0.002	1.864	4.317
Fundy 2	0.048	0.044	0.038	0.005	0.003	1.818	5.149
Fundy 3	0.049	0.044	0.039	0.005	0.003	2.033	5.202
Fundy 4	0.05	0.045	0.039	0.005	0.003	1.858	5.492
Fundy 5	0.05	0.045	0.039	0.007	0.003	2.057	6.696

APPENDIX 5

Command lines and annotation for beta diversity for Bray-Curtis ecological distance analysis of fungal communities used in the study*.

```
core_diversity_analyses.py -i final_otu_tables/otu_table_776.biom -o
cdout/ -m map_edited.txt -e 776 --nonphylogenetic_diversity
   -i, - input biom file
   -o, - output directory
   -m, - mapping file path
   -e, - sequencing depth in this study 776 was used for fungal ITS.
   -nonphylogenetic_diversity – refers to non-phylogenetic alpha and
      beta (bray_curtis) diversity, this become useful when working
      with non-amplicon BIOM tables or if a reliable tree is not
      available (e.g., if you're working with ITS amplicons)
beta_diversity_through_plots.py -m map_edited.txt -t clustering -i
final_otu_tables/otu_table_776.biom -o plots/bdiv_otu
```

-i, - input biom file

-o, - output directory

-m, - mapping file path

-e, - sequencing depth in this study 776 was used for fungal ITS.

```
* Similar command lines were used for 16s rRNA data analysis.
```

APPENDIX 6

The command lines and annotation for Adonis test based on Bray-Curtis beta-diversity distances

```
a. How to build Adonis using Bray-Curtis dissimilarity matric for sample
and location*.
Here is an example of the command line used to build Adonis using Bray-
Curtis dissimilarity matric for sample type
   compare_categories.py -i final_otu_tables/otu_table_776.biom --method
   adonis -i cdout/bdiv_even776/bray_curtis_dm.txt -m map.txt -c
   Sample_type -o adonis_Sample_type -n 999
      -compare_categories.py - This is the script being run.
      --method - statistical method to used in this case it was Adonis.
      -i, - input, in this case Bray-Curtis distance matrix,
         bray_curtis_dm.txt, and biom. table, otu_table_776.biom
      -m, - metadata mapping file
      -c, - categories from the mapping file
      -o, - output directory
      -n, - number of permutations default: 999
* You can use "location" or "time" as a category to test for the
significance instead of sample type.
```

APPENDIX 7

How to build Adonis using Weighted UniFrac dissimilarity matric (only for 16S rRNA data analysis

```
Here is an example of the command line used to build Adonis using Weighted
unifrac dissimilarity matric for sample type
   compare_categories.py -i final_otu_tables/otu_table_5545_map.biom --
   method adonis -i plots/bdiv_otu/weighted_unifrac_dm.txt -m map.txt -c
   Sample_type -o adonis_Sample-type -n 999
      --method - statistical method to used in this case it was Adonis.
      -i, - input, in this case weighted unfrac distance matrix,
         weighted_unifrac_dm.txt, and biom. table, otu_table_776.biom
      -m, - metadata mapping file
      -c, - categories from the mapping file
      -o, - output directory
      -n, - number of permutations default: 999
      -n, - number of permutations default: 999

* You can use "location" or "time" as a category to test for the
significance instead of sample type.
```

APPENDIX 8

To perform Adonis test on the significance of grouping for microbial communities from individual locations by sample type.

```
To perform Adonis test on the significance of grouping for microbial
communities from individual locations by sample type, biome tables with
sequencing data for each location were created:
1. Subset the biome table
1.1. Create a text file with samples IDs you want to include in sub-biome
table. In this example the name of the file with the list of samples
collected in Fundy compost facility was map_sampleID.txt, the name of
original biome table is otu_table_5545.biom and the sub-biome table with
sequencing data from samples from Fundy compost facility was named
otu_table_5545_map_Samplefundy.biom. For example, to create a sub-biom
table with only samples from Samplefundy the line below was ran.
      biom subset-table -i final_otu_tables/otu_table_5545.biom -a sample -
      s map_sampleID.txt -o
      final_otu_tables/otu_table_5545_map_Samplefundy.biom

After sub-biome tables were created we used protocols in Text 2. a and 2.b
```