

# **The Microbial Ecology of Urban Organic Solid Waste Treatment (Compost)**

by

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# Abstract

Urban organic solid waste is increasing due to human population growth. Currently, in Australia most organic waste is disposed of into landfills, where it decomposes anaerobically, producing greenhouse gases such as methane and carbon dioxide, which contribute to global warming. However, organic waste can be diverted from landfill and treated by composting, which is a more sustainable strategy.

In this thesis, the efficacy of two different medium-sized, in-vessel commercial units have been evaluated for their ability to address this problem. Different features of each vessel contributed to their inability to produce compost. One vessel (from Closed Loop Environmental Solutions Pty. Ltd.) failed because of external heating controlled by the moisture in the waste, excessive mixing with internal paddles and the mode and strength of aeration. The result of these operational features produced dehydrated, partially degraded organic waste. During the operation the pH slightly declined, which was correlated with a high abundance of lactic acid bacteria. A Closed Loop inoculum that was to be used in the initial vessel cycle contained ~35% *Alicyclobacillus* sp., by 16S rRNA gene metabarcoding. This bacterium was never found in any further metabarcoding analyses of any Closed Loop experiments.

The other commercial vessel, On-Site Composting Apparatus (OSCA currently available from Global Composting Solutions Ltd.) was designed with excessive mixing via rotation of the barrels. This led to “balling” of the organic waste facilitating development of anaerobic centres that produced highly odourous gases. Reduced mixing improved the composting process; however, the overall machine design requires further modifications to address excessive moisture condensation in the interior of the vessel.

Due to the failure of the commercial units, a prototype in-vessel composter called Cylibox (cylinder in a box) was designed and constructed. Critical attributes of effective composting were insulation of the cylinder, once per day mixing with internal paddles, and appropriate aeration. Insulation ensured that microbially generated heat was retained in the cylinder leading to temperatures of ~65°C in

the treatment bed during the active phase of the composting. An optimal carbon:nitrogen ratio (~30:1) of the organic waste and sawdust mixture mitigated lactic acid producing bacterial growth in the active phase and accelerated the maturation phase. When Cylibox's composting process was optimised, the active phase was complete in ~nine days. *Bacillus coagulans* was the most abundant bacterium during this phase. During the curing phase, *Sphingobacteraceae* dominated the bacterial community, and in total, approximately two months was required to produce mature compost ready for land application.

**Keywords:** Compost, Microbial Ecology, Organic waste, Pathogens, In-vessel composting, Acidulo<sup>TM</sup>.

*To my parents, Lida Castillo and Amancio Jaimes.*

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Finally, I would like to thank my family who was always supportive during my studies.

# Declaration

I hereby declare that this investigation entitled "The Microbial Ecology of Urban Organic Solid Waste Treatment (Compost)" is my original work and to the best of my knowledge. This thesis has not been previously submitted, published, or written by myself or any other person for the award of any degree or professional qualification.

I confirm that the intellectual content of this research is the product of my own, except where due appropriate acknowledgment has been given within this thesis to the contribution of collaborators.

Signed:

A handwritten signature in blue ink, appearing to read "Kishan".

Date: 26 December 2020

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## Chapter 1

# Introduction

## 1.1 Statement of the problem

The human population is increasingly established in urban areas. Thus, as urban cities grow they require many goods and services, generating a large quantity and diversity of waste. The main urban solid waste generated in cities is called Municipal Solid Waste (MSW) and ~50% of it is comprised of urban organic solid waste (Edjabou et al., 2015). Globally, 37% of MSW is disposed of in landfills, 33% is disposed in rubbish dumps, 11% is incinerated, and 19% are recycled or treated by composting (Kaza et al., 2018). In developing countries most of the MSW is disposed of in rubbish dumps and in developed countries it goes to landfills (Ferronato and Torretta, 2019, Abdel-Shafy and Mansour, 2018). Disposing of MSW in rubbish dumps or landfills is not a sustainable technique, because when organic matter decomposes in anaerobic conditions it generally produces the greenhouse gases, methane and carbon dioxide. Globally, MSW management is producing more than ~5% of total greenhouse gases which contribute to global warming (Kaza et al., 2018).

Composting organic matter is not a novel technique. In fact, it has been done for centuries. Treating organic matter by windrow composting takes a long time (six to nine months) (Cooperband, 2000), to produce mature compost because it is affected by environmental conditions. In contrast, in-vessel composting can accelerate organic matter decomposition (Makan et al., 2013). Although it is more costly, manufacturers of commercial in-vessel units claim their units can produce compost in as little as 24 hours or 14 days and that the end-product is ready to be applied to the soil.

In this investigation, to test the claims of the in-vessel unit manufacturers, two different commercial units were used to study food waste degradation, and the produced material was then physicochemically and biologically characterised. In addition and based on the short-comings of the commercial units, an in-vessel composter prototype was developed.

This research was focused on treating the organic waste in the urban Australian context, however, the outcome of this investigation can be implemented in any urban city. Although the collection, treatment, and disposal of organic waste is the responsibility of city councils, to make the implementation of a efficient and sustainable management of organic waste possible, it is important to work with a multidisciplinary approach. For instance, there are still enormous gaps between the waste generator, city councils, policymakers and technology developers which have to be bridged. Therefore, to minimise organic waste negative impacts on the environment and human health, it is necessary to implement integrated MSW management projects, where all stakeholders contribute to mitigate this problem.

## **1.2 Research gaps and contributions**

Currently, in attempting to tackle organic waste management and treatment, certain manufacturers have developed in-vessel units. However, producing compost in a 24 hour to 14 day window does not follow the normal composting profile. The active phase of the composting process can be accelerated by providing optimal conditions for microbial activity, however, cellulosic organic material continues to decompose during the curing phase, and consequently takes a long time to reach maturity. Hence, is important to understand the science and technology behind in-vessel organic waste composting.

There is abundant literature regarding traditional or windrow composting. In contrast, little research has been conducted to assess the performance of commercial in-vessel units for treating organic waste. This investigation responds to this gap in knowledge, testing two commercial in-vessel units and one in-vessel composter prototype, in a systematic organic waste experimental review.

To determine whether the treatment process follows a typical composting profile, the physical, chemical and microbial parameters were analysed. As a results of the evaluation of these in-vessel units, a comprehensive dataset was obtained, which are discussed and explained in the research chapters.

### 1.3 Research aims, objectives and questions

The premise of this research is that composting is an option (compared to other processes) for treating organic waste that has the ability to mitigate greenhouse gas emissions, reduce (or remove) pathogens, and generate a humic material (material formed by the microbial degradation of dead plant matter or compost) for nutrient recycling to plant production systems. In addition, compost could facilitate carbon sequestration in amended soils (Trevisan et al., 2010) and provide a social benefit by enabling urban communities to play a central role in their waste treatment.

The process performance (organic material bioconversion and pathogen remediation) of compost in-vessel units depends upon the physicochemical balance of material parameters. The processing conditions applied (time, temperature, mixing, etc.) and the indigenous microbial communities (structure and function) who carry out the bioconversions and contribute to some process conditions (e.g., heat generation, production of toxins that might kill pathogens, etc.) are essential components during composting.

The aims of this research are: First, measurement of the main operational physical, chemical and microbial community (by 16S rRNA gene metabarcoding) parameters during organic waste treatment in three in-vessel units. Second, test the pathogen remediation capacity of the processes (via detection of the indicator organisms *Escherichia coli*, *Salmonella* spp. and *Enterococcus* spp.). Third, determine the maturity (via the Solvita® test) of the end-product.

To reach key operational objectives for treating organic waste, the following questions were raised in regard to the stated objectives:

**Objective 1.** The in-vessel composter should provide enough airflow for the aerobic microbial growth and the vessel should be insulated to maintain the metabolically-generated heat. The vessel, food inputs and applied processing conditions all play a role in the length of time that it takes to produce stable, mature compost.

- *What are the operating conditions and time necessary for the commercial in-vessel units and in-vessel composter prototype to produce stable, mature compost? (Chapters: 4, 5 and 6).*
- *Does the Acidulo™ microbial inoculum play an important role in organic waste treatment? (Chapters: 4 and 6).*

**Objective 2.** The organic waste should have an optimal carbon to nitrogen (C:N) ratio and moisture content to rapidly attain a stable compost.

- *How does the C:N balance impact the organic waste treatment process? (Chapters: 5 and 6).*

**Objective 3.** Maintaining ideal physical and chemical parameters should improve the performance of the microbial activity during the composting process.

- *What are the main parameter drivers in microbial diversity changes during the composting process? (Chapter: 6).*

**Objective 4.** The presence or absence (and in the latter, also the abundance) of target pathogens (*Escherichia coli*, *Salmonella* spp., or *Enterococcus* spp.) should be determined to ascertain the suitability of the composting process to generate a safe final compost.

- *Is the final product from commercial in-vessel units and in-vessel composter prototype free of pathogens? (Chapters: 4, 5 and 6).*

## 1.4 Theses structure

This thesis has seven chapters.

**Chapter 1:** Based on a brief literature overview, inappropriate management of organic waste in urban cities is identified as a critical problem. Also, research gaps and contribution of the thesis are described. Furthermore, the aim, objectives and research questions are outlined. Finally, the thesis structure is explained.

**Chapter 2:** A literature review is presented to provide relevant information about the management and treatment of organic waste. This chapter also explains how the main physical and chemical parameters can contribute to the important development of microbial activity. Finally, it focuses on the attributes and uses of compost as a natural soil amendment.

**Chapter 3:** The materials and methods are described in detail. To analyse the physical and chemical parameters of degradation products, Test Method for the Examination of Composting and Compost (TMECC) and Australian Standard AS 4454–2012 protocols were followed. The microbial analyses were performed by 16S rRNA gene metabarcoding and pure culture methods.

**Chapter 4:** Two systematic experiments of organic waste treatment were conducted in the in-vessel unit, Closed Loop (CL). The first experiment, CL1, comprised five sub-experiments (CL1.1, CL1.2, CL1.3, CL1.4 and CL1.5), which each ran for 24 hours. Based on the CL1.2 organic waste composition and processing conditions, a second experiment CL2 was run for seven days in an effort to improve the organic waste degradation. Also, the microbes in the Acidulo<sup>TM</sup> proprietary bacterial inoculum were determined.

**Chapter 5:** A commissioning experiment was performed to investigate the performance of the On-Site Composting Apparatus (OSCA) in default mode before commencing systematic experiments. Based on the results of this experiment, two further experiments were conducted wherein OSCA was run for 23 days (OSCA7) and four days (OSCA8).

**Chapter 6:** Once designed and built, the in-vessel composter prototype Cylibox (CX) was used to run five experiments (CX3, CX4, CX5, CX6, CX7). CX3 used the same waste composition as CL2 (C:N of ~17.5:1), while all other CX experiments had the C:N adjusted to ~30:1 with sawdust.

**Chapter 7:** Conclusions are drawn by comparing the performance of the two commercial in-vessel units and the in-vessel composter prototype. Limitations of this research and proposed future direction for further research are discussed.

## Chapter 2

# Literature review

## 2.1 Introduction

Global estimations of MSW generation in 2016 were 2.01 billion tonnes, and by 2050, it is estimated that this will increase to 3.40 billion tonnes (Kaza et al., 2018). On average, each person generates 0.74 kg of MSW every day. However, it can vary from 0.1 kg to 4.54 kg depending on the socioeconomic level of people. Generally, the generation of MSW in high-income countries is higher than in lower-income counties. Green and food waste represent 44% of total MSW (Kaza et al., 2018).

Urban organic solid waste (composed of green and food waste), disposed of in landfills or open dumps, decomposes anaerobically, producing methane gas (Edjabou et al., 2015, Kaza et al., 2018). Methane is a powerful greenhouse gas (GHG), 34 times more powerful in trapping heat from solar radiation than carbon dioxide. Hence, methane has a negative environmental impact because it contributes to global warming (IPCC, 2013). In 2016, MSW management emitted approximately 1.6 billion tonnes of carbon dioxide equivalent (CO<sub>2</sub>-e), which represents ~5% of total GHG gas emitted to the environment, and by 2050 it is projected to increase to ~2.6 billion tonnes of CO<sub>2</sub>-e (Kaza et al., 2018).

To minimise MSW management negative impacts, organic waste could be diverted from landfills and treated biologically by composting (Klimas et al., 2016) or anaerobic digestion (Gaur et al., 2017). Composting is a natural process wherein microorganisms such as bacteria, archaea and eukarya (e.g., fungi) degrade organic matter and transform it into a humus-like material called compost (Tiquia et al., 2002).

There are two main techniques for composting. One is open windrow composting, where organic waste is mixed in long piles generally remote from its generation (Atalia et al., 2015). While effective, this process has certain disadvantages because it is affected by environmental conditions and it is difficult to control the composting parameters (Epstein, 2011). The second, is in-vessel

composting, involving full control of aeration, temperature, and mixing of the organic matter inside the composter vessel, generally close to the source (Atalia et al., 2015). This can facilitate more rapid and more efficient composting compared to windrow composting (Makan et al., 2013).

Organic waste may be treated by anaerobic digestion (AD). However, due to the waste's often non-homogeneous composition and rapid putrescibility, AD produces volatile fatty acids (VFAs), which reduces the pH, negatively affecting the digestion process (Patil and Deshmukh, 2015). Hence, AD works better with homogenous inputs such as sewage sludge (Bratina et al., 2016).

In-vessel composting may be one of the best current alternative for treating organic waste. Nevertheless, the composting process requires optimal conditions for the development of appropriate microbial activity, to satisfy the requirements of compost standards.

## **2.2 Municipal solid waste (MSW) management**

### **2.2.1 Global management of MSW**

In 2016, high-income counties were generating large amounts of MSW (683 millions of tonnes per year) (Kaza et al., 2018). As human populations increase, MSW generation also increases (Hoornweg and Bhada-Tata, 2012). By 2030 it is projected that lower-middle income (827 millions of tonnes per year) and upper-middle income (835 millions of tonnes per year) countries will increase MSW. By 2050, lower-middle income countries will likely be leading MSW generation (1,233 millions of tonnes per year) (Kaza et al., 2018). By 2100, if MSW is generated under 'business-as-usual' conditions, it is projected that more than 3,500 million metric tonnes per year of MSW will be generated (Hoornweg et al., 2013).

The World Bank's estimates for the composition of 2.01 billion tonnes of MSW generated in 2016 are shown in Figure 2.1 (Kaza et al., 2018). Organic waste was a large proportion, and food waste represents almost half of it (Edjabou et al., 2015).

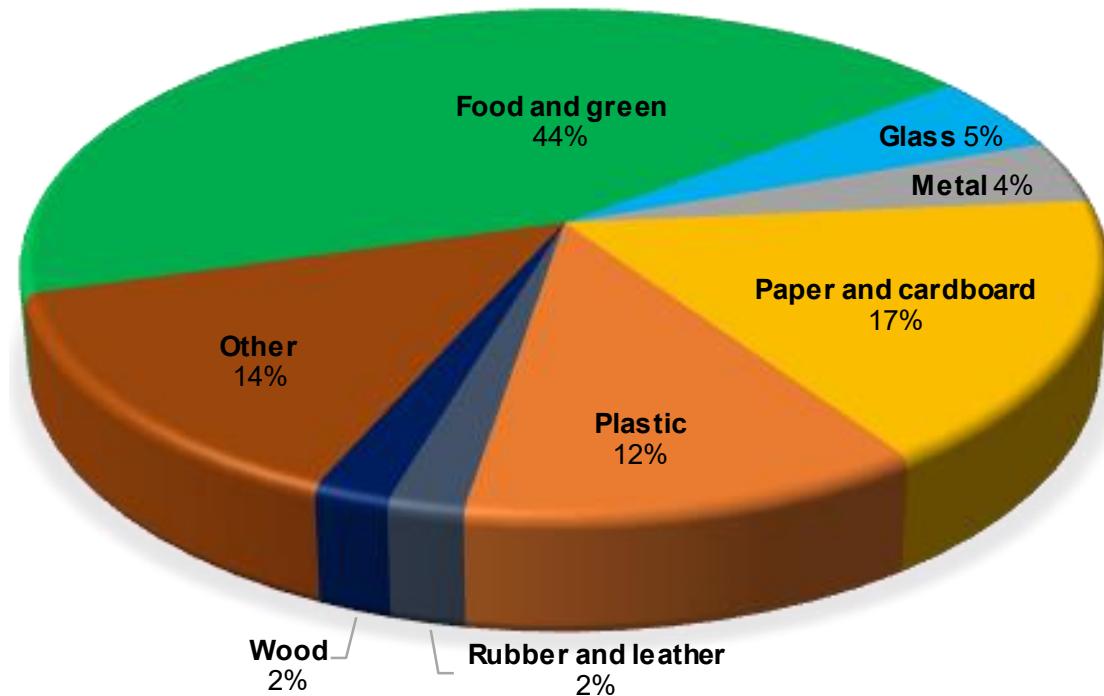


Figure 2.1 The composition of global MSW, adapted from (Kaza et al., 2018).

The amount and type of MSW generated are not the same in every region. Primarily, it depends on the human population and consumption of goods. In terms of food and green waste generation, upper-middle (353.7 million tonnes per year) and lower-middle (310.58 million tonnes per year) income countries generate a higher amount of organic waste which represent more than 50% of the total MSW in these regions (Kaza et al., 2018).

Globally, in all parts of the supply chain, ~1.2 billion tonnes per year of food produced is being wasted (FAO, 2011). In North America and Oceania, ~42% of the food supply was wasted, with a substantial part of it (61%) discarded in the consumption stage (Figure 2.2). In low and middle-income countries, food losses occur more in production, handling and storing stages due to inadequate facilities and technology (Lipinski et al., 2013). Wasting food may have negative environmental impacts by contributing to global warming, acidification of soils and eutrophication of streams (Scherhauser et al., 2018)

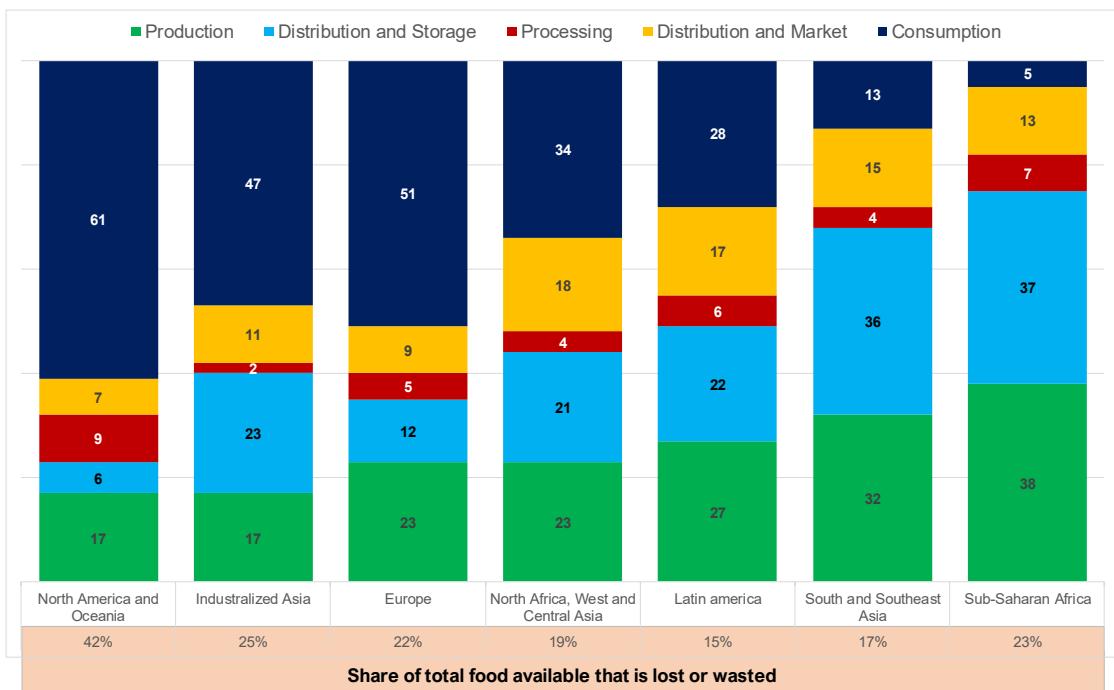


Figure 2.2 Global food loss and waste during food supply chain, adapted from (Lipinski et al., 2013).

The main components of food waste are carbohydrates (C, H, and O), which are water soluble and can be found in foods such as bread, pasta, corn and potato (Ovando-Martínez et al., 2013), and lipids (C, H, and O), which are not water soluble and can be classified as triglycerides, waxes, phospholipids and steroids. Lipids are present in oils, avocados, almonds, eggs, nuts and dairy products (Gajera and Golakiya, 2008). Proteins (C, H, O, and N – sometimes S and P) are comprised of amino acids, which are organic molecules with carboxyl and amino groups. Proteins are important because they are used for growth, cell restoration and replacement, and some function as enzymes and antibodies (Lamond, 2002). Proteins can be found in products such as fish, egg white, cheese, and meat, among many others (Moran et al., 2011).

The organic waste components facilitate biological metabolism, generate heat which increases the temperature during composting (Paritosh et al., 2017). However, using food waste for energy production may be challenging due to the material's heterogeneity and low calorific value (Adhikari et al., 2008). In addition, due to the improper MSW segregation at the generation stage, food waste is routinely mixed with other impurities, which increase contamination of the organic waste (Puig-Ventosa et al., 2013).

### 2.2.2 Australian management of MSW

According to the Australian National Waste Report 2018 (Pickin et al., 2018), it was estimated that in 2016 Australia generated 13.8 million tonnes of MSW and 31.2% (4.3 million tonnes) of it was food waste. From total food waste generated in Australia, 87% was disposed into landfills, 11% was recycled, and around one percent was used for energy production (Pickin et al., 2018). Food waste disposed of inappropriately in landfills, open dumps, soil, water streams, can generate environmental problems such as water contamination, air pollution and GHG gas emissions (Chan et al., 2016). In total, in Australia, MSW disposal emitted 8.7 million tonnes of CO<sub>2</sub>-equivalent (Pickin et al., 2018).

There are several organic waste treatments, for instance, using aerobic decomposition by composting, where organic waste can be considered a resource to produce “nutrient rich organic matter for soil” (Alvarenga et al., 2017). Treating food waste by anaerobic decomposition (AD or landfilling) produces methane gas, which can be used for “fuel, energy and heat” (Lam and Lin, 2014).

## 2.3 Biological treatment of organic municipal solid waste

Globally, MSW is not well managed. In 2016, 13.5% of it was recycled and only 5.5% was composted. The rest of the MSW was disposed of in either a controlled landfill (4%), unspecified landfill (25%), sanitary landfill with gas collection (7.7%), or open dump (33%) (Kaza et al., 2018). There are several options for treating organic municipal solid waste. This section focuses on the major biological procedures of anaerobic digestion, landfilling and composting.

### 2.3.1 Biomethanation

Biomethanation also called AD, is a technique for treating of organic waste for methane production (Gaur et al., 2017). Biomethanation is a biochemical process in the absence of oxygen and it has four main phases. In the hydrolysis phase, complex organic matter (carbohydrates, proteins and fats) is catabolised into soluble organic molecules such as sugar, amino acids, and fatty acids (Adekunle and Okolie, 2015). In the acidogenesis phase, the temperature rises to 70°C, pH is in the range from 3 to 5, and VFAs are created in concentrations

exceeding 6,000 mg L<sup>-1</sup> (Wang et al., 2002). Under these conditions, the organic molecules are transformed into carbonic acids, alcohols, hydrogen, carbon dioxide and ammonia. In the acetogenesis phase, VFAs are converted into acetic acid, carbon dioxide and hydrogen. Finally, in the methanogenesis stage, certain archaea produce methane and carbon dioxide (Adekunle and Okolie, 2015).

Generally, biomethanation has better performance and higher biogas production with high moisture and homogeneous inputs, such as sewage sludge and livestock waste (Bratina et al., 2016). The large particle size of food waste as input, and rapid biodegradation of certain food wastes in the hydrolysis phase generates high concentrations of VFAs, reducing the pH and inhibiting methanogenesis (Bong et al., 2017).

Some organic waste can be high in lipids, which inhibit methanogenesis (Dasa et al., 2016). An excess of carbohydrates can also reduce the accumulation of VFAs facilitating methanogenesis (Neves et al., 2008). When biomethanation is successful, the produced methane gas may be used for electricity generation or, after purification, can be used as a fuel (Paolini et al., 2018). The remaining solids (digestate) can be applied as a soil conditioner (Mir et al., 2016). However, certain regulatory requirements must be met or digestate must be landfilled (Patil and Deshmukh, 2015). Another barrier for digestate application to land is the lack of acceptance in the market (Amir et al., 2016).

### 2.3.2 Landfilling

Landfilling is one of the most common techniques for disposing of MSW worldwide (US-EPA, 2019). Highly engineered landfills are used in developed countries (Abdel-Shafy and Mansour, 2018). However, in many low-income countries, most of the MSW is disposed of in open dumps and often burnt (Ferronato and Torretta, 2019). Disposing of MSW in landfills has several advantages such as low operational cost, low investment, and the ability to handle large amounts of MSW (Li et al., 2017), but there can also be disadvantages. The decomposition of organic matter in landfills, produces GHGs (methane, carbon dioxide) (US-EPA, 2019). Using landfilling as a MSW treatment, it contributes approximately 3% to 5% of global GHGs (UNEP, 2010).

The accumulation of methane gas may lead to explosions, and presents a fire risk to the nearby population (Ma et al., 2014). For instance, between 2013 to 2016, there was registered fire in Australian landfills such as Wingfield, Somerton, Broadmeadows, Pialligo and Chester Hill (Fattal et al., 2016). Another problem from landfilling is leachate production, if it not well managed, it has negative impacts on groundwater, water bodies, aquifers, soils, and ecosystems (Crowley et al., 2003).

In contrast, if the landfills are well managed by using geomembrane and clay liners, the negative environmental impacts can be minimised, and methane generated can be used as an energy source (Shakeri et al., 2012). Certain landfills based on the Kyoto Protocol are implementing Clean Development Mechanism (CDM) projects to recover methane and generate electricity (Leme et al., 2014). However, landfills might recover 75% of methane, with the rest being emitted as fugitive methane (US-EPA, 2019). A sustainable option to minimise the negative impacts of GHG emissions and leachate production is the diversion organic waste from landfill (Mason et al., 2011). Treating organic waste by composting rather than by landfilling contributes to reductions in GHG emissions (Yang et al., 2009).

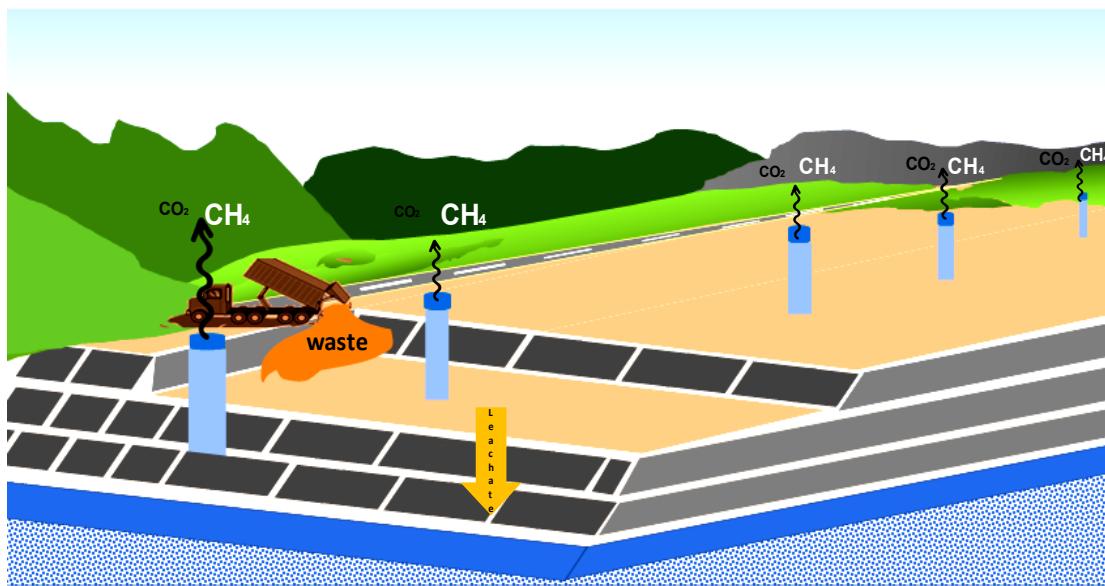


Figure 2.3 Green house gas production in the landfill, adapted from (Freed et al., 2004).

### 2.3.3 Composting

Diverting organic waste from landfill and treating it by composting can reduce negative environmental impacts and produce compost (Levis et al., 2010, He et al., 2011). Although, carbon dioxide is released during composting (Haug, 1993), it is biogenic carbon dioxide, produced from organic matter degradation under aerobic conditions (Sánchez-García et al., 2015). These biogenic carbon dioxide emissions are not counted in global warming effects (US-EPA, 2019). Therefore, according to the life cycle assessment, composting has a lower negative environmental impact in comparison to landfilling or incineration (Saer et al., 2013).

Composting is an oxidative biological process (Elorrieta et al., 2002), where the organic waste matter is transformed into stable a humus-like material via biochemical processes (Tiquia, 2010). During the composting process, the continuously changing parameters, such as temperature, moisture content, oxygen and carbon dioxide gas exchange, gradually drive the abundance of a microbial community and the degradation of complex organic matter (Tiquia et al., 2002). The endogenous bio-heat produced during the organic matter decomposition, increases the temperature, which inhibits the growth of non-thermotolerant microorganisms (Xiao et al., 2017). Figure 2.4 shows a general in-vessel composting scheme.

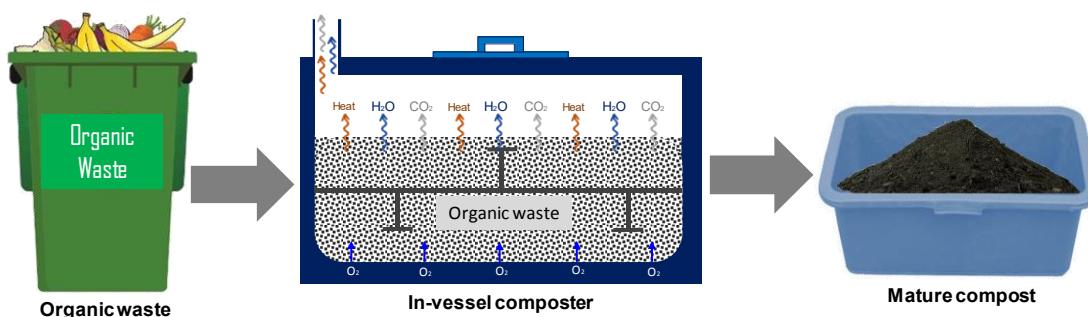


Figure 2.4 In-vessel composting scheme, adapted from (Cooperband, 2000, Rynk et al., 1992).

Composting is divided into two main phases. The first is the active phase, which includes the early mesophilic (ambient to 40°C) and thermophilic stages (>40°C). The second is the curing phase, which includes the natural cooling and the maturation stage (<40°C) (Cooperband, 2000, Bernal et al., 2009,

Mehta et al., 2014, Sánchez et al., 2017). The early mesophilic phase lasts between one and three days, where microorganisms mineralise organic compounds producing heat, organic acids, carbon dioxide and ammonia (Bernal et al., 2009).

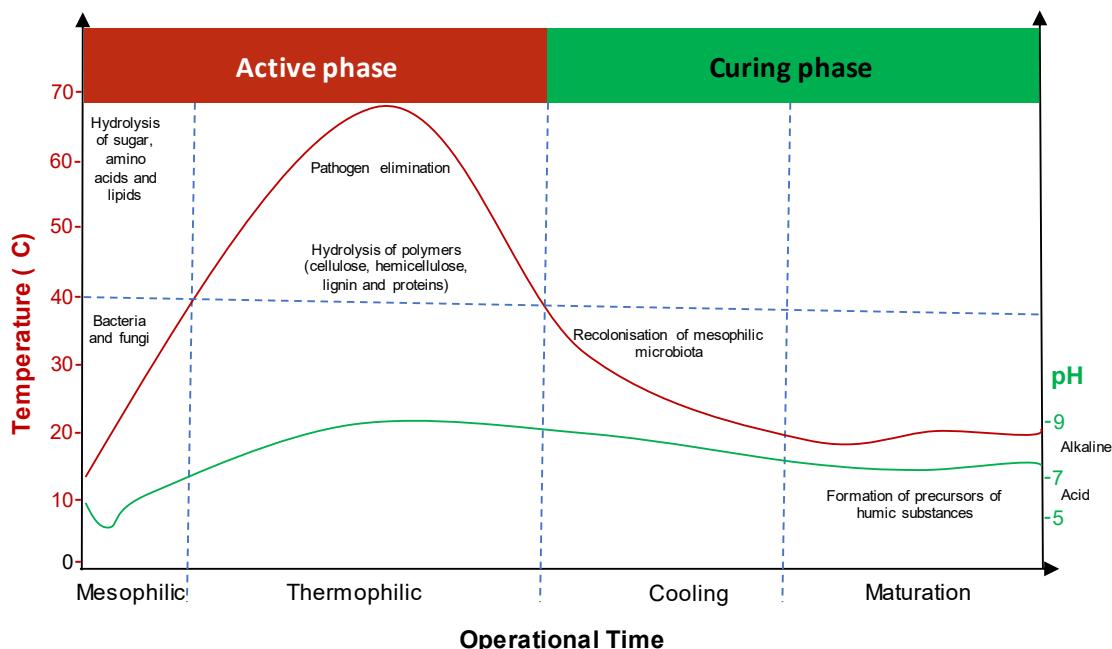


Figure 2.5 Temperature and pH profile during the composting process, adapted from (Sánchez et al., 2017) and (Cooperband, 2000).

During the active phase, conditions are initially mesophilic which is when the microorganisms start breaking down the organic matter and using the nutrients in their growth, particularly readily degradable components (Fourti, 2013). This process generates endogenous heat, due to the metabolism (Singh and Kalamdhad, 2014), which leads to thermophilic conditions and thermophilic microorganisms continue to rapidly consume the organic matter (Mehta et al., 2014). During the thermophilic stage, the temperature increases and the optimum range is between 40°C and 65°C (de Bertoldi et al., 1983). However, if the temperature goes above 70°C, some beneficial microorganisms may be inhibited, which can affect the compost quality (Lynch et al., 2005).

The temperature profile depends on the composition of the initial input, and on the provided and generated physical and chemical parameters during composting (Pasda et al., 2005). By mixing organic matter in the optimal carbon

to nitrogen (C:N) ratio, and by providing ideal temperature, moisture and air flow conditions, the organic matter will rapidly decompose.

For composting, a C:N ratio of ~30:1 by weight and a continuous air supply greater than 10% of oxygen are optimal for microbial activity (Epstein, 2011). If the temperature reaches above 55°C, it can inactivate pathogenic microorganisms (de Bertoldi et al., 1983). To pasteurise the compost, the temperature in the thermophilic stage must be maintained above 55°C for at least three days (Australian-Standard, 2012). During the thermophilic stage, bacteria are the main decomposer, while at high temperatures (>60°C), fungi are generally inhibited (Klamer and Bååth, 1998).

Finally, the active phase substantially slows due to the unavailability of readily degradable organic matter. The microbial activity is reduced, the temperature decreases gradually, and the cooling and maturation stage starts (Insam and de Bertoldi, 2007, Mehta et al., 2014). The composted material is stored in a dry aerated location for slow decomposition of cellulose, lignin, and hemicellulose, which may take weeks to months (Chen et al., 2011). Once, the compost achieves maturity, it is considered that this end-product is stable, free of pathogens and phytotoxicity (Wichuk and McCartney, 2007).

### **Windrow composting**

The classical composting process is the windrow method, where organic matter is placed in long physical rows or piles (Sánchez-Monedero et al., 2005), on the ground and periodically turned over by mechanical methods to ensure aeration (Epstein, 2011). The size of the piles is determined by the volume of the organic matter to be treated and the available working space (Shammas and Wang, 2007, Sharma et al., 1997). The most important parameter to be controlled during windrow composting is aeration. The windrow piles can be aerated by frequent turning of the piles (Bertoldi et al., 1982) or by forced aeration, which does not require turning to maintain porosity and drainage (Waldron and Nichols, 2009).

To maintain aerobic conditions, the turning frequency of the piles should be once or twice per week (Shammas and Wang, 2007). In windrowing, there is a trade-off between piles that are too large (and might generate anaerobic

conditions in certain parts) and those that are too small (which lose heat, thus reducing microbial activity and pathogen pasteurisation) (Rynk et al., 1992).

### In-vessel composting

In-vessel composting was developed as an alternative to windrowing for more efficient control of the parameters such as airflow, moisture, and temperature (Makan et al., 2013), such that the process could be accelerated. All composting phases, including the curing phase are still required (Spencer, 2007). There are several types of in-vessel composters, from very basic (e.g., bin composter) (Rynk et al., 1992), to more sophisticated (e.g., stainless steel construction, forced airflow, control over evaporation, a turning mechanism, and operation and monitoring by computers). Generally, these latter technologies are used in large-scale compost production (Spencer, 2007).

In-vessel composters can be of varying sizes and made from concrete, shipping containers, stainless steel, etc. Depending on space, in-vessel composters can be made as modular vertical units (which can have compaction problems), or horizontal units (Mamta et al., 2017). The amount and type of organic matter, location, cost and local or national regulations, impact the type and size (small ( $50 \text{ kg d}^{-1}$  – e.g., household), medium ( $50\text{-}600 \text{ kg d}^{-1}$  – e.g., cafes) large ( $> 600 \text{ kg d}^{-1}$  – e.g., residential complexes)) of in-vessel composter chosen (Epstein, 2011).

### Applications and uses of compost

Compost is the product of microbial decomposition of organic matter, and can be applied as a soil amendment in home gardening or the agriculture sector (Cooperband, 2000). Mature compost is a humic substance which is comprised of colloidal humin, humic acids and fulvic acid, that can be applied as a stable material to soil (Trevisan et al., 2010).

Compost provides mineral nutrients including N, P, K, Mg, Ca and other micronutrients for soil food web (Cheng and Grewal, 2009). Microorganisms mineralise organic nutrients to inorganic forms, ready for plant uptake and growth (Farrell and Jones, 2009). Since mineralisation is a slow process, it regulates nutrient accessibility into the soil environment (Larney et al., 2008).

Thus, the leaching of nutrients from soil after compost application is minimised (Gale et al., 2006).

Stable compost has great advantages for sustainable agriculture (Scotti et al., 2015). Compost enhances soil water-holding capacity and mitigates soil erosion and moisture evaporation (Curtis and Claassen, 2005). Due to the micro and macroporosity of compost, it can also improve soil structure and increase water content by 58% to 86% (Celik et al., 2004). Although soil-type determines the optimal application of compost (Yuksek et al., 2009). Finally, composting organic MSW creates new business opportunities that can improve the economy (Pandyaswargo and Dickella, 2014).

## 2.4 Physical parameters during composting and compost production

### 2.4.1 Particle size

During the preparation of the composting input, the particle size of the organic matter must be reduced. If the particle size of the organic matter is too large, it could negatively affect the decomposition rate. However, if the particle size is too small, it will reduce the porosity (Bernal et al., 2009). The optimum particle size should be in the range from 0.3 to 5 cm in diameter (Rynk et al., 1992). Hence, to maintain the optimum particle size, a sieve within these dimensions may be used (Ge et al., 2015).

### 2.4.2 Mixing frequency

Periodically mixing composting materials has several advantages such as maintenance of the porosity for better air distribution, reduced compaction (Lazcano et al., 2008), making raw organic waste available for microbial decomposition (Kalamdhad and Kazmi, 2009), and improving compost homogenization (Petric et al., 2012). Mixing, changes the physical and chemical parameters. For example, the temperature, moisture content, C:N ratio, and pH are all affected by the mixing regime (Getahun et al., 2012).

In order to improve aeration, the frequency of mixing may be set to once a day (Li et al., 2015), every four to five days (Ros et al., 2006), or weekly (Mohee et al., 2015). The mixing regime could be optimised based on the specific purpose of the composting process (Kalamdhad and Kazmi, 2009). In one composting

experiment with MSW and poultry manure, the mixing regime was once a day for 30 minutes (Petric et al., 2015). In another MSW composting experiment, the mixing frequency was weekly (Mohee et al., 2015). Comparing the mixing frequency of windrow composting, weekly mixing contributed significantly to increase the bacterial population. In contrast, once a day mixing generated a lower bacterial population (Awasthi et al., 2014). Mixing has an effect on the temperature in uninsulated vessels; a higher mixing regime may reduce the temperature such that it may not reach the appropriate temperature to inactivate pathogenic microorganisms (Kalamdhad and Kazmi, 2009). If mixing is insufficient, the decomposition rate could slow due to depleted oxygen (Rynk et al., 1992).

#### **2.4.3 Temperature**

The temperature profile during composting may be used as an indicator of the composting stages (Haug, 1993). The composting process occurs primarily in mesophilic and thermophilic stages (Pace et al., 1995). To a point, as the temperature increases, the microbial activity increases (López-González et al., 2015). This is crucial as a higher microbial population typically results in a higher decomposition rate (Kumar et al., 2010). However, every microbe has its optimum and maximum temperature for growth, so above its maximum, though a higher temperature, would be fatal for that microbe.

At temperatures  $<40^{\circ}\text{C}$ , mesophilic microorganisms effectively decompose the organic matter and produce compost. However, if a higher temperature is attained, pathogenic microorganisms and weed seeds can be destroyed (Pace et al., 1995). The Australian Standard AS 4454 (2012), recommends that maintaining the temperature of a bed/pile  $>55^{\circ}\text{C}$  for three or more days deems the compost product to be pasteurised. Moreover, Wichuk and McCartney (2007) reported that maintaining the temperature  $>55^{\circ}\text{C}$  for three to four days inactivated pathogenic microorganisms. However, increasing the temperature higher than  $70^{\circ}\text{C}$  may also inhibit most of the beneficial compost microorganisms (Lynch et al., 2005, Imbeah, 1998, Huang et al., 2004).

Once the thermophilic stage ends, the temperature decreases progressively in the cooling stage and continues to decrease close to ambient temperature in

the maturation stage (curing phase) (Sanchez-Monedero et al., 2010). The decrease in temperature during composting could be an indicator of the lack of biodegradable material (Ravindran and Sekaran, 2010).

Self-heated composting is based on the decomposition of organic matter by aerobic microorganisms (Li et al., 2013). During exothermic oxidation, biogenic carbon dioxide and heat are released (Bialobrzewski et al., 2015, Kulikowska, 2016). The bio-heat continuously increases the temperature, which progressively changes the microbial community (Bhatia et al., 2013, Pepe et al., 2013). In contrast, using a quick external heating in-vessel unit to increase the temperature rapidly and maintain it at thermophilic, may be detrimental for the growth of microorganisms (Li et al., 2013). A comparison of self-heated and externally heated composting experiments demonstrated that gradual heating from aerobic activity resulted in three to four times more microbial biomass than external heating (Sundh and Rønne, 2002).

#### 2.4.4 Moisture content

Moisture content is important in the composting process because water is the medium for biochemical reactions where carbohydrates, lipids and proteins are broken down (Paritosh et al., 2017), and all microbes rely upon moisture to survive. During composting, through the moisture content, the dissolved nutrients are distributed to the microorganisms for their metabolic activities (Liang et al., 2003, Guo et al., 2012).

Water maintains the osmotic balance through dissolved salt concentrations, where water diffuses from low to high salt concentration (Lynch et al., 2005). The microbial cell is in a positive water balance when the water diffuses from the environment into the microbial cell, as generally, the microbial cell has a higher salt concentration. If the environment has a higher salt concentration, the water from the microbial cell diffuses to the environment, which generates dehydration, hence, the microbial growth is affected (Madigan et al., 2018). Also, the respiratory gases (e.g., oxygen and carbon dioxide) dissolve in the water (Park et al., 2001). However, having high moisture content (>60%) in the composting process, affects the rate of oxygen uptake and gas diffusion, which

may create anaerobic zones (Abdullah and Chin, 2010, Mohammad et al., 2012).

The type of organic waste will determine the optimum moisture content (Bernal et al., 2009). For composting food waste or green waste, the optimum moisture content was calculated to be ~60% (Kumar et al., 2010). However, during the composting process, (Luangwilai et al., 2011), a broad range (40 to 70%) has been reported for optimum microbial activity. The minimum moisture content for microbial activity should have a moisture content of ~30% (Zameer et al., 2010).

Water has a cooling effect by removing heat through evaporation (Margesin et al., 2006). Therefore, when the temperature increases, moisture content decreases. An observation of this inverse relationship during the composting process could be interpreted as an indicator of high microbial activity (Varma and Kalamdhad, 2015). However, allowing the moisture content to decrease below the optimum range can promote dehydration and reduce microbial activity (Makan et al., 2013).

## 2.5 Chemical parameters during composting and compost production

### 2.5.1 pH

During the first days of composting, bacteria and fungi produce short-chain organic acids which decrease the pH (Sundberg et al., 2004, Beck-Friis et al., 2001). In the early mesophilic stage of the composting process, at acidic conditions, fungi are particularly active at degrading organic matter (Klamer and Baath, 1998). The decomposition and volatilization of short-chain organic acids contribute to a progressive increase in pH during the active phase, that should stabilize close to neutral during the curing phase (Hellmann et al., 1997, Sundberg et al., 2004). pH varied more in a self-heated composting experiment than in a quick external-heating organic waste treatment (Beck-Friis et al., 2001).

During the composting process, the optimal pH should be in the range from 5 to 8 (Thompson et al., 2001). However, if the pH falls outside this range, microbial activity could decline (Sundberg et al., 2004) and the organic matter decomposition time prolonged (Smårs et al., 2002, Beck-Friis et al., 2001).

There are microbes that grow below pH 5 and above pH 8, but they are not commonly found in food waste (Madigan et al., 2018). During composting food waste, the highest decomposition rate was achieved at pH levels between 6 to 8 (Smårs et al., 2002). Mature compost with a pH in the range from 6.0 to 8.5 (Hachicha et al., 2009), can be applied to most soils (Epstein, 2011).

### **2.5.2 Electrical Conductivity (EC)**

Electrical conductivity (EC) is an indirect measure of soluble salt concentration and can be defined as a quantitative measure of the electrical current conducted through an aqueous solution (Johnson et al., 2006). During composting, EC values could increase due to moisture losses (Yadav et al., 2012). There are several studies which suggest an optimum EC range for compost. According to Tognetti, Mazzarino, and Laos, (2007), the optimum EC in a mature compost should be in the range from 1 to 3 mS cm<sup>-1</sup>, which could then be applied to most plants. Awasthi et al., (2014), set 4 mS cm<sup>-1</sup> as the maximum limit for EC. The TMECC recommend that the EC of the final compost should be <6 mS cm<sup>-1</sup> (Thompson et al., 2001). The Australian Standard AS 4454 (2012) uses a broader range of EC maximum value (10 mS cm<sup>-1</sup>).

High salt levels in compost can indicate salinity, which may affect the growth of salt sensitive plant (Grattan and Grieve, 1998). Although salt concentration in compost can be reduced by leaching techniques, nutrients may be reduced as well (Eghball et al., 1997). The application of compost can also depend on the soil type as compost with a high EC value can be mixed with soil with low a EC value (Gao et al., 2010).

### **2.5.3 Aeration**

Composting is an aerobic process, and during organic matter oxidation, oxygen is used and water vapour and biogenic carbon dioxide are released to the environment (Awasthi et al., 2014, Petric and Selimbasic, 2008). When moisture content is optimal, oxygen dissolves in water and is available for microbial use (Luangwilai et al., 2011). However, low oxygen levels during composting affect the activity of aerobic microorganisms and the efficiency of organic matter decomposition (Nakasaki et al., 2009). Increasing oxygen levels

during composting, for example by aeration in the presence of suitable moisture content, helps microbes break down organic acids, which in turn more alkaline (Sundberg and Jönsson, 2008). Hence, providing continuous aeration, with >10% of oxygen during the thermophilic phase is considered optimal for microbial activity (Epstein, 2011), as long as the moisture content is suitable. However, the specific composting process and the type of organic matter defines the optimum range of aeration (Gao et al., 2010).

Proper aeration can also facilitate increases in temperature to the thermophilic stage (Raut et al., 2008). During windrow composting, frequent turning of piles provides aeration for organic matter decomposition (Li et al., 2015). If the concentration of oxygen falls below 5%, the composting process becomes anaerobic, which could produce hydrogen sulfide gas and VFAs, which generates offensive odours (Chen et al., 2011). Forced aeration provides air at different rates (Table 2.1).

Table 2.1 Forced aeration rate during composting.

Composting	Forced aeration rate (L min <sup>-1</sup> kg <sup>-1</sup> OM)	Reference
Composting organic waste	0.19 to 0.38	(Kim et al., 2008)
Composting food waste	0.43 to 0.86	(Lu et al., 2001)
Composting MSW and poultry manure	0.9	(Petric et al., 2015)
Composting food and green waste	1	(Kumar et al., 2010)
Composting vegetable and fruit waste	0.62	(Arslan Topal et al., 2011)

In contrast, excessive aeration can lead to higher evaporation and heat loss, which cools down the composting process and inhibits the thermophilic stage (Gao et al., 2010, Sundberg and Jönsson, 2008).

#### 2.5.4 Essential nutrients

During composting, the main mineral nutrients required for microbial activity are, C, N, P and K (Pace et al., 1995). Macronutrients are required in larger amounts, and micronutrients in trace amounts. If the phyto-availability of these elements are low, the crop yield could be affected (White and Brown, 2010). Macro and micronutrients are important for cellular function (Table 2.2).

Table 2.2 Macro and micronutrients during microbial activity.

Element	Absorbed form	Cellular function
<u>Primary macronutrients</u>		
Nitrogen (N)	$\text{NO}_3^-$ , $\text{NH}_4^+$	In the chlorophyll, it forms part of proteins and nucleic acids. Nitrogen is absorbed by plants as $\text{NO}_3^-$ then it is reduced to $\text{NH}_4^+$ before it is incorporated into organic molecules <sup>2</sup> .
Phosphorus (P)	$\text{H}_2\text{PO}_4^-$ , $\text{HPO}_4^{2-}$	Phosphorus is part of nucleic acids, phospholipids and co-enzymes and provides energy through the pyrophosphate bond in ATP <sup>2</sup> .
Potassium (K)	$\text{K}^+$	Potassium act as a cofactor in several proteins which changes their configuration and activate enzymes. Also, $\text{K}^+$ can stabilise the pH by neutralising the anions in the cytoplasm <sup>2</sup> .
<u>Secondary macronutrients</u>		
Sulfur (S)	$\text{SO}_4^{2-}$	The sulfur incorporated into methionine, amino acid and cysteine, facilitate protein synthesis <sup>2</sup> .
Magnesium (Mg)	$\text{Mg}^{2+}$	Magnesium contributes to the activity of enzymes, in particular in enzymes that transfer phosphate. Also, magnesium participates in the ATP synthesis and it is a linker in the addition of ribosome subunits <sup>2</sup> .
Calcium (Ca)	$\text{Ca}^{2+}$	During the hydrolysis of the ATP, calcium acts a cofactor in the enzymes <sup>2</sup> .
<u>Micronutrients</u>		
Iron (Fe)	$\text{Fe}^{2+}$ , $\text{Fe}^{3+}$	Iron is present in cytochromes; oxygenases; catalases; iron–sulfur proteins; peroxidases; and all nitrogenases <sup>1</sup> .
Manganese (Mn)	$\text{Mn}^{2+}$	Activates several enzymes; part of certain superoxide dismutases and of the water splitting enzyme in oxygenic phototrophs (photosystem II) <sup>1</sup> .
Zinc (Zn)	$\text{Zn}^{2+}$	Zinc is required carbonic anhydrase; alcohol dehydrogenase; RNA and DNA polymerases; and many DNA binding proteins <sup>1,2</sup> .
Copper (Cu)	$\text{Cu}^+$ , $\text{Cu}^{2+}$	In respiration, in plastocyanin, in photosynthesis; cytochrome oxidase; some superoxide dismutases <sup>1</sup> .
<u>Other elements</u>		
Cobalt (Co)	$\text{Co}^{2+}$	Vitamin B12; transcarboxylase (only in propionic acid bacteria) <sup>1</sup> .
Nickel (Ni)	$\text{Ni}^{2+}$	Most hydrogenases; coenzyme F430 of methanogens; carbon monoxide dehydrogenase; urease <sup>1</sup> .
Carbon (C)		Constituent of all organic cell material <sup>1</sup> .

<sup>1</sup>. (Madigan et al., 2018); <sup>2</sup>. (George et al., 2007).

## 2.5.5 Carbon to Nitrogen ratio (C:N)

The most important elements during composting are C (as a source of energy for microorganisms) and N (as building blocks of cell structure) (Iqbal et al., 2015, Chen et al., 2011). Nitrogen is also a constituent of enzymes, amino acids, DNA and proteins (Madigan et al., 2018). The C:N ratio of composting inputs is strongly dependent on the chemical properties of the organic matter

(Adhikari et al., 2008), and the right mix of inputs based on the optimum C:N ratio will deliver the right amount of carbon and nitrogen required for microbial activity (Chen et al., 2011).

During the composting process, the C:N ratio changes continuously because the mineralisation rate of organic C is faster than organic N (Yang et al., 2013). Furthermore, the microorganisms consume C at a faster rate (30 to 35 times faster) than N (Hilkiah Igoni et al., 2008). During the oxidation process, C is released as carbon dioxide and the C:N ratio decreases, which can be used as an indicator of the decomposition rate (Lazcano et al., 2008).

The initial optimum C:N ratio for composting should be 25:1 to 30:1 (Huang et al., 2004). If the initial C:N ratio is higher than the optimum, it will affect microbial growth due to N limitation, which will slow down the decomposition rate and lower the temperature (Hilkiah Igoni et al., 2008). In contrast, if the C:N ratio is lower than the optimum and the pH is above 8, the excess N can be transformed into ammonia (in large percentages) or nitrous oxide (small amounts), which also changes the C:N ratio (Xiying and Benke, 2008). Denitrification may also contribute to nitrogen losses when nitrates are transformed into nitrous oxide or nitrogen gas (Martins and Dewes, 1992). The release of most of these gases generates undesirable odours (Mohee et al., 2015). As denitrification is an anaerobic process, providing enough aeration to the composting process, denitrification can be minimised (Mahimairaja et al., 1995).

Before starting the composting process, the initial C:N ratio can be adjusted by mixing materials with lower and higher C:N ratio. To increase the C:N ratio, materials such as sawdust, wood chips, rice husks, among other bulking agent can be used (Wang et al., 2015, Zhang and Sun, 2016). The bulking agent not only raises the C:N ratio, but also reduces excessive moisture and controls odours (Imbeah, 1998). Composted organic matter with a low initial C:N ratio takes longer to mature (Huang et al., 2004). Hence, during the curing phase, the higher the decomposition of the organic matter, the lower is the C:N ratio (Lazcano et al., 2008). When the organic matter is fully metabolised, the bacterial activity slows and reproduction ceases (Lowenfels and Lewis, 2010).

The C:N in the final compost is reduced to ~10:1 to ~15:1 and this stable mature compost does not release odours (Chen et al., 2011).

## **2.6 Microbial ecology during composting**

### **2.6.1 Composting microbiome**

Cellular living organisms are classically divided into three domains on the basis of gene sequence data comparisons and are known as bacteria and archaea, collectively referred to as prokaryotes, and eukarya (Madigan et al., 2018) (see Figure 2.6). Prokaryotes can colonise all livable spaces such as soil, water, plants, and animals (Whitman et al., 1998). Bacteria are single-celled organisms with cell membranes surrounded by walls of varying chemicals, but largely peptidoglycan (Madigan et al., 2018). Generally, the dimensions of these microorganisms are between 0.5 µm to 5.0 µm and they can be found in spherical, spiral or rod shapes (Woese et al., 1990).

To identify microorganisms from environmental samples, pure-culture-dependent and non-pure-culture-dependent methods can be used. The culture-dependent methods have limitations because more than 99% of prokaryotes are unculturable (Amann et al., 1995), however, this percentage is debated today. In contrast, using non-culture-dependent methods, also largely modern molecular methods, the culturable and unculturable microorganisms can be identified (Mullis et al., 1986, Woese, 1987). In most microbial studies, including composting studies, molecular biological analyses are currently being used (Neher et al., 2013, Franke-Whittle et al., 2014).

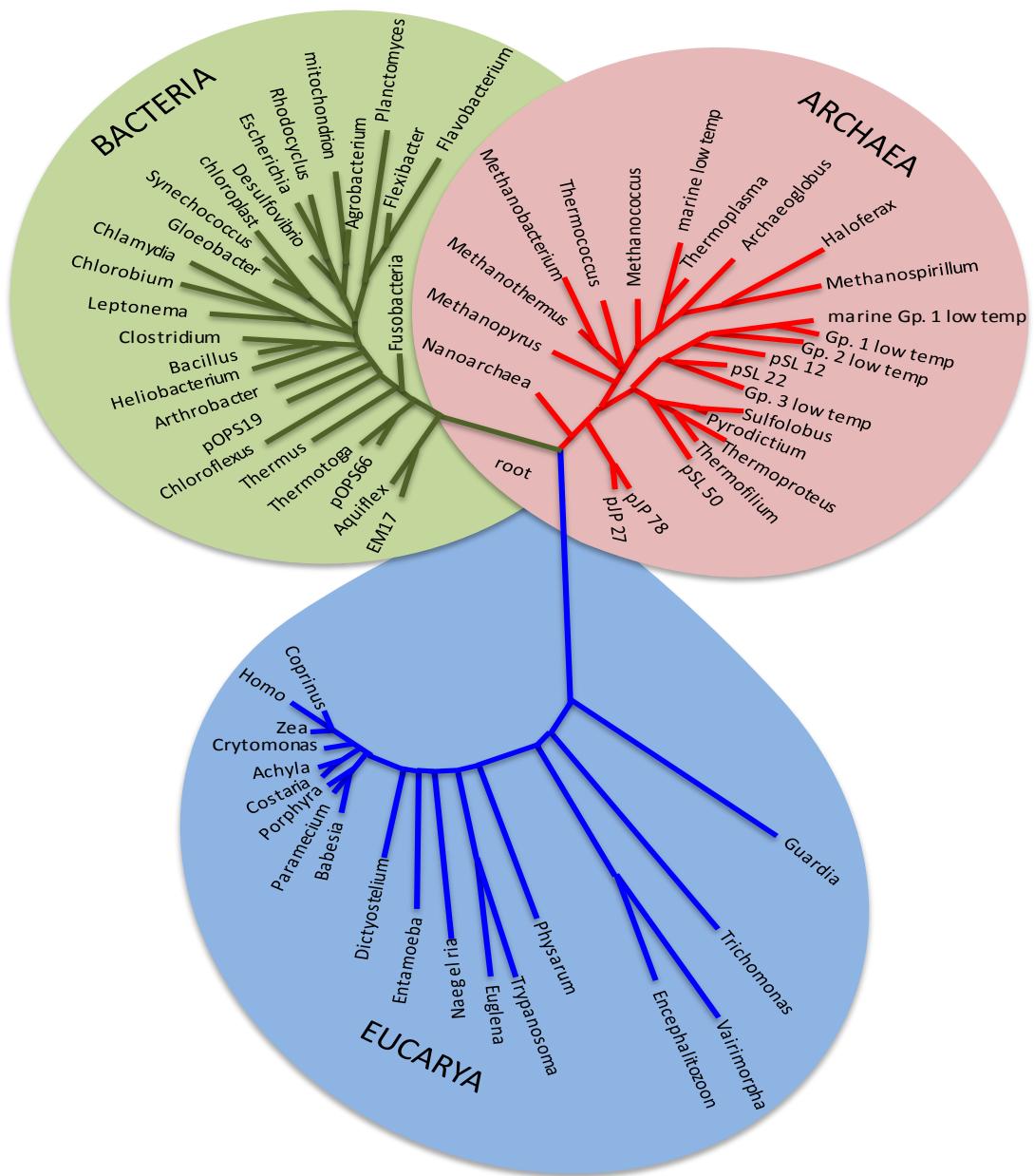


Figure 2.6 An evolutionary tree of the three domains of cellular life as revealed by genetic analyses, adapted from (Lineweaver and Chopra, 2011).

The identification of microorganisms during composting is conducted in order to understand the compost microbiome (Franke-Whittle et al., 2014, López-González et al., 2015), and to determine which organisms are most functionally relevant. The main challenges in obtaining representative composting samples for microbial analysis pertain to the heterogeneity of the organic material and the large sample size of a composting process (López-González et al., 2015). Composting is a complex environment, where physical and chemical factors influence the microbial diversity and abundance, and vice-versa (Li et al., 2019).

Table 2.3 Microbial diversity during composting.

Microbes during composting	Microbiota	Reference
Higher diversity of mesophilic	Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria	(Wang et al., 2017)
Nitrogen-fixing	<i>Pseudomonas</i> , <i>Xanthomonas</i> , <i>Klebsiella</i> , <i>Alcaligenes</i> , <i>Caulobacter</i> , <i>Achromobacter</i> and <i>Stenotrophomonas</i>	(Pepe et al., 2013)
Thermotolerant	<i>Bacillus</i> strains	(Mayende et al., 2006)

During the mesophilic and thermophilic stage, bacteria dominate other microorganisms in the composting environment because they are more successfully competitive in degrading simple carbohydrates, proteins and lipids (Ryckeboer et al., 2003). Temperature plays an important role in composting since mesophilic microorganisms are resilient to temperature changes while thermophilic microorganisms are sensitive (Cooper et al., 2002).

Generally, at the beginning of the composting process acid-producing bacteria dominate, which drops the pH, affecting the microbial growth (Sundberg et al., 2011). Under acidic conditions, microorganisms produce odours, which can be minimised by providing aeration (Sundberg et al., 2013). Different members of the complex microbial community (bacteria and fungi) may rapidly degrade certain components of organic matter (Lowenfels and Lewis, 2010). However, complex materials such as hemicellulose and lignin are relatively slow to be degraded by bacterial enzymes. At this point, fungi apparently dominate the community and continue decomposing in the curing phase (Amir et al., 2008). However, prokaryotes will definitely play a major role and the role of fungi has not been clarified yet. During the composting process, microorganisms including bacteria release enzymes such as dehydrogenase, beta-glucosidase, arylsulfatase, phosphatase, urease (Castaldi et al., 2008). These enzymes catabolise organic matter polymers to their component monomers (e.g., sugars, amino acids) into small electrically charged nutrients such as nitrogen compounds (ammonium, nitrate), phosphorus, sulfur, and potassium (Lowenfels and Lewis, 2010).

### **Microbial inoculation**

Although organic matter is naturally decomposed by indigenous microorganisms, adding specific inocula may improve the decomposition rate (Karnchanawong and Nissaikla, 2014, Onwosi et al., 2017). The characteristics of the organic matter determine the type of inoculum to be used during composting (Ke et al., 2010). By inoculating anti-acidification bacterial consortia such as *Pseudomonas*, *Bacillus*, and *Lactobacillus saliva* (sic), among others, the initial composting acidification would be minimised (Ding et al., 2016). However, contrary to this, *Lactobacillus* spp. are strong acid producers (particularly lactic but also acetic), so it is not possible that they could be deemed “anti-acidification”. Adding *Thermoactinomycetes vulgaris* A31 during the thermophilic stage of food waste composting has been shown to accelerate the decomposition rate (Ke et al., 2010). During the curing phase, fungal consortia including *Aspergillus flavus* MTCC 1425, *Aspergillus niger* MTCC and *Trichoderma viride* MTCC 793 can be added to enhance the degradation rate of MSW (Awasthi et al., 2014).

According to Karnchanawong and Nissaikla, (2014), commercial microbial inocula might not be essential to improve the composting process. Instead, adding mature compost enhances the decomposition rate. Furthermore, by adding different types of organic matter, indigenous microbes would be naturally inoculated (Zainudin et al., 2013). For example, in a study of composted kitchen waste, no significant differences in the final C:N ratio were found between composting processes with or without a lignocellulosic microbiota inoculum (Nair and Okamitsu, 2010). Finally, the microbial communities present depend on the type of organic waste, composting technique, and environmental conditions, hence, one study cannot be used as a model for others (Karnchanawong and Nissaikla, 2014).

#### **2.6.2 Pathogenic microorganisms**

Along with beneficial organic matter degradative microbes, compost can also harbour pathogenic microorganisms (Kim et al., 2009). For instance, food waste might contain the pathogenic bacteria like *Campylobacter*, *Listeria*, *Shigella*, *Yersinia*, *Vibrio*, *Salmonella*, Shiga toxin-producing (STEC) O157 and non-

O157 *Escherichia coli* as well as parasitic eukaryotes like *Cyclospora* and *Cryptosporidium*, (Holley, 2011).

Generally, products such as poultry meat, eggs, lettuce, sprouts, spinach and cantaloupe can be associated with *Salmonella* spp. (Beuchat, 1996). The genus *Salmonella* contains two species (*S. enterica* and *S. bongori*), but there are more than 2400 serotypes, and most *S. enterica* can affect human health (Blaser and Newman, 1982) *E. coli* can be found in beef, lettuce, sprouts, spinach, and cantaloupe. *E. coli* bacteria live in the digestive tract of mammals where they generally have beneficial roles. However, enterohaemorrhagic *E. coli* of the O157 serotype group (specifically *E. coli* O157:H7) can have negative effects on human health (Lim et al., 2010). *Salmonella* spp. and *E. coli* O157:H7 can survive outside their mammalian hosts for days and even months and are commonly found in animal and human waste (Stephens et al., 2007).

Different feedstocks have varying concentrations of pathogenic bacteria (Manyi-Loh et al., 2016), and depending on the composting process, pathogenic microbes can decay or grow rapidly (Lemunier et al., 2005). During the first days of composting (still mesophilic), indigenous microbes compete strongly against pathogens for nutrients and can outcompete them (Millner et al., 1987).

Sewage sludge, also called biosolids, may be treated by composting. High concentrations of potentially pathogenic microorganisms are typically present in biosolids (e.g., *Salmonella* spp. and *E. coli* O157:H7 and the eukaryote *Giardia*). However, composted biosolids must also comply with the requirements of compost standards before being used as fertiliser (Zaleski et al., 2005).

National and international standards (e.g., Australian Standard AS 4454–2012) require that the composting process must be kept at 55°C for at least three consecutive days to eliminate non-spore forming pathogenic microbes. It is generally considered that pathogenic microbes cannot grow at temperatures above 55°C (Jones and Martin, 2003). However, some pathogens generate spores to survive this phase, and thermotolerant coliforms (able to grow at the lower thermophilic temperatures of 44°C to 45°C) must be considered as these

are typically used as sanitation indicators in environmental samples (Lemunier et al., 2005).

Most pathogenic microbes can survive at mesophilic and low thermophilic temperatures. The optimum growth temperature for *E. coli* is around 37°C, and temperatures above 48°C are lethal, thus demonstrating how high temperature mitigates pathogenic microbes (Madigan et al., 2018). Pathogenic microbes must be eliminated in the active phase of the composting process because, in the curing period, where the temperature is below 40°C, the pathogenic microbes might proliferate (Millner et al., 1987).

### **Conclusions**

Composting has naturally occurred for millennia. It has also been employed by humans in a directed fashion, and some of the physicochemical parameters are well understood. Much of the knowledge of the microbes in compost come from application of traditional pure-culture methods. In recent times with increasing generation of urban organic waste, sustainable solutions for its treatment are needed and composting is the most applicable. There are many gaps in knowledge, particularly around small-medium in-vessel composting systems and this thesis focusses on bridging these gaps.

## Chapter 3

# Materials and Methods

## 3.1 Introduction

Physical, chemical and microbial analytical techniques are all employed to understand the composting process, and in this research all of these approaches were used. The compost texts, Test Method for the Examination of Composting and Compost (TMECC) (Thompson et al., 2001), and the Australian Standard: Composts, soil conditioners and mulches – AS 4454–2012, were used for most physical, chemical and microbial tests carried out. Comparative information of the optimum parameters is shown in Table 3.1.

The Australian Standard – AS 4454 (2012) describes the parameters that facilitate safe pasteurising or composting of organic wastes in medium and large scale, but it does not apply to home composting. According to AS 4454–2012, compost is defined as: “*An organic product that has undergone controlled aerobic and thermophilic biological transformation through the composting process to achieve pasteurisation and reduce phytotoxic compounds, and achieved a specific level of maturity required for compost*”. It categorises the organic mature product after composting as: “*Raw mulch; pasteurised product; composted product; and mature compost*”.

In this research, two commercial in-vessel units were used to treat urban organic solid waste collected mostly from university café precincts and mulches obtained from a suburban food market in Melbourne, Australia. A 20 kg d<sup>-1</sup> capacity in-vessel unit from Closed Loop (Chapter 4) and a 100 kg d<sup>-1</sup> capacity unit On-Site Composting Apparatus (OSCA) obtained from Worms Downunder - WDU Sustainability Pty. Ltd. (now operated by Global Composting Solutions) (Chapter 5) and a prototype in-vessel composter called Cylibox (Cylinder in a box) (Chapter 6) were all studied and the details of the input waste are all described in the relevant chapters.

Table 3.1 Optimum physical, chemical and microbial parameters of compost and the composting process according to the two given texts.

Parameters	TMECC 2001	AS 4454-2012
<b>PHYSICAL</b>		
Temperature (°C)	Mesophilic: Ambient to 40°C Thermophilic: 40°C to 70°C Pasteurization: ~55°C for three days	Pasteurization: Keep at ~55°C for three days
Moisture (%)	Initial: ~60% (wet) End: ~30% (Dry)	Min. 25% Max. 50%
Particle size	<5 cm	5 mm to 16 mm
<b>CHEMICAL</b>		
pH	5.0 to 8.0	Min. 5
Electrical Conductivity (mS cm <sup>-1</sup> )	< 6	Max. 10
C:N ratio	Initial: 30:1 End: ~21:1	Level appropriate for application specific products
C:P ratio	Initial: ~100:1 to 140:1 End: ~85:1 to 90:1	---
<u>Primary macronutrients</u>		
Total Nitrogen (%)	0.5 to 6.0	≥ 0.8
Phosphorus	0.2 to 3.0 (%)	≤ 5 (mg L <sup>-1</sup> )
Potassium (%)	0.1 to 3.5	---
<u>Secondary macronutrients</u>		
Sulfur (mg kg <sup>-1</sup> )	Small quantities	---
Magnesium (mg kg <sup>-1</sup> )	Small quantities	---
Calcium (mg kg <sup>-1</sup> )	Small quantities	---
<u>Micronutrients</u>		
Iron (mg kg <sup>-1</sup> )	Small quantities	---
Manganese (mg kg <sup>-1</sup> )	Small quantities	---
Zinc (mg kg <sup>-1</sup> )	27 to 150	---
Copper (mg kg <sup>-1</sup> )	5 to 30	150
<u>Other elements</u>		
Cobalt (mg kg <sup>-1</sup> )	0.02 to 1.0	---
Nickel (mg kg <sup>-1</sup> )	< 50	---
Organic Carbon (%)	---	≥ 20
<b>BIOLOGICAL</b>		
<u>Pathogens</u>		
<i>Escherichia coli</i> (MPN g <sup>-1</sup> total compost)	< 1000	< 1000
<i>Salmonella</i> spp.	< 0.75 MPN g <sup>-1</sup>	Absent in 50g (dry weight equivalent)
<i>Enterococcus</i> spp. (MPN g <sup>-1</sup> total compost)	< 1000	< 1000
<b>MATURITY</b>		
Solvita® maturity test	≥ 6 (For CO <sub>2</sub> ) ≥ 5 (For NH <sub>3</sub> )	≥ 7 or 8 (For CO <sub>2</sub> ) ≥ 5 (For NH <sub>3</sub> )

## 3.2 Input preparation, sampling and sample preparation

### 3.2.1 Particle size of the organic waste

Prior to in-vessel treatment, large pieces of urban waste were cut to reduce the particle size to <5 cm in diameter (Rynk et al., 1992). The organic matter was cut manually with a knife on a chopping board. The reduction of the particle size of organic matter contributes to a better distribution of moisture and nutrients during composting. However, if the particle size is too small, it might reduce the air space and create anaerobic zones due to the lack of oxygen (Cooperband, 2000).

### 3.2.2 Sampling

Samples were obtained from in-vessel units during the treatment process while wearing nitrile gloves. They were taken in triplicate from different locations in the treatment bed after the bed had been automatically mixed at mixing regimes in the case of Closed Loop and OSCA in-vessel units, and manually in case of Cylibox, as detailed in Chapters 4, 5 and 6. Each sample was collected in triplicate in sterile 50 mL polypropylene tubes (Falcon™), then stored immediately at -80°C.

### 3.2.3 Sample preparation

Samples stored at -80°C were defrosted at ambient temperature and sieved with a 9.5 mm sieve to reduce the particle size, with only large particles like bones not passing through the sieve. A total of 4 g of sieved sample was added to 20 mL of distilled water in a 50 mL polypropylene tube and, shaken for 30 min in an orbital shaker incubator at 180 rpm. The slurry was used to measure, pH, electrical conductivity (EC) and water-soluble elements.

## 3.3 Physical and chemical analysis

### 3.3.1 Temperature

The temperature was measured by using a PT100 waterproof thermometer with a probe (CENTER® RTD C370-IC), measuring in the range from -100°C to 300°C and with an accuracy of +/-0.4°C). The probe was carefully plunged into

the compost bed at three different locations, each temperature was recorded, and the average temperature was calculated.

Continuous temperature monitoring during urban food waste treatment employed a temperature data logger called Tinytag Splash 2 TG-4105 (-30 to +105°C) and Tinytag Explorer Data Logging Software. The organic food waste and the Tinytags were placed into the in-vessel unit. The temperature data were downloaded using the Explorer software. The recorded data are shown in a graphical form, displaying the maximum, minimum and average temperature measured during the food waste treatment.

### 3.3.2 Moisture content

The moisture content of slurries was measured by the oven-dry method (TMECC 03.09–A Total Solids and Moisture at 70±5°C). The incubator (Memmert INE600) was set at 70±5°C, since higher temperatures could facilitate loss of some volatile organic compounds, which would affect the correct moisture measurement. The wet weight of the sample in a 50 mL glass beaker was determined (Cheetah JA2603B balance with 1 mg precision); the beaker and sample were dried in the oven for 24 hours; and the dry weight was measured.

The following equation was used to calculate the moisture content percentage.

$$\text{Moisture content (\%)} \quad [\text{Equation 1}]$$

$$MC = \left[ \frac{W_w - W_d}{W_w} \right] 100$$

**Where:**

$MC$ : Moisture content (%) of the sample

$W_w$ : Wet weight of the sample

$W_d$ : Dry weight of the sample

### 3.3.3 pH

The pH of the slurry was measured using a TPS smartCHEM pH meter. The pH meter was calibrated each 10-12 sample measurements (Method 04.11 section at TMECC). The pH probe was cleaned with 70% ethanol between each

sample measurement to preclude buildup of films from sample organic acids, which could reduce accuracy.

### 3.3.4 Electrical conductivity

The EC of slurries in  $\text{mS cm}^{-1}$  was measured with an electrical conductivity probe using a TPS smartCHEM meter, which was calibrated using a standard solution of  $2.76 \text{ mS cm}^{-1}$  (Method 04.10 section at TMECC). The EC probe was cleaned with 70% ethanol between each sample measurement to preclude buildup of films from sample organic acids, which could reduce accuracy.

### 3.3.5 Digestion technique - water soluble elements

The slurries were centrifuged at  $8000 \text{ g}$  for 15 minutes. The supernatant was filtered through a  $0.45 \mu\text{m}$  membrane (Gilson SupaTop™ nylon syringe filter 25 mm pink) and  $500 \mu\text{L}$  of filtrate was added to a  $25 \text{ mL}$  glass volumetric flask. It was made to volume with 2% nitric acid ( $\text{HNO}_3$ ) and mixed by inversion. The chemical elements in the shaken mixture were determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) iCAP 7000 SERIES Thermo Scientific (Method 04.12-D section at TMECC). To calibrate the ICP-AES equipment, standards at  $0.05 \text{ ppm}$ ,  $0.1 \text{ ppm}$ ,  $0.5 \text{ ppm}$ , and  $1 \text{ ppm}$ , were prepared by mixing  $\text{HNO}_3$  with milli-Q® water. The results were reported on a dry weight basis as  $\text{mg g}^{-1}$  (Method 04.12-D section at TMECC).

### 3.3.6 Carbon to nitrogen ratio (C:N)

Organic carbon is the potentially biodegradable carbon of organic matter and total nitrogen comprises organic and inorganic nitrogen. The total carbon and total nitrogen analyser “LECO” TruMac™ 928 Series Macro Determinator was used to determine the total organic carbon and total nitrogen. In this research  $0.5 \text{ g}$  of sample was used to determine the carbon/nitrogen. Under ultra-high purity oxygen, the samples are oxidised at temperatures up to  $1,450^\circ\text{C}$ , where they become carbon dioxide and nitrogen gases, which go through an infrared detector. The moisture generated during the combustion gas is removed by a thermoelectric cooler (Method 05.02-A section at TMECC).

To calculate the C:N ratio of the mixture of more materials the following equation was used.

**C:N ratio of the mixture***[Equation 2]*

$$C:N(mixture) = \frac{\sum_{n=1}^{\infty} (Q_n [C_n (100 - M_n)])}{\sum_{n=1}^{\infty} (Q_n [N_n (100 - M_n)])}$$

**Where:***C:N (mixture)*: C:N ratio of the resulting materials to compost*Q<sub>n</sub>*: Quantity of the fresh material (*n*)*C<sub>n</sub>*: Total carbon content of the dry material (*n*)*M<sub>n</sub>*: moisture content of the fresh material (*n*)*N<sub>n</sub>*: Total nitrogen content of the dry material (*n*)

The quantity of the fresh material (organic waste) (*n*) will be determined based on the type of organic waste to be treated in each experiment.

This equation was used to adjust the C:N ratio mixture of the inputs in the composting experiments.

### 3.3.7 Solvita® maturity test

The Solvita® Maturity Test was used to determine treatment end-product maturity. It measures the production of carbon dioxide and ammonia due to microbial respiration in material like compost. Carbon dioxide production in the range from 2 - 30 mg CO<sub>2</sub> g<sup>-1</sup> sample day<sup>-1</sup> and ammonia production in the range from 200 - 20,000 mg NH<sub>3</sub> + NH<sub>4</sub><sup>+</sup>-N kg<sup>-1</sup> sample day<sup>-1</sup> can be measured. A compost sample of 40-60% moisture content is added to a test jar (re-moisturisation could be required) and incubated for 24 hours to facilitate regrowth of microbes, then Solvita test probes or paddles (one each for carbon dioxide and ammonia) are inserted into the sample, the jar is tightly sealed and left for four hours at 20°C to 25°C out of sunlight before reading. The paddles contain gels that are highly reactive and respond rapidly to carbon dioxide and ammonia gases as they are released naturally from a sample into the headspace of the test jar.

A colourimetric comparative scale is used to determine the carbon dioxide and ammonia values after the four hours period (Figure 3.1). For carbon dioxide, the values range from 1 (highest carbon dioxide) to 8 (lowest carbon dioxide) and for ammonia, values range from 1 (highest ammonia) to 5 (lowest ammonia) (Method 05.02-A section at TMECC).

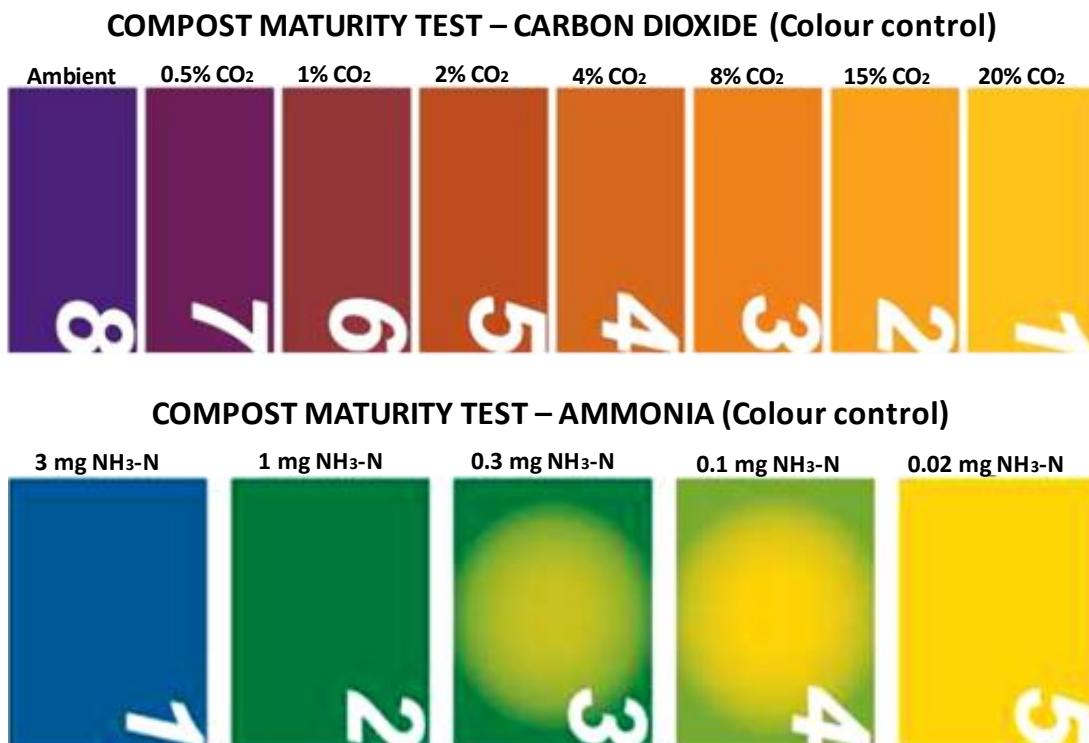


Figure 3.1 Solvita® compost maturity colourimetric comparative scale, adapted from Solvita®.

The Compost Maturity Index (CMI) is determined from the Solvita Calculator and the material deemed to be fresh mix; ideal active or ideal curing; or mature compost (Table 3.2).

Table 3.2 Compost maturity index calculator.

		Solvita® Carbon Dioxide test results								
		1	2	3	4	5	6	7	8	
Solvita® Ammonia test results	5	Very low/ No NH <sub>3</sub>	1	2	3	4	5	6	7	8
	4	Low NH <sub>3</sub>	1	2	3	4	5	6	7	8
	3	Medium NH <sub>3</sub>	1	1	2	3	4	5	6	7
	2	High NH <sub>3</sub>	1	1	1	2	3	4	5	6
	1	Very high NH <sub>3</sub>	1	1	1	1	1	2	3	4
	Example: if the NH <sub>3</sub> result is 2, and the CO <sub>2</sub> result is 6, then the Maturity Index is: 4.									

Additionally, to know the status of the composting process are given (Figure 3.2).

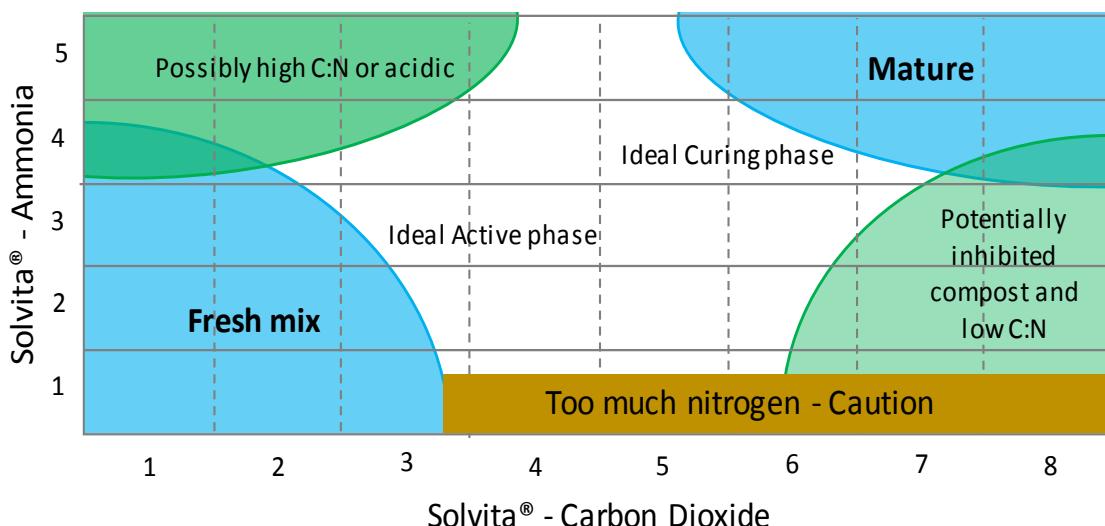


Figure 3.2 Status of composting process, adapted from Solvita®.

The Solvita test measures the biological production of carbon dioxide and ammonia in a batch test setting and since it determines compost maturity, it should only be used with the material during the curing phase so that its maturity can be determined.

### 3.3.8 Data analysis

The open source software R-Studio (R v3.6.0) (R-Core-Team, 2018) was used to analyse the physical and chemical data. The results for temperature, moisture content, pH, electrical conductivity and mineral nutrients (water soluble elements) were analysed using the Principal Component Analysis (PCA) statistical procedure. To plot the PCA graphs a ggplot2 (v3.2.1) based biplot ggbio (v0.55) package was used.

## 3.4 Molecular microbial analysis

### 3.4.1 Metabarcoding of the 16S rRNA genes

Prior to identification of prokaryotic microorganisms, the samples stored at -80°C, were defrosted at ambient temperature and then used in metabarcoding analysis.

#### Genomic DNA extraction

Genomic DNA (gDNA) was extracted from 0.25 g of thawed samples using the DNeasy® PowerLyzer® PowerSoil® kit (DNA kit) according to the manufacturer's

instructions (QIAGEN). To detect contaminations during gDNA extraction, negative controls such as sterilised Milli-Q® water and no DNA template in the DNA extraction were used. DNA was checked for purity and roughly quantified by 1% agarose gel electrophoresis using TAE buffer (Tris-acetate-EDTA (Ethylenediaminetetraacetic acid (EDTA)).

### Library Preparation

Samples from Closed Loop experiments (Chapter 4) were sequenced at the Australian Centre for Ecogenomics (ACE), The University of Queensland, Australia from extracted DNA. Samples from OSCA (Chapter 5) and Cylibox (Chapter 6) experiments were sequenced at the Walter and Eliza Hall Institute (WEHI). The adapted Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) overhang sequencing protocol of Mr. Stephen Wilcox, WEHI was followed for metabarcoding of the 16S rRNA genes from DNA extracted from OSCA and Cylibox samples (see Appendix A). The V3-V4 region of the 16S rRNA genes from sample genomic DNA (Klindworth et al., 2013) was analysed.

#### 3.4.2 Data analysis

##### Sequence data analysis

Generated partial 16S rRNA gene amplicon sequence data were bioinformatically analysed by the Quantitative Insights Into Microbial Ecology (QIIME) pipeline, version qiime2-2019.10 (see Appendix B) (Bolyen et al., 2019). Through the terminal emulator MobaXterm, QIIME2 was installed on the National Collaborative Research Infrastructure Strategy (NCRIS) cloud facility via the National eResearch Collaboration Tools and Resources project (Nectar), which is an online high-speed cloud computing system. An instance with Ubuntu 18.0 (the operating system is based on Linux) was created in Nectar, then using the instance's IP was interconnected with the MobaXterm. The QIIME2 tutorial was followed to install Miniconda and QIIME2-2019.10.

Demultiplexed raw amplicon sequence data were uploaded through MobaXterm and imported into QIIME2, the WEHI overhang sequences were removed with cutadapt version 2.6 (Martin, 2011), then the sequence quality assessment was performed. The quality sequenced reads were shown in a two-

dimensional chart, then the better-quality sequence reads were filtered, denoised and chimera checked using DADA2 (Callahan et al., 2016). The amplicon sequence variants (ASVs) were obtained by grouping the sequences. The ASVs table generated was rarefied to normalise the number of reads to analyse based on sequence data from all the samples. The Greengenes database (McDonald et al., 2012) classifier (Greengenes 13\_8 99% OTUs) was used to carry out taxonomic analysis. The ASVs corresponding to mitochondria and chloroplast were removed.

To continue with the data analysis, the ASV table, the taxonomic assignment and metadata generated in QIIME2-2019.10 were imported to R-studio with a threshold significance level at  $\alpha = 0.05$  (see Appendix C). Before processing the data, the packages, phyloseq (McMurdie and Holmes, 2013), ape, vegan (Oksanen et al., 2018), decontam (Davis et al., 2018), ggplot2 (Wickham, 2019), microbiome (Lahti and Shetty, 2017), gridExtra (Auguie and Antonov, 2017), car (Fox et al., 2019), and mvabund (Wang et al., 2012) were installed.

First the QIIME2 outputs were transformed into a phyloseq compatible format (McMurdie and Holmes, 2013). To know if the sequencing captured all the microbial diversity, the rarefaction curve was plotted (ASVs vs Reads) in the vegan package. Contaminating DNA might come from reactants (DNA kit), water or could come from inappropriate laboratory practices. Applying the 'prevalence' script in the decontam package, the contaminant DNA sequences were identified and removed (Davis et al., 2018). The bacterial community composition was visualised in the stacked taxabarplots which were generated in ggplot2 at different taxonomic levels.

To compare the alpha diversity, the bacterial community richness was calculated based on the observed ASVs. The species evenness was assessed by Simpson's Index (Simpson, 1949), and the overall species diversity was measured by Shannon's Index (Shannon and Weaver, 1949). Applying the vegan package and based on Bray-Curtis dissimilarity of Hellinger transformed data, the Non-Metric Multidimensional Scaling (NMDS) ordination was represented for beta diversity (Buttigieg and Ramette, 2014). Finally, a heatmap was plotted for the 20 most abundant bacterial taxa at the genus level.

## 3.5 Pure culturing

### 3.5.1 Indicator bacteria

TMECC protocols were used to determine the presence of *Escherichia coli* (Method 07.01-C section at TMECC), *Salmonella* spp. (Method 07.02 section at TMECC), and *Enterococcus* spp. (Method 07.03 section at TMECC) in samples at the completion of processing. Control cultures for these three targets were *Escherichia coli* O157:H7, ATCC 43895, *Salmonella typhimurium*, ATCC 13311 and *Enterococcus faecalis*, ATCC 19433.

The -80°C stored samples were thawed at ambient temperature and 1 g of sample was added to 9 mL of Difco™ peptone water (15 g in 1 L of Milli-Q® water) and vortexed for two min. One mL of the 10<sup>-1</sup> dilution was added to 9 mL of peptone water to create a 10<sup>-2</sup> dilution, which was vortexed for two min. Attempts were made to isolate *E. coli*, *Salmonella* spp., and *Enterococcus* spp. on the selective media Eosin Methylene Blue Agar (Merck KgaA EMB Agar), Xylose Lysine Deoxycholate agar (BD Difco™ XLD agar) and Kenner Fecal Agar (Oxoid Ltd. KF Agar), respectively. The media were prepared according to the manufacturer's instructions and 100 µL of diluted samples were spread inoculated and incubated at 37°C for 24 to 48 hours.

Different colony types, based on color and morphology, were sub-cultured to purity and then grown on Nutrient Agar (Oxoid Ltd. NA).

#### Pure culture DNA extraction

The genomic DNA of isolated bacterial pure cultures was extracted by using a salting out DNA extraction protocol (see Appendix D).

#### 16S ribosomal RNA gene amplification by PCR

The 16S rRNA genes were amplified by using 25 µL MangoMix™, 1 µL 27F primer (5' AGAGTTTGATCCTGGCTCAG 3') and 1 µL 1492R primer (5' GGTTACCTTGTACGACTT 3') (both at 0.1 µM) (Lane, 1991), and, based on the concentration of the genomic DNA, Y µL genomic DNA (to achieve up 200ng DNA in 50 µL) and X µL of sterile Milli-Q® water to generate a final volume of 50 µL per reaction was prepared. PCR was carried out with a Bio-Rad thermalcycler with the following program: 95°C for 5 min; 30 cycles of 95°C

for 40 s, 55°C for 40 s, 72°C for 1 min; 72°C for 10 min; and 15°C indefinite. PCR products were checked by 1% agarose gel electrophoresis.

### Purification of PCR products

The Wizard® SV Gel and PCR Clean-Up protocol generated purified PCR products that were evaluated by 1% agarose gel electrophoresis. When amplicates generated clean, distinct single bands of ~1,500 nucleotides (according to a molecular weight ladder) on the gel, they were sent for sequencing to the Australian Genome Research Facility or Macrogen (Korea), using 27F primer to determine the 16S rRNA gene by Sanger sequencing.

#### 3.5.2 Data analysis

Sequence chromatograms were visualised in the BioEditor sequence alignment Editor software and corrected sequences prepared. The DNA sequences were analysed through the National Center for Biotechnology Information (NCBI) website by the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) to determine the closest matches. Sequences were phylogenetically analysed by Molecular Evolutionary Genetics Analysis (MEGA 7.0.26) (Kumar et al., 2016) software and phylogenetic trees generated. For sequence alignment, ClustalW was used and by applying the Neighbor Joining clustering method (Saitou and Nei, 1987) the dendograms were drawn with 1000 bootstrap resamplings (Felsenstein, 1985).

#### 3.5.3 Counting bacterial colonies

Plate Count Agar BD Difco™ (PCA) was prepared to facilitate determination of total bacterial colony counts in -80°C stored samples, which were thawed at ambient temperature. Due to the different concentrations of bacteria present during treatment, a suitable dilution factor for all samples had to be determined by trial and error. Once the optimal dilution factor was found, it was applied to all the samples and 1 mL of various dilutions were inoculated to ~20 mL PCA, plates were poured, allowed to solidify and incubated at 37°C for 24 hours. Colonies were counted and reported in colony forming units (CFU) g<sup>-1</sup> sample.

## Chapter 4

# Treatment of organic waste in the in-vessel unit Closed Loop (CL)

### 4.1 Summary

A commercial 20 kg d<sup>-1</sup> capacity in-vessel unit (CLO-10) from Closed Loop Environmental Solutions Pty. Ltd. (CL) was used. Organic waste was collected from university caf  s and, for most experiments, the starting material comprised ~55% coffee grounds, ~20% noodles and rice, ~14% vegetables, ~12% fruits, ~0.5% chicken meat, and ~0.5% of food plate scrapings (called “off plate”).

CLO-10 was used to treat organic waste following the manufacturer’s instructions and, during the first operational cycle, was dosed with their proprietary bacterial inoculum. Based on the literature, the C:N ratio of the input was calculated to be ~10:1 to ~20:1 and not adjusted for any CL experiments. The first experiment, CL1, comprised five sub-experiments (CL1.1, CL1.2, CL1.3, CL1.4 and CL1.5), in which each ran for 24 hours. In most of the five sub-experiments, the operating temperature was in the range from 35°C to 55°C. However, as the moisture content increased after process initiation due to water released from the organic matter, the external heating system automatically activated, and the temperature, though erratic, occasionally reached ~80°C. Despite this, the temperature did not follow conventional composting stages of mesophilic, thermophilic, mesophilic. Continuous mixing via internal paddles, high operating temperature and strong air ventilation caused the moisture content of the in-vessel material to be reduced below the optimal range of 40% to 60%.

During the five sub-experiments, the initial pH of ~4.90 to 5.20 dropped slightly, and the electrical conductivity (EC) increased from 1.8-2.7 mS cm<sup>-1</sup> to 2.7-4.5 mS cm<sup>-1</sup>. Each CL1 sub-experiment showed differences in microbial communities during processing, but all were initially dominated by (in decreasing relative abundance) Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. A trend of increasing lactic acid bacteria (~33%) in the waste bed during treatment

correlated with a reduction in pH. No sub-experiment showed the presence of indicator pathogens after 24 hours.

A seven day CL2 experiment, based on the CL1.2 organic waste composition and processing conditions was performed in an effort to improve the organic waste degradation. Due to continuous moisture loss in CL1 operations, five L of tap water were added every seven hours into the CLO-10 chamber. Despite this, the moisture content of the compost bed after seven hours was close to 40%, and after 30 hours, it was below 15%. The temperature went from 26°C to 48°C after two days of operation, then remained at 35°C to 45°C. Over the seven days of operation, the EC increased from 2.5 to 5.0 mS cm<sup>-1</sup> and the pH continuously dropped from an initial 5.20 to 4.75. Again, lactic acid bacteria were present in high abundance (~49%), which was correlated with the decreasing pH during treatment. There was no presence of indicator pathogens. According to the Solvita® maturity test, CLO-10 output was determined to be mature. However, after two weeks, CLO-10 output was covered by what was visually concluded to be fungal mycelium. This suggests the output is not mature and that it might be classified as partially degraded organic waste.

According to 16S rRNA gene metabarcoding the Closed Loop proprietary bacterial inoculum (Acidulo™) comprised ~35% *Alicyclobacillus* sp. (phylum Firmicutes), ~13% *Dyella* sp. (Gammaproteobacteria), and ~12% *Bacillus* sp. (Firmicutes). The former two genera (comprising nearly 50% of all bacteria) were not present in any of the samples from the CL1 or CL2 experiments.

## **4.2 Introduction**

Oklin International Ltd. (2020) sell in-vessel units worldwide for the treatment of organic waste. In Australia, these units are marketed by Closed Loop Environmental Solutions Pty. Ltd. One Closed Loop program is organic recycling, where in-vessel units treat organic waste. According to the manufacturer, the in-vessel units can reduce the volume of organic waste by 90% and produce "highly concentrated compost" within a highly desirable 24 hour. The in-vessel units are automated machines that control the temperature, agitation, and airflow. In addition, Closed Loop supplies a "proprietary starter material" (a bacterial inoculum that Oklin calls Acidulo<sup>TM</sup>) to commence the process. These units accept all types of organic waste except for large bones, oyster shells, bulk cooking oil, and scallop (Closed-Loop-Environmental-Solutions-Pty-Ltd., 2020).

The end-product (CL output) which comes from the in-vessel units after 24 hours, looks more homogenous and dry compared to the starting material. However, it is not clear how to classify this product. Closed Loop use the Oxford Dictionary definition of compost as "decayed organic material used as a fertiliser for growing plants", and claim that the end-product is "compost" after 24 hours. They also use the definition of the Environmental Protection Authority from New South Wales (EPA-NSW), which defines compost as "a process of managed biological transformation", where the composting process should achieve pasteurisation of the material, and the curing phase should maintain appropriate moisture until it reaches stability.

Closed Loop say that the end-product from the in-vessel units does not meet the EPA-NSW definition of compost. Therefore, describing the end-product from the Closed Loop vessels as "compost", is misleading or ambiguous. In this research CLO-10 in-vessel unit was used in several experiments and the results demonstrated that the end-product is not compost. It may be classified as partially degraded organic waste.

### **4.3 Operational conditions of the in-vessel unit Closed Loop**

The commercially available Closed Loop in-vessel units range from  $4 \text{ kg d}^{-1}$  to  $200 \text{ kg d}^{-1}$  capacity, and they follow the same principle for controlling the temperature, agitation, and airflow. The model CLO-10 in-vessel unit, designed for  $20 \text{ kg d}^{-1}$  was used. This in-vessel unit is exogenously heated via a thermostat-controlled hot oil tank in the base of the chamber. Internal paddles rotate constantly at one rpm; seven min clockwise, seven min anticlockwise, three min stop. A strong external fan pulls air into the vessel and removes gases and moisture. The maximum power consumption for moving the paddles, heating the chamber, and venting for the  $20 \text{ kg}$  in-vessel unit is  $500 \text{ kWh month}^{-1}$ .

#### **Technical specification of Closed Loop in-vessel unit:**

- Capacity  $\text{d}^{-1}$ :  $20 \text{ kg}$
- Electricity usage/month:  $500 \text{ kWh}$  (maximum)
- Electricity requirements: AC  $240 \text{ V}$
- Power rating:  $50\text{Hz}, 2.1 \text{ kW}$
- Dimensions (mm):  $1160 (\text{w}) \times 620 (\text{d}) \times 1030 (\text{h})$
- Weight:  $240 \text{ kg}$



Figure 4.1 External view of the CLO-10 in-vessel unit Closed Loop.

## 4.4 Experimental design

The Acidulo™ inoculum was mixed with water and then added to CLO-10, which was operated for at least 12 hours. Gradually, organic waste was added and after one to two weeks, the in-vessel unit reached its normal operating efficiency. The inoculum is sufficient for about eight to ten months of operation according to Oklin.

The CLO-10 had been running for about three months before starting any experiments in this research. According to the manufacturer, part of the completed cycle output is left in the vessel prior to initiating the next cycle with organic waste. This comprises the “operational inoculum” and was determined to be ~35% by weight of the CLO-10 output. The operational inoculum “CLa” used for CL1.1 was the treated organic waste from the previous operational run. After finishing the CL1 experiment, the in-vessel unit was continuously operating as the café owners continued adding waste into the unit.

Three months after of the CL1 experiment, the CL2 experiment ran. As in the CL1.1, the operational inoculum “CLb” was the treated organic waste from the previous days. The data are shown in more detail in Figure 4.2 and Table 4.1.

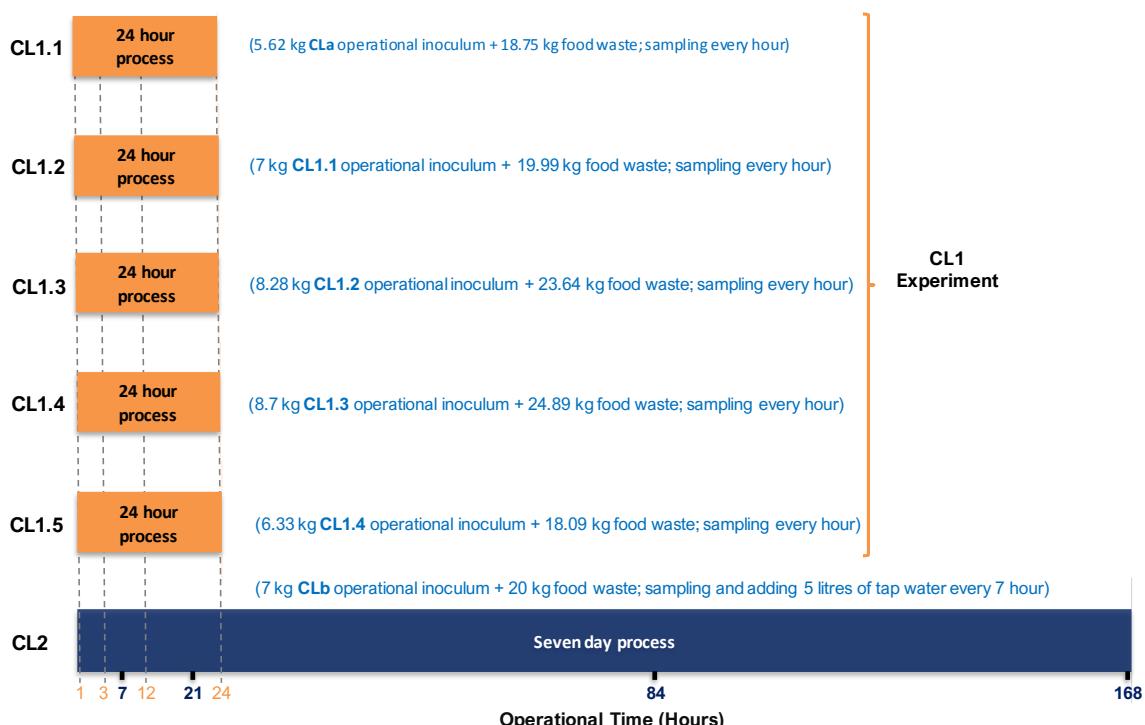


Figure 4.2 Operational time of the experiments CL1 and CL2, organic waste composition is given in Table 4.1.

#### 4.4.1 Collection and audit of organic waste

Organic waste was collected from the Swinburne Place South café precinct located at Swinburne University of Technology. Organic waste was audited for the first Closed Loop 1 (CL1) experiment, which had five sub-experiments (CL1.1, CL1.2, CL1.3, CL1.4, and CL1.5). Prior to running each sub-experiment, 35% of the former process end-product (CL operational inoculum) was left in the chamber (see Table 4.1).

Table 4.1 Organic waste composition in kg – Experiment Closed Loop 1- (CL1).

Organic Waste	Experiment CL1				
	CL1.1	CL1.2	CL1.3	CL1.4	CL1.5
Noodles & rice	2.07	3.9	6.09	5.79	2.7
Vegetables	0.77	2.7	2.85	5.47	1.81
Bread & pizza	1.05	0	0	1.02	0
Fruits	0.97	2.34	3.96	1.05	0.34
Chicken meat	0.23	0.06	0.57	1.51	0.2
Coffee grounds	13.66	10.9	9.02	5.18	12.87
Off plate	0	0.09	0	0.67	0
Eggshells	0	0	0.16	0.35	0.17
Seafood	0	0	0.01	3.36	0
Red meat	0	0	0.98	0.49	0
TOTAL	<b>18.75</b>	<b>19.99</b>	<b>23.64</b>	<b>24.89</b>	<b>18.09</b>
CL operational inoculum (35%)	5.62	7	8.28	8.7	6.33

#### 4.4.2 Organic waste treatment experiment Closed Loop 1 (CL1)

CLO-10 was operated according to Closed Loop's instructions, with each experiment running for 24 hours and sampling every hour. The in-vessel unit was completely emptied, then the operational inoculum was mixed with organic waste (Table 4.1) prior to starting the operation. Temperature was monitored by the in-vessel unit, and additional wireless temperature data loggers (Tinytag) were added to the chamber.

Once the experiment was started, nitrile gloves were worn for acquisition of triplicate ~30 g sub-samples every hour, which were placed into sterile 50 mL polypropylene tubes (Falcon®) and immediately stored at -80°C for later analyses.

#### **4.4.3 Organic waste treatment experiment Closed Loop 2 (CL2)**

After operating CL1, it was determined that 24 hours processing might have been insufficient since the output was very dry (less than optimal moisture content in all cases) and the operational pH was low (see Appendix E, Figure E2 – E6 and Figure 4.3). Hence, a second experiment (CL2) was carried out for seven days with the addition of water at sampling times into the vessel in an effort to re-moisten the dehydrated organic waste. The same proportion of organic waste and inoculum was used as for CL1.2 and samples were collected every seven hours generating 24 samples over seven days. Five L of water was added after each sampling.

The physical and chemical analyses were carried out on all samples of CL1 (24 samples from each sub-experiment) and CL2 (24 samples). Microbial metabarcoding and pathogen determinations were carried out on the 1 hour, 3 hour, 12 hour and 24 hour CL1 samples and on the seven hours, 21 hour, 84 hour and 168 hour CL2 samples. Figure 4.2 shows the codes of the experiments and the operational time of organic waste treatment.

### **4.5 Results**

#### **4.5.1 Time course of organic waste treatment - CL1 experiment**

In general, the organic waste had high moisture content, low pH and highly variable EC (see Table 4.2).

Table 4.2 Physical and chemical parameters of organic waste prior to treatment.

<b>Organic Waste</b>	<b>Moisture content (%)</b>	<b>pH</b>	<b>Electrical conductivity (<math>\text{mS cm}^{-1}</math>)</b>
Noodles & rice	61	5.73	3.93
Vegetables	88	4.87	8.42
Bread & pizza	39	5.94	5.19
Fruits	83	4.40	4.97
Chicken meat	57	6.21	3.69
Coffee grounds	57	5.23	2.07
Off plate	65	5.65	5.76
Eggshells	18	9.01	0.66
Seafood	67	6.08	14.46
Red meat	49	6.28	6.54
CLa	9	4.85	3.26
CLb	16	5.34	2.58

Overall, the CL1 operational temperature did not follow the conventional composting stages of mesophilic, thermophilic, mesophilic (Sánchez et al., 2017, Cooperband, 2000); it was mostly in the low thermophilic range ( $> 40^{\circ}\text{C}$ ). The maximum temperature attained, the maximum moisture content and time attained, the final moisture content, the initial and final pH, and initial and final EC of each CL1 sub-experiment are shown in Table 4.3.

Table 4.3 Physical and chemical parameters during CL1 experiment.

Experiment CL1 Subexperiments	Physical and chemical parameters – 24 hour process						
	Max T ( $^{\circ}\text{C}$ ) at Time (hr)	Max MC (%) at Time (hr)	Final MC (%)	Initial pH	Final pH	Initial EC ( $\text{mS cm}^{-1}$ )	Final EC ( $\text{mS cm}^{-1}$ )
CL1.1	60 at 4	35.57 at 1	2.04	5.20	4.97	2.77	3.50
CL1.2	50 at 3	51.90 at 2	31.50	5.27	5.17	1.91	2.43
CL1.3	>60 at 19	51.05 at 1	16.09	5.21	5.14	2.34	4.13
CL1.4	80 at 5	60.25 at 1	17.58	5.44	5.26	2.73	4.14
CL1.5	<50 at 4	26.26 at 1	2.01	5.23	5.15	3.36	3.70

Where: Temperature (T), moisture content (MC), electrical conductivity (EC).

Although the moisture content of organic waste was high at the beginning (Table 4.2), once it was mixed with the dry operational inoculum, it dropped. In all CL1 experiments, the pH slightly decreased and the EC slightly increased. Full results of CL1.2 are in Figure 4.3, while all CL1 results are given in Appendix E (Figure E2 – E6).

Phyla Proteobacteria, Firmicutes and Actinobacteria (in decreasing abundance) substantially dominated all CL1 samples; Bacteroidetes were often present in lower abundances (Figure 4.3 and Appendix E, Figure E2 – E6). Proteobacteria were progressively reduced as Firmicutes and Actinobacteria increased in abundance. As there were numerous similarities between all the CL1 sub-experiments (see Appendix E, Figure E2 – E6), a description of CL1.2 only is presented below as a representative example.

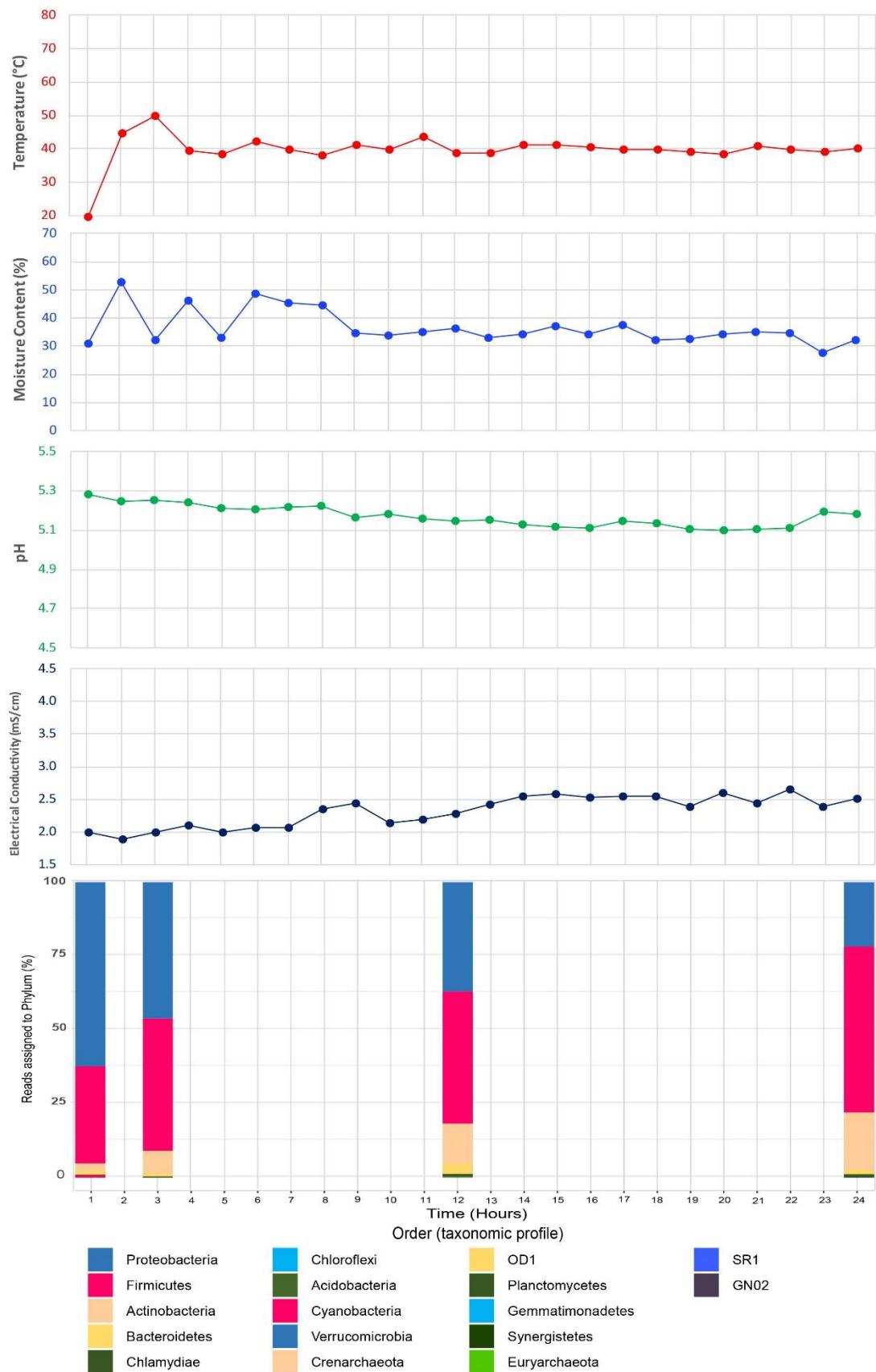


Figure 4.3 Physical, chemical and microbial measurements during organic waste treatment in Closed Loop in-vessel unit - CL1.2 experiment.

#### 4.5.2 Time course of organic waste treatment – CL2 experiment

CL2 ran for seven days, and at the seven hour sampling times five L of tap water was added to the vessel. The amount of tap water to add into the in-vessel unit during organic waste treatment was calculated based on the moisture losses in the CL1.2 experiment. However, due to the external heating activation, the moisture content was reduced rapidly.

The organic waste of CL2 had a similar composition as CL1.2 (Table 4.1). Physical and chemical parameters are given in Figure 4.4. Throughout the operation, the temperature was only slightly above 40°C. Despite re-moisturising the unit contents, the moisture content decreased from ~40% and after 63 hours, it ranged between 5% and 15%. Concomitantly, the EC increased; from 63 hours it was ~4.0 mS cm<sup>-1</sup>. The pH consistently dropped even until the last day of the experiment. The initial pH was 5.2 and the pH of the last sample was 4.75 (Figure 4.4).

Table 4. 4 Physical and chemical parameters during CL2 experiment.

Experiment CL2	Physical and chemical parameters – seven days process						
	Max T (°C) at Time (hr)	Initial MC (%) at Time (hr)	Final MC (%)	Initial pH	Final pH	Initial EC (mS cm <sup>-1</sup> )	Final EC (mS cm <sup>-1</sup> )
CL2	<50 at 49	60.83 at 0	10.72	5.20	4.75	2.71	4.65

At these physical and chemical conditions, the phyla in higher abundance during the CL2 experiment were Firmicutes, Proteobacteria (in decreasing abundance), and Actinobacteria. Bacteroidetes was below 4% in abundance, the rest of the phyla were below 0.5% (Figure 4.4).

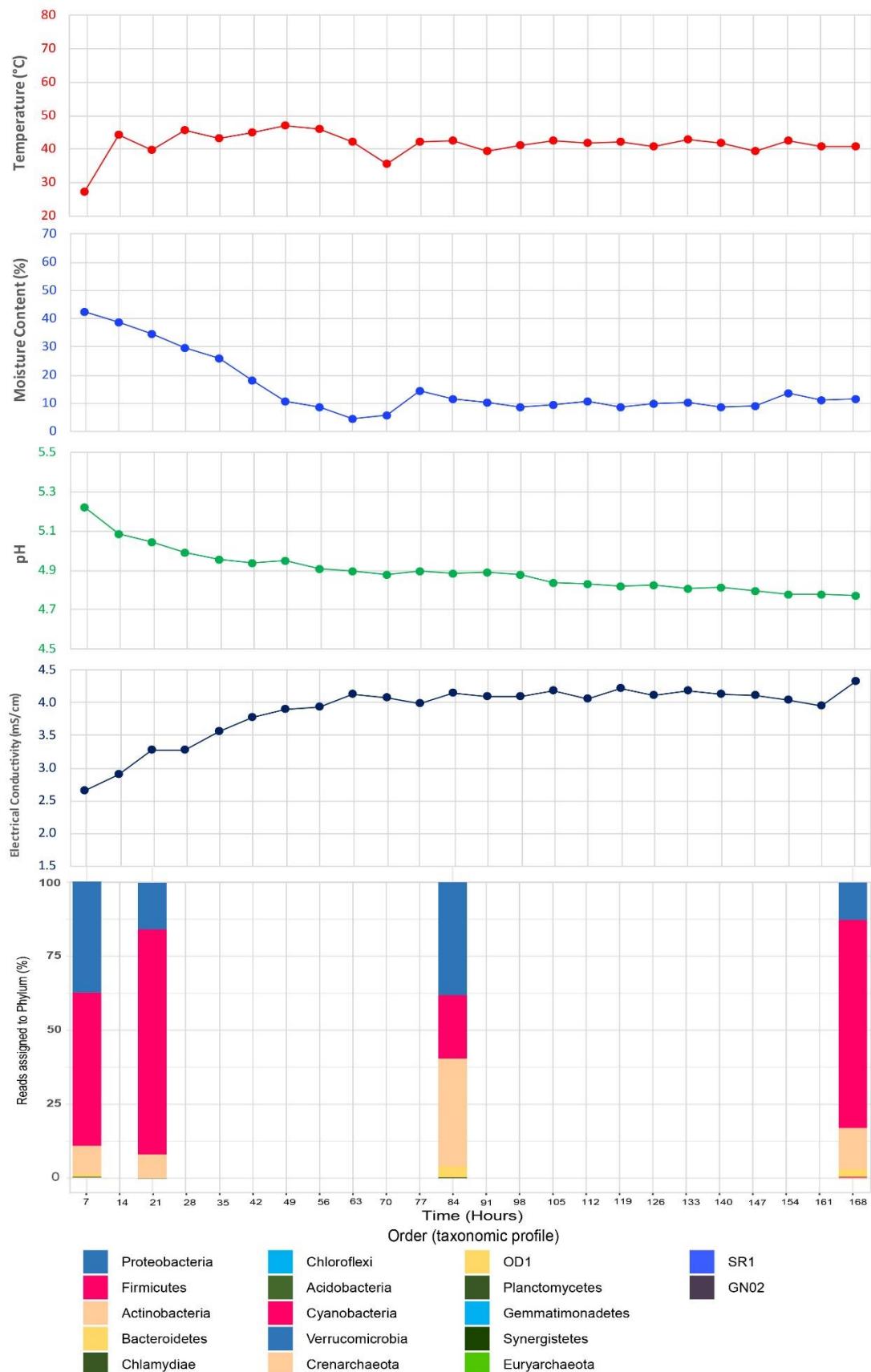


Figure 4.4 Physical, chemical and microbial changes during organic waste treatment in Closed Loop in-vessel unit - CL2 experiment.

### 4.5.3 Physical and chemical analysis

Principal Component Analysis (PCA) of the physical and chemical data are shown in Figure 4.5. Results which group closer are more similar than others that are far apart; the variables differentiate one group from another. Figure 4.5a shows the analysis of four parameters (temperature, moisture content, pH, and EC) of CL1; a total of 120 data points was plotted. Figure 4.5b shows the analysis of CL2 (seven day process) and CL1.2 (24 hour process), with 48 data-points; 24 from CL2 and 24 from CL1.2.

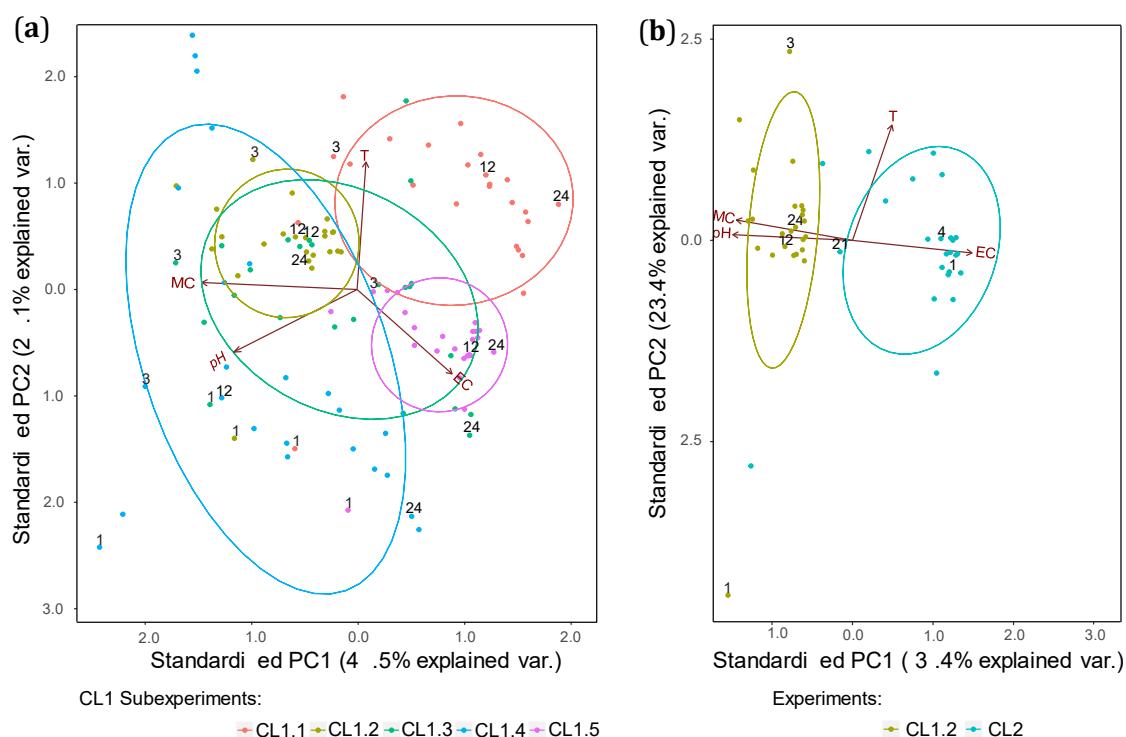


Figure 4.5 Physical and chemical analysis by Principal Component Analysis (PCA) **(a)** PCA of CL1 and **(b)** PCA of CL2 and CL1.2. Where T = temperature ( $^{\circ}\text{C}$ ), MC = moisture content (%), pH, and EC = electrical conductivity ( $\text{mS cm}^{-1}$ ).

In Figure 4.5a, the highest eigenvector and eigenvalue for PC1 was 46.5% and the second highest was for PC2 representing 26.1%. CL1.2 and CL1.5 have lower variability (samples grouped closer) in comparison to the samples of CL1.1, CL1.3, and CL1.4. The main contributor of the CL1.1 data-point location was temperature; for CL1.2 it was moisture content; for CL1.4 it was moisture content and pH, and for CL1.5 it was EC. CL1.3 had no main contributing physical or chemical parameter. From Figure 4.5b, CL1.2 samples were

correlated with moisture content and pH, while CL2 samples were more correlated with temperature and EC.

### **Carbon to nitrogen (C:N) ratio – CL1.2**

Total organic carbon and total nitrogen were measured in triplicate for all 24 samples of CL1.2 and the C:N was calculated on a dry matter basis. Figure 4.6 shows that the C:N ratio of the organic waste and during its subsequent treatment during CL1.2 was below the optimum for composting, which is 30:1.

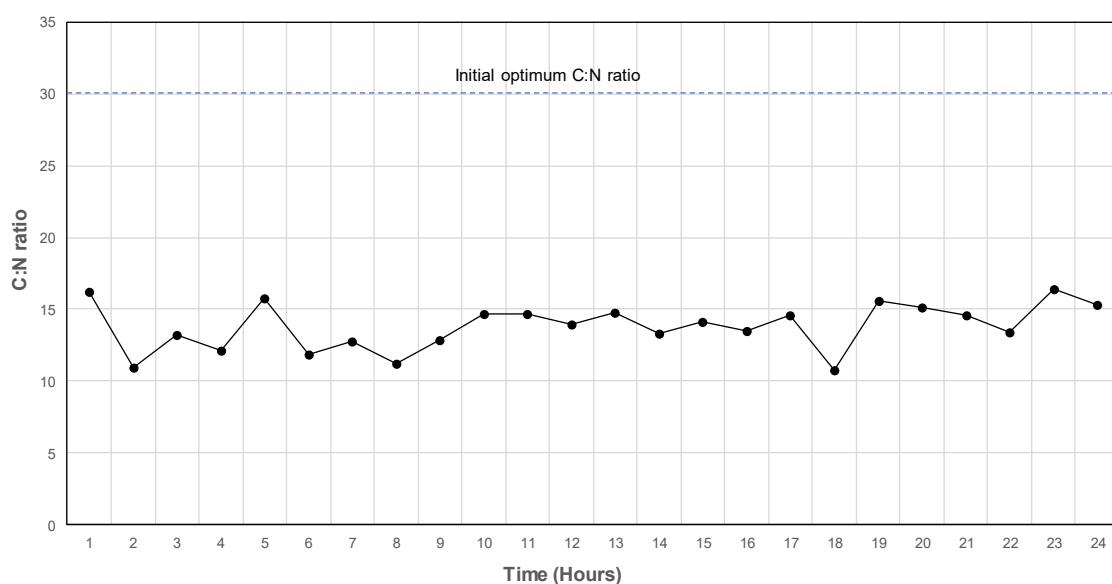


Figure 4.6 The measured carbon to nitrogen ratio of CL1.2, which was below the optimum of 30:1 for composting.

### **Compost maturity by the Solvita® test – CL2**

The maturity of the CL2 output at 168 hours was determined by the Solvita® test, which analyses carbon dioxide and ammonia evolution. At this time, the moisture content was ~10% (Figure 4.4). According to the Solvita® instructions, the samples had to be adjusted to ~50% moisture content and then incubated for 24 hours to reactivate the microbes. This was done, then the Solvita® paddles for carbon dioxide and ammonia measurement were inserted into the modified CL2 output in a Solvita® jar, the lid was closed, and the jar incubated for four hours at lab temperature.

Since the colours of both carbon dioxide and ammonia paddles did not change, the colour chart (see Chapter 3) showed that for carbon dioxide, the reading

was 8 and for ammonia it was 5. The conclusion, based on the colour comparator chart alone, is that the CL2 output was mature.

#### 4.5.4 Metabarcoding microbial analysis

The V3-V4 region of the 16S rRNA gene was PCR amplified from extracted DNA, which on occasion required dilution. The primers 515F-806R (the numbers refer to nucleotides in the *E. coli* 16S rRNA gene (Walters et al., 2015)) were used, and products were observed by agarose gel electrophoresis where a band at ~300 nucleotides (compared to a molecular weight ladder) would be positive. These PCR products were sent to the Australian Centre for Ecogenomics at the University of Queensland (ACE - UQ) for amplicon sequencing using the Illumina MiSeq machine. Five CL1 24 hour process samples (taken at 1 hour, 3 hours, 12 hours, and 24 hours), and the CL2 samples (seven day process) samples (taken at 7 hours, 21 hours, 84 hours, and 168 hours) and one sample from the Acidulō™ inoculum (in total 25 samples) were amplicon sequenced.

Bioinformatic analyses followed methods described in Section 3.2.2. A total of 503,209 raw reads from all 25 samples was obtained, and per sample, the reads were: minimum 1,830, mean 20,128.36 and maximum 74,083. After denoising and chimeric filtering with DADA2, the total number of reads was reduced to 169,995 and per sample, the reads were: minimum 840, mean 6,800 and maximum 20,574. A total of 731 ASVs were revealed in the samples.

From the rarefaction curve, the lowest sequencing depth was determined to be 1281 reads (from CL2 at 84 hours). The choice of this read depth, could allow small losses of data and consequently minimal loss of sample diversity. However, it ensures that most of the samples are included in downstream analyses. For all samples the rarefaction curves plateaued (see Appendix E, Figure E1).

Running decontam (Davis et al., 2018) at the default threshold of  $p = 0.1$ , five putative contaminant ASVs (representing 0.491% of relative abundance of the bacterial community) were found which were removed from the data (see Appendix E, Table E1). The remaining 663 ASVs were further analysed.

Overall, seventeen phyla were identified; fifteen were bacteria and two were archaea (Figure 4.7). Bacteria in CL1 and CL2 samples were composed (in abundance order) of the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Percentages of microbial groups are presented, and they refer to the abundance of those groups relative to 100% as determined by metabarcoding. Over the five 24 hour CL1 experiments, the percentages of Proteobacteria generally decreased (from ~70% to ~20%). Generally, Firmicutes increased over the 24 hours operation (Figure 4.7); e.g., in CL1.1, Firmicutes were initially low in abundance (~4 to ~15%), then increased at 12 hours and 24 hours (~20 to ~70%). Actinobacteria fluctuated between ~1 to ~20% abundance, and Bacteroidetes were ~1 to ~10% of prokaryotic abundance. Both these latter phyla tended to rise in abundance over the operational time. The remaining prokaryotic phyla were <2% abundant. CL2 prokaryotic phyla followed a similar trend as CL1.

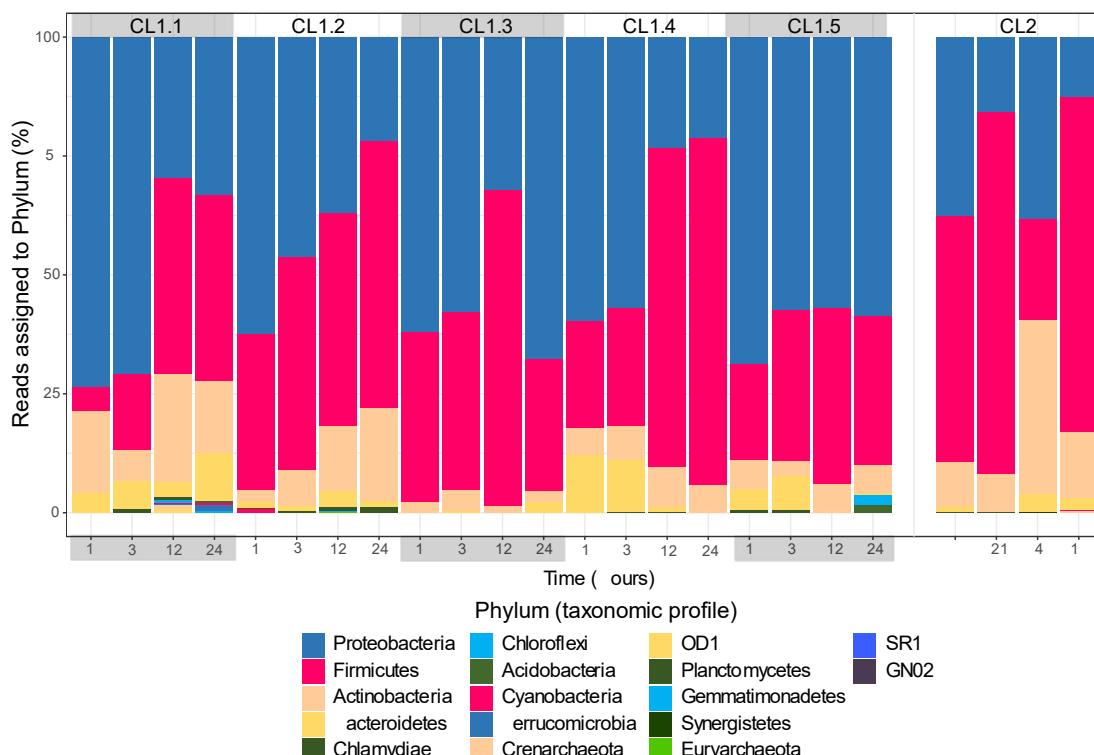


Figure 4.7 Phyla of bacteria and archaea in the CL1 and CL2 experiment.

The archaeal phyla Crenarchaeota (CL1.1 at 12 hours, CL2 at seven days, and in the Acidulot<sup>TM</sup> inoculum) and Euryarchaeota (CL1.2 at 12 hours) were in low abundance (<1%). Unassigned phyla were present at <0.5%, and only in the CL1.4 at four hours (Figure 4.7).

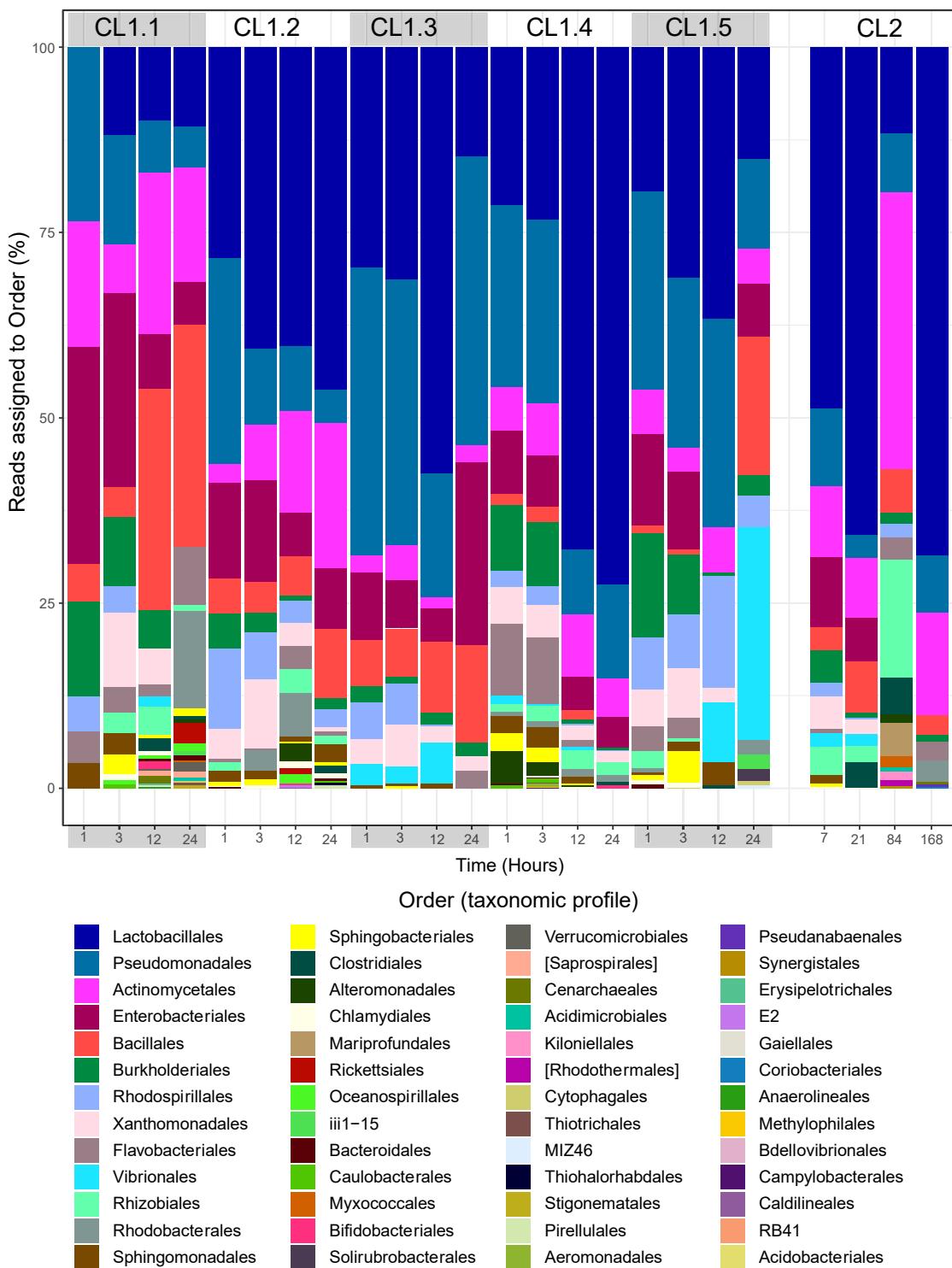


Figure 4.8 Order level prokaryotes in CL1 and CL2.

The most abundant bacterial order during CL1 and CL2 experiments was Lactobacillales maximum (~72%) (Figure 4.8). In CL1.2, CL1.3 and CL1.4, which started with a moisture content in the optimum range in or near 40% to 60%, there was higher abundance of Lactobacillales, between three and 12 hours of operation. In contrast, in CL1.1 and CL1.5, which started with a

moisture content below the optimal, Lactobacillales were comparatively low in abundance. In the first 21 hours of CL2, Lactobacillales increased, similar to in CL1, and by seven days, Lactobacillales were still in high abundance (~65%) (Figure 4.8).

The second most abundant order was Pseudomonadales; in CL1 they ranged from ~4% to ~39%, and in CL2 from ~3% to ~10% (Figure 4.8). Pseudomonadales generally declined during process operation. Actinomycetales were more abundant in CL2 (~3% to ~39%) than in CL1 (~1% to ~21%) (Figure 4.8). Enterobacteriales were present throughout the operation of CL1 (ranging from ~4% to ~28%), and less so in CL2 (ranging from ~5% to ~9%). However, by the end CL2, no Enterobacteriales were present. In CL1.1 and CL1.5, Bacillales increased over the 24 hour operation. Overall, they ranged between ~1% and ~29% in CL1 and between ~2% and ~7% in CL2. Burkholderiales and Rhodospirillales were present but in low abundance in CL1 (<13% and <10%) and CL2 (<1% and <2%). Xanthomonadales and Flavobacteriales were also present but in low abundance in CL1 and CL2. Vibrionales were generally <5% in CL1 and CL2, except that at 12 hour and 24 hour, CL1.5 had ~6 and ~26%, respectively.

The 20 most abundant bacterial genera are shown in heatmaps (Figure 4.9). *Pseudomonas* were in highest abundance in CL1 (Figure 4.9a). *Leuconostoc* and *Weissella* were present in all CL1 sub-experiments, except CL1.1. The heatmap in Figure 4.9b shows clear differences in microbial abundances between the 24 hour process (CL1.2) and the seven day process (CL2). Microbial diversity is higher in CL1.2 compared to CL2. The most abundant bacteria were lactic acid bacteria; CL1.2 was dominated by *Weissella*, *Leuconostoc*, and *Lactobacillus* and CL2 was dominated by *Weissella*, *Leuconostoc*, *Propionibacterium* and *Lactococcus*. The food spoilage bacteria *Acinetobacter*, *Pseudomonas*, and *Brochothrix* were quite abundant in both CL1.2 and CL2 (Figure 4.9b).

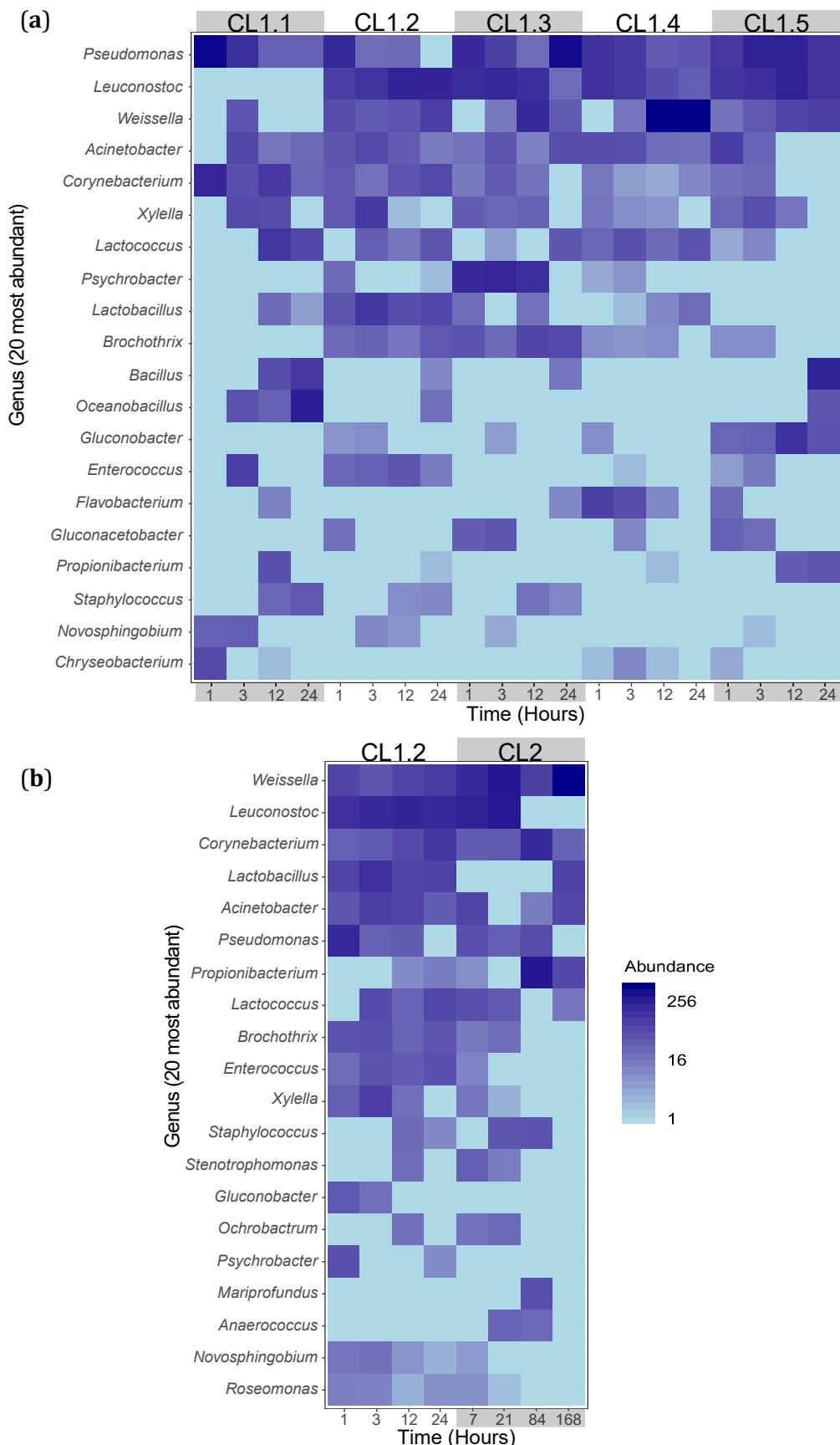


Figure 4.9 Heatmap of 20 most abundant actelial genera: **(a)** CL1 experiment and **(b)** CL1.2 and CL2 experiment.

### Acidulo<sup>TM</sup> inoculum

The microbial composition of the Acidulo<sup>TM</sup> inoculum was determined by methods described in Section 3.2.2. *Alicyclobacillus* dominated the microbial community (~35% abundance), followed by *Dyella* (~13%); neither of these were found in CL1 or CL2 samples. The Acidulo<sup>TM</sup> inoculum contained the bacteria *Nocardiooides* (<3%), *Streptomyces* (<2%), *Streptococcus infantis* (1.5%), *Sphingomonas changbaiensis* (<1.5%) and *Elizabethkingia* (<0.5%), and the archaea *Cenarchaeum symbiosum* (<1%), but these were not found in CL1 or CL2 samples. In contrast, *Bacillus* (~12%), *Propionibacterium* (<2%), *Pseudomonas fragi* (<1.5%) and *Aminobacterium* (<0.5%) were found in the Acidulo<sup>TM</sup> inoculum, and also in CL1 and CL2 samples.

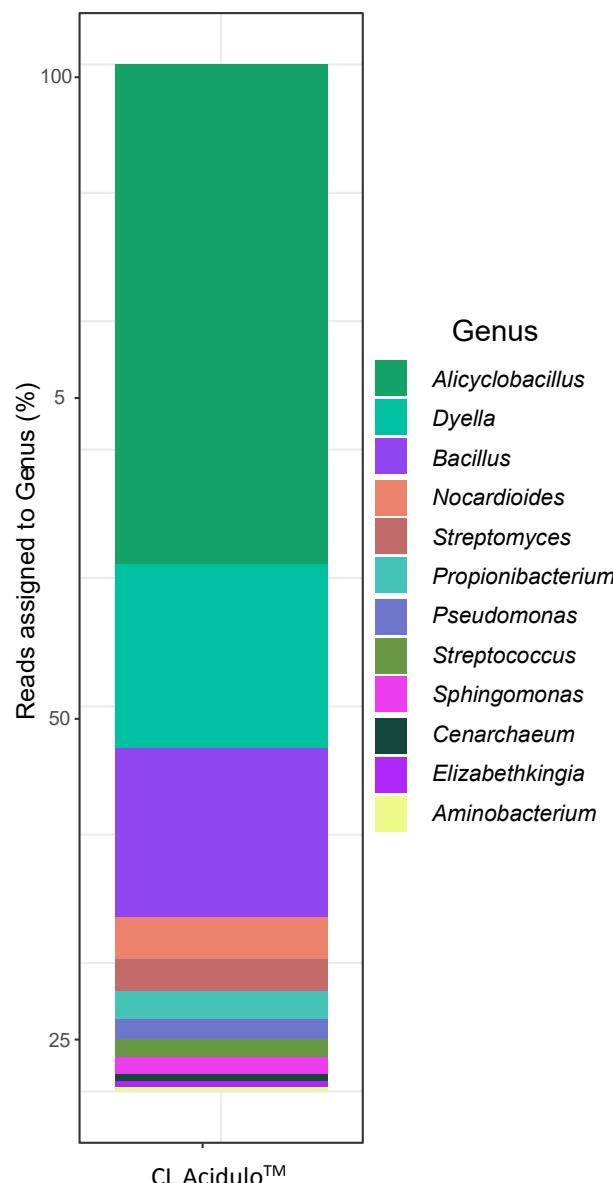


Figure 4. 10 The bacterial genera in “organic starter material” (Acidulo<sup>TM</sup> inoculum).

## Alpha diversity

The alpha diversity, community evenness (heterogeneity), and overall quantitative microbial community richness were determined as described in Section 3.2.2 and are presented in Figures 4.11 and 4.12. There was no consistency in ASV numbers (Figure 4.11a) among CL1 sub-experiments. Throughout the unit operations, some sub-experiments trended to more ASVs (CL1.1 and CL1.3), CL1.4 and CL1.5 trended to fewer ASVs and the number of ASVs in CL1.2 was erratic. Evenness according to Simpson's Diversity Index (Figure 4.11b) was somewhat consistent among the different sub-experiments, apart from two samples (CL1.1 at 1 hour and CL1.5 at 12 hour). Richness according to Shannon's Index (Figure 4.11c) was similarly consistent as evenness among the different sub-experiments, apart from three samples (CL1.1 at 1 hour, CL1.4 at 3 hour and CL1.5 at 12 hour).

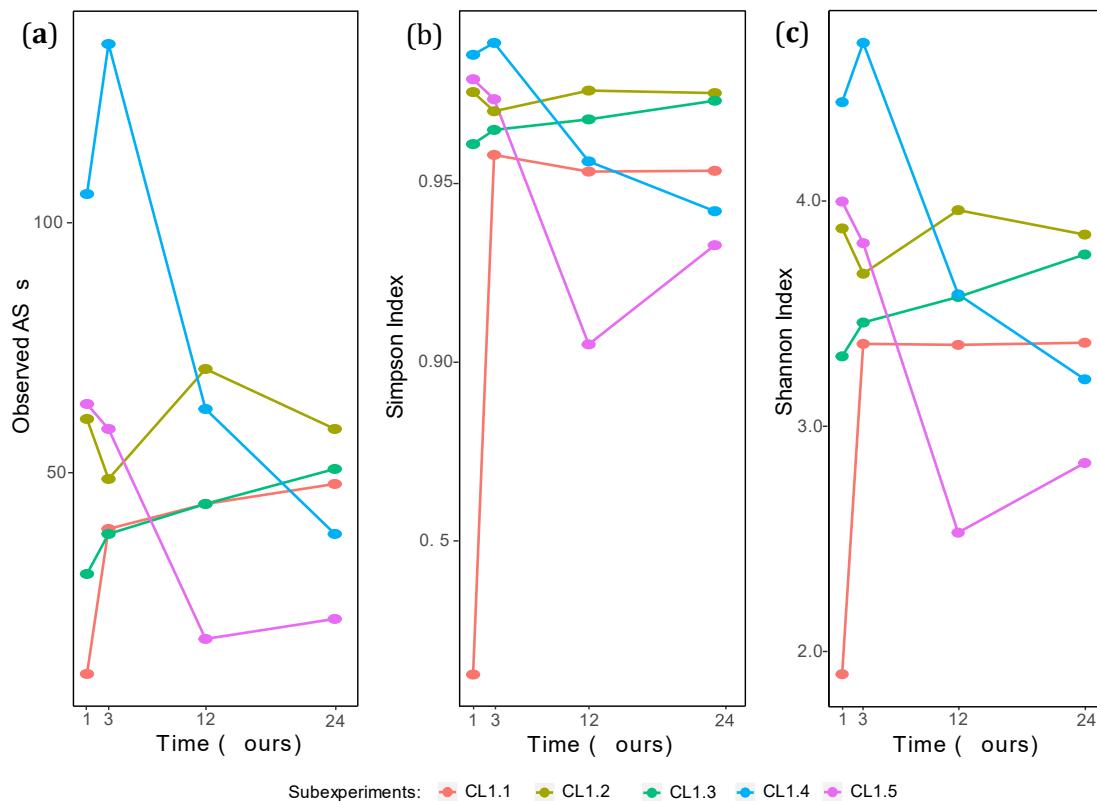


Figure 4.11 Alpha diversity of CL1 experiment. **(a)** Observed ASVs, **(b)** Simpson's diversity index and **(c)** Shannon's diversity index.

All diversity measures including ASV numbers, community evenness and community richness dropped sharply throughout the seven day CL2 operation; CL1.2 is shown for comparison (Figure 4.12).

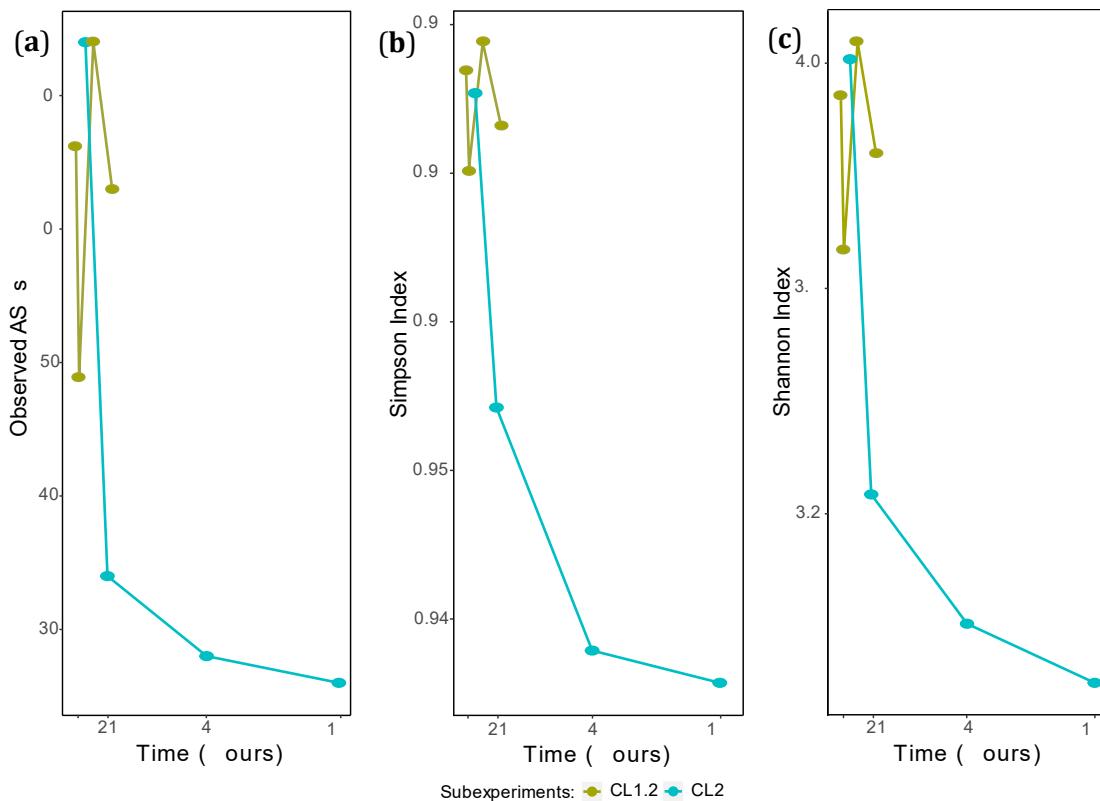


Figure 4.12 Alpha diversity of CL1.2 and CL2 experiment. **(a)** Observed ASVs, **(b)** Simpson's diversity index and **(c)** Shannon's diversity index.

### Beta diversity

Beta diversity was determined by methods described in Section 3.2.2 and plotted in a non-metric multidimensional scaling (NMDS) ordination. The similarities or differences among the microbial communities present during the different CL1 sub-experiments were determined (Figure 4.13a). Although the same CLO-10 vessel was used in all experiments, it is likely that the bacterial communities would have been shaped by the different compositions of starting organic waste (Table 4.1).

Figure 4.13a shows the distribution of the data-points from all CL1 experiments, which are grouped in ellipses according to their sub-experiment. In this case, four samples (1 hour, 3 hour, 12 hour and 24 hour) from each sub-experiment were analysed. The data-points of CL1.2, CL1.3 and CL1.4 are more similar to

each other and less variable compared to CL1.1 and CL1.5 (Figure 4.13a). GLM-based analysis revealed that the bacterial communities differed significantly based on the sub-experiment (manyGLM, LRT = 597.1,  $p = 0.002$ ) and time (manyGLM, LRT = 436.4,  $p = 0.001$ ) (see Appendix E, Table E2).

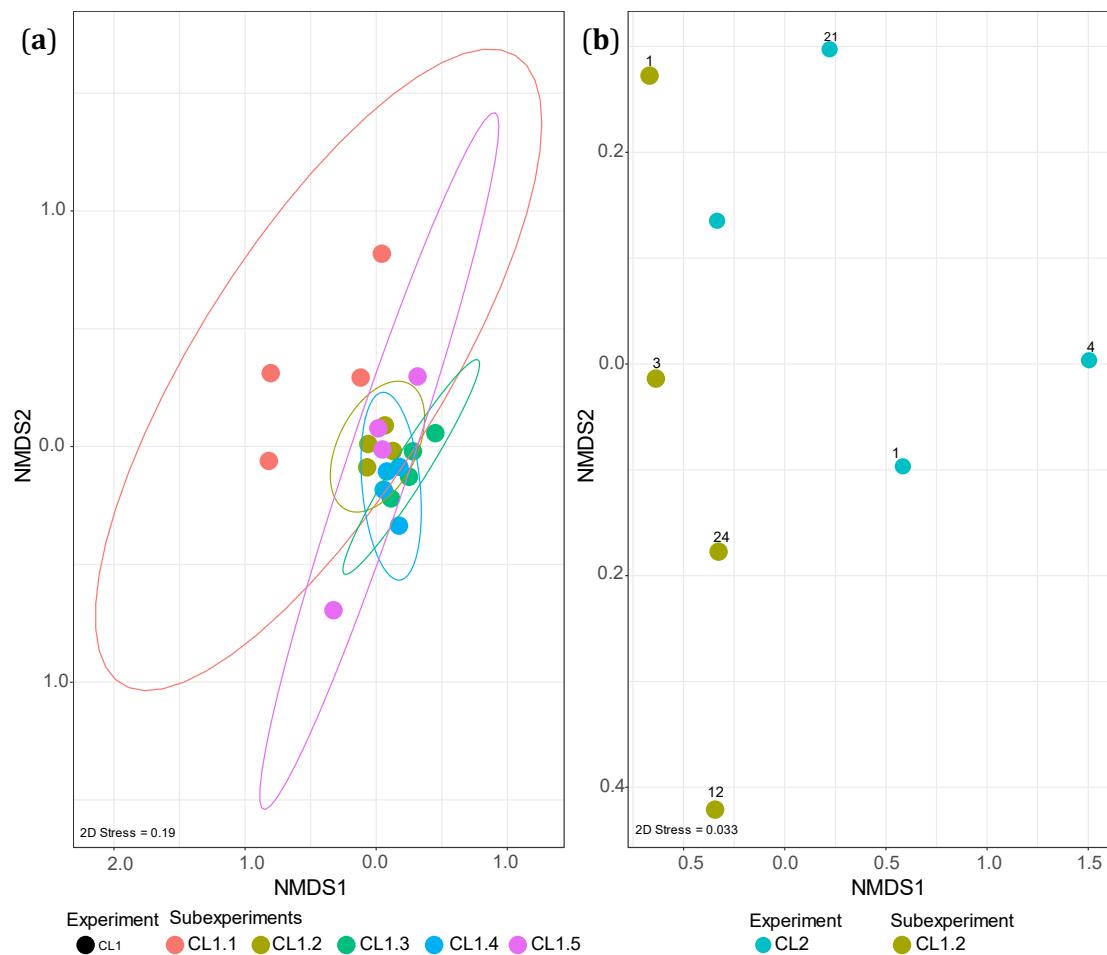


Figure 4.13 Bacterial community comparison by nMDS ordination based on Bray–Curtis distances, ellipses indicate 95% confidence intervals: **(a)** NMDS ordination of CL1 experiment, and **(b)** NMDS ordination of CL1.2 and CL2 experiments.

Figure 4.13b shows that the data-points from CL1.2 and CL2 mostly group with their own experiment; an exception is CL2 at seven hour which is more similar to CL1.2 samples. GLM-based analysis revealed that there was no significant difference in community composition based on sub-experiment (manyGLM, LRT = 155.5,  $p = 0.382$ ). However, the bacterial communities differed significantly based on the time (manyGLM, LRT = 1482.2,  $p = 0.005$ ) (see Appendix E, Table E3).

#### 4.5.5 Pathogenic microbial analysis

Efforts to isolate pathogens on suitable media followed methods described in Section 3.3.1. Although several bacteria (approximately  $3 \times 10^5$  CFU g<sup>-1</sup>) were isolated on selective and differential media, no pathogenic *Escherichia coli*, *Salmonella* spp. or pathogenic *Enterococcus* spp. were isolated. Controls for these latter three bacteria were grown, and their colonies were compared with those isolated from CL1 experiments, since none grew from CL2. The 16S rRNA genes from CL1 isolates were Sanger sequenced generating 44 nucleotides. Some non-pathogenic *Escherichia* sp. and *Enterococcus* spp. along with other bacteria were isolated from CL1 samples at different operational hour (Figure 4.14).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.65066068 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 44 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 324 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

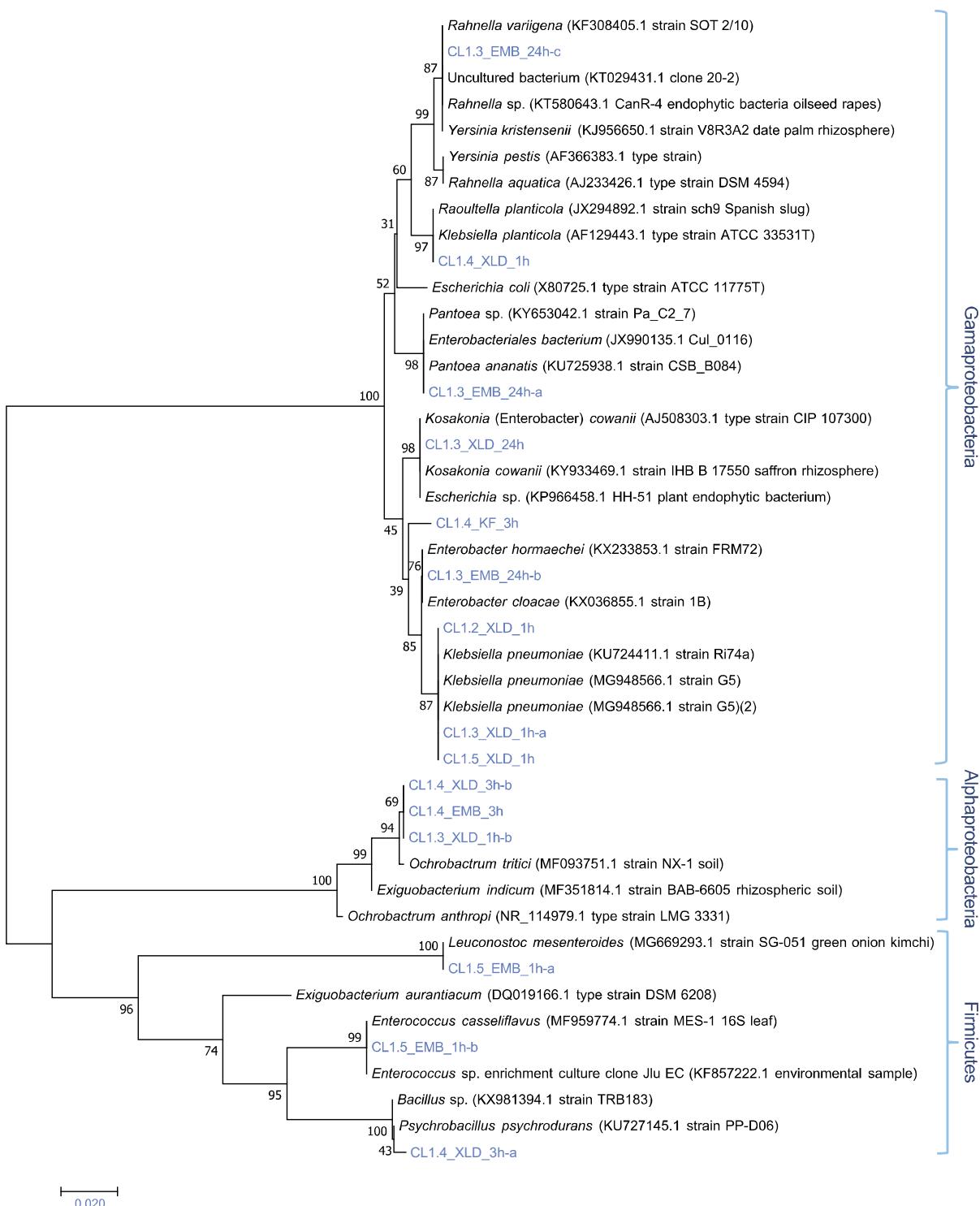


Figure 4.14 Phylogenetic tree of bacterial pure cultures obtained from CL1 samples on pathogenic bacterial selective media. The tree was drawn in Molecular Evolutionary Genetics Analysis 7.0.26 software. Numbers at the nodes indicate the percent of resamplings (1000 replicates) that supported that node. Bacterial isolate codes: CL1 – closed loop experiment 1 with sub-experiment 1, 2, 3, 4, 5 indicated; letters following are the media used for isolation – EMB = Eosin Methylene Blue Agar, KF = Kenner Fecal Agar and XLD = Xylose Lysine Deoxycholate agar; numberh = sample collected at hour of operation. Letters a, b, and c are the replicates of the isolates.

Table 4.5 Highest matches according to BLAST used to identify microorganisms isolated from CL1 experiments.

Identified microorganisms - BLAST	Sequence length (bp)	Match score	Percentage similarities	Accession number	CL1.1 (Hour)	CL1.2 (Hour)	CL1.3 (Hour)	CL1.4 (Hour)	CL1.5 (Hour)
<i>Rahnella variigena</i> (strain SOT 2/10)	1345	1345/1345	100	KF308405.1	---	---	24	---	---
Uncultured bacterium (clone 20-2)	1445	1445/1445	100	KT029431.1	---	---	24	---	---
<i>Rahnella</i> sp. (CanR-4 endophytic bacteria oilseed rapes)	1400	1340/1345	99.63	KT580643.1	---	---	24	---	---
<i>Yersinia kristensenii</i> (strain V8R3A2 date palm rhizosphere)	980	980/980	100	KJ956650.1	---	---	24	---	---
<i>Yersinia pestis</i> (AF366383.1 type strain)	146	1461/1461	100	AF366383.1	---	---	24	---	---
<i>Rahnella aquatica</i> (AJ233426.1 type strain DSM 4594)	1473	1473/1473	100	AJ233426.1	---	---	24	---	---
<i>Raoultella planticola</i> (strain sch9 Spanish slug)	1402	843/845	99.76	JX294892.1	---	---	---	1	---
<i>Klebsiella planticola</i> (type strain ATCC 33531T)	1507	1423/1429	99.58	AF129443.1	---	---	---	1	---
<i>Escherichia coli</i> (type strain ATCC 11775T)	1450	1450/1450	100	X80725.1	---	---	---	1	---
<i>Pantoea</i> sp. (strain Pa_C2_7)	1420	1420/1420	100	KY653042.1	---	---	24	---	---
Enterobacteriales bacterium (Cul_0116)	1408	1408/1408	100	JX990135.1	---	---	24	---	---
<i>Pantoea ananatis</i> (strain CSB_B084)	1350	1350/1350	100	KU725938.1	---	---	24	---	---
<i>Kosakonia (Enterobacter) cowanii</i> (type strain CIP 107300)	1362	1362/1362	100	AJ508303.1	---	---	24	---	---
<i>Kosakonia cowanii</i> (strain IHB B 17550 saffron rhizosphere)	1507	1507/1507	100	KY933469.1	---	---	24	---	---
<i>Escherichia</i> sp. (HH-51 plant endophytic bacterium)	1444	1444/1444	100	KP966458.1	---	---	24	---	---
<i>Enterobacter hormaechei</i> (strain FRM72)	1421	1421/1421	100	KX233853.1	---	---	24	---	---
<i>Enterobacter cloacae</i> (strain 1B)	1412	1412/1412	100	KX036855.1	---	---	24	---	---
<i>Klebsiella pneumoniae</i> (strain Ri74a)	1102	910/911	99.89	KU724411.1	---	---	---	---	1
<i>Klebsiella pneumoniae</i> (strain G5)	1474	1474/1474	100	MG948566.1	---	1	---	---	---
<i>Ochrobactrum tritici</i> (strain NX-1 soil)	1382	790/792	99.75	MF093751.1	---	---	---	3	---
<i>Exiguobacterium indicum</i> (strain BAB-6605 rhizospheric soil)	1552	1552/1552	100	MF351814.1	---	---	---	3	---
<i>Ochrobactrum anthropi</i> (type strain LMG 3331)	1388	1388/1388	100	NR_114979.1	---	---	1	---	---
<i>Leuconostoc mesenteroides</i> (strain SG-051 green onion kimchi)	1406	831/831	100	MG669293.1	---	---	---	---	1
<i>Exiguobacterium aurantiacum</i> (type strain DSM 6208)	1552	1552/1552	100	DQ019166.1	---	---	---	3	---
<i>Enterococcus casseliflavus</i> (strain MES-1 16S leaf)	1502	861/862	99.88	MF959774.1	---	---	---	3	---
<i>Enterococcus</i> sp. enrichment culture clone Jlu EC (environmental sample)	1413	1413/1413	100	KF857222.1	---	---	---	3	---
<i>Bacillus</i> sp. (strain TRB183)	1295	1295/1295	100	KX981394.1	---	---	---	3	---
<i>Psychrobacillus psychrodurans</i> (strain PP-D06)	1422	871/874	99.66	KU727145.1	---	---	---	3	---

Most of the bacterial isolates were found in the CL1.3 at the end of 24 hour process. In the case of CL1.4, isolates were found in samples at one and three hours. In CL1.2 and CL1.5, few isolates were found in samples from the first hour. No bacteria were isolated from CL1.1 on the selective/differential media.

#### 4.5.6 Total colony counting

Bacterial colony forming units (CFU) per gram of sample from CL experiments are shown in Table 4.6. CL1.1 and CL1.2 samples had the most CFUs in the first hour. However, in most CL1 experiments, the number of colonies decreased through the treatment process, and some samples (CL1.3 at 3 hour, CL1.5 at 1 hour, and CL2 at 84 hour) generated no CFUs. CL2 samples had extremely low CFUs.

Table 4.6 Number of colony forming units per g of CL1 and CL2 sample during organic waste treatment.

Experiment		CL1					CL2	
Subexperiment		CL1.1	CL1.2	CL1.3	CL1.4	CL1.5	Time (Hours)	(CFU g <sup>-1</sup> )
	Time (Hours)			Colony forming unit (CFU g <sup>-1</sup> )				
1		7.3x10 <sup>3</sup>	6x10 <sup>3</sup>	2x10 <sup>3</sup>	1.7x10 <sup>3</sup>	0	7	6x10 <sup>1</sup>
3		3.2x10 <sup>3</sup>	8.8x10 <sup>2</sup>	0	1.1x10 <sup>3</sup>	3.5x10 <sup>2</sup>	21	4x10 <sup>1</sup>
12		1.3x10 <sup>2</sup>	2.5x10 <sup>2</sup>	1.2x10 <sup>2</sup>	1.4x10 <sup>2</sup>	6x10 <sup>1</sup>	84	0
24		3x10 <sup>1</sup>	1.4x10 <sup>2</sup>	1.5x10 <sup>3</sup>	4x10 <sup>1</sup>	5x10 <sup>1</sup>	168	2x10 <sup>1</sup>

## 4.6 Discussion

### 4.6.1 CL1 and CL2 experiments – physical and chemical data

The microbial decomposition of organic matter is an exothermic process. The biologically generated heat leads to an increase in the temperature of the material being composted (Finstein and Morris, 1975). Composting goes through three thermal phases. In the beginning, the compost is mesophilic, typically ranging from ambient temperature to 40°C. This is followed by a thermophilic phase with temperatures around 40°C to 70°C; this is the active metabolic phase and microbial metabolism leads to the elevated temperatures. When the rapidly biodegradable organic matter is decomposed, the microbial

metabolic activity declines and the temperature decreases to the final mesophilic or curing phase (Mehta et al., 2014).

Each of the five CL1 sub-experiments were run for 24 hours, while CL2 was run for seven days. The CLO-10 is exogenously heated as described in Section 4.3. Heating is controlled by an in-built moisture sensor, with higher and sustained temperatures at higher moisture levels. The moisture level of most CL1 sub-experiments was relatively low at ~30% and CL2 was ~40%; throughout CLO-10 operation, moisture content trended downwards. This low moisture level would partly explain the lower than optimal composting temperatures of ~40°C to 50°C being achieved by exogenous heating of CLO-10.

At optimum conditions, the composting process is self-regulated, while the temperature increases due to the endogenous heating, the microbial population and diversity changes progressively (Bhatia et al., 2013, Pepe et al., 2013). Increasing the temperature rapidly to thermophilic in the externally heated CLO-10, may affect the microbial population (Li et al., 2013). Treating organic waste by self-heated composting has higher (three to four times more) microbial biomass than an externally heated one (Sundh and Rønne, 2002). Clearly, in CLO-10 experiments, the microbes did not substantially contribute to temperature increases.

CL1.4 had a higher initial moisture level (~60%) compared to other sub-experiments, and was heated to 80°C at hour five. The high initial moisture content in CL1.4 likely triggered the vessel to heat leading to the high temperature, compared to other CLO-10 experiments. However, CL1.3 started at a somewhat higher moisture level of ~50% but did not heat as in CL1.4.

The operation of CLO-10 is not favourable for the normal development of mesophilic through to thermophilic composting microbes. This is because the exogenous heating and temperature maintenance works against the natural selection for specific microbes. Typically, mesophilic microbes start decomposing the most readily degradable organic matter, then due to natural heat build-up in the compost bed, thermophiles are selected for. Finally, as readily degradable organic matter is depleted, microbes utilise less readily degradable compounds like cellulose, hemicellulose, and lignin, and

metabolism slows, leading to less heat production and a return to mesophilic conditions (Chen et al., 2011).

Moisture content plays an essential role in microbial metabolism and gas movement during the composting process (Madigan et al., 2018). Therefore, if the initial moisture content is below the optimum (40%-60%), the microbial activity and thus decomposition rate will be negatively affected (Zameer et al., 2010). One contributor to low moisture in the CL1 experiments was the mixing of moist organic waste (Table 4.2) with the relatively dry operational inoculum, leading to an overall drier mixture. The operational inoculum in all CLO-10 experiments had a moisture content <30% and occasionally <5% e.g., CL1.1 and CL1.5.

During both CL1 and CL2 experiments, the temperature, moisture content, and pH were not in the optimal ranges for composting organic waste. The final product was very dry (always less than 30%) and of a low pH (always less than 5.5). The low pH has been deemed responsible for low ammonia gas evolution (Asano et al., 2010), although ammonia was not measured in this thesis. Ammonia is considered a major odour problem for composting, and also the loss of this important nutrient from compost outputs is a negative feature. According to the Solvita® test, the CLO-10 did not generate human-perceptible odours during experiments, so ammonia might not have been lost or it may have been <0.02 mg NH<sub>3</sub>-N. The olfactory odour detection threshold for ammonia is between 0.0266-39.6 mg m<sup>-3</sup> (Michaels, 1999).

The low measured pH could have been responsible for preventing ammonia loss. Several others have reported low moisture and low pH of acidulocomposting outputs (Asano et al., 2010, Hemmi et al., 2004, Suematsu et al., 2012). It was shown in this thesis that addition of tap water simply triggered more heating (CL2) and facilitated the drying of the compost. Therefore, the end-product of Closed Loop may be classified as partially degraded organic waste. More importantly, the process, rather than being composting should be considered dehydration.

The C:N ratio is an important composting parameter. It was measured during CL1.2 as between 10:1 to 17:1. The optimal C:N ratio at the beginning of

composting should be ~30:1 (Epstein, 2011), while that of mature compost should be in the range from 10:1 to 15:1 (Chen et al., 2011). The CLO-10 experiments all had suboptimal starting C:N ratios, and the low end-product C:N would not allow it to be classified as mature compost.

The Solvita® maturity test of CL2 sample shows that this output was apparently mature. However, the results of the colony counting at 168 hours showed only 20 CFU g<sup>-1</sup>, likely due to the moisture content being so low at ~10%, that microbial viability would be severely impaired. Although the CL2 output was re-moistened to ~50% and left for 24 hour before doing the Solvita® test, the microbial population was not able to recolonise as rapidly as was expected. The Solvita® result showed that there was no microbial carbon dioxide production, which was very likely correct. However, the low microbial activity was not because the CL2 output was mature, but because there were not enough microbes to degrade the raw organic matter. According to the Solvita® test, the ammonia evolution was undetectable (<0.02 mg NH<sub>3</sub>-N), the ammonia paddles did not changed colour.

After finishing the CL2 Solvita® test, the jars were left open at room temperature, and after a week fungal growth was macroscopically visible. By the end of two weeks, the surface was fully covered, which confirms that the CL2 output was not mature. Mature compost is a stable, humic-like material (Tiquia et al., 2002). Hence, 24 hour or seven day processes are not sufficient to produce mature compost in the CLO-10 in-vessel unit. Nitrification during the curing phase to produce nitrates is another indicator of compost maturity (Rynk et al., 1992), but that was not employed in this research.

#### 4.6.2 Acidulo™ inoculum and acidulocomposting

The Acidulo™ inoculum is required at the initiation of Closed Loop machine operations and it must be re-added every eight to 10 months of operation (Oklin-International-Ltd., 2020). Closed Loop Environmental Solutions Pty. Ltd. call the Acidulo™ inoculum “a proprietary starter material pack with microbes”, which was provided mixed with sawdust. According to 16S rRNA gene metabarcoding, *Alicyclobacillus* was the most abundant bacterium present comprising ~35% of the inoculum starter material.

*Alicyclobacillus sendaiensis* (phylum Firmicutes, order Bacillales) was described by Tsuruoka et al., (2003) after it was isolated from soil in Sendai, Japan. *A. sendaiensis* is “an acidophilic, slightly thermophilic bacterium, that produces a thermostable extracellular acid collagenase activity with potential industrial applications”. It has a temperature range for growth of 40°C to 65°C, an optimum at 55°C, and a pH range for growth of 2.5 to 6.5, with an optimum at pH 5.5. Physico-chemical conditions in both CL1 and CL2 experiments would have been suitable for the growth of *A. sendaiensis*, however, this bacterium was never detected.

Nishino et al. (2003) developed acidulocomposting as an autonomously sustained thermophilic food refuse treatment process. In a composting apparatus, *A. sendaiensis* strain NTAP-1 (Acidulo®) was inoculated to food waste and operated exogenously at 50°C to 70°C, air was removed at 0.5 L/min and it maintained a pH of 4 to 6 over a two year period. A heated sheet below the composting vessel, maintained at 85°C, supplied heat to the vessel. Limited information about the process output was given (nothing on maturity), but odour emissions were low, unsanitary vermin were not present and the moisture content was between 25% to 40% when organic waste was added daily. When organic waste was not added, the moisture content decreased to 10% (Nishino et al., 2003). Acidulocomposting was concluded to be a low-maintenance process where “isual inspection suggested that the degradation of the added substrate was completed within 24 hours at high temperatures”.

When acidulocomposting was applied to cattle manure on a small scale, it was concluded that the low pH is maintained by an abundance of lactic acid bacteria, which were demonstrated by isolating *Pediococcus* sp., *Weissella* sp. and *Lactobacillus* sp. (Asano et al., 2010, Hemmi et al., 2004). Suematsu et al. (2012) indicated that acidulocomposting does not need the addition of starter microorganisms, but the initial report by Nishino et al. (2003) did add *A. sendaiensis*. Typical acidulocomposting conditions of 45°C to 60°C, pH 4 to 6, and low moisture content of 20% to 30% were used to treat university café waste (Suematsu et al., 2012). The food waste was mixed with high concentrations of *Bacillus subtilis* and *Pseudomonas putida*, but both these

bacteria were outcompeted within a week of operation, and the high amounts of lactic acid bacteria were present in the microbial community.

Kliopova, (2016) used the Acidulo<sup>TM</sup> microbes in the in-vessel unit GreenGood (from Oklin, Hong Kong) in Lithuania to compost organic waste from a resort location. External heating increased the temperature to 75°C for one hour to eliminate microbes and parasites and primary compost was produced in 24 hours. However, no information on the process operation was provided and the quality of the final product was not described. In any case, this report demonstrates the full-scale application of acidulocomposting. oběrková et al., (2020) conducted an organic waste treatment experiment using the commercial GreenGood Model GG-02 in-vessel unit (from Oklin, Hong Kong) in the Czech Republic. Mixing organic waste with Acidulo<sup>TM</sup> inoculum the treatment process ran for 28 days inside the vessel and 35 days outside the vessel (for maturation). The temperature ranged from 23°C to 79°C, the pH was in the acidic range ~4.2, EC was between 5 to 6 mS cm<sup>-1</sup>, the C:N ratio was ~20:1, and according to the phytotoxicity test, the end-product was toxic to plants. Finally, they concluded that the GG-02 unit was not capable of composting organic waste, and the end-product was not compost.

The inoculation of certain microorganisms in specific composting situations has some merit; e.g., to overcome acid inhibition (Cheung et al., 2010). However, inoculating microbes (e.g., Acidulo<sup>TM</sup> microbes) into complex environments such as organic waste treatment, may not be suitable, as indigenous microbes could outcompete the inoculum in this rapidly changing environment (Hosseini and Abdul Aziz, 2013, Gabhane et al., 2012). This happened in all the CL experiments.

Closed Loop's in-vessel units (from Oklin, Hong Kong) in Australia, are based on the acidulocomposting process as described in Section 4.3. Although the Acidulo<sup>TM</sup> inoculum was used according to Closed Loop's instructions, and the inoculum was proven to be dominated by *Alicyclobacillus* (~35%) and *Dyella* (phylum Proteobacteria, class Gammaproteobacteria, order Xanthomonadales) (~13%), neither of these were present in any of the CL1 or CL2 experiments. Therefore, during organic waste treatment, the inoculated *Alicyclobacillus* did

not adapt to the environment which was provided by Closed Loop's in-vessel unit. Several researchers have commented on the dry nature of acidulocomposting output and the results in this thesis concur. The end-product of acidulocomposting after 24 hours has the appearance of dry "brown pulverulent material" (Nishino et al., 2003), which supports the notion that the process is dehydration with limited biodegradation.

#### 4.6.3 CL1 and CL2 experiments – microbiological results

##### Bacterial numbers

The CFUs recovered on Plate Count Agar at 37°C were extremely low; the maximum was  $\sim 7 \times 10^3$  CFU g<sup>-1</sup> sample (CL1.1 at 1 hour), but the vast majority of CL1 samples contained  $< 2 \times 10^3$  CFU g<sup>-1</sup>, and CL2 samples grew only 20 to 60 CFU g<sup>-1</sup>. Other composting results for bacteria in compost were 10<sup>5</sup>-10<sup>9</sup> CFU g<sup>-1</sup> compost (Chandna et al., 2013). During the mesophilic stage, bacteria increased from 10<sup>8</sup> cells g<sup>-1</sup> to 10<sup>10</sup> cells g<sup>-1</sup>. At the thermophilic stage, the bacteria (from 10<sup>7</sup> to 10<sup>9</sup> cells g<sup>-1</sup>) and actinomycetes (10<sup>8</sup> g<sup>-1</sup>) increased rapidly. Fungi were  $\sim 10^6$  cells g<sup>-1</sup> in the mesophilic phase and was reduced during the thermophilic phase (Epstein, 1997). The provided substrates (Table 4.1), are very suitable composting materials so the CLO-10 results showing low and declining bacterial numbers over the process time, suggest that the process was unsuitable for bacterial growth.

##### Metabarcoding

The Firmicutes, which are typically low mol% G+C, Gram positive cocci or bacilli, generally increased in abundance (CL1.1, CL1.2, CL1.4) or maintained their abundance (CL1.5 and CL2) throughout CLO-10 processes. Drilling down into this phylum showed that order Lactobacillales dominated. Lactobacillales are Gram positive, acid-tolerant, generally non-sporulating, non-respiring, either rod or coccus-shaped bacteria that share common metabolic and physiological characteristics. These bacteria are usually found in decomposing plants and milk products, produce lactic acid as the major metabolic product of carbohydrate fermentation (Madigan et al., 2018).

*Leuconostoc* sp., *Weissella* sp. *Lactococcus* sp. and *Lactobacillus* sp. were the abundant Lactobacillales in CLO-10 experiments. The abundance and increase

of Lactobacillales throughout the 24 hour and seven day experiments was correlated with the maintenance of low pH. Other researchers (Sundberg et al., 2013, Ishii et al., 2000, Tran et al., 2019) have also reported particularly the detrimental effects of lactic acid bacteria causing low pH in early stages of composting. Asano et al. (2010) cite the role of high and stable Firmicutes concentrations (*Bacillus* sp., *Pediococcus* sp., *Weissella paramesenteroides* and *Lactobacillus salivarius*) in maintaining low ammonia emissions during acidulocomposting.

*Pseudomonadales* was the second most abundant order of bacteria in the CLO-10 experiments; typically reducing in abundance over the 24 hour operation but being of varying abundances in CL2. The *Pseudomonadales* genera *Pseudomonas* sp. and *Psychrobacter* sp. were common. *Pseudomonas* sp. have wide metabolic activities and contribute to organic matter degradation in the mesophilic phase of food waste composting (Xie et al., 2017).

Sundberg et al. (2011) note that *Bacillales* and *Actinobacteria* are needed for efficient composting. Both these groups were quite abundant in most CLO-10 runs. Sundberg et al. (2011) also state that recycling bulk material could prevent start-up problems. The CLO-10 experiments did recycle ~35% of the previous cycle, and *Bacillales* and *Actinobacteria* were abundant; however, the processes could not be considered efficient composting according to temperature profiles, pH and moisture content.

### **Potential indicator microorganisms**

During CL1 experiments no pathogenic *Escherichia coli*, *Salmonella* spp. or pathogenic *Enterococcus* spp. were found during the 24 hour or seven day processes. None of the isolated colonies were similar to the reference pathogenic colonies. Most of the microbes identified by culturing in CL1 samples were most closely related to *Enterobacter cloacae*, *Enterococcus casseliflavus*, and *Klebsiella pneumoniae* according to partial 16S rRNA gene analysis. *Enterococcus casseliflavus* was found by metabarcoding in all CL1 experiments except CL1.3 and in the seven hour CL2 sample. These microorganisms are common nosocomial pathogens, which can cause urinary tract infections and pneumonia. These bacteria may cause serious invasive

infections (Moellering, 1998, Jondle et al., 2018, Aghamohammad et al., 2020), but their low abundance and absence from the final compost, suggest these potential nosocomial pathogens in Closed Loop output to be a minimal risk for human health.

#### 4.6.4 Application of Closed Loop output

Mature compost should not have negative impacts on plant growth (Epstein, 1997). In contrast, immature compost can have harmful effects on crop growth (Hue and Liu, 1995). Furthermore, immature compost can cause anaerobic conditions to occur due to the oxygen consumption of remaining organic matter (Mathur et al., 1993). Acidulocomposting end-product may be phytotoxic for plants if it contains high concentrations of acetic, butyric, and propionic acids, or other organic compounds (Yu et al., 2010). *Weissella* sp. have been reported to produce acetic acid in the early stages of composting and this can inhibit other composting organisms (Tran et al., 2019). These bacteria were abundant in CLO-10 experiments and the pH was low at the end, so the CLO-10 generated compost might not be suitable for plant application.

Yamamoto et al. (2014), evaluated acidulocomposting output for cabbage growth. Adding 700 g m<sup>-2</sup> of this output did not have negative effects on the growth of cabbage. However, adding 1,400 g m<sup>-2</sup> inhibited the normal growth and yield. Partially degraded organic waste might be phytotoxic for plants (Alvarenga et al., 2017), and it may have the capacity to affect seed germination and root elongation (Majlessi et al., 2012, Bernai et al., 1998). Acidulocomposting output has been suggested as a biological weed control strategy (Asagi et al., 2016). However, more tests to determine optimal doses for weed suppression without affecting plant growth are required (Yamamoto et al., 2014).

## **4.7 Conclusions**

Composting is a natural process where microorganisms decompose organic matter into compost. Manipulating the composting conditions can improve or affect the indigenous or added microbes and their activity. If the microbes have sufficient nutrients, and the physical and chemical conditions are optimal, the microbes will rapidly decompose the organic matter. If the composting parameters are not optimal, the microbial proliferation will likely be limited.

The Closed Loop in-vessel unit CLO-10 does not provide optimum conditions for microbial development because the temperature, pH, and moisture content profiles are not those required for efficient composting. Consequently, lactic acid producing-bacteria were mostly selected for. The external heating and strong suction ventilation, make the end-product dry and the semi-continuous rotation of paddles reduce the particle size generating a dusty output. Therefore, based on the physical, chemical and microbial results of CL1 and CL2 experiments, the output could be classified as dehydrated, partially degraded organic waste which requires further treatment.

## Chapter 5

# Treatment of organic waste in the in-vessel unit On-Site Composting Apparatus (OSCA)

## 5.1 Summary

OSCA bite-size, a two-barrel composting unit, was operated following the manufacturer's instructions. The two barrels cannot be operated separately, and in the default mode they rotate at one rpm for three minutes hour<sup>-1</sup> (= once hour<sup>-1</sup>). In a commissioning experiment with Barrel 1, the once hour<sup>-1</sup> rotation mode and the cylindrical structure of the barrel, caused the organic matter to roll around in the barrel eventually forming balls which increased in size when more organic matter was added. These tennis ball sized organic matter balls became anaerobic, evidenced by the intense odour from OSCA.

The OSCA7 experiment was carried out in Barrel 2 over 23 days where the carbon content of suburban food market waste was increased with mulches and shredded paper; the C:N was ~18:1. Rotation of the two barrels was reduced and set to three minutes once day<sup>-1</sup> at one rpm (= once day<sup>-1</sup>), since once hour<sup>-1</sup> rotation was considered too frequent and the reason for balling. In the less frequent rotation mode, a mild temperature increase (from ambient to 45°C) and pH increase (from ~5.4 to ~7.3) were recorded in seven days. At this time, the anaerobically-generated odour from the balled waste in Barrel 1 intensified.

The rotation was increased to once hour<sup>-1</sup> in an effort to ameliorate odour. After a further 14 days operation, the composting process in Barrel 2 had to be terminated. The balling of the waste generated internal anaerobic zones, which facilitated anaerobic metabolic microbes. In addition, the once hr<sup>-1</sup> mixing regime led to excessive moisture (water condensed on the vessel lifting hoods) and heat loss (the vessels are not sealed or insulated properly), both of which are unfavourable for optimal composting.

In OSCA8, the C:N ratio had to be adjusted to ~30:1 by mixing suburban food market waste with sawdust and Barrel 2 rotation was set at once day<sup>-1</sup>. After three days, the processing had to be terminated again due to excessive odour from the continuing Barrel 1 commissioning experiment. The parameters of temperature (ambient to ~51°C), pH (4.1 to 5.0), moisture content (75% to 67%) and EC (1.9 to 3.1 mS cm<sup>-1</sup>) in Barrel 2 followed suitable composting profiles before compulsory process termination. There was substantial moisture condensation on the OSCA internal lifting hoods. An exhaust mechanism would improve operation.

It was concluded that the default rotation mode of once hour<sup>-1</sup> generated balling of the organic matter. OSCA7 and OSCA8 experiments were operated with mixing at once day<sup>-1</sup>, in an attempt to avoid organic matter balling. This marginally improved the composting process as concluded from an increase in temperature of the vessel contents to lower thermophilic levels. However, due to no air exhaust, the water vapour condensed on the lifting hoods of the OSCA internal cabinet. In default mode, OSCA bite-size did not produce compost. Suggestions for OSCA redesign are made.

## 5.2 Introduction

The decomposition rate of organic matter can be accelerated by manipulating physical and chemical conditions, which select the most suitable microbes. The main parameters to be controlled are C:N, moisture content and temperature. The On-Site Composting Apparatus (OSCA) is an organic waste treatment in-vessel unit. The former manufacturer Worms Downunder (WDU) Sustainability Pty. Ltd. (current manufacturer, Global Composting Solutions Ltd.), claims that OSCA reduces the input of organic waste by up to 80%, and it can produce “immediately usable compost” within 10 to 14 days. This in-vessel unit can process different types of organic waste including food waste, paper, cardboard, and green waste. The commissioning experiment of OSCA employed the manufacturer’s instructions.

### **5.3 Operational conditions of in-vessel unit OSCA**

The WDU Sustainability in-vessel unit can process  $100 \text{ kg d}^{-1}$  of organic waste and is called OSCA bite-size 100; this was used in this research (Figure 5.1 and Figure 5.2).



Figure 5.1 External view of the in-vessel unit, OSCA bite-size 100.

#### **Technical specification of in-vessel unit OSCA bite-size 100:**

- Capacity:  $100 \text{ L d}^{-1}$  mixed waste
- Dimensions:  $2.4 \text{ m long} \times 1.2 \text{ m wide} \times 1.7 \text{ m high}$
- Electricity requirements: 240V, 10A or Solar option available
- Electricity usage/month: 30 kWh
- Electricity:  $\$7.00 \text{ month}^{-1}$  (based on  $23 \text{ c.kW}^{-1}.\text{h}^{-1}$ )
- Options: Solar, plug & play additional modules for extended capacity, color & cladding, extreme climate version

OSCA bite-size 100 is an automated and regularly (once or twice day $^{-1}$ ) fed in-vessel composting unit. It has two barrels connected to the same central axis. The default rotation of the barrels is once hour $^{-1}$ , which is suggested as being suitable for most waste streams. Continuous forced ventilation goes through two holes of ten cm diameter, located in the upper and lower part of the side edge of each barrel. OSCA has three carbon filters which should remove offensive emissions if produced.

OSCA can be loaded once or twice per day to a maximum of 100 kg of organic waste. The company recommends adding a small amount of organic waste and progressively increasing it. The maximum waste to be added is 75% of the total capacity of the barrel. Once the first barrel reaches the limit, the second barrel can be filled. On average, the period of feeding takes one week, and while one barrel is being fed, the other barrel is processing the organic waste for one week more. Thus, after 14 days the end-product is an “immediately usable compost”. To download the end-product, a container is placed below the barrel, the hatch panel is opened, and the barrel is rotated until the end-product drops into the container.

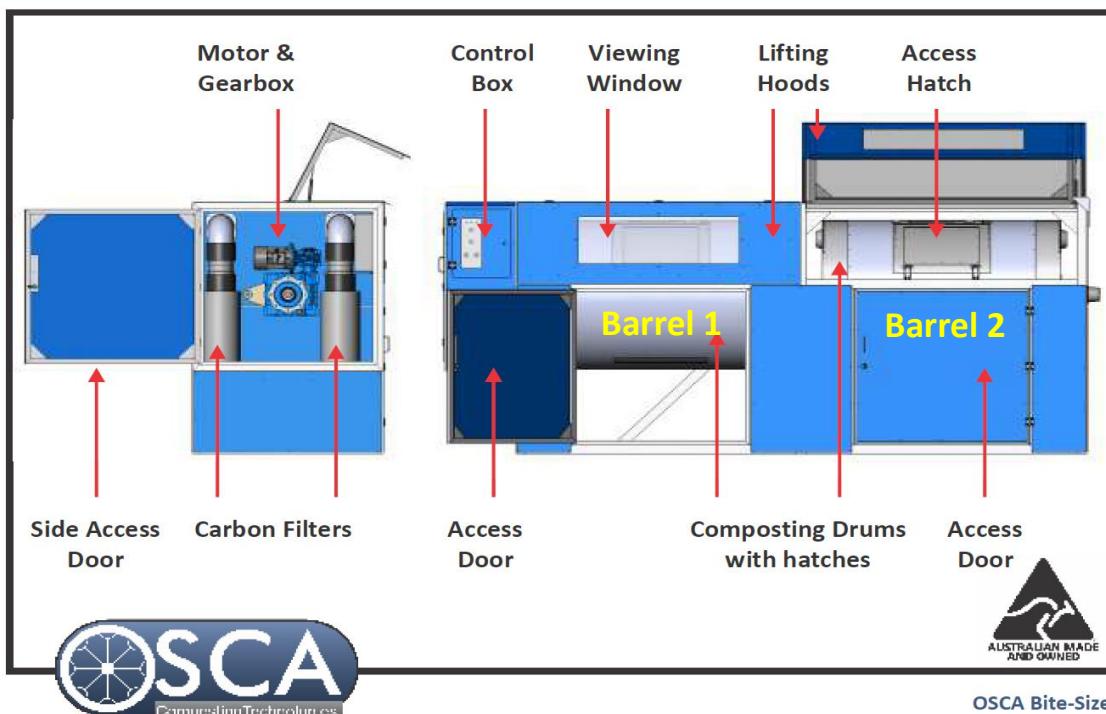


Figure 5.2 Internal diagrammatic view of the in-vessel unit, OSCA bite-size 100.

## 5.4 Experimental design

### 5.4.1 OSCA commissioning

Once the OSCA unit was installed at Swinburne University, it operated continuously for 168 days, and as part of this initiative, during the commissioning experiment, the cafes and the bookshop were adding waste every day to the OSCA unit, ~10 kg organic waste and two kg paper respectively (Barrel 1). This unit was operated at the default rotation rate of three minutes

hour<sup>-1</sup> at one rpm. The only exception to this was when OSCA7 and OSCA8 experiment ran at one rotation per day (see Figure 5.3).

### 5.4.2 Collection and audit of food waste

Food waste composed of vegetables, fruits, chicken meat, and bread, were collected from the Camberwell market, Hawthorn, Melbourne. Newspapers were collected from Swinburne bookshop and mulches were provided by Craig Hudson (C. R. Hudson & Associates Pty. Ltd.). In OSCA7, 54.5 kg of organic matter was placed into the Barrel 2 and the calculated C:N was ~18:1. In OSCA8, the C:N was adjusted to 30:1 by mixing 2.21 kg of sawdust with 50 kg of organic waste which was treated in Barrel 2 (Table 5.1).

Table 5.1 Characterisation of organic waste – OSCA.

Organic waste	pH	Electrical conductivity (mS cm <sup>-1</sup> )	<i>Q<sub>n</sub></i>	<i>Q<sub>n</sub></i>	<i>M<sub>n</sub></i>	<i>C<sub>n</sub></i>	<i>N<sub>n</sub></i>	C:N*
			OSCA7 (kg)	OSCA8 (kg)	Moisture content (%)	Carbon* (%)	Nitrogen* (%)	
Mulches	6.21	3.15	30.00	0.00	53.00	57.80	3.40	17.00
Vegetables	4.98	1.55	10.00	3.33	91.72	37.50	2.50	15.00
Fruits	4.53	0.98	12.50	16.67	88.66	56.00	1.40	40.00
Newspaper	7.23	0.55	2.00	0.00	2.20	23.88	0.06	398.00
Food waste (Chicken meat, bread)	4.98	1.55	0.00	30.00	59.00	23.50	1.25	18.80
Sawdust	4.63	0.82	0.00	2.21	5.30	106.10	0.20	530.50
<b>TOTAL</b>			<b>54.5</b>	<b>52.21</b>				
<b>C:N (mixture)</b>			<b>18.6</b>	<b>30</b>				

**Note:** Values of carbon and nitrogen taken from \*(Rynk et al., 1992), and \*(Ballesteros et al., 2014). For C:N ratio calculation for OSCA7 and OSCA8, *n* = 4 (see Section 3.3.8; equation 2).

### 5.4.3 OSCA7

The organic waste was chopped to reduce the particle size to <5 cm in diameter, then it was mixed with the shredded paper and mulches. The mixture was added into Barrel 2 (empty barrel) since the commissioning experiment was on-going in Barrel 1. Three temperature data loggers were added to the organic material. Samples were taken every day as per Section 3.2.

#### 5.4.4 OSCA8

A total of 50 kg of food waste (Table 5.1) was treated by chopping the large organic matter to <5 cm in diameter. The 50 kg of treated organic waste and 2.21 kg of sawdust were mixed inside Barrel 2 to achieve a C:N of ~30:1 (see Table 5.1 and Section 3.1.8). Three temperature data loggers were added into the compost mix and samples were taken every day as per Section 3.1.3.

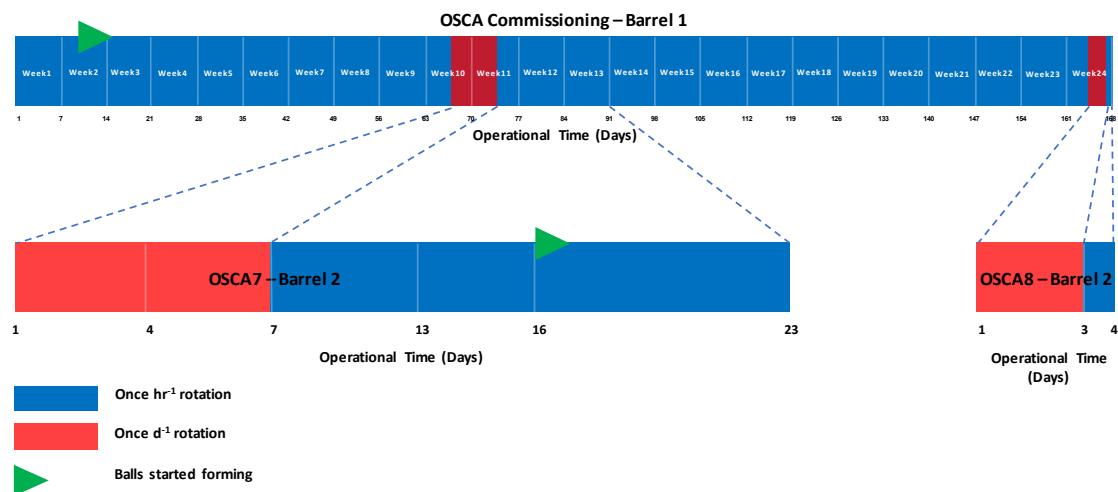


Figure 5.3 Timeline of commissioning of OSCA (Barrel 1) and of OSCA7 and OSCA8 (both in Barrel 2) experiments

## 5.5 Results

### 5.5.1 OSCA commissioning

When the barrel was operated at the default rotation mode of once hour<sup>-1</sup>, the organic material moved up then fell by rolling down the cylindrical vessel wall, subsequently forming different sized balls, some of which were bigger than a tennis ball (Figure 5.4). Ball formation commenced in week 2 (Figure 5.3), which coincided with the generation of offensive odours.



Figure 5.4 Images of OSCA during operation in Barrel 1. The organic matter is shown forming balls during the commissioning of OSCA bite-size 100.

The default rotation facilitated frequent exposure of the organic material to the barrel surfaces, which was hypothesised to provoke heat loss and water condensation, and the temperature of the composting material was mostly below 40°C. The ventilation holes became covered with organic material, which would have impeded aeration (Figure 5.5 left). At each rotation, small particles of organic matter fell through the barrel lid holes (Figure 5.5 right) to rain on the floor below. A container was placed there to collect the waste.



Figure 5.5 Images of OSCA during operation. Left - Ventilation hole covered by organic waste. Right - Small particles falling through the barrel lid holes.

These observations were communicated to the manufacturer. OSCA7 was operated in Barrel 2 starting in week 10 of the commissioning process, with the barrels at once day<sup>-1</sup> rotation.

### 5.5.2 Time course of organic waste treatment in OSCA7 and OSCA8

For the first four to five days, when OSCA7 was operated at once day<sup>-1</sup> rotation, the microbial activity endogenously generated heat and increased the compost bed temperature to a maximum of ~45°C (Figure 5.6). As the temperature rose, the moisture condensed on the barrel wall and ceiling of the lifting hood and condensed water fell to leak outside the OSCA unit (Figure 5.7). The organic matter in OSCA7 formed balls on day 16, similar to those in the commissioning barrel (Barrel 1) (Figure 5.3).

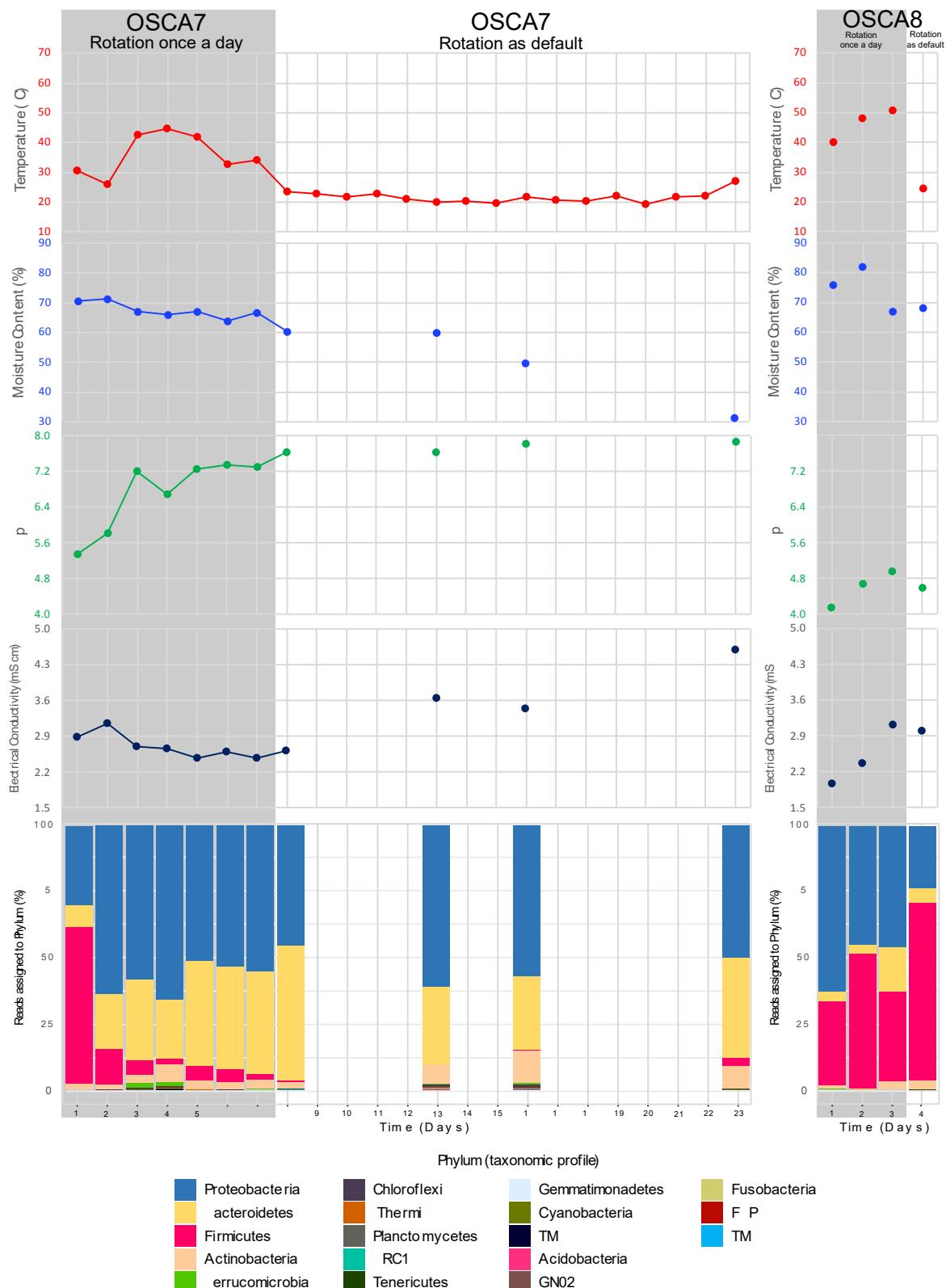


Figure 5.6 Physical, chemical and microbial changes during OSCA experiments.

The formation of waste balls in the commissioning Barrel 1 coincided with the generation of offensive odours. In an effort to more effectively disperse the odours from Barrel 1, at the end of day seven of OSCA7, the mixing was

increased back to three minutes hour<sup>-1</sup> (the default mode). Over the following 16 days, the moisture content dropped to 30%, which is below the optimum range of 40% to 60% (Figure 5.6). The pH increased from acidic (5.4) to alkaline (7.9), and the EC was in the range from 2.5 mS cm<sup>-1</sup> to 4.5 mS cm<sup>-1</sup> (Figure 5.6). After a total of 23 days, the final product from OSCA7 was removed from Barrel 2.



Figure 5.7 OSCA7 and OSCA8 operation. Left and centre - vapour condensation on the lifting hoods; and right, condensate leaking from the base.

The organic waste for OSCA8 was adjusted to a C:N ratio of 30:1 by sawdust addition (Table 5.1). After adding the amended organic waste to Barrel 2, it was operated at once d<sup>-1</sup> rotation for three minutes. Over three days, the temperature increased rapidly to be ~50°C, moisture content decreased from ~75% to ~65% (these are both relatively high), and both pH and EC rose (Figure 5.6).

These promising composting trends were short-lived as OSCA8 had to be modified on the evening of the third day, because of the anaerobically generated offensive odours being produced in Barrel 1, where balling of the waste material was still occurring. The increased rotation mode of once hour<sup>-1</sup>, initiated at the end of the third day, had a dramatic impact on the physical and chemical parameters. The temperature dropped to 25°C, moisture content slightly increased, and the pH and EC decreased (Figure 5.6).

### 5.5.3 Physical and chemical analysis

Principal Component Analysis (PCA) of the physical and chemical data are shown in Figure 5.8. The temperature, moisture content, pH and EC were determined as described in Section 3.3.

Figure 5.8a compares the effect of barrel rotation (once day<sup>-1</sup> for seven days and once hour<sup>-1</sup> for 16 days) in OSCA7. The highest eigenvector and eigenvalue of the OSCA7 PCA biplot (Figure 5.8a) represent 62.8% for the PC1, and 22.2% for the PC2. The reduced mixing samples were correlated to temperature, while the more frequent mixing generated high variability with correlation to EC.

Figure 5.8b shows the data for the first four days of OSCA7 and OSCA8 experiments and compares the effect of sawdust addition in OSCA8 with its absence, in OSCA7. The highest eigenvector and eigenvalue of the Figure 5.8b biplot represent 50.0% for PC1 and, 29.1% for PC2. In the first four days of OSCA7, the samples from day one and two were more similar and the samples from day three and four were more similar between each other. In contrast, the samples of OSCA8 had high variability and dissimilarities among them.

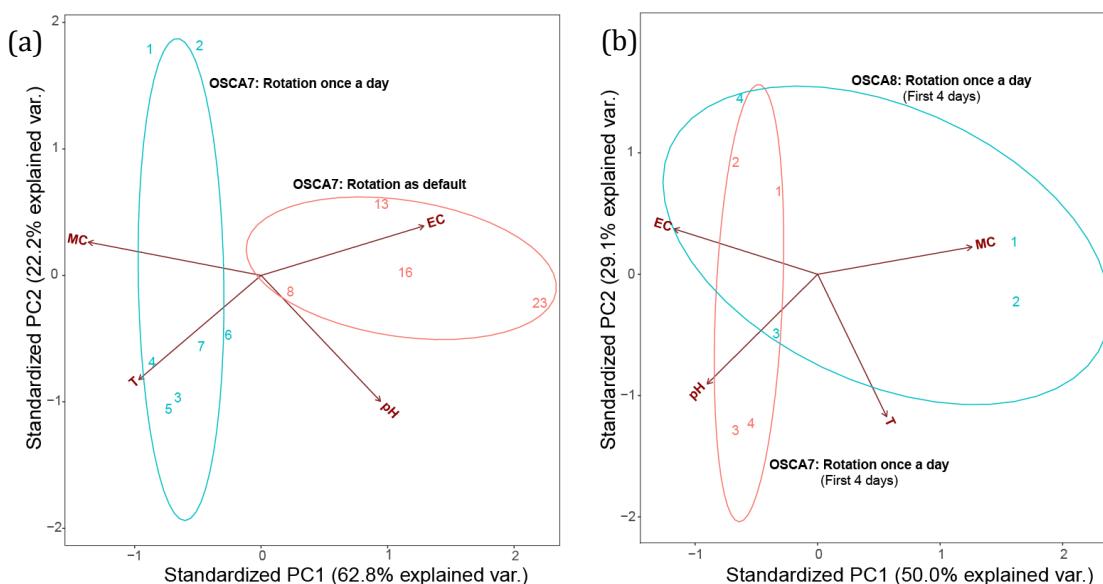


Figure 5.8 Physical and chemical analysis **(a)** Principal Component Analysis (PCA) of OSCA7 experiment and **(b)** PCA of OSCA7 and OSCA8 (first four days) experiment. Where T = temperature ( $^{\circ}\text{C}$ ), MC = moisture content (%), pH, and EC = electrical conductivity ( $\text{mS cm}^{-1}$ ). Ellipses indicate 95% confidence intervals.

### Compost maturity by Solvita® test – OSCA7

Samples from 13, 16 and 23 days were evaluated by the Solvita® compost maturity test (Section 3.1.9; Figure 5.9). Only the sample from 23 days (30% moisture content) required re-moisturisation, and incubation for 24 hours. Samples from 13 days and 16 days were equal to a carbon dioxide reading of 1 translating to 20% carbon dioxide (Figure 3.1, Section 3.1.9) and the sample from 23 days equaled 2 translating to 15% carbon dioxide (Figure 3.1, Section 3.1.9). The paddle for ammonia for all samples was equal to a reading of 5 which translates to  $\leq 0.02$  mg ammonia (Figure 3.1, Section 3.1.9).

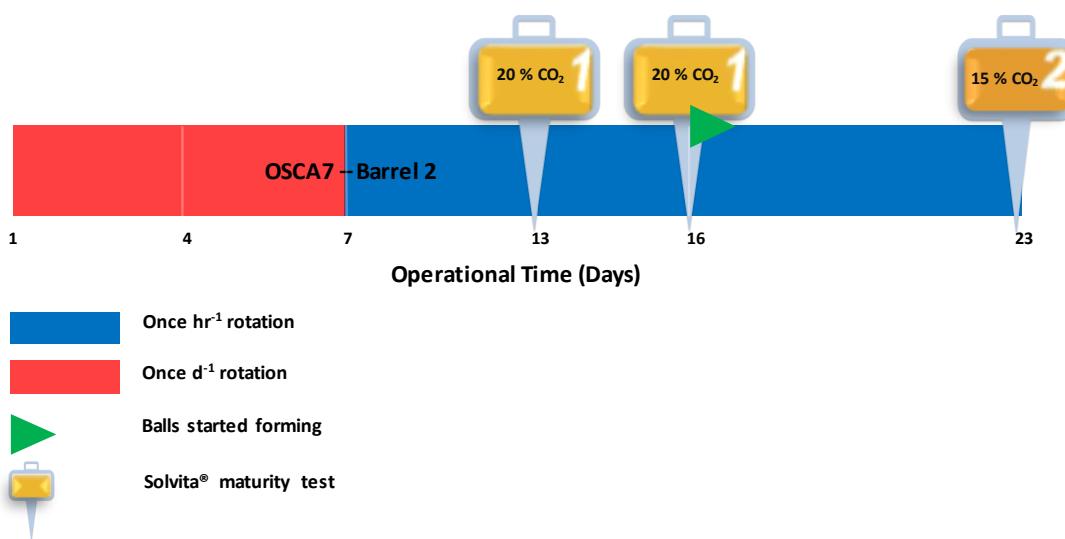


Figure 5.9 Maturity test via Solvita® of compost during - OSCA7.

#### 5.5.4 Metabarcoding microbial analysis

The V3-V4 region of the 16S rRNA gene was PCR amplified from extracted DNA, which on occasion required dilution. The primers 515F-806R (the numbers refer to nucleotides in the *E. coli* 16S rRNA gene (Walters et al., 2015)) were used and products were observed by agarose gel electrophoresis where a band at ~300 nucleotides (compared to a molecular weight ladder) would be positive. The PCR products were processed as described in Section 3.2.1 at the WEHI and amplicon sequenced by the Illumina MiSeq machine. A total of 11 OSCA7 samples and four OSCA8 samples were metabarcoded (Figure 5.6). One sample from sawdust, a DNA extraction kit sample and Milli-Q water were also analysed as negative controls. In total 18 samples were sequenced.

Bioinformatic analyses followed methods described in Section 3.2.2. A total of 640,837 raw reads from 18 samples were obtained, and per sample the reads were: minimum 23,952, mean 35,602.1 and maximum 50,655 reads. After denoising and chimeric filtering with DADA2, the total number of reads was reduced to 294,231 and per sample the reads were: minimum 153 (number of reads of sawdust), mean 16,346.2 and maximum 27,324. A total of 4,153 ASVs were revealed in the samples.

From the rarefaction curve (see Appendix F, Figure F1), the sequencing depth chosen for further analyses was 9,500 reads which meant that OSCA8 day one was not analysed since it only had ~5,000 reads, where several samples (e.g., OSCA7 three days and four days) had not reached their asymptote. The choice of this read depth, could allow small losses of data and consequently minimal loss of sample diversity. However, it ensures that most of the samples are included in downstream analyses. Decontam (Davis et al., 2018) software at the default threshold of  $p = 0.1$ , showed no contamination of the sequences.

### **Microbial diversity and abundance at phylum level – OSCA7 and OSCA8**

Four phyla represented >95% of bacteria; they were Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria (Figure 5.6) A few other phyla were identified in extremely low abundances (<2%) and only occasionally (e.g., Chloroflexi and Verromicrobia) (Figure 5.6).

During OSCA7 at once day<sup>-1</sup> barrel rotation, phylum Proteobacteria was initially ~30% abundant, then increased to ~45% from day two to day seven. Firmicutes were initially ~59% abundant but dropped substantially over seven days to be ~1% abundant. Bacteroidetes was initially low at <10% abundance, but they gradually increased to be ~50% abundant by seven days. On day eight, barrel rotation was changed to once hour<sup>-1</sup> and the bacterial phyla Proteobacteria were ~50 to 55% and Bacteroidetes 30 to 50% abundant at different times, although there was no consistent trend in their abundances. Actinobacteria increased to be ~7 to 12% abundant, while other phyla were <2%, (Figure 5.6).

During the first three days of OSCA8 at once day<sup>-1</sup> rotation, the abundances of Firmicutes fluctuated between ~30% to ~50%, and Proteobacteria decreased from ~60% to ~45%. Bacteroidetes was <4% in the first two days, then

increased to ~17%, Actinobacteria was <3%, and other phyla were <0.3%. On day four, barrel rotation was changed to once hour<sup>-1</sup> and Firmicutes increased in abundance to ~67%, while Proteobacteria decreased to ~23%, as did Bacteroidetes to ~5.7% (Figure 5.6).

### **Microbial diversity and abundance at order level - OSCA7 and OSCA8**

On the first day of OSCA7 with barrel rotation once day<sup>-1</sup>, Bacillales (~32%), Lactobacilales (~27%), Pseudomonadales (~12%) and Sphingobacteriales (~7%) dominated the microbial community (Figure 5.10). Day two to four revealed lots of fluctuations and no abundance trends in bacterial orders (Figure 5.10). From day five to eight, several trends were observed in Sphingobacteriales, Flavobacteriales, Actinomycetales, Pseudomonadales, Burkholderiales, Rhodobacteriales and Rhizobiales (Figure 5.10).

Xanthomonadales (at day one <5%) increased but fluctuated between 18% and 26% from day two to seven; Sphingobacteriales increased but fluctuated from ~7% on day one to ~18-20% on day five to eight; Pseudomonadales increased from day two (~11%) to day four (~20%), then decreased to be <10%. In contrast, Flavobacteriales and Rhodobacteriales were <5% abundant in the first four day, then increased from day five to seven to ~20% and 8%, respectively. The remaining bacterial orders were <10%. When OSCA7 was operated at once hour<sup>-1</sup> rotation, Flavobacteriales dominated the bacterial orders at ~30% but fluctuated; Alteromonadales increased in abundance on day 13 and 16 (~12 and 21%), but on day 23, were very low in abundance (Figure 5.10).

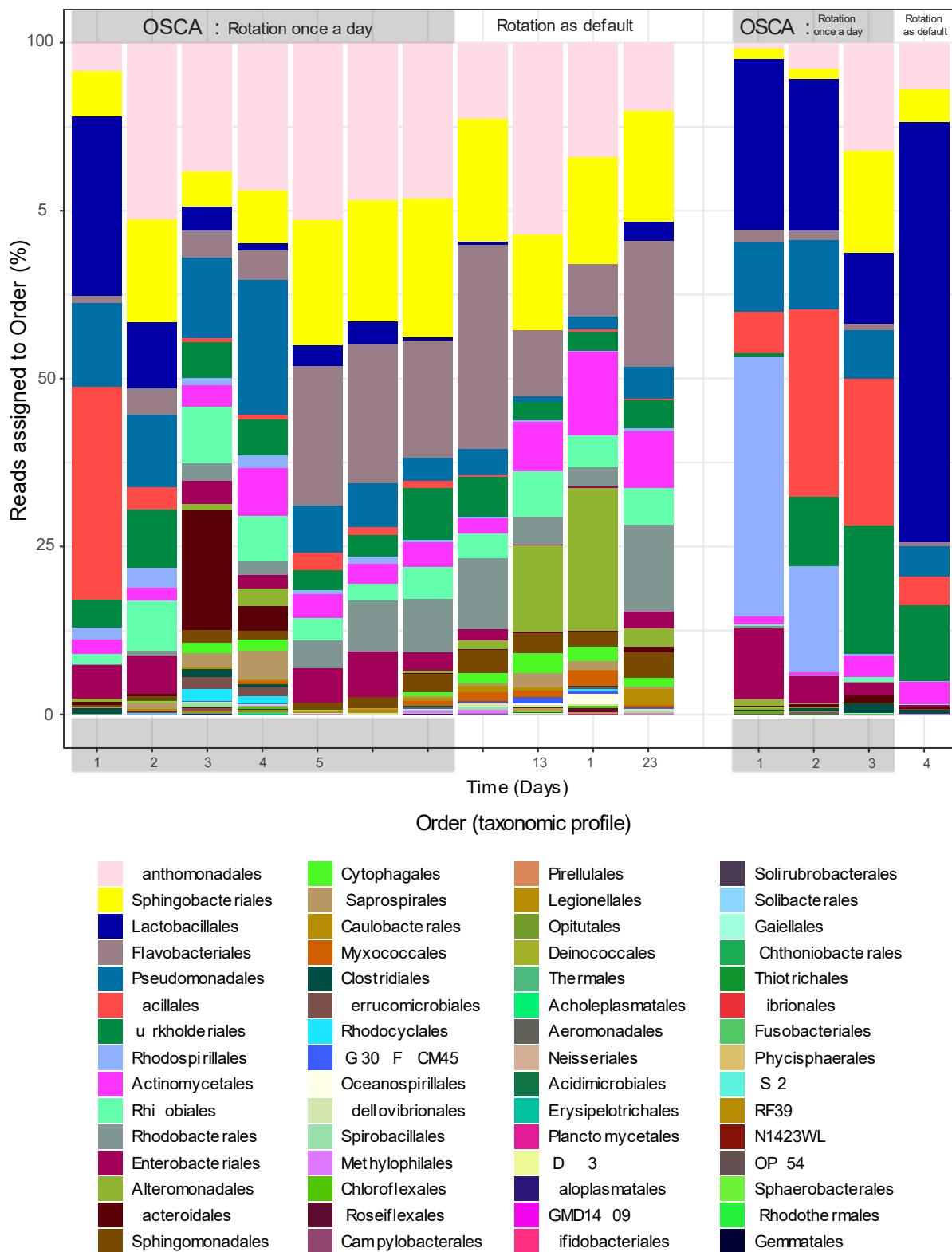


Figure 5.10 Microorganisms at order level of OSCA7 and OSCA8 experiments.

In OSCA8 on day one and three when the mixing was once day<sup>-1</sup> rotation, Rhodospirillales was in high abundance (~38%) but sharply declined; Lactobacillales (~25%) also declined (to ~10%); and Rhodospirillales

decreased from ~16% to ~0.1%. *Pseudomonadales* (~10%) remained about the same abundance in the three operational days; *Bacillales* rose from ~6% abundance to ~25%; *Burkholderiales* increased from <1% to ~19%; and *Xanthomonadales* increased from ~4% to ~16%. After changing mixing to once hour<sup>-1</sup> rotation at the end of the day three and only for one day, the physical and chemical operational parameters were impacted (Figure 5.6), as was the microbial diversity (Figure 5.6 and Figure 5.10). *Lactobacillales* substantially increased in abundance from ~10% to ~63%; *Burkholderiales* declined from ~19% to ~11%; *Xanthomonadales* decreased from ~16% to ~7%); and the remaining bacterial orders were <5% (Figure 5.10).

### **Microbial diversity and abundance at genus level - OSCA7 and OSCA8**

At the genus level, only few patterns in microbial communities and different barrel rotation phases in OSCA7 (Figure 5.3) and OSCA8 (Figure 5.3) were obvious, but focus can be drawn from the heatmaps in Figure 5.11. For OSCA7 (23 days monitoring), there was a trend in the abundance of some genera over the first four days compared to after four days of OSCA7 (Figure 5.11a):

- sharp decline till day four, then absent/low - *Lactobacillus*,
- steady/low through day four then increasing - *Sphingobacterium*, a *Weeksellaceae* genus, *Parapedobacter*, *Paracoccus*, *Thermomonas*
- increasing/steady through day four then decreasing – *Pseudoxanthomonas*, *Pseudomonas*, an *Enterobacteriaceae* genus, *Xanthomonas*.

*Cellvibrio* increased in abundance on day 13 and 16 and *Bacillus* was high (~21%) on day one, but very low (<1.5%) thereafter.

During the four days of OSCA8 operation, *Lactobacillus* increased sharply in abundance from ~7% to ~62%; *Sphingobacterium* and a *Weeksellaceae* genus were always <1.5% abundant, and collectively two *Comamonadaceae* genera were negligible on day one, then consistently ~10-15% abundant on day two, three and four; and there were sharp falls abundance of *Acetobacter* (from ~34% to be negligible), *Weissella* (from ~8% to be negligible) *Pseudomonas*

(from ~7% to ~3%), one Enterobacteriaceae genus (from ~7% to <0.5%) and *Bacillus* (from ~7% on day two to <1%).

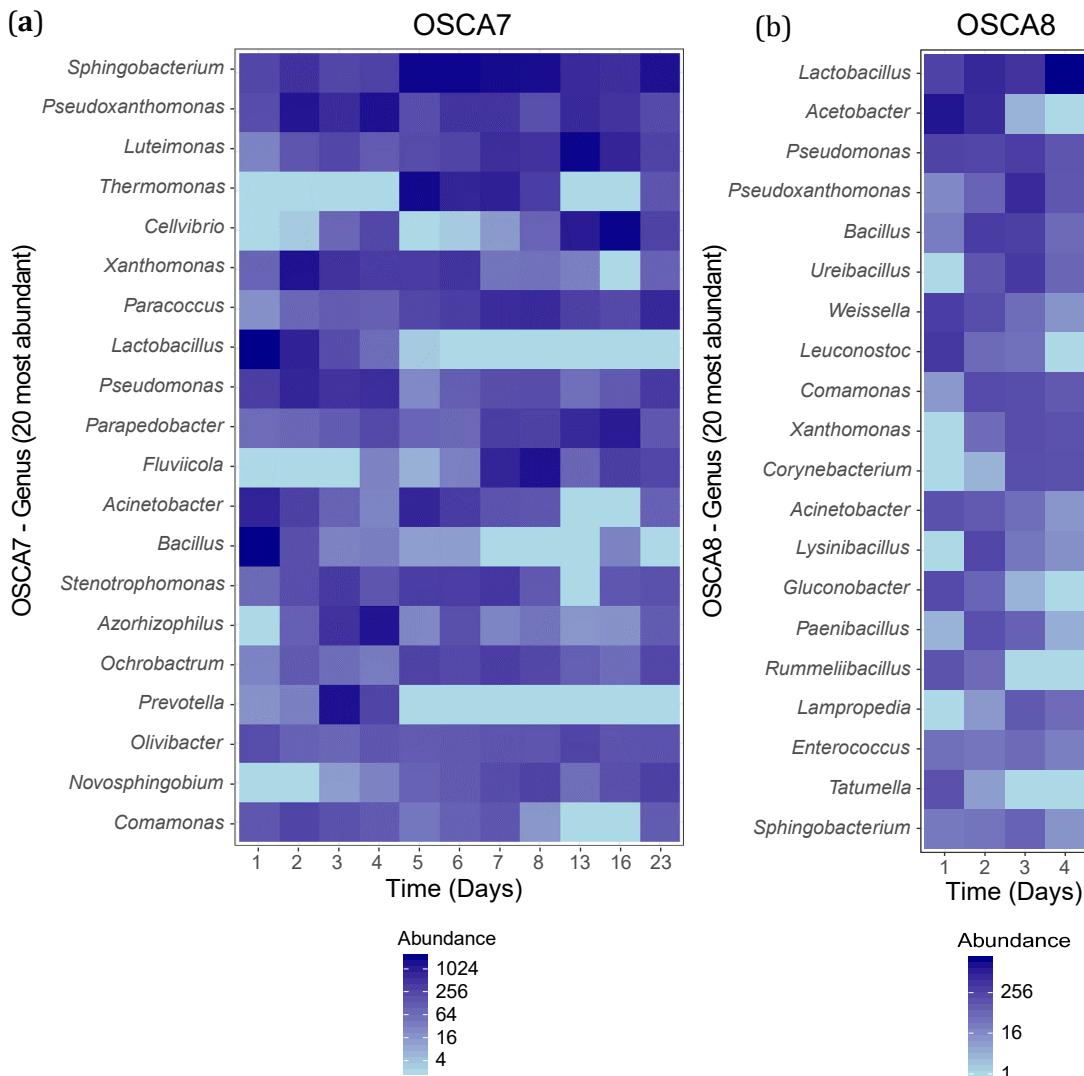


Figure 5.11 Heatmap of 20 most abundant bacterial genera in: (a) OSCA7, and (b) OSCA8.

## Sawdust

The microbial composition of the sawdust was determined by methods described in Section 3.2.2. The sawdust was used to amend OSCA8 urban waste to achieve a C:N of ~30:1. In the sawdust sample, *Kerstersia* was in higher abundance (~33%), *Fulvimonas* was represented by ~18%, *Rathayibacter*, *Asticcacaulis* were at the same proportion of ~10%. None of these genera were present in the OSCA7 or OSCA8 samples. *Solimonas* was in low abundance in sawdust (~7%). Also, it was found in extremely low abundance (<0.5%) in the first four days of OSCA7. Lactic acid producing

bacteria *Weissella* (~13%) and *Lactobacillus* were present in sawdust (~10%). Also, these microorganisms were present in the early composting of OSCA7 and OSCA8.

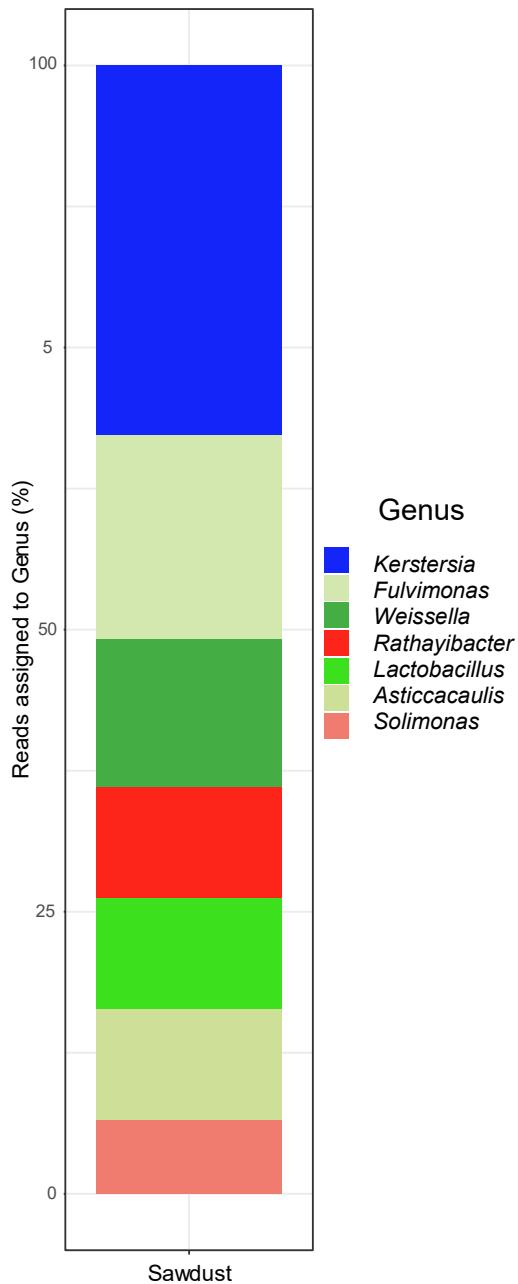


Figure 5.12 The bacterial genera in sawdust used to amend OSCA8.

### 5.5.5 Microbial diversity analysis

#### Alpha diversity

The alpha diversity, community evenness (heterogeneity), and overall quantitative microbial community richness were determined as described in Section 3.2.2 and are presented in Figure 5.13. Consistent with the day four

breakpoint observed for genus abundances in OSCA7 (Section 5.5.4), the observed ASVs achieved the highest richness on day three then dipped slightly on day four, before decreasing dramatically on day five (Figure 5.13a). ASV numbers increased between day six and eight before plateauing through day 23. Evenness according to Simpson's Diversity Index (Figure 5.13b) was somewhat consistent over the 23 days run, albeit with the day three/four dip then recovery. Richness according to Shannon's Index (Figure 5.13c) was similarly consistent as evenness.

Over the four days of OSCA8 observed ASVs were frequently lower than those from OSCA7. The highest of all alpha diversity indices was on day two; they all then decreased after day three when mixing was increased from once day<sup>-1</sup> to once hour<sup>-1</sup> (Figure 5.13). Microbial community evenness and richness were substantially lower on day four compared to day one to three, which is consistent with dramatic bacterial genus changes as described in Section 5.5.4.

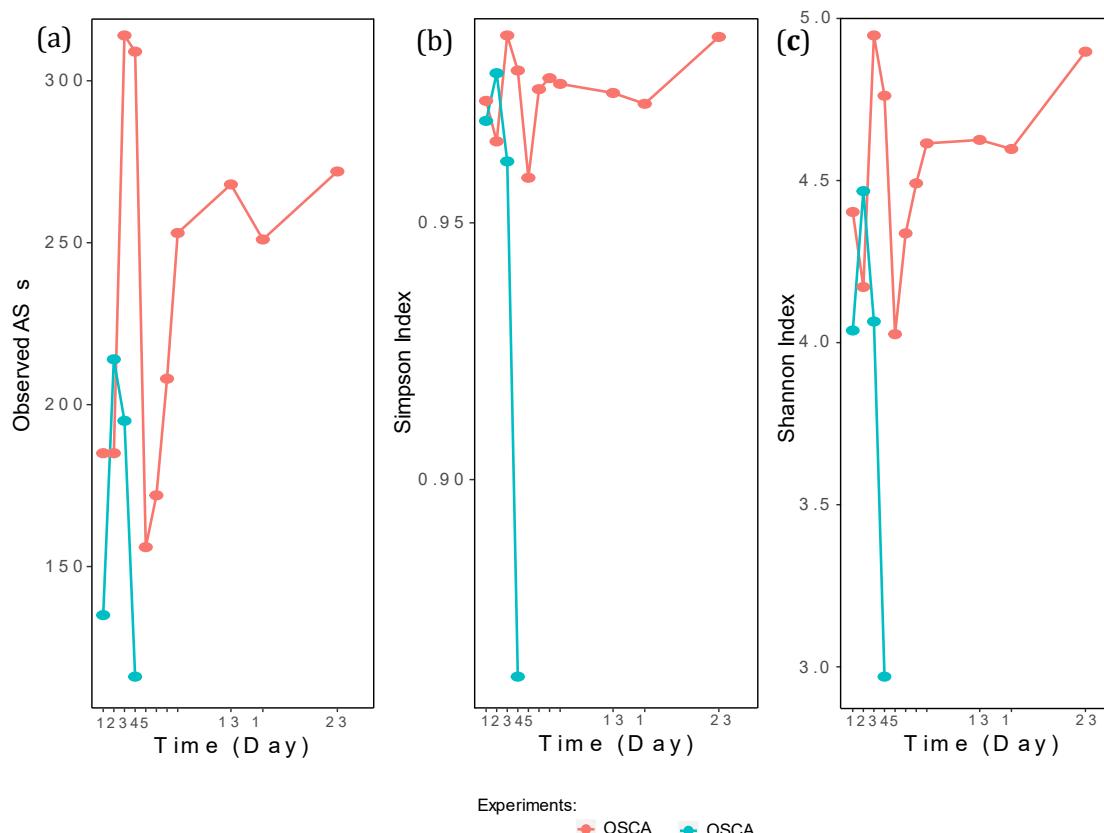


Figure 5.13 Alpha diversity of OSCA experiments. **(a)** Observed ASVs, **(b)** Simpson's diversity index and **(c)** Shannon's diversity index.

## Beta diversity

Beta diversity was determined by methods described in Section 3.2.2 and plotted in a non-metric multidimensional scaling (NMDS) ordination. Figure 5.14a shows that the data-points from the two different rotation regimes of OSCA7 grouped based on the mixing with low variability in the first seven days, and higher variability from day eight onwards.

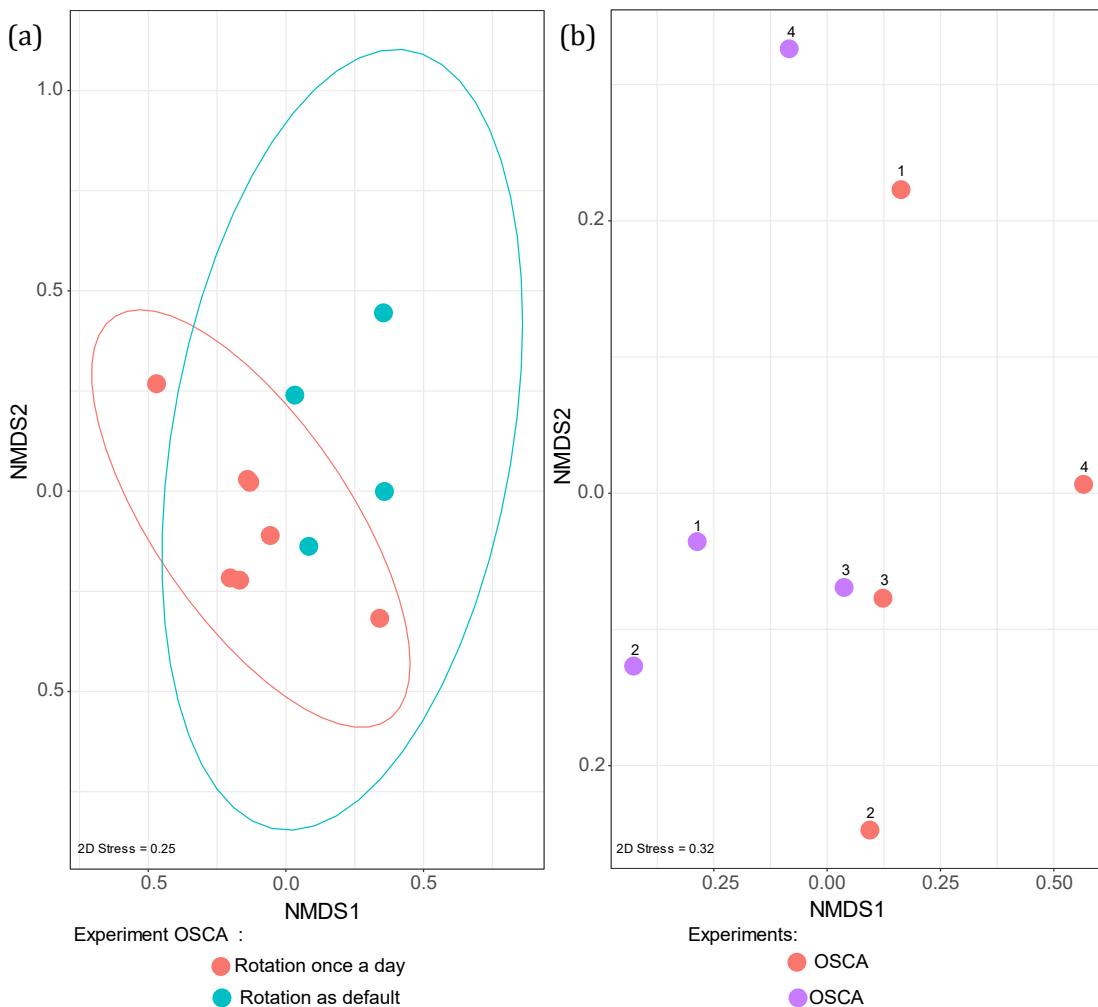


Figure 5.14 Bacterial community comparison by non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis distances, where ellipses indicate 95% confidence intervals: **(a)** OSCA7 – different rotation modes, **(b)** days one to four for OSCA7 and OSCA8.

The OSCA7 bacterial communities were tested with a Generalized Linear Model (GLM). GLM-based analyses revealed that there were significant differences in community composition based on rotation mode (manyGLM, LRT = 0,  $p = 0.001$ ), and composting time (manyGLM, LRT = 480.6,  $p = 0.021$ ) (see

Appendix F, Table F1). Figure 5.14b shows that data-points from day one to three (mixing once day<sup>-1</sup>) clustered together to the exclusion of day four data (mixing once hour<sup>-1</sup> introduced at the end of day three).

Comparing the distribution of the first four samples by NMDS (from day one to day four) of the non-C:N adjusted (OSCA7) and C:N adjusted (OSCA8) experiments, the data-points were plotted. GLM-based analysis revealed that there was a significant difference in the community composition based on the different experiments (manyGLM, LRT = 0,  $p = 0.004$ ), however, the bacterial communities did not differ significantly based on the time (manyGLM, LRT = 424.7,  $p = 0.157$ ) (see Appendix F, Table F2).

### 5.5.6 Pathogenic microbial analysis

Attempts to isolate potential pathogens on suitable media followed methods described in Section 3.3.1. No bacterial colonies identical to the reference *Escherichia coli* O157:H7 (ATCC43895), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 13311; *S. typhimurium*) or *Enterococcus faecalis* (ATCC 19433) were recovered on their differential/selective media from the OSCA experiments. Isolates were acquired on these media of *Klebsiella oxytoca* strain JM117 from day one of OSCA7; *Enterococcus cloacae* strain B3X8 was isolated from all OSCA7 and OSCA8 samples.

Bacterial presumptive identity of the isolates was determined through NCBI by BLAST of partial 16S rRNA gene sequences determined by Sanger sequencing (Section 3.3.2). Metabarcoding showed that none of *E. coli*, *Salmonella* spp. or *Enterococcus* spp. were isolated from the samples. However, *Enterococcus cecorum* and an unresolved *Enterococcus* sp. were present in low abundance (<1%) in the first four days in OSCA7 and OSCA8, according to metabarcoding.

Table 5.2 Pure cultured bacteria during OSCA7 and OSCA8 experiment as identified by BLAST of partial 16S rRNA gene sequences.

Identified microorganisms - BLAST	Sequence length (bp)	Match score	Percentage similarities	Accession number	OSCA7 (Day)	OSCA8 (Day)
<i>Klebsiella oxytoca</i> strain MJ117	1438	698/698	100	MN758877.1	1	1
<i>Enterobacter cloacae</i> strain B3X8	987	870/870	100	MN330012.1	2-23	2-4

### 5.5.7 Colony counting

Total bacterial colony forming units (CFU) g<sup>-1</sup> sample from OSCA7 during the once day<sup>-1</sup> barrel rotation mode (one to seven days) were between 1.5x10<sup>6</sup> to 3.4x10<sup>7</sup>. OSCA7 was set to once hour<sup>-1</sup> rotation at the end of day seven; day eight contained 2.3x10<sup>5</sup> CFU g<sup>-1</sup> and day 23 had 1.1x10<sup>2</sup> CFU g<sup>-1</sup>. On day one of OSCA8 (once day<sup>-1</sup> barrel rotation), there were 1.5x10<sup>6</sup> CFU g<sup>-1</sup> in the compost mix; and on day four (once hour<sup>-1</sup> barrel rotation) there were substantially more at 4.1x10<sup>8</sup> CFU g<sup>-1</sup>.

Table 5.3 Number of colony forming units g<sup>-1</sup> of OSCA7 and OSCA8 sample during organic waste treatment.

Experiment	OSCA7	OSCA8
Time (Days)		Colony forming unit (CFU g <sup>-1</sup> )
1	1.5x10 <sup>6</sup>	1.5x10 <sup>6</sup>
3	1.2x10 <sup>7</sup>	2.6x10 <sup>7</sup>
4	---	4.1x10 <sup>8</sup>
5	3.2x10 <sup>5</sup>	---
7	3.4x10 <sup>7</sup>	---
8	2.3x10 <sup>5</sup>	---
16	1.4x10 <sup>3</sup>	---
23	1.1x10 <sup>2</sup>	---

## 5.6 Discussion

### 5.6.1 OSCA7 and OSCA8 - physical and chemical data

OSCA functions as a drum composter, which has a horizontal barrel with continuous rotation. Varying the rotation frequency from four rpm to six rpm, provides mixing and aeration for composting (Haug, 1993). The default rotation of OSCA is once hour<sup>-1</sup>, which was found to produce organic matter balls. An anoxic inner-ball environment was likely created, and anaerobic microbes were selected for. These anaerobes decompose organic matter and produce carbon dioxide, methane (Haug, 1993), volatile fatty acids, organic sulfides, amines and mercaptans (Miller, 1993, Goldstein, 2002), alcohols, terpenes, ammonia, and hydrogen sulfide which all contribute to foul odours (Epstein, 1997).

Kalamdhad and Kazmi, (2009) ran several experiments to evaluate the optimum rotation frequency in a drum composter and found that once  $d^{-1}$  rotation maintains high temperature ( $58^{\circ}C$ ) during the active phase and reduces moisture losses. A rotary drum composter was successfully operated with one complete rotation day $^{-1}$  (Varma et al., 2018, Varma and Kalamdhad, 2015). In an effort to improve organic matter degradation, and potentially mitigate balling of organic matter and odours, OSCA7 and OSCA8 were initially operated with reduced mixing compared to manufacturer's instructions; i.e., rotation once day $^{-1}$ . In OSCA7, the temperature increased for the first three to four days to be above  $40^{\circ}C$ , but then decreased. OSCA8 also heated in the first three days of operation at the once day $^{-1}$  rotation.

Heat loss was hypothesised to partially be due to waste being in contact with the cool barrel walls and excessive moisture condensation. The barrels in OSCA are not well insulated and it has been found that insulation of small scale, particularly rotary drum composters, has a major effect on heat maintenance (Alkoai et al., 2019). Due to excessive foul odours from the commissioning experiment in Barrel 1, OSCA7 and OSCA8 were changed to more frequent rotation (once hour $^{-1}$ ), but this continued to facilitate heat loss; in OSCA7 to  $\sim 20^{\circ}C$  by day eight, and the moisture content continuously, gradually dropped to be  $\sim 30\%$  at day 23. However, both these parameters were already in a downward trajectory from the once day $^{-1}$  rotation operation. During the active composting phase, the heat production contributes to vaporisation, which reduces the moisture content, hence, moisture loss can be an indicator of decomposition rate (Liao et al., 1997); in OSCA7, there was no heat generation accompanying moisture loss from day eight to day 23. Rotation is highly favourable for improving the decomposition rate as it mixes the feed material with the microbes and ensures oxygen entrainment and dissolution (Chandna et al., 2013). Excessive rotation might have contributed to poor composting in OSCA7 from day eight, but it was already not functioning optimally.

OSCA8 had an adjusted C:N of  $\sim 30:1$  and the rotation mode was set to once day $^{-1}$ ; this lead to improvement in the decomposition rate compared to OSCA7 as reflected in the rapid increase of the temperature from  $40^{\circ}C$  on day one to  $50^{\circ}C$  at the end of day three. Microbes use carbon as an energy source and

nitrogen for growth (Chen et al., 2011) with concomitant heat production. At the end of the third day, when the rotation was changed to once hour<sup>-1</sup>, the physical and chemical parameters dramatically changed; the temperature dropped rapidly from 51°C to 25°C, the pH decreased from 5.0 to 4.6, the moisture content slightly increased perhaps due to condensation, and the EC decreased as a direct result of moisture increasing.

The excessively short operational time of OSCA8 make it impossible to draw major conclusions from it. OSCA7 could not maintain heat and lack of insulation was hypothesised to be a contributor. However, OSCA8 was able to generate a good heating profile until day three. It could be that the initial days operation sets the scene for subsequent days and the process's mixing disturbance led to microbial disturbances that precluded OSCA 's ability to develop a good composting profile.

According to the Solvita® compost maturity test, the OSCA7 samples from days 13 and 16 were producing 20% carbon dioxide due to microbial activity, clarifying that the OSCA product is not mature compost after 13 or 16 days. The sample from the day 23, after being re-moistened and left for 24 hours, was still producing 15% carbon dioxide, which could be classified as an immature product. Immature compost is an unstable material and can cause phytotoxicity (Wichuk and McCartney, 2007). Therefore, OSCA7 output could not be applied to plants as mature compost. The Solvita® test ammonia concentration reported undetectable levels.

Parameters such as temperature, waste C:N and pH were significantly affected by the amount of vessel rotation which mixes organic matter during composting (Getahun et al., 2012). It was concluded that the rotation duration of the OSCA barrels at once hour<sup>-1</sup> is excessive and could alter the physical and chemical parameters during organic matter decomposition. In contrast, rotating the barrels once day<sup>-1</sup> improved some of the aspects of composting. However, the OSCA in-vessel unit requires substantial redesign to effectively collect the condensed water vapour, better carry out gas exhaust, and facilitate improved aeration. When this is done, a thorough investigation of OSCA as an efficient composter can be carried out.

## **5.6.2 OSCA7 and OSCA8 experiments – microbiological results**

### **Bacterial numbers**

The amount of rotation of the OSCA barrels impacted bacterial CFU g<sup>-1</sup>. OSCA7 contained ~10<sup>6</sup> to 10<sup>7</sup> CFU g<sup>-1</sup> at once day<sup>-1</sup> rotation and ~10<sup>5</sup> at once hour<sup>-1</sup> rotation, which are both at the lower end of composting of 10<sup>5</sup>-10<sup>9</sup> CFU g<sup>-1</sup> (Chandna et al., 2013). OSCA8, with very limited operational duration, harboured conventional numbers of bacteria. The reasons for mediocre composting in OSCA7 (concluded from limited temperature increase) cannot be due to substrates provided (Table 5.1) or the numbers of bacteria but are concluded to be mostly due to process design features that need improvement.

### **Metabarcoding**

Generally, at the early stage of composting, lactic acid producing bacteria dominate the microbial community (Sundberg et al., 2011, Tran et al., 2019) and Lactobacillales were abundant in both OSCA7 (~27%) and OSCA8 (~25%). Their metabolic end-products clearly did not impact the pH which initially rose in both experiments; and Lactobacillales abundances dropped in both experiments. The day four temperature peak in OSCA7 (albeit relatively low and unsustained) was correlated with several bacterial order and genus abundance changes and was reflected in the alpha diversity. Classical composting thermophiles like Bacillales (Strom, 1985, Tang et al., 2004), were ~32% abundant at day one and dropped dramatically in OSCA7. Mesophiles will be responsible for initial temperature increases in composting, then thermophiles will be selected by the higher temperatures (Chen et al., 2011); but Bacillales were not selected as the temperature rose.

The day four breakpoint of OSCA7 was not correlated with the barrel rotation change or any other process modification, so is likely due to some microbial competition. The barrel rotation changes after day seven, did not substantially alter the trends of temperature or moisture content (which were both trending downwards), pH (which was maintained around 8), EC (which trended upwards and was negatively correlated with moisture content) or microbial alpha diversity. However, a major composting parameter (temperature) was not correlated with good composting from day four onwards. It could be concluded that the combined poor management of water vapour, gas exhaust and aeration

in OSCA negatively affected the ability of suitable composting microbes to be selected for.

Bhatia et al. (2013) determined microbial communities in a rotary drum composter using culture dependent methods and by cloning of the full 16S rRNA genes. Heterotrophic bacterial counts were quite low ( $<10^6$  CFU g $^{-1}$ ), indicators of potential pathogens were present but mostly declined through 100 days of operation, and a majority of identified isolates were *Bacillus* spp. The cloning methods are not very definitive but Varma et al., (2018) explored the microbial communities in a rotary drum composter by the same metabarcoding methods as in this thesis. Composting over 20 days was very efficient according to several parameters; e.g., temperature rose to at least 50°C (maximum was 65°C) for seven days. A total of ~144 species were reported, which is lower than the number of ASVs found in OSCA7. So again, OSCA seems able to maintain good composting microbes but the vessel operation was unsuitable for composting.

At the phylum level, ~25% were Bacteroidetes, ~22% were Firmicutes and ~15% were Proteobacteria, however, whether different locations or compost phases were analysed, or a composite sample was used, was not clear (Varma et al., 2018). Although OSCA7 had the same major phyla, their abundances were different at ~32% Bacteroidetes (day one to seven 29%; day eight to 24%), ~9% Firmicutes (day one to seven 13%; day eight to 23%), and ~54% Proteobacteria (day one to seven 62%; day eight to 24%). It is difficult to compare the data from OSCA7 with those from Varma et al., (2018) as the composting process in OSCA7 was so poor.

The first three days of OSCA8 at once day $^{-1}$  rotation generated good physical and chemical composting parameters. However, the moisture content was quite high. Dramatic microbial changes occurred on day four after the rotation of OSCA8 was increased to once hour $^{-1}$ . Microbes, which modulate the chemical parameters (especially temperature and pH), are influenced by mixing regime (Kalamdhad and Kazmi, 2009). The physical and chemical parameters also dramatically changed on day four.

The once day<sup>-1</sup> rotation favoured Lactobacillales, which dominated the microbial orders. The high abundance of Lactobacillales correlated positively with pH reduction, likely as a result of lactic acid production. These organic acids can affect the growth of all microbes, because they can penetrate the cell membranes and adversely change intracellular pH (Brinton, 1998, Warnecke and Gill, 2005). Bacillales declined dramatically; they could have been the thermophiles selected for by the high temperature on day one to three. The change that occurred on day four was substantial and extremely rapid, reflected in most physical, chemical (moisture content did not vary that much) and microbial parameters. However, it is difficult to conclude much from the data as the time of operation was too short.

### 5.6.3 Potential pathogenic microorganisms

According to the Australian Standard AS 4454–2012, pasteurisation of the compost requires maintenance at 55°C for three consecutive days. Neither OSCA experiment fulfilled this criterion. However, microbial pathogenic indicators such as *E. coli* O157:H7, *S. typhimurium* or *E. faecalis* were not detected during OSCA experiments. This could have been because these microorganisms may not have been present in the organic waste (Table 5.1). If they were present in the food being composted, they might not have been eliminated. It is important to reach temperatures of 55°C to eliminate pathogenic microorganisms (Australian-Standard, 2012).

### 5.6.4 End-product application as soil amendment

During composting, organic matter is decomposed naturally by bacteria, archaea, fungi and other microorganisms, producing compost, which is a humus-like material (Tiquia et al., 2002). The OSCA end-product at once hour<sup>-1</sup> rotation mode (generated balling) is not compost. The balled organic matter facilitates anaerobic zone formation allowing anaerobes to grow and produce offensive odours. The provision of optimal physical and chemical conditions for compost microbes facilitates their rapid growth and they generate high-quality compost (Cooperband, 2000). It was hypothesised that once day<sup>-1</sup> barrel rotation would be better for composting compared to once hour<sup>-1</sup>. Composting was not optimal at all, and it could have been that the less frequent rotation

generated other problems such as water vapour condensation, generating leachate and leakage. OSCA may produce compost, however, its design requires substantial improvement if it is to follow a good composting profile and produce compost.

## **5.7 Conclusions**

Treating organic waste in the OSCA bite-size did not produce compost. Instead, the organic matter formed into balls, which decomposed anaerobically, creating odours. The OSCA7 and OSCA8 experiments showed that the default mode's rotation frequency negatively impacted the system's physical and chemical parameters, which in turn affected microbial activity. Loss of heat and moisture prevented the operating temperature from increasing, hence the composting material was not pasteurised. The in-vessel OSCA bite-size unit could be improved by reducing the frequency of rotation and creating an exhaust for gases and water vapour. It requires a redesign to provide optimum conditions for composting.

## Chapter 6

# Composting organic waste in the in-vessel composter, Cylibox (CX)

## 6.1 Summary

Five composting experiments (coded CX3, CX4, CX5, CX6, and CX7) were run in the Cylibox (cylinder in a box) composter with mixing once per day for two minutes by manual rotation of the internal paddles, twice clockwise and twice anticlockwise at ~four rpm. Air was pumped into the vessel, which was insulated, and moisture generated during composting was collected via an external condensation system.

CX3 used the same waste composition as CL1.2 (see Section 4.4.1; Table 4.1), while all other CX experiments had the C:N adjusted to ~30:1 with sawdust. The active phase for CX3 and CX4 lasted 14 days as determined by the temperature falling to  $\leq 40^{\circ}\text{C}$ . According to the Solvita® test, the compost maturation for CX4 was faster than CX3 (69 days versus 94 days). Heat loss occurred when the lid of Cylibox was opened for sampling from CX3 and CX4, which was considered significant for the active phase taking 14 days, relative to CX5, CX6 and CX7 operations, which had shorter active phases.

In the first ten days of the active phase of CX3, lactic acid bacteria such as *Leuconostoc* (Day 1, ~50%, Day 2, ~40%), and *Lactobacillus* (Day 5, ~62%, Day 7, ~50%, Day 8, ~33%, Day 10, ~37%) dominated the microbial community, while *Weissella* was  $\leq 6\%$ . In contrast, in the first three days of CX4, *Weissella* (Day 1, ~73%, Day 2, ~75%, Day 3, ~52%) dominated the microbial community during composting, after which, lactic acid bacteria were  $\leq 10\%$ .

Lactic acid bacteria were highly abundant for a longer period of time in CX3 compared to CX4. It was concluded that adjusting the C:N ratio in CX4, was detrimental to lactic acid bacteria. CX5 was largely not mixed and the temperature did not follow a typical composting profile, presumably due to

microbes not having ready access to the organic matter. CX6 was operated with once a day mixing, but no opening, and therefore no regular sampling during the active phase. The process was completed in nine days, according to the daily measured temperatures. This shorter (nine days for CX6 versus 14 days for CX3 and CX4) active phase was concluded to be due to effective retention of the endogenously generated heat, due to Cylibox not being opened. CX7 replicated CX6, except that sample collection during the active phase was from a 5 cm hole in the lid, and it was done as rapidly as possible in an effort to preclude heat loss. Again, the active phase was nine days.

The CX experiments demonstrate that efficient composting relies on a suitable carbon and nitrogen balance in the input (e.g., C:N of ~30:1). The CX experiments followed the profile of typical composting according to physical and chemical analyses. The temperature increased during the active phase due to the endogenous heat production from microbial activity, the pH decreased initially then increased and stabilised in the curing phase. The moisture content was 40% to 60% and the electrical conductivity was below the phytotoxicity level. The CX3 compost reached maturity by day 94; the CX4, CX5 and CX6 compost reached maturity by day 69; and the CX7 compost achieved maturity by day 60. The microbial analyses showed that Firmicutes, Proteobacteria, and Actinobacteria dominated during the active phase, and Bacteroidetes increased in abundance during the curing phase. Figure 6.1 summarises the CX composting process.

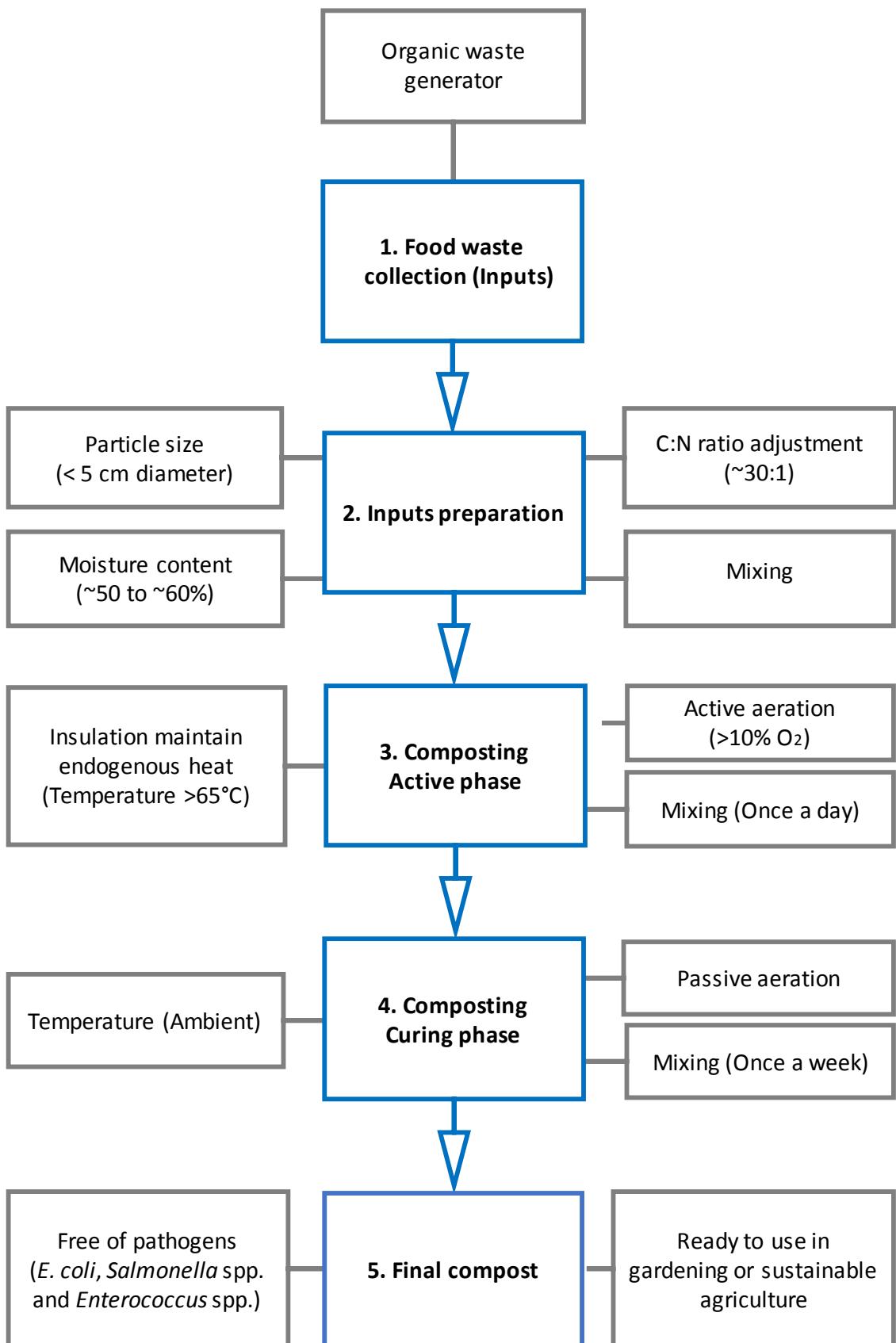


Figure 6.1 Optimum in-vessel composting process. Based on the TMECC.

## **6.2 Introduction**

There are different types of commercial in-vessel composting technologies in the market, such as bin composting, agitated beds, rotation drums, transportable containers, among others. The main advantages of using in-vessel composters are to accelerate the composting process, facilitate more effective physicochemical parameter control, reduce labour and production of better quality, consistent compost. The main disadvantage is the cost of the in-vessel composter unit (Mishra and Rao, 2003), which is much higher than windrow composting.

The treatment of organic waste was carried out in a newly designed, small-scale eco-efficient in-vessel composter called Cylibox. Its capacity is 28 L where 10 kg of organic waste can be composted. Cylibox is composed of an insulated cylinder (facilitating maintenance of biogenic temperature production to  $\sim 65^{\circ}\text{C}$ ), manual rotating paddles (rotated twice clockwise and twice anticlockwise at  $\sim$ four rpm once per day), a small air pump providing continuous airflow (max  $9 \text{ L min}^{-1}$ ), and moisture condensate collection ( $\sim 450 \text{ mL}$  to  $\sim 1 \text{ L week}^{-1}$ ) with recirculation if deemed necessary. The only energy requirement is for air pumping, calculated to be  $\sim 87.6 \text{ kWh yr}^{-1}$ , or approximately \$A10.00 per year at \$A0.11 kWh $^{-1}$ . This in-vessel composter was designed to provide optimum conditions for microbial activity to pasteurise compost in the active phase and produce mature compost.

## **6.3 The in-vessel composter Cylibox**

A desire to create an in-vessel composter able to provide optimal physical and chemical conditions for microbial activity inspired the design and construction of Cylibox. Most components of the unit were recycled materials. The only parts purchased were the air pump, thin hose for air-flow, internal metallic bar paddles, and a plastic container (3 L) for condensed water collection. All other materials were collected from the streets or waste skips at a suburban building site. A cylindrical Hygena three-shelf toy cabinet was transformed into an empty cylinder with three lids, each with a 5 cm diameter hole (Figure 6.2c). A bicycle pedal was used as a mixing handle (Figure 6.2d). Shower caddy display rack baskets were used to support the water vapour condenser (Figure 6.2d).



Figure 6.2 Building process of in-vessel composter prototype: **(a)** Water recirculation system, **(b)** Insulation box, **(c)** Insulated cylinder, **(d)** In-vessel composter Cylibox with water vapour condenser.

The plastic cylinder was placed into foam moulded into a styrofoam container (Figure 6.2b; 6.2c) to provide cushioning and insulation from the external environment. Composting occurs in the cylinder, which also contains gas and moisture collection tubes for condensate removal from the composting process and water tubes for recirculation of condensate to the compost bed (Figure 6.2a). Airflow for oxygenation is facilitated by an air pump (Hydropro Z4000 Air pump, <https://aquatecequipment.com>). The insulation provides endogenous heat retention, which facilitates temperature increases. Three paddles on the central rod are manually rotated for compost mixing. The total volume of the cylinder is 44 L and the working capacity is approximately 28 L.

The gases and water produced during composting passed through tubes as described in Figure 6.3. Water was condensed and collected by gravity into a three litre water container. This can be recirculated to the compost bed through water-flow tubes, but this depends on how much moisture is required for decomposition of the organic matter. The cylinder has three lids that slide up and down to close and open the cylinder. Small holes of 5 cm diameter, were in each lid, which were used for sampling in the CX7 experiment. For sampling through the holes, modified barbecue tongs were used to facilitate rapid sample collection (taking ~5 seconds). The internal 3D view of the in-vessel composter Cylibox is shown in the Figure 6.3.

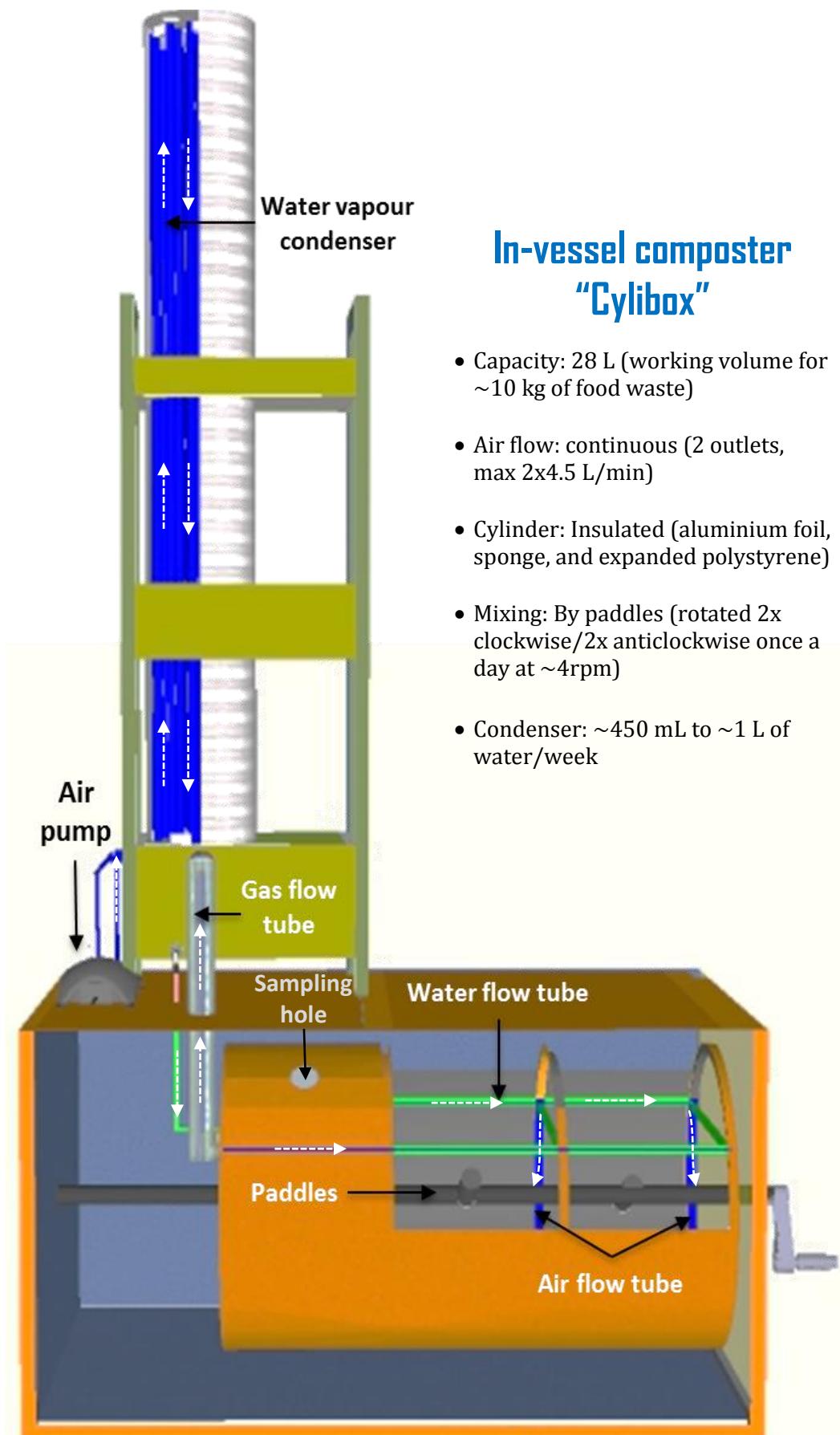


Figure 6.3 Internal view of the in-vessel composter Cylibox.

## 6.4 Experimental design

The Cylibox in-vessel composter prototype was used to run all CX experiments at different times. The CX3 ran from October to December 2017, CX4 ran from January to March 2018, CX5 ran from March to May 2018, CX6 ran from May to July 2018, and CX7 ran from May to July 2019. The Cylibox composter was located in a building protected from the outside environment.

### 6.4.1 Collection and audit of food waste

Organic waste was collected from the Swinburne Place South (SPS) cafe precinct located at Swinburne University of Technology. The organic waste composition and proportion for Cylibox 3 (CX3) (Table 6.1) was similar to that used in CL1.2 and CL2 experiments carried out in the Closed Loop in-vessel unit (see Section 4.4.1; Table 4.1). For Cylibox 4 (CX4), the C:N ratio was adjusted with sawdust material which contained the Acidulo<sup>TM</sup> inoculum (called “proprietary starter material” by Closed Loop Pty Ltd) (see Section 4.5.4 and Figure 4.10). For Cylibox 5 (CX5), Cylibox 6 (CX6), and Cylibox 7 (CX7), the C:N ratio was adjusted with plain sawdust which was also used in OSCA8 (see Section 5.5.4 and Figure 5.12). The C:N ratio adjustment is shown in Table 6.1, and the overview of each experiment is shown in Figure 6.4.

Table 6.1 Characterisation of organic waste and Carbon to Nitrogen ratio adjustment.

Organic waste	pH	Electrical conductivity ( $\text{mS cm}^{-1}$ )	$Q_n$	$Q_n$	$M_n$	$C_n$	$N_n$	C:N*
			CX3 (kg)	CX4-CX7 (kg)	Moisture content (%)	Carbon* (%)	Nitrogen* (%)	
Coffee grounds	5.66	1.08	5.45	4.90	58	47.10	2.70	17.44
Vegetables	4.98	1.55	1.35	1.22	92	37.50	2.50	15.00
Fruits	4.53	0.98	1.17	1.05	89	56.00	1.40	40.00
Food waste (Noodles, rice, chicken meat, off plate)	5.74	4.15	2.03	1.84	69	46.40	2.90	16.00
Sawdust	4.63	0.82	0.00	1.00	5	106.10	0.20	530.50
<b>TOTAL</b>			<b>10</b>	<b>10</b>				
<b>C:N (mixture)</b>			<b>~17.5</b>	<b>~30</b>				

**Note:** Values of carbon and nitrogen taken from \*(Rynk et al., 1992), and \*(Ballesteros et al., 2014). For C:N ratio calculation for CX3,  $n = 4$ , and from CX4 to CX7,  $n = 5$  (see Section 3.3.8; equation 2).

The mixing of 4.1 kg organic waste (food waste, vegetables, and fruits), 4.9 kg coffee grounds and 1 kg of sawdust generated a calculated C:N of ~30:1 and this was used for the CX4, CX5, CX6, and CX7 experiments (for the C:N ratio calculations, see Section 3.3.8; equation 2).

#### 6.4.2 Composting organic waste experiments

The Cylibox composter was loaded with 10 kg of organic waste with a particle size of <5 cm in diameter, three Tinytag temperature data loggers were added and the cylinder lid was closed and sealed by sticky taping the lids (see Figure 6.2c), before finally covering with moulded foam. The paddles were rotated once per day for mixing and triplicate samples were taken by opening the lid, and sampling at three different locations in the composting bed (from cylinder).

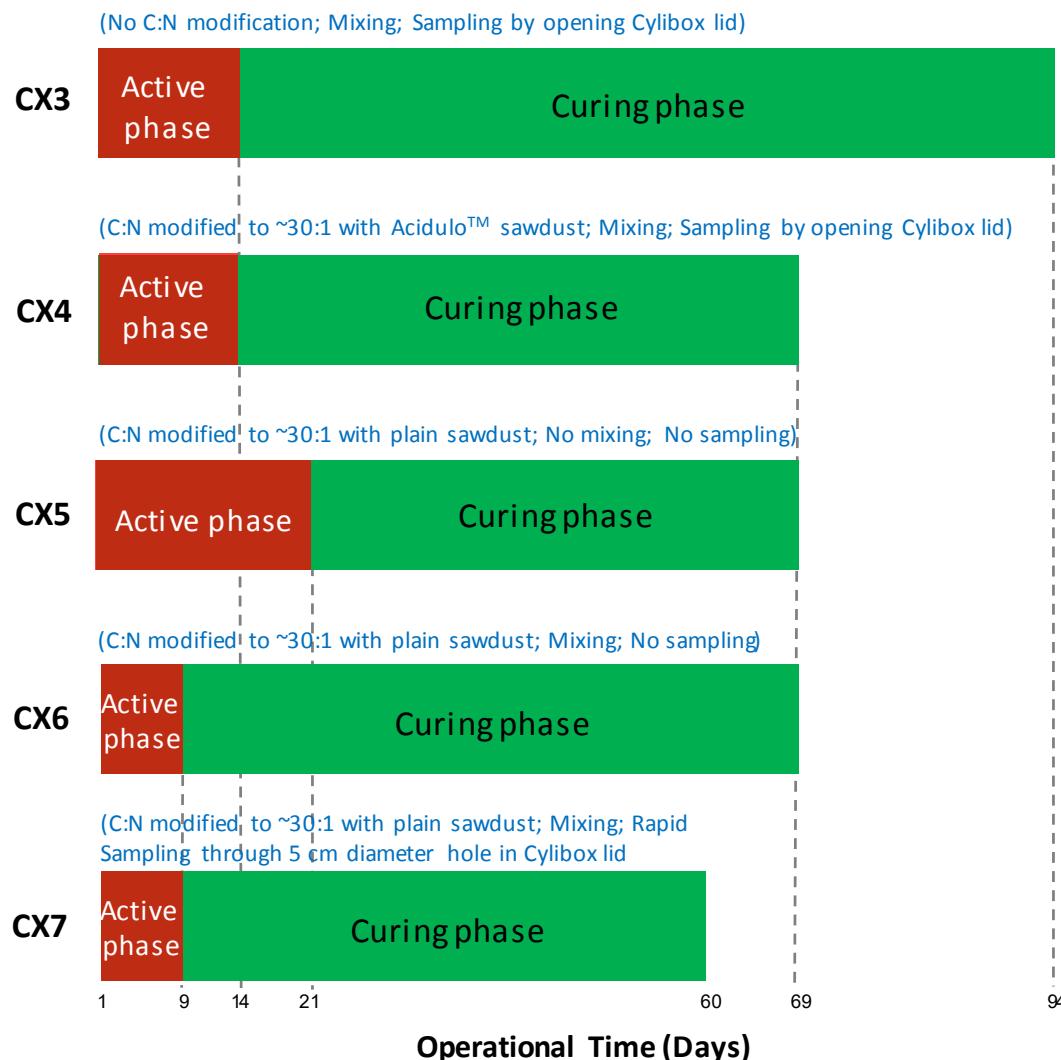


Figure 6.4 Timeline of Cylibox (CX) composting experiments.

Physico-chemical (temperature, moisture, pH, electrical conductivity and nutrients – Section 3.3), compost maturity (see Section 3.3.9) and microbial (community analysis by 16S rRNA gene metabarcoding – Section 3.4, colony counting and pathogen determination by selective enrichment – Section 3.5), analyses were carried out on samples at different times of Cylibox operation.

## **6.5 Results – composting overview**

### **6.5.1 CX3 and CX4 experiments**

Both CX3 (no C:N adjustment) and CX4 (C:N adjusted to ~30:1 with Acidulo<sup>TM</sup> sawdust), showed a thermophilic ( $> \sim 40^{\circ}\text{C}$ ) active phase from days two to 14 (Figures 6.5 and 6.6). The fluctuation in the compost bed temperature was likely due to Cylibox being opened for sampling, leading to bed cooling and lowered microbial activity. This was followed by 55 days of curing, when the temperature was close to ambient. The moisture content of the compost bed remained relatively high (~65%) and stable during the active phase and dropped in the curing phase to be in the optimum range of 40% to 60%.

In the first five days of CX3, the pH dropped to ~5, after which it increased to ~6.9, and during the curing phase, the pH stabilised to ~6. Initially, during CX4 the pH was ~4.5; during the active phase it increased to ~6.3 and, during the curing phase, it stabilised to ~6. The electrical conductivity (EC) during CX3 experiment trended upwards throughout the active and curing phases. The EC of the CX4 experiment remained around  $1.7 \text{ mS cm}^{-1}$  (Figures 6.5 and 6.6).

### **6.5.2 CX5 and CX6 experiments**

CX5 was operated without mixing from days 1 to 13, as the paddles broke from the central bar precluding mixing (Figure 6.7). Although the temperature declined, it was always in the thermophilic range of  $>40^{\circ}\text{C}$ . After 13 days, the compost bed was manually mixed, which accelerated microbial activity, leading to endogenous heating and a temperature of  $59^{\circ}\text{C}$  on day 14; and then temperature declined to  $\sim 40^{\circ}\text{C}$  by day 23 (Figure 6.7). Samples were not taken in the active phase so the effect of compost bed cooling on the process due to lid opening was unable to be explored. Samples were taken from day 19 onwards.

The physical, chemical and microbial parameters were measured for the samples from the curing phase. The moisture content of CX5 was ~65%, which is slightly higher than the optimum. However, during the composting process the moisture content was reduced. Due to the moisture content reduction, the electrical conductivity increased. The pH was in the optimum range and it stabilized close to neutral. The difference between mixing and no mixing affected the composting in the CX5 experiment.

CX6 was operated with mixing once a day (Figure 6.8) and the active phase finished in nine days; and the temperature in the curing phase was close to ambient. The operational parameters of good insulation and mixing once a day facilitated the temperature to rise to >40°C by day two. Inadvertently, no mixing occurred on day six, causing the temperature to drop to ~30°C on day seven. However, mixing on day seven facilitated accelerated organic matter degradation and a rapid temperature increase to >65°C. The temperature dropped close to 30°C then below 20°C on days nine and ten respectively, indicating the end of the active phase. The moisture content declined during the curing phase to finally be 35.5%. The electrical conductivity increased concomitantly with moisture lowering but was always within the effective compost range. The pH stabilized at ~6, which is in the range of optimum compost pH.

### **6.5.3 CX7 experiment**

The active phase of the CX7 experiment lasted for nine days and achieved maturity by day 60 (Figure 6.9). During the active phase of composting, the temperature increased rapidly from ~30°C on day one to 60°C on day six. During the curing phase, the temperature rose to >40°C only on day 17 (temperature continuous recorded in the data logger TinyTag). This might have been due to undecomposed material on the walls of Cylibox, which might have reinitiated decomposition once it became available to the microbes. The moisture, pH and electrical conductivity profiles were all consistent with stable compost (Figure 6.9).

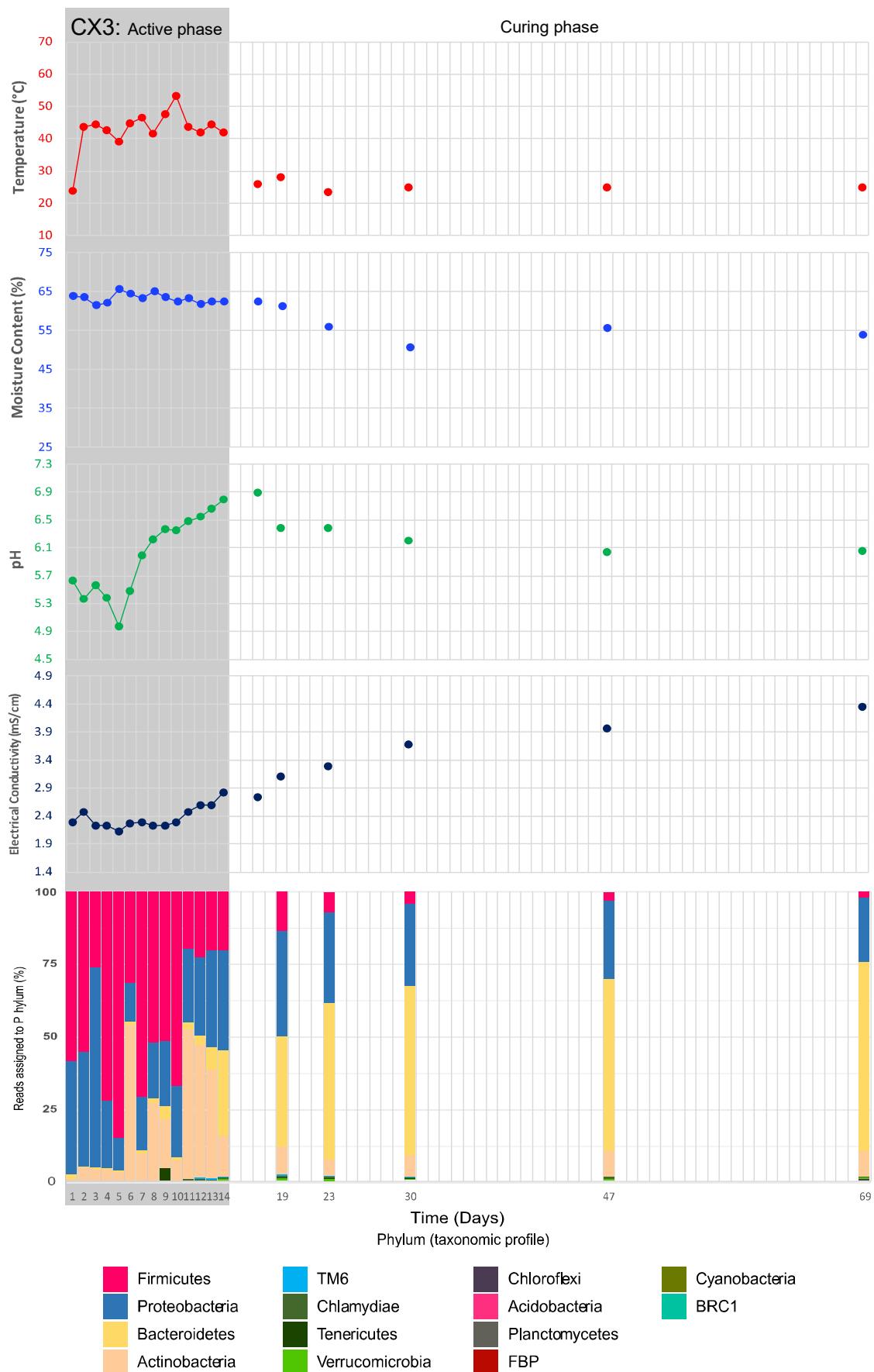


Figure 6.5 Physical, chemical and microbial changes during composting organic waste in Cylibox during CX3.

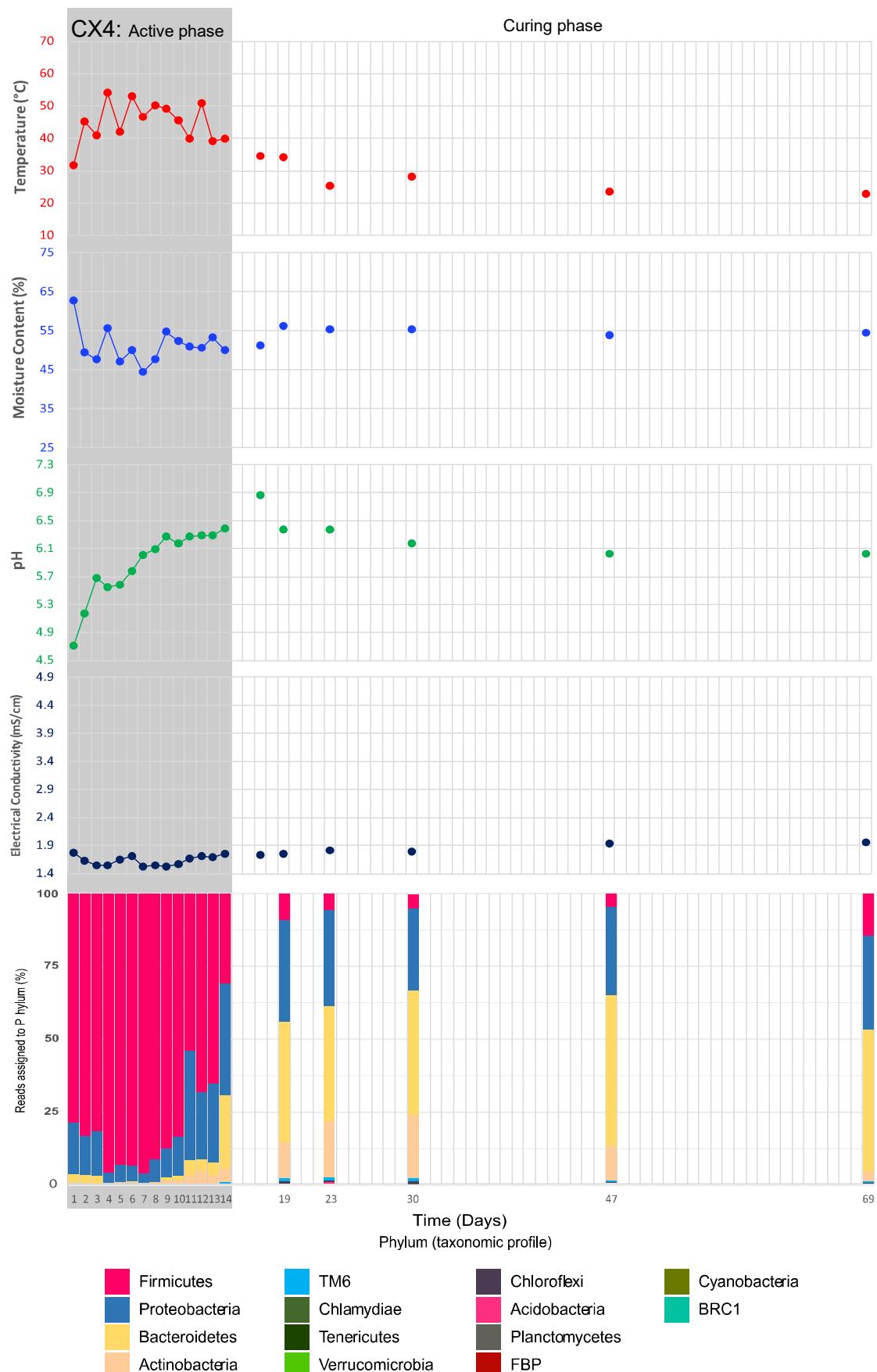


Figure 6.6 Physical, chemical and microbial changes during composting organic waste in Cylibox during CX4.

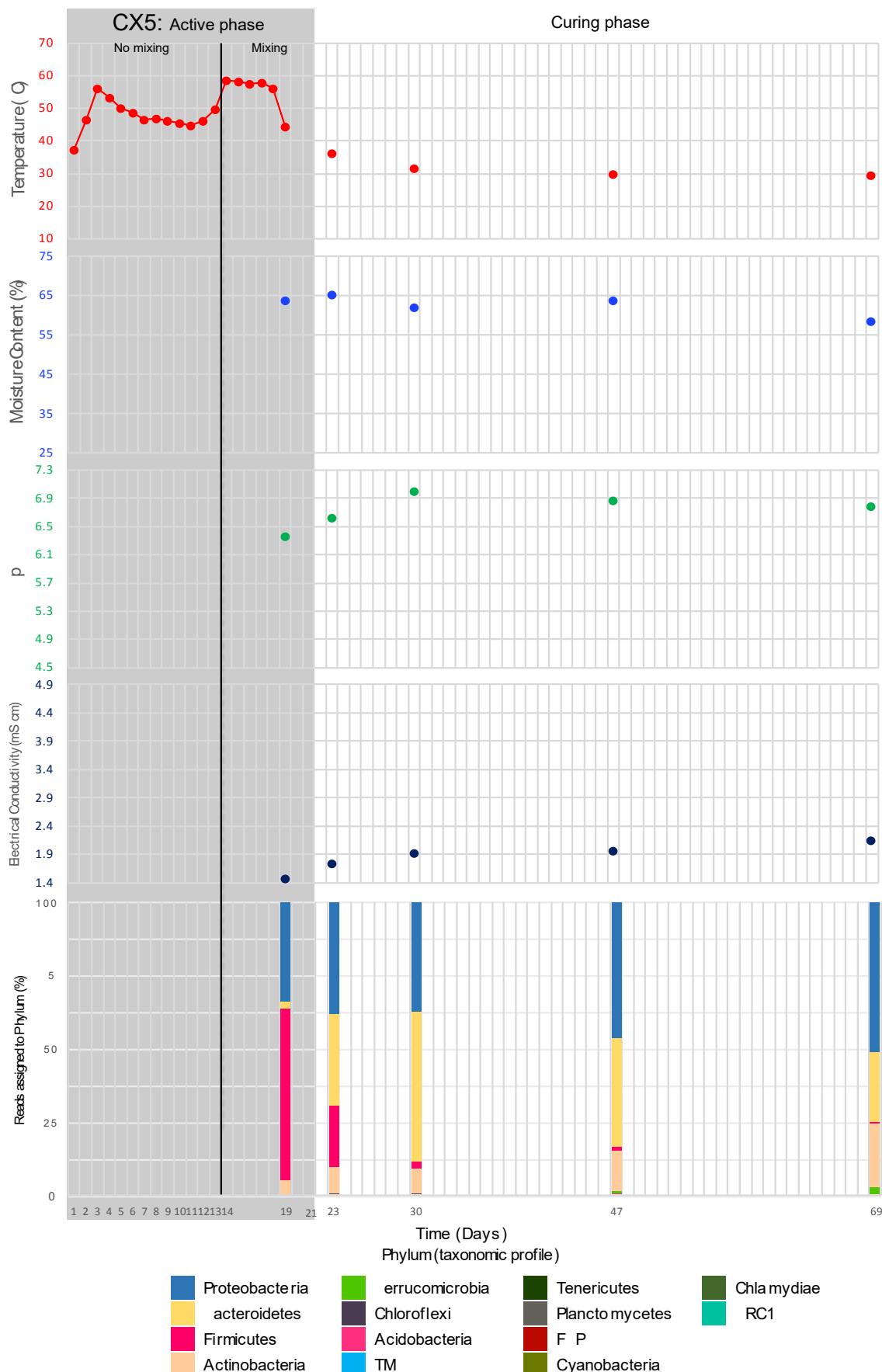


Figure 6.7 Physical, chemical and microbial changes during composting organic waste in Cylibox during CX5.

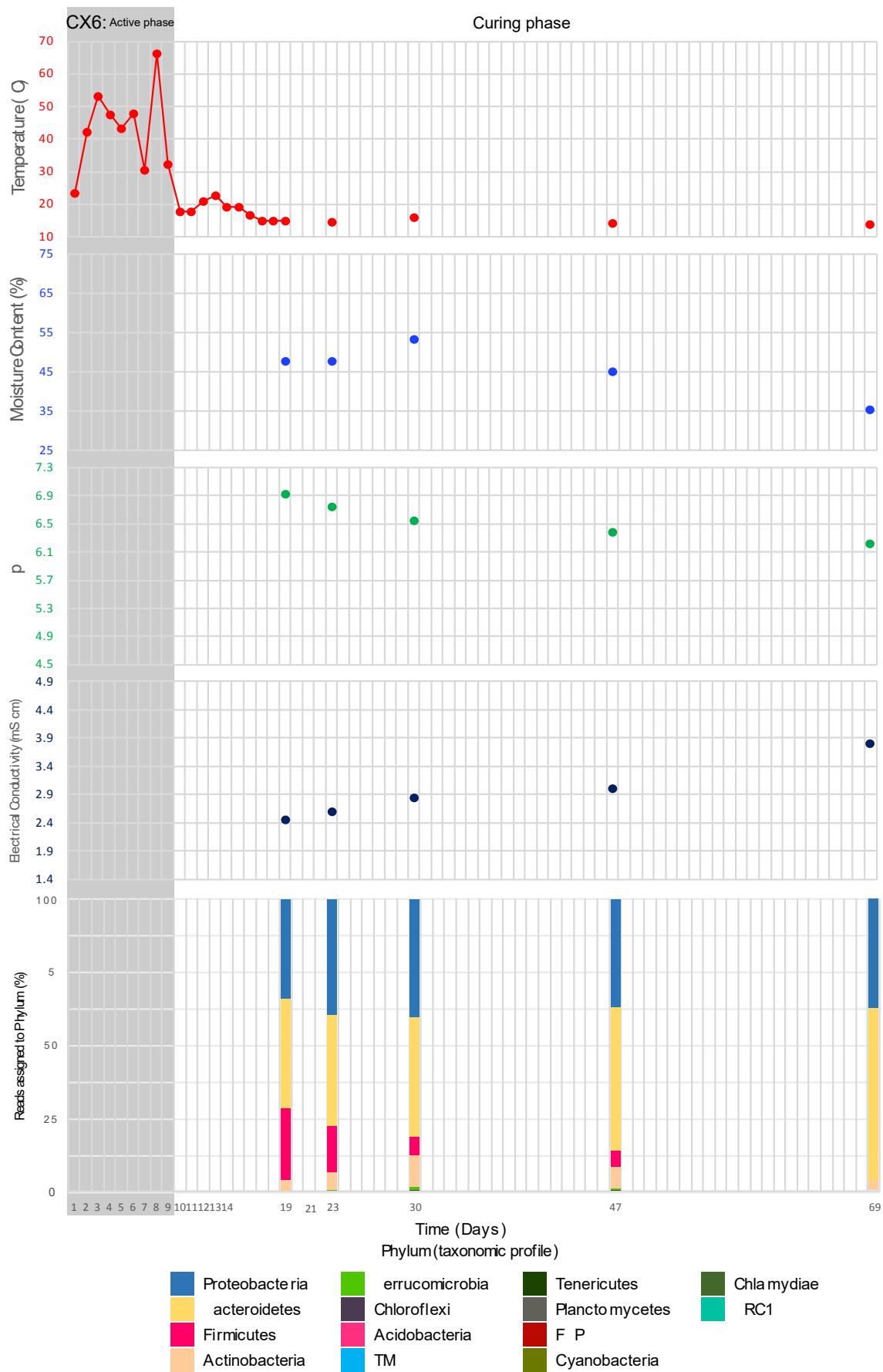


Figure 6.8 Physical, chemical and microbial changes during composting organic waste in Cylibox during CX6.

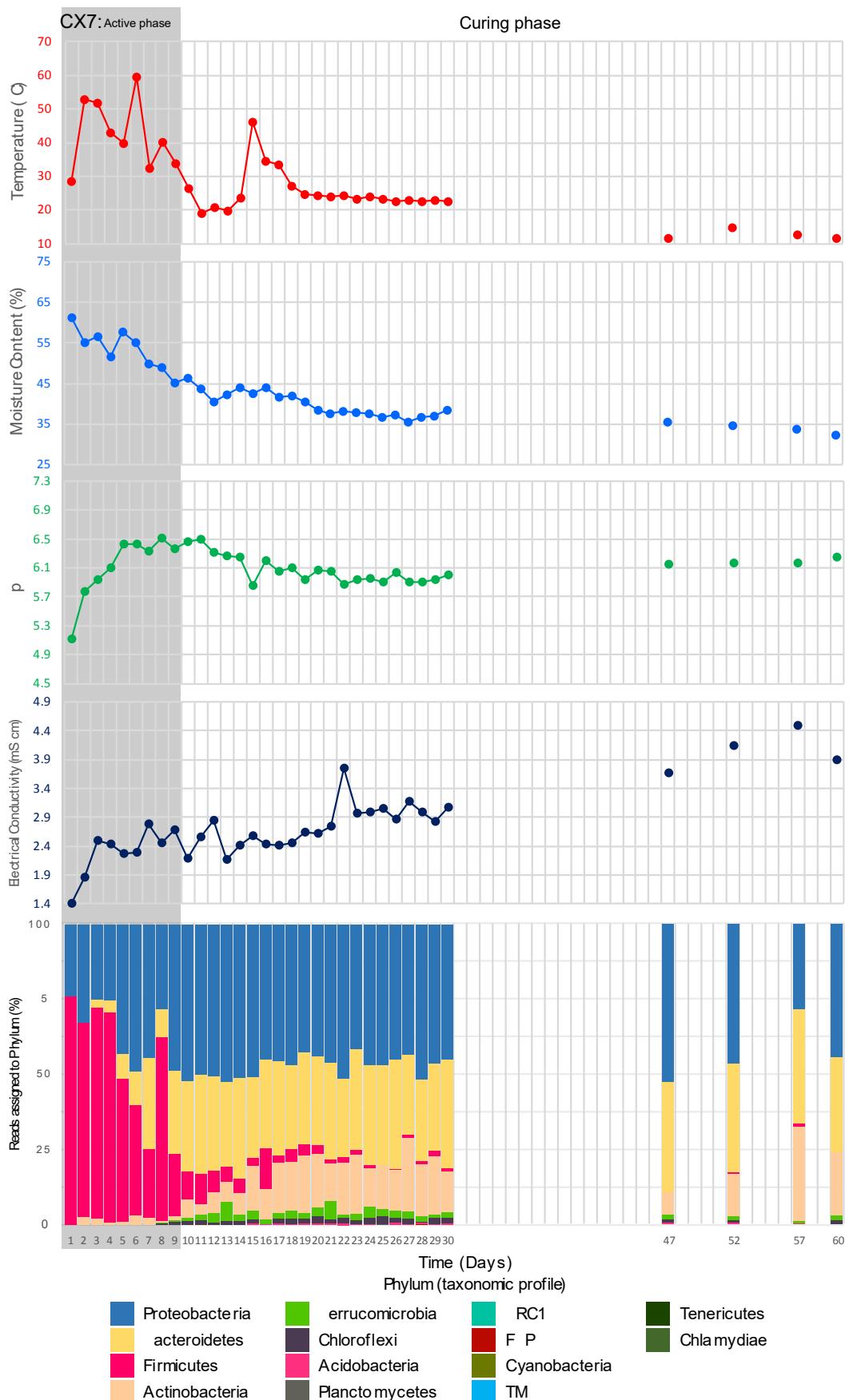


Figure 6.9 Physical, chemical and microbial changes during composting organic waste in Cylibox during CX7.

## 6.6 Results – physical and chemical analyses

Temperature (T), moisture content (MC), pH and electrical conductivity (EC) were evaluated during the active and curing phases of composting. Principal Component Analyses (PCA) of the physical and chemical data during composting phases were carried out. CX5 and CX6 did not generate enough data to analyse by PCA due to limited sampling.

### 6.6.1 Principal Component Analyses (PCA)

PCA biplots (Figure 6.10a, Figure 6.10b) of physicochemical data are from CX3 and CX4. In total 20 data-points were plotted for each experiment. The first 14 data-points correspond to the 14 days of the active phase and data-points from 19 to 69, correspond to the curing phase days; there was a clear distinction between these two phases. The highest eigenvector and eigenvalues are on the figure axes. There was higher variability among the active phase samples compared to the curing phase samples. For both CX3 and CX4, the active phase samples are correlated to temperature, the curing phase samples are correlated to EC, and the samples between the active and curing phases are correlated to pH. The CX3 active phase is also correlated to MC; in contrast, in CX4 the phase between active and curing is correlated to MC.

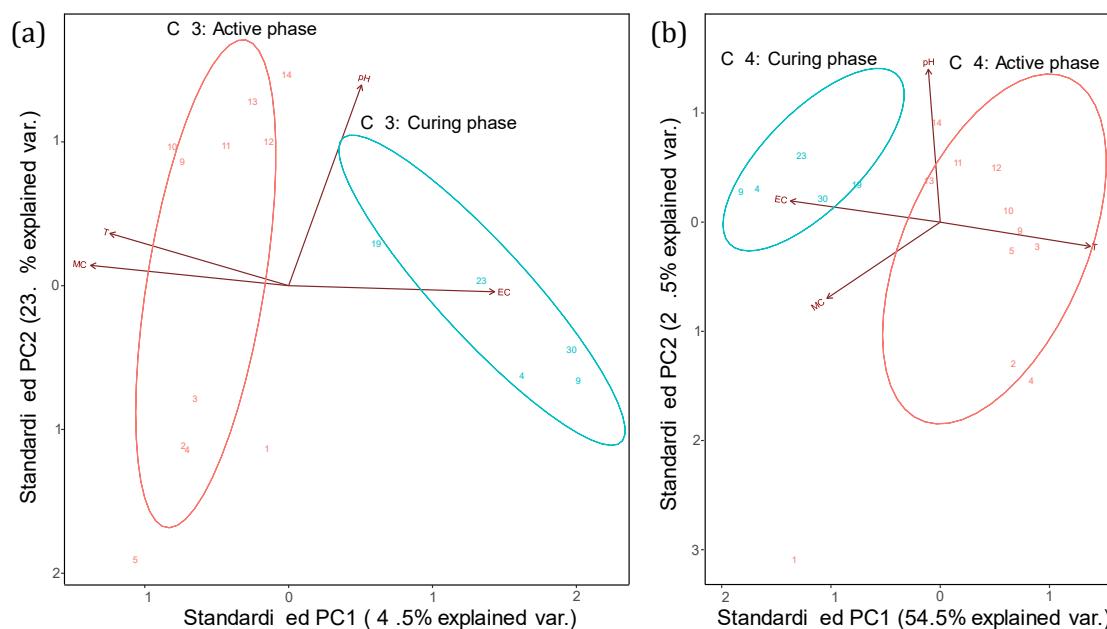


Figure 6.10 Physical and chemical analysis by Principal Component Analysis (PCA). **(a)** CX3 and **(b)** CX4. Where T = temperature ( $^{\circ}\text{C}$ ), MC = moisture content (%), pH, and EC = electrical conductivity ( $\text{mS cm}^{-1}$ ). Ellipses indicate 95% confidence intervals.

Sample data followed a trend with time and the closer the data-points are, the greater the similarities. In CX3 the samples of the first six days clustered closely, the samples from day seven to 14 were clustered closely, and the curing phase samples grouped together. The data-points of CX4 experiment were closer in the active and curing phase than the data-points of CX3 experiment

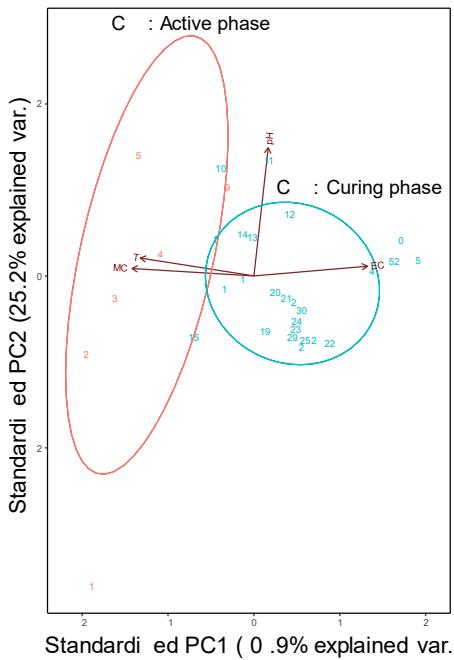


Figure 6.11 Physical and chemical analysis by Principal Component Analysis (PCA) of CX7.  
Where T = temperature ( $^{\circ}\text{C}$ ), MC = moisture content (%), pH, and EC = electrical conductivity ( $\text{mS cm}^{-1}$ ). Ellipses indicate 95% confidence intervals.

From CX7, 34 compost data-points were plotted (Figure 6.11); the first nine (samples from day one to day nine) correspond to the active phase of composting, and the remaining 25 samples (samples from day 10 to 60) were from the curing phase. As for CX3 and CX4, data-points from the active phase of CX7, showed high variability relative to the curing phase. The active phase samples were correlated to temperature and MC, while the curing phase samples were correlated to EC; specially from days 47 to 60. The samples between the active and curing phases were correlated to pH.

In the early stage of CX3, compost samples were more correlated with the micronutrients (Fe, Mn, Zn, Cu), while the primary macronutrients (P and K) were not correlated (Figure 6.12a). The secondary macronutrients such as Ca and Mg were correlated to the early active phase. Other elements such as Ni

and Co were correlated to the active phase and the early curing phase, respectively.

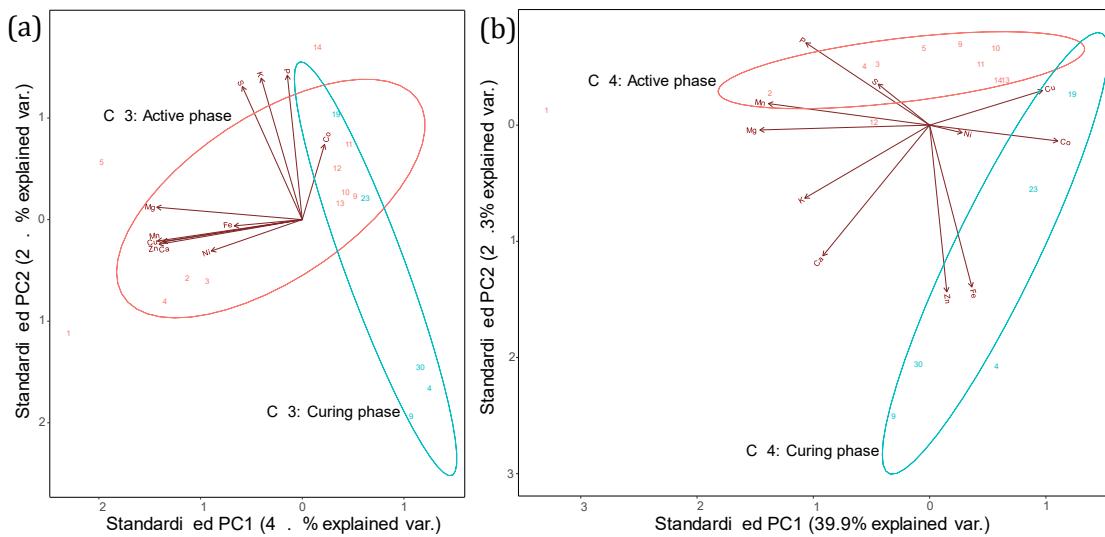


Figure 6.12 Essential nutrients analyses by Principal Component analysis (PCA), **(a)** CX3 and **(b)** CX4. Primary macronutrients (P, K); secondary macronutrients (S, Mg, and Ca); micronutrients (Fe, Mn, Zn, Cu); and other elements (Co, and Ni). Ellipses indicate 95% confidence intervals.

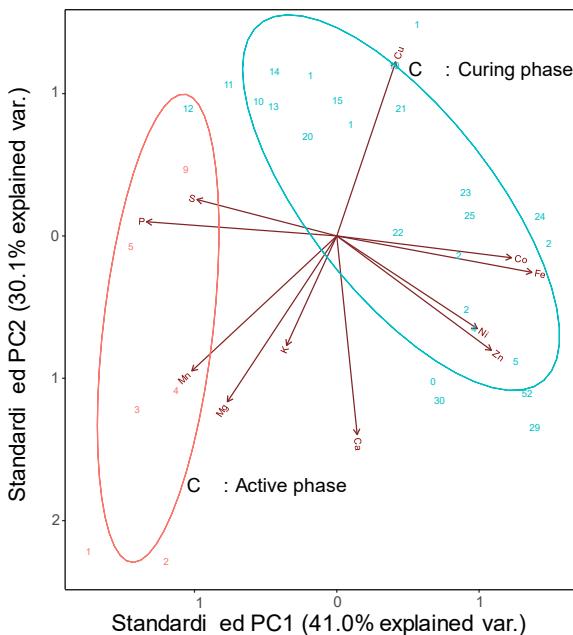


Figure 6.13 Essential nutrients analyses by Principal Component Analysis (PCA) of CX7. Primary macronutrients (P, K); secondary macronutrients (S, Mg, and Ca); micronutrients (Fe, Mn, Zn, Cu); and other elements (Co and Ni). Ellipses indicate 95% confidence intervals.

The mineral nutrient Mn in CX4 (Figure 6.12b) was correlated to the early active phase, Cu was correlated to the early curing phase, and Zn and Fe were correlated to the end of the curing phase. The primary macronutrients P was

correlated with the early active phase, and K was not correlated with the samples. The secondary mineral nutrient S, was correlated to the early active phase, Ca and Mg were not correlated to the active or curing phases, and Co was correlated to the early curing phase.

The results from CX7 for nutrients (Figure 6.13) follow similar trends as those of CX4; particularly, phosphorus was correlated with both active phases. However, in CX7, there were clear differences between the active phase and curing phase (Figure 6.13). In CX7, the macronutrients (P, K, S, Mg, Ca) in a soluble form were correlated positively with the active phase samples, and the micronutrients (Fe, Mn, Zn, Cu) and other elements (Co and Ni) were correlated positively with the curing phase samples, except Mn, which was present in the active phase (Figure 6.13). In all of CX3, CX4 and CX7, there is a clear difference in mineral nutrients correlations between the active and curing phases. Primary and secondary macronutrients were in higher concentration during the active phase, then decreased in the curing phase (see Appendix G; Figure G4). The trend of the micronutrients (water soluble elements) such as Mn and Cu started slightly high in the active phase, then those decreased in the curing phase. In contrast, Fe and Zn were in higher concentration during the curing phase than in the active phase. Co and Ni, slightly increased in the curing phase (see Appendix G; Figure G5).

In general, the physical and chemical parameters of the composting experiments CX3, CX4 and CX7, follow similar trends.

### **6.6.2 Compost maturity test**

The Solvita® test was used to measure compost maturity during the curing phase of the composting process (see Section 3.3.9).

In CX3, the maturity test was performed on samples from days 19, 23, 30, 47, 69, and 94. By days 19 and 30, the CO<sub>2</sub> production was high (20%), however, it decreased progressively and by day 94, the compost was considered to be mature. In CX4, there was high CO<sub>2</sub> production (20%) on day 19, which gradually decreased, and the compost reached maturity at day 69.

Although the active phase of the CX5 took 21 days, due to mixing issues, the compost achieved maturity by day 69. The CO<sub>2</sub> concentration was reduced from 8% on day 30 to 2% on day 47. At day 69, the CO<sub>2</sub> concentration was 1%, which means that the compost is mature. During CX6, the active phase finished in nine days, however, the curing phase took another 60 days to achieve maturity.

The CO<sub>2</sub> concentration was reduced from 20% on day 19 to 8% on day 30, and then it was reduced to 2% on day 47, and at day 69, the CO<sub>2</sub> concentration was 1%. In CX7, the active phase finished in nine days; and maturity was attained by the day 60, where the CO<sub>2</sub> concentration was 1% (Figure 6.14). In all experiments the concentration of NH<sub>3</sub> was always low ( $\leq 0.02 \text{ mg N } 3\text{-N}$ ).

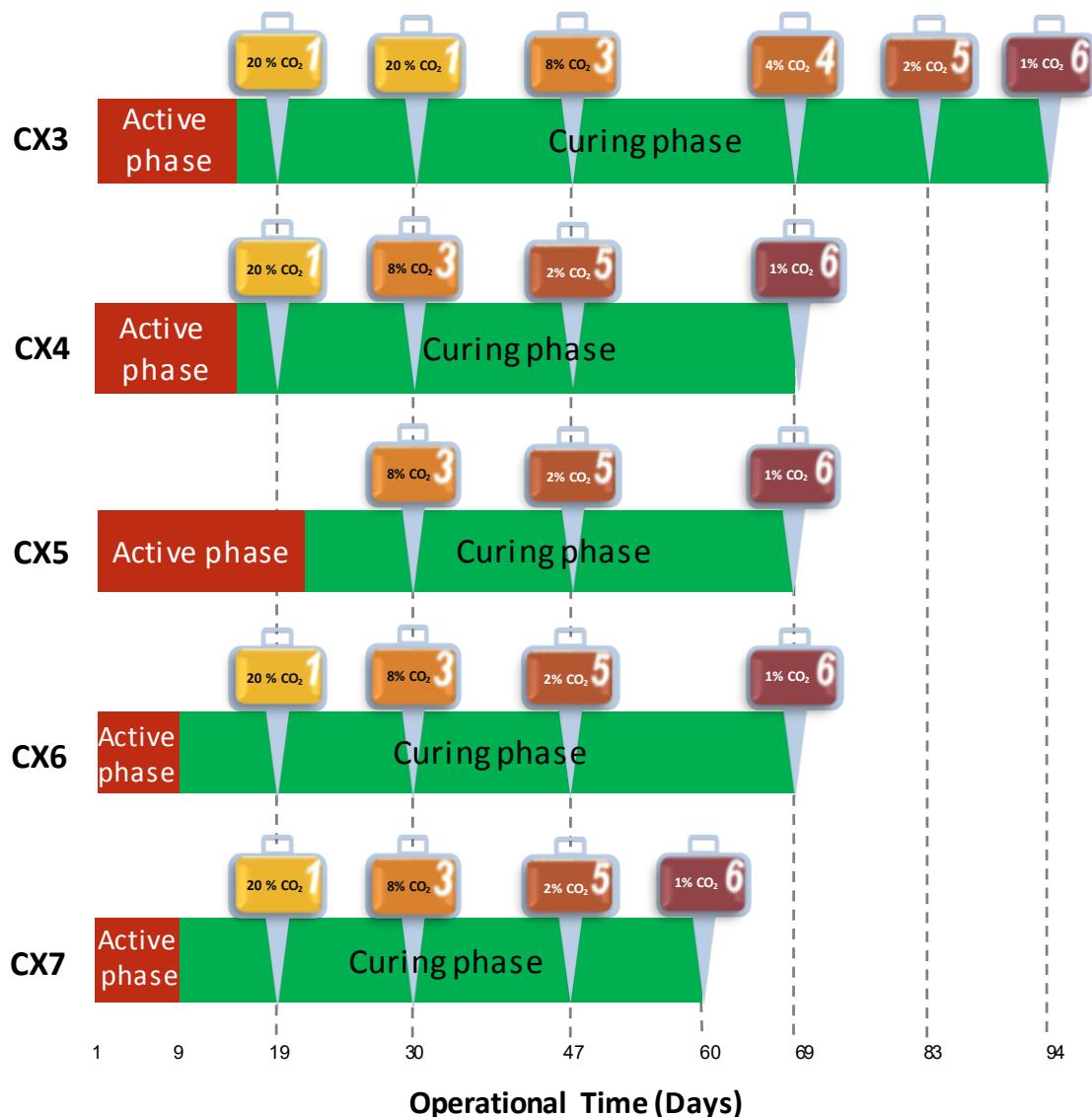


Figure 6.14 Schedule of compost Solvita® maturity testing during curing phase.

## 6.7 Results – microbiological analyses

### 6.7.1 Metabarcoding - microbial communities

From CX3 and CX4, 19 samples (14 from the active phase and five from the curing phase), from CX5 and CX6 five curing phase samples, and from CX7 34 samples (nine from the active phase and 25 from the curing phase) were analysed by 16S rRNA gene metabarcoding (see Section 3.4.1). One sample of sawdust, and negative controls (the DNA extraction kit and Milli-Q water) were also analysed by metabarcoding. In total 85 samples were analysed.

Methods in Section 3.4.2 were used to bioinformatically analyse the generated sequence data. There was a total of 10,919,523.00 raw reads of partial 16S rRNA gene sequences, with a minimum of 23,073.00 reads, mean of 125,511.75 reads and maximum of 355,186.00 reads per sample. After denoising and chimeric filtering with DADA2, 6,735 ASVs were found and the total number of reads was reduced to 899,686, the minimum was 153 (from the sawdust sample), the mean was 6,846, and the maximum was 25,316 reads per sample.

Rarefaction via the R-Studio rarecurve function in the vegan package was used to determine the cut-off for data analysis at 2,500 reads (see Appendix G; Figure G1). Running the decontam (Davis et al., 2018), at the default threshold of  $p = 0.1$ , three putative contaminant ASVs (representing 0.113% relative abundance of the bacterial community) were found which were removed from the bacterial communities (see Appendix G; Table G1). The remaining 6720 ASVs were analysed.

### 6.7.2 CX3 and CX4 – Bacterial Phyla

Eleven and fourteen bacterial phyla were identified in CX3 and CX4 samples, respectively. During the active phase of CX3, Firmicutes (minimum ~19% and maximum ~85%; Figure 6.5) dominated, however, in contrast during the curing phase, Firmicutes were of decreasing then low abundance; ~20% at the onset of curing and ~2% at the end of curing (Figure 6.5). Proteobacteria were more variable during the active phase (minimum 11.4% and maximum ~69%; Figure 6.5). During the curing phase, Proteobacteria were variable and in the range

~36% (early curing) to ~22% (end of curing). Bacteroidetes was in low abundance during the active phase (<~1% in the early active phase and ~30% at the end). However, during the curing phase Bacteroidetes dominated the microbial community; in the early curing ~38% rose to a maximum of ~66% at the end of the curing phase. Actinobacteria rose in abundance during the mid-active phase to be in the range ~38% to ~52%. During the curing phase, Actinobacteria were in low abundance. The remaining ten phyla were <1% abundant (Figure 6.5).

Firmicutes dominated the microbial community in the active phase (Figure 6.6), being ~79% at day one of CX4, increasing to ~97% at mid active phase, then reducing to ~31% by the end of the active phase. Firmicutes were always in low abundance in the curing phase (~15% at early curing and ~5% from day 30). Proteobacteria decreased from ~15% in the active phase as Firmicutes increased in abundance; by the end of the active phase Proteobacteria were ~38%, and during days 19 to 69 of the curing phase they were ~30%. Bacteroidetes were in low abundance during most of the active phase but increased to ~26% at the end of active phase. In the curing phase, Bacteroidetes dominated the microbial community; in the early curing phase being ~39% rising to ~52% at the end of the curing phase. Actinobacteria were in low abundance during the active phase (<5%) and were highest in the mid curing phase (~22% at day 47). The remaining ten phyla were <1% abundant (Figure 6.6).

### 6.7.3 CX3 and CX4 – Bacterial Orders

During the active phase of CX3, the most dominant Order was Lactobacillales (~70% in mid active phase (day five) declining to ~5% by the end of the active phase) (Figure 6.12a). The second most abundant Order was Actinomycetales which fluctuated throughout the active phase (maximum ~54% on day six) (Figure 6.15a). Bacillales was the third most abundant Order at ~58% on day four, but more typically Bacillales were in the range 10-15% in the active phase. Burkholderiales and Pseudomonadales were generally ~5% or greater in abundance, particularly at the end of active phase (Figure 6.15a). Pseudomonadales were higher at ~18% in the first 2 days of the active phase. Xanthomonadales, Rhizobiales and Enterobacterales were generally >5%; but

there were some dramatic fluctuations as evidenced by the latter being ~56% only on day three. The remaining Orders were <5% (Figure 6.15a).

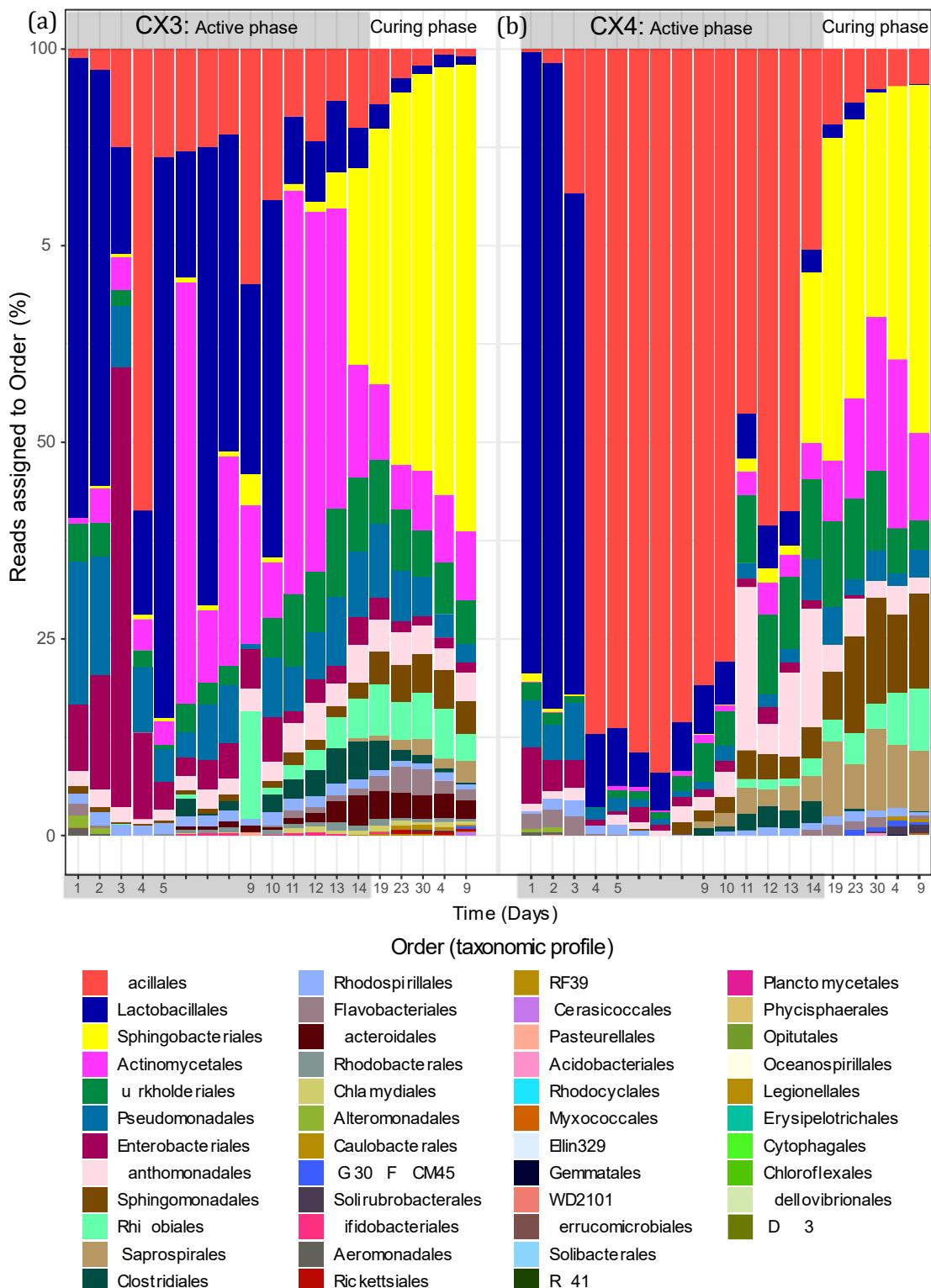


Figure 6.15 Microorganisms at the Order level during the composting in Cylibox during (a) CX3 and (b) CX4.

During the curing phase of CX3, the main bacterial Order was Sphingobacteriales (Bacteroidetes), which steadily increased from ~25% on day 14 to ~59% on day 69. All other Orders were substantially lower than Sphingobacteriales and none showed an increasing trend throughout curing as this Order did (Figure 6.15a).

Lactobacillales dominated the microbial community (~64 to ~82%) from days one to three of the active phase in CX4; after day four they were always <10% (Figure 6.15b). Bacillales was always abundant from day four to day 10 (~78% to ~92%; Figure 6.15b), decreasing to ~25% at day 14. Pseudomonadales, Burkholderiales, Xanthomonadales and Enterobacteriales were of low but noticeable abundance (<10%) through most of the active phase of CX4, with the former three tending to rise somewhat from day 11 (Figure 6.15b).

In CX4, Sphingobacteriales, dramatically rose from <2% in the late active phase to ~22% on day 14 and ~44% on day 69. Actinomycetales also quite dramatically rose from day 14 (~5%) to peak on day 47 (~21%) and fall to ~11% on day 69. Other Orders that were also notably present in the curing phase of CX4 were Burkholderiales, Pseudomonadales, Xanthomonadales, Rhizobiales, Sphingomonadales and Saprospirales (Figure 6.15b).

#### 6.7.4 CX3 and CX4 – Bacterial Genera

The abundant Lactobacillales genera present in the active phase were *Leuconostoc* (50% and 40% on days one and two) and *Lactobacillus* (fluctuating dramatically from days four (~6% on day four and ~62% on day five) to ten (~37%)). *Weissella* was also present but in very low abundance (always <2% after day two). *Bacillus* was mostly ~5 to ~10% abundant (apart from ~54% on day four). *Corynebacterium* fluctuated dramatically – e.g., ~3% on day five and ~56% on day six but was always present in the mid-late active phase (Figure 6.16a). In contrast, from day 10 to 14, most of the microbial genera were in a similar proportion (Figure 6.16a). During the curing phase, the microbial diversity was higher in comparison to the genera from the active phase (Figure 6.16b).

Family Sphingobacteriaceae (Order Sphingobacteriales, Phylum Bacteroidetes) genera that were unable to be resolved to genus by QIIME2

analysis dominated the microbial community of CX3 in the curing phase, rising fairly steadily from ~5% on day 13 to ~56% on day 69. To resolve the Sphingobacteriales ASVs to genera, the basic local alignment search tool (BLAST) was used to compare the ASV sequences (76 in total) with those in GenBank. It was revealed that 45 were *Sphingobacterium*, 23 were *Olivibacter*, six were *Parapedobacter*, and the other two gave very low percent identities to any genus in GenBank.

The C:N ratio of CX4 was adjusted to ~30:1 with Acidulo<sup>TM</sup> sawdust. As described in Section 4.5.4 and Figure 4.10, the Acidulo<sup>TM</sup> inoculum on sawdust contains ~35% *Alicyclobacillus* and ~13% *Dyella*. From days two to five and on day nine, <1% of the bacterial community comprised *Alicyclobacillus* and no *Dyella* were discovered. *Weissella* (~75% falling to ~50%), dominated the first three days of the CX4 active phase; *Pseudomonas*, *Leuconostoc*, and *Acinetobacter* were also present during these days, but at relatively low levels. On day three of CX4 active phase, *Bacillus* comprised ~12% of the bacterial community, rose to ~82% on day four and stayed above ~90% until day nine, declining to ~8% on day 12. There was several *Bacillus* spp. of which the most abundant was *B. coagulans* (e.g., day three, ~5%; day four to nine, ~73% to ~55%). *B. alkalinitrilicus* was between ~2% to ~6% during the active phase; an unresolved *Bacillus* sp. and *B. acidicola* were collectively between ~2% to ~6% in this phase. However, *B. coagulans* dominated most of the active phase. Other genera were in low abundance during the active phase of the composting process (Figure 6.16c). *Tuberibacillus* increased in the late active phase (days 9 to 12), comprised ~34% of the bacterial community of CX4 on day 13, but then substantially declined to ~1% to 2% by day 17. *Paenibacillus* followed a similar trend, albeit lower percentages compared to *Tuberibacillus*. Sphingobacteriaceae (Order Sphingobacteriales, Phylum Bacteroidetes) genera that were unable to be resolved by metabarcoding dominated the microbial community of CX4 in the curing phase; rising fairly steadily from ~18% on day, to ~43% on day 17 then fluctuating and being ~32% on day 69.

Heatmaps for QIIME2 resolved genera in the curing phases of CX3 and CX4 are shown in Figure 6.16b to Figure 6.16d. Note that there were abundant unresolved genera, which were resolved only to the family

Sphingobacteriaceae, and these were not captured by the heatmaps. These were abundant, but declining in the curing phase of both CX3 and CX4.

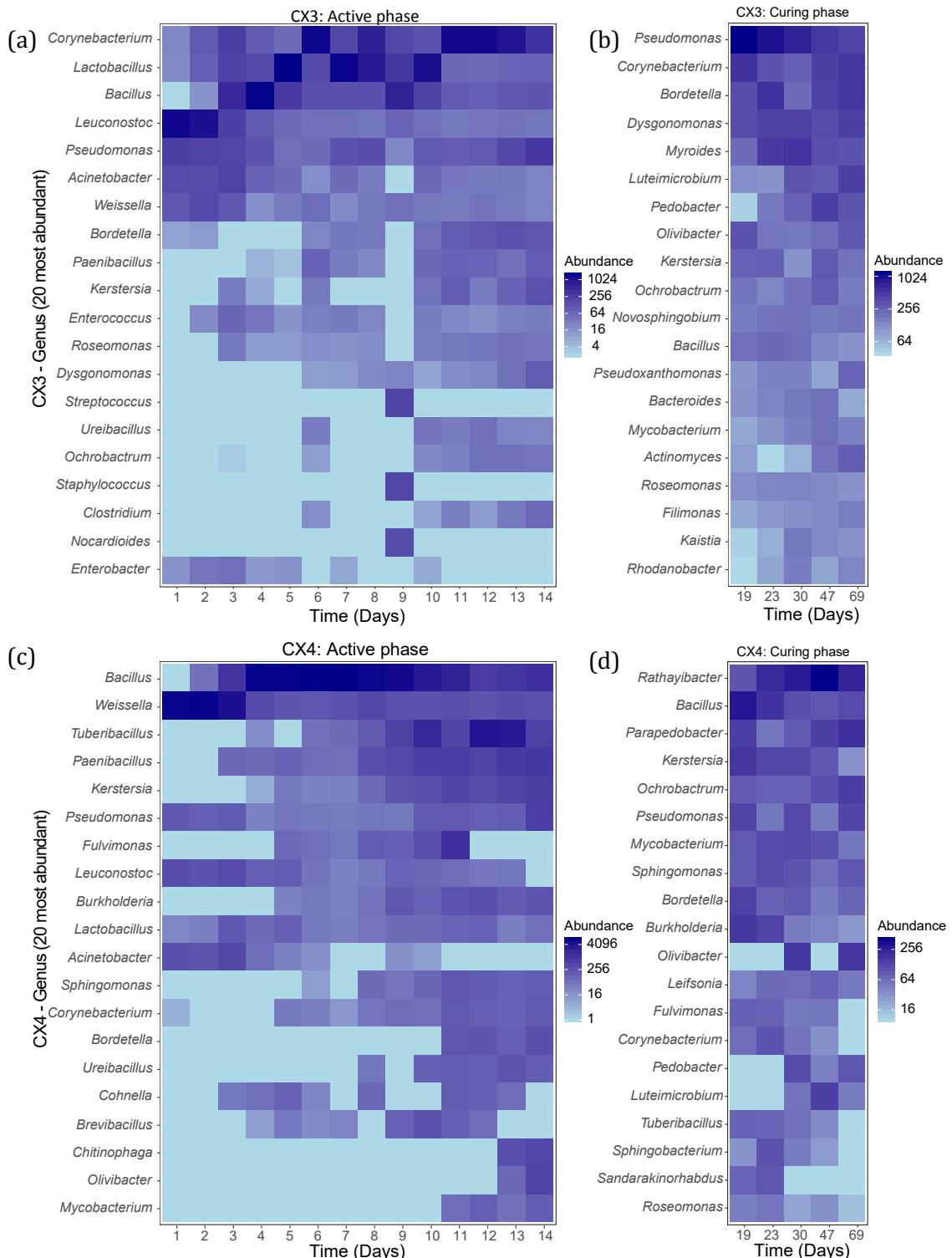


Figure 6.16 Heatmap of the 20 most abundant bacterial genera: **(a)** Active phase - CX3, **(b)** Curing phase - CX3, **(c)** Active phase - CX4, **(d)** Curing phase - CX4.

### 6.7.5 CX5 and CX6 – Bacterial Phyla, Orders, Genera (curing phase)

The active phase in CX5 lasted for 21 days (Figure 6.7) and one active phase sample was analysed by metabarcoding. Phylum Firmicutes (~58%), which were all Order Bacillales were present on day 19, which is the end of the active phase (Figure 6.17a). However, they declined to ~21% by day 23 and to <1% by day 69. More than half of the Bacillales (~28% of the bacterial genera) in the day 19 sample were *Bacillus* sp. (Figure 6.18a) and the majority of these (~19% of the bacterial species) were *B. coagulans*.

During the curing phase, four samples (days 23, 30, 47 and 69; Figure 6.14) were analysed by metabarcoding (Figure 6.17a). The dominant bacterial Order was Sphingobacteriales which fluctuated in abundance (~5%, ~33%, ~19% and ~12%) for the four sample days in the curing phase (Figure 6.17a). Actinomycetales increased from ~9% on days 23 and 30, to ~13% on day 47, and to ~22% on day 69. Over these four sample days, Burkholderiales decreased from ~13%, to ~10%, ~7% and finally to ~4%. Rhizobiales increased to ~27% by day 69, Saprospirales decreased from ~23% (Day 23) to ~10% on day 69. Sphingomonadales were ~10% throughout the curing phase, while the remaining Orders were <10% and often <2% (Figure 6.17a).

During the curing phase of the CX6, Sphingobacteriales dominated the bacterial Orders at ~25% on day 19, then they increased to ~47% on day 69 (Figure 6.17b). Bacillales decreased from ~23% on day 19 to non-detectable on day 69. Actinomycetales increased from ~5% on day 19 to ~22% on day 69, Burkholderiales, Pseudomonadales and Xanthomonadales fluctuated but were never >12%. Rhizobiales, Enterobacteriales, Saprospirales and Flavobacteriales were always <10%, while the remaining Orders were <1% (Figure 6.17b).

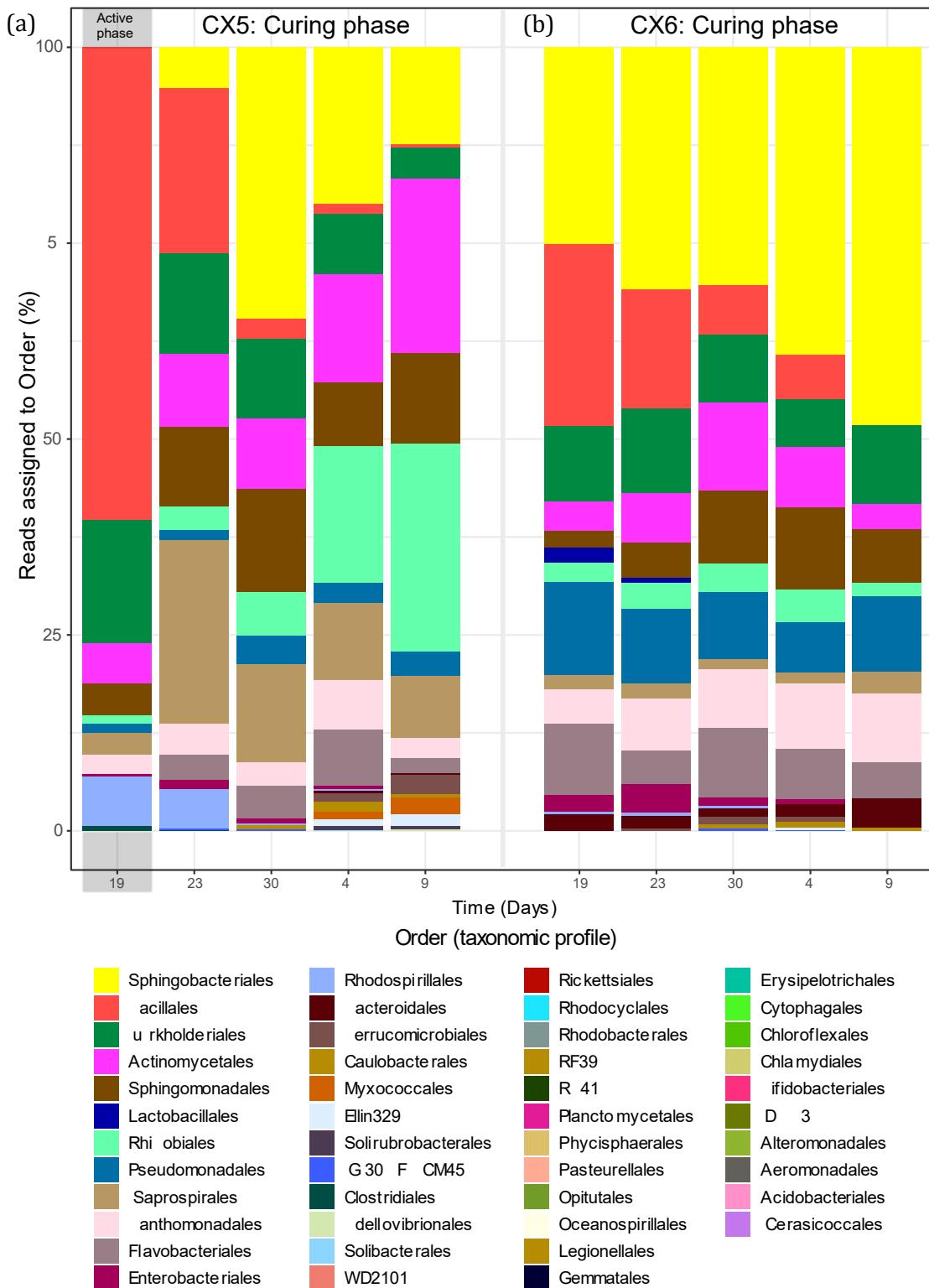


Figure 6.17 Microorganisms at the Order level during the composting in Cylibox during **(a)** CX5 and **(b)** CX6.

Heatmaps for QIIME2 resolved genera in the curing phases of CX5 and CX6 are shown in Figure 6.18a and 6.18b, respectively. In CX6, *Olivibacter*, *Sphingobacterium*, *Pseudomonas*, *Bordetella*, *Pseudoxanthomonas* were generally between 5 to 10%, while *Flavobacterium*, *Bacillus*, *Parapedobacter* and *Pedobacter* were generally <5% abundant. Note that there were abundant unresolved Sphingobacterales genera in the curing phase, that will not have been captured by these heatmaps.

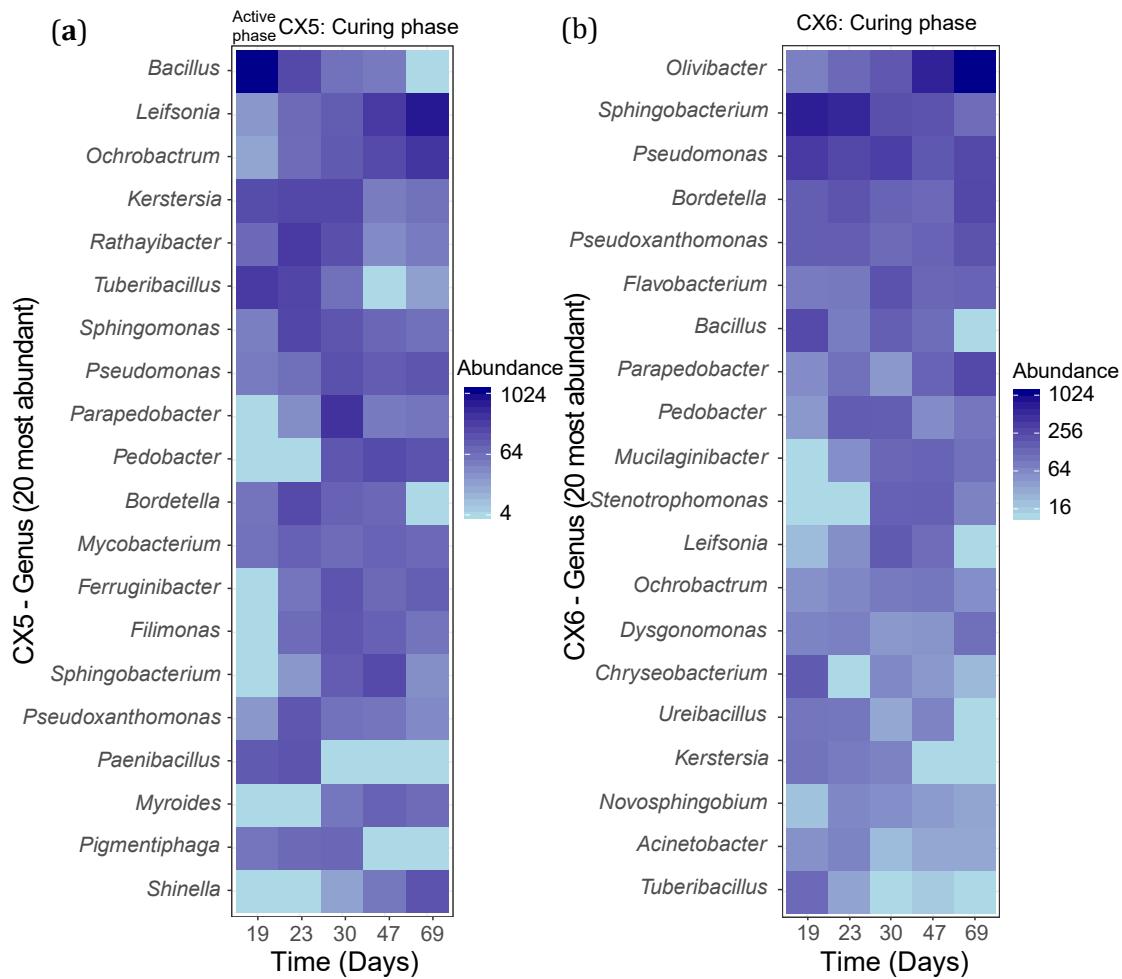


Figure 6.18 Heatmap of the 20 most abundant bacterial genera: **(a)** Curing phase CX5 and **(b)** Curing phase CX6.

### 6.7.6 CX7 - Bacterial Phyla, Orders, Genera

Firmicutes dominated the nine days of the active phase of composting; on day two they were ~76%, then they generally decreased to ~21% on day nine (Figure 6.19). Proteobacteria generally trended upwards in abundance, ranging from ~24% on day one to ~48% on day nine (Figure 6.19). Bacteroidetes started

in low abundance (on day three ~3%), then generally increased to ~28% on day nine. Actinobacteria were always <3% during the active phase and other phyla were <1%. During the curing phase, Firmicutes continuously declined in abundance from ~9% on day 10 to undetectable on day 60 (Figure 6.9). Proteobacteria were mostly between ~41% to ~52%, Bacteroidetes were between ~25% to ~37%, Actinobacteria increased from ~3% to ~32%, and Verrucomicrobia fluctuated but ranged between ~1% to ~6%. Three other phyla were in <3% (Figure 6.9).

At the Order level, Lactobacillales comprised ~73% on day one to be undetectable by the end of the active phase. Bacillales increased in a largely fluctuating manner from ~4% on day one (e.g., day three and four ~63% and 64%, day five ~45%, day eight ~55% and day nine ~20%). Over the active phase, Pseudomonadales decreased from ~10% to <1%, Xanthomonadales increased from ~1% to ~19%, Enterobacteriales decreased from ~8% to ~1%, Rhizobiales mostly increased from ~2% to ~4%, and the remaining Orders were <3% (Figure 6.19).

During the curing phase of the CX7 (Figure 6.19), Lactobacillales (most days undetectable) and Bacillales (from ~10% to undetectable) were in very low abundance. The ranges of different Orders throughout the curing phase were: Sphingobacteriales ~8% to ~20%, Actinomycetales ~3% to ~32%, Burkholderiales, Sphingomonadales and Saprospirales ~3% to ~11%, Pseudomonadales and Flavobacteriales ~1% to ~10%, Xanthomonadales and Bacteroidales ~2% to ~13%, Rhizobiales ~6% to ~12%, Enterobacteriales ~3% to <1% and Alteromonadales ~1% to ~6%. The remaining bacterial Orders were <3% during the curing phase (Figure 6.19).

The abundant lactic acid bacterial genera in the early active phase were *Weissella* (~35% on day one) and *Leuconostoc* (~27% on day one); both dramatically decreased after day one (Figure 6.20a). *Bacillus* increased through the active phase to be ~33% to 34% on days three and four (Figure 6.20a).

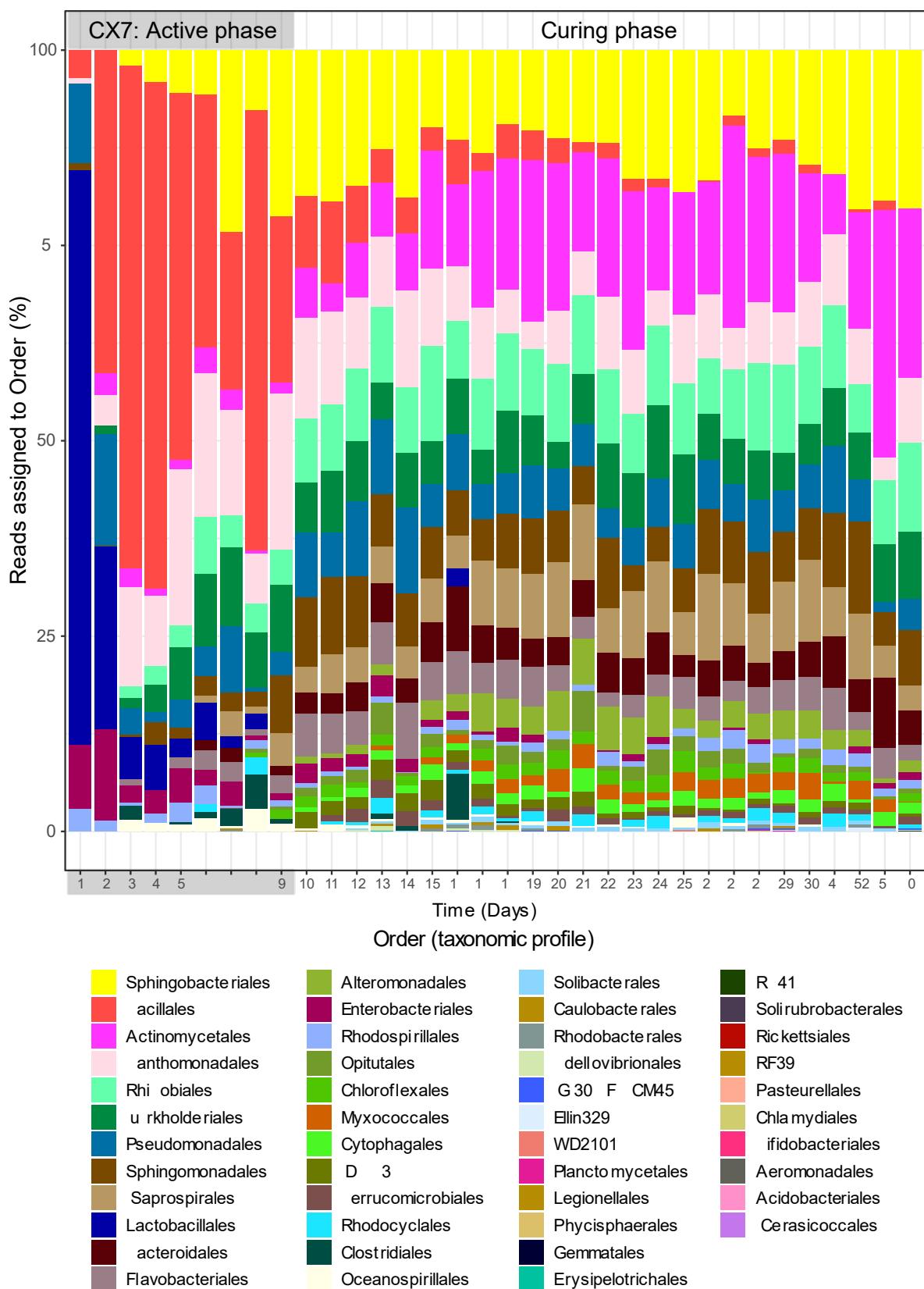


Figure 6.19 Microorganisms at the Order level during the composting in Cylibox during CX7.

In the curing phase of CX7, unresolved genera from families Sphingobacteriaceae (Sphingobacterales) and Chitinophagaceae (Saprospirales), *Pseudoxanthomonas*, *Parapedobacter*, *Pseudomonas*, and *Sphingobacterium* were found as more abundant genera (Figure 6.20b). In general, there were fewer microbial genera in the active phase compared to the curing phase (Figure 6.20a and 6.20b).

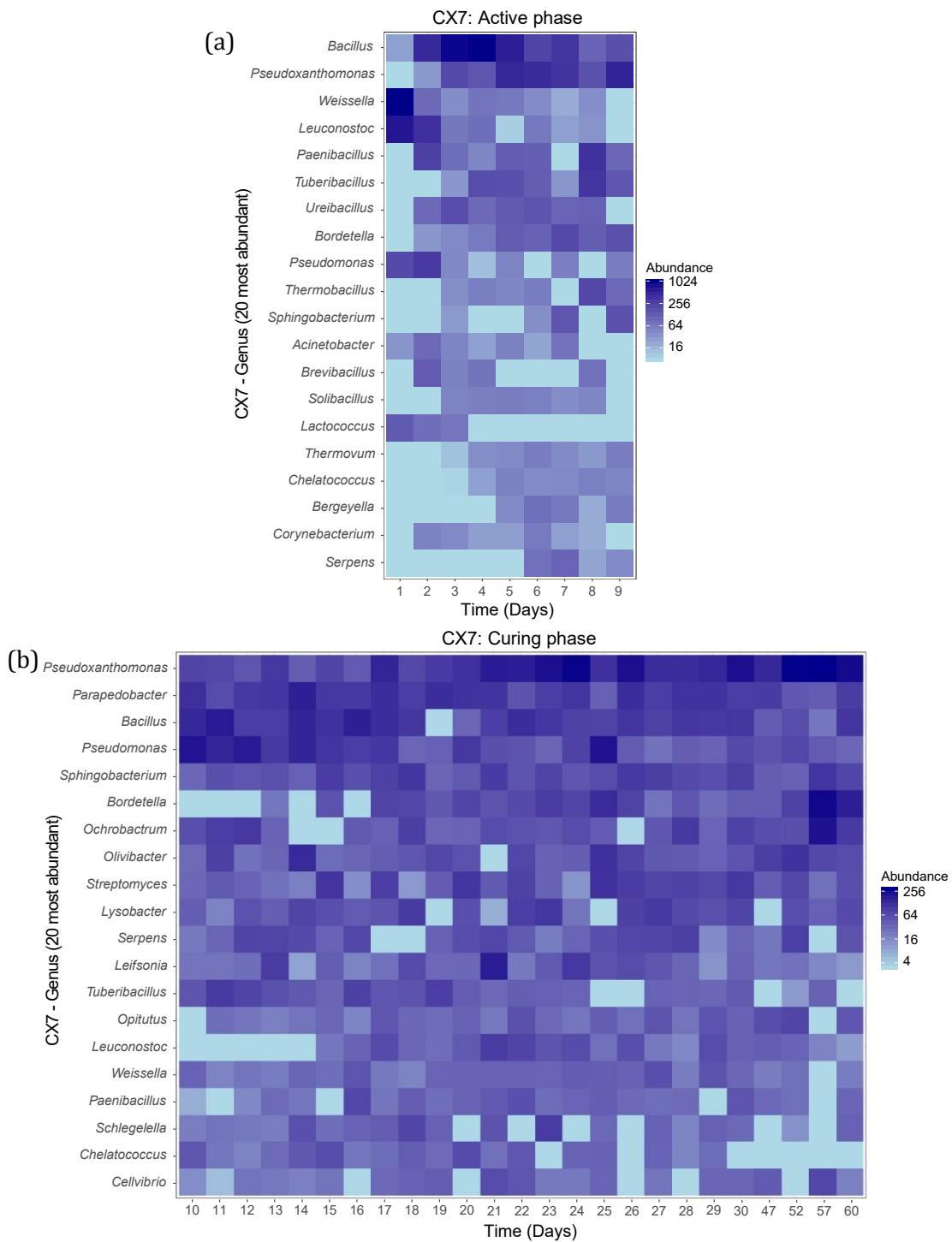


Figure 6.20 Heatmap of the 20 most abundant bacterial genera in CX7 **(a)** Active phase and **(b)** Curing phase.

### 6.7.7 Metabarcoding - microbial diversity

#### Alpha diversity – diversity within a sample

The microbial community diversity by all indices tended to increase during the active phases, then plateau during the curing phases (Figure 6.21).

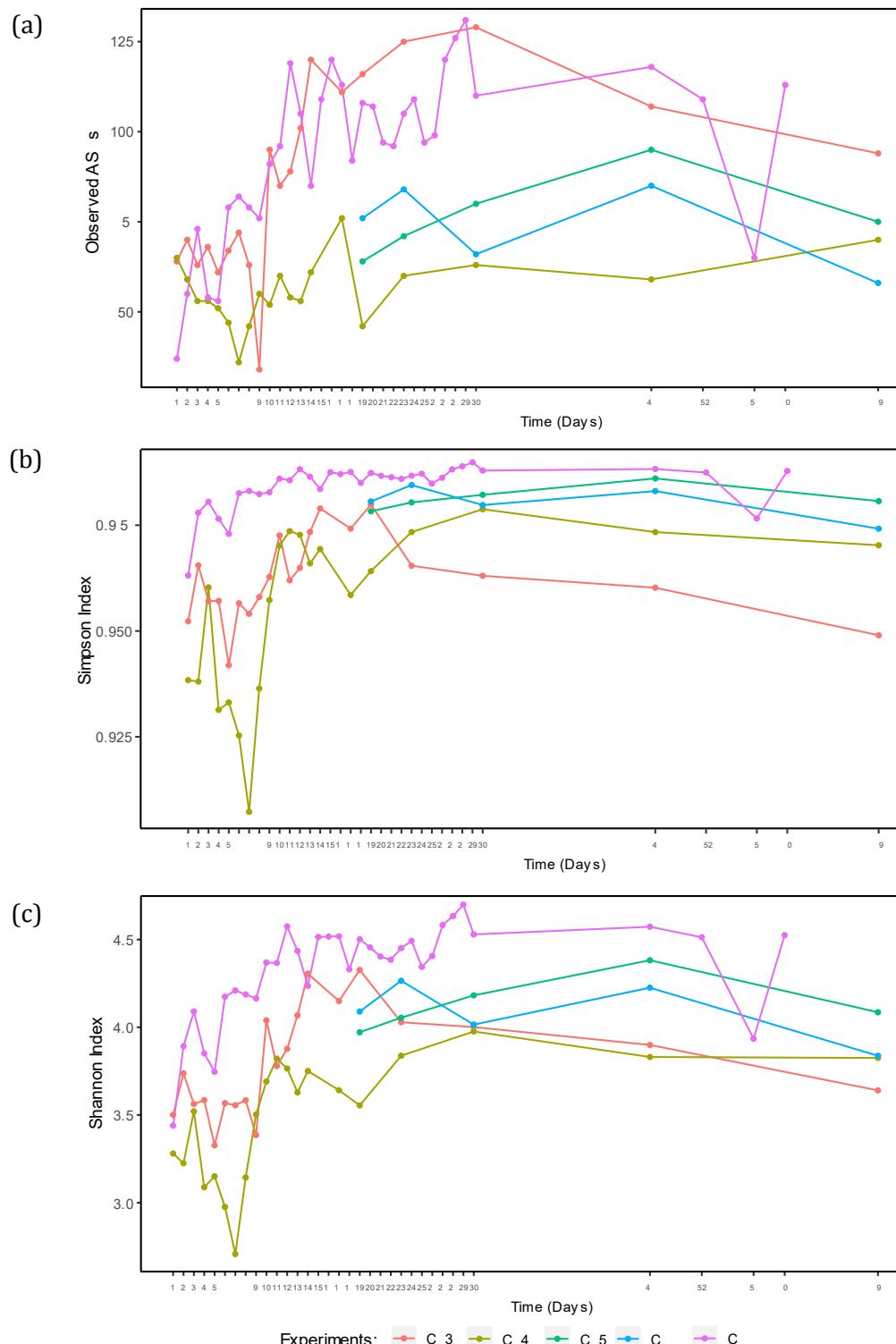


Figure 6.21 Alpha diversity of CX experiments. **(a)** Observed ASVs, **(b)** Simpson's diversity index and **(c)** Shannon's diversity index.

### Beta diversity – diversity between samples

CX3 and the C:N ratio adjusted CX4 beta diversity results are shown in Figure 6.22a; the curing phases of non-mixed CX5 and mixed CX6 are in Figure 6.22b, and the active and curing phases in CX7 are in Figure 6.22c.

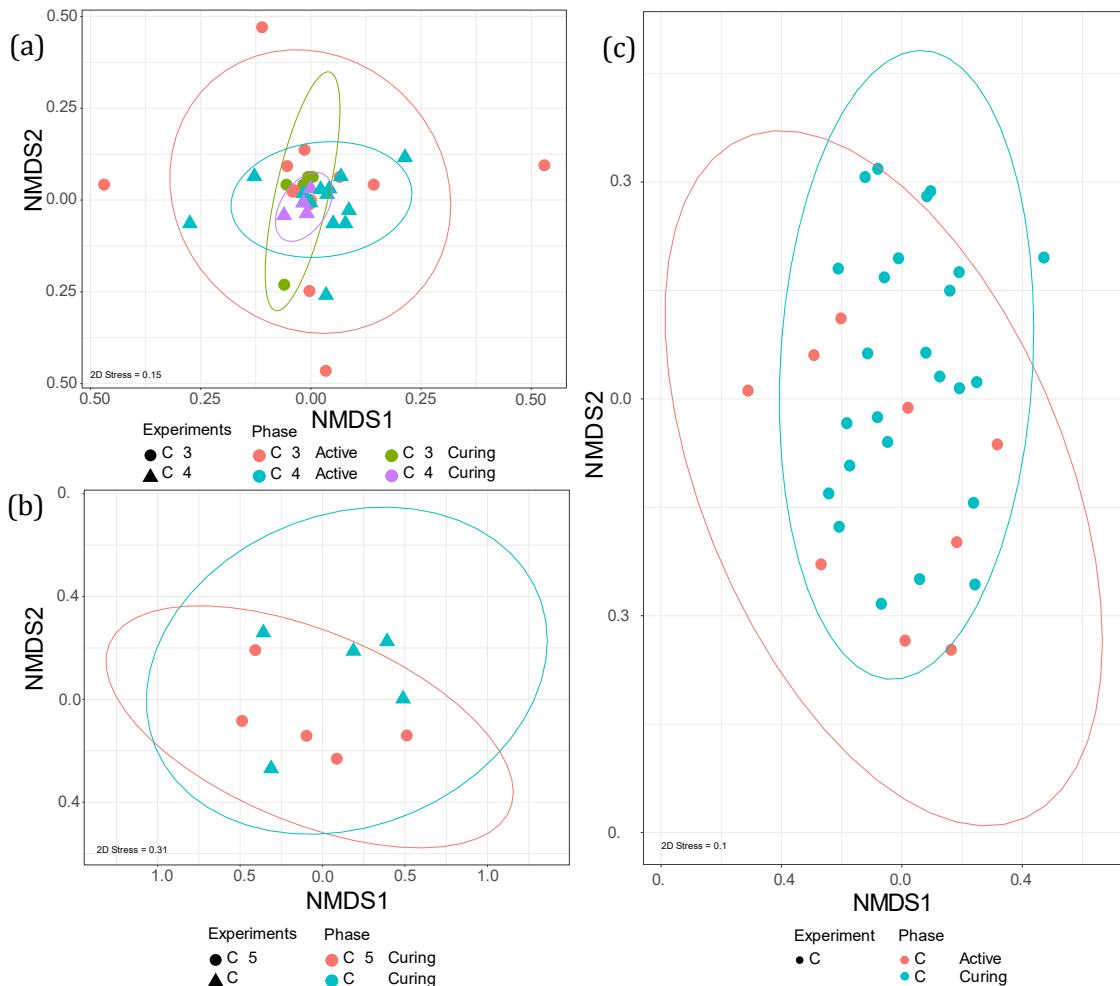


Figure 6.22 Bacterial community comparison by NMDS ordination based on Bray–Curtis distances. Ellipses indicate 95% confidence intervals: **(a)** CX3 and CX4, **(b)** CX5 and CX6, and **(c)** CX7.

High distribution of data-points for the active phases of both CX3 and CX4 compared to the curing phases was observed (Figure 6.22a). The similarity of the CX3 and CX4 bacterial communities was demonstrated with a Generalised Linear Model (GLM) analysis that revealed there was a significant difference in community composition based on experiment (manyGLM, LRT = 318,  $p = 0.001$ ), but that the bacterial communities did not differ significantly based on time (manyGLM, LRT = 4192,  $p = 0.201$ ) (see Appendix G; Table G2).

In the curing phases of CX5 and CX6 (Figure 6.22b), the data-points are distributed relatively distantly, which means that the microbial communities were not similar during this phase. GLM-based analysis revealed that there was a significant difference in the community composition based on the experiment (manyGLM, LRT = 0,  $p = 0.019$ ), but not on time (manyGLM, LRT = 1858,  $p = 0.061$ ) (see Appendix G; Table G3).

GLM-based analyses of CX7 data (Figure 6.22c) shows that there was a significant difference in community composition based on the experiment (manyGLM, LRT = 84,  $p = 0.008$ ) but not on the time (manyGLM, LRT = 3708,  $p = 0.194$ ) (see Appendix G; Table G4). The data-points of the CX7 active phase had high variability compared to those of the curing phase.

In general, in all experiments, the data-points of the active phases had higher variability and were more dissimilar compared to the data-points from the curing phase, which had relatively low variability.

#### 6.7.8 Pathogenic microbial analysis

Isolation of the targeted pathogenic indicator bacteria, *Escherichia coli*, *Salmonella* spp., and *Enterococcus* spp. was carried out on specific selective media. Neither *E. coli* nor *Salmonella* spp. were isolated from any of the CX samples. *Enterococcus faecalis* was isolated from samples from the early curing phases but was not isolated from samples from mature compost.

Most of the isolates from CX3 were Firmicutes, including *E. faecalis* and *Leuconostoc mesenteroides*, and the Gammaproteobacteria *Klebsiella pneumoniae*. By the end of the curing phase, these microorganisms were not isolated. From experiments carried out with feed C:N ratio adjustment (i.e., all but CX3), all of the isolates were from phylum Proteobacteria.

*Bordetella petrii*, *Burkholderia* sp., *K. pneumoniae* and *Ochrobactrum intermedium* were isolated from the curing phase of CX4. *B. petrii*, *Burkholderia* sp., *O. intermedium* and *Pseudomonas aeruginosa* were isolated from the curing phase of CX5. *Klebsiella* sp., *O. intermedium*, and *P. aeruginosa* were isolated from the curing phase of CX6. *B. petrii*, *Enterobacter* sp., *O. intermedium* and *P. aeruginosa* were isolated from the curing phase of CX7.

The majority of these identified isolates were also discovered by metabarcoding, with the exception of *Klebsiella* sp. (Table 6.2).

Table 6.2 Identification of isolated bacteria from samples during curing phase of CX experiments. Bacteria named have the highest % identity to the isolates according to BLAST.

Isolated microorganisms - BLAST	Percentage similarities	CX3 (Day)	CX4 (Day)	CX5 (Day)	CX6 (Day)	CX7 (Day)	QIIME2-2019.10 Metabarcoding
<i>Bordetella petrii</i> strain BSN20	95%	---	30; 47; 69	30	---	60	<i>Bordetella, Bordetella Petrii</i>
<i>Burkholderia</i> sp. strain 785H	100%	---	19; 23	19; 23	---	---	<i>Burkholderia, B. gladioli, B. tuberum</i>
<i>Enterobacter</i> sp. strain FYP1101	100%	---	---	---	---	23	<i>Enterobacter</i>
<i>Enterococcus faecalis</i> MF582810.1 strain CAU170	100%	19; 30	---	---	---	---	<i>Enterococcus, Enterococcus casseliflavus</i>
<i>Klebsiella pneumoniae</i> MG461515.1 strain N1a1	100%	19; 30	---	---	---	---	---
<i>Klebsiella pneumoniae</i> strain MV36808	100%	---	19; 23; 69	---	---	---	---
<i>Klebsiella pneumoniae</i> strain Sum2	100%	---	19; 23; 30; 47	---	---	---	---
<i>Klebsiella</i> sp. strain HWS3	100%	---	---	19; 23; 30; 69	---	---	---
<i>Leuconostoc mesenteroides</i> MG669293.1 strain SG-051 green onion kimchi	100%	19; 23; 30	---	---	---	---	<i>Leuconostoc mesenteroides</i>
<i>Ochrobactrum intermedium</i> strain L22	99%	---	19; 23; 69	---	---	23; 30	<i>Ochrobactrum, Ochrobactrum pseudointermedium</i>
<i>Ochrobactrum intermedium</i> strain OZK3	100%	---	---	19; 23; 30; 47; 69	47; 69	30	<i>Ochrobactrum pseudogrignonense</i>
<i>Pseudomonas aeruginosa</i> strain ST11	100%	---	---	19; 23; 30; 47; 69	19; 23; 30; 47; 69	23	<i>Pseudomonas, Pseudomonas citronellolis, Pseudomonas aeruginosa, Pseudomonas fragi, Pseudomonas syringae, Pseudomonas alcaligenes</i>

### 6.7.9 Colony counting

The bacterial colony forming units (CFUs) were determined for a few representative active and curing phase CX composting samples (Table 6.3). Generally, the CFUs increased from the active to the early curing phase, then they decreased through the curing phase (Table 6.3).

Table 6.3 Number of colony forming units per gram of sample during composting organic waste.

Experiment	CX3	CX4	CX5	CX6	CX7
Time (Days)	Colony forming unit (CFU g <sup>-1</sup> )				
1	2.6x10 <sup>7</sup>	4x10 <sup>6</sup>	---	---	2.4x10 <sup>6</sup>
5	9.1x10 <sup>6</sup>	3.8x10 <sup>7</sup>	---	---	3.1x10 <sup>7</sup>
9	2.1x10 <sup>7</sup>	1.7x10 <sup>7</sup>	---	---	3x10 <sup>8</sup>
19	7.2x10 <sup>8</sup>	3.9x10 <sup>8</sup>	2x10 <sup>8</sup>	1.4x10 <sup>8</sup>	2.9x10 <sup>8</sup>
23	4.2x10 <sup>8</sup>	3.5x10 <sup>8</sup>	5.5x10 <sup>7</sup>	2.8x10 <sup>8</sup>	3.4x10 <sup>8</sup>
30	2.1x10 <sup>8</sup>	3.8x10 <sup>8</sup>	1.6x10 <sup>8</sup>	3x10 <sup>8</sup>	2.3x10 <sup>7</sup>
47	3.1x10 <sup>7</sup>	2.2x10 <sup>7</sup>	1.1x10 <sup>7</sup>	2.4x10 <sup>7</sup>	4.6x10 <sup>6</sup>
60	---	---	---	---	5.2x10 <sup>5</sup>
69	3.6x10 <sup>6</sup>	4.1x10 <sup>5</sup>	3x10 <sup>6</sup>	2.1x10 <sup>6</sup>	---

## 6.8 Discussion

### 6.8.1 General composting features

The main physical and chemical parameters which regulate the microbial decomposition rate and the operational time are: starting waste C:N ratio and particle size, operational temperature, moisture content and pH, and amount of mixing and aeration (Sharma et al., 1997, Azim et al., 2018, Choi, 1999). These parameters are interdependent, hence, changing one likely affects others (Pichtel, 2014). Generally, organic waste has a high moisture content, which is necessary to transport nutrients and energy sources through the cell wall and membrane of microbes (Azim et al., 2018). Mixing the food waste allows the material to become homogenous (Getahun et al., 2012), exposes the organic matter to the microbes (Kalamdhad and Kazmi, 2009), facilitates growth of

microbes and leads to endogenous heat and subsequent moisture losses (Epstein, 2011).

Microbial communities compete with each other for organic matter during its decomposition and will go through several community shifts (Madigan et al., 2018) as a result of changes in composting physical and chemical parameters that affect microbial development (Mehta et al., 2014).

### 6.8.2 Cylibox

A novel composter called Cylibox was designed and constructed after considering several composting parameters including insulation, moisture content, mixing (intensity and amount) and aeration. These were improved in Cylibox by flexible design features and after optimisation of other parameters like C:N ratio and particle size of the feed material, the operation of CX7 lead to extremely effective active composting in a short time of nine days.

### 6.8.3 Lactobacillales transition to Bacillales in active phase

All Cylibox experiments where active phase samples were acquired (CX3, CX4 and CX7) showed high levels of Order Lactobacillales (~60% to 70% of bacterial Orders; Figure 6.12; Figure 6.19) in the first two to three days of operation. The types of Lactobacillales differed between CX3 (mostly *Leuconostoc*), CX4 (largely *Weissella*) and CX7 (about equal *Leuconostoc* and *Weissella*). These lactic acids producing microbes would likely have been responsible for the low pH in the early CX active phases. Microbial growth is affected by organic acids like lactic acid since the undissociated acid molecules pass freely through microbial membranes, where they dissociate and modify cellular internal pH (Brinton, 1998, Warnecke and Gill, 2005). Tran et al., (2019) and Sundberg et al., (2011) note that almost all food waste composting has lactic acid bacteria (LAB) present in the early stages. The ratio of lactic acid producing LAB to acetic acid producing LAB apparently impacts composting success (Tran et al., 2019). Since the specific organic acids were not measured in this thesis, no comment can be made about this feature relative to CX operations. However, since successful composting occurred in each CX experiment, it is concluded that the LAB ratio must have suitable.

The lactic acid bacteria were quickly replaced by other microbes, substantially by Order Bacillales (Figure 6.12; Figure 6.19) most of which were *Bacillus coagulans*, and this genus dominated the central part of the active phases of CX3 (maximum ~50%), CX4 (maximum ~81%) and CX7 (maximum ~26%). Only in CX3 did high proportions of Lactobacillales persist into the mid active phase (Figure 6.12; Figure 6.19), where *Lactobacillus* largely replaced *Leuconostoc*. Other *Bacillus* spp. and close relatives like *B. thermoamylovorans*, *Paenibacillus barengoltzii*, *Paenibacillus* sp., *Ureibacillus* sp., and *Bacillus acidicola* and *Thermobacillus* sp. (both latter ones were higher in CX7, respective maxima of ~13% and ~6%) were individually typically at least ~2-6% of the bacterial genera in the active phases of CX3, CX4 and CX7. Bacillales frequently collectively represented substantially more than 50% of the bacteria during the active phases (especially in CX4 and CX7) and were very likely metabolically important to the active composting (Liu et al., 2015).

#### 6.8.4 Transition away from Bacillales in curing phase

Bacillales typically increased in abundance through the mid active phases of CX3, CX4 and CX7 (Figure 6.12; Figure 6.19), then decreased in abundance towards the end of the active phases. In all five CX experiments, Sphingobacteriales was a markedly abundant Order the curing phase. Abundant bacterial Orders that concomitantly developed or persisted through the curing phases of all experiments were Actinomycetales (in CX3, they were more abundant in the active phase), Burkholderiales, Rhizobiales, Xanthomonadales, Pseudomonadales, Sphingomonadales, and Saprospirales. Due to their overwhelming abundance, these eight families were clearly responsible for most of the transformations in the curing phase.

#### 6.8.5 CX3 – C:N = ~17.5:1 and CX4 – C:N modified to ~30:1

Initially and throughout the active phase of CX3, LAB dominated the microbial communities in the compost process, and the pH values were initially ~5 to 5.6. In the first two days *Leuconostoc* was in high abundance (~40% to ~50%), and although *Leuconostoc* can produce acetic acid (Wu et al., 2016) which is detrimental for beneficial indigenous composting microbes (Tran et al., 2019), this bacterium was quickly reduced in abundance (to ~4% by day three, and

subsequently even lower) mitigating its potentially damaging metabolic features. LAB frequently dominated the microbial community after the first two days of the active phase; often being ~40% (e.g., on days eight and ten) to ~70% (e.g., on day five) and they were largely *Lactobacillus*. It could have been that *Lactobacillus* produced lactic acid which has the ability to inhibit acetic acid production, facilitating the beneficial microbes' growth (Tran et al., 2019). Another abundant bacterium in the active phase of CX3 was *Corynebacterium* (e.g., ~52% on day six and ~33% to ~48% on days 11 to 13), which has been previously reported as abundant in compost (Zhong et al., 2020).

The initial waste material contributes to the degradation rate and the quality of the compost (Biddlestone and Gray, 1985, Azim et al., 2018). One of the most important parameters in composting organic matter is the C:N ratio (Saber et al., 2011, Choi, 1999). The optimum initial composting C:N ratio is ~30:1, and the C:N ratio of mature compost is ~15:1 (Brito et al., 2008). In CX4, the C:N ratio of the food waste was adjusted with Acidulo<sup>TM</sup> sawdust in an effort to improve composting efficiency. It is important have an initial food waste C:N ratio of ~30:1, because carbon provides the energy source for microbial activity and nitrogen forms part of microbial cells (Chen et al., 2011). Choi (1999) reported that microbes use 30 parts of cellulose for each part of nitrogen during composting. Although Acidulo<sup>TM</sup> sawdust (Closed Loop inoculum) was used as the C:N modifier, the microbes in the inoculum did not contribute in the organic waste decomposition in CX4, because abundant inoculum bacteria (see Section 4.5.4 and Figure 4.10) were in low abundance (<1%) and only in a few samples. Hence, the sawdust of the Acidulo<sup>TM</sup> inoculum was considered the main contributor of additional carbon for microbes.

At the early active phase of CX4, lactic acid producing bacteria were in high abundance. In the first two days of CX4, *Weissella hellenica* (~73% to ~76%) and on day three, an unresolved *Weissella* sp. (~47%), dominated the microbial communities. These are typically acetic acid producing LAB. *W. hellenica* is a common food fermenter (Panthee et al., 2019). It is not clear what facilitated the out-competition of *Weissella* spp. after day three. In any case, the temperature rose to be >40°C on day two and *Weissella* spp. were in low abundance. The use of optimised C:N ratio feed in CX4 might have facilitated higher abundances

of *B. coagulans* and lower abundances of LAB, compared to CX3. Overall, the temperature profiles of the active phases in CX3 and CX4 are quite similar, albeit a bit higher in CX4. *Bacillus* spp. have the ability to generate endospores facilitating better survival at high temperatures (Ishii et al., 2000, Kumar et al., 2010), but many of them have growth and optimum temperatures in the thermophilic range. Both these features (endospore production and thermophilic growth capacity) likely contributed to their abundances in the active phases of both CX3 and CX4.

There were differences in microbial communities of the active phases between CX3 and CX4. CX3, operated with a starting C:N of ~17.5, supported substantially higher abundances of *Leuconostoc*, *Corynebacterium* and *Lactobacillus* and a substantially lower abundance of *Bacillus* into the mid-active phase, compared to CX4 with an initial C:N of ~30:1. It could have been that the differences in the C:N of the waste input influenced the development of the microorganisms. Despite the starting C:N differences in the feed wastes, the active phases in both CX3 and CX4 lasted 14 days as determined by the operational temperature falling below 40°C. Several operating features during the active and the curing phases of CX3 and CX4 were similar, but there were differences in the profiles of moisture content, pH and EC. Unresolved genera in the family Sphingobacteriaceae increased in abundance during the curing phase in both CX3 and CX4.

### 6.8.6 How to accelerate composting – mixing and insulation

Due to a technical issue, CX5 was operated without mixing after day one, but this gave the opportunity to test this parameter on composting. The low operational temperature (~40°C) during no mixing indicated limited microbial activity. After 13 days, the compost bed of CX5 was again mixed, the temperature rose rapidly to ~60°C, and the active phase was completed in a further eight days as determined by the bed temperature of <40°C. This clarified that mixing is an important compost operational parameter, which had been previously reported as due to enhanced decomposition rate (Chandna et al., 2013). Getahun et al., (2012) reported that during composting municipal solid waste, mixing frequency significantly affects operational temperature, pH, C:N ratio (via microbial activity), but not electrical conductivity.

During CX3 and CX4, the lid of Cylibox was opened for daily sampling. This process led to heat loss in the compost bed, which was considered to negatively impacted the composting process. CX6 was operated without sampling during the active phase (no lid opening) and with mixing once a day. The active phase in CX6 was completed in nine days (compared to 14 days in CX3 and CX4) as determined by the bed temperature declining to ~30°C on day nine. The active phase temperature profile of CX6 showed a rapid increase compared to CX4 or CX5. This was concluded to be due to a suitable mixing regime (once per day) and reduced opportunities for temperature loss due to no sampling.

### **6.8.7 Optimised Cylibox operations**

The results from CX3, CX4, CX5 and CX6 provided “know how” on how to optimise the in-vessel composting process in Cylibox. A final systematic CX7 experiment was operated by combining the collective “know how”. The active phase of CX7 lasted for nine days and the compost was mature by day 60. Features employed included chopping the organic waste to reduce the particle size, to <5 cm in diameter (Rynk et al., 1992), and adjusting the C:N ratio to ~30:1 with plain sawdust.

As the microbes degrade the complex organic waste, they change their environment and at the same time, this continuously changing environment changes the microbial diversity and abundance (Li et al., 2019, Cayuela et al., 2009, Partanen et al., 2010). The simple action of continuously opening Cylibox for sampling during CX3 and CX4, generated changes in the temperature profile of the organic waste composting, leading to a longer active phase (14 days) compared to in CX6 and CX7 (nine days) with no or reduced opening. This was likely contributed to by differences in the selected microbial communities as a result of better temperature retention in CX6 and CX7.

In the first days of CX6 and CX7, mesophilic microbes initially grew to begin the endogenous temperature increase. This facilitated the selection for thermophilic microbial organic waste degraders, which further increased the operational temperature to ~50°C to ~60°C. The active phase microbial community of CX7 was more evenly diverse, compared to that in CX3 and CX4 (see Appendix G; Figure G2 and Figure G3). The lack of an even diversity of other bacteria in the

microbial communities of CX3 and CX4 (Figure 6.12) could have been detrimental to rapid active phase composting, which was recorded in CX7.

Temperature is a main driver in modulating microbial community structure during the active phase (Awasthi et al., 2015, Gao et al., 2010). The temperature was correlated positively with all CX active phase samples by PCA. The knock-on effect of increasing temperature during the active phase is evaporation leading to reduced moisture content, which was inversely correlated with increasing electrical conductivity – see CX7 (Section 6.7.6). It has previously been reported that during composting, the electrical conductivity is affected by moisture loss, reduction in total mass and mineralisation of organic matter (Yadav and Garg, 2011).

CX3, CX4 and CX7 all had an abundance of *Bacillus* in the active phase, which very likely is an effective, thermophilic composting bacterium. However, CX3 was also dominated by LAB and *Corynebacterium*, and CX4 had an overwhelming abundance of *B. coagulans* (~55% to 70% during days four to nine) and *Tuberibacillus calidus* (~20% to 35% during days 10 to 13) with very few other bacteria. *B. coagulans* is a lactic acid producing, spore-forming, thermophile (optimum temperature 50°C) that has been often isolated from and associated with compost (Chen et al., 2005, Miyamoto et al., 2013, Ö ü sağlam and Aksaray, 2010), and it is a common probiotic ingredient (Majeed et al., 2016). *T. calidus* is a thermophile that was also isolated from compost (Hatayama et al., 2006) but very little information is available on this bacterium.

During the curing phase, the physicochemical changes were slowed due to lack of readily biodegradable materials, and the microbes were dominated, particularly in CX3 and CX4 by several Sphingobacteriaceae genera (increasing in abundance from ~24% on day 14 to ~56% on day 69 in CX3 and from ~18% on day 14 to ~32% on day 69 in CX4; Figure 6.12), along with a more diverse microbial community compared to the active phase. Sphingobacteriaceae increased through the curing phases in CX5, CX6 and CX7, but was substantially less abundant in CX7 compared to in CX3 and CX4.

The pH of the compost bed is microbially controlled via biological metabolism; e.g., lactic acid producers were responsible for low pH in the early phases of

Cylibox operations. In CX3, LAB persisted through most of the active phase and the pH remained low until day 5 to 6. In CX4 and CX7, LAB were only abundant in the first three or one days (respectively), and the pH increased rapidly from ~4.5-5 to ~6.5 during the active phase, then it stabilised above 6 by the end of the curing phase. According to Awasthi et al. (2015), acids metabolically produced during the first days of composting are used by other microbes to break down proteins and amines, which facilitate an increase in the pH. The pH of CX4 and CX7 was in the optimum range for composting, and other measured physical and chemical parameters also followed favourable composting trends; all of these were due to optimised composting microbe development during these operations. The difference between CX4 and CX7, was that Cylibox was opened minimally in CX7, compared to CX4.

During the early active phase of composting, high levels of enzymes and adenosine triphosphate (ATP) are recorded, and low levels are found the curing phase (Garcia et al., 1992). There was a higher concentration of macronutrients (water soluble elements) during the active phase of CX3, CX4 and CX7, then it decreased during the curing phase (see Appendix G; Figure G4).

Organic waste is composed of several complex components including starch, sugars, proteins, lipids, cellulose, and lignin among other compounds generally in lower concentration (Pichtel, 2014). Most of the minerals (P, K, Mn, Mg, Fe, S, Ca, Zn, Cu, and Co) are present in organic waste and together with carbon, nitrogen and oxygen, play essential roles in the growth of microbial cells (Pichtel, 2014). The complexity of the carbon sources varies; the more complex, the slower the degradation and sugars and starches are more easily degraded than cellulose or lignin (Pichtel, 2014). The starting organic waste and the associated microbes (no inoculum was used) along with the provided composting environment of Cylibox, facilitated a rapid active phase in CX7 of nine days and a typical time length curing phase of 51 days. All required nutrients (organic and inorganic) and the physical setup of Cylibox were necessary for the optimized CX7 operation.

In all CX experiments, spent coffee grounds from the cafes represented ~50% of the treated organic waste. Neu et al. (2016) isolated *B. coagulans* from

rapeseed meal and found the bacterium to be a good source of lactic acid production when grown on mucilage, which is a residue from coffee production. Mucilage is a liquid suspension consisting of glucose, galactose, fructose, xylose and sucrose, all as free sugars up to  $60\text{ g L}^{-1}$ . It could be that the large component of coffee grounds, which are high in arabinose, mannose and galactose (collectively  $\sim 50\text{ g L}^{-1}$ ) (Ballesteros et al., 2014), in the organic waste, strongly selected for *B. coagulans* during CX3, CX4 and CX7 active phases.

The sawdust used for adjusting the C:N ratio is comprised of cellulose (~40%), hemicellulose (~30%) and lignin (~30%) (Zuriana et al., 2016). Since ~10% of the Cylibox treated material was sawdust, the proportion of these components would have been diluted and much of the cellulose at least could have been available into the curing phase. Several cellulolytic and ligninolytic bacteria were present in the curing phase like *Thermobifida* (Zhang et al., 2016), *Cellvibrio*, *Mycobacterium*, *Rhodococcus* and *Streptomyces* (Li et al., 2019), though they were all in relatively low abundance.

### 6.8.8 Potential pathogenic microorganisms

According to the TMECC–2001 and the Australian Standard 4454–2012, the pathogenic microbial indicators *Escherichia coli* and *Enterococcus* spp. should be  $<1,000$  most probable number (MPN)  $\text{g}^{-1}$  (note that MPN = colony forming units, which was measured in this thesis) of compost. *Salmonella* spp. should be  $<0.75$  MPN  $\text{g}^{-1}$  (TMECC) or absent in 50 g (AS 4454–2012).

During the active phase of CX experiments, due to the endogenous heating, the highest temperatures was in the range of 55°C to 65°C, which pasteurised the compost. Neither *E. coli* nor *Salmonella* spp. were isolated from any of the CX composting samples. *Enterococcus* spp. was isolated in the early curing phase but undetectable at compost maturity. So, the compost from Cylibox satisfies these pathogen remediations.

Other bacteria were found in the curing phase. *Klebsiella pneumoniae*, isolated from CX3 and CX4 has been reported in wood or composting ecosystems, where it can fix nitrogen, and degrade cellulose and hemicellulose (Doolittle et al., 2008, Droffner et al., 1995). However, *K. pneumoniae* can cause infections, such as liver abscesses, bacteremia, urinary tract infections, and pneumonia,

mostly in immunocompromised individuals (Paczosa and Mecsas, 2016). *L. mesenteroides*, isolated from CX3, is a lactic acid producing bacterium that can be associated with crop plants (Mundt et al., 1967), vegetables and fruits (Pederson and Albury, 1969). However, it is also associated with certain nosocomial infections (Bou et al., 2008). *Enterobacter* sp. isolated from CX7, is commonly found in soil, compost and water (Murray et al., 1990). In general, most of these identified bacterial isolates were in low abundance, and typically undetectable by metabarcoding. Nevertheless, using enrichment and selective media, some of these bacteria were able to be grown.

### 6.8.9 Compost maturity and colony counting

The Solvita® test evaluated compost maturity. Maturity was considered to be achieved between 60 and 69 days. As the compost reached maturity, the numbers of CFUs declined. At the early curing phase, CFUs were in the range  $2.9 \times 10^8$  to  $7.2 \times 10^8$  CFU g<sup>-1</sup>, and by the end of the curing phase, the CFUs fell to be between  $4.1 \times 10^5$  and  $3.6 \times 10^6$  CFU g<sup>-1</sup>. These results agree with other reports from composting agricultural by-products (Chandna et al., 2013), where there were  $10^9$  CFU g<sup>-1</sup> at early curing, decreasing to  $10^5$  CFU g<sup>-1</sup> at the end of curing.

Although at the end of the curing phase, the compost is considered to be mature, the bacterial population will continue to slowly degrade recalcitrant organic matter to form humus and to progressively decrease the biodegradable organic matter. The mature compost is a humic-like end product which is a stabilised organic matter (Tiquia et al., 2002). Compost via mineralization can regulate and make available nutrients for plants (Farrell and Jones, 2009).

## 6.9 Conclusions

To accelerate the composting process, an in-vessel composter prototype called Cylibox was designed and constructed. To find the optimum conditions for microbial activity, one parameter was modified in each of five experiments. Based on the previous experiment, the following ones were modified to become more optimised. The most optimised composting process was achieved when the particle size of the input was reduced to <5 cm in diameter,

the C:N ratio was adjusted to ~30:1 with sawdust, the in-vessel composter was well-insulated, and the compost bed was mixed once a day. The active phase finished in nine days, the temperature biogenically increased to ~60°C to ~65°C, and the final compost reached maturity in 60 days. This research contributes with knowledge on how to improve the eco-efficiency of the in-vessel composting process.

The majority of bacteria in the active phases were in order Bacillales (*Bacillus*, *Tuberibacillus*, *Paenibacillus*, *Ureibacillus*, and unresolved Bacillales genera). In the curing phases the most abundant bacteria were from Sphingobacteriaceae (*Sphingobacterium*, *Olivibacter*) and Actinomycetales (no outstandingly abundant genus), though other Orders were also quite abundant particularly in CX5 and CX7 like Rhizobiales.

# Overall conclusions and future directions

## 7.1 Conclusions

### 7.1.1 Operational conditions for treating organic waste

The profiles of composting parameters are well defined in the literature and mainly comprise an active phase followed by a curing phase (Cooperband, 2000, Bernal et al., 2009, Mehta et al., 2014, Sánchez et al., 2017). Both phases are important because during the active phase, the readily biodegradable organic matter is rapidly decomposed by a complex microbial community that generates endogenous heat, increasing the compost bed temperature to  $\sim 55^{\circ}\text{C}$ , which pasteurises the material (de Bertoldi et al., 1983). During the subsequent curing phase, the cellulosic material continues to decompose more slowly by mesophilic microorganisms (Amir et al., 2008). During composting, the internal environment changes continuously due to the microbial activity, which has an impact on the microbial diversity and the abundance of different microbial groups (Li et al., 2019).

This research proved that the in-vessel commercial unit called Closed Loop (CLO-10), does not facilitate a typical composting profile. The internal environmental conditions are not favorable for composting microbial activity because external heating (not endogenous) is provided (via a heated oil bath, controlled by compost bed moisture content), mixing is continuous, and ventilation is vigorous. After the company-recommended composting time of 24 hours, or even seven days as was carried out in this research, the final product is a dry and dusty material. The Closed Loop process is considered to be dehydration, not composting; and generates partially degraded food waste.

The second commercial in-vessel unit evaluated was OSCA. At the default rotation mode (once hour<sup>-1</sup> for three minutes at one rpm), this unit triggered the treated organic material to form  $\sim$ tennis ball sized dense masses, whose interior was anaerobic. This biomass form created an offensive stench forcing

termination of the experiment. Changing the rotation mode to once per day facilitated some composting process improvement and some balling mitigation. If composting did occur, the temperature of the unit contents rose endogenously. However, since the OSCA unit does not have an exhaust mechanism, water condensed on the unit lifting hoods and pooled extensively on the floor. The unit does have a ventilation system, but during operation, that was covered with fine organic matter, precluding aeration. Additionally, on each rotation, small organic matter particles fell through the unit, sprinkling to the ground. Hence, many features of the OSCA unit have to be improved and redesigned to operate properly as a composter.

The in-vessel composter Cylibox was designed and built based on a self-heating composting process. In order to maintain the endogenous heat released from microbial activity, an insulation system was used to enclose the vessel. Continuous air flow was provided through an air pump, the organic waste was mixed once a day with internal paddles. The gases and the water vapour were conducted through an exhaust, where it was condensed and collected into a container, with the option to reintroduce this liquid if desired. These features of Cylibox provided optimum conditions for microbial activity and composting was completely self-regulated. Experiments carried out in Cylibox followed the typical composting profile and produced pasteurised, mature compost in a relatively short time – nine days of active composting and additional ~50 days of curing to reach maturation.

Neither the Closed Loop commercial in-vessel unit nor OSCA were considered suitable units for composting. Closed Loop does not provide appropriate conditions for microbial organic matter decomposition and OSCA needs to be redesigned in order to provide appropriate conditions for microbial metabolism. In contrast Cylibox provided optimal design features of insulation and mixing that offers excellent conditions for rapid microbial activity.

### **7.1.2 Physical, chemical and microbial parameters**

The external heating system of the Closed Loop in-vessel unit increased the temperature to be thermophilic, independent of microbial metabolism. This exogenous heating rapidly reduced the moisture, such that it was below the

optimal composting range of 40% to 60%; this was concomitant with electrical conductivity rising. The measured pH decreased, most likely as a result of lactic acid bacterial growth – these bacteria were present in high abundance throughout all CL operations except CL1.1. The abundant Lactobacillales were *Weissella*, *Leuconostoc*, *Lactobacillus* and other unresolved genera. Lactic acid bacteria produce large amounts of organic acids, particularly lactic and acetic. Other microorganisms did not grow to be anywhere near as abundant as Lactobacillales. The Acidulo<sup>TM</sup> starting inoculum microorganisms (~35% *Alicyclobacillus* and ~13% *Dyella*), which are presented as being critical for Closed Loop operation, and which were inoculated at start up as per instructions, were never present during the organic waste treatment experiments with Closed Loop.

In the OSCA experiments, with the rotation mode set to once per day for three minutes at one rpm, the temperature of the vessel contents increased. However, the temperature only reached low thermophilic range, hypothesised to be due to heat losses from the uninsulated vessel, and likely also because of limited microbial activity. Consequently, the pasteurisation time/temperature levels were not achieved. The moisture content decreased via water vapour condensation on the lifting hoods, generating substantial water leakage to the floor. The pH increased to alkaline levels and the electrical conductivity decreased as a result of moisture loss during processing. At the default rotation mode (once hour<sup>-1</sup> for three min at one rpm), the moisture content decreased even more rapidly than when rotation was less frequent; the electrical conductivity concomitantly increased. Therefore, to improve the composting process, the OSCA in-vessel unit needs substantial redesign and evaluation. Lactobacillales were present initially in OSCA7 and through all of the few days of OSCA8. In OSCA 7, Xanthomonadales, Spingobacteriales, and Flavobacteriales dominated.

In Cylibox experiments, different parameters were sequentially modified – C:N of the starting waste material (CX3 and CX4), mixing (CX5 and CX6) and insulation (CX6 and CX7). During Cylibox experiments, the temperature increased rapidly due to vigorous microbial metabolism of the readily degradable carbon substrates, leading to endogenous heating, and

maintenance of that heat by effective insulation. During the active phase, the temperature was  $>45^{\circ}\text{C}$  and often  $50^{\circ}\text{C}$  to  $55^{\circ}\text{C}$  (especially in CX7), satisfying compost pasteurisation. The active phase was considered finished when the temperature returned to  $\leq 40^{\circ}\text{C}$ . The moisture content decreased, and was maintained in the optimum composting range, the pH increased to nearly neutral and the electrical conductivity increased but was always below phytotoxic levels. All of the measured parameters were in the optimal range for composting. Microbial diversity and abundance were high in CX7, which was operated with all conditions adjusted to optimum, when the active phase was completed in nine days and maturity achieved in a further 50 days. Lactobacillales were common in the first couple of days of CX operations; only in CX3 (non optimised C:N of the waste), did these bacteria persist into the active phase. The abundant active phase microbes were from order Bacillales (*Bacillus* (largely *B. coagulans*), *Tuberibacillus*, *Paenibacillus*, *Ureibacillus*, and unresolved Bacillales genera). In the curing phases the most abundant bacteria were from Family Sphingobacteriaceae (*Sphingobacterium*, *Olivibacter*) and Actinomycetales (there was no outstandingly abundant genus). Other Orders were also quite abundant particularly in CX5 and CX7 like Rhizobiales.

The two commercial in-vessel units (Closed Loop and OSCA) did not provide suitable conditions for composting organic matter. The physical and chemical parameters were not favourable for the development of composting microorganisms. In contrast, Cylibox facilitated the growth of complex microbial communities whose metabolism led to rapid composting. Different microbial communities were selected for in the active and curing phases.

### 7.1.3 End-product from organic waste treatment

Processing organic waste for 24 hours or seven days in the Closed Loop unit, produced a dry dusty end-product. This material is not compost because the in-vessel unit did not provide suitable conditions for microbes to carry out the composting process. Hence, this dehydrated end-product requires further treatment to become compost. The output from the OSCA unit, also cannot be classified as compost. The raw organic waste was trapped in dense balls, considered to be due to the excessive rotation. The anaerobic centres of the balls produced offensive odours. This material should definitely not be applied

to soil. The end-product from the eco-efficient in-vessel unit Cylibox, is compost. The process followed an active ( $\sim 55^{\circ}\text{C}$ , pasteurisation) and a curing phase, and achieved maturity. This output is safe to use as compost.

## 7.2 Future directions

### 7.2.1 In-vessel composting technology

Currently there are several commercial in-vessel units available with the purpose of rapid production of compost. However, the end-product of Closed Loop and OSCA, cannot be classified as compost. These materials require further treatment or could be disposed into landfills. The latter activity is anathema to the high potential for recycling nutrients from urban food waste.

The main recommendation from this research is to integrate science, engineering and technology to produce better in-vessel units for treating organic waste. Based on this study, further research could be done to improve the operations of Cylibox, such that it could enter the commercial in-vessel market. Similarly, lessons learned from the improved performance of the Cylibox system could be adapted to improve the performance of existing in-vessel processing units.

Additionally, this research was carried out with funding from the Cooperative Research Centre for Low Carbon Living and it included a large social science component. Educating the community to better embrace household composting, or at least to be sure that the community members place the correct items in the compost/recycling bins for council recycling activities, is a major aspect that needs more work. Devices like Cylibox (easily transportable) could be major demonstration tools for community education and communication.

### 7.2.2 A new way of municipal solid waste management

MSW generated at small and medium scale could be pre-treated onsite. Applying an integral MSW management system will reduce negative impacts from its current management. Classifying and reducing the volume of the recyclable materials could facilitate bigger capacity to store more material and keep it for longer at the generation place. This would reduce the transportation

of these heavy materials to a treatment plant. Recyclable materials can clearly be used to manufacture in-vessel composters (as was done in this thesis) for treating urban organic waste.

Onsite composting may be a solution for treating organic waste generated in households, restaurants, cafes, markets, supermarkets, among other organic waste generators. To implement composting programs, a holistic approach is required to engage all stakeholders so that they contribute to better organic waste management. In the first step, the organic waste generator should separate and prepare the input for composting. The manufacturers should provide an effective, simple, in-vessel composter. The waste management company should collect the composted material and provide it to farmers or gardeners, who would use it as a soil amendment. The council or local government should regulate for proper organic waste management. In the Australian context, the Environmental Protection Authority (EPA) must promote and regulate the new way of MSW management and certify with green-labeling to the companies, councils, and generators of MSW that are applying this new approach of MSW management.

Based on this research and other new investigations the Australian Standard 4454-2012 should be updated, where onsite composting must be included. The main component for onsite composting is to have an efficient in-vessel composter. From the technological point of view, to facilitate the control of the physicochemical parameters, sensors could be used. Automation could control the mixing frequency and aeration rate. A more sophisticated in-vessel composter could be controlled from a mobile application. However, it is important to understand the science of composting, where the indigenous microorganisms play a crucial role in converting the organic matter to compost. It is critical that appropriate physical, chemical and microbiological properties are maintained if composting is to become a successful technique for treatment of MSW.

The in-vessel composter Cylibox is a small-scale prototype with 28 L capacity for 10 kg of organic waste. However, it can be scaled up to medium size (~100 kg per day) to compost more organic waste.

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## 9. Appendices

### Appendix A: Library preparation protocol - WEHI

The extracted genomic DNA from samples was prepared for sequencing based on the adapted CRISPR overhang sequencing protocol of Mr. Stephen Wilcox at WEHI.

#### The first PCR.

Make the master mix by adding into each well of a 96-well plate:

- 10 µL of GoTag Green (or NEB 2x Taq enzyme mix or MangoMix™)
  - 0.5 µL Primer (515F 5'-CTGAGACTTGCACATCGCAGCGTGYCAGCMCCGCGTAA-3') (10 µM)
  - 0.5 µL Primer (0 R 5'-GTGACCTATGAACTCAGGAGTCGGACTACNVGGGTWTCTAAT-3') (10 µM)
  - 8 µL Nuclease-Free water
  - 1 µg of compost genomic DNA (concentration of ~100 ng/µL)
- TOTAL 20 µL per reaction

The primer overhang adapters are underlined (Penington et al., 2018).

Run 'O 1 ' program (1 cycles) on i oRad i osystems™ SimpliAmp™ thermalcycler.

- Heat the lid to 100°C
- At 95°C for 3 min
- 18 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s
- 72°C for 7 min
- Hold at 10°C

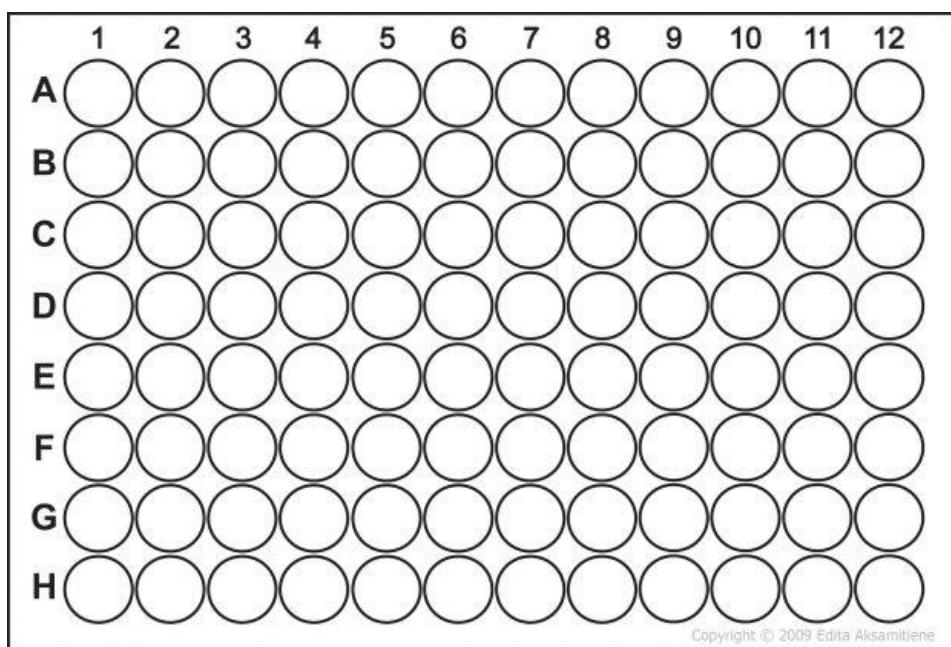
#### **Clean up.**

- Add 20 µL of next generation sequencing (NGS) beads CLEANNA to each well (giving a 1:1 ratio, NGS-beads: DNA-sample) using a multichannel pipettor, mix well by vortexing, wait 5 min for beads to bind DNA. (total 40 µL)
- Put the plate onto magnetic rack and wait until beads can be seen attaching to the side of the wells.
- Carefully aspirate the supernatant – taking care not to disturb the beads.
- Carefully add 150 µL of 70% ethanol and pipette up and down gently.
- Remove as much ethanol as possible without disturbing the pellets.
- Let the plate air dry ethanol evaporation. When beads are dry, the pellets will have a dry flakey appearance.
- Add 40 µL of Nuclease-Free water and mix well, this will elute the DNA from the beads (a plate shaker can be used after resuspension). (total volume is 40 µL).
- Place plate back onto the magnetic rack and wait for the liquid to become clear (indicating the beads have bound to the magnet leaving DNA in suspension).

Prepare a primer dilution plate (Pre-PCR room)

Into a new 96-well plate, dilute F and R overhang (NGS primers to 10 µM (stock = 100 µM).

Setting up Fwd ad Rev indexing primer dilution plate as in the image below (use multichannel pipettor).



### The second PCR.

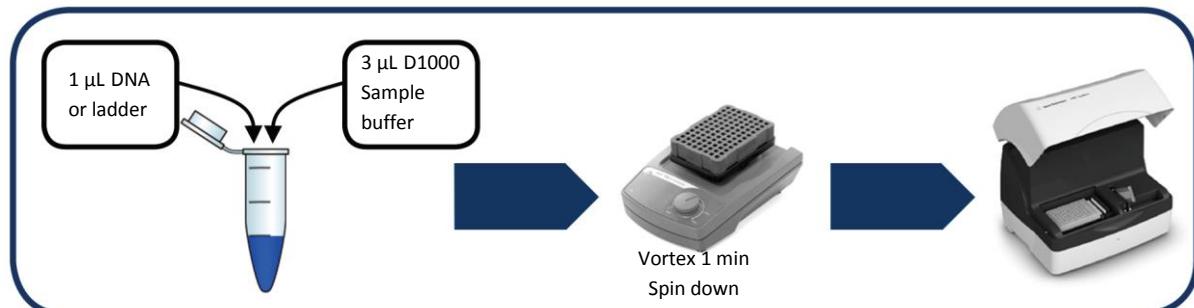
- From the last step of the cleanup, transfer 10 µL of DNA from each well to wells in a new plate.
  - Add 10 µL of GoTaq Greenmix (or NEB 2x Taq enzyme mix)
  - Add 0.5 µL of Fwd indexing primer (10 µM) (Aubrey et al., 2015)
  - Add 0.5 µL of Rev indexing primer 10 µM) (Aubrey et al., 2015)
- Run 'OH 24' program (24 cycles) on BioRad PCR machines Genomics Lab.
- (heated lid 100°C).
  - 95°C for 3 min
  - 24 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s
  - 72°C for 7 min
  - Hold at 10°C.

### **PCR band verification**

Electrophorese some random samples from each plate on a 1% agarose gel to confirm correct sized bands with minimal non-specific bands. Run a few random samples representing each first pair. The expected size will depend on the original PCR amplicon size.

By using the Tapestation, verify PCR bands. For more details, review the following link.

[https://www.agilent.com/cs/library/usermanuals/public/ScreenTape\\_D1000\\_QG.pdf](https://www.agilent.com/cs/library/usermanuals/public/ScreenTape_D1000_QG.pdf)



**Prepare library pool**

- Take 5 µL from each well including negative controls and place in a 25 mL reservoir.
- Mix pool with a P1000 pipettor.
- Transfer whole pool volume into new 1.5 mL microcentrifuge tube (For all 96 wells, this will be ~500 µL in total volume).
- Transfer 50 µL of the pool to a fresh 1.5 mL Eppendorf tube.

**Bead cleanup of a pool**

- Add 40 µL of NGS beads ('A' labelled bottle) to 50 µL library pool.
- Leave at room temperature for 5 min.
- Put tube onto magnetic rack and wait until beads are visible attaching to the side of the wells.
- Carefully aspirate the supernatant being careful not to disturb the beads.
- Carefully add 180 µL of 80 % ethanol.
- Remove ethanol.
- Carefully add another 180 µL of 80 % ethanol.
- Remove ethanol.
- Using a P20 pipettor take care to remove as much of the remaining ethanol solution as possible without disturbing the pellet.
- Transfer tube to a rack, and let tube air dry
- Add 105 µL of nuclease-free water and gently mix using a vortexer. Briefly spin.
- Leave for 5 min and then place on magnetic rack
- Once beads have migrated to the magnet, transfer 90 µL to new 1.5 mL microcentrifuge tube.

**Sequencing**

- An Illumina® MiSeq® instrument at WEHI was used to sequence samples.

## Appendix B: QIIME2-2019.10 script

*Script code:*

```
#QIIME2-2019.10 activation-----
ubuntu
source activate qiime2-2019.10
#Obtaining and importing data -----
qiime tools import \
--type SampleData[PairedEndSequencesWithQuality] \
--input-path ~/Data/ \
--input-format CasavaOneEightSingleLanePerSampleDirFmt \
--output-path ~/demuxed.qza
#Demultiplexing sequences-----
qiime demux summarize \
--i-data demuxed.qza \
--o-visualization demuxed.qzv
#Still primers attached - they need to be removed before denoising-----
qiime cutadapt trim-paired \
--i-demultiplexed-sequences ~/demuxed.qza \
--p-front-f CTGAGACTTGCACATCGCAGCGTGYCAGCMGCCGCGTAA \
--p-front-r GTGACCTATGAACTCAGGAGTCGGACTACNVGGGTCTAAT \
--p-error-rate 0.20 \
--output-dir ~/Data/trim \
--verbose
qiime demux summarize \
--i-data ~/Data/trim/trimmed_sequences.qza \
--o-visualization ~/Data/trim/trimmed_sequences.qzv
#Option 1: Denoising and QC filtering with DADA2
(https://docs.qiime2.org/2019.10/tutorials/atacama-soils/)-----
#Paired-end read analysis commands
nohup qiime dada2 denoise-paired \
--i-demultiplexed-seqs ~/Data/trim/trimmed_sequences.qza \
--p-trim-left-f ... \
--p-trim-left-r ... \
--p-trunc-len-f ... \
--p-trunc-len-r ... \
--o-table table.qza \
--o-representative-sequences rep-seqs.qza \
--o-denoising-stats denoising-stats.qza
qiime metadata tabulate \
--m-input-file denoising-stats.qza \
--o-visualization denoising-stats.qzv
#FeatureTable and FeatureData summarie-----
qiime feature-table summarize \
--i-table table.qza \
--o-visualization table.qzv \
--m-sample-metadata-file sample-metadata.tsv
qiime feature-table tabulate-seqs \
--i-data rep-seqs.qza \
--o-visualization rep-seqs.qzv
#Generate a tree for phylogenetic diversity analyses-----
qiime phylogeny align-to-tree-mafft-fasttree \
--i-sequences rep-seqs.qza \
--o-alignment alignedrep-seqs.qza \
--o-masked-alignment masked-alignedrep-seqs.qza \
--o-tree unrooted-tree.qza \
--o-rooted-tree rooted-tree.qza
#Taxonomic analysis-----
wget \
-O "gg-13-8-99-515-806-nb-classifier.qza" \
https://data.qiime2.org/2019.10/common/gg-13-8-99-515-806-nb-classifier.qza
qiime feature-classifier classify-skllearn \
--i-classifier gg-13-8-99-515-806-nb-classifier.qza \
--i-reads rep-seqs.qza \
```

```
--o-classification taxonomy.qza
qiime taxa filter-table \
--i-table table.qza \
--i-taxonomy taxonomy.qza \
--p-exclude mitochondria, chloroplast \
--o-filtered-table table-no-mitochondria-no-chloroplast.qza
qiime metadata tabulate \
--m-input-file table-no-mitochondria-no-chloroplast.qza \
--o-visualization taxonomy.qzv
qiime taxa barplot \
--i-table table-no-mitochondria-no-chloroplast.qza \
--i-taxonomy taxonomy.qza \
--m-metadata-file sample-metadata.tsv \
--o-visualization taxa-bar-plots.qzv
#Exporting data for R-Studio analysis-----
qiime tools export \
--input-path ~/unrooted-tree.qza \
--output-path ~/output/
qiime tools export \
--input-path ~/taxonomy.qza \
--output-path ~/output/
qiime tools export \
--input-path ~/table-no-mitochondria-no-chloroplast.qza \
--output-path ~/output/
biom convert -i ~/output/feature-table.biom \
--to-tsv \
-o ~/output/table.tsv
```

## Appendix C: R-Studio script

### *Script code - Phyloseq:*

```

# import data-----
otu <- read.table( "table.tsv", header = TRUE, sep = "\t", row.names = 1)
tax <- read.table( "tax.tsv", sep = "\t", fill = TRUE, row.names = 1)
met <- read.table( "meta.txt", header = TRUE, sep = "\t", row.names = 1)
tre <- read_tree("tree.nweeks")
# convert to phyloseq-----
# add levels of taxonomy to taxonomy table
colnames(tax) <- c("Domain", "Phylum", "Class", "Order", "Family", "Genus", "Species")
# convert data frames to matrices for compatibility with phyloseq
otu_mat <- as.matrix(otu)
tax_mat <- as.matrix(tax)
# combine OTU, taxonomy, and metadata files into a phyloseq object
phy <- phyloseq(otu_table(otu_mat, taxa_are_rows = T), tax_table(tax_mat),
sample_data(met))
# add tree data
phy <- merge_phyloseq(phy, tre)
# remove zero sum ASVs
phy <- prune_taxa((taxa_sums(phy) > 0), phy)
rm(otu_mat)
rm(tax_mat)
#Rarefaction curve -----
rarecurve(t(otu), step = 250, sample = 2500, ylab = "ASVs", xlab = "Reads", col="blue", cex = 0.6, main = "Compost samples")
# identify the contaminants-----
consList <- isContaminant(seqtab = phy, neg = "neg", method = "prevalence")
# get the names of contaminants
cons <- rownames(consList)[consList$contaminant=="TRUE"]
# what percentage were the contaminants in the compost samples?
compost <- subset_samples(phy, sample_type == "compost")
# merge the compost samples
compost <- merge_samples(compost, "sample_type", fun = sum)
# transform counts to percentages
compostPer <- transform_sample_counts(compost, function(x) 100 * x/sum(x))
# subset the contaminants
conSub <- prune_taxa(x = compostPer, taxa = cons)
# write otu table to dataframe
conData <- data.frame(t(conSub@otu_table))
# add tax data to dataframe
conData <- cbind(conData, conSub@tax_table[,2:7])
# # write data to file for reference
# write.csv(file = "contaminants.csv", x = conData, row.names = FALSE)
# remove the contaminants from the main phyloseq file
phy <- remove_taxa(phy, taxa = cons)
#Barchart colours-----
barColours1 <- c(
"#0000A6", "#006FA6", "#FF34FF", "#A30059", "#FF4A46", "#008941", "#8FB0FF", "#FFDBE5", "#997D87",
"#1CE6FF", "#63FFAC", "#809693", "#7A4900", "#FFFF00", "#004D43", "#1B4400", "#FEFFEE", "#B79762",
"#BA0900", "#4CF823", "#4DE052", "#5A0007", "#4FC601", "#D16100", "#FF2F80", "#4A3B53", "#61615A", "#FFAA92",
"#6B7900", "#00C2A0", "#FF90C9", "#B903AA", "#CFC6E6", "#7B4F4B", "#DDEFFF", "#000035", "#BF
AE1B", "#D3E8AF", "#8FB530", "#622FB7", "#B58C02", "#53C190", "#C476EC", "#E2E0D3", "#137EBE", "#2A9
E12", "#FBC902", "#E0B1C8", "#4F116D", "#905B9D", "#F99B70", "#E3DD6D", "#9EB5FB", "#72E6AD", "#6294
8D", "#1B24A5", "#5956A3", "#4DA339", "#1D53E7", "#41473B", "#878A10", "#0EFA07", "#7205D9", "#B3A18
9", "#3FADF2", "#DD482A", "#1B2D09", "#FCDF41", "#463219", "#A8C892", "#78FB49", "#939482", "#7B161D
", "#AC1528", "#FE21A6", "#3B3FB2", "#7D997D", "#FAC28E", "#EB2F75", "#B4FD09", "#D2BC87", "#D2F8C0
", "#5D5A15", "#91BD3E", "#42A77E", "#F7153E", "#48E1E9", "#795BC4", "#B270CC", "#7BC1A8", "#A5B81B",
"#2DC72F", "#B9FB34", "#C51640", "#3E8AF7", "#430578", "#50C770", "#63FAC8", "#70FE60", "#53EC96",
"#EBBCCB", "#51FAF2", "#75F72B", "#1325F9", "#DCF40A", "#C442D1", "#C87DC1", "#66AC25", "#A136C8", "#5774C6",
"#D427CC", "#E8B122", "#073F6C", "#444FC7", "#706F49", "#D17346", "#1266DD", "#9045F0", "#21
A13A", "#4ECF87", "#3D8A45", "#A32FF2", "#92DBB0", "#C5C2CD", "#D118CF", "#1100BA", "#E4E479", "#9E8
BD5", "#34A80E", "#8BC4EA", "#ED3DEF", "#67D983", "#7E6012", "#7BE0D2", "#6F664E", "#B397C0", "#DA4C
77", "#8B0284", "#8D8883", "#777D7D", "#8EDB04", "#EB7DE2", "#392845", "#2BACEA", "#F97F53", "#D6140
")

```

```

8", "#FECDF8F", "#09226E", "#0C05A3", "#C44526", "#F5A057", "#CD3F9D", "#60CB63", "#E73858", "#CEA0C8
", "#699844", "#1239FE", "#DBA182", "#253480", "#FDD7F5", "#542766", "#51A342", "#5DB8BE", "#14A269"
, "#EB6006", "#6D5806", "#560898", "#AD5AA0", "#2A34F1", "#4FD23D", "#D37BA9", "#84B038", "#65BFF6",
"#EBAFA2", "#3E1E48", "#AD1B15", "#7AB294", "#8D69A8", "#B1B5F5", "#C65128", "#363B15", "#697D96",
"#871C48", "#788F3F", "#BCFC65", "#172C74", "#E06A7A", "#E8C55A", "#2197CD", "#41744E", "#83F79E",
"#2EC828", "#444D15", "#61C153", "#14473C", "#059D78", "#44AD43", "#52C60D", "#981749", "#951F40",
"#E CFA88", "#AE2BC4", "#ABF417", "#6B0A53", "#200691", "#559C4B", "#ACADAB", "#3A52AD", "#1851C1", "#08
FE88", "#93B577", "#40F35F", "#8E1668", "#6FE83E", "#4C7FFA", "#B09F1D", "#ED22C5", "#34F10F", "#EB3
944", "#7157CB", "#DE39C0", "#854C07", "#6D758A", "#22DE03", "#6E76CC", "#CBFBC8", "#62159D", "#3A41
C9", "#E2EC0E", "#C7B32A", "#6984B7", "#702BC1", "#5ACC16", "#FBB73B", "#9CCCAC", "#BF34A9", "#C13DA
9", "#F3891D", "#B0B713", "#58EE67", "#21C007", "#660FF7", "#7E007D", "#924FF0", "#11FF09", "#D7009A
", "#6C1304", "#9C67AE", "#34C2E7", "#30048F", "#444011", "#CD1346", "#D4E25E", "#6C423A", "#27FEBE
", "#0B61F3", "#8D9108", "#7B62F4", "#735CEB", "#E98555", "#F88053", "#67EFA1", "#BEAFF5", "#20B5FE",
"#1F4895", "#DB0CF5", "#E2213E", "#B068C8", "#BCA4E8", "#DE18F7", "#15B459", "#76B548", "#092B5B
", "#16BDC7", "#E91406", "#DE0DAB", "#7A0681", "#E03AE8", "#52D8C0", "#35DDD2", "#53F9FF", "#4B6362
", "#6B87D0", "#F46E7E", "#86E5C7", "#A45CA9", "#BB3F5B", "#36F141", "#BB509F", "#C16A69", "#E
1EA34", "#BB9E2D", "#807BB3", "#A6BCEC", "#B2CFD2", "#02E67F", "#D0284C", "#C64A10", "#EE3426", "#A3
04B4", "#698A67", "#B66DDD", "#94A6C3", "#FF4FF3", "#ED826C", "#91FAA0", "#1D675F", "#C2B461", "#965
F80", "#57C3D4", "#76208B", "#3D3300", "#3A615C", "#9F1A04", "#44C2B5", "#3CE01D", "#64FFF3", "#6423
95", "#1BFD36", "#85A8DC", "#9B8C1A", "#1798CF", "#9BC6D1", "#925147", "#C2122B", "#DB2961", "#980BE
2", "#728042", "#10AEE7", "#717BC9", "#00E898", "#DDAE31", "#4DDD4A", "#94FF54", "#9CABA7", "#529EE7
", "#69E6F9", "#5CFDD6", "#94E9E8", "#430296", "#7525AC", "#127BFD", "#D94CB5", "#0DDC77", "#AF26F8
", "#08EC5A", "#7DCD11", "#E1E5B1", "#031C43", "#A04937", "#1D84FC", "#D6DA00", "#497D23", "#E3550E
", "#66E559", "#CEE098", "#151852", "#93C336", "#C0266F", "#FBA4E0", "#E84B9", "#26C9B5", "#69ECB0
", "#4DDEF", "#FFCB23", "#733A4B", "#D8D08B", "#35E586", "#EFF847", "#512B50", "#C14AA5", "#B23BA9
", "#EB0C6E", "#F76FAD", "#B52A10", "#0BD851", "#3E712E", "#616009", "#6404CE", "#AB1C40", "#B26E5F
", "#41A393", "#A0205E", "#4FD6DD", "#06751B", "#F8AD2B", "#2F6367", "#8748B1", "#97D7DC", "#9F133D
", "#796770", "#6C96C6", "#D70BEA", "#BBF072", "#2F3624", "#BA67B6", "#EFDC98", "#5F893A", "#F56281
", "#0B1A9C", "#F1F7A5", "#44C3CD", "#83FC2F", "#4A6D46", "#B3040E", "#038A22", "#131F34", "#C20896
", "#514B
CF", "#CEA6C6", "#4FE8E2", "#11B250", "#F74368", "#AEDF11", "#4516B5", "#A8F9DB", "#F8A0FA", "#B0EFB
3", "#2B9CE4", "#873DE2", "#268EF9", "#393655", "#9B55C4", "#F07C18", "#3EF77B", "#1D929B", "#E2B12B
", "#C99AB6", "#7DF5E0", "#6E44E0", "#7C91E1", "#9098FF", "#16EBC3", "#F60407", "#59EDA5", "#F61AA8
", "#64E891", "#72A5C8", "#581475", "#5CB000", "#030AF6", "#FD2419", "#0FC43E", "#71D1A9", "#BC7D81
", "#757A43", "#EF4ABA", "#8138A9", "#E01E4", "#ECC8AA", "#B57E46", "#2FE7DB", "#7DED8B", "#AF53F1
", "#42BCE9", "#59F0A0", "#D7D5896", "#E4A53E", "#E5CA5E", "#A8FC8C", "#C55EDD", "#56A167", "#55A147
", "#D89F9E", "#A9CE69", "#B002E6", "#CE14DB", "#6CC803", "#824A70", "#46DF21", "#4A57AE", "#3DB03A
", "#E6C322", "#A6799B", "#8D0128", "#23232D", "#76FA59", "#7D3124", "#B7D12D", "#BF1BFC", "#FBE4C8
", "#8789B2", "#B90014", "#3366FA", "#A7B0F4", "#8C78B5", "#B7C3D7", "#0A4900", "#75B5CE", "#2571DE
", "#C89EBC", "#29FF1F", "#B4F362", "#25F03A", "#6DF139", "#655051", "#841506", "#5AF1DD", "#51C766
", "#EB3038", "#10962E", "#19AE55", "#A1FFDD", "#F405ED", "#2A1779", "#EDF994", "#107545", "#D4AF96
", "#00F171", "#4DB97E", "#A2B125", "#DC8759", "#91456F", "#5E0B2F", "#DE77FD", "#9AE0AB", "#EF7C6E
", "#DBBF69", "#739B2A", "#1F1FCE", "#6B545A")
barColours2 <- sample(barColours1)
barColours3 <- sample(barColours2)
# Barchart subset compost samples-----
compost <- subset_samples(phy, sample_type == "compost")
# Collapse taxa to specified level of taxonomy
compostOrder <- tax_glm(compost, "Order")
# Transform counts to percentages
compostPer <- transform_sample_counts(compostOrder, function(x) 100 * x/sum(x))
# Melt phyloseq data
q <- psmelt(compostPer)
# change stacking so most abundant (overall) are at top
q$Order <- reorder(q$Order, q$Abundance)
q$Order <- factor(q$Order, levels = rev(levels(q$Order)))
# change order of samples along x axis
q$Subexperiment <- factor(q$experiment, levels = c("code1", "code2", "code3", .... "codeN"))
# Generate ggplot2 object
p <- ggplot(q, aes_string(x = "experiment", y = "Abundance", fill = "Order"))
# Customise and output ggplot2 object
p + geom_bar(stat = 'identity', position = "stack") + ylab("Reads assigned to Order") +
  theme_bw() + theme(legend.position = "bottom") + scale_fill_manual(values = barColours1)
+ guides(fill = guide_legend(ncol = 4)) + theme(axis.text.x = element_text(angle = 90))
# diversity metrics-----
# subset compost samples
compost <- subset_samples(phy, sample_type == "compost")
compost <- prune_taxa((taxa_sums(compost) > 0), compost)

```

```

# rarefy at a level equal to the sample with the fewest reads
compost <- rarefy_even_depth(compost, min(sample_sums(compost)), rngseed = 1)
# create diversity metric plot object of "average observed ASVs" experiment/d
ob <- plot_richness(CLoutput, "subGrouping", measures = c("Observed"))
# specify box-plot
ob <- ob + geom_boxplot(data=ob$data, aes(x=subGrouping, y=value), show.legend = FALSE) +
  theme_bw() + theme(axis.title = element_blank())
# reorder data along x-axis
ob$data$subGrouping <- factor(ob$data$subGrouping, levels = c("code1",
  "code2""code3",....."codeN"))
# create diversity metric plot object of "Simpson's Index" evenness per experiment/d
sm <- plot_richness(compost, "subGrouping", measures = c("Simpson's"))
# specify box-plot
sm <- sm + geom_boxplot(data=sm$data, aes(x=subGrouping, y=value), show.legend = FALSE) +
  theme_bw() + theme(axis.title = element_blank())
# reorder data along x-axis
sm$data$subGrouping <- factor(sm$data$subGrouping, levels = c("code1",
  "code2""code3",....."codeN"))
# create diversity metric plot object of "Shannon's Index" alpha diversity per experiment/d
sh <- plot_richness(compost, "subGrouping", measures = c("Shannon's"))
# specify box-plot
sh <- sh + geom_boxplot(data=sh$data, aes(x=subGrouping, y=value), show.legend = FALSE) +
  theme_bw() + theme(axis.title = element_blank())
# reorder data along x-axis
sh$data$subGrouping <- factor(sh$data$subGrouping, levels = c("code1",
  "code2""code3",....."codeN"))
# print plots on one page
grid.arrange(ob, sm, sh, nrow = 1)
#Observed ASVs, Simpson's, and Shannon's for each sample
obs <- ggplot(ob$data, aes(x=samplingD, y=value, color=experiment)) + geom_line(size=1) +
  geom_point(size=3) + scale_x_continuous(breaks = c(1,2,3,...))
smp <- ggplot(sm$data, aes(x=samplingD, y=value, color=experiment)) + geom_line(size=1) +
  geom_point(size=3) + scale_x_continuous(breaks = c(1,2,3,...))
shg <- ggplot(sh$data, aes(x=samplingD, y=value, color=experiment)) + geom_line(size=1) +
  geom_point(size=3) + scale_x_continuous(breaks = c(1,2,3,...))
# print plots on one page
grid.arrange(obs, smp, shg, nrow = 1)
# 2D ordination to assess beta diversity-----
# subset compost samples
compost <- subset_samples(phy, sample_type == "compost")
compost <- prune_taxa((taxa_sums(compost) > 0), compost)
# convert samplingDay variable from numeric to factor
compost@sam_data$samplingDay <- factor(compost@sam_data$samplingD)
compost.nmds <- ordinate(compost, method = "NMDS", k = 2, distance = "bray")
p <- plot_ordination(compost, ordination = compost.nmds, type = "samples", axes = c(1, 2),
  color = "samplingD", shape = "experiment") + theme_bw() + geom_point(size = 8) +
  stat_ellipse()
print(p)
# subset compost samples-----
compost <- subset_samples(phy, sample_type == "compost")
compost <- prune_taxa((taxa_sums(compost) > 0), compost)
# collapse the data to Genus to reduce noise and accelerate computation for this demo
compostGen <- tax_glm(compost, 'Genus')
# get the OTU table & transpose it
otuTab <- t(compostGen@otu_table)
# subset the metadata
mvaMeta <- met[1::::,2:3]
# combine the metadata & OTU table
combo <- cbind(mvaMeta, otuTab)
# create mvabund object from count data & check it
comboMva <- mvabund(combo[,3::])
is.mvabund(comboMva)
# check mean vs variance relationship
meanvar.plot(comboMva)
# variance increases with the mean = heteroscedasticity
# create model of data
mod <- manyglm(comboMva ~ combo$experiment * combo$time, family="poisson")

```

```

plot(mod)
mod <- manyglm(comboMva ~ combo$experiment * combo$time, family="negative_binomial")
plot(mod)
anova(mod)
# Heatmap subset non-rarefied data-----
phycompost <- subset_samples(phy, sample_type == "compost" & neg == "FALSE" & "TRUE" != "kaust")
# Collapse by 'phase'
phycompost <- merge_samples(phycompost, "phase")
order1 <- c("code1", "code2""code3",....."codeN")
# Genus level
# Collapse to genus
phycompost <- tax_glom(phycompost, taxrank = "Genus")
phycompostg <- rarefy_even_depth(phycompostg, sample.size = min(sample_sums(phycompostg)), rngseed = 1)
# Identify 20 most abundant taxa
genus20 <- top_taxa(phycompostg, n = 20)
# Retain only the top 20
phycompostg20 <- prune_taxa(phycompostg, taxa = genus20)
# Get full taxa names
temp <- apply(phycompostg20@tax_table, 1, paste, collapse = ";")
# Replace OTU IDs with full taxa names
taxa_names(phycompostg20@tax_table) <- temp
taxa_names(phycompostg20@otu_table) <- temp
# get total number of each otu
sumz <- taxa_sums(phycompostg20)
# Plot heatmap with most abundant taxa at top
plot_heatmap(phycompostg20, sample.order = order1, taxa.order = names(sort(sumz, decreasing = F)), low = "lightblue", high = "darkblue", na.value = "lightblue") + theme_bw()
# theme(axis.text.x = element_text(angle = 0, hjust = 0.5, vjust = 1))

```

**Script code – Principal Component Analysis (PCA):**

```

#Import data
#Install ggbioplot package
#Activate ggbioplot
library(ggbioplot)
Data.pca <- prcomp(Data [, -1], center = TRUE, scale. = TRUE)
ggbioplot(Data.pca)
ggbioplot(Data.pca, labels=rownames(Data))
Data.experiment <- c(rep("Data ", ...), rep("Data ",...))
ggbioplot(Data.pca, ellipse=TRUE, groups= Data.experiment)

```

## Appendix D: Salting out DNA extraction protocol library

### Bacterial DNA Extraction Protocol of Swinburne University of Technology Teaching Laboratory

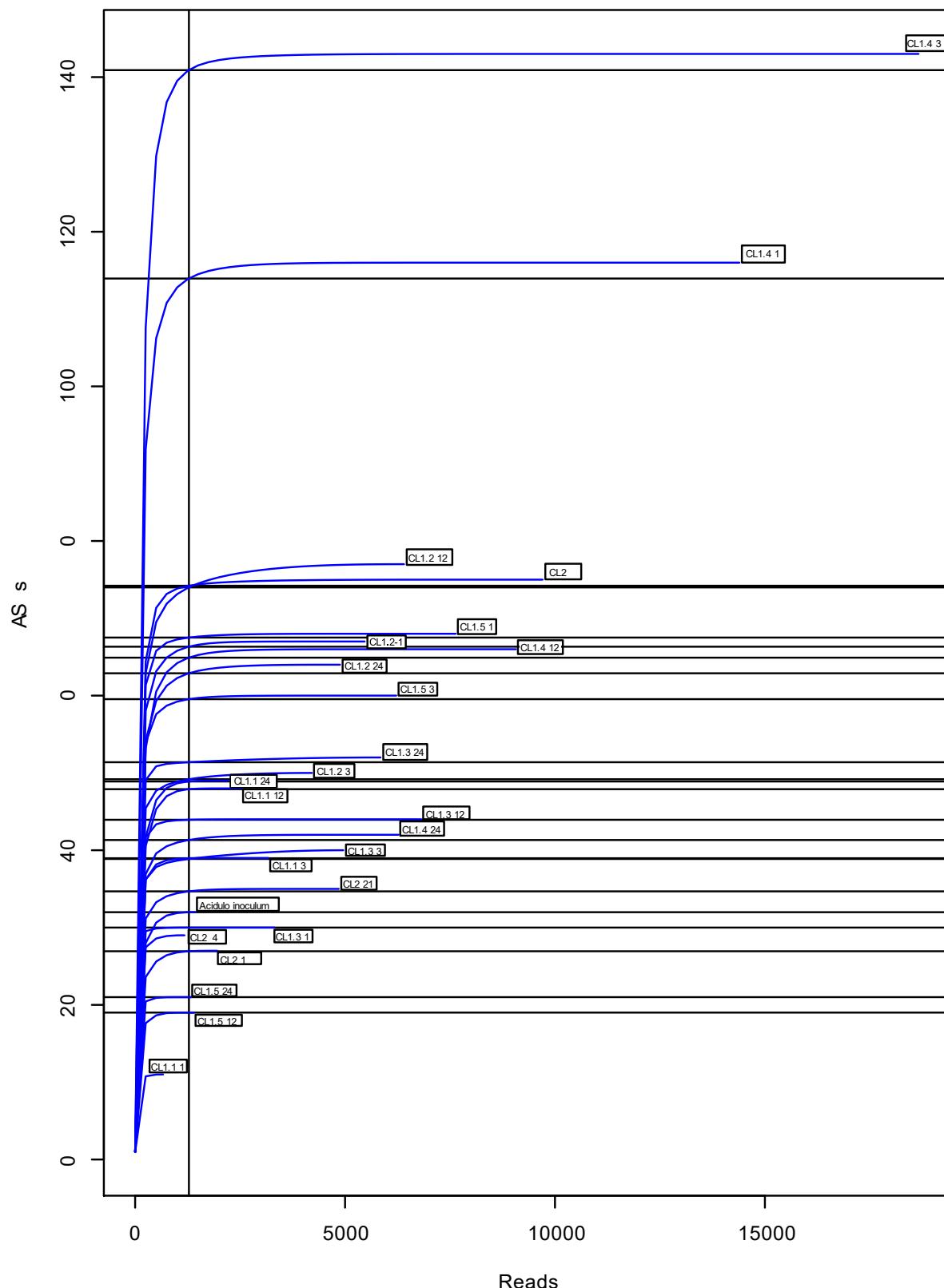
#### Material:

- Extraction buffer (0.1M Tris-HCl pH 7.5, 0.05 EDTA pH 8.0, 1.25% SDS)
- 10 mg/mL lysozyme (keep on ice)
- 6M ammonium acetate (stored in fridge)
- Iso-propanol
- 70% ethanol
- RNase (keep on ice)
- 80°C waterbath
- Bead tubes
- Microcentrifuge tubes

#### Method:

1. Add 1.8 mL overnight bacterial culture into a 2 mL collection tube or suspend a large loopful of bacterial colonies from an-agar plate in 1 mL sterile water. Centrifuge for 1 min at 14,100 g, discard supernatant.
2. Re-suspend pellet in 500 µL of extraction buffer and transfer to a bead tube
3. Add 20 µL Lysozyme (10 mg/mL) to each tube, seal and shake/vortex thoroughly. Incubate on ice for 15 min and then disrupt cells in the bead beater (FastPrep24) for 20 s at default speed 4.0 m/s. Incubate tubes at 80°C for 5 min.
4. Cool tubes on ice for 5 min before adding 250 µL cold 6M ammonium acetate. Shake/vortex vigorously to mix in the ammonium acetate and then leave to stand for 10 min on ice.
5. Centrifuge tubes for 5 minutes at 14,100 g to collect the precipitated proteins and bacteria
6. Pipette 600 µL of the supernatant into new microcentrifuge tubes containing 360 µL of iso-propanol. Mix thoroughly by inverting the tubes 4-5 times and allow the DNA to precipitate for 5 min at room temperature.
7. Pellet the DNA by centrifuging the tubes for 5 min at 14,100 g and then tip off the supernatant. Allow the remaining fluid to drain off the DNA pellet by inverting the tubes onto a piece of paper towel for 1 min.
8. Gently wash the pellet by adding 500 µL of 70% ethanol.
9. Centrifuge the tubes for 5 min at 14,100 g and again discard the supernatant. Leave the tube open and allow the DNA pellet to dry at room temperature for 10 mi.
10. Resuspend the pellet in 100 µL Milli-Q® and add 2 µL RNase (10 mg/mL).
11. Quantify DNA using spectrophotometry (e.g., Nanodrop) and check quality by agarose gel electrophoresis (mix 2 µL with 2 µL loading dye).

## Appendix E: Closed Loop - physical, chemical and microbial analysis



**Figure E1:** Rarefaction curve of observed ASVs vs Reads at 99% sequence similarity for CL1, CL2 and Acidulo™ samples.

**Table E1:** The five abundant contaminant ASVs identified by decontam across all CL1 and CL2 samples.

N	Kingdom	Phylum	Class	Order	Family	Genus	Relative abundance (%)
1	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		0.08
2	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	<i>Propionibacterium</i>	0.24
3	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	0.15
4	Bacteria						0.018
5	Bacteria						0.003
<b>Total</b>							<b>0.491</b>

**Table E2:** Generalized Linear Models analysis of differences in beta diversity based on the CL1 sub-experiments of organic waste treatment and time.

Analysis of Deviance Table

Model: manyglm(formula = comboMva ~ combo\$subexperiment \* combo\$time, family = "negative\_binomial")

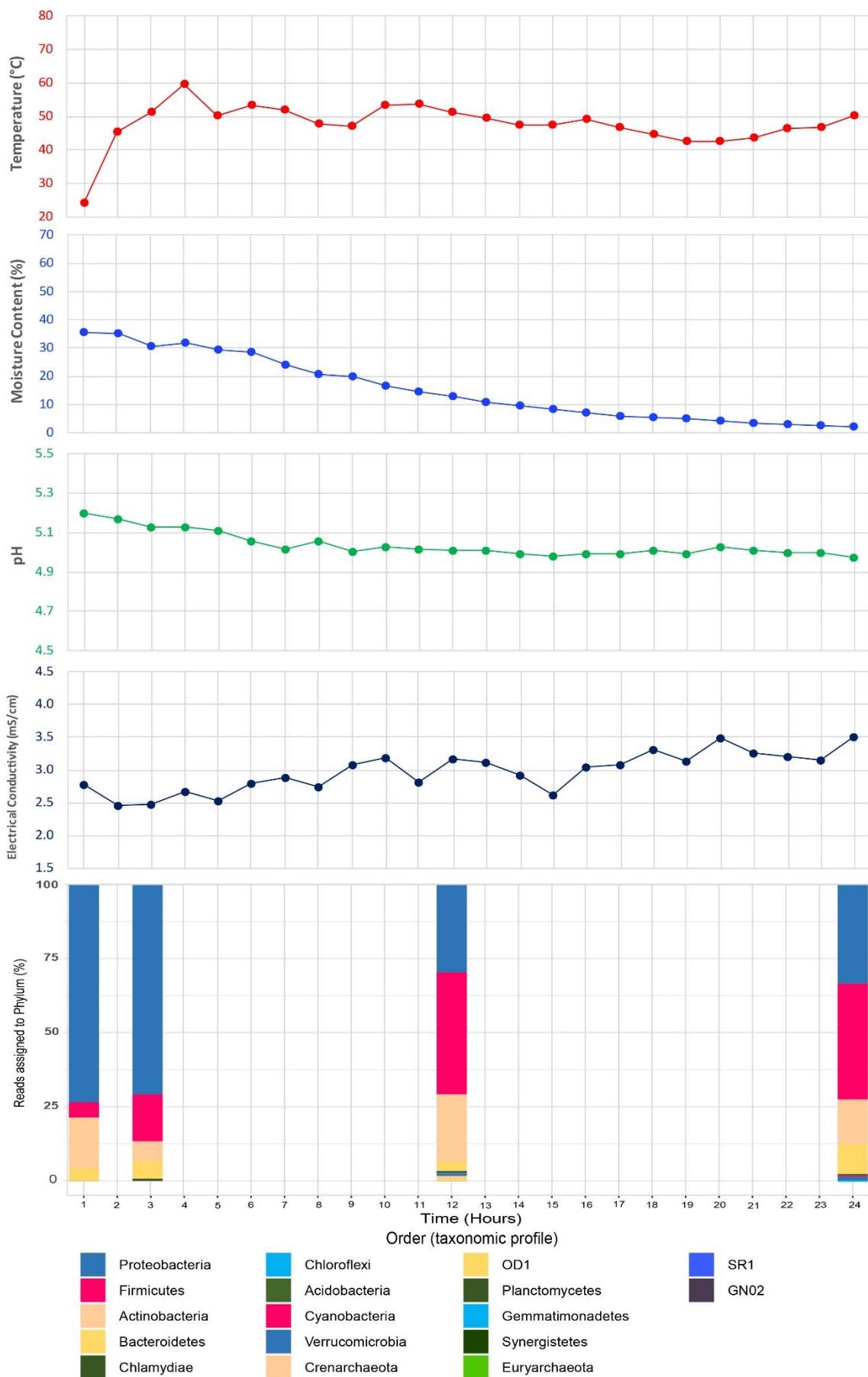
Multivariate test:		Res.Df	Df.diff	Dev	Pr(>Dev)
(Intercept)		19			
combo\$subexperiment		13	6	597.1	0.002 **
combo\$time		12	1	436.4	0.001 ***
combo\$subexperiment: combo\$time		10	5	394.7	0.126
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1					

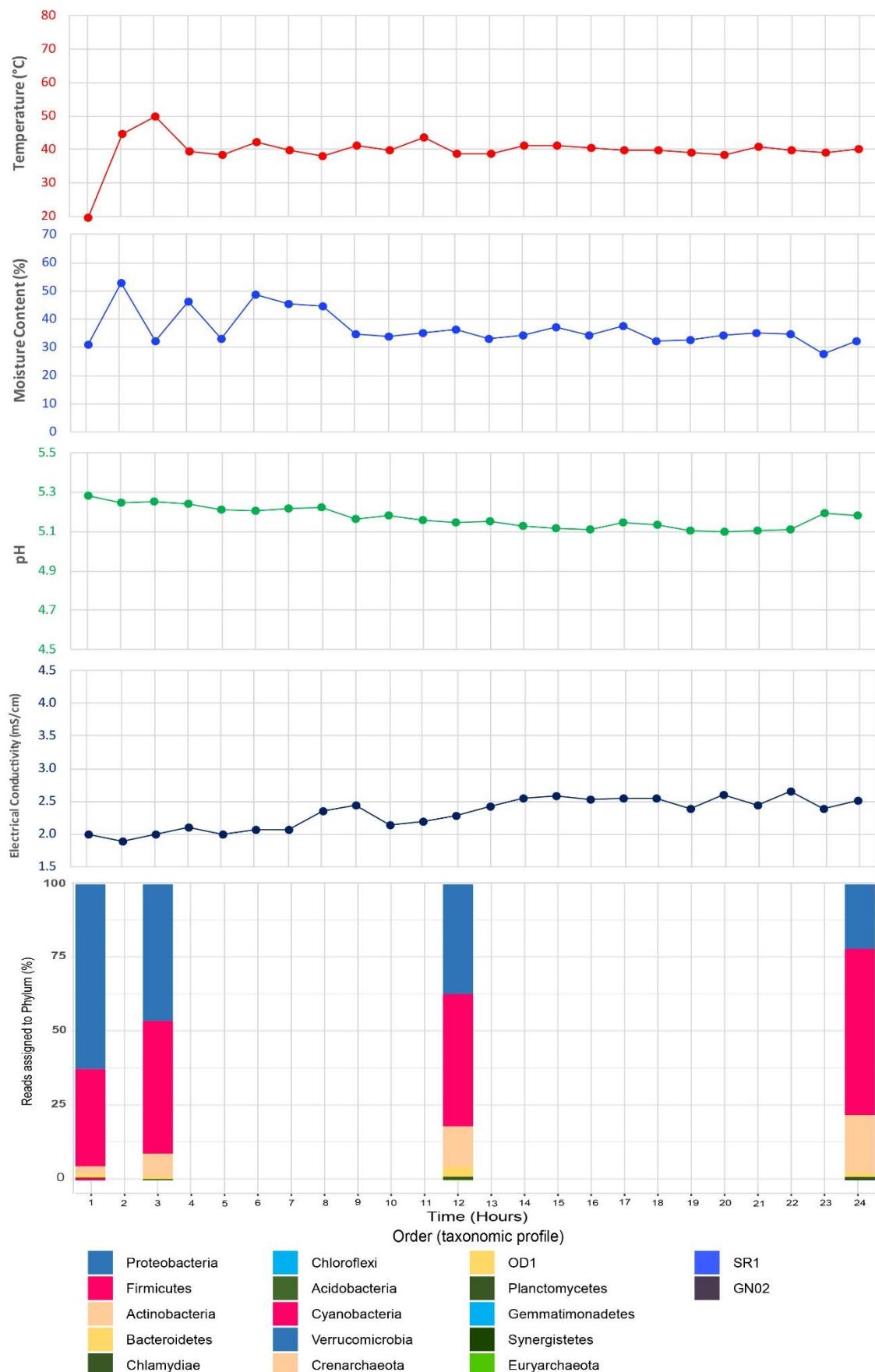
**Table E3:** Generalized Linear Models analysis of differences in beta diversity based on the operational time (CL1.2 vs CL2).

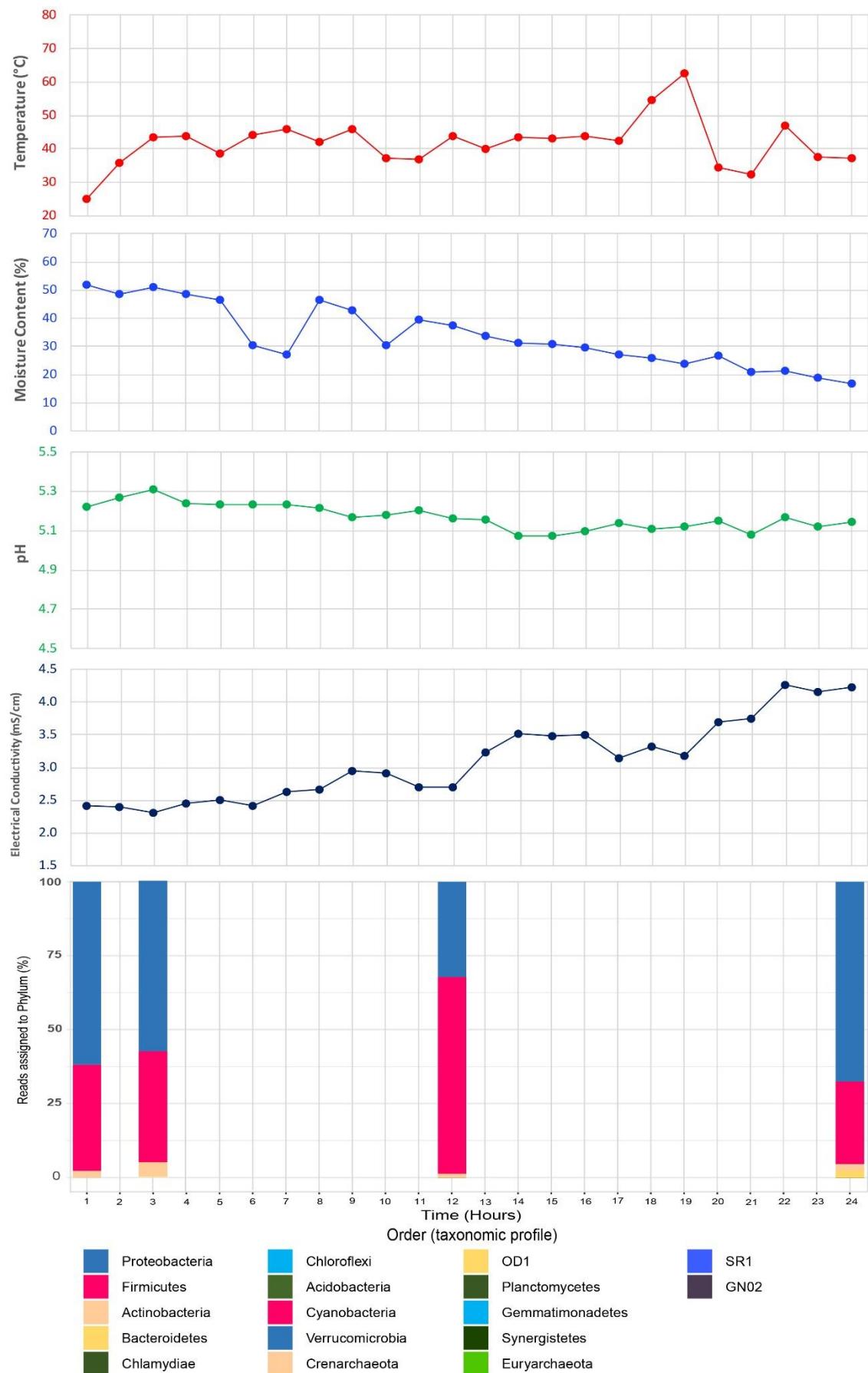
Analysis of Deviance Table

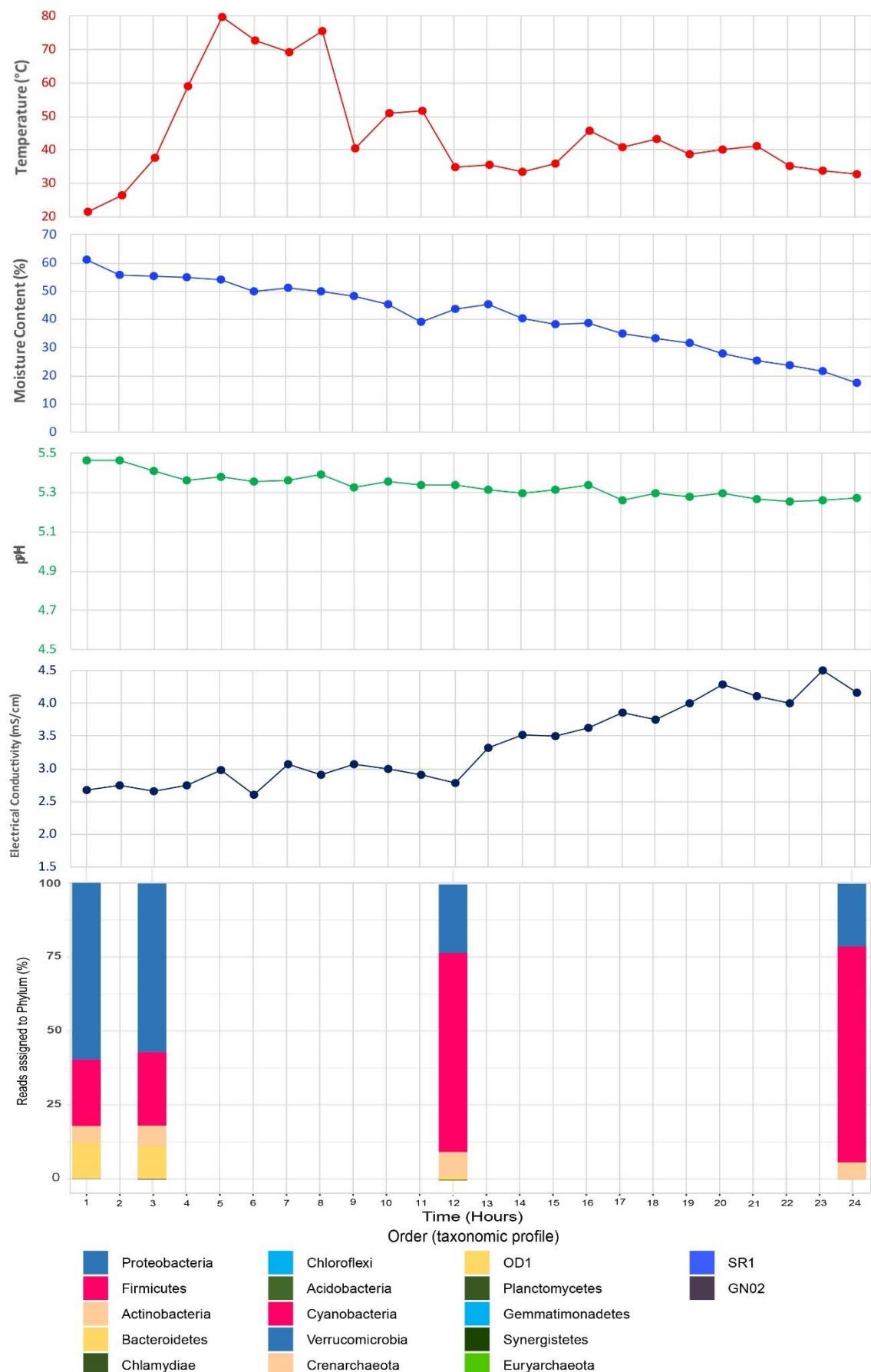
Model: manyglm(formula = comboMva ~ combo\$subexperiment \* combo\$time, family = "negative\_binomial")

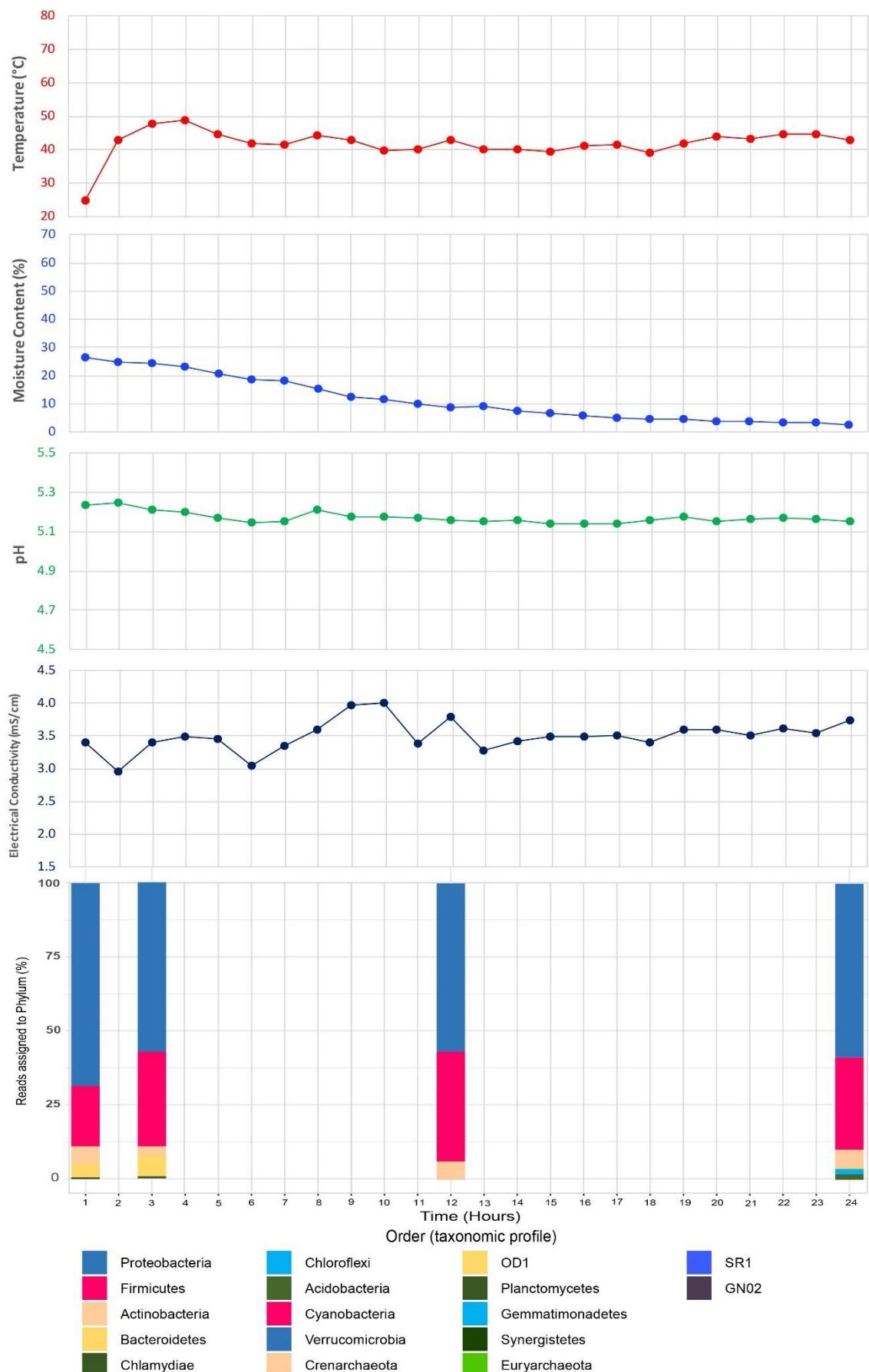
Multivariate test:		Res.Df	Df.diff	Dev	Pr(>Dev)
(Intercept)		8			
combo\$subexperiment		6	2	155.8	0.382 **
combo\$time		-2	8	1482.2	0.005 ***
combo\$subexperiment: combo\$time		0	1	0.0	0.693
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1					

**Figure E2:** Physical, chemical and microbial analysis - CL1.1

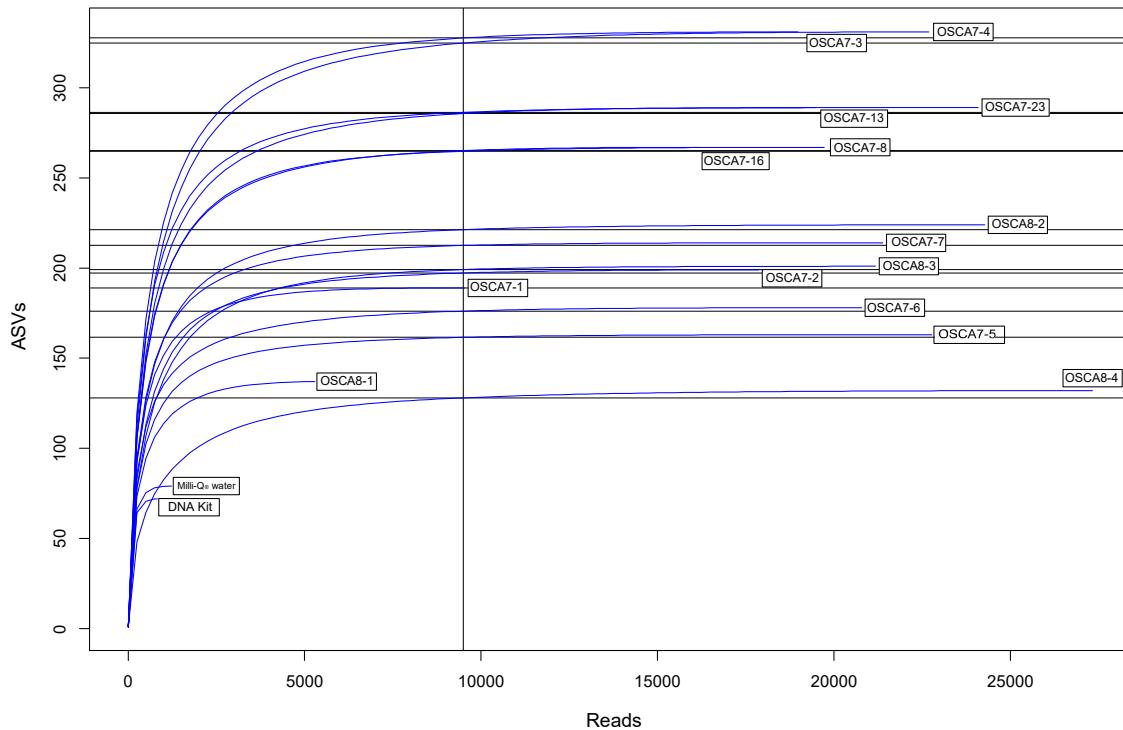
**Figure E3:** Physical, chemical and microbial analysis - CL1.2

**Figure E4:** Physical, chemical and microbial analysis - CL1.3

**Figure E5:** Physical, chemical and microbial analysis - CL1.4

**Figure E6:** Physical, chemical and microbial analysis - CL1.5

## Appendix F: OSCA - Physical, chemical and microbial analysis



**Figure F1:** Rarefaction curve of observed ASVs vs Reads at 99% sequence similarity for all OSCA samples.

**Table F1:** Generalized Linear Models analysis of differences in beta diversity based on the rotation frequency (OSCA7) and time.

Analysis of Deviance Table

Model: manyglm(formula = comboMva ~ combo\$rotation-mode * combo\$time, family = "negative_binomial")				
Multivariate test:				
	Res.Df	Df.diff	Dev	Pr(>Dev)
(Intercept)	10			
combo\$ rotation-mode	9	1	0.0	0.001 ***
combo\$time	9	1	480.6	0.021 *

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 '' 1

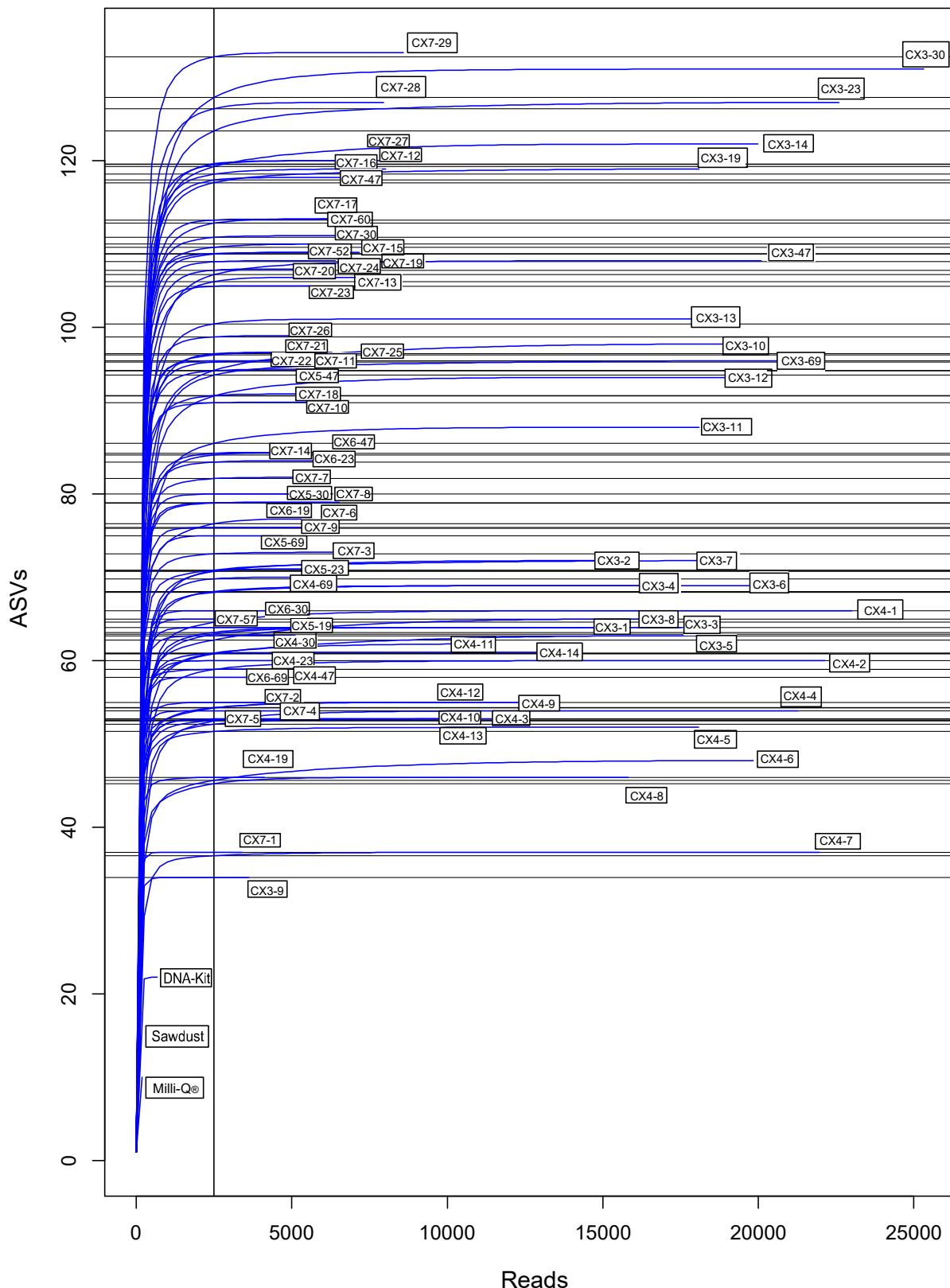
**Table F2:** Generalized Linear Models analysis of differences in beta diversity based on the C:N ratio adjustment (OSCA7 vs OSCA8 ) and time (first four days).

Analysis of Deviance Table

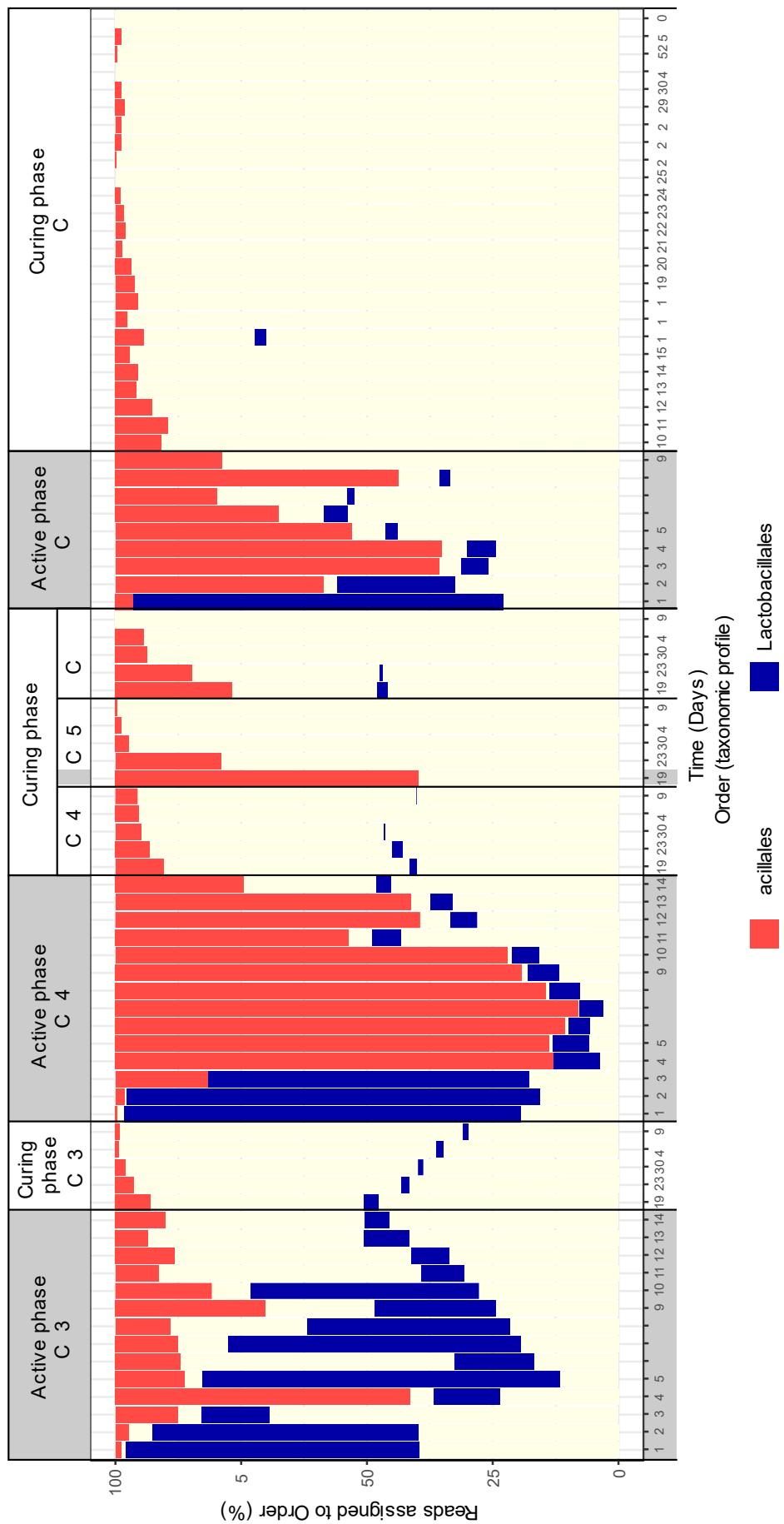
Model: manyglm(formula = comboMva ~ combo\$experiment * combo\$time, family = "negative_binomial")				
Multivariate test:				
	Res.Df	Df.diff	Dev	Pr(>Dev)
(Intercept)	7			
combo\$ experiment	6	1	0.0	0.004 **
combo\$time	6	1	424.7	0.157

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 '' 1

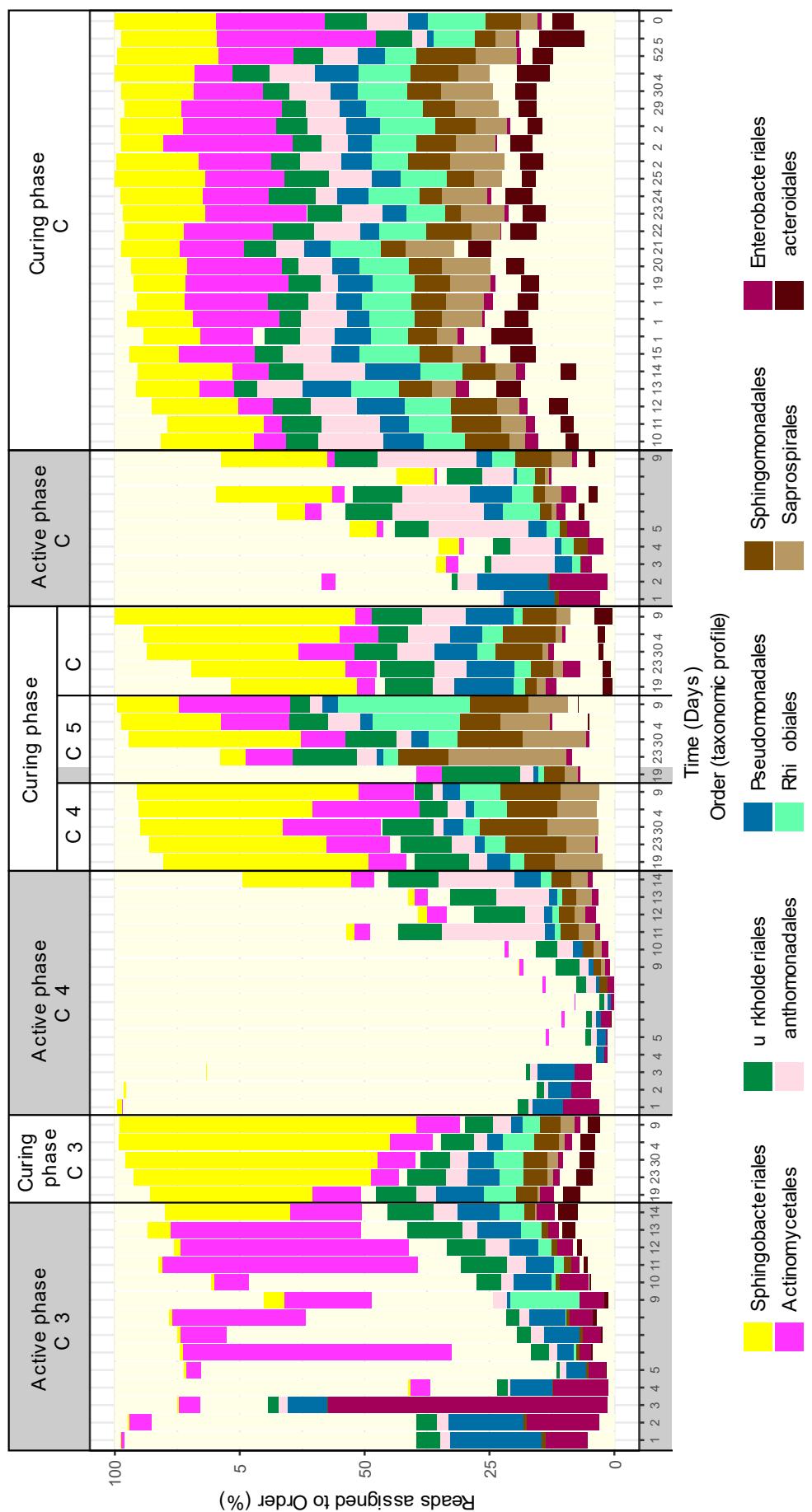
## Appendix G: Cylibox - Physical, chemical and microbial analysis



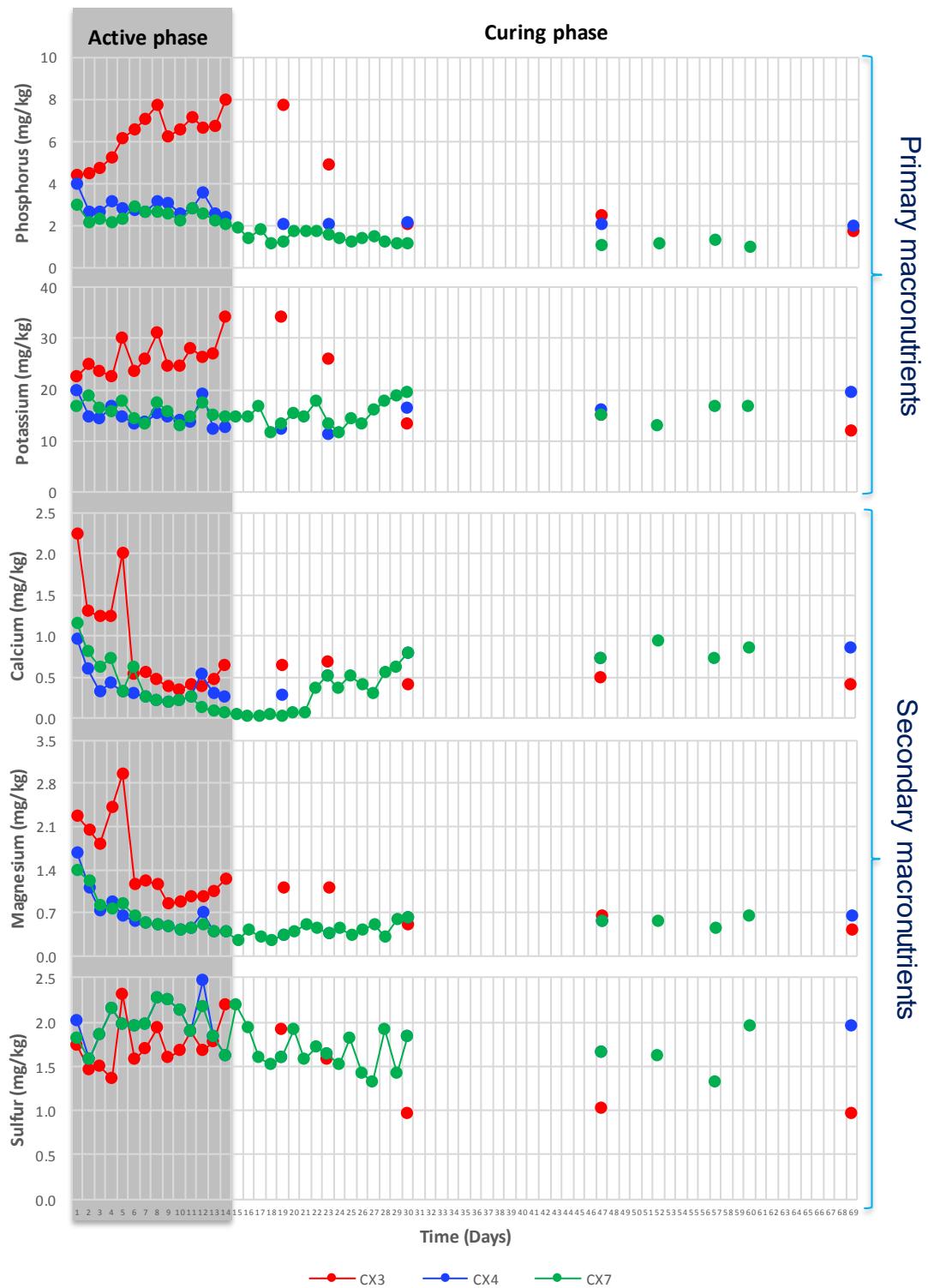
**Figure G1:** Rarefaction curve of observed ASVs vs Reads at 99% sequence similarity for all CX samples.

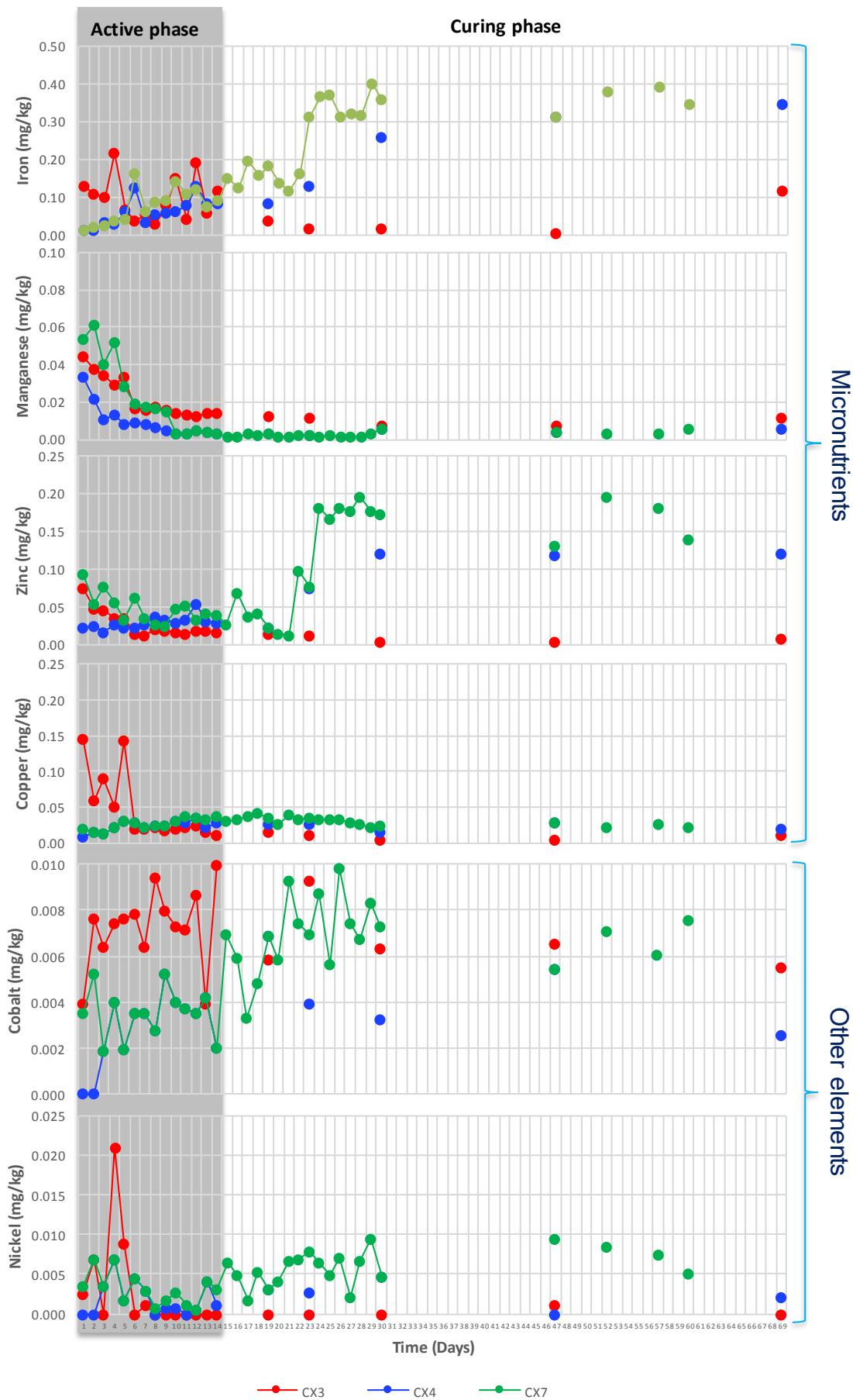


**Figure G2:** Two most abundant bacterial Order during active phase of CX composting.



**Figure G3:** Ten most abundant bacterial Order during curing phase of CX composting.





**Figure G5:** Micronutrients and other elements (water soluble elements) during CX3, CX4 and CX7 composting.

**Table G1:** The three-abundant contaminant ASVs identified by decontam across all CX samples.

N	Phylum	Class	Order	Family	Genus	Species	Relative abundance (%)
1	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	<i>coagulans</i>	0.028
2	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	<i>Kerstertia</i>	<i>gyiorum</i>	0.067
3	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Pseudoxanthomonas</i>	<i>byssovorax</i>	0.018
<b>Total</b>							<b>0.113</b>

**Table G2:** Generalized Linear Models analysis of differences in beta diversity based on the C:N ratio of initial input (CX3 vs CX4) and time.

Analysis of Deviance Table

Model: manyglm(formula = comboMva ~ combo\$experiment \* combo\$time,family = "negative\_binomial")

Multivariate test:		Res.Df	Df.diff	Dev	Pr(>Dev)
(Intercept)		37			
combo\$ experiment		35	2	318	0.001 ***
combo\$time		0	37	4192	0.201

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 '' 1

**Table G3:** Generalized Linear Models analysis of differences in beta diversity based on the mixing and non-mixing (CX5 vs CX6) and time.

Analysis of Deviance Table

Model: manyglm(formula = comboMva ~ combo\$experiment \* combo\$time,family = "negative\_binomial")

Multivariate test:		Res.Df	Df.diff	Dev	Pr(>Dev)
(Intercept)		9			
combo\$ experiment		8	1	0	0.019 *
combo\$time		0	9	1858	0.061

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 '' 1

**Table G4:** Generalized Linear Models analysis of differences in beta diversity based on the composting phases (CX7 active phase vs CX7 curing phase) and time.

Analysis of Deviance Table

Model: manyglm(formula = comboMva ~ combo\$experiment \* combo\$time,family = "negative\_binomial")

Multivariate test:		Res.Df	Df.diff	Dev	Pr(>Dev)
(Intercept)		33			
combo\$ experiment		31	2	84	0.008 **
combo\$time		0	33	3708	0.194

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 '' 1

# **List of publications**

## ***List of abstracts, posters and oral presentations***

JAIMES-CASTILLO, A., BLACKALL, L., ELDRIDGE, D., ZAFERANLOO, B. & WEATHERLEY, A. 2019. Microbial diversity during composting food waste in the novel in-vessel composter "Cylibox". Oral presentation. Microbial Ecology-Environmental Microbiology (MEEM) in Victoria, 16 May 2019 La Trobe University city campus, 360 Collins Street, Melbourne, Australia.

<http://victoria.theasm.org.au/assets/2019-05-16-Microbial-Ecology-Environmental-Microbiology-MEEM.pdf>

JAIMES-CASTILLO, A., BLACKALL, L., ELDRIDGE, D., ZAFERANLOO, B. & WEATHERLEY, A. 2018. The microbial ecology of urban organic waste treatment (compost). Oral presentation. Urban Composting Research Symposium, 27 August 2018 Hawthorn Arts Centre, Melbourne, Australia.

[http://www.lowcarbonlivingcrc.com.au/sites/all/files/publications\\_file\\_attachments/urban\\_composting\\_roadshow\\_2018\\_outcomes\\_report.pdf](http://www.lowcarbonlivingcrc.com.au/sites/all/files/publications_file_attachments/urban_composting_roadshow_2018_outcomes_report.pdf)

JAIMES-CASTILLO, A., BLACKALL, L., ELDRIDGE, D., ZAFERANLOO, B. & WEATHERLEY, A. 2018. A new small-scale composter for urban organic solid waste treatment. Poster presentation. International Society for Microbial Ecology (ISME), 12 to 17 August 2018 Leipzig, Germany.

<https://next.morressier.com/article/1449--new-smallscale-composter-urban-organic-solid-waste-treatment/5b5199c3b1b87b000ecf00c6?>

JAIMES-CASTILLO, A., BLACKALL, L., ELDRIDGE, D., ZAFERANLOO, B. & WEATHERLEY, A. 2017. RP: 2019: The microbial ecology of urban organic waste treatment (compost). Poster presentation. CRC for Low Carbon Living's Annual Forum, 22 to 23 November 2017 Melbourne, Australia.

[http://www.lowcarbonlivingcrc.com.au/sites/all/files/publications\\_file\\_attachments/rp2019 - swin - alex jaimes castillo - updated.pdf](http://www.lowcarbonlivingcrc.com.au/sites/all/files/publications_file_attachments/rp2019 - swin - alex jaimes castillo - updated.pdf)

JAIMES-CASTILLO, A., BLACKALL, L., ELDRIDGE, D., ZAFERANLOO, B. & WEATHERLEY, A. 2017. The microbial ecology of urban organic waste treatment (compost). Australian Microbial Ecology Conference - AusME 2017, 13 to 15 February 2017 Melbourne, Australia.

<http://ausme-2017.p.asnevents.com.au/days/2017-02-14/abstract/42555>

JAIMES-CASTILLO, A., BLACKALL, L. & ELDRIDGE, D. 2016. RP 2019: The microbial ecology of urban organic waste treatment (compost). Poster presentation CRC for Low Carbon Living's Annual Forum, 15 to 16 November 2016 Sydney, Australia.

[http://www.lowcarbonlivingcrc.com.au/sites/all/files/publications\\_file\\_attachments/rp2019 - swinburne - alex castillo.pdf](http://www.lowcarbonlivingcrc.com.au/sites/all/files/publications_file_attachments/rp2019 - swinburne - alex castillo.pdf)

BLACKALL, L., JAIMES-CASTILLO, A. & ELDRIDGE, D. 2016. The microbial ecology of urban organic waste treatment (compost). Oral presentation. IBS 2016, the 17th International Biotechnology Symposium and Exhibition, 24 to 27 October 2016 Melbourne Convention Centre, Australia.

***List of videos***

*Towards Zero Carbon - The Compost Project CRC LCL – RP2019 project*, 2019.

Directed by DART, C. Melbourne, Australia.

<https://www.youtube.com/watch?v=hvOvwDB4kx4&t=8>

*The microbial ecology of urban organic waste treatment (compost)*, 2019. Directed by

GANLY, J. Melbourne, Australia.

[https://www.thinkable.org/submission\\_entries/Jqo4oJ3r?fbclid=IwAR2gTes5z5Kwa6cj3\\_zNb31EBw3DcxHNb2VX\\_UrDj9SRhrQQ6jEkgYr5u7g](https://www.thinkable.org/submission_entries/Jqo4oJ3r?fbclid=IwAR2gTes5z5Kwa6cj3_zNb31EBw3DcxHNb2VX_UrDj9SRhrQQ6jEkgYr5u7g)

***Part of annual reports***

CRC-FOR-LOW-CARBON-LIVING-LTD 2016. Annual report highlights 2015–16

(Page 20). In: CRC-FOR-LOW-CARBON-LIVING-LTD (ed.). Tyree Energy Technologies Building UNSW Sydney NSW 2052 Australia.

[http://www.lowcarbonlivingcrc.com.au/sites/all/files/publications\\_file\\_attachments/crccl\\_2016\\_annual\\_highlights\\_report\\_final.pdf](http://www.lowcarbonlivingcrc.com.au/sites/all/files/publications_file_attachments/crccl_2016_annual_highlights_report_final.pdf)

SWINBURNE-UNIVERSITY-OF-TECHNOLOGY 2016. Annual report 2016. Case

study 1: Brewing a sustainable future (Page 32). Melbourne Australia: Swinburne-University-of-Technology.

<http://www.swinburne.edu.au/media/swinburneeduau/about-swinburne/docs/pdfs/swinburne-annual-report-2016.pdf>