Microbial population dynamics during windrow composting of broiler litter

by

Pieter Hermanus Myburgh
20266677

Submitted in fulfilment of the requirements for the degree
MAGISTER OF SCIENCE in
ENVIRONMENTAL SCIENCE

School for Biological Sciences
North-West University: Potchefstroom Campus
Potchefstroom, South Africa

Supervisor: Prof. C.C. Bezuidenhout
Co-supervisor: Dr. J.J. Bezuidenhout

November 2012

ABSTRACT

South Africa produces an average of 154 million broilers (*Gallus gallus domesticus*) annually, arising to an estimated 886 million kg of broiler litter. The largest population of broilers are reared in the North West province. Various applications for this largely underexploited resource have been published, including forming part of ruminant diets and direct land application. This however has several disadvantages, as it could lead to eutrophication of fresh water sources and faecal contamination of produce. Windrow composting of broiler litter has previously been studied, and found to deliver a stabilized product free of pathogenic and phytotoxic effects, therefore making it an excellent soil conditioner. This study aimed to characterize the microbial community present during the windrow composting of broiler litter. Four different formulations of substrate were tested; these being broiler litter (Windrow 1), Windrow 1 with previously composted material (Windrow 2), Windrow 2 amended with woodchips (Windrow 3) and Windrow 3 with an additional 12.5% (w/w) zeolite (Windrow 4). Broiler litter used in this experiment had a C:N ration of 10.3:1, whilst the blue gum woodchips added as an amendment had a C:N ratio of 172:1. Windrow and environmental temperatures were monitored on a regular basis. Windrow 1 largely mimicked environmental temperature, and could not sustain a true thermophilic phase during the experimental period. Windrow 2 did achieve a short lived thermophilic phase during the first few days of the composting process, however could not sustain its temperature over the whole period. In contrast Windrows 3 and 4 sustained temperature above 40°C for the largest part of the experimental period, regardless of environmental temperature. No significant difference ($p < 0.05$) could be observed between average moisture levels in the 4 windrows. Internal moisture profiles were however found to differ significantly, especially on the surface of the windrows. Moisture was also lost faster in Windrows 1 and 2 compared to Windrows 3 and 4. Chemical analysis showed differences between the four windrows constructed. A higher amount of nitrogen was lost in Windrows 1 and 2, mostly due to a sub-optimal initial C:N ratio in these windrows. Windrow 2 contained the highest values for plant nutrients P, Mg, Ca, Mn and Cu. Microbial population dynamics were observed using PCR-DGGE of samples collected throughout the composting of various treatments. Various commercial DNA extraction kits where tested in a previous study for their ability to remove PCR inhibitory substances, such as humic acids. The Machery-Nagel Soil DNA isolation kit was used as it gave amplifiable DNA from all samples. Samples were amplified using a nested PCR approach primer sets 27f-1492r \ 341f(GC)-907r and EF3-EF4 \ EF4(GC)-fung5 (where “GC” indicates a GC-rich clamp) for prokaryotic and eukaryotic species respectively. The PCR products were analyzed by agarose gel electrophoresis, and equal amounts of product were subjected to denaturing gradient gel electrophoresis (DGGE). Bands obtained from these polyacrylamide gels where then re-amplified using the same secondary primer sets (without the GC-clamp), and sequenced. A total of 454 prokaryotic bands in 55

distinct rf-positions were observed. Seven distinct rf-positions were observed in eukaryotic DGGE profiles. Prokaryotic profiles were aligned and the microbial diversity was analyzed by means of Ward's clustering algorithm and the dice coefficient of similarity, as well as Simpson's reciprocal, Shannon-Weaver and Species richness indices. Canonical correspondence analysis (CCA) was also performed on both the banding patterns as well as the bands present, together with the physico-chemical results obtained. Several bands were successfully identified as being influenced by physico-chemical parameters. Temperature, C:N ratio, ash, and moisture showed a correlation on CCA bi-plots. Sixteen bands were sequence identified. These sequences were compared to two different databases. The 16S rRNA database for Bacteria and Archaea gave identities to genus level, however maximum identity scores were low. Of the 16 sequences, 12 sequences were identified as uncultured bacteria when compared to the nucleotide collection database. In comparing the sequences with sequences collected in the nucleotide collection database, 12 were either first described in composts and soils, or animal manures. Results indicated mostly members of the genus *Bacillus* and *Paenibacillus.* The addition of a carbon source greatly affected the microbial metabolism, resulting in a thermophilic phase being achieved in amended windrows. As no thermophilic phase was observed in windrows that were not amended with woodchips, it could be concluded that the use of a carbon source is irremissible when composting broiler litter. A zeolite amendment is also strongly advised, as this further increased temperatures within the windrow.

Keywords: broiler litter, windrow, compost, C:N ratio, thermophilic phase, zeolite, microbial diversity, microbial population dynamics

“Earth knows no desolation.

She smells regeneration in the moist breath of decay.”

George Meredith

~ooOoo~

DEDICATION:

I dedicate my work to He who Was, who Is and always Will Be, the Creator of this small, perfect, self-sustainable planet that we call our temporary home.

Aan U kom toe al die eer en die heerlikheid,
tot in alle ewigheid.
Amen

~ooOoo~

ACKNOWLEDGEMENTS

The author would like to thank the following persons and companies:

Prof C.C. Bezuidenhout for his support and encouragement (and sometimes his scolding) during the course of this project. Also for the countless hours he spent on “The Famous Five”.

Dr J.J. Bezuidenhout for his help with the statistical analysis, and support.

To Charné Myburgh, for being my friend, my companion, and my wife. I love you dearly my Skapie.

My parents, Francois and Annette, for always being supportive and understanding when weekends are spent on work rather than family. Your loving support and guidance throughout my life, I could never forget.

My brother, Hendri, for his help and support over a lifetime.

Ouma Annetjie, for climbing countless steps to see me work, and inspiring curiosity through her youthful soul.

My in-laws, Dries and Leoni van Coller, for countless prayers and motivational speeches

National Research Foundation - Scarce Skills for a bursary received. Without their financial support I would not have been able to complete this project.

Alewyn Carstens for convincing me that writing is important, and for always being willing to have a braai, and discuss the future.

Hermoine Venter and Karen Jordaan, my molecular buddies, for filling up the tip boxes, for discussing methods and madness, and creating a whole lot of chapters in the soon to be published book: “101 Things that could go wrong in a molecular lab (and how to act as if it never happened)”

Galltec Pty. Ltd. for funding aspects of this project.

All the countless friends I have made during my tenure at Microbiology. I would have thanked each of you, but if I keep this dissertation under 100 pages, it’s cheaper to bind. Therefore, you are thanked.

PREFACE

Any opinion, findings, and conclusions or recommendations expressed in this material are those of the author and therefore the NRF does not accept any liability in regards thereto.

DECLARATION

The experimental work conducted and discussed in this dissertation was carried out at the School of Biological Sciences, Microbiology, North-West University, Potchefstroom Campus. This study was conducted under the supervision of Prof. C. C. Bezuidenhout and Dr. J.J. Bezuidenhout.

The study represents original work undertaken by the author and has not been previously submitted for degree purpose to any other university. Appropriate acknowledgements have been made in text where the use of work conducted by other researchers has been included.

_____

Pieter H. Myburgh

November 2012

TABLE OF CONTENTS

ABSTRACT i
DEDICATION: iv
ACKNOWLEDGEMENTS v
PREFACE vi
DECLARATION vii
LIST OF FIGURES xi
LIST OF TABLES xiii
CHAPTER 1 1
LITERATURE OVERVIEW 1
1.1 General Overview 1
1.2 Poultry Production And Current Management Systems 1
1.3 Broiler Litter Composition 3
1.4 Uses Of Broiler Litter 4
1.4.1 Ruminant Feed 5
1.4.2 Biofuel Source 6
1.4.3 Direct Land Application 7
1.5 Composting 9
1.5.1 Importance Of Composting 9
1.5.2. Commercial Microbial Composting Techniques 10
1.5.3. Optimal Physico-Chemical And Microbiological Parameters 12
1.5.3.1 C:N Ratio: 12
1.5.3.2 Moisture: 13
1.5.3.3 Temperature 13
1.5.3.4 Microbial Populations In Composting 15
1.5.3.5 Zeolite Amendment 16
1.6 Compost Quality Standards 17
1.7 Microbiological Identification Methods Used In Studying Compost 19
1.8 Summary of Literature 22
1.9 Aims and Objectives: 23
CHAPTER 2 24
MATERIALS AND METHODS 24
2.1 Commissioning Of Study 24
2.2 Study Site 24
2.3 Windrow Composition 24
2.4 Windrow Monitoring and Composting 25
2.5 Environmental Monitoring 25
2.6 Chemical Monitoring 25
2.7 Sample Collection 25
2.8 Genomic DNA Isolation 26

2.8.1 Genomic DNA Isolation 26
2.8.2 Agarose Gel Electrophoresis Of Genomic DNA 26
2.9 DNA Amplification 27
2.9.1 16S rRNA PCR amplification 27
2.9.2 18S rRNA PCR amplification 28
2.9.3 Agarose gel electrophoresis of PCR products 29
2.10 Denaturing Gradient Gel Electrophoresis (DGGE) 29
2.10.1 16S and 18S rDNA DGGE analysis 29
2.10.2 Staining and visualizing of DGGE profiles 29
2.10.3 Excision Of Bands 30
2.11 PCR Amplification Of DNA From Excised Bands 30
2.12 Sequencing Of Excised Band PCR Product 30
2.12.1 PCR Cleanup 30
2.12.2 Sequencing Reaction 30
2.12.3 Sequencing And Sequence Analysis 31
2.13 Statistical And Diversity Analysis 31
CHAPTER 3: 33
RESULTS 33
3.1 Introduction Of Results 33
3.2 Environmental And Windrow Temperature And Moisture Data 34
3.3 Chemical Analysis 42
3.4 DNA Isolation 45
3.5 DNA Amplification 46
3.5.1 16S rDNA Amplification 46
3.5.2 18S rDNA Amplification 47
3.6 Denaturing Gradient Gel Electrophoresis (DGGE) 48
3.6.1 16S rDNA profiles 48
3.6.2 18S rDNA profiles 48
3.7 Microbial Community Dynamics 50
3.8 Influence Of Average Temperature And Moisture On Microbial Dynamics 57
3.9 Prokaryotic Sequencing Results 60
3.10 Summary Of Results 66
CHAPTER 4 67
DISCUSSION 67
4.1 Introduction 67
4.2 Physico-Chemical And Microbiological Parameters That Influence Composting 67
4.2.1 Composting Broiler Litter (Windrow 1) 68
4.2.2 Using An Inoculum In Broiler Litter Composting (Windrow 2) 69
4.2.3 Chemical Analysis Of Broiler Litter Composting 71
4.2.4 Adjusting The C:N Ratio (Windrow 3) 73

4.2.4 Adjusting The C:N Ratio And Amending With Zeolite (Windrow 4) 75
4.2.5 Chemical Analysis Of Co-Composting Broiler Litter 76
4.2.6 Prokaryotic Diversity Compared To The 16S rRNA Gene Database 77
4.2.7 Prokaryotic Diversity Compared To The Nucleotide Collection Database 81
4.2.8 Eukaryotic Diversity During The Thermophilic Phase Of Co-Composting Broiler Litter 81
CHAPTER 5 83
CONCLUSION AND RECOMMENDATIONS 83
5.1 Conclusions 83
5.1.1 Compiling Four Different Windrows For Evaluation 83
5.1.2 Evaluation Of The Four Different Formulated Windrows On Physical Attributes 84
5.1.3 Characterizing Of The Microbial Community Succession By Means Of PCR-DGGE Analysis 84
5.1.4 Evaluation Of The Microbial Population Dynamics In Windrows 85
5.2 Recommendations 86
REFERENCES 88
APPENDICES: 109
APPENDIX A – TECHNICAL DATA SHEET FOR TYPICAL CHEMICAL COMPOSITION OF MINED ZEOLITE 109
APPENDIX B - NITROGEN CONTRIBUTIONS OF DIFFERENT SUBSTRATES DURING THE COMPOSTING PROCESS. 110

LIST OF FIGURES

Figure 1.1: Broiler production in South Africa for the first decade of this millennium (SAPA, 2010).....**1**

Figure 1.2: Distribution of layer and broiler birds across South Africa. (SAPA, 2011).....**2**

Figure 1.3: Theoretical temperature profiles during the composting process (Parr *et al.,* 1994).....**14**

Figure 2.1: (a) Schematic representation of windrows and sampling point. (b) indicates the three points used for sampling. **25**

Figure 3.1: Average windrow temperature for the first two days.....**34**

Figure 3.2: Average windrow temperature during the composting process **36**

Figure 3.3: Differences in average windrow temperature **37**

Figure 3.4: Average temperature during the composting period at different distances from the windrow surface.....**38**

Figure 3.5: Average moisture % during the composting period **39**

Figure 3.6: Average moisture content % during the composting period at different distances from the windrow surface. **41**

Figure 3.7: A 1 % (w/v) ethidium bromide stained agarose gel containing genomic DNA isolated using a Machery-Nagel NucleoSpin Soil kit. **45**

Figure 3.8: An inverted photograph of a 1.5% (w/v) agarose gel stained with ethidium bromide containing product from the first PCR.....**46**

Figure 3.9: An inverted photograph of a 1.5% (w/v) agarose gel stained with ethidium bromide with the product from the nested PCR **46**

Figure 3.10: An inverted composite 1.5% (w/v) agarose gel, stained with ethidium bromide, of samples from Windrow 4.....**47**

Figure 3.11: Inverted 1.5% (w/v) agarose gel image of nested eukaryotic samples from Windrow 4 stained with ethidium bromide.....**47**

Figure 3.12: Negative composite image of DGGE profiles obtained by matching intergel reference lane makers in Photoshop CS3. .....**49**

Figure 3.13: A negative image of an ethidium bromide stained linear denaturing polyacrylamide:bisacrylamide (37.5:1) (30-50% denaturing) gel of eukaryotic PCR samples obtained from Windrow 4.....**48**

Figure 3.14: Dendrogram obtained from composite DGGE profiles using Ward's clustering algorithm and the Dice coefficient of similarity. ..**51**

Figure 3.15: Species Richness as obtained from bands present in DGGE lanes for all four windrows.....**52**

Figure 3.16: Simpson's Reciprocal Index (SRI) values for DGGE profiles from Windrows 1–4.....**53**

Figure 3.17: Shannon-Weaver indices for DGGE profiles for Windrows 1 – 4.....**54**

Figure 3.18: Canonical Correspondence Analysis (CCA) performed on the (a) Bands and (b) DGGE lane patterns obtained from the four windrows studied, physico-chemical analysis results, moisture and temperature data **55**

Figure 3.19: Species richness, Shannon-Weaver and Simpson's Reciprocal indices for eukaryotic species. **57**

Figure 3.20: Graphical summary of temperature, moisture and prokaryotic diversity results obtained in the study.....**58**

Figure 3.21: Graphical summary of temperature, moisture and eukaryotic diversity results.....**59**

LIST OF TABLES

Table 1.1: Approximation of the composition of South African broiler litter **4**

Table 1.2: Comparison of different composting techniques **11**

Table 1.3: European Compost Network Quality Assurance Scheme **17**

Table 2.1: Windrow composition **24**

Table 2.2: Lysis conditions used for compost samples subjected to DNA extraction with the MN – NucleoSpin Soil Kit **26**

Table 2.3: List of primer sequences used for 16s rRNA amplification.....**27**

Table 2.4: List of primer sequences used for 18s rRNA amplification.....**28**

Table 3.1: Comparative table of Student t-test values for average temperature at various depths within the four different windrows **40**

Table 3.2: Comparative table of windrow moisture data.....**41**

Table 3.3: Comparative table of Student t-test values for average moisture content at various depths within the four different windrows **43**

Table 3.4: Primary and secondary plant macronutrients at the end of the composting process **42**

Table 3.5: Plant micronutrients at the end of the composting process.....**44**

Table 3.6: Ash, pH, C:N and moisture content at the end of the composting process **44**

Table 3.7: Sequence identities, statistical probability, sources and presence in current study's windrows. **62-65**

CHAPTER 1
LITERATURE OVERVIEW

1.1 General Overview

Agriculture is the economic backbone of any country, not only ensuring food security but also creating jobs, and in turn establishing a micro economy in rural areas. In South Africa primary agriculture has a share of about 7 % of the formal employment sector, and contributes 3 % of the gross domestic product (GDP) (GCIS, 2010). Animal rearing is the main agricultural activity in South Africa, contributing 42 % of the total farming activities in the census conducted in 2007 (Statistics South Africa, 2009).

In the first decade of this millennium, the total South African poultry livestock had increased 40.6 %, from 109 650 000 to 154 136 000 birds per day (SAPA, 2010). Broilers (chicken, *Gallus gallus domesticus*) contributes the largest proportion of these birds, with more than 60 % of the total birds being reared for slaughtering purposes (SAPA, 2010).

1.2 Poultry Production And Current Management Systems

Poultry products are the largest source of protein to the South African consumer (SAPA, 2012a) and this may be due to the fact that it is the cheapest source of animal derived protein (FNB, 2012). The demand has steadily increased in recent years, with the South African Poultry Association estimating a total of 931 443 000 broilers being produced in 2009 and further increasing to more than a milliard birds being placed per annum in 2010 (SAPA, 2010). Figure 1.1 shows the steady growth in broiler production in South Africa in the first decade of this millennium.

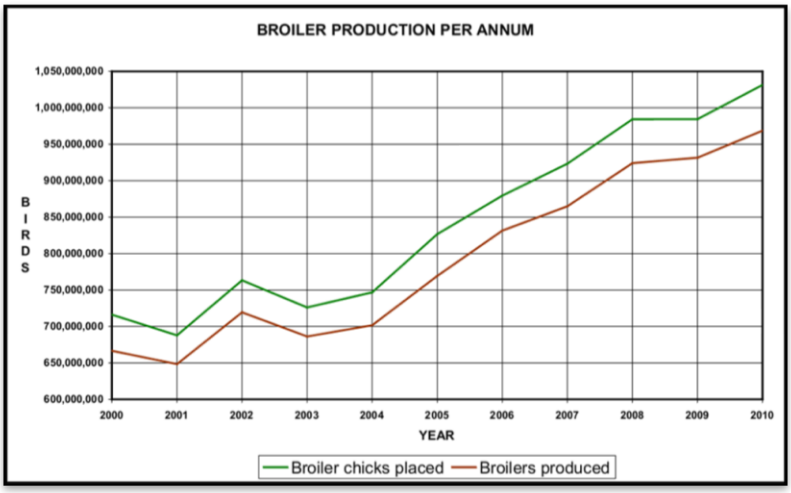


Figure 1.1: Broiler production in South Africa for the first decade of this millennium (SAPA, 2010). An increase is observed in both the amount of broiler chicks placed and broilers produced.

The overall trend in the broiler industry seems to be a steady increase of 3.9 % in production to meet consumer demands. Broiler deaths were also constant during the preceding decade, indicating that the current disease management systems and broiler house guidelines within South Africa are beneficial to the broiler production sector. No national outbreaks of disease (i.e. Newcastle disease, Avian influenza etc.) were reported for the period, although sporadic and localized outbreaks still occurred (Abolnik, 2007). In the North-West province a localized outbreak of Newcastle disease was however reported in 2012 in the Greater Taung Local municipality, however the spread was contained (SAPA, 2012b).

The above estimates are only for broiler birds. A broiler is defined as “a young chicken suitable for roasting or grilling” (Cambridge, 2012). These birds are normally reared to the age of 42 to 49 days with an expected live mass of 1.86 kg (NDA, 2000).

The North West Province has the highest population of broiler birds according to a South African Poultry Association survey of birds in the 2010-2011 fiscal year (SAPA, 2011). Figure 1.2 shows the distribution of both broiler and layer birds across South Africa. Layer birds amounted to 26.9 million birds, with 24.5% of these found in the Gauteng Province. The broiler bird population for the same period was 107.6 million birds.

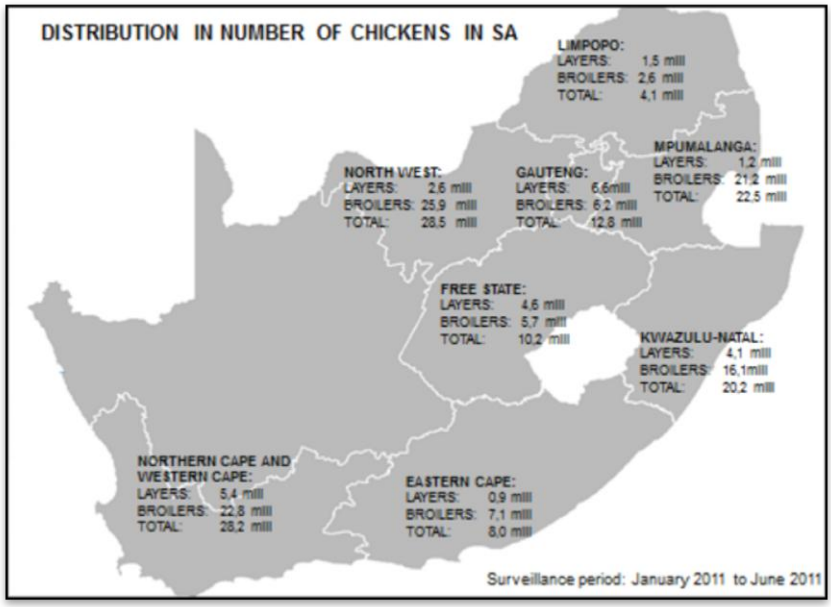


Figure 1.2: Distribution of layer and broiler birds across South Africa. (SAPA, 2011).

The North West provinces has a land area of 106 512 square kilometres (South Africa, 2012) with the highest broiler farms near Potchefstroom and Rustenburg. This translates to 243 broiler birds per square kilometre, the third highest broiler population per square kilometre after Gauteng and Mpumalanga (375 and 277 broilers per square kilometre respectively). The Limpopo province has the lowest broiler population.

According to the United States Poultry and Egg Association a broiler produces 2 pounds of litter in its lifetime (USPEA, 2012). This relates to more than 86 million kg of broiler litter produced annually in South Africa, with roughly 24 million kg being produced in the North West Province if the numbers in Figure 1.2 is taken into consideration. For both the country and the province this creates a large waste management problem. If this agricultural waste where to be mismanaged on a large scale it could lead to increased emissions of various nitrogen based gasses, including ammonia (NH_4) (Miles *et al.*, 2008) and nitrous oxide (N_2O) (Thorman *et al.,* 2006).

1.3 Broiler Litter Composition

Broiler litter consists mainly of manure mixed with bedding material (woodchips, wheat hulls, sunflower hulls, peanut hulls, maize stalks *etc.*), feathers and waste feed, and has a low water content (Szogi & Vanotti, 2009). The bedding material used in most broiler production operations have a high cellulose component, and a very low moisture content. This enables the absorption of spilled water and moisture, that would contribute to the spread of diseases within the broiler flock. It also provides an insulating barrier between the floor (normally a solid cement slab) and the birds, whilst diluting the excreta, therefore minimizing the contact between manure and birds (Casey *et al.*, 2005).

Bedding material is of utmost importance to the broiler producer, as it creates a crucial part of the broiler's environment over the entire lifespan of the bird. In a South African context, Jordaan (2004) reported that peanut hulls as bedding material resulted in the highest production number (an index value measuring the efficiency of performance), however he did not observe any significant difference between using a bedding material and not using a bedding material ($p < 0.05$). Furthermore, his results showed that no significant difference could be observed between different bedding materials used in the rearing of broiler birds. The same result was obtained by Tohgyani *et al.,* (2010), except for reporting that rice hulls resulted in a significant lower body weight, antibody titer and feed intake ($p < 0.05$).

The material used as bedding material is dictated by the availability of the resource, as well as the cost to the broiler producer. South African broiler producers normally use peanut- and sunflower hulls, wheat straw or wood shavings (Jordaan, 2004). Broiler houses in South Africa are cleaned after every cycle, as opposed to American broiler houses which are only cleaned once a year (Jordaan, 2004).

Broiler litter composition therefore differs according to the bedding material used. Table 1.1 sketches the composition of broiler litter in a South African context. It is evident from this

composition that broiler litter can be utilized in various applications including soil conditioning, ruminant feed and energy production.

Table 1.1: Approximation of the composition of South African broiler litter

Moisture[1]	10-24	%	Potassium[2]	13±3.4	g/kg
Crude protein (CP) [1]	10-26	%	Aluminium[2]	834±1196	mg/kg
True protein (% of CP) [1]	40-60	%	Copper[2]	43.6±17.7	mg/kg
Crude fibre[1]	22-25	%	Iron[2]	1335±1878	mg/kg
Ash[1]	10-17	%	Zinc[2]	254±59	mg/kg
Totally digestible nutrients (TDN) [1]	45-65	%	Manganese[2]	317±128	mg/kg
Metabolisable Energy (ME) [1]	6-7.3	%	Cadmium[2]	0.32±0.34	mg/kg
Calcium[1]	1.5-3.0	%	Cobalt[2]	1.08±0.96	mg/kg
Phosphorus[1]	1.2-1.8	%	Chromium[2]	11.21±18	mg/kg
Magnesium[2]	5.8±1.1	g/kg	Arsenic[2]	4.92±13.8	mg/kg
Sodium[2]	5.6±1.6	g/kg	Lead[2]	0.55±2.02	mg/kg
Mercury[2]	0.48±0.67	mg/kg	Vanadium[2]	12.1±8.38	mg/kg
Selenium[2]	0.62±0.24	mg/kg	Molybdenum[2]	1.5±1.06	mg/kg
Nitrogen (organic)[3*]	22	$kg.t^{-1}$	Nitrogen (Inorganic)[3*]	7	$kg.t^{-1}$

1 (Van Ryssen, 2001); 2 (Van Ryssen *et al.,* 1993); 3 (Mkhabela, 2004)

All samples were tested on a dry basis, except those indicated by an asterix (*)

A common indicator of a soil enhancers' capabilities to supply plant nutrients is the ratio between Nitrogen (N), Phosphorous (P) and Potassium (K). These elements are also referred to as the macronutrients of plants. Chemical fertilizers mainly contains N, P, and K, in different ratios (Brinton, 2000). Broiler litter on the other hand also contains several trace elements, such as aluminium (Al), copper (Cu), iron (Fe), zinc (Zn) and manganese (Mn), and low levels of possible toxic elements such as mercury (Hg), copper (Cu), arsenic (As) and lead (Pb). Arsenicals are added to broiler feed to control coccidiosis, whilst copper sulphate promotes broiler growth (Kpomblekou-A *et al.,* 2002).

1.4 Uses Of Broiler Litter

Various uses of broiler litter have been documented, including direct land application, ruminant feedstuff, energy generation and composting. Each application has its own set of advantages and disadvantages, which will briefly be discussed in subsequent sections.

1.4.1 Ruminant Feed

The use of broiler litter as a relatively cheap nitrogen source for ruminant feed has been extensively investigated during the last 60 years (Noland *et al,* 1955; Bhattacharya & Fontenot, 1965; Bakshi & Fontenot, 1998; Mavimbela, 1999; Jordaan, 2004 and Azizi-Shotorkhoft *et al.,* 2012 *i.a.*). In South Africa, the practice can be traced back at least to the 1960's (Van Ryssen, 2001).

Poultry litter, including broiler litter, could be used as a major dietary component in rearing ruminants especially during times when reduced forage is available. Therefore broiler litter would be a valuable source of nitrogen during droughts and when pastures were destroyed in veldfires or are sub-optimum in winter (Mavimbela *et al.*, 1997; Hoon *et al.,* 2011). Between 1.5-2.5 kg/head/day for cattle and 0.2-0.4 kg/head/day for sheep is recommended if broiler litter is used as winter supplement, whilst during drought up to 6 kg/head/day and 1 kg/head/day can be given for cattle and sheep respectively (Van Ryssen, 2001).

The main non-protein nitrogen (NPN) compound in broiler litter is urea (Bhattacharya & Fontenot, 1966) contributing roughly 0.7-2.4% of the dry weight (Bhattacharya & Fontenot, 1965; Nicholson *et al.,* 1996). Rumen bacteria use NPN compounds, such as urea, to synthesize protein compounds that can be accumulated by ruminants. Therefore it is possible to raise and sustain ruminants on a protein limited diet, using NPN sources such as broiler litter (FAO, 1968; Ribeiro *et al.,* 2011). Using broiler litter instead of urea can lead to a reduced possibility of urea toxicity, as overdosing is unlikely to be a problem.

The use of broiler litter as a ruminant feed however still remains a controversial subject, polarizing farmers, scientists and consumers alike. Some of the arguments against the use of broiler litter as a ruminant feed pertain to possible pathogenic organisms present (Lu *et al.,* 2003; Aury *et al.,* 2011), heavy metal toxicity (Suttle & Price, 1976; Mavimbela *et al.,* 1997), antimicrobial and antiprotozoal agents present in litter (Shlosberg *et al.,* 1992*;* Mavimbela *et al.,* 1997) and an overall negative perception of this practice by the general public. In a South African context the use of broiler litter as a ruminant feed has been highly debated in an agricultural publication, particularly by Prof. Chrisjan Cruywagen of the University of Stellenbosch and Dr. Dietmar Holm of the University of Pretoria's Veterinary Faculty, Holm being for the use whilst Cruywagen against it (Kriel, 2012). Holm proposed that the use of broiler litter is safer than other NPN sources, such as urea, as over-dosing is less likely. He also stated that broiler litter contains other micronutrients that could help ensure animal health. Cruywagen warned that the same micronutrients could be assimilated in too large quantities,

leading to toxicity, especially in sheep. The use of poultry litter as ruminant feed had been applied for centuries, and is still a worldwide practice (Holm, 2011)

One of the most serious concerns when feeding broiler litter to ruminants is the amount of antimicrobial substances present. Furtula *et al.,* (2010) found that antimicrobial residues in litter can range between 0.07 – 66 mg/L depending on the compound. This could create antibiotic resistance in several species present in the broiler litter, including *Escherichia coli.*

The use of unsterilized broiler litter as an animal feed is prohibited in South Africa under Act 36 of 1947, and suppliers of broiler litter should register it as an animal feed with the Department of Agriculture. Stacking of broiler litter at a low moisture content for at least 11 days results in pathogen reduction, especially of *Salmonella enteriticis, E. coli* and *Shigella sonnei* (Kwak *et al.,* 2005). The reduction is attributed to interspecies competition and low moisture levels.

1.4.2 Biofuel Source

Poultry litter could also be implemented in energy conversion, acting as a primary energy source. Dávalos *et al.,* (2002) calculated the massic energy combustion at 14 447kJ / kg (3450.52 kcal / kg) poultry litter. As a biofuel source, broiler litter can be used in producing biogas (Kelleher, 2002; Gelegenis *et al.,* 2007) or can be directly combusted to produce heat energy.

Biogas is mainly produced by means of anaerobic digestion, involving the degradation and stabilisation of organic material under anaerobic conditions. Methane is the main gas produced, together with several other inorganic products:

$$\text{Organic matter} + H_2O \xrightarrow{\text{anaerobes}} CH_4 + CO_2 + \text{New Biomass} + NH_3 + H_2S + \text{heat}$$

(Kelleher *et al.*, 2002)

Anaerobic digestion of broiler litter is a relatively efficient conversion process, resulting in a collectable biogas with 60% methane being reported (Kelleher *et al.,* 2002). The process, however, needs biofermenters to be erected near the location of the source product. The initial set-up costs costs are high, and the system needs constant monitoring to ensure optimum biogas formation (Biogas Energy Inc., 2008).

Direct combustion of poultry litter can also be used. However, there are several possible negative impacts on the environment, including emission of NO_x species, SO_2 and HCl. A company in the United Kingdom has patented and implemented three power plants with poultry

litter as a fuel source. The plants processes 670 000 metric tonnes of chicken litter per annum, generating 61 MW of electricity as well as a high quality fertilizer (EPRL, 2012). Waste from these facilities produce poultry litter ash as waste. The waste product is processed to a commercial fertilizer that is high in P and K (Fibrophos, 2012). Field experiments indicated that both P and K are largely available to crops and grasslands (Richardson, 1993).

1.4.3 Direct Land Application

The nutrient content (Table 1.1) makes the direct land application of broiler litter as a soil conditioner appealing to agriculturalists. Not only does broiler litter have a high nitrogen (N) content of over 29 $kg.ton^{-1}$ (Mkhabela, 2004), but it also contains an ample amount of phosphorous (P) and potassium (K). The precise ratio of N:P:K (also known as the primary macronutrients of plants) was published as 6:2:3 by Nicholson *et al.,* (1996) for samples collected in the United Kingdom. Furthermore, poultry manure can aid in supplying micro-nutrients to crops, greatly increasing plant vitality and growth.

The use of poultry litter in direct land application on economically important crops has been well documented, including application on maize (Moss *et al.,* 2001), soybean (Adeli *et al.,* 2005), horticultural species (Rubeiz *et al.,* 1998) and cotton (Sistani *et al.,* 2004; Mitchell & Tu, 2005; Tewolde *et al.,* 2009).

From Table 1.1 it could also be observed that the largest portion of nitrogen is in organic form. Organic nitrogen-containing compounds cannot be easily assimilated by plants (Jones *et al.,* 2005), resulting in a vast array of microbiological processes needed to convert the organic nitrogen into its inorganic counterparts, especially NH_4^+ and NO_3^- species. Therefore, applying broiler litter to the soil results in a slow-release nitrogen source, as opposed to the rapid nitrogen availability of synthetic fertilizers (Sistani *et al.,* 2008). The risk of excessive nitrogen induced stresses in plants are consequently limited. It could be argued that as the release is more consistent a reduced application for fertilizer substances is needed.

The application of manures, especially broiler litter, ensures optimal soil nutrient levels for plants as well as improving the soil's physical attributes. Soil pH can be altered, mainly by compounds containing calcium (Ca) and magnesium (Mg) present in broiler litter. For South African broiler litter the amount of Ca and Mg can be as high as 30 $kg.ton^{-1}$ and 69 $kg.ton^{-1}$ respectively (as calculated from Table 1.1). Raising the pH of acidic soils have been shown to improve P availability and reducing aluminium (Al) toxicity (Materechera & Mkhabela, 2002).

Broiler litter also increases the soil organic matter fraction which in turn enhances soil physical properties such as tilth, structure, water holding capacity and -filtration rate, and also microbial activity (Sweeten and Mathers, 1985; Bolan *et al.,* 2010). Additionally the use of broiler litter could increase the natural microbial biomass and species diversity. This would increase the number of predators as well as ensure competition between consumers. This could result in a lowered dependence of crops on pesticides. Broiler litter is an excellent nitrogen source resulting in high ammonia and/or nitrous acid concentrations in the soil. This could have a further suppressing effect on plant pathogens, depending on the soil's pH, buffering capacity, organic matter content and nitrification rate of the soil (Lazarovits, 2001).

The use of broiler litter as a soil conditioner does seem to be a method for utilizing this otherwise waste-product. Several considerations needs to be kept in mind before applying vast amounts of litter to agricultural land. Although broiler litter can be applied directly to farmland, it could potentially create human and animal health risks as well as pollute fresh water systems (Ogunwanda *et al.,* 2008). The impact of broiler litter application on contaminating fresh water sources are of greatest concern. The very chemical composition of broiler litter that makes it such a desirable soil amendment, could lead to eutrophication of water sources. Also the presence of potential pathogens, such as *Salmonella* and faecal coliforms, could cause dire effects on agricultural crops such as tuber vegetables, animals and humans.

Various strategies needs to be applied to ensure responsible use of manures, including broiler litter, in agricultural applications. The application rate of broiler litter is mainly a function of the input of N and P, as these nutrients are the largest contributors to the eutrophication process. Risse *et al.,* (2001) puts forth a nutrient management plan to minimize the eutrophication process from various literature sources:

- Nutrient composition of broiler litter needs to be determined.
- Crop demand should be determined and met by applying correct amounts of broiler litter.
- Watering and precipitation should be monitored and application of broiler litter should not coincide with heavy watering of crops.
- Land management should be optimized to reduce leaching of nutrients and resulting in contamination of fresh-water sources.

These considerations should be well understood by farmers and agronomists alike, to ensure responsible use of broiler litter. As broiler litter is normally applied in its raw form, these considerations could also help to curb the spread of pathogenic organisms to the surrounding environment and water sources (Nicholson *et al.,* 2005).

The use of manures as soil conditioners are further limited by the great variability that could exist between different manures, or different batches of the same type of manure. The nutrient value of manures are mainly a function of animal feed, animal health and storage conditions of the manure in question (Bolan *et al.,* 2010).

Many countries, including South Africa, may produce more broiler litter than can successfully be used in direct soil applications (Kelley *et al.,* 1997; Lichtenberg *et al.,* 2002). Broiler rearing practices are mainly condensed near slaughtering facilities that can facilitate large numbers of birds. Transport of broiler litter would greatly escalate the price, and this combined with the possible negative impacts and variability of the chemical composition of broiler litter could influence it's reliability as a good soil conditioner (Bolan *et al.,* 2010).

1.5 Composting

Another use for broiler litter is to use its high nitrogen content in a composting process. Bernal *et al.,* (2009) defines composting of organic wastes as "a bio-oxidative process involving the mineralisation and partial humification of the organic matter, leading to a stabilised final product, free of phytotoxicity and pathogens and with certain humic properties". Though composting is a natural occurring process, various studies have been done to better understand it. In doing so procedures could be optimized to create an economically viable product.

1.5.1 Importance Of Composting

Various authors have described composting of manures as a practical approach to discard of an otherwise waste product (Tiquia & Tam, 2000; Gomez *et al*., 2005; Dong & Tollner, 2003). The composted product has various ecological and economical values, is easier to handle, and can aid in the rehabilitation of exhausted and depleted areas of land, especially in an agricultural milieu.

During the first phase of the composting process, simple organic carbon compounds are mineralised and metabolised by a biologically diverse microbial community. This phase produces various inorganic gases, including CO_2, NH_3 and H_2O. Organic acids and heat are also produced. The latter product is the reason why this phase is called the thermophillic phase. The thermophillic phase is often seen as the most important phase of the composting process. (Bernal *et al*., 2009).

During the thermophillic phase, pathogens, as well as various plant seeds, are inactivated, thus rendering a safe end-product. Immature composts can have serious negative influences on

crop production (Cambardella *et al.,* 2003). This includes nitrogen starvation, delay in plant growth, phytotoxicity, and the introduction of weed seeds and harmful bacteria that survived the composting process (Kato & Miura, 2008).

Composting has several advantages, which outweighs the disadvantages. Milligan *et al*., (2008) stated that composting addresses food safety issues, as the high temperatures reached during the composting process reduces pathogen numbers. These high temperatures deactivates weed seeds that could be incorporated into the broiler litter during storing and transport, as well as insects such as fly and other larvae. Odours could be better managed, especially during application. Farming equipment have less wear and tear, as compost has a uniform consistency. This also leads to a more uniform application of nutrients being possible, as thoroughly composted and mixed compost has a unvarying nutrient content. A major disadvantage of the composting process is the loss of C and N. This can be greatly reduced by adjusting the starting C:N ratio in turn reducing the formation of ammoniac compounds (See Section 1.5.3.1).

1.5.2. Commercial Microbial Composting Techniques

Various composting techniques exists, with main differences between techniques being the biota used (i.e. earthworms, microorganisms etc.) and the physical parameters. Three mainstream microbiologically facilitated composting techniques are used worldwide by composting facilities, namely windrow composting, static pile composting and forced aeration static pile composting. Windrows are made up of the material to be composted that are formed in triangular prismal form. These windrows are frequently turned by specialised agricultural implements and additional water is added. The physical homogenization increases oxygen within the piles, and thus leads to an aerobic process, whilst producing a much more consistent product (Brodie *et al.,* 2000).

Static piles are in contrast not turned and are formed into conical piles. Water still needs to be added to ensure optimal microbial activity. However, the process often becomes anaerobic and thus takes longer to complete (Brodie *et al.,* 2000). A variation of the technique is to force air through perforated pipes within these static piles. This increases aerobic microorganisms activity, and is indicated as an extremely fast process for producing a stable final product (Gao *et al.,* 2010).

The economic feasibility of these techniques can be summarized using the energy input needed, labour requirements, operational costs and time until completion (Brodie *et al.,* 2000; Gao *et al.,* 2010). Energy requirements refers to the amount of input energy needed, whether it

be electrical or mechanical (i.e. having specialized turning equipment, or forming of the composting parameters *etc.*). All composting practices need at least to some small extent staff to tend to the composting process, monitoring the process, adding water when needed, aerating the compost and forming and homogenizing the substrate to be composted.

Both the energy and labour requirements influence the operational costs and composting time needed. Often, in a commercial environment, the composting time needs to be as quick as possible, within a reasonable cost framework whilst not reducing the end-product quality. The comparison between windrow composting, static pile composting and forced aeration composting is given in Table 1.2.

Table 1.2: Comparison of different composting techniques

Criteria	Windrows[a]	Static piles[a]	Forced Aeration Static Piles[b]
Energy requirements	High	Low	High
Labour requirements	High	Low	Medium
Operational Costs	Medium	Low	High
Time	100 days	300 days	60-70 days

a) Reworked from (Brodie *et al.,* 2000*)*
b) Reworked from (Gao *et al.,* 2010)
c) Energy requirements refer to amount of energy needed to produce the product. These include fuel for equipment, electricity etc.

Brodie *et al.,* (2000) published a study conducted on a commercial scale, with nearly 300 tons of material being composted. They reported that, all conditions being the equal, the composting process is extremely forgiving. No marked difference in compost quality could be observed between the two methods investigated.

The main difference between static piles and turned windrows are in the amount of energy and labour required as well as the composting time. Static pile systems requires less energy and labour, thus making it a somewhat cheaper technology (Table 1.2). There were no advantages found for passively aerated static piles above that of normal static piles. However, passively aerated static piles had a higher start-up cost, and labour costs involved.

Even though static piles are more cost effective, these piles were only completely composted after 300 days (Table 1.2). Machine turned windrows were mature within 100 days (Table 1.2). This results in machine turned windrows being the most economically feasible production method of the mentioned composting techniques. Production in machine turned windrows are

66 % faster, which could justify higher production costs as annual income could be increased (Table 1.2).

Gao *et al.,* (2010) used a forced aeration composting method in the composting of poultry manure with great success (Table 1.2). They coupled the aeration system to temperature probes. When the temperature was found to be above 65°C a blower forced cool air through the piles, thus lowering the temperature. When the temperature was between 55°C and 65°C, an intermitted aeration was followed, with 5 minutes of aeration, followed by a 5 minute pause. When the temperature was below 55°C, a 5 minute aeration took places, followed by a pause of 10 minutes. They reported that the manure was completely composted on day 62. Thus leading to the conclusion that this method is 38% faster than windrow composting (Gao *et al.,* 2010).

1.5.3. Optimal Physico-Chemical And Microbiological Parameters

Successful composting is influenced by various physico- chemical attributes. These include the initial carbon-nitrogen ratio (C:N ratio), moisture content, thermal profile and the use of amendments to fulfil in the environmental requirements that microbial communities need for optimal succession to take place.

1.5.3.1 C:N Ratio:

Composting can also lead to nitrogen losses in the form of volatized gasses. However, the nitrogen losses can be mitigated if the initial setup of compostable material are done on a scientifically sound method. This leads to a commercially viable organic fertilizer (Kelleher *et al.,* 2001).

Various authors have stated that a optimized carbon to nitrogen (C:N) ratio is crucial for an optimized composting process delivering a standardized end-product (Bernal *et al., 2009*; Silva *et al.,* 2009 and Ramaswamy *et al.*, 2010). Carbon is used by most microorganisms as an energy source, whilst nitrogen is needed for the formation of proteins and cell constituents. To successfully select for a optimized composting consortium, the C:N ratio needs to be optimized at the start of the composting process (Ogunwande *et al.,* 2008).

Fresh poultry manure has a relatively low C:N ratio (Petric *et al.,* 2009) of between 5.8:1 (Silva *et al.,* 2009) and 9:1 (Milligan *et al.*, 2008). Due to this low C:N ratio it has to be amended with an organic carbon source for microbial communities to optimally execute the composting process. Peanut and sunflower hulls, sawdust and wood shavings are commonly used as

bedding manure in the rearing of poultry, as this creates a dry surface for the birds, while reducing odours and certain pathogens (Jordaan, 2005). However, this only marginally increases the C:N ratio of broiler litter to between 10:1 – 15:1 (Milligan *et al.,* 2008).

The blue gum tree (*Eucalyptus globulus)* has been listed as an invasive plant in South Africa, threatening water sources with its high water requirements (DWAF, 2005). Woodchips from these trees could be used as a carbon source in the composting process, thus reducing these invaders' numbers and creating a use for the carbon captured in the tissues of these trees.

A C:N ratio of between 25:1-35:1 (Bernal *et al.,* 2009; Silva *et al.,* 2009 and Ramaswamy *et al.*, 2010) is said to have sufficient energy to sustain composting organisms, and reduce the selection of organisms involved in volatizing nitrogen to for instance ammonia that can be leached to the environment. If however the C:N ratio is higher than 35:1, the process can be extremely slowed, due to the excess of degradable substrate available.

1.5.3.2 Moisture:

The vast numbers of microorganisms present in the composting process requires large quantities of water. Metabolic activity also increases the temperature of especially windrows, therefore leading to an increased evaporation of water (Gajalakshmi & Abbasi, 2008). Water thus needs to be added to the piles.

A moisture content of between 50-60% saturation ensures optimum composting, especially with regards to windrow composting (Gajalakshmi & Abbasi, 2008; Bernal *et al.,* 2009 ; Ramaswamy *et al.,* 2010). If the moisture content is too high, oxygen movement is inhibited and this leads to an anaerobic process. If, however the moisture content is too low, this can inhibit microbial activity and slow down the humification process (Gajalakshmi and Abbasi, 2008). Optimum moisture content is therefore important for compost processes to occur in the minimum time.

1.5.3.3 Temperature

During the composting process temperatures should increase due to the breakdown of organic material by beneficial protozoa, fungi, actinomycetes and bacteria (Ichida *et al*., 2001). Compost temperature is often used to assess the progress of decomposition (Yu *et al.,* 2008).

The composting process is usually characterized by a short lag phase, followed by mesogenic heating and thermogenic heating. During the thermogenic phase compost temperature is normally maintained at temperatures above 40°C, regardless of the ambient temperature. The

thermogenic phase can last up to 80 days. Figure 1.3 is a graphic representation for the temperature profile during the composting process. The thermogenic phase is often described as the most important phase, as pathogen reduction (Hassen *et al.,* 2001; Wichuk & McCartney, 2007), weed seed destruction (Tompkins *et al.,* 1998) and degradation of large organic constituents (Nakasaki *et al.,* 2005) all take place during this phase.

The temperature profile could also be directly correlated to the C:N ratio (Huang *et al.,* 2004) with higher temperatures obtained with a higher C:N ratio. After the thermogenic phase a stabilization of the temperatures follows, known as the curing phase. The temperature profile is summarized in Figure 1.3

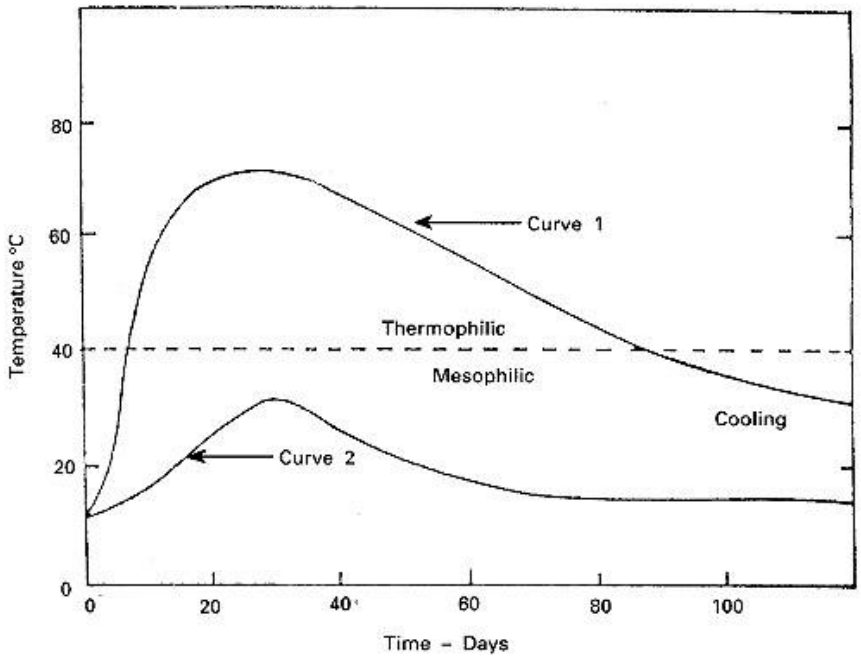


Figure 1.3: Theoretical temperature profiles during the composting process (Parr *et al.,* 1994).

Curve 1 (Figure 1.3) shows the expected temperature profile for more than 100 days in a compost that is correctly formulated (i.e. water added and optimized C:N ratio). Curve 2 (Figure 1.3) in turn shows the temperature profile for a compost with suboptimal C:N ratio and moisture content (Parr *et al.,* 1994, Bernal *et al.,* 2009). Optimizing C:N ratio therefore would theoretically increase the metabolic rate within the compost, transforming carbon and nitrogen to increase biomass and therefore metabolic activity (Ogunwande *et al.,* 2008). The thermogenic profile (Curve 1) is the benchmark for composting facilities, as it ensures optimized composting of substrate.

In compostable substrates that are poorly formulated (Curve 2, Figure 1.3) thermogenic temperature are not achieved. This could lead to pathogen and weed seed survival, that could have detrimental effects on environmental as well as animal and human health. The

thermophilic temperature-time (i.e. time that compost is within thermophilic range) relationship is therefore of utmost importance (Wichuk & McCartney, 2007).

During the different phases of the composting process, differences in the microbial community can be observed. The mesogenic phase is characterized by mesophilic organisms with a optimum temperature range of between 0°C and 40°C (Trautmann & Olynciw, 1996). A short secondary lag phase is followed by the thermogenic phase, in which thermophilic organisms dominate. During the curing phase the diversity of microorganisms increase and further biotransformations of organic matter occurs due to the re-establishment of fungi, actinomycetes and mesophilic bacteria (Beffa, 2002).

1.5.3.4 Microbial Populations In Composting

Some of the early work on microbial organisms present during the composting process were performed as far back as the 1920's (Sanborn, 1925). In the following decade various articles appeared on thermophilic organisms isolated from soils and composts (Waksman *et al*, 1939a; Waksman *et al.,* 1939b cited by Golueke *et al.,* 1953). The thermophilic organisms was mainly attributed to the use of stable manure added to composting mixtures (Golueke *et al.,* 1953).

Culture based techniques is still widely used for the determination of microorganisms present during different stages of the composting process. Devi *et al.,* (2012) used such methods to cultivate organisms present during the co-composting of poultry litter and paddy straw wastes. Using different culture media they found enteric-, spore-forming and diazotrophic bacteria, as well as actinomycetes, fungi and algae. Diazotrophic bacteria, actinomycetes and algae were more prominent towards the end of the composting process (Devi *et al.,* 2012).

The amount of data obtained from culture based techniques are limited. This is mainly due to the selective nature of growth media used in the cultivation of microorganisms (Giraffa & Neviani, 2001). Culture independant methods are able to identify a larger population of microorganisms. Partanen *et al.,* (2010) for instance estimated bacterial diversity of over 2000 different phylotypes in compost from source separated municipal biowaste using a cloning and sequencing approach.

Broiler litter predominantly consists of mainly (87%) Gram-positive bacteria (Lu *et al.,* 2003; Lovanh *et al.,* 2007). Of these 62-67% were low G+C Gram-positive bacteria (*Bacillales* and *Lactobacillales)*. High G+C Gram-positive bacteria comprises between 25-33% of microbial diversity, whilst gram-negatives has a low abundance at between 0% and 13% (Lu *et al,* 2003; Enticknap *et al.,* 2006).

1.5.3.5 Zeolite Amendment

Zeolites are aluminosilicates, formed by tetrahedras of aluminium orthophosphate (AlO_4) and SiO_4. This forms a framework structure enclosing cavities occupied by large ions and water molecules, which have a considerable freedom of movement, permits ion exchange, diffusion, dehydration, reversible dehydration and catalysis. (Eleroğlu & Yalçin, 2005). Zeolite can also act as a molecular sieve (Davis & Lobo, 1992). It is therefore an excellent adsorbent in various industrial applications. The sieving properties of Zeolite can be altered by chemical processes creating pore sizes of varying diameter (3, 4, 5 or 8) (Ángstrom; Anon, 2012).

Zeolite could be successfully implemented as part of the bedding material used by broiler farmers. Eleroğlu and Yalçin (2005) reported that adding 25% zeolite to the bedding material (wood shavings) improved feed efficiency significantly from 1.83 g feed/g gain to 1.71 g feed/g gain. Similar results were obtained using 50% and 75% zeolite cover (1.74 and 1.73 g feed/g gain respectively). Furthermore adding zeolite to the bedding material did not cause any leg or body abnormalities, and decreased moisture from 36.2% to 25.2, 23.6 and 21.8% for the 25, 50 and 75% treatments respectively (Eleroğlu and Yalçin, 2005). The reduced available moisture could decrease pathogen prevalence. Furthermore, the heat-absorbent characteristic of zeolites could reduce heat induced deaths of broilers in poultry houses that are insufficiently ventilated.

Karamanlis *et al.,* (2008) also investigated the use of zeolite as a bedding material when used in combination with sawdust. They also found that zeolite had a positive effect on broiler health, and that adding 2% zeolite to the feed together with a 2 kg zeolite / m^2 cover significantly increased growth rate.

Barrington *et al.,* (2002) describes how Witter and Lopez-Real (1988) observed that a cover of zeolite over compost piles provided an effective adsorbing effect of volatized ammonia. Elwell *et al.,* (1998) quantified this adsorptive effect, and reported a reduction of 44% in ammonia losses with a 38% zeolite layer placed on the surface of the manure.

Salt content could have detrimental effects on plants, as plants differs in sensitivity towards salinity of soils (Brinton, 2000). Gamze-Turan (2007) successfully used a zeolite amendment to reduce the salinity of poultry litter compost. His results showed that a 10% addition of zeolite reduced the salinity of the end-product by 88% (Gamze-Turan, 2007).

1.6 Compost Quality Standards

Compost quality is an important issue as compost is used in both agriculture and environment rehabilitation (Baffi *et al.*, 2007). Further, Baffi *et al.,* (2007) defines that compost quality has to adhere to four standards, namely plant and soil friendliness, environmental friendliness and compost must be a socially responsible product.

One of the most important aspects is that compost has to be absolutely pathogen free. Various pathogens and potential pathogenic organisms have been isolated from compost. These pathogenic organisms included *Salmonella* sp., *Yersinia* sp., and *Escherichia coli* (Gong *et al,* 2005; Yun *et al.,* 2007). The presence of pathogenic organisms could be mainly attributed to the absence of a thermogenic heating phase. Moral *et al.,* (2000) presents a review where composts have presented characteristics described as limiting factors for horticultural use. These factors include hazardous levels of heavy metals, poor physical properties such as a high pH, phytotoxic effects and a compost with a high electrical conductivity.

Some European countries have collectively created the European Compost Network (ECN). Members subscribing to the ECN Quality Assurance Scheme (ECN-QAS) needs to comply to several standards put forth. Some of the European composting standards are summarized in Table 1.3.

Table 1.3: European Compost Network Quality Assurance Scheme

Substance	Value	Unit
Organic matter	>15 %, declaration	
Alcaline effective materials (CaO)	Declaration	
Nutrients (N, P, K, Mg)	Declaration	
Plant Compatibility	Benchmark	
Water content	Declared	
pH	Declaration	
Grain Size	Declaration	
Electric conductivity	Declared	
Salmonellae	0 in 25g	
Manmade foreign matter	<0.5% Dry matter	% of dry weight
Cadmium	1.3	mg/kg
Chromium	60	mg/kg
Copper	300	mg/kg
Mercury	0.45	mg/kg
Nickel	40	mg/kg
Lead	130	mg/kg
Zinc	600	mg/kg

Reworked from (ECN, 2012)

The values for N, P, K and Mg needs to declared on the packaging material (Table 1.3). This is the case for chemical fertilizers as well (Brinton, 2000). The pH, plant compatibility, electrical conductivity, organic matter, grain size and alkaline effective materials also needs to be declared. This enables the consumer to make an educated decision on the application rate of the compost.

No Salmonellae may be present in the end-products. Salmonellae is used as an indicator species, and the absence ensures consumer safety. The amount of impurities such as manmade foreign objects and stones should be kept to a minimum. This ensures that the product weight is not artificially increased by substances that could harm both the consumer or the environment (ECN, 2012).

The values for permissible heavy metals is also indicated by the ECN-QAS. These values are a precautionary measure to ensure that heavy metal toxicity or excessive heavy metal uptake does not result from the application of composts. Although copper and zinc are classified as essential elements, declaration of values above 110 $mg.kg^{-1}$ and 400 $mg.kg^{-1}$ for copper and zinc respectively is required (ECN, 2012).

Further, some countries have implemented regulations pertaining the thermogenic phase of composting. The general consensus seems to be that two to three weeks at temperatures above 55°C are needed for sufficient reduction of plant pathogens and ensure compost quality (Brinton, 2000).

South Africa lacked legislation regarding compost and compost product standards at least until 2010 (Mefane, 2009) and no quality assurance guidelines were in place (Manungufala *et al.,* 2008). On 10 September 2012 Regulation Gazette No. 35666 was published pertaining to regulations regarding fertilizers in the Gazette. In this document compost, defined as a "stabilised, homogenous, fully decomposed substance of animal or plant origin to which no plant nutrients have been added and that is free of substances or elements that could be harmful to man, animal, plant or the environment", has to be registered and must meet a set of requirements. Accordingly compost must be sold in containers. The product must be relatively fine, with 100% passing through a 12 mm standard sieve. The ash content may not exceed 670 g/kg on a dry matter basis, and moisture has to be below 400 g/kg. It may also not contain any visible undecomposed organic matter, or foreign material (i.e. inorganic matter such as rubber, plastic etc.). A phytotoxic test also needs to be performed, with at least 80% of seeds planted under controlled conditions germinating. These plants have to show no abnormal growth when planted according to the manufacturer's instructions. The regulation also makes provision for composted poultry manure. Accordingly no macro- or micro-elements may be added to the

compost without the written approval of the Registar of Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies of the Republic of South Africa.

The South African regulation still lacks the level of detail that compost quality assurance standards of other countries have. No mention is made of temperatures that should be reached during the composting process, nor the levels of macro- and micro nutrients and heavy metals permissible. The South African Bureau of Standards has no internal compost standards that could be applied (Mefane, 2009).

1.7 Microbiological Identification Methods Used In Studying Compost

Various culture dependant and culture independent techniques have been applied to further our understanding of the microbiology in the composting process (Sanborn, 1925; Golueke *et al.,* 1953; Lu *et al.,* 2003; Enticknap *et al.,* 2006; Partanen *et al.,* 2010; Devi *et al.,* 2012) . Culture dependant methods for estimating microorganisms however are somewhat biased, as currently the complex nutritional needs of all organisms cannot be fulfilled (Giraffa & Neviani, 2001). Culture dependant techniques is therefore selective in the organisms that can be isolated and cultured. Some estimates are that only 1.4% of all soil microbial life can be cultivated using media preparations (Janssen *et al*., 2002).

The problem has been overcome, in part, by using molecular techniques based upon the amplification and isolation of differences in molecular composition present in different organisms (Giraffa & Neviani, 2001). Molecular based techniques can be applied to organisms cultivated on growth media, or directly on environmental samples. Various molecules have been successfully used in the classification and identification of microorganisms. These include DNA, RNA and to a lesser extent proteins (Cardenas & Tiedje, 2008). For microbial diversity studies from environmental samples DNA is especially well suited, as it can be easily isolated, is more stable than RNA, and diversity could be estimated based upon differences in nucleotide sequence (Hirsch *et al*., 2010).

The amount of DNA obtained, especially from environmental samples, are subject to the biomass within the sample (Aoshima *et al.,* 2006), as well as the isolation method used (Bürgmann *et al.,* 2001). Various isolation techniques have been described for DNA isolation from soil samples (Zhou *et al.,* 1996; Hurt *et al.,* 2001). These techniques include various laborious and time-consuming procedures such as phenol/chloroform extraction, caesium chloride density gradient centrifugation and column chromatography (Whitehouse & Hottel, 2007).

Various commercial kits are available for DNA isolation, each pertaining to a specific type of sample (i.e. soil, water, biofilm, *etc.*). Most of these kits are based upon a combination of both mechanical and chemical lysis (Ettenauer *et al.,* 2012), followed by binding of DNA to specially designed membranes in the presence of a high salt solution. The DNA can then be successfully purified from PCR inhibitory substances and eluted in a suitable buffer for storage and subsequent analysis.

Composts normally have a very high level of organic matter (30 – 70 %) that is a particular challenge in obtaining PCR-amplifiable DNA (LaMontagne *et al.,* 2002). Humic acids are the most predominant PCR-inhibitory substance present in DNA isolated from composts (Reuter *et al.,* 2009). Any DNA isolation technique applied to compost samples should therefore be able to sufficiently reduce the amount of humic acids in the eluted DNA to ensure successful use in downstream applications.

The advent of the polymerase chain reaction (PCR) technique has reshaped our understanding of the complexities that are present in environmental samples, especially with regards to microbial populations (Rodriquez *et al.,* 2009). Using PCR, minute quantities of DNA can be amplified logarithmically with each amplification cycle.

Diversity studies are mainly based on regions within the genome of taxa that are present in all individuals. The 16S rRNA gene is widely used in studying the diversity of prokaryotes and archaea, and the 18S rRNA gene for eukaryotic species. Both the 16S and 18S rRNA genes are present in the majority of individuals of these organisms (Mignard & Flandrois, 2006). However these genes contain differences at either species or genus level, which can be used to identify individuals within an environmental sample (Van Damme *et al.,* 1996). In the 16S rRNA gene organisms have a 97 % sequence identity. The remaining 3 % (approximately 45 bp) are not evenly scattered along the primary structure of the molecule, but are contained in several hyper-variable regions (Stackebrandt & Goebel, 1994). These hyper-variable regions give information regarding the specific strain of an organism.

Various primers have been published that amplify specific regions within the 16S and 18S rRNA genes. The primer binding sites are highly conserved regions within the genes studied. Lane *et al.,* (1991) is attributed with the development of primer set (27f and 1492r) that amplifies a 1465bp amplicon within the 16S gene. Smit *et al.,* (1999) was the first to report primer set EF3 – EF4 that amplified only fungal members of eukaryotes. This primer set was also found to be the most successful in analysing eukaryotic diversity in composts (Marshall *et al.,* 2003).

Various downstream applications exist that can be applied to amplified DNA to obtain information regarding the diversity within a sample. One such technique is denaturing gradient gel electrophoresis (DGGE), first described by Muyzer *et al.* (1993).

DNA fragments obtained from PCR amplification of the genes studied can be separated in a linear denaturing gradient polyacrylamide gel (Fisher & Lerman, 1983). Originally developed to identify point mutations of purified cultured samples, DGGE could also be used to study microorganisms in their natural habitat, such as compost, without the need to first culture these organisms (Muyzer & Smalla, 1998). DGGE provides an indication of the diversity and succession of the dominant individuals within a community of microorganisms, based on a comparison of the ribosomal DNA sequences of different microbes (Hori *et al.,* 2006).

The denaturing conditions within PCR-DGGE are provided by urea and formamide, and a constant temperature of 60°C. The melting profile of an amplicon during electrophoresis is mainly due to the nucleotide composition of the amplicon. Guanine (G) and cytosine (C) have stronger hydrogen bonds opposed to the bonds between adenine (A) and thymine (T). Based on this a GC-rich tail (often called a GC-clamp) is added to the amplified product. As the amplicon migrates within the gel, it is subjected to increasing denaturing stresses. Once denaturing starts the amplicon partially loses its double stranded nature. The GC-clamp remains intact resulting in the mobility of the partially denatured molecule being retarded. This manifests as a band on the DGGE gel. Therefore the differences in nucleotide composition results in each amplicon having a different position within the polyacrylamide gel (Myers *et al.,* 1985; Sheffield *et al.,* 1989; Muyzer *et al.,* 1993). .

Visualization of amplicons under UV light can be achieved using various staining methods, including ethidium bromide (EtBr) (Muyzer & Smalla, 1998). Silver staining (Felske *et al.,* 1996) can also be used, but has a limited efficiency as single stranded DNA can also be stained (Heuer & Smalla, 1997). Bands can be excised from PCR-DGGE gels and DNA retrieved from these bands could be sequenced to obtain the nucleotide composition. These results can be compared to results captured in public domain databases, such as Genebank.

Yamamoto *et al.,* (2009) used PCR-DGGE to study bacterial communities from animal manure composts. DNA was extracted from samples obtained from various composting facilities in Japan. They successfully showed that the bacterial community could be identified using PCR-DGGE, and also noted changes in the microbial consortia present at different temperatures.

Novinscak *et al.,* (2009) also used PCR-DGGE analysis to identify bacterial and fungal communities in composted biosolids. During the two year period they identified 53 bacterial species belonging to 10 phyla, and 21 fungal species belonging to 4 phyla.

Although PCR-DGGE can be utilized to identify key organisms present within a sample, it is not without limitations. The PCR-DGGE technique is subject to variations in DNA extraction method used, PCR-biased amplification and successful gradient formation (Muyzer & Smalla, 1998). To study microbial populations a standardized procedure is needed, therefore limiting the effect of these restrictions. Co-migration of bands could also lead to an underestimation of the true biodiversity within a sample, whilst partial migration (or "interband" regions) can result in the amplification of bands already present (Muyzer & Smalla, 1998).

Other techniques used in studying microbial diversity in composts include single-strand conformation polymorphism (SSCP) (Peters, *et al.,* 2000), terminal restriction fragment length polymorphism (T-RFLP) (Székely *et al.,* 2009) and metagenomic sequencing (Pang *et al.,* 2009). Currently the most data could be obtained using a metagenomic approach, but it a capital intensive approach.

1.8 Summary of Literature

A steady increase in broiler production lead to an increase in the amount of broiler litter produced annually. The problem especially affects the North West province, as the highest population of broiler birds are being reared in this province. Broiler litter is mainly comprised of manure, bedding material, feathers and waste feed. The high content of primary macronutrients results in various uses for this otherwise waste product that could be explored.

Broiler litter has been successfully used as a ruminant feed and a bio-fuel source. In South Africa it is mostly directly applied to agricultural land. The practice of direct land application could lead to eutrophication of fresh-water sources, as well as spread several disease causing organisms including *E. coli, Salmonella spp.,* and certain viruses that could cause infection in other broiler houses.

Composting of broiler litter has been indicated as an environmentally sound method of stabilizing the material, whilst creating a economically viable product for application in agriculture and horticulture. Broiler litter compost has been successfully applied to a large variety of agricultural crops, including maize, wheat and other monocultures.

Various techniques for stabilizing broiler litter could be implemented, with windrow composting being the preferred method for a company that is in the initial phases of production. To ensure pathogen destruction and successful composting, certain physico-chemical and microbiological parameters needs to be monitored and controlled. Most importantly the initial C:N ratio needs to be formulated to ensure optimum composting. Moisture and temperature within the compost needs to be constantly monitored, therefore ensuring the development of a truly thermogenic phase within the composting process. As the composting process is based upon microbial activity, the establishment of a diverse group of organisms are of utmost importance. This could be done by ensuring optimum conditions within the substrate. The effect of zeolite was also investigated, with regards to its capability to capture nitrogen compounds and retain water.

It was only recently that compost standards were published in South Africa. Although the implementation is still in its early phases, it could lead to a more regulated compost industry in South Africa.

Various methods exist for identifying and characterizing the microbial consortium of microorganisms that are present during the composting process. Culture independent methods have a somewhat lower bias as opposed to culture dependent methods. Although PCR-DGGE has certain limitations, these can be overcome in part by establishing a standard method for sample taking, DNA isolation, PCR and DGGE conditions.

In conclusion it could be stated that a diverse group of microorganisms take part in the complex bio-oxidative process that is composting. This work aims to gather some working knowledge on these organisms in this interesting naturally occurring process.

1.9 Aims and Objectives:

The aim of this study was to characterize the microbial community succession in four differently formulated broiler litter windrows by means of PCR-DGGE.

Objectives were :

- To compile 4 different windrows for evaluation during the composting process
- To evaluate 4 differently formulated windrows on physical attributes
- To optimize the DNA extraction process and to characterize the microbial community succession by means of PCR-DGGE
- To evaluate microbial population dynamics in windrows formulated according to different standards

CHAPTER 2
MATERIALS AND METHODS

2.1 Commissioning Of Study

The study formed part of contractual work commissioned by Galltec (PTY) Ltd. performed by the North-West University Potchefstroom campus. The experimental design was formulated according to the specifications by the company.

2.2 Study Site

Windrows were formed and samples were collected at the Ventersdorp plant of Galltec (PTY) Ltd. The plant was located at latitude -26.413969° and longitude 26.822807°, in the North West Province of South Africa.

2.3 Windrow Composition

Four windrows where constructed for this study. These were formed according to the information in Table 2.1:

Table 2.1: Windrow composition

Windrow 1	Windrow 2	Windrow 3	Windrow 4
5000 kg Broiler Litter	5000 kg Broiler Litter 500 kg Previously Composted Material	2500 kg Broiler Litter 2500 kg Blue gum Woodchips 500 kg Previously Composted Material	2500 kg Broiler Litter 2500 kg Blue gum Woodchips 500 kg Previously Composted Material 500 kg Zeolite

Windrow 1 simulated the composition of windrows used by the company. In Windrow 2 previously composted material (PCM) was used to evaluate the effect on composting time and community succession. The PCM was composted in windrows for 8 weeks and contained only composted broiler litter. Windrows 3 and 4 had a higher C:N ratio, due to the addition of Blue gum woodchips. Zeolite, an alluminosilicate, was added to Windrow 4 as an enhancer substance.

2.4 Windrow Monitoring and Composting

Windrow temperature and moisture level was measured using a 1.2 m long DFM temperature and moisture probe (DFM, South Africa). The probe measured 6 different values within the windrow. Data was logged and data was used to create graphs in Microsoft Excel 2007.

Windrows were turned using an industrial self propelled compost turner whilst simultaneously watering the windrows. Windrows were composted for 8 weeks upon request of the funding company.

2.5 Environmental Monitoring

Environmental temperature was obtained using a Oregon Scientific WMR 100 weather station (Oregon Scientific, USA). Data was logged on a dedicated computer.

2.6 Chemical Monitoring

Broiler litter and bluegum woodchips C:N ratio was determined by NviroTek Labs (Brits, South Africa). After 8 weeks samples from all four windrows were again analysed for chemical composition.

2.7 Sample Collection

Samples were collected every 3 days for the first 2 weeks, and weekly thereafter. The middle of the windrow was used, as indicated in Figure 2.1(a) and three samples were collected per sampling trip (Figure 2.1 (b)). Three sampling points were used to ensure maximum biodiversity sampled.

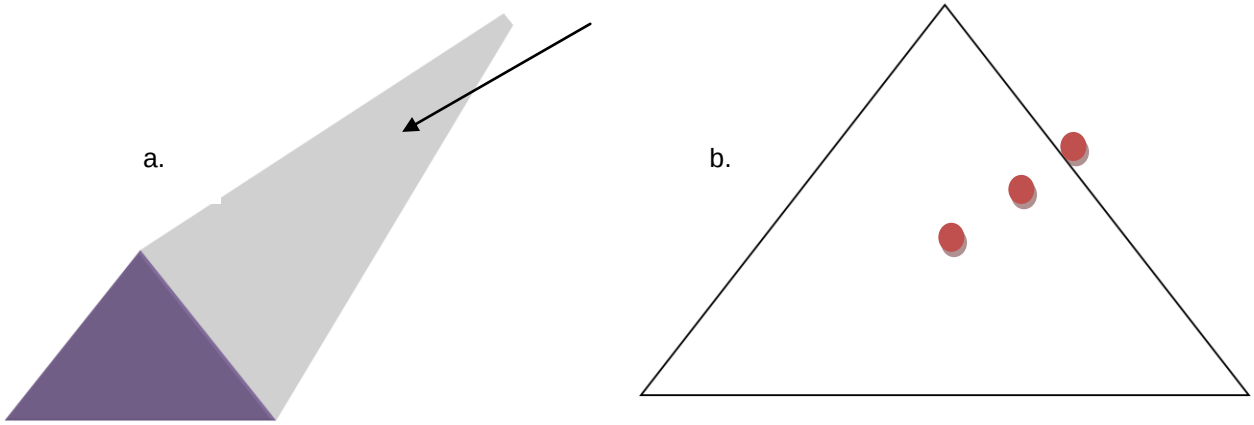


Figure 2.1: (a) Schematic representation of windrows and sampling point. **(b)** Indicates the three points used for sampling.

Samples were placed in plastic bags and stored on ice during transport. The three samples per windrow were combined and mixed.

2.8 Genomic DNA Isolation

2.8.1 Genomic DNA Isolation

Direct DNA extractions from compost samples were done using Machery-Nagel Nucleospin Soil Extraction kit (Macherey-Nagel, Germany). Two-hundred and fifty milligrams (250 mg) of sample material were subjected to the manufacturer's instructions.

The Machery-Nagel NucleoSpin Soil kit has a choice of two lysis buffers and an addition enhancer that can be used in combination with these buffers. All four possible lysis conditions were tested during the optimization process, as indicated in Table 2.2

Table 2.2: Lysis conditions used for compost samples subjected to DNA extraction with the MN – NucleoSpin Soil Kit

Condition 1	Condition 2	Condition 3	Condition 4
Lysis buffer SL1	Lysis buffer SL1 Enhancer SX	Lysis Buffer SL2	Lysis Buffer SL2 Enhancer SX

Isolated DNA quantity and quality (i.e. A_{260}/A_{280} and A_{260}/A_{230}) were determined by a NanoDrop 1000 Spectrophotometer (Thermo FischerScientific, USA).

2.8.2 Agarose Gel Electrophoresis Of Genomic DNA

Extracted genomic DNA quality was further determined by horizontal electrophoresis of 10 µL of eluded DNA mixed with 5 µL of 6 x Orange Loading dye (Fermentas Life Science, USA). A 1.5% (w/v) agarose (Seakem, US) gel was made in 1 x TAE buffer (40 mM Tris (Sigma Aldrich, US), 20 mM Acetic acid (Merck, USA) and 1 mM EDTA (Merck, US), pH 8.01) .

Gel solution was heated in a microwave to dissolve agarose, cooled and 10 µL of a 10 mg/mL Ethidium Bromide (EtBr) (Bio-Rad, UK) was added and mixed to enable visualisation of double stranded DNA under UV light. Gels were eletrophoresed for 45 minutes at 80 V in a Bio-Rad Wide MiniSub Cell (Bio-Rad, UK). Gel images were captured using a Gene Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 7.09) software.

2.9 DNA Amplification

2.9.1 16S rRNA PCR amplification

A nested approach was used for 16s rRNA amplification. Primer sets 27f / 1492r and 341f-GC / 907r were used. Primer sequences are listed in Table 2.3

Table 2.3: List of primer sequences used for 16s rRNA amplification

Primer Set	Sequence	Expected Product Size	Reference
27f	5'-AGA GTT TGA TCM TGG CTC AG-3'	1465 bp	Lane, 1991
1492r	5'-TAC GGY TAC CTT GTT ACG ACT T-3'		
341f-GC*	5'-CCT ACG GGA GGC AGC AG -3'	566 bp	Muyzer *et al.*, 1993
907r	5'-CCG TCA ATT CCT TTG AGT TT-3'		

A GC-rich clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGG-3' was added to primer 341f enabling separation of amplicons during DGGE.

Genomic DNA was amplified using primer set 27f-1492r (Table 2.3) in an iCycler thermal cycler (Bio-Rad, UK) . The final reaction volume of 50 µL consisted of 25 µL double strength PCR mastermix (0.05 U/µL *Taq* DNA Polymerase in reaction buffer, 0.4 mM of each dNTP, 4 mM $MgCl_2$) (Fermentas Life Science (Now ThermoFisher), US), 1 U of additional *taq* polymerase (5 U/µl) (KAPA Biosystems, South Africa), 0.2 µL of a 25 mM $MgCl_2$ (KAPA Biosystems, South Africa), 25 pmol of each primer (Inqaba Biotech, South Africa), 2 µL isolated genomic DNA (<100ng) and 22.1 µL PCR-grade water (Fermentas Life Science (Now ThermoFisher), US).

Cycling conditions for the first amplification were set at 95°C for 180 seconds an initial denaturing cycle, followed by 27 cycles of denaturing at 95°C for 60 seconds, annealing at 52°C for 60 seconds and extension at 72°C for 120 seconds. A final extension cycle was done at 72°C for 240 seconds.

A nested PCR was then performed on the product obtained. The final reaction volume of 25 µL contained 12.5 µL of the same PCR mastermix mentioned above, 0.2 µL of PCR product, 0.2 µL of both primers 341f-GC and 907r (Table 2.3) and 12.1 µL PCR grade water.

Cycling conditions for the nested amplification were set at 95°C for 180 seconds as an initial denaturing cycle, followed by 15 cycles of denaturing at 95°C for 35 seconds, annealing at 56°C for 40 seconds and extension at 72°C for 50 seconds. A final extension cycle was done at 72°C for 180 seconds.

2.9.2 18S rRNA PCR amplification

A nested approach was also used for 18s rRNA amplification. Primer sets EF-3 / EF-4 and EF-4 – GC / fung5 were used. Primer sequences are listed in Table 2.4

Table 2.4: List of primer sequences used for 18s rRNA amplification

Primer Set	Sequence	Expected Product Size	Reference
EF-3 **EF-4**	5′-TCCTCTAAATGACCAAGTTTG-3’ 5′-GGAAGGG[G/A]TGTATTTATTAG-3′	1400 bp	Smit *et al.,* (1999)
EF-4 – GC	5′- [G/A]TGTATTTATTAG-3′	500bp	Smit *et al.,* (1999)
Fung 5	5′-GTAAAAGTCCTGGTTCCCC-3′		

***A GC-rich clamp (5’- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGAAGGG-3’) was added to primer EF4 enabling separation of amplicons during DGGE.**

Genomic DNA was amplified using primer set EF-3\EF-4 (Table 2.4) in an ICycler thermal cycler (Bio-Rad, UK) . The final reaction volume of 50 µL consisted of 25 µL double strength PCR mastermix (0.05 U/µL *Taq* DNA Polymerase in reaction buffer, 0.4 mM of each dNTP, 4 mM $MgCl_2$) (Fermentas Life Science (Now ThermoFisher), US), 1 U of additional *taq* polymerase (KAPA Biosystems, South Africa), 0.2 µL of a 25 mM $MgCl_2$ (KAPA Biosystems, South Africa), 40 pmol of each primer (Inqaba Biotech, South Africa), 2 µL isolated genomic DNA (<100ng) and 22.1 µL PCR-grade water (Fermentas Life Science (Now ThermoFisher), US).

Cycling conditions for the first amplification were set at 94°C for 240 seconds an initial denaturing cycle, followed by 40 cycles of denaturing at 95°C for 60 seconds, annealing at 48°C for 60 seconds and extension at 72°C for 120 seconds. A final extension cycle was done at 72°C for 600 seconds.

A nested PCR was then performed on the product obtained. The final reaction contained 25 µL of the same PCR mastermix mentioned above, 0.4 µL of PCR product, 25 pmol of both primers EF4-GC and fung5 (Table 3.4) and PCR grade water to a final volume of 50 µL.

Cycling conditions for the nested amplification were set at 95°C for 180 seconds as an initial denaturing cycle, followed by 25 cycles of denaturing at 95°C for 45 seconds, annealing at 48°C for 45 seconds and extension at 72°C for 60 seconds. A final extension cycle was done at 72°C for 420 seconds.

2.9.3 Agarose gel electrophoresis of PCR products

PCR success was determined by horizontal electrophoresis of 5 µL of PCR product mixed with 2 µL of 6 x Orange Loading dye (Fermentas Life Science, USA). A 1.5% (w/v) agarose (Seakem, US) gel was made in 1 x TAE buffer (40 mM Tris (Sigma Aldrich, US), 20 mM Acetic acid (Merck, USA) and 1 mM EDTA (Merck, US), pH 8.0) .

Gel solution (100 mL) was heated in a microwave to dissolve agarose, cooled and 10 µL of a 10 mg/mL Ethidium Bromide (EtBr) (Bio-Rad, UK) was added and mixed to enable visualisation under UV light. Gels were eletrophoresed for 45 minutes at 80V in a Bio-Rad Wide MiniSub Cell (Bio-Rad, UK). Gel images were captured using a Gene Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 7.09) software.

2.10 Denaturing Gradient Gel Electrophoresis (DGGE)

2.10.1 16S and 18S rDNA DGGE analysis

DGGE analysis was performed using a DCode Universal Mutation Detection System (Bio-Rad, UK) . For 16S rDNA samples a 7.5% (w/v) acrylamide:bis-acrylamide (37.5:1) gel was used, whilst for 18S rDNA samples a linear gradient between 6-8 % (w/v) acrylamide:bis-acrylamide (37.5:1) gave better results. A 30-55% and 30-50% (where 100% denaturant is equivalent to 7 $M.L^{-1}$ urea and 40 % (v/v) formamide) linear gradient of denaturing was used in the DGGE for 16S and 18S rDNA samples respectively. Polymerization of the gels were due to the addition of ammonium persulphate (Bio-Rad, UK) and TEMED (N,N,N',N'-Tetramethulethane- 1,2-diamine) (Sigma-Aldrich, USA).

Twenty microliters of PCR sample were loaded onto the gels. Prokaryotic and archeae (i.e. 16S) samples were electrophoresed for 16 hours at 100 V and a constant temperature of 60°C. Fungal samples were electrophoresed for 5 hours at 200 V at a constant temperature of 60°C.

2.10.2 Staining and visualizing of DGGE profiles

Gels were immediately stained for 15 minutes after electrophoreses was completed in a EtBr TAE buffer solution. The gels were then visualized using a Gene Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 7.09) software and stored as Tagged Image File Format (.tiff) files.

2.10.3 Excision Of Bands

Bands were excised using a scalpel on a UV transilluminator. The scalpel was sterilized by insertion into ethanol followed by flaming of the ethanol.

Excised bands were suspended in 50 µL sterile proteonomics grade water (Bio-Rad, UK) and stored at 4°C for 24 hours. Thereafter samples were frozen, theoretically causing the polyacrylamide gels to contract and release the DNA contained within. The samples were then thawed and refrozen for a second time.

2.11 PCR Amplification Of DNA From Excised Bands

After repeating the freeze thaw step, samples were re- amplified using the same primers but without the GC-rich clamp. Reaction setup was as follows: 12.5 µL Mastermix (as described in section 3.8.1 and 3.8.2), 0.2 µL of both primers, 0.5 µL excised band water and PCR-grade water to 25 µL. PCR cycling conditions were: an initial denaturing step at 95°C for 240 seconds, followed by 29 cycles of denaturing at 95°C for 35 seconds; annealing at 58°C for 35 seconds and extension at 72°C for 45 seconds. Final extension was done at 72°C for 420 seconds. A higher annealing temperature was used to increase specificity and reduce non-specific amplification of DNA isolated from the polyacrylamide gels. PCR success was again evaluated using agarose gel electrophoresis as discussed in section 3.8.3.

2.12 Sequencing Of Excised Band PCR Product

2.12.1 PCR Cleanup

PCR products were cleaned using a Machery-Nagel NucleoSpin Gel and PCR Cleanup Kit (Machery-Nagel, Germany). The cleanup was done using manufacturer's instructions, with the following alterations. Two wash steps were performed using 700 µL of the supplied wash buffer. The NucleoSpin columns were then dried by centrifuging for 5 minutes instead of 2 minutes, and then placed in a dry heat bath set at 70°C for 5 minutes. Elution was with 20 µL of nuclease free water. Cleanup success was determined by NanoDrop 1000 Spectrophotometer (Thermo FischerScientific, USA).

2.12.2 Sequencing Reaction

The sequencing reaction was done in a iCycler thermal cycler (Bio-Rad, UK). Reaction setup was 4 µL of a 1:10 dilution BigDye Terminator v3.1 Cycle Sequencing Reaction Mix (Applied

Biosystems, USA) together with 2 µL BigDye Terminator v1.1, v3.1 5x Sequencing buffer (Applied Biosystems, USA), 3.2 pmol primer, 1 µL cleaned PCR product and nuclease-free water (Fermentas, USA) to 20 µL.

Cycling conditions were an initial denaturing step at 96°C for 60 seconds followed by 25 cycles of denaturing at 96°C for 10 seconds, annealing at 50°C for 5 s and extension at 60°C for 240 seconds.

2.12.3 Sequencing And Sequence Analysis

Sequence reaction was cleaned using a ZymoResearch DNA Sequencing Cleanup Kit (ZymoResearch, USA) according to the manufacturer's instructions. Cleaned sequencing product were sequenced by Hermoine Venter (North-West University, Potchefstroom Campus, South Africa) using an ABI 3130 Genetic Analyzer (Applied Biosystems, UK).

Sequence chromatograms were viewed and edited using Geospiza FinchTV (v 1.4). In cases where sequences for both the forward and the reverse primers were sequenced, a consensus sequences was created after alignment in BioEdit software (v 7.1.3.0) (Hall, 1999). BLASTn searches (http://www.ncbi.nlm.nih.gov/BLAST) were used to identify sequences obtained. BLASTn searches were conducted using both the nucleotide collection and 16S ribosomal RNA gene (for Bacteria and Archaea) databases.

2.13 Statistical And Diversity Analysis

Temperature and moisture values were plotted using Microsoft® Office® Excel 2007 (Microsoft, USA). Student's T-test was performed on data from different depths and different windrows and used to evaluate similarities or differences that are present in the datasets.

A composite gel was created from the separate DGGE images using Adobe Photoshop CS3. Images were aligned visually using the intergel marker. The composite DGGE image (hereafter referred to as the DGGE image) was analyzed using Phoretix 1D software-trial version (Nonlinear Dynamics, Durham, USA). Lane and band selection was performed manually. Bands were aligned to reduce inter- and intragel distortion. Band similarities was quantified as a Dice similarity coefficient (Dice, 1945) followed by cluster analysis using Ward's method (Ward, 1963).

A presence/absence table was created from the relative migration (rf) values using a 0.1 vector for alignment. Peak height was used as an indication of the presence of a specific band within

the gel. Shannon-Weaver indices were constructed from the peak height data by applying Equation 3.1:

$$H' = -\sum P_i \ln P_i$$

Equation 3.1

(Shannon & Weaver, 1963)

Where P_i is the importance of the band within the lane, determined by Equation 4.2:

$$P_i = \frac{n_i}{N}$$

Equation 3.2

(Eichner *et al.,* 1999)

where

n_i = height of peak

and N = sum of all peak heights in lane.

Using the same data, the Simpson index of dominance concentration (*D*) was calculated using Equation 3.3:

$$D = \sum P_i^2$$

Equation 3.3

(Simpson, 1949)

The peak height values, together with the physico-chemical data (i.e. temperature, moisture and chemical analysis data) was used to perform multivariate analysis, specifically Canonical Correspondence Analysis (CCA), using Canoco for Windows 4.5 (Jongman *et al.,* 1995).

CHAPTER 3:
RESULTS

3.1 Introduction Of Results

The research represented in Chapter 2 was done on a contractual basis for Galltec (Pty.) Ltd. Four windrows were formed using broiler litter and amendments, including previously composted material (PCM), woodchips and zeolite. The formation of windrows was done according to Table 2.1 and to the specifications of Galltec (Pty.) Ltd.

Windrows were monitored for internal temperature and moisture by the company. Environmental temperature was also monitored and logged on a computer. Moisture content and temperature data were evaluated both on average of the windrow, and on different depths within the same windrow.

A chemical analysis of the finished compost product was performed by Nvirotek Labs. This was then compared to both local standards and international standards, especially the European standards for composts.

For microbial community analysis three samples per windrow were obtained and transported to the laboratory in plastic bags. These samples were pooled and thoroughly mixed. Deoxyribonucleic acid (DNA) was isolated from all samples, and amplified using a nested-PCR process for both prokaryotic (16S rRNA) and eukaryotic (18S rRNA) species. The amplicons were subjected to a linear denaturing gradient polyacrylamide gel electrophoresis at 60°C to obtain a community profile for the specific windrow. These gels were two dimensional, with sample profile in vertical lanes and time of composting horizontally.

Prokaryotic gels were visualized and digitized, then combined according to an intergel marker. Low eukaryotic representation resulted in only the end of the composting process of Windrow 4 being evaluated. Statistical analysis combining banding properties, temperature and moisture data as well as chemical analysis was performed to obtain isolates crucial to the composting process, especially the thermogenic phase. Lane profiles were also used to create a dendrogram of similarity on the basis of the Dice coefficient of similarity with Ward clustering algorithm.

Bands isolated from the original DGGE gels were re-amplified, cleaned and sequenced. Sequence data was edited to remove background noise (if present) and identified using BLASTn searches.

3.2 Environmental And Windrow Temperature And Moisture Data

Temperatures within the windrows were monitored by Galltec (PTY) Ltd. Windrows were monitored 6 hourly for the first two weeks of composting, and then at random intervals thereafter. Due to different individuals performing the measurements, the first depth reading was omitted from statistical analysis. This was done as temperature measurements from measurement level 1 closely resembled the ambient temperature, indicating the possibility that the probe was not always completely inserted into the windrow.

Two different measurements (i.e. two different locations within the same windrow) were performed resulting in 10 depth temperature and moisture measurements. Average temperature was then calculated for a specific depth using the two values obtained from these measurements.

Figure 3.1 - 3.3 represents data obtained from monitoring temperatures of Windrows 1 – 4. The average temperatures at the start of this project are highlighted in Figure 3.1, whilst Figure 3.2 indicates the temperature profile over the whole composting process. Figure 3.3 shows the average differences observed in temperature during the composting period.

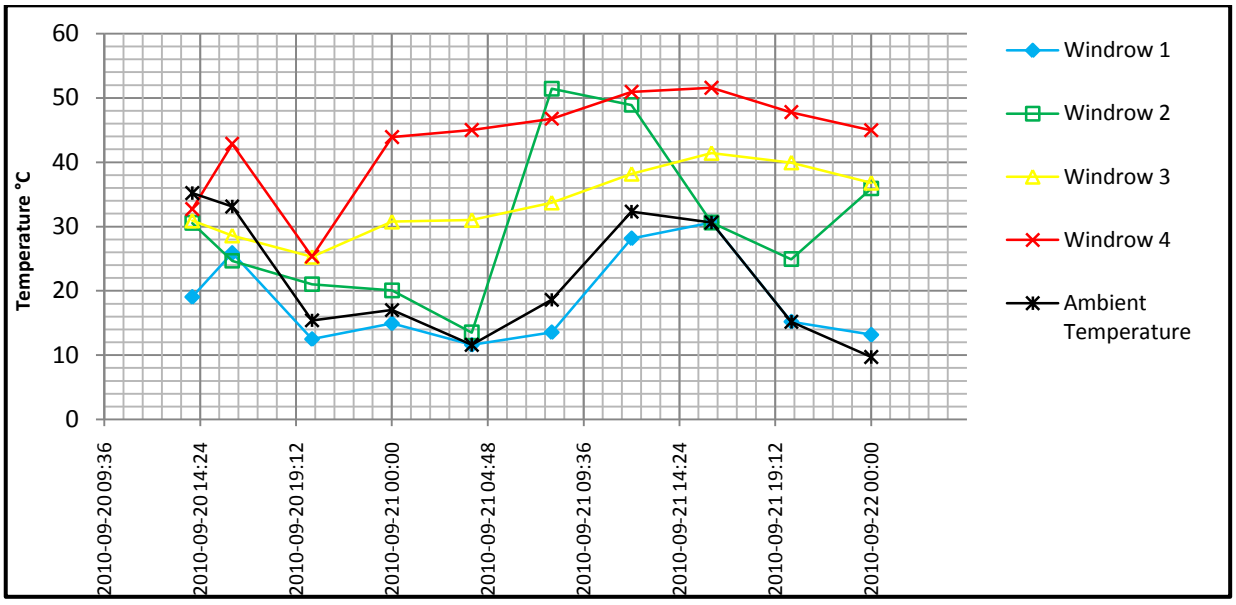


Figure 3.1: Average windrow temperature for the first two days. Windrows were given specific colours for identification on all figures. Windrow 1 – blue (as it had the coldest average temperature profile), Windrow 2 – green, Windrow 3 – yellow, Windrow 4 – red (as it had the highest average temperature). Ambient temperature is always indicated in black.

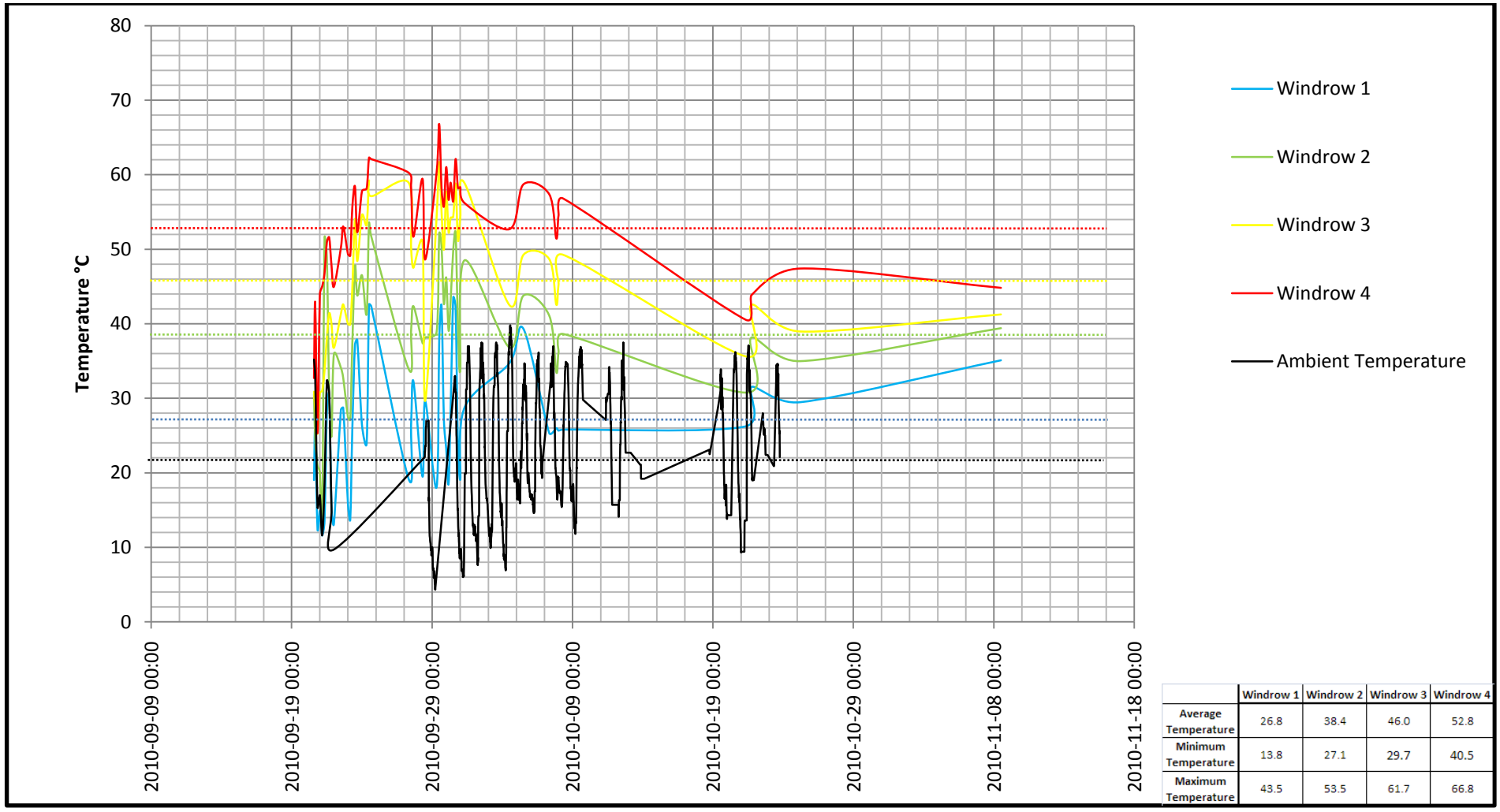
All windrows had temperatures lower than the ambient temperature during the first measurement. Windrow 1 started with the lowest temperature (19.0°C) followed by Windrows 2 - 4 with 24.6, 24.7 and 30.9°C respectively. Ambient temperature at the start of the experiment was 35.2°C (Figure 3.1).

During the start up process the temperature profile of Windrow 1 followed that of the ambient temperature (Figure 3.1). The temperature profile of Windrow 2 was slightly higher during the first 48 hours, with an increase on day 2 of composting (21-09-2010). The temperature profile of this windrow mimics the circadian temperature rhythm. Elevated temperatures could not be maintained during the lower evening ambient temperatures (19:12) of the first night. At the end of this initial period, Windrow 2 had the same temperature as Windrow 3.

Both Windrow 3 and 4 reached high temperatures during the afternoon of day 0 of the composting process. This higher temperatures could not be maintained and it decreased during the evening (20:00) to approximately 25°C respectively. After this an increase could be observed for both windrows. Temperatures above 30°C for Windrow 3 (Woodchips and broiler litter) and 40°C for Windrow 4 (zeolite, woodchips and broiler litter) was maintained for the remainder of the starting 48 hour period. This increase is in strong contrast to the ambient temperature which reached temperatures as low as 11.8°C (04:00).

Over the course of the composting process temperatures in Windrow 1 was generally lower profile (Figure 3.2). Temperatures of above 40°C were only reached on two occasions in Windrow 1. This was dependent on the ambient temperature. Windrow 1 had only one temperature measurement that exceeded that of Windrow 2 (20-09-2010 16:00), and did not have any temperature readings higher than Windrows 3 or 4 on any occasion. The lowest temperature observed was in Windrow 1 (13.8°C) after the initial two days.

The higher temperatures observed towards the end of the monitored composting process in Windrow 1, indicates development of a thermophilic phase. However the temperature observed at the end (35.2°C; 08-11-2012) was still lower (in the mesophilic range) than the corresponding temperature measurement of Windrow 2 (39.4°C).



	Windrow 1	Windrow 2	Windrow 3	Windrow 4
Average Temperature	26.8	38.4	46.0	52.8
Minimum Temperature	13.8	27.1	29.7	40.5
Maximum Temperature	43.5	53.5	61.7	66.8

Figure 3.2: Average windrow temperature during the composting process. Temperature data was obtained from random measurements towards the end of the composting process. Due to chronic interruptions in electrical supply, ambient temperature data values were lost between the periods of 2010-10-09 and 2010-10-19. The average temperatures for the composting period are indicated by coloured dotted lines. The average, maximum and minimum temperatures are given in inserted table. The minimum temperature selection excluded the 2 days at the start of the composting process.

Windrow 2 had a temperature profile that was constantly slightly higher than that of Windrow 1 (Figure 3.2). Windrow 2 had a maximum observed temperature of 53.5°C and a minimum observed temperature 27.1°C. The average temperature of Windrow 2 was 39.4 ± 9.2°C. The internal temperatures obtained from Windrow 2 were higher than the ambient temperature for the largest part of the composting process evaluated. Windrow 2 reached temperatures well above that of Windrow 3 on at least three separate occasions (21-09-2010 08:00 and 12:00; 28-09-2010 12:00). The temperature of Windrow 2's was at one stage higher than Windrow 4 (21-09-2010 08:00). On average Windrow 2's temperature was 7.5 and 14.4°C lower than that of Windrows 3 and 4 respectively (Figure 3.3).

Windrow 3 achieved temperatures well above 40.0°C (Figure 3.2), that could be maintained for several days. At the end of the evaluation period, temperatures were still above 40.0°C. Highest temperature observed in Windrow 3 was 61.7°C, and the lowest temperature in this windrow was 29.7°C. Average temperature was 46.0°C. Windrow 3's temperature was however always lower than the internal temperature of Windrow 4, averaging 6.8°C lower (Figure 3.3).

The temperature of Windrow 4 remained above the thermogenic temperature of 40.0°C (Figure 3.2) for the composting process. Even after termination of the study, temperatures were higher than 45.0°C. The lowest temperature observed in Windrow 4 (excluding the initial 2 days) was 40.5°C, well above thermophilic ranges. Maximum temperature was 66.8°C, with an average temperature of 52.8°C.

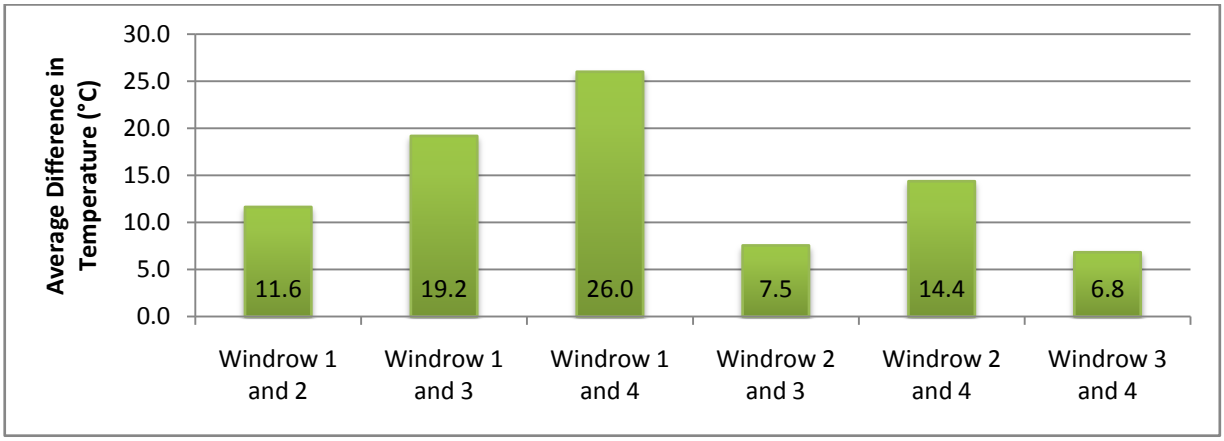


Figure 3.3: Differences in average windrow temperature. Differences between windrows are indicated within the bars.

Average temperatures at different depths were mainly constant indicating a relatively uniform temperature distribution within the windrows. The average temperature data obtained from the

composting period is presented in Figure 3.4. The average ambient temperature during the period of composting was 21.9°C.

Temperatures between depth 3 and 4 were consistently the highest for all windrows (Figure 3.4). Temperatures gradually decreased to depth 1, closest to the surface of the windrow. Depth 5 was located near the compacted earth surface on which the windrows were constructed.

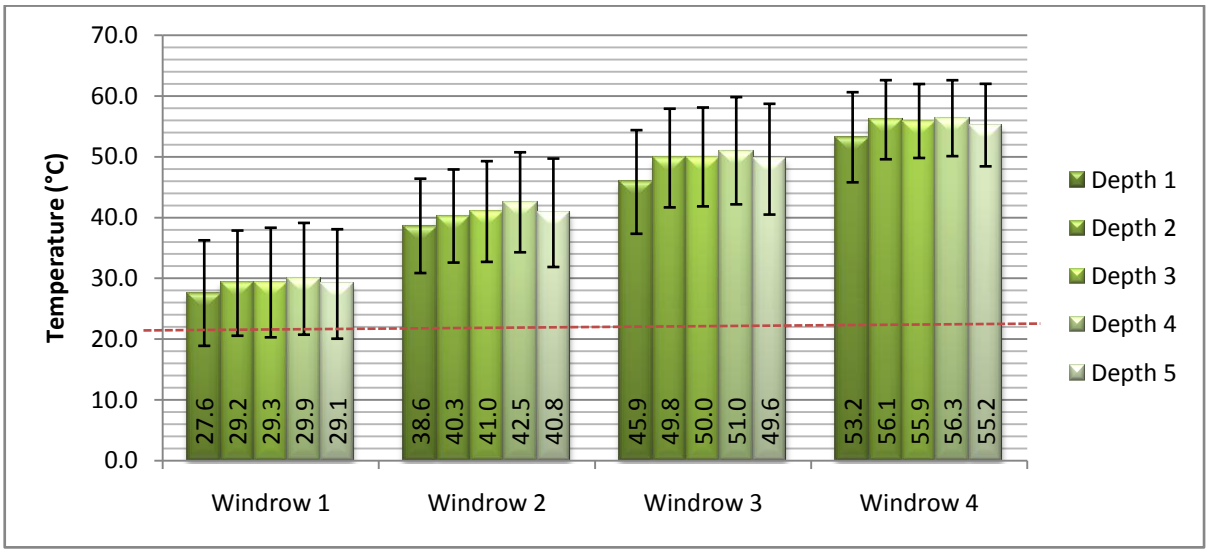


Figure 3.4 – Average temperature during the composting period at different distances from the windrow surface. Depth 1 indicates the surface of the windrow, with distance from the surface increasing to depth 5. Standard deviations are depicted as both positive and negative error bars. Average temperature at depth is indicated within the bars. The red dotted line indicates the average ambient temperature (21.9°C**)**

Windrow 1 had the lowest average temperature during the course of the composting process for all five depths evaluated in this study (Figure 3.4). No significant differences ($p < 0.05$) could be observed between the temperatures measured at different depths within Windrow 1 (Table 3.1).

Windrow 2 achieved an average temperature of 40.6°C (Fig 3.4). With the exception of depths 1 and 4 in Windrow 2, no significant difference ($p < 0.05$) could be observed between different depths of measurement (Table 3.1). Furthermore, Windrow 2 differed significantly ($p < 0.05$) from Windrow 1 in all depth categories (Table 3.1).

Significant differences ($p < 0.05$) could be observed between measurements made in Windrow 3 at depth 1 and all the other depths. No significant differences could be observed between depths 2 - 5 (Table 3.1). Windrow 3 had an average temperature of 49.2°C during the 8 weeks of composting (Figure 3.4). Windrow 3 had a significant different temperature profile compared to Windrows 1 and 2 (Table 3.1).

The highest average temperatures were observed in Windrow 4 (Fig. 3.4), averaging 55.3°C across all 5 depths for the composting period. Significant differences ($p > 0.05$) were observed between measurements near the surface and bottom of the windrow on the one hand and the core of the windrow on the other (i.e. between depths 1 (surface) and 5 (bottom) and 2-4) (Table 3.1). With the exception of measurements made in Windrow 3 at depth 4 and Windrow 4 at depth 1, the temperature profiles differed significantly ($p < 0.05$) between Windrow 4 and all the other windrows.

All four windrows achieved average temperatures above the average ambient temperature. The greatest difference between ambient temperature and windrow temperature was observed in Windrow 4. No statistical significant similarities ($p > 0.05$) were observed between the four windrows and ambient temperatures (Table 3.1).

The data presented in Figure 3.5 shows the average moisture content of the four windrows. This was measured at different times during the 8 week composting process implemented by the company.

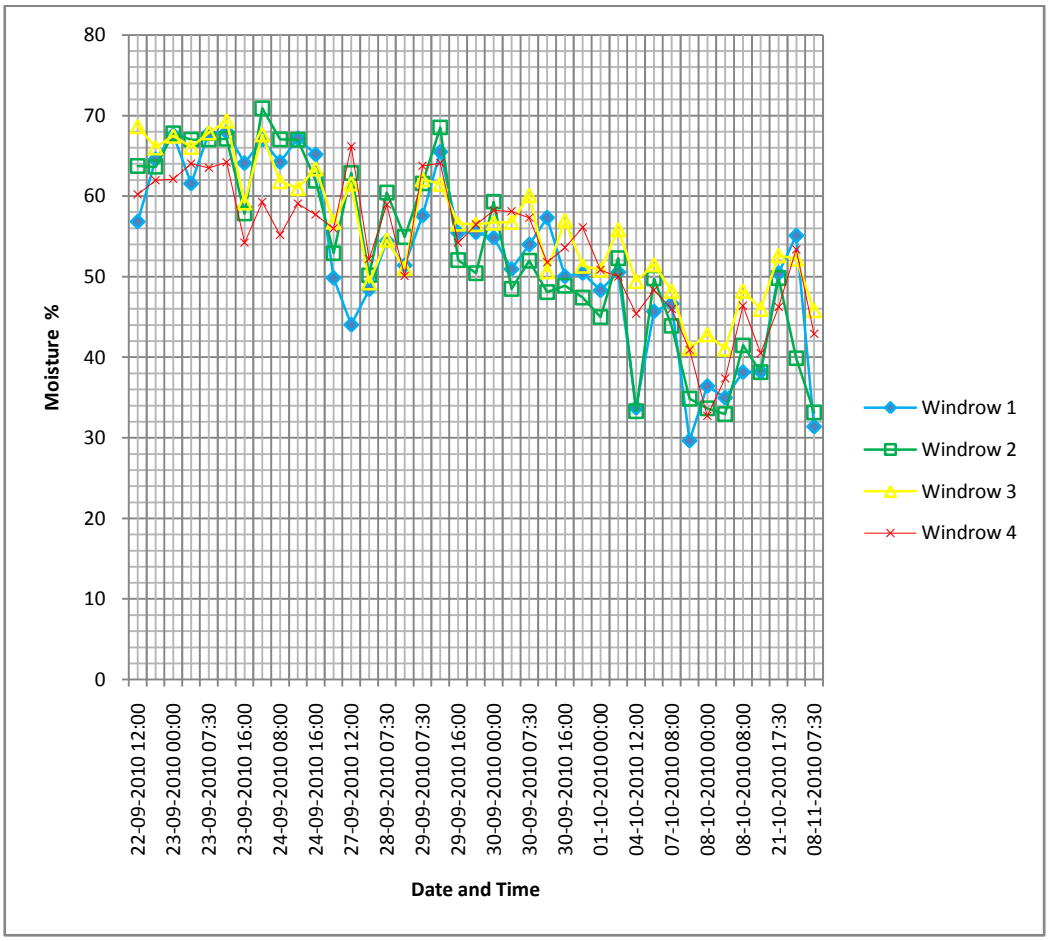


Figure 3.5 – Average moisture % during the composting period. No significant differences ($p < 0.05$) was observed, and therefore averages were not plotted in this figure

Table 3.1: Comparative table of Student t-test values for average temperature at various depths within the four different windrows

	Windrow 1: Depth 1	Windrow 1: Depth 2	Windrow 1: Depth 3	Windrow 1: Depth 4	Windrow 1: Depth 5		Windrow 2:Depth 1	Windrow 2:Depth 2	Windrow 2:Depth 3	Windrow 2:Depth 4	Windrow 2:Depth 5		Windrow 3:Depth 1	Windrow 3:Depth 2	Windrow 3:Depth 3	Windrow 3:Depth 4	Windrow 3:Depth 5		Windrow 4: Depth 1	Windrow 4: Depth 2	Windrow 4: Depth 3	Windrow 4: Depth 4	Windrow 4: Depth 5
Windrow 1: Depth 1	-																						
Windrow 1: Depth 2	0.246	-																					
Windrow 1: Depth 3	0.229	0.946	-																				
Windrow 1: Depth 4	0.107	0.624	0.678	-																			
Windrow 1: Depth 5	0.298	0.923	0.871	0.565	-																		
Windrow 2:Depth 1	0.000	0.000	0.000	0.000	0.000		-																
Windrow 2:Depth 2	0.000	0.000	0.000	0.000	0.000		0.198	-															
Windrow 2:Depth 3	0.000	0.000	0.000	0.000	0.000		0.072	0.569	-														
Windrow 2:Depth 4	0.000	0.000	0.000	0.000	0.000		0.003	0.082	0.258	-													
Windrow 2:Depth 5	0.000	0.000	0.000	0.000	0.000		0.115	0.694	0.882	0.216	-												
Windrow 3:Depth 1	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.015	0.000		-										
Windrow 3:Depth 2	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.004	-									
Windrow 3:Depth 3	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.003	0.888	-								
Windrow 3:Depth 4	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.000	0.382	0.459	-							
Windrow 3:Depth 5	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.010	0.895	0.791	0.340	-						
Windrow 4: Depth 1	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.000	0.007	0.011	0.096	0.008		-				
Windrow 4: Depth 2	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.012	-			
Windrow 4: Depth 3	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.016	0.838	-		
Windrow 4: Depth 4	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.005	0.807	0.645	-	
Windrow 4: Depth 5	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.001	0.000		0.084	0.415	0.521	0.286	-
Ambient Temperature	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000

Water was added to the windrows by means of water pipes attached to the windrow turner. Input of water could not be controlled directly, due to the watering method used. From Figure 3.5 the general trend seems to be that moisture is lost during the composting process, even when additional water was added. The average moisture content of all four windrows was above 50% (52.64, 53.0, 56.08 and 53.83 for Windrows 1 - 4 respectively).

Although the water added to the various windrows could not be measured directly, Figure 3.5 and Table 3.2 indicates that the watering was fairly constant in all windrows. No significant difference ($p < 0.05$) could be observed in the average windrow moisture levels for the whole period (Table 3.2). On average the moisture content was maintained within a narrow range, also indicated by Table 3.2.

Table 3.2: Comparative table of windrow moisture data *

	Windrow 1	Windrow 2	Windrow 3	Windrow 4
Windrow 1	-	0.36	3.44	1.19
Windrow 2	0.89	-	3.08	0.83
Windrow 3	0.12	0.17	-	-2.25
Windrow 4	0.59	0.71	0.22	-

***Black text indicates p-values obtained using Student t-test on all moisture content measurements made during the composting cycle.**
Yellow highlighted red text indicates the difference between average windrow moisture content in %

From Figure 3.5 it seems that moisture was lost more rapidly from Windrows 1 and 2, whilst retention was higher in Windrow 3 and 4. At the end of the composting process (i.e. the last week) Windrows 1 and 2 lost nearly 20% of its moisture, whilst Windrows 3 and 4 only lost about 10%.

Windrow 3 had the highest moisture content, followed by Windrows 4, 2 and 1 in sequence (Table 3.2). Moisture distribution however was not constant within the same windrow. Figure 3.6 represents the data obtained from varying depths within the same windrow.

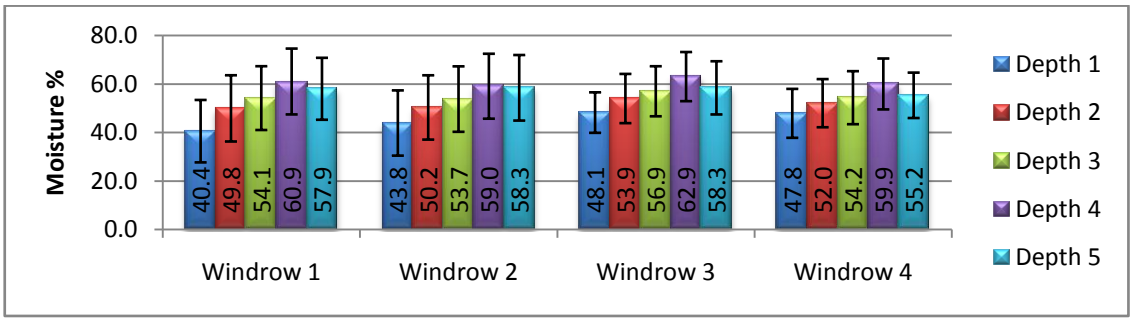


Figure 3.6: Average moisture content % during the composting period at different distances from the windrow surface**.** Depth 1 indicates the surface of the windrow, with distance from the surface increasing to depth 5. Standard deviations are depicted as both positive and negative error bars.

The lowest moisture content was observed in all four windrows at depth 1, therefore closest to the surface of the windrow. Moisture content increased towards the centre of the windrows, with a maximum average moisture content measured at depth 4. Table 3.3 compares the moisture measurements obtained using the Student's t-test.

Within Windrow 1 measurements obtained at depths 3 – 5 were statistically comparable ($p > 0.05$). No significant difference could be observed between corresponding depths in Windrow 1 and the other windrows, with the exception of depth 1. Depth 1 in Windrow 1 was only comparable to Windrow 2 (broiler litter with PCM) (Table 3.3). This phenomenon is further demonstrated by the fact that Windrow 3 and 4 showed no significant difference in values obtained from depth 1 ($p = 0.81$).

With the exception of depth 1, windrow moisture content showed comparable results. Moisture levels of the core (depths 2 – 5) corresponded well with each other, with the exception of depth 4 values measured in Windrows 2 and 3 ($p = 0.04$).

3.3 Chemical Analysis

Broiler litter and woodchips were analysed by NviroTek Labs (Brits, South Africa) for their respective N and ash value as well as carbon to nitrogen ratios. Ash values were 2.51 % and 14.07 %, while N values were 0.33 % and 4.87 % for woodchips and broiler litter respectively. The broiler litter used in this project had a C:N ratio of 10.3:1 and blue gum woodchips had a C:N ratio of 172:1.

After completion of the composting protocol used by Galltec (PTY) Ltd. samples of all four windrows were taken and elemental composition, ash and moisture percentage as well as pH and final C:N ratio were determined. Table 3.4 summarizes macronutrient levels obtained at the end of the composting process.

Table 3.4: Primary and secondary plant macronutrients at the end of the composting process

	Primary Macronutrients			**Secondary Macronutrients**		
	N	**K**	**P**	**Mg**	**S**	**Ca**
	%					
Windrow 1	2.56	3.31	1.58	0.72	0.61	2.77
Windrow 2	2.28	3.09	1.64	0.79	0.59	2.92
Windrow 3	1.69	2.31	1.11	0.55	0.45	2.18
Windrow 4	1.1	2.22	0.74	0.46	0.33	1.64

Table 3.3: Comparative table of Student t-test values for average moisture content at various depths within the four different windrows

	Windrow 1: Depth 1	Windrow 1: Depth 2	Windrow 1: Depth 3	Windrow 1: Depth 4	Windrow 1: Depth 5		Windrow 2:Depth 1	Windrow 2:Depth 2	Windrow 2:Depth 3	Windrow 2:Depth 4	Windrow 2:Depth 5		Windrow 3:Depth 1	Windrow 3:Depth 2	Windrow 3:Depth 3	Windrow 3:Depth 4	Windrow 3:Depth 5		Windrow 4: Depth 1	Windrow 4: Depth 2	Windrow 4: Depth 3	Windrow 4: Depth 4	Windrow 4: Depth 5
Windrow 1: Depth 1	-																						
Windrow 1: Depth 2	0.00	-																					
Windrow 1: Depth 3	0.00	0.04	-																				
Windrow 1: Depth 4	0.00	0.00	0.00	-																			
Windrow 1: Depth 5	0.00	0.00	0.07	0.12	-																		
Windrow 2:Depth 1	0.11	0.00	0.00	0.00	0.00		-																
Windrow 2:Depth 2	0.00	0.88	0.05	0.00	0.00		0.00	-															
Windrow 2:Depth 3	0.00	0.07	0.84	0.00	0.05		0.00	0.09	-														
Windrow 2:Depth 4	0.00	0.00	0.02	0.31	0.61		0.00	0.00	0.01	-													
Windrow 2:Depth 5	0.00	0.00	0.05	0.19	0.83		0.00	0.00	0.03	0.77	-												
Windrow 3:Depth 1	0.00	0.26	0.00	0.00	0.00		0.02	0.18	0.00	0.00	0.00		-										
Windrow 3:Depth 2	0.00	0.06	0.68	0.00	0.02		0.00	0.09	0.86	0.00	0.01		0.00	-									
Windrow 3:Depth 3	0.00	0.00	0.20	0.02	0.47		0.00	0.00	0.14	0.21	0.35		0.00	0.06	-								
Windrow 3:Depth 4	0.00	0.00	0.00	0.36	0.01		0.00	0.00	0.00	0.04	0.02		0.00	0.00	0.00	-							
Windrow 3:Depth 5	0.00	0.00	0.03	0.14	0.83		0.00	0.00	0.02	0.73	0.98		0.00	0.00	0.30	0.01	-						
Windrow 4: Depth 1	0.00	0.21	0.00	0.00	0.00		0.05	0.15	0.00	0.00	0.00		0.81	0.00	0.00	0.00	0.00		-				
Windrow 4: Depth 2	0.00	0.41	0.14	0.00	0.00		0.00	0.50	0.22	0.00	0.00		0.02	0.22	0.00	0.00	0.00		0.01	-			
Windrow 4: Depth 3	0.00	0.04	0.87	0.00	0.04		0.00	0.06	0.95	0.01	0.02		0.00	0.79	0.12	0.00	0.01		0.00	0.15	-		
Windrow 4: Depth 4	0.00	0.00	0.00	0.53	0.26		0.00	0.00	0.00	0.60	0.40		0.00	0.00	0.04	0.08	0.32		0.00	0.00	0.00	-	
Windrow 4: Depth 5	0.00	0.01	0.61	0.00	0.11		0.00	0.01	0.46	0.04	0.08		0.00	0.28	0.35	0.00	0.04		0.00	0.02	0.45	0.00	-

Table 3.4 indicates that for all samples potassium was higher than both nitrogen and phosphorous for all windrow composts. Nitrogen was lowest in Windrow 4, and highest in Windrow 1. Phosphorous was also the lowest in Windrow 4. However, it was higher in compost obtained from Windrow 2. Magnesium, sulphur and calcium were consistently the lowest in Windrow 4, whilst Mg and S were highest in Windrow 1. Windrow 2 had the highest calcium levels.

Phytotoxicity and plant wellbeing is also dependant on several plant micro-nutrients. Values for plant micro-nutrients are represented by Table 3.5:

Table 3.5: Plant micronutrients at the end of the composting process

	Fe	Mn	Cu	Zn	Mo	B	Na
	mg/kg						%
Windrow 1	4474	739	99	531	11.65	69.80	0.46
Windrow 2	6849	826	101	520	10.72	61.80	0.42
Windrow 3	7849	597	79	373	7.83	49.36	0.34
Windrow 4	6374	491	58	265	5.74	12.86	0.37

Iron was present in large amounts in all compost samples, with Windrow 3 having the highest amount and Windrow 1 the lowest. Again, Windrow 4 consistently had the lowest amount of micronutrients, except for sodium which was slightly lower in Windrow 3. The highest amount of Mn and Cu was present in Windrow 2, with Windrow 1 having the highest values for Zn, Mo, B, and Na.

Table 3.6: Ash, pH, C:N and moisture content at the end of the composting process

	Ash	pH	C : N	Moisture
	%			%
Windrow 1	31.54	8.51	15.5 : 1	28.42
Windrow 2	37.13	8.79	16.1 : 1	26.72
Windrow 3	37.93	9.27	21.4 : 1	31.52
Windrow 4	39.57	8.99	31.9 : 1	30.13

Ash values were lower in Windrow 1 when compared to Windrows 2 – 4. The pH for all samples was slightly alkaline, with Windrow 3 having the highest pH. Windrow 4 had the highest C:N ratio at the end of the composting process, followed by Windrows 3, 2 and 1 in sequence. Moisture was the lowest in Windrow 2, and highest in Windrow 3.

Overall, Windrows 1 and 2 contained higher amounts of primary and secondary plant macronutrients. The results obtained for nitrogen was further analysed to evaluate nitrogen dynamics within the windrows. These calculations are presented in Appendix B. The largest amount of Nitrogen (2.38%) was lost from Windrow 2 during the composting period observed. Windrow 1 lost 2.31% of its initial nitrogen. Windrows 3 and 4 had a greatly reduced nitrogen loss, estimated at 0.91% and 1.28% respectively. The chemical characterization of the composts concludes the physico-chemical characterization of the four windrows for the composting period observed.

Another crucial consideration is that the differences in chemical values arose from the differences windrow formulations. Windrows 3 and 4 had half the amount of broiler litter at the start of the composting process, resulting in differences in especially the micronutrient content.

3.4 DNA Isolation

Genomic DNA (gDNA) was extracted from 9 samples of each windrow, resulting in 36 genomic DNA samples. Figure 3.7 is an example image of an ethidium bromide stained 1% (w/v) agarose gel. This illustrates the quality and quantity of DNA extracted from compost samples.

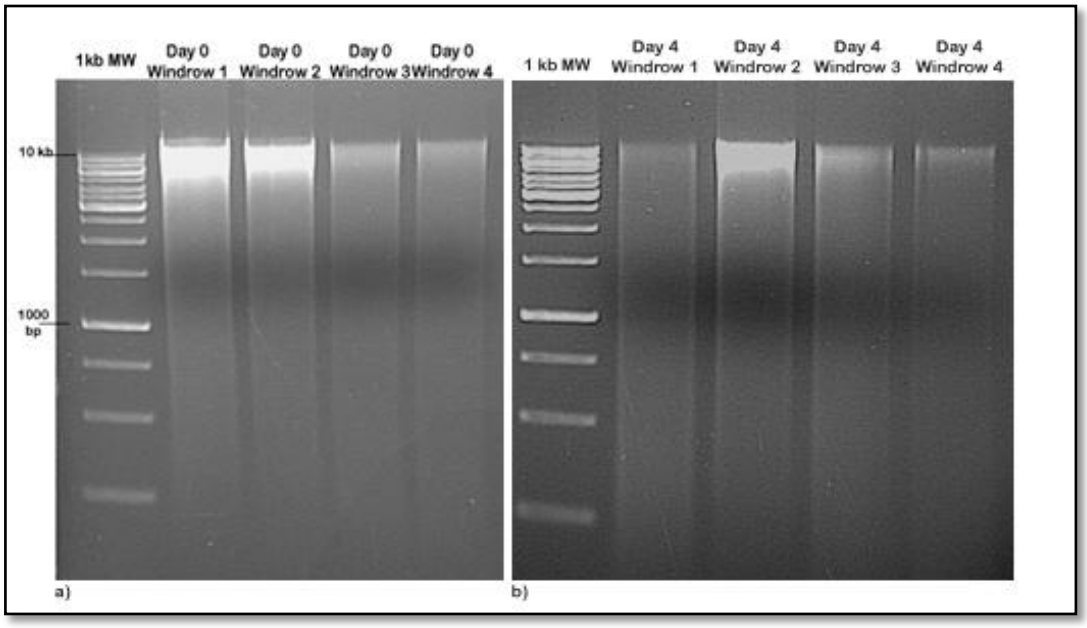


Figure 3.7: A 1 % (w/v) ethidium bromide stained agarose gel containing genomic DNA isolated using a Machery-Nagel NucleoSpin Soil kit. Samples were from the first day of composting. A 1 kb molecular size marker (O'GeneRuler 1 kb DNA Ladder, Fermentas Life Science, USA) was loaded in lane 1 (1 kb MW).

Figure 3.7a represents gDNA isolated from day 0 of all windrows, whilst Figure 3.7b represents gDNA from day 4. A small amount of DNA shearing is visible on the gel in both Figure 3.7a and b. This was also present in all other samples subjected to DNA extraction using this kit.

Overall the results from spectrophotometric analysis of the isolated DNA indicated that the DNA was of good quality. The average yield for all samples were 35.06 ± 15.01 ng.μL^{-1}, with average 260/280 and 260/230 values being 1.77 and 1.09 respectively.

3.5 DNA Amplification

3.5.1 16S rDNA Amplification

Isolated genomic DNA was amplified using the optimized PCR conditions described in Section 2.8.1. Figure 3.8 is an example the results obtained for primer set 27f and 1492r.

The product obtained from this first PCR was then subsequently subjected to another amplification, using primers 341f-GC and 907r. An example of the results obtained from this nested PCR are given in Figure 3.9.

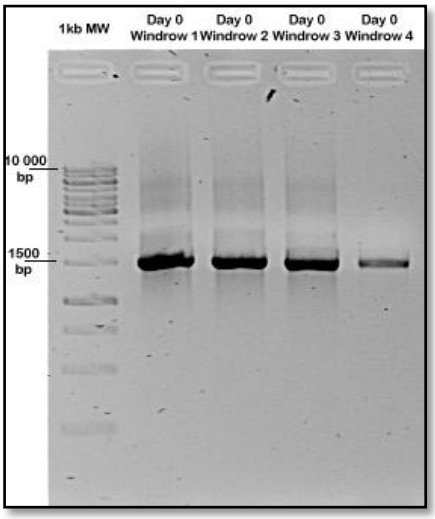


Figure 3.8: An inverted photograph of a 1.5% (w/v) agarose gel stained with ethidium bromide containing product from the first PCR. A 1kb moleculer weight marker (O'GeneRuler 1 kb DNA Ladder, Fermentas Life Science, USA) is indicated by 1kb MW. Amplicon length was approximately 1500 bp.

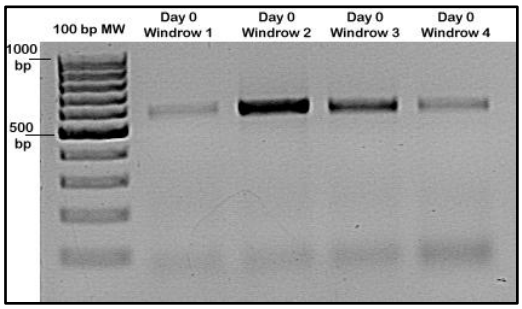


Figure 3.9: An inverted photograph of a 1.5% (w/v) agarose gel stained with ethidium bromide with the product from the nested PCR. A 100 bp moleculer weight marker (O'GeneRuler 100 bp DNA Ladder, Fermentas Life Science, USA) is indicated by 100 bp MW. Amplicon length was approximately 600 bp.

The product obtained from the nested PCR did not contain any smearing. There were, however, a small amount of primer-dimers present. These products were suitable for DGGE analysis.

3.5.2 18S rDNA Amplification

Amplification of the 18S rDNA from genomic DNA samples obtained from compost was problematic. Figure 3.10 is an example of the PCR product from Windrow 4 samples using primer set EF3-EF4. This is the first PCR in the nested PCR sequence.

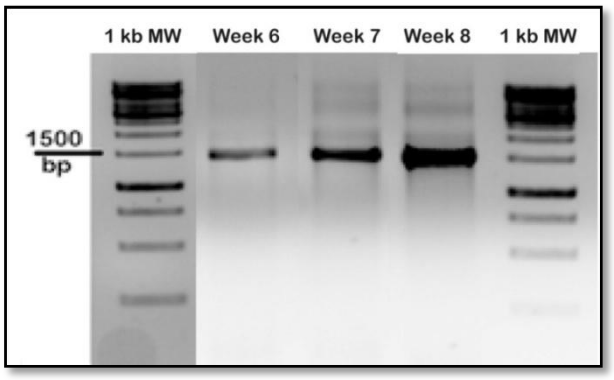


Figure 3.10: An inverted composite 1.5% (w/v) agarose gel, stained with ethidium bromide, of samples from Windrow 4. A 1kb Molecular weight marker is indicated by 1 kb MW.

Sporadic appearance/disappearance of amplifiable 18S genetic information was observed for samples from Windrows 1, 2 and 3. Although various different optimization steps were performed (i.e., increasing *Taq* polymerase, increasing $MgCl_2$, increasing cycle number) no consistent amplification could be obtained. It was thus decided that for the purpose of this study the focus should be on eukaryotic species present in Windrow 4. Samples from day 0, day 5, weeks 2, 6, 7 and 8 gave strong bands with primer set EF3-EF4 and were thus used for further analysis.

These samples were subjected to a second amplification cycle, as described in Section 2.8.2. Figure 3.11 shows some of the samples that amplified in Figure 3.8. Amplification was achieved using primer set EF4-GC and fung5.

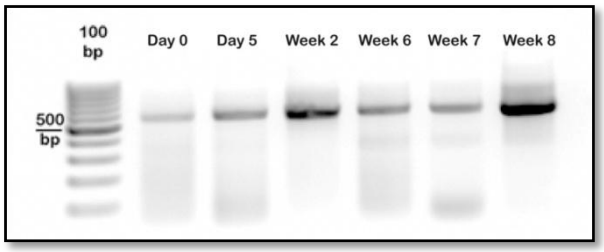


Figure 3.11: Inverted 1.5% (w/v) agarose gel image of nested eukaryotic samples from Windrow 4 stained with ethidium bromide. A 100 bp moleculer weight marker (O'GeneRuler 100 bp DNA Ladder, Fermentas Life Science, USA) is indicated by 100 bp MW. Amplicon length was approximately 550 bp.

3.6 Denaturing Gradient Gel Electrophoresis (DGGE)

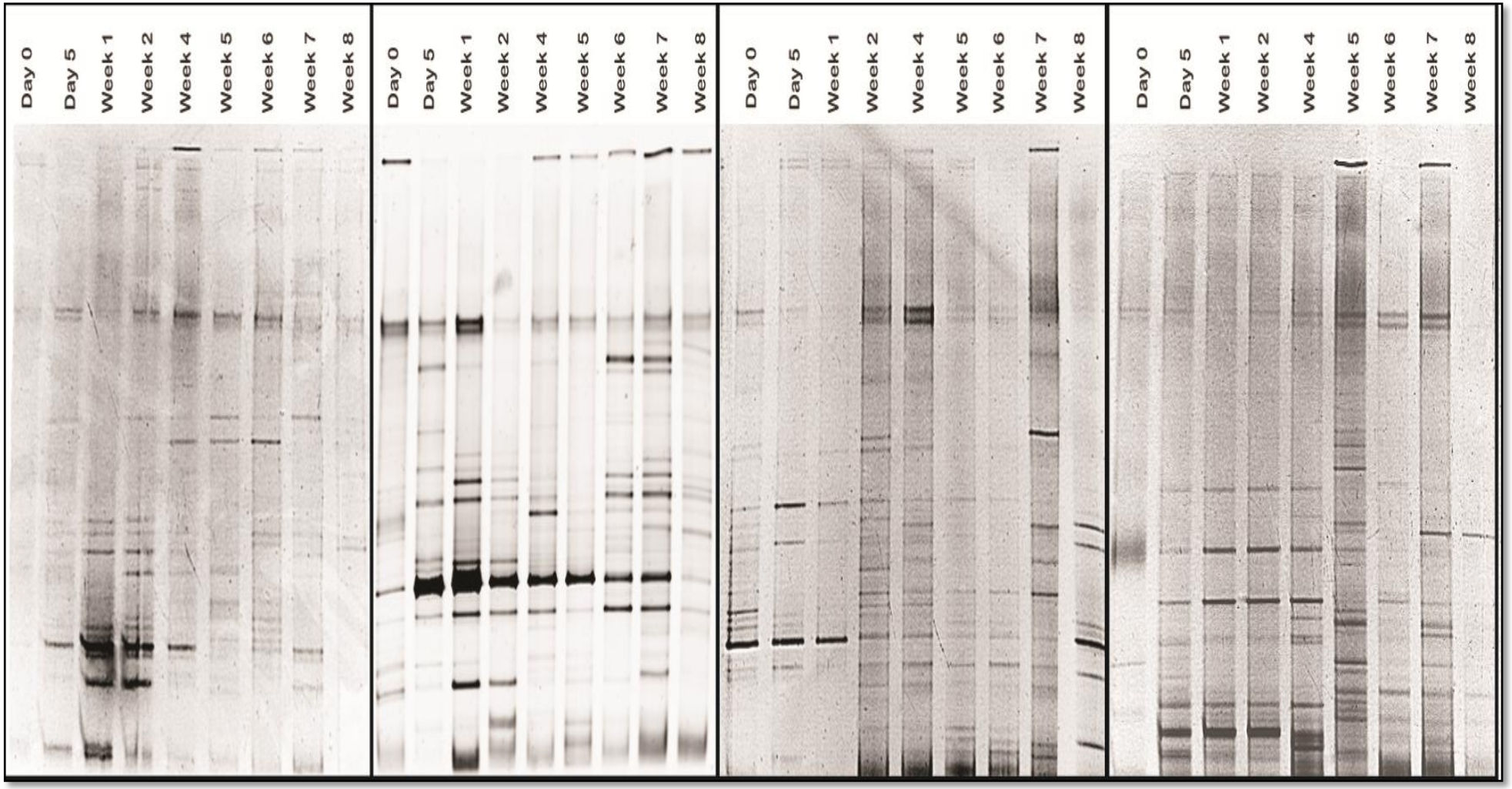
3.6.1 16S rDNA profiles

Samples were taken on Day 0, Day 5, Week 1, 2, 4, 5, 6, 7 and 8 of the composting process. Day 0 of Windrow 4 was loaded onto all the gels as a reference to allow for inter-gel comparisons. The gel images obtained were aligned in Photoshop CS3 using the inter-gel marker as reference. Special care was taken to ensure that gel dimensions remained intact. Figure 3.12 gives the prokaryotic 16S rDNA composite gels over all periods for Windrow 1 (Figure 3.12a), Windrow 2 (Figure 3.12b), Windrow 3 (Figure 3.12c) and Windrow 4 (Figure 3.12d).

3.6.2 18S rDNA profiles

Samples were amplified from Day 0, Day 5, Week 2, Week 6, Week 7 and Week 8. These samples were electrophoresed for 5 hours at 200V. The number of bands present is much less than the rich species diversity observed in prokaryotic samples. The DGGE results are shown in Figure 3.13. From Figure 3.13, seven distinct bands could be excised.

Figure 3.13: A negative image of an ethidium bromide stained linear denaturing polyacrylamide:bisacrylamide (37.5:1) (30-50% denaturing) gel of eukaryotic PCR samples obtained from Windrow 4. The gel also had an increasing gradient of polyacrylamide:bisacrylamide from 6%-8%.



a.) b.) c.) d.)

Figure 3.12: Negative composite image of DGGE profiles obtained by matching intergel reference lane makers in Photoshop CS3**.** A.) corresponds to Windrow 1, b.) Windrow 2, c.) Windrow 3 and d.) Windrow 4. In essence gels are two dimensional, as samples were loaded horizontally according to the time of sampling.

3.7 Microbial Community Dynamics

Figure 3.12 was imported into Phoretix 1D pro (evaluation licence) and analyzed. A total of 454 prokaryotic bands were identified in the 36 samples. A presence\absence matrix table was constructed, using relative migration values (rf-values). The identified bands were categorized into 55 distinct rf locations. Subsequently a dendrogram was constructed to investigate profile similarity. The Dice coefficient of similarity was used together with Ward's clustering algorithm. Results are shown in Figure 3.14

Figure 3.14 contains two clusters (A and B), with 5 sub-clusters (A1, A2, B1, B2 and B3). Cluster A contains most of the lanes from Windrows 3 and 4, whilst cluster B contains lanes primarily from Windrows 1 and 2. Sub-cluster A1 contains 5 lanes of Windrow 3, with most of these samples being from the end of the composting process (lanes 4-8 corresponding to weeks 4, 5, 6 and 7). Also grouped in sub-cluster A1 is one lane from Windrow 1 (corresponding to week 5) and Windrow 4 (corresponding to week 5). Sub-cluster A2 grouped samples from Windrow 4 together. Lanes 5, 7 and 8 grouped well together, and a similarity is also present between lanes 3 and 4 of Windrow 4 is also observed.

Cluster B was separated into 3 sub-clusters. Sub-cluster B1 contained mainly samples from Windrow 2. The starting period of Windrow 2 (i.e. samples 1 and 2) grouped together with the end of the period (i.e. samples 7, 8 and 9). Windrow 4 sample 2 and Windrow 3 sample 1 was also included in the grouping of this sub-cluster. With the exception of Windrow 4 sample 1, sub-cluster B2 only contained samples from Windrow 1. Windrow 4 sample 1 and Windrow 1 sample 1 clustered together. Sub-cluster B3 contained samples from Windrows 2, 3 and 4. These samples were from the start of the composting process for Windrow 2, the start and end of Windrow 3, and the end profile for Windrow 4.

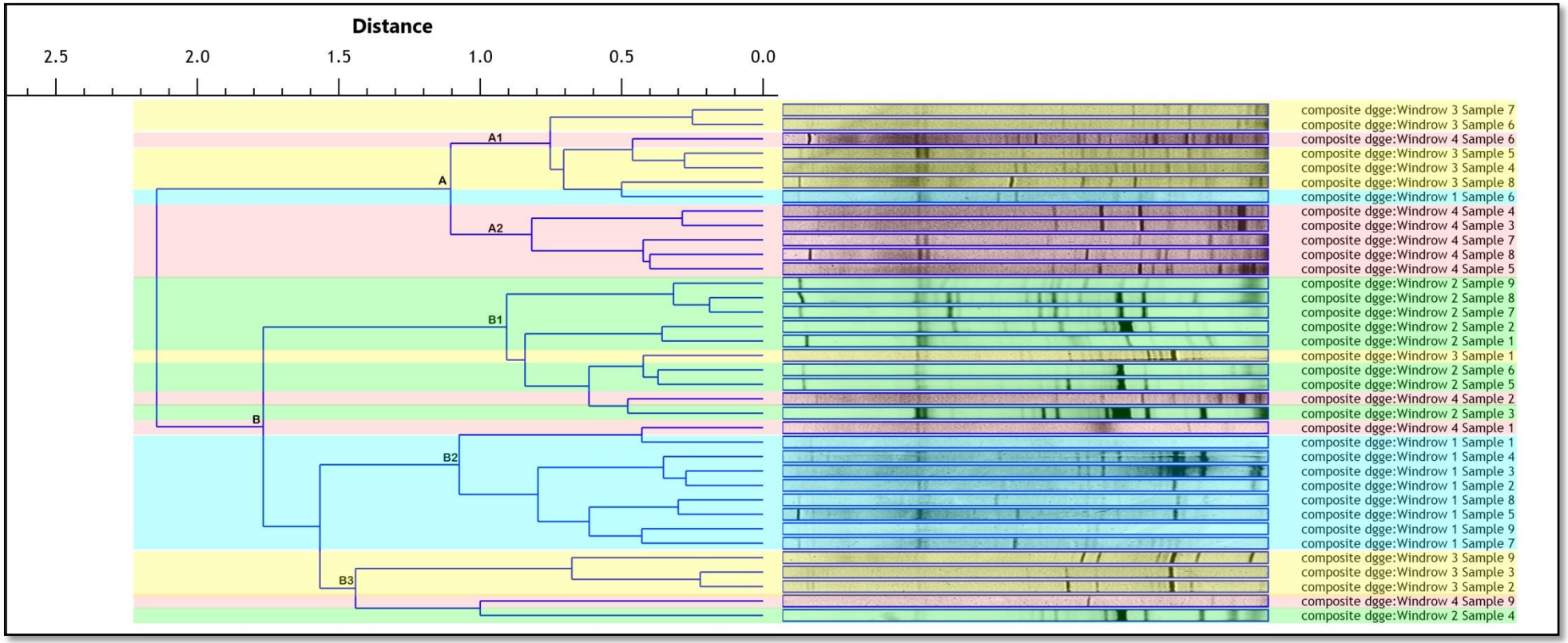


Figure 3.14: Dendrogram obtained from composite DGGE profiles using Ward's clustering algorithm and the Dice coefficient of similarity. Results from Windrow 1 are highlighted in blue, Windrow 2 in green, Windrow 3 in yellow and Windrow 4 in red.

Species richness was determined by counting the number of bands present within each DGGE lane (time frame). Figure 3.15 gives the species richness values for all the windrows studied.

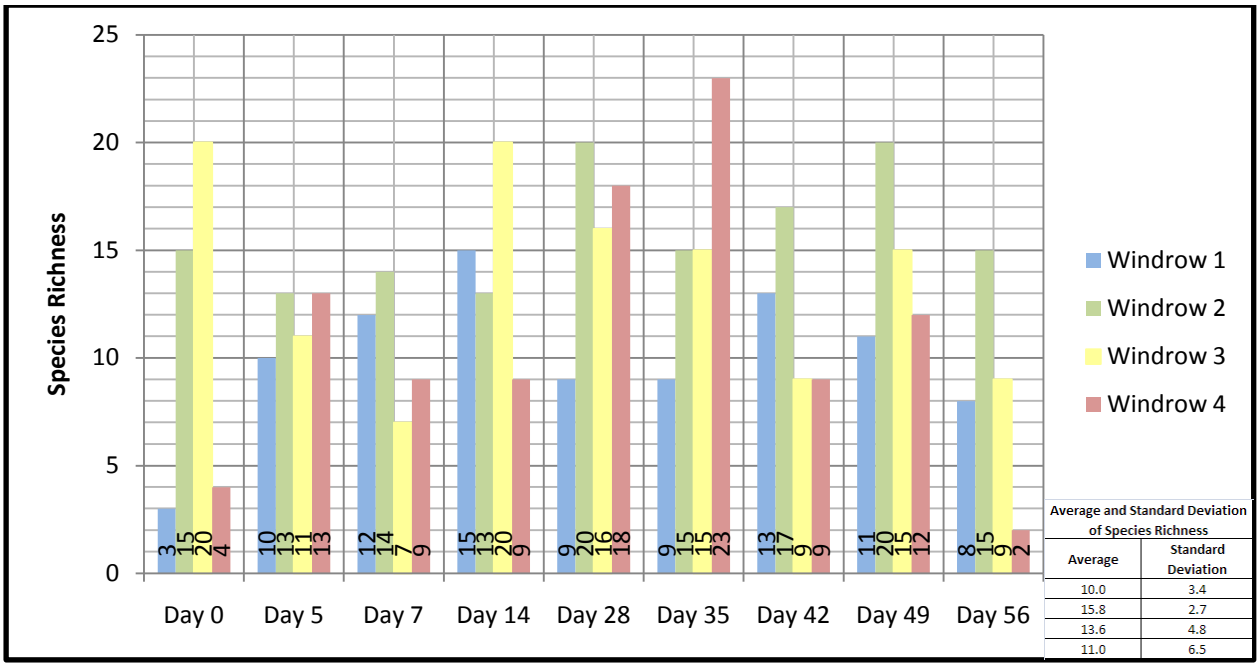


Figure 3.15: Species Richness as obtained from bands present in DGGE lanes for all four windrows. Each band was considered as a unique DNA sequence, corresponding to a single organism.

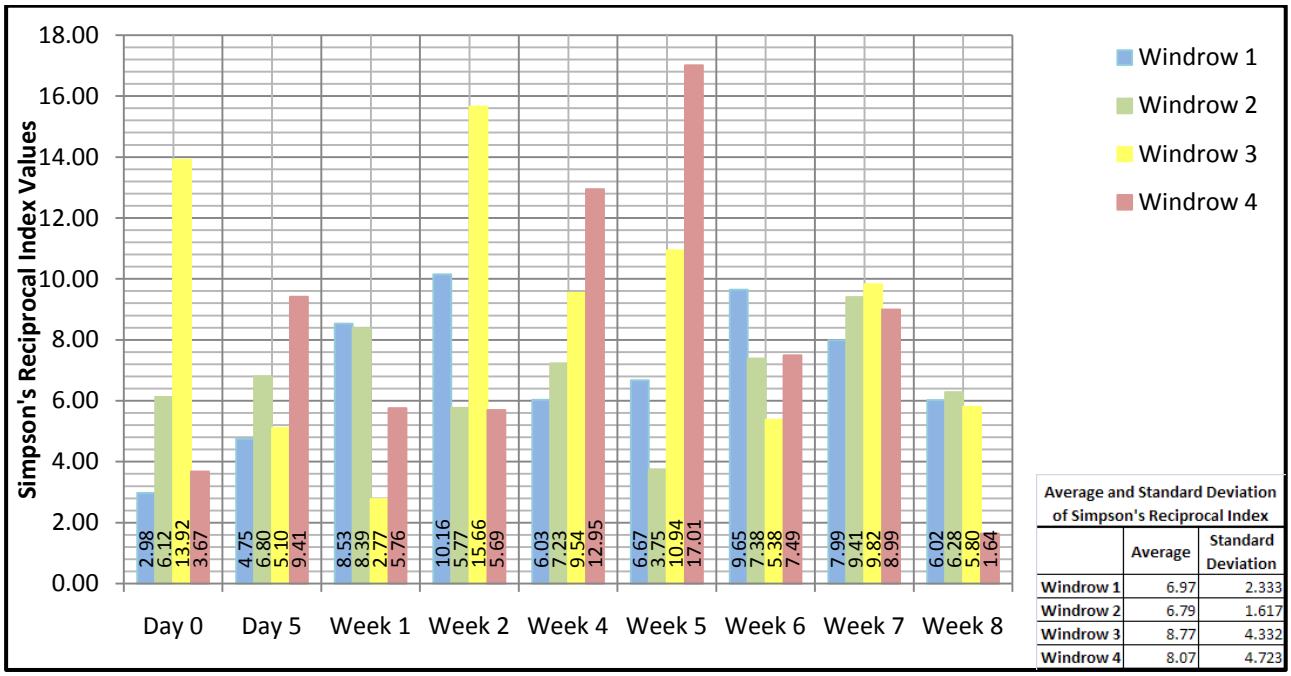
Species richness (as indicated in Figure 3.15) was low for day 0 in Windrows 1 and 4, whilst higher for Windrow 2 and 3. Species richness increased for the first 14 days in Windrow 1, decreased during the period of 14 days to 42 days, increased again for the period days 42 to 49, and returned to almost the same value observed at day 42 for day 56.

Windrow 2 species richness remained fairly constant for the first 14 days, and then increased by 5 species in the period that lasted from day 14 to day 28. Diversity remained above 15 for the remainder of the period studied.

Windrow 3 had a high number of species at day 0. This decreased within the first 7 days of composting. After 1 week of composting (i.e. the time frame Day 7 – Day 14) species richness increased again. Thereafter species richness varied each week with no specific trends.

Windrow 4 had an increase in species richness during the first 5 days of composting. This then stabilized for days 7 to 14. Thereafter an increase in the number of species could be observed, increasing to 23 species observed in samples from day 35. For the last three weeks of composting species richness decreased, and the lowest value was observed at the end of the composting process.

The banding patterns and peak height data were used to calculate Simpson's Reciprocal Index for all samples. These values are plotted in Figure 3.16.



Average and Standard Deviation of Simpson's Reciprocal Index

	Average	Standard Deviation
Windrow 1	6.97	2.333
Windrow 2	6.79	1.617
Windrow 3	8.77	4.332
Windrow 4	8.07	4.723

Figure 3.16: Simpson's Reciprocal Index (SRI) values for DGGE profiles from Windrows 1–4. The inserted table indicates the average and standard deviation for composting period.

Windrow 1 had the lowest bacterial diversity based on Simpson's reciprocal index at the start of the composting process. This increased during the first two weeks of composting. A decrease in diversity was observed that occurred during weeks 3 and 4, however the diversity increased again during week 5 – 6. The final two weeks of composting resulted in a decrease in diversity for Windrow 1.

The diversity of Windrow 2 was more than double that of Windrow 1 at the start of the composting process. A steady increase was observed in SRI values during the first two weeks of composting (i.e. from day 0 – end of week 1 in figure 3.16). During week two a decrease in diversity was observed. After an increase during week 3 and 4, the diversity was reduced to the lowest value obtained in week 5. During the fortnight that lasted from week 6 to week 7, a steady increase in diversity was observed for Windrow 2. Similar to Windrow 1, diversity in windrow DGGE profiles decreased during the last week of composting.

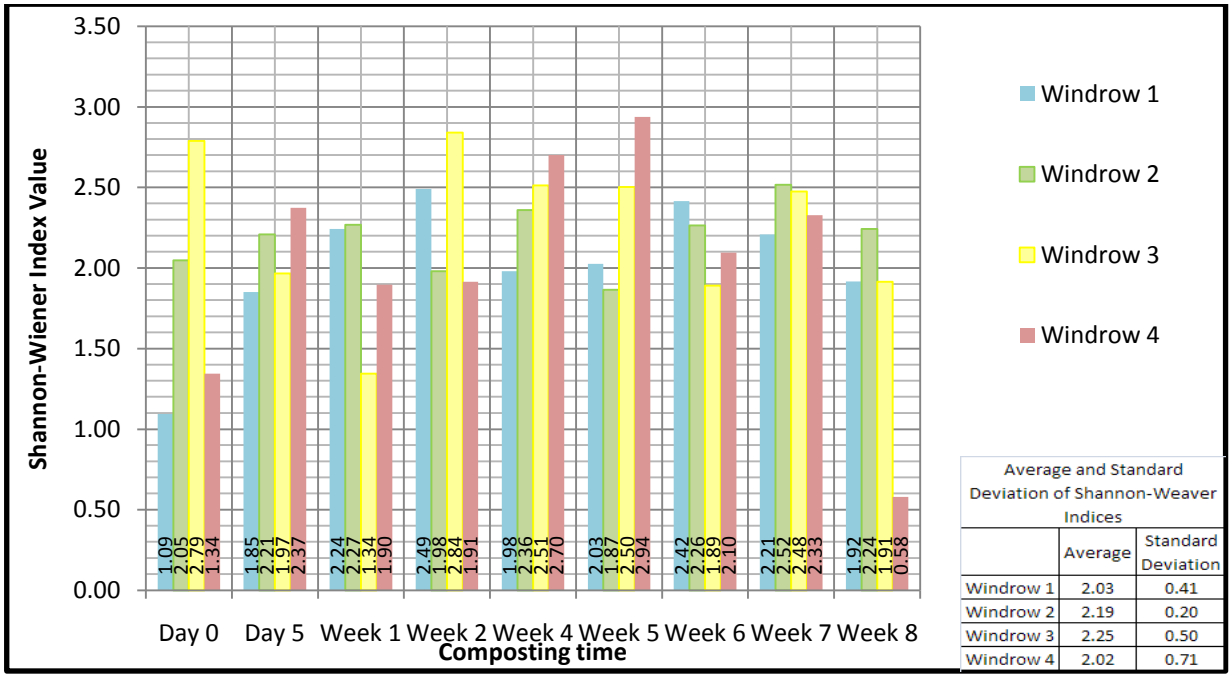
When compared to all the other windrows, Windrow 3 showed a different pattern during the first three weeks of composting. Diversity decreased to the lowest value obtained. A marked increase in diversity, resulting in the highest diversity observed for the windrow, was achieved within during week 2. During weeks 3 and 4 a decrease occurred and after a slight recovery,

the diversity decreased in week 6 of composting. During the last two weeks, diversity increased in week 7 and decreased in week 8. This trend was also observed in all other windrows.

In Windrow 4 the diversity increased during the first 4 days of composting. After one week of composting the diversity decreased and remained at the same level for two weeks. During the following three weeks of composting diversity increased to the highest diversity observed for all windrows (17.01). During week 6 diversity decreased, followed by a slight increase in week 7 and then dropped to the lowest value observed for all windrows for the composting period.

The Simpson's Reciprocal Index (SRI) values indicate that overall the highest diversity could be observed in Windrow 3 (Average = 8.77). The highest diversity estimate was obtained in Windrow 4 during week 5. Lowest value was obtained also in Windrow 4 at the end of the composting process (week 8). Windrow 2 had the smallest shifts in microbial diversity, if the standard deviation is taken as an account of the range within which the index values are distributed.

Peak height data was also used to calculate Shannon-Weaver index. These values are presented in Figure 3.17. The same general trends that could be observed in the Figure 3.16 are also present in Figure 3.17, with the exception of diversity being slightly higher in Windrow 2 during week 7 of the composting process when compared to Windrow 3.



Average and Standard Deviation of Shannon-Weaver Indices

	Average	Standard Deviation
Windrow 1	2.03	0.41
Windrow 2	2.19	0.20
Windrow 3	2.25	0.50
Windrow 4	2.02	0.71

Figure 3.17: Shannon-Weaver indices for DGGE profiles for Windrows 1 – 4. The inserted table indicates the average and standard deviation for the composting period.

The table in Figure 3.17 indicates the averages and standard deviations of the Shannon-Weaver values. Highest average diversity was observed in Windrow 3 (2.25). On average, Windrow 1 and 4 had similar diversity indices. The lowest standard deviation (0.2) was obtained from Windrow 2, whereas Windrow 4 had the highest standard deviation (0.71).

Multivariate analysis was performed using Canoco software for Windows 4.5. Temperature and moisture results, physico-chemical data as well as peak height data were included in the analysis. Figures 3.18a and b are bi-plots of the resultant Canonical Correspondence Analysis (CCA).

In Figure 3.18 windrow physico-chemical parameters are represented as vectors radiating from the centre of each bi-plot. These vectors indicates the influence of the parameter on the windrow DGGE banding patterns (Figure 3.18a, indicated as small circles) and the bands rf-values as an indication of the species implied by the band position (Figure 3.18b, indicated by triangles).

Large variances, indicated by the length of the vector, was observed for C:N ratio, temperature and most of the chemical parameters tested (with the exception of Na, Fe, pH). Moisture had the smallest variance (Figure 3.18).

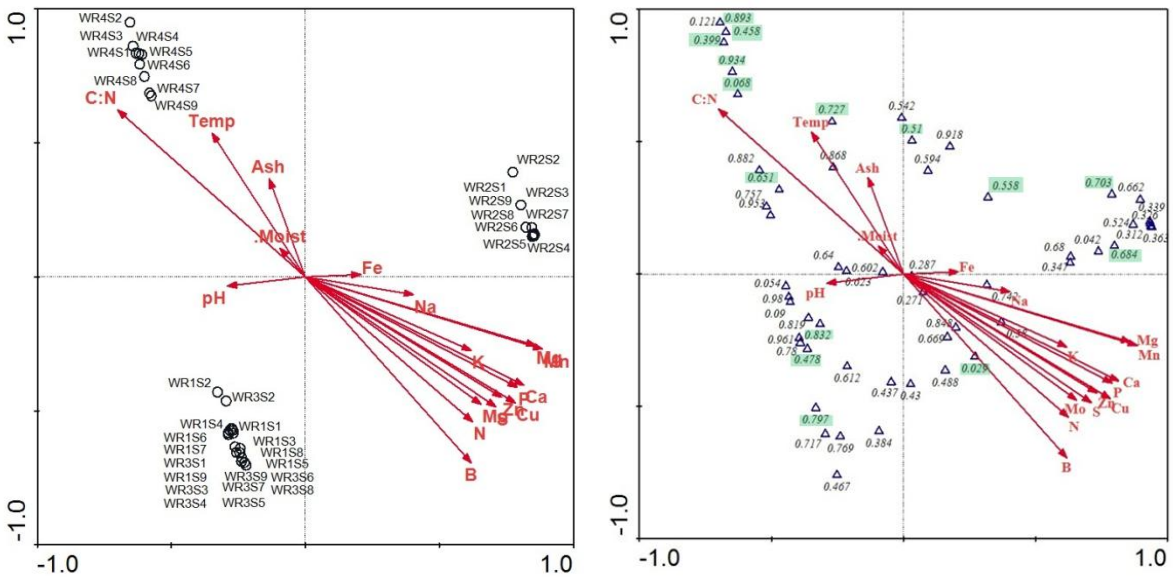


Figure 3.18: Canonical Correspondence Analysis (CCA) performed on the (a) Bands and (b) DGGE lane patterns obtained from the four windrows studied, physico-chemical analysis results, moisture and temperature data. Eigen-values for the first two axes were 0.59 and 0.26 respectively. The abbreviation WR indicates windrow number, whilst S is an indication of the sample number; S1- indicating day 0 and S9 indicating day 56. Bands were identified using their respective rf-values. Green highlighted bands were sequenced to obtain their identities (Section 3.8).

The cosine of the angle between two vectors is an indication of the correlation between the two variables. The smaller this angle is, the greater is the correlation between the two variables. C:N ratio, moisture, temperature and ash content all had a strong correlation with one another (Figure 3.18). This indicates that these parameters influenced one another. These parameters also had a negative correlation with the chemical data obtained.

In Figure 3.18a banding patterns were evaluated against the physico-chemical results. The banding patterns from Windrows 1 and 3 were indistinguishable using this technique. The profiles grouped closely together. Windrows 2 and 4 could be distinguished as distinct groupings. The banding patterns observed in Windrow 4 were influenced mostly by C:N ratio (Table 3.6), windrow temperature (Figure 3.2) and to a lesser degree the ash content (Table 3.6). Moisture did not have a significant effect on DGGE banding patterns. This is supported by the fact that no significant differences were observed between the average moisture content of the various windrows.

In Figure 3.18b the same internal windrow parameters and physico-chemical results were used. However instead of analyzing DGGE banding patterns, individual bands were analyzed. Each band was associated with its corresponding rf-value and labelled accordingly. The distance of a perpendicular line from the band to a variable (vector) indicates the influence of the variable on the specific organism.

Figure 3.18b implies that the C:N ratio had an effect on bands located at several rf-values (0.068, 0.121, 0.399, 0.458, 0.651, 0.757, 0.882, 0.893, 0.934 and 0.953). Temperature largely influenced two organisms, located at rf-values of 0.727 and 0.868 respectively. The latter band was also influenced by ash value and C:N ratio. A large group of bands (organisms) were influenced by the pH of the compost.

A temperature response curve (not shown) indicated that organisms located at 0.727; 0.934, 0.893 and 0.797 were present only at high temperatures, whilst 0.684 completely disappeared at high temperatures.

The gel image presented as Figure 3.13 was analysed to produce an presence/absence matrix table, using the same technique employed for Figure 3.12. Diversity indices for eukaryotic species present in Windrow 4 are presented in Figure 3.19. Species richness was lower for eukaryotic species. Diversity and species richness increased towards the later stage of the composting process. Lowest diversity was observed on day 14, while the highest diversity was observed on day 56 of composting.

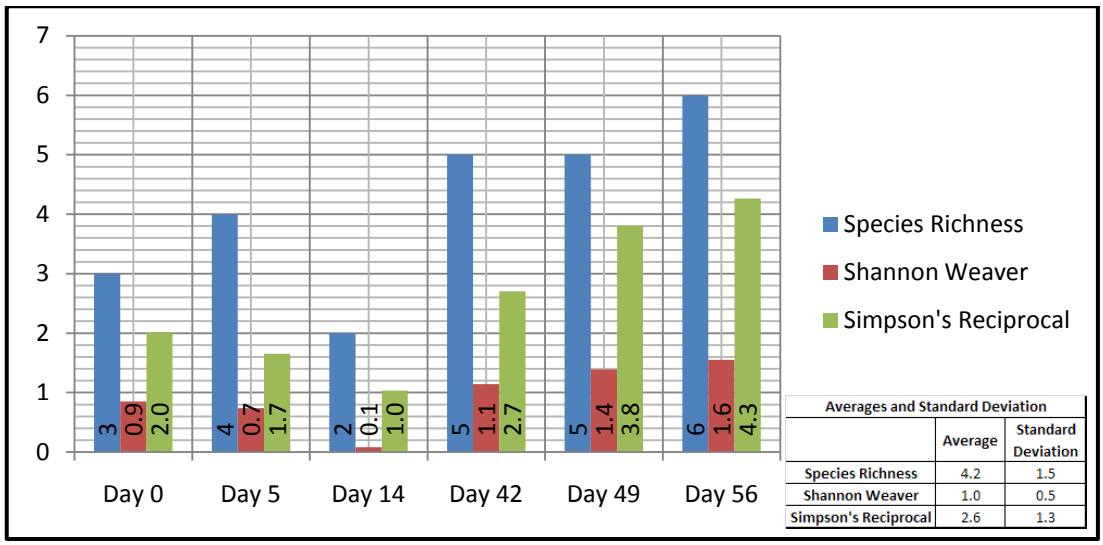


Figure 3.19: Species richness, Shannon-Weaver and Simpson's Reciprocal indices for eukaryotic species. The inserted table indicates average and standard deviation values.

Both the Shannon-Weaver index and the Simpson's Reciprocal index follow the same pattern. Between days 14 – 42 an increase in the number of species resulted in an increase in the diversity indices. Between days 42 – 49 species richness was similar, followed by an increase on day 56.

Microbial dynamics could be influenced by various factors. The effect that the environment in which the organisms presides are one of the main influences. Preceding results should therefore be investigated in a combined manner.

3.8 Influence Of Average Temperature And Moisture On Microbial Dynamics

A graphical summary of the average temperature and moisture profiles plotted against diversity indices are presented in Figure 3.20. The plots for average temperature and moisture were smoothed using the average temperature for the period preceding the sampling date. Shannon-Weaver values are plotted as $H \; x \; 10$.

In Windrow 1 microbial diversity and temperature was low at the commencement of this study. Average temperatures increased during the first five days, as did the microbial diversity. From day 5 to day 14 microbial diversity increased further, and average temperature stabilized. Moisture content was greatly reduced on day 14. Water was added to the windrow, and microbial diversity decreased in the following 14 days. From day 28 onwards diversity increased and was sustained until day 42. Temperatures started to increase, but was still below the thermogenic temperature range (indicated by temperatures above 40.0°C). Moisture was lost from the windrow from day 35 until the end of the composting process. Towards the end of the evaluation period (day 42 – day 56) prokaryotic diversity decreased.

Figure 3.20: Graphical summary of temperature, moisture and prokaryotic diversity results obtained in the study. Diversity Index values are presented on the secondary axis (i.e. right-axis)

Average temperatures rapidly increased in Windrow 2 during the first week of composting. The microbial diversity also increased during the first week of composting. Windrow 2 had similar diversity indices values to Windrow 1 on day 7 of composting. Moisture was also lost from the windrow. On day 14, moisture was below 40%, and diversity declined. Diversity increased on day 28, but decreased on day 35. The last three weeks of composting (day 42 – day 56) was characterized by an increase in average temperature, and large shifts in diversity. Overall Windrow 2 had a slightly higher average temperature profile compared to Windrow 1.

Windrow 3 had the highest diversity indices at the start of the windrows. Diversity quickly decreased, as average temperature increased. Again moisture was lost from the windrow during the first week of composting. Prokaryotic diversity increased during the second week of composting, and temperatures were within thermophilic ranges. As temperatures decreased during the period that lasted from day 28 – day 35 microbial diversity increased. The last three weeks was characterized by large shifts in diversity observed on the PCR-DGGE profiles. Temperatures remained above were on average 40°C for the last four weeks of composting. Windrow 3 had a significantly higher temperature profile when compared to Windrows 1 and 2.

The first week of composting in Windrow 4 was characterized by an increase in temperature and prokaryotic diversity. During the first two weeks, Windrow 4 reached its highest temperatures. Similar diversity indices where obtained for the period that lasted from day 7 – day 14. As temperatures started to decrease, microbial diversity increased. The highest diversity index score was obtained on day 35. Diversity then decreased to the lowest score on Day 56. Windrow 4 had the highest temperature profile, and temperatures were still within thermophilic ranges at the end of the composting period.

Figure 3.21 presents the possible effects that average temperature and moisture could have on the eukaryotic species present in Windrow 4. As the temperature increased and moisture decreased during the first two weeks of composting, eukaryotic diversity declined. During the last 3 weeks temperature and moisture stabilized, in turn resulting in a slight increase of the eukaryotic diversity observed.

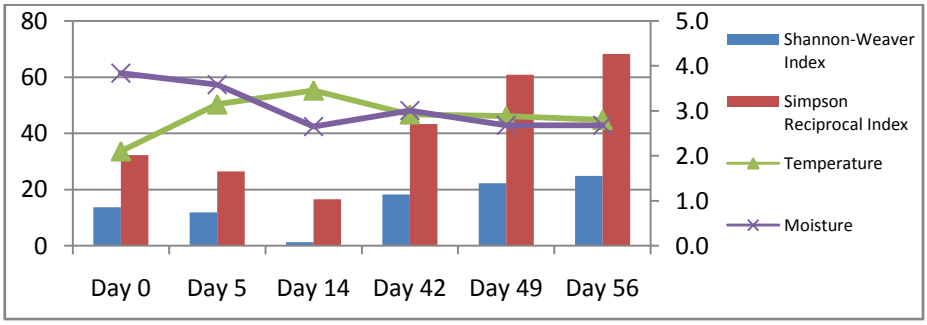


Figure 3.21: Graphical summary of temperature, moisture and eukaryotic diversity results. Diversity Index values are presented on the secondary axis (i.e. right-axis)

3.9 Prokaryotic Sequencing Results

Not all bands on the various gels were sequenced. The CCA analysis and response curves were used to select bands that were re-amplified and sequenced. Some additional bands were also included. The sequence results obtained are presented in Table 3.7.

Table 3.7 indicates the accession number for the BLASTn results, a description of the organisms, statistical probabilities for identification success and the source of the isolated bacterium. The presence or absence of sequenced organisms are also given.

Sequences were BLASTn searched using both nucleotide collection (clear rows in Table 3.7) and 16S ribosomal RNA sequences for Bacteria and Archaea (grey rows in Table 3.7). Overall sequences from 16 excised bands were obtained.

The highest identity scores with the lowest E-value were obtained from the nucleotide collection database (clear rows in Table 3.7). Of the 16 sequences analysed, 12 closely matched the sequences of uncultured bacteria. Furthermore, 12 (not necessarily the same) were from either composts or animal manures.

The average amplicon length that was sequenced was 467 bp. This translates to an average sequencing success rate of 85 %, if the average length of the amplicons are taken at 550 bp. Average E-value was 0, which indicates that the statistical chance of obtaining a wrong identification was very low.

Organisms that were the closest matches were obtained using the 16S ribosomal RNA sequence database (grey rows in Table 3.7) for Bacteria and Archaea. Dominating species were indicated as members of the order Bacillales, including members from the Genus *Bacillus* and *Paenibacillus.* *Pedobacter lentus*, *Ulvibacter antarcticus*, *Mechercharimyces mesophilus* and *M. asporophorigenens* were also identified.

Windrow 1 was largely dominated by members of *Bacillus* spp. and *Paenibacillus* spp. None of the bands identified were present on day 0 of composting in Windrow 1. In Windrow 2 ten of the sixteen sequences were identified. Fifteen of the sixteen sequences identified were from Windrow 3, whilst twelve were from Windrow 4.

An uncultured bacterium, originally isolated from pig slurry, was only present in Windrow 2 during the last 4 weeks of composting (rf-position -0.1). The closest match to this bacterium was *Ulvibacter antarcticus*, a bacterium previously found in Antararctic seawater. The last 4 weeks of the composting process in Windrow 4 was characterized by temperatures that started

to increase, however the temperatures were still within mesophilic ranges. Moisture was below 40%.

Six identities were obtained for members of *Bacillus* spp. These included *B. aidingensis, B. vireti, B. thermoamylovorans, B. hwajinpoensis, B. thermocloaceae* and *B. niacini.*

B. aidingensis was present from day 14 in Windrow 1, and was identified from each of the subsequent samples taken from this windrow. *B. aidingensis* was observed for three samples in Windrow 3, mainly during the first week of composting and then on day 35. *B. vireti* was present on one occasion in Windrows 2, 3 and 4, and twice in Windrow 1 (day 28 and day 42). *B. thermoamylovorans, B. hwajinpoensis* and *B. thermocloaceae* was present throughout the composting process in Windrow 2. *B. hwajinpoensis* was only detected on day 0 in Windrow 3, and not detected in Windrow 4. *B. thermocloaceae* and *B. thermoamylovorans* was were absent during the final two weeks of composting in Windrows 3 and 4.

Six of the sequenced bands were indicated as members from the *Paenibacillus* spp. With the exceptions of *P. illinoisensis* and *P. woosongensis strain ZL*, the presence of *Paenibacillus* spp. was short-lived in the composting process. *P. illinoisensis* was present throughout most of the composting process in all four windrows, whilst *P. woosongensis strain ZL* was only detected in windrows with an increased C:N ratio (Windrows 3 and 4).

Mechercharimyces spp. was also only identified from Windrow 3 and Windrow 4. *M. asporophorigenens* was detected during the later stages of the composting process of the windrows. *M. mesophilus* was only detected on two occasions, in Windrow 3 on day 14 and Windrow 4 day 35.

Pedobacter lentus was identified as rf-position 0.068. It was not detected in Windrow 1, but was present in all samples of Windrows 2 and 4, and the mostly also during the composting process of Windrow 3.

Table 3.7: Sequence identities, statistical probability, sources and presence in current study's windrows.

Band ID	rf Position	Query Length	Accession Number	Description	Max Score/ Total Score	Query Coverage	E-value	Max Identity	Source	Presence Windrow 1	Presence Windrow 2	Presence Windrow 3	Presence Windrow 4
281	-0.1	514 bp	AM982608.1	Uncultured bacterium partial 16S rRNA gene, clone PISD-ALB10	816/816	100%	0	95%	Pig slurry	Not detected	Last 4 weeks	Not Detected	Not Detected
			NR 044279.1	Ulvibacter antarcticus strain IMCC3101	654/654	100%	0	88%	Antararctic Seawater				
12	0.029	526 bp	DQ129366.1	Uncultured bacterium clone AKIW942 16S ribosomal RNA gene, partial sequence	940/940	99%	0	99%	Urban aerosol	From day 14 onward	Not detected	Present Day 5, 7 and 35	Not detected
			NR 043884.1	Bacillus aidingensis strain 17-5 16S ribosomal RNA, complete sequence	841/841	99%	0	96%	Ai-Ding salt lake China				
251	0.068	451 bp	GQ175344.1	Uncultured bacterium clone GD18 16S ribosomal RNA gene partial sequence	751/751	100%	0	97%	Poultry litter	Not detected	Present throughout the period	Day 5 - Day 35	Present throughout the period
			NR 044218.1	Pedobacter lentus	535/535	100%	4E-152	86%	Culture collection				
463	0.399	499 bp	DQ358734.1	Paenibacillus sp. GA5 16S ribosomal RNA gene, partial sequence	580/580	98%	3E-162	87%	Unpublished	Not detected	Not detected	Day 14	Day 35
			NR 042092.1	Paenibacillus peoriae strain DSM 8320	575/575	98%	3E-164	85%	Paperboard				

Band ID	rf Position	Query Length	Accession Number	Description	Max Score/ Total Score	Query Coverage	E-value	Max Identity	Source	Presence Windrow 1	Presence Windrow 2	Presence Windrow 3	Presence Windrow 4
286	0.458	541 bp	HM036055.1	Uncultured bacterium clone 23s-58 16S ribosomal RNA gene, partial sequence	958/958	100%	0	99%	Compost	Not detected	Not detected	Day 49	Day 35
			NR 041345.1	Mechercharimyces asporophorigenens strain YM11-542	711/711	97%	0	90%	Salt water				
111	0.478	428 bp	428AB699228.1	Uncultured bacterium gene for 16S rRNA partial sequence, isolate: DGGE band 10	686/686	100%	0	96%	Compost	Days 28 and 42	Day 28	Day 21	Day 35
				Bacillus vireti strain R-15447 16S ribosomal RNA, partial sequence	680/680	99%	0	96%	Grassland				
223	0.51	508 bp	HM036046.1	Uncultured bacterium clone 23d-64 16S ribosomal RNA gene, partial sequence	872/872	100%	0	98%	Compost soil	Not detected	Day 5, 7 and 14	Day 0	Not detected
			NR 043229.1 NR 025666.1 NR 025489.1	Paenibacillus spp. (P. woosongensis, P. ehimensis, P. agarexedens)	740/740	100%	0	92%	Forrest soils				
224	0.558	377 bp	GU067470.1	Bacillus thermoamylovorans strain S2 16S ribosomal RNA gene, partial sequence	360/360	98%	6E-96	83%	Hot Spring, Gujarat, India	Not detected	Throughout the period	Absent in last two weeks	Day 5, 7 and 14
			NR 029151.1	Bacillus thermoamylovorans strain DKP 16S ribosomal RNA, complete sequence	345/345	98%	3E-95	82%	Palm wine fermentation				

Band ID	rf Position	Query Length	Accession Number	Description	Max Score/ Total Score	Query Coverage	E-value	Max Identity	Source	Presence Windrow 1	Presence Windrow 2	Presence Windrow 3	Presence Windrow 4
432	0.651	478 bp	HM036046.1	Uncultured bacterium clone 23d-64 16S ribosomal RNA gene, partial sequence	832/832	99%	0	99%	Compost soil	Day 5- 28, Day 42-56	Day 0-42	Day 0 - 28	Day 0 - Day 42
			NR 040884.1	Paenibacillus illinoisensis strain JCM 9907	675/675	99%	0	92%					
225	0.684	408 bp	FN667436.1	Uncultured compost bacterium partial 16S rRNA, clone PS3139	587/587	100%	2E-164	92%	Compost	Days 14, 28, 35, and 49	Day 14	Day 14	Not detected
			NR 042092.1 NR 042009.1 NR 037006.1	Paenibacillus spp. (P. peoriae, P. jamilae, P. polymyxa)	506/506	87%	2E-143	92%	Paperboard, Olive-mill Wastewater				
212	0.703	382 bp	AM930318.1	Uncultured bacterium partial 16S rRNA, clone SMQ1	645/645	99%	0	97%	Cooling stage of composting	Not detected	Present throughout the period	Day 0	Not detected
			NR 025264.1	Bacillus hwajinpoensis strain SW-72	630/630	100%	0	96%	East and Yellow Seas, Korea				
226 \ 433	0.727	423 bp	FN667432.1	Uncultured compost bacterium partial 16S rRNA gene, clone PS3144	690/690	98%	0	95%	Compost	Day 35, 42	Throughout the period	Absent Days 7 and 56	Day 5-Day42
			NR 036986.1	Bacillus thermocloaceae	612/612	98%	2E-175	91%	Culture collection				

Band ID	rf Position	Query Length	Accession Number	Description	Max Score/ Total Score	Query Coverage	E-value	Max Identity	Source	Presence Windrow 1	Presence Windrow 2	Presence Windrow 3	Presence Windrow 4
454	0.797	543 bp	FN667048.1	Uncultured compost bacterium partial 16S rRNA gene, clone PS71	1044/1044	100%	0	100%	Compost	Not detected	Not detected	Day 14	Day 35
			NR 041344.1	Mechercharimyces mesophilus	760/760	99%	0	91%	Salt water				
227	0.832	518 bp	AF 252325.1	Uncultured bacterium pPD14 16S ribosomal RNA gene, partial sequence	928/928	99%	0	100%	Compost	Not detected	Day 0-5	Absent day 56	Sporadic, Day 0, Day 35, Day 49
			NR 024695	Bacillus niacini strain IFO15566	773/773	99%	0	93%	Culture collection				
426	0.893	507 bp	JQ 796007.1	Paenibacillus sp. ASC 668 16S ribosomal RNA gene, partial sequence	717/717	99%	0	92%	Compost	Not detected	Not detected	Day 35	Day 5-35
			NR 043229.1	Paenibacillus woosongensis strain YB-45	700/700	99%	0	91%	Compost				
427	0.934	372 bp	HE586338.1	Paenibacillus sp. R-27422 partial 16S rRNA gene	590/590	100%	2E-165	95%	Spoilage of dairy products	Not detected	Not detected	Day 14, Day35-42	Day 0 - Day 42
			NR 043861.1	Peanibacillus woosongensis strain ZL	581/581	100%	2E-166	95%	Warm Spring				

3.10 Summary Of Results

Windrows 1 and 2 had lower average temperatures observed during the course of the composting process, when compared to Windrows 3 and 4. Windrow 4 had the highest average temperatures, and also reached the thermogenic stage of the composting process within the initial period evaluated in this study. Temperatures were evenly distributed within the windrow. Temperatures between the windrows differed significantly.

No significant difference could be observed in the average moisture content of the windrows. This indicates a similar treatment for the windrows. Moisture loss was higher in Windrows 1 and 2 as opposed to Windrows 3 and 4.

Windrow 1 had the highest values for N, K, S, Zn, Mo, B, and Na. Lowest values for ash, pH and C:N ratio was observed in Windrow 1. Windrow 2 contained the highest P, Mg, Ca, Mn and Cu levels. Overall, Windrow 4 had the lowest value for all plant macro- and micronutrients. Highest C:N ratio was observed in Windrow 4.

Prokaryotic DNA was amplified using a nested-PCR approach. Amplification was of high quality, observed on agarose gel electrophoreses. Eukaryotic DNA amplification was troublesome, as many samples did not amplify even with several optimization steps applied. Amplified DNA was subjected to denaturing gel electrophoresis. Profiles obtained were successfully analyzed, using Species Richness, Shannon-Weaver and Simpson's Reciprocal Indices.

Bands, indicating the probable presence of a single organism, were grouped into 55 rf-positions. Overall, 454 prokaryotic bands were identified and some were excised. Identification of the DNA present within the band was also successful, with an average sequence length of 467 base pairs. Seven unique eukaryotic bands were identified on the obtained profile.

Statistical analysis also included CCA, indicating the influences of windrow physico-chemical parameters on banding patterns and specific bands. Several bands could be identified that was influenced by C:N ratio, temperature and pH. Chemical parameters mostly had a negative to no correlation with banding patterns.

Identities obtained from sequence analysis included six members of both *Bacillus* spp. and *Paenibacillus* spp. Two identifications closely to *Mechercharimyces asporophorigenens* and *M. mesophilus* were identified from windrows that contained woodchips. *Pedobacter lentus* was present for most of the observed period in windrows containing the inoculum.

CHAPTER 4
DISCUSSION

4.1 Introduction

This study was concerned with the physico-chemical and microbiological aspects surrounding the windrow composting of broiler litter. Four windrows were formulated, with Windrows 1 being a control windrow that contained only broiler litter; Windrow 2 contained broiler litter and previously composted material. The latter served as an inoculums. Windrow 3 had the same substrate as Windrow 2 with the addition of a carbon source in the form of woodchips. Windrow 4 had the same composition as Windrow 3 but it also had a zeolite amendment.

Temperature and moisture of the windrows were monitored by the company (Galltec Pty. Ltd). Chemical analyses were conducted on the raw material (broiler litter and woodchips) and the end compost products by Nvirotek laboratories. Regular samples were taken and subjected to DNA isolation according to the method described in Section 3.4. The eluted DNA was used for PCR-DGGE analysis to obtain microbial population profiles, as well as to identify key organisms in the composting process.

4.2 Physico-Chemical And Microbiological Parameters That Influence Composting

All windrows were constructed using the same batch of broiler litter obtained from the same farm. Windrows were evaluated based on the internal temperatures achieved during the composting period, moisture content, chemical data and microbial diversity. Influences of the physico-chemical analysis on the microbial dynamics was also analysed by means of Canonical Correspondence Analysis (CCA).

Any change in the physical or chemical composition of the windrows could lead to an alteration in the microbial community. Adjustments in moisture level (Elwell *et al.,* 1998), aeration (Steger *et al.,* 2005), the use of inoculums (Ichida *et al.,* 2001) and altering the C:N ratio (Eiland *et al.,* 2001) are a few possibilities for altering the microbial population within windrows. The effect that the environment has on windrows can also not be neglected. As all four windrows were composted at the same time, in the same facility, the effect of the environment was minimized. As no significant differences ($p < 0.05$) were observed when comparing all the moisture measurements made, differences in moisture content may have had only a very slight influence between the various individual windrows.

The subsequent sections discusses these parameters as represented in the results from Chapter 3. Each of the four windrows that were constructed differed in only one substrate or

amendment. It is therefore possible to compare these windrows based upon the effects (or lack thereof) that said substrate or amendment could have had.

4.2.1 Composting Broiler Litter (Windrow 1)

Windrow 1 consisted only of broiler litter that was obtained from a local farm in the North West Province, South Africa. It served as the control windrow for this experiment as it mimicked the formulation and approach used by Galltec (Pty.) Ltd.

Microbial diversity was relatively low in the broiler litter at the start of the process. The intestinal tract of broiler birds is an ecological niche that harbours a wide variety of microorganisms (Cressman *et al.,* 2010). It could be that these organisms were greatly reduced by litter management practices prior to the windrow constitution. In South Africa, the reuse of broiler litter as a bedding material is not advised. Broiler houses are cleaned after every batch of broilers that has been reared (Jordaan, 2004). These broiler houses are also thoroughly sanitized using a combination of physically removing all litter and treatment with chemical antimicrobials (Jordaan, 2004; SAPA, 2012c).

Cressman *et al.,* (2010) applied PCR-DGGE to both fresh litter and reused litter. During a 42 day period they observed two dominant bands pertaining to fresh litter samples, while more bands were observed in reused litter. Older broiler litter therefore had a more complex diversity pattern. Microbial diversity present in South African broiler litter was expected to have a lower diversity as opposed to other broiler houses that reuses broiler litter as a bedding material. Furthermore, collected broiler litter had a low moisture content (personal observation), which could have induced a moisture shortage for microorganisms and reduced microbial diversity. Lovanh *et al.,* (2007) found that reduced moisture content in fresh litter in broiler houses had a lower diversity as opposed to litter found near watering areas. This is substantiated by Williams (2012) who also found that older, dryer broiler litter had a lower microbial diversity and a greatly reduced microbial population.

During the first hours of the composting process, Windrow 1 showed no increase in average internal temperature. However, an increase in average temperature was observed from day 0 to day 5. During this period the microbial community diversity increased, with a further increase observed between days 5 to day 14. The initial increase in microbial diversity could be explained by the addition of water, and thus an increase in the moisture content.

Water could have increased microbial diversity in two ways. Firstly, the water was obtained from a nearby dam, and therefore contained a microbial consortium characterized by the dam's

own intrinsic physico-chemical characteristics. The added water would also have reduced water shortage stresses present within the broiler litter. This could have resuscitated dormant species (Griffiths *et al.,* 2003; Whiteley *et al.,* 2003), increasing the relative abundance of DNA from these species and allowed for PCR amplification to be possible.

The average temperature remained fairly constant during the period that lasted from day 14 – day 35. Microbial diversity decreased around day 28 most likely due to a response to decreased average moisture content observed in the period leading up to the sampling date. Schimel *et al.,* (1999) reported that decreased moisture for 2 weeks, reduced microbial biomass and diversity in birch litter. Although not explicitly mentioned in their paper, the results of Adams & Frostick (2009) also indicated a reduced diversity obtained from DGGE profiles when the moisture in compost was either very low (44-36 %) or extremely high (71-75 %).

In the present study water was added resulting in microbial diversity increases, which lead to an increase in temperature during the period 28-42 days. Average moisture rapidly decreased, and no water was added towards the end of the composting process. This was done as part of the composting procedures implemented by the company. Windrows were not watered at least 2 weeks prior to the packaging of the material. This was done to reduce the water content and ensure that subsequent heating did not occur in the package material

Temperature profiles constructed from the average internal temperatures showed that the windrow temperatures closely resembled that of the ambient temperature during the composting process observed. This observation leads to the conclusion that no composting occurred in this windrow. The microbial community present within Windrow 1 lacked the metabolic activity to increase temperatures to within thermophilic temperature ranges. The possibility therefore exists that pathogenic organisms could survive in composts made from only broiler litter.

4.2.2 Using An Inoculum In Broiler Litter Composting (Windrow 2)

Initial reports indicated that inoculums did not alter the composting process and at most would only decrease the lag-phase before composting starts (Golueke *et al.,* 1953). More recently organisms that could increase the humification during composting, increasing compost quality and decrease composting time were described and successfully used as inoculums (Ohtaki *et al.,* 1997; Ichida *et al.,* 2001; Vargas-Garcia *et al.,* 2006; Vargas-Garcia *et al.,* 2007; Kalemelawa *et al.,* 2012). The use of an inoculum was therefore investigated.

At commencement of this study it was thought best to use previously composted material as an inoculum. The microbial community present at the end of a previous composting process was

thought to increase the microbial diversity, decrease the lag-phase and therefore shorten the composting period needed.

The addition of previously composted material did increase the species richness of Windrow 2 at day 0. The species richness was five times higher than the sample obtained from Windrow 1, and for the largest part of the composting process remained higher than the observed number of species in Windrow 1.

Although the temperature did increase in Windrow 2 during the first few hours of the composting process, the temperatures could not be maintained. This indicates that the consortium of organisms that was added was able to somewhat increase internal windrow temperatures. The same set of events that occurred in Windrow 1 for the first week, was also prevalent in Windrow 2. Moisture again could have been a limiting factor for microbial diversity, as was the case in Windrow 1. The amount of moisture added to the later stages of this windrow seems to be lower than that observed in Windrow 1. This could explain the decrease in microbial diversity observed at day 35 of the composting process in Windrow 2. As temperature increased the diversity also increased, however diversity according the Simpson's Reciprocal index was almost the same at the end of the observed process.

Over the course of the composting process, internal windrow temperatures were at thermophilic ranges for several measurements. These temperatures could, however, not be maintained and were affected by ambient temperatures. Based on cluster analysis of DGGE profiles it was observed that profiles from Windrow 2 clustered into the same major cluster as Windrow 1. However, the DGGE profiles were still distinctively separated from that of Windrow 1. This separation was also observed in the CCA analysis and indicates that the microbial communities differed to some extent from those of other windrows.

The use of an inoculum increased the average temperature in Windrow 2 significantly ($p < 0.05$) above the temperatures observed in Windrow 1. On average Windrow 2 was 11.6°C warmer than Windrow 1. This could be due to an increased microbial biodiversity and biomass, resulting in an increased internal metabolic rate (Beffa *et al.,* 1995) and thus increased temperatures within the windrows (MacGregor *et al.,* 1981).

The lack of a true thermogenic phase indicates that even with an increase in the microbial diversity, true composting of broiler litter did not occur. Even though the temperatures were above 40°C, the time spent above 40°C was intermittent and could not be sustained. A possible reason for the sustained temperature could be due to the low carbon content of the windrow. Huang *et al.,* (2004) studied the effect of C:N ratio on composting pig manure and

sawdust. They found that temperature increases were delayed in substrates that were formulated to a 15:1 C:N ratio. An immediate increase in temperatures were observed if the substrate was formulated to a 30:1 C:N ratio.

Average moisture content differed only 0.36 % points between Windrows 1 and 2. Moisture levels at the same average depth had no significant difference, indicating that the windrows had mainly the same internal water distribution. The use of an inoculum therefore did not significantly alter the moisture dynamics within the windrow.

The addition of an inoculant also lead to a more stable microbial consortium present within the windrow. Standard deviation for both the Shannon-Weaver and Simpson's Reciprocal indices were the lowest in Windrow 2 indicating the lowest shifts in diversity. Microbial diversity was also the highest in Windrow 2 according to the Shannon-Weaver index. Temperature influences the microbial community. As Windrow 2 had an average temperature within the mesophilic temperature range, this could have lead to the stabilized community dominating.

Based on these observations the use of previously composted material as an inoculum is advised when broiler litter is composted. However, the use of an inoculant alone could not ensure maintained thermophilic temperatures, and the possibility of pathogen and weed seed survival is a probability (Kato & Miura, 2008).

4.2.3 Chemical Analysis Of Broiler Litter Composting

As both Windrows 1 and 2 contained only broiler litter, the discussion on the chemical analysis for these two windrows are combined. Both windrows contained higher amounts of primary plant macronutrients N, K, and P compared to the other two windrows. In Windrow 1 nitrogen (2.56 %) and potassium (3.31 %) values were elevated above that of Windrow 2 by 0.28 and 0.22 percentage points, respectively. Windrow 2 had a P value (1.64 %) that was only 0.06 percentage points higher than that of Windrow 1. The differences would suggest that the use of an inoculant did not have a large effect on the primary plant macronutrients.

In both Windrows 1 and 2 the C:N ratio increased. Normally the C:N ratio is reduced by the microbial activity. This is due to the metabolic utilization of carbon at a higher rate than nitrogen. The C:N ratios at the end of the process was estimated at 15.5:1 and 16.1:1 for Windrows 1 and 2 respectively, while the C:N ratio for broiler litter was given as 10.3:1.

The increase in the C:N ratio is attributed to the loss of nitrogen from the two windrows. The broiler litter used as substrate had an initial N value of 4.87 %, therefore a loss of 2.31

percentage points of N (or 52.6 % of the total N) in Windrow 1 and 2.59 percentage points N in Windrow 2 (or 46.8 % of the total N) was observed. Tiquia & Tam (2000) reported a 59 % loss of the initial N in poultry litter in forced aeration piles. As aeration plays a role in ammonia volitization, the lower values obtained from windrow composting can be justified by the reduced aeration frequency. Goyal *et al.,* (2005) obtained the similar results, reporting an increase in C:N ratio during the composting of unamended broiler litter. They measured the C:N ratio at 30 day intervals, and the C:N ratio increased from 13.9:1 at the start of the composting process, to 22.7:1 sixty days later.

Volatilization of N mainly occurs as the formation of high amounts of ammoniac compounds (Elwell *et al.,* 1998, Tiqua & Tam, 2000). Ammonia has a distinctive smell, and could be smelt near Windrows 1 and 2, indicating concentrations above 50 ppm (Busca & Pistarino, 2003). Ammonia is lost to the atmosphere, reducing the nitrogen value of the compost and therefore increasing the C:N ratio. It could also have a potential negative impact on the environment. Deaths of Blacksmith plover birds (Bont kiewiet, *Vanellus armatus)* were observed during the course of this project near other windrows with the same formulation. It is speculated that these birds, which had nests between the windrows, had succumbed to the ammonia emissions. This was, however, not tested.

Ammonia volatilization during the composting of poultry litter was investigated in the work of DeLaune *et al.,* (2004). They reported a loss 382 g NH_3 m^{-2} lost on an annual basis in unamended poultry litter. Higher NH_3 emissions was observed using an undisclosed microbial mixture (406 NH_3 m^{-2}). However, their results indicated a 44 % reduction in ammonia volatilization when the compost was amended with H_3PO_4 and a 62 % reduction when amended with alum.

In the microbial dynamics, the clustering of Windrows 1 and 2 in the dendrogram indicated that these windrows shared a similarity in microbial communities' structure present. It is possible that the consortium of organisms are comprised of many ammonia producing bacteria.

Plant micronutrients were within the ranges indicated for compost by European countries. Iron was almost 53 % higher in Windrow 2 when compared to the value obtained for Windrow 1. It would be possible for Fe concentration to increase as the mass of the substrate is reduced, therefore having a cumulative effect when previously composted material is added to a new windrow. The amount of iron in Windrow 1 is more than the approximate iron values published by Van Ryssen *et al.,* (1993). This could also be due to volume reduction of the broiler litter leading to a increased concentration of iron within the windrow.

4.2.4 Adjusting The C:N Ratio (Windrow 3)

Carbon is needed by all life forms on earth, as it forms the basis of the chemistry of life as we know it. Broiler litter has an intrinsically low carbon level, indicated by the low C:N ratio of the material (Milligan, 2008). Therefore, to maintain the nutritional requirements of microorganisms present during the composting process, the initial value for C needs to be increased. This in turn reduces competition between organisms, resulting in an increased diversity.

Adjusting the carbon content during the initial formulation of the substrate to be composted was included in this study (Silva *et al.,* 2009; Bernal *et al.*, 2009; Ramaswamy, 2010). A target C:N ratio of between 25:1-35:1 was selected based upon the C:N values for broiler litter and Blue gum woodchips.

Windrow 3 was able to maintain temperatures above 25°C for the first 48 hours of composting. The windrow was thermogenic after 24 hours of composting, and temperatures could be maintained, even with changes in ambient temperature. As temperature increased in Windrow 3, moisture content and microbial diversity decreased in the first week of composting. The decrease in diversity is in contrast to diversity indices observed in Windrows 1, 2 and 4. Temperatures remained above 40°C even after the termination of the study.

On average, Windrow 3 was 19.2°C and 7.5°C warmer than Windrows 1 and 2 respectively. Ogundwande *et al.,* (2008) also reported this correlation between C:N ratio of broiler litter composting and temperature. In their results temperatures were found to increase with 10°C if the C:N ratio is increased from 20:1 to 25:1. Average temperature only increase by 1.08°C if the C:N was changed from 25:1 to 30:1

The high microbial diversity observed at the start of the composting process of Windrow 3 could be explained by the cumulative biodiversity of the three substrates. Profiles from Windrows 2 and 3 (Day 0) clustered together on the dendrogram presented in Figure 3.14. This implies that, although differences exists in the community at Day 0, similarities also occurred. The difference in microbial community was probably influenced by the microbial community present in the woodchips.

Noll & Jirjis (2012) reviewed literature on the microbial diversity present within stored woodchips. From their study of available literature they concluded that a diverse consortium of microorganisms are present within woodchips.

The decrease in biodiversity in the first week of the composting process could be interpreted by the work of Yu *et al.,* (2008). Mesophilic organisms predominate the substrate at the start of the composting process. As the temperature increases in the compost, mesophiles became stressed and the microbial community shifted to a more thermophilic consortium of organisms. This lead to an initial decline in biodiversity (Yu *et al.,* 2008).

The high microbial biodiversity observed in Windrow 3 in the first week could also infer a large bacterial population. Due to the high moisture content at the start of the composting process, the diffusion of oxygen (O_2) could have been limited within the substrate (Beffa, 2002). The combination of a large bacterial population and high moisture content could have resulted in the process becoming anoxic, decreasing the bacterial diversity and selecting for organisms capable of surviving these thermophilic, anoxic conditions. The grouping of samples from Days 5, 7 and 56 within cluster B3 of the dendrogram presented in Figure 3.14 could potentially be explained by this effect.

Atkinson *et al.,* (1996), estimated that about 1% of bacterial species present in the composting process were obligatory anaerobes. Further, all the obligatory anaerobic bacteria were found in the thermophilic stage of the composting process, whilst facultative anaerobic organisms dominated the mesophilic stage. All thermophilic obligatory anaerobic bacteria were highly cellulolytic, which would indicate the possibility for a similar selection within the windrows of the present study that contained woodchips (Atkinson *et al.,* 1996).

The remainder of the composting process observed were dominated by large shifts in microbial diversity. Internal moisture content differed significantly within Windrow 3, with only significant observations being between depths 2 and 3 and depths 3 and 5. This indicates that the moisture distribution was not uniform within Windrow 3. No significant differences could be observed at depths 2 – 5 between Windrows 1, 2 and 3. The surface moisture content differed significantly between Windrows 1 and 2 and Windrow 3. This indicates that the addition of woodchips altered the surface moisture loss. Windrow 3 had a higher average moisture content at its surface (48.1 % opposed to 40.4 and 43.8 % for Windrows 1 and 2 respectively).

By adding woodchips, the physical characteristics of the windrow was changed. This lead to an increase in the internal free air space of the windrow. The addition of woodchips creates inter particle voids that could be occupied by water (Tuomela *et al.,* 2000) resulting in an altered internal moisture profile.

4.2.4 Adjusting The C:N Ratio And Amending With Zeolite (Windrow 4)

Due to high ammonia volatilization observed during the composting of broiler litter, the effect of adding natural zeolites was also investigated. The use of zeolites as adsorbents of ammoniac compounds have been well documented. Bernal *et al.,* (1993) successfully showed that zeolite could be applied in the composting process. In their work, they found that 80 % of ammonia could be retained within the compost at an application rate of 53 $g.kg^{-1}$, in a pig-slurry/chopped straw co-composting system. Cooney *et al.,* (1999) also showed that zeolite could be used as an effective method for removing high amounts of ammonia from water. Zeolite is also applied in industrial applications as an effective ammonia adsorbent material (Busca & Pistarino, 2003)

Windrow 4 contained a 12.5 % (w/w) zeolite amendment, mixed into the same substrate used in Windrow 3. In Windrow 4 temperatures above 40°C was maintained throughout the composting process, except for a few hours during the first night of composting. The highest average temperature obtained in Windrow 4 was 67°C. Windrow 4 never reached a secondary mesophilic stage, also known as the curing phase. Temperatures at the surface (depth 1) were significantly lower than temperature measurements from the deeper measuring sites, indicating a non-uniform distribution in temperature. Also, no significant comparison could be made between varying depths in Windrows 1 and 2 and Windrow 4. Temperatures at corresponding depths were also different between Windrows 3 and 4, with the exception of temperature measurements made at depth 4 in Windrow 3 and Depth 1 in Windrow 4. Temperature increased rapidly, most likely due to an increase in biodiversity observed between Days 0 – 5. An increase in biodiversity implies an increase in the biomass present. This in turn leads to an increase in metabolic activity, which increases temperatures (Tiquia *et al.,* 1996)..

Moisture distribution in Windrow 4 was not uniform, and significant average values were only obtained between Depths 2 and 3 and Depths 3 and 5. Windrow 4 had no significant differences between corresponding depths in comparison to the moisture content of Windrow 3. This indicates that the moisture profiles were comparable and that a zeolite amendment did not alter the moisture characteristics of the windrow.

At the surface (depth 1), moisture content Windrows 1 and 2 differed significantly from Windrows 3 and 4. It could be postulated that the use of wood chips altered the surface-atmosphere interaction (Tuomela *et al.,* 2000). Although the windrows did not show any significant difference in average moisture content over the composting period observed, one could only speculate about the moisture dynamics within the windrows, as the water added to the windrows were not controlled. In theory, the addition of woodchips would increase the internal surface area of the windrow (Cornell University, 1996), therefore increasing water

retention . Also, the woodchips used had a low moisture content, and would absorb water more readily.

The low diversity observed at Day 0 and Day 56 in Windrow 4 is an anomaly, as Windrow 3 had a higher diversity observed on the same days. It may be explained by low amplification rate due to human error in the set up of the PCR, or biased amplification within the PCR reaction. Microbial diversity is thought to increase towards the end of the thermophilic phase as temperatures decline.

At Day 5 the highest diversity for the specific sampling day is observed. The values decreased for Day 7 and stabilized until Day 14. These organisms are largely involved in the thermophilic composting at high temperatures. Thereafter temperatures somewhat stabilized to within the 45 - 48°C range. On Day 35 microbial diversity had increased to the highest value obtained during this experiment.

Taking the whole composting process into consideration, Windrow 4 had the highest average temperature observed. The addition of zeolite effectively increased the temperature above that of Windrow 3, by 6.8°C. A large difference in maximum temperature between these two windrows were also obtained. The work of Venglovsky *et al.,* (2005), corroborates the heating effect that zeolite could have in processing animal manures.

As the addition of zeolites increased internal temperatures of the windrows, it would be a valuable amendment to the compost. The use of zeolites in agriculture has been extensively investigated. Ramesh & Reddy (2011) reviewed available literature on zeolite application in agriculture. Their summary concluded that zeolites can be successfully applied to agricultural soils. This study did not take into account the economical feasibility of incorporating zeolites, or effect of different application rates on the composting process.

4.2.5 Chemical Analysis Of Co-Composting Broiler Litter

The addition of zeolite greatly affected all the chemical parameters tested for in this study. All these values, except Na and ash, were higher in Windrow 3 with regards to Windrow 4. As ash percentage is an indication of the mineral content of a substance, it would be expected that the addition of zeolite would increase this value. The difference in sodium is very small, and could be accounted for by the natural Na content of zeolite (Appendix A).

Woodchips contained a very low N value of only 0.33 %. If the same N value is taken for previously composted material as was observed in Windrow 1 at the end of the composting

process, Windrow 3 had an approximate N value of 2.6 % N at the start of the composting process (Appendix B). Increasing the C:N ratio resulted in lower N losses. Windrow 1 and 2 lost 2.31 % and 2.38 % of the initial N respectively, while Windrow 3 had the lowest loss in N of only 0.91 %. No values for N in zeolite were available (Appendix A), therefore contributions could not directly be determined for Windrow 4. If the initial contribution of N from zeolite is not taken into account, Windrow 4 lost 1.1 % of its initial N content. This is corroborated by the higher C:N ratio observed in Windrow 4 compared to Windrow 3.

The high losses in N during the composting of poultry litter was also observed by Elwell *et al.,* (1998). Their results also indicated that the rate of ammonification increases as the pH increases (Sun, 2006). This could be due to selective competition that arises as pH rises in the compost between different organisms (Thomas & Wimpenny*,* 1993). The addition of carbon decreases the interspecies competition within the composting process.

Differences in all the other nutrients tested could be explained by the differences in mass between Windrows 3 and 4 as well as the chemical composition of zeolite (Appendix A). Windrow 3 had an initial mass of 5 500 kg, and Windrow 4 a mass of 6 000 kg.

At the end of the composting process, Windrow 4 still had a very high C:N ratio of 31.9:1, while Windrow 3 had a C:N ratio of 21.4:1. Based on the C:N ratio, Windrow 4 would have lost a large amount of N in relation to Windrow 3. However, the total N loss from samples only differed 0.37 percentage points from one another, resulting in a difference of approximately 20 kg of Nitrogen. This is an anomaly that could not be explained, other than by the possibility that human error could have played a role.

4.2.6 Prokaryotic Diversity Compared To The 16S rRNA Gene Database

During the composting process observed, large shifts in microbial diversity was observed by means of PCR-DGGE analysis. Using the 16S rRNA gene database gave the description of the closest related organisms that has already been characterized .

Canonical correspondence analysis indicated several band positions that were influenced by C:N ratio and temperature. Sequenced bands were mostly from members of the *Bacillus* spp. and *Paenibacillus* spp. As each compost system differs, so does the inherent microbial community utilizing the substrates present. Various authors have described the presence of members of *Bacillus* spp. and *Paenibacillus* spp*.* during the composting process (Ryckeboer *et al.*, 2003; Wakase *et al.,* 2008).

4.2.6.1 *Bacillus* species

At rf-position of 0.029 a dominant species in Windrow 1 was found to be closely related (96 %) to *Bacillus aidingensis.* Xue *et al.,* (2008) first identified *B. aidingensis* in the Ai-ding salt lake in China. *B. aidingensis* is a moderately halophilic organism. *B. aidingensis* was also identified in Windrow 3 at days 5, 7 and 35. The organism's dominance during the composting process of Windrow 1 could be due to the high Na content of the windrow. It was present both in mesophilic temperatures and temperatures above 40°C, with moisture content being between 40% and 60% on days detected. *B. aidingensis* is capable of utilizing a very wide variety of substrates, but unable to produce NH_3 (Xue *et al,* 2008). Canonical correspondence analysis implied that boron concentration influenced *B. aidingensis* significantly.

An organism very closely (96%) related to *Bacillus vireti* was also isolated from samples taken on days 28 and 42 in Windrow 1, day 28 in Windrow 2, day 14 in Windrow 3 and Day 35 in Windrow 4. These periods were all characterized by moisture levels above 40%. The presence of *B. vireti* on day 42 in Windrow 1 and its absence in Windrow 2 on the same day could be explained by low moisture in Windrow 2. Windrow 1 had an average moisture content of 43.3% whilst Windrow 2 moisture was 36.5% on day 42. From CCA plots it could be concluded that pH only had a slight influence on *B. vireti. B. vireti* was first proposed as a new species by Heyrman *et al.,* (2004). This species was first isolated from soil. It is able to occur at maximum temperatures between 40 – 45°C (Heyrman *et al.,* 2004). *B. vireti* is urease negative and therefore not implicated in the production of ammonia.

A band located at rf-value 0.558 was identified from Windrows 2, 3 and 4. However, the maximum identity score was low (82%). The E-value was also relatively high (3×10^{-95}). The true identity of this organism could therefore only be speculated as that of *Bacillus thermoamylovorans.* The absence of *B. thermoamylovorans* from Windrow 1 indicates that it had to be one of the organisms present in the previously composted material. First proposed in 1995 by Combet-Blanc *et al.* (1995) *, B. thermoamylovorans* is a moderately thermophilic and amylolytic bacterium. This species was not able to grow at temperatures above 58°C. It was also absent during certain periods of composting in Windrow 4 which reached temperatures above 58°C. From the CCA bi-plot it could be concluded that C:N ratio, temperature and ash had only a very slight influence, if any, on *B. thermoamylovorans.*

Bacillus hwajinpoensis was identified as a dominant organism in the composting of Windrow 2 with a maximum identity score of 96 %. It was absent in Windrow 1, indicating that it probably was inoculated with the previously composted material. It was first identified from the East and Yellow seas in Korea by Yoon *et al.,* (2004). It is a slightly halophilic, endospore-forming

organism. The sequence data also indicated a compost bacterium that is present during the cooling stages of composting. *B. hwajinpoensis* is urease-negative, but could reduce nitrates (Yoon *et al.,* 2004). No correlation was observed between the bacterium and the physico-chemical results obtained.

An organism closely related (91%) to *Bacillus thermocloaceae* was dominant in Windrows 2, 3 and 4, and present during the latter stages of the composting process observed in Windrow 1. Demharter & Hensel (1989) were the first to characterize *B. thermocloaceae* from sewage sludge. *B. thermocloaceae* is a thermophilic organism with an optimal growth temperature between 50 and 60°C (Demharter & Hensel, 1989). *B. thermocloaceae* was present at varying temperatures that ranged from 29.5 – 57.3°C. It was absent in the last week of composting in Windrow 3 and Windrow 4. This could be due to a decrease in substrate. Temperatures decreased by 4.2°C and 6.0°C for Windrows 3 and 4 respectively in times when *B. thermocloaceae* was not detected. Although *B. thermocloaceae* could not increase the metabolic rate alone, it could be implied as one of the contributing organisms to the thermogenicity of Windrows 3 and 4. This is further enforced by CCA bi-plots, which shows that temperature and *B. thermocloaceae* had a high correlation .

Bacillus niacini (maximum identity of 93%) was a dominating band in the composting process observed in Windrow 3, and was only detected sporadically in Windrows 2 and 4. This species was detected within a wide set of environmental parameters, and was not influenced by temperature, moisture or C:N ratio. *B. niacini* is capable of growing on nicotinate (niacin) as a sole source of nitrogen, carbon and energy. It also produces a blue pigment. *B. niacini* is capable of growth under anaerobic conditions in the presence of KNO_3 (Nagel & Andreesen, 1991). Felske *et al.,* (2004), stated that *Bacillus niacini* is among the most abundant soil Bacillus.

4.2.6.2 *Paenibacillus* species

In 1993 the proposal was made to create a new genus called *Paenibacillus* (Ash *et al.,* 1993). This was done due to the high diversity observed within the genus *Bacillus*, and the physiological differences observed. The genus *Paenibacillus* is facultative anaerobic spore-forming bacteria found in diverse environments.

Two bands that occurred only once during the composting of Windrows 3 and 4 was identified as *Paenibacillus peoriae* (maximum identity of 85%). *P. peoriae* was first described as *Bacillus peoriae* by Montefusco *et al.* (1993). The organism is capable of reducing nitrate to nitrite, and grows well at 45°C (Montefusco *et al.,* 1993). *P. peoriae* is also known to produce antimicrobial

substances, therefore making it a possible biocontrol agent (Von der Weid *et al.,* 2003). Within the context of this study, *Paenibacillus peoriae,* was identified on day 14 in Windrow 3 and Day 35 in Windrow 4. On both occasions temperatures were close to 45°C.

The band at rf-position 0.51 gave similar values for three different species when compared to sequences in the 16S rRNA gene database for Bacteria and Archaea. *P. woosongensis, P. ehimensis* and *P. agarexedens* (maximum identity score of 92 %) are therefore proposed identities for the uncultured clone 23d-64 sequence obtained during the comparison in the nucleotide collection. The latter was isolated from compost, whilst all three *Paenibacillus* species were found in forest soils. These soils are rich in decomposing material and humic acids, therefore fitting the possible role that these organisms could play during mesophilic composting observed in Windrow 2. They grow well at 37°C (Lee & Yoon, 2008) which was close to the temperature observed in Windrow 2 for the period.

P. illinoisensis (maximum identity score of 92%), identified from rf-position 0.651, was first proposed by Shida *et al.,* (1997). From the CCA bi-plots presented it could be implied that C:N ratio greatly influenced *P. illinoisensis.* This species is capable of utilizing ammonia, explaining its dominance in all the windrows and the influence implied by the CCA bi-plots. It could be beneficial to agriculture processes that involves high nitrogen levels. Jung *et al.,* (2004) showed that *P. illinoisensis* could be successfully used as a biocontrol agent for Phytophora blight in pepper plants. During the composting it may have played a vital role, as it was present in most samples taken from the windrows at different times in all four windrows.

The *Paenibacillus* spp*.* identified at rf-position 0.684 was identified as sensitive to temperature fluctuations, and is closely related to *P. peoriae, P. jamilae* or *P. polymyxa* (maximum identity of 92 %). The latter is widely used as a plant-growth-promoting rhizobacteria (PGPR) (Raza *et al.,* 2008) and was identified by *Ryckeboer* et al., (2003) as an organism present during the composting process.

4.2.6.3 Other prokaryotic species

Pedobacter lentus (maximum identity score of 86%) was a dominating species in all windrows that contained the previously composted material (i.e., Windrows 2, 3 and 4). Canonical correspondence analysis indicated that the organism was greatly influenced by C:N ratio. The occurrence of this organism at temperatures above 37°C could not be explained, as the maximum temperature is was published as 37°C (Yoon *et al.,* 2007). As *Pedobacter lentus* only obtained a maximum identity score of 86 %, it is possible that the organism indicated by the

band located at rf-value 0.068 has still to be fully cultivated and named. Therefore, it is concluded that the organism is not *P. lentus,* but rather an organism that needs to be defined.

Two members of the genus *Mechercharimyces* was identified at rf-position 0.458 and 0.797. This genus forms part of the family *Thermoactinomycetaceae* (Matsuo *et al.,* 2006). These bands (organisms) were only detected in windrows containing woodchips. Both *M. asporophorigenens* and *M. mesophilus* were previously isolated from salt water. Maximum identity scores were 90% and 91% respectively.

4.2.7 Prokaryotic Diversity Compared To The Nucleotide Collection Database

The 16S rRNA database contains organisms previously published. These organisms were previously cultivated on nutrient media, and their physiological needs were already determined. Overall, maximum identity scores were lower using this technique. This implies a low level of confidence in the identity proposed. Petti (2007) indicates that taxonomist only accepts maximum identity levels of ≥97% for genus and ≥99% for species level.

Based on this observation, the sequence information was also compared against sequences contained within the nucleotide collection. Utilizing the information contained in this database, twelve organisms were identified as uncultured bacteria. The source for these uncultured bacteria were either compost or manure related for twelve of the bands sequenced. The other four contained one unpublished member of the *Paenibacillus* spp., a bacterium present in urban aerosol, a *Bacillus* present in hot springs and dairy products.

Estimates are that only 1.4% of all soil microbial life can be cultivated using media preparations (Janssen *et al*., 2002). Although most of the identified organisms from this study could be related to the composting process, they remain unidentified in name and function when using only the nucleotide collection. This is one of the pitfalls of our current sequence driven research (Janda & Abbott, 2007).

The possibility that some of these bands were from previously unpublished microorganisms is also a possibility. This is the first study on broiler litter composting done in the North West province, and environmental factors could have played a role in evolutionary terms.

4.2.8 Eukaryotic Diversity During The Thermophilic Phase Of Co-Composting Broiler Litter

This study also investigated the diversity of eukaryotes during the thermophilic phase of composting. Eukaryotic diversity was low, and results from PCR-DGGE analysis identified only

7 bands that could be identified during the composting process of Windrow 4. Towards the later stages of the composting process diversity increase by 2 dominant species. Windrow 4 remained within the thermophilic temperature range during the composting process observed.

The low diversity of eukaryotic species during the thermophilic cycle of composting was also reported by Ryckeboer *et al.,* (2003). They reported that as temperatures start to decrease, both the biodiversity and biomass of fungi increases.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The aim of this study was to characterize the microbial community succession in four differently formulated broiler litter windrows by means of PCR-DGGE analysis.

Five well-structured objectives were formulated to achieve the aim of the study. These objectives could be divided into two main goals: to analyse the microbial diversity during the composting of broiler litter based substrates, and to evaluate the constructed windrows on their physico-chemical characteristics.

5.1.1 Compiling Four Different Windrows For Evaluation

Four windrows were constructed with broiler litter as the main constituent. An initial literature review indicated that the use of inoculants, increasing the C:N ratio and adding zeolite could alter the composting process. The company commissioned the study to increase production whilst decreasing the composting time. Another goal was to evaluate their current operational procedures.

Windrow 1 acted both as a control to evaluate the other windrows against, as well as to evaluate the company's composting processes. Windrow 2 included an inoculum of previously composted material. An inoculum was used as from the initial literature review it followed that inoculums could decrease the initial lag phase, and therefore decrease the composting time. This could have lead to increased production and would have increased the annual income generated by the company. Windrows 3 and 4 both had a increased carbon content at the start of the composting process. Increasing the C:N ratio was shown in literature to increase internal temperatures, as it acts as an energy source that could be utilized by the microbial consortium of organisms present. As the company observed large losses in nitrogen due to the volatization of ammonia, zeolite was added to the substrate as a mitigation effort.

The four windrows were successfully subjected to windrow composting, and no alterations were needed for the management practices applied by the company. No problems were observed during the physical manipulation of the windrows, therefore indicating that any of the compositions could be used by the company without altering the machinery used.

5.1.2 Evaluation Of The Four Different Formulated Windrows On Physical Attributes

The study was successful in monitoring both physico and chemical parameters of the windrows. Windrow 1 had the lowest average temperature during the course of the composting process observed, and never entered a true thermophilic phase. Although Windrow 2 had temperatures on several occasions that were above 40°C, these temperatures could not be maintained. Based on these observations, no composting occurred in both Windrow 1 and Windrow 2.

Nitrogen was lost in all four windrows, however Windrows 1 and 2 had losses that were far above that of Windrows 3 and 4. These losses were mainly in the form of ammonia compounds and a pungent odour was quite evident in the vicinity of the composting facility.

These observations led to the conclusion that the current composting process used by the company does not entail a true composting process. The loss of nitrogen to the atmosphere is of concern, as this results in a lower nitrogen content within the end product. The product obtained from these facilities using only broiler litter should be marketed as dried, pelleted broiler litter and not as composts.

Windrow 3 and Windrow 4 both had significantly higher temperatures that were maintained for an extended period. Both entered a true thermogenic phase. Windrow 4 had a longer thermogenic phase and this could potentially be due to the addition of zeolite. This is an aspect that has to be experimentally determined in subsequent tests.

Nitrogen losses were significantly less in these windrows, with the lowest nitrogen loss observed in Windrow 3. Based on these observations, it is concluded that composting of broiler litter should entail an adjustment to the C:N ratio of the initial substrate. This would lead to a more commercially sustainable product that has both increased soil nutritional values, and is free (or at least has a greatly reduced level) of pathogenic organisms and weed seeds. The period of composting should be extended beyond the 8 weeks currently used by the company. Data should be obtained on a daily basis about the performance of the windrows, and using a combination of temperature profiles and C:N ratio as indication of compost maturity. The inclusion of a curing phase is crucial to the stabilization of the end-product.

5.1.3 Characterizing Of The Microbial Community Succession By Means Of PCR-DGGE Analysis

The successful extraction of DNA from compost is limited by the high amounts of humic-acids present within the compost. This normally greatly reduces the quality of DNA, especially if the

DNA is subjected to downstream applications such as PCR. Various commercial isolation kits were evaluated as part of a previous study. DNA was successfully isolated using the Machery-Nagel Soil DNA Extraction kit. Amplification using different primer sets acted as proof of the efficiency of the DNA isolation technique, together with the spectrophotometric values obtained from NanoDrop measurements and agarose gel electrophoresis.

PCR-DGGE analysis was successfully applied to isolated DNA samples from the four different windrows. These profiles were complex, and large shifts could be observed in the microbial community. Overall 454 prokaryotic bands in 55 different positions were identified, indicating a large community present during the composting process.

For this study, sixteen bands were identified as important in the composting process. The sequences obtained from these bands were compared to two databases. The 16S rRNA database for Bacteria and Archaea was used to a possible identity for the bands excised. Results from using this database mainly belonged to either *Bacillus* spp. or *Paenibacillus* spp. Other organisms identified were *Pedobacter lentus, Mechercharimyces asporophorigenens, M. mesophilus* and *Ulvibacter antarcticus.* This database however, gave low maximum identity scores for the identifications, resulting in a low confidence in the identities obtained.

The nucleotide collection was also employed in comparing the sequences obtained. Overall, higher maximum identifications were obtained using this database. Twelve of the sequences were also found to be related to either composting processes or animal manures. Although the names of these organisms could not be concluded from the nucleotide collection, most of the sequences were previously found in composting related materials.

5.1.4 Evaluation Of The Microbial Population Dynamics In Windrows

Using PCR-DGGE analysis showed that the microbial community is dynamic during the composting and co-composting of broiler litter. Windrows 1 and 2 never reached a true thermophilic temperature range. Thermophilic phases were observed in Windrows 3 and 4. This resulted in a selection in microbial diversity that was observed in the dendrogram constructed.

From CCA it could be concluded that temperature and C:N ratio played significant roles in the composting process. Several bands could also be identified as sensitive to changes in physico-chemical parameters observed in this study.

Applying Simpson's Reciprocal, Shannon-Weaver and species richness indices to DGGE profiles were completed successfully. Large shifts were observed during the composting

process. These were mainly related to moisture content and temperature within the windrows. Altering the compost substrate resulted in significant changes in the microbial community present during the composting process.

5.2 Recommendations

The following points should be considered as recommendations for future studies on broiler litter composting:

- Constant monitoring of the physico- chemical characteristics during the composting process should be performed. This would allow for the monitoring of chemical dynamics within the windrows and provide more data points that could be used for analysis.
- Inclusion of a windrow with the same composition as Windrow 3, but without the previously composted material, would have tested only the effect of C:N ratio on the natural composting process. The use of previously composted material could have lead to a selection of ammonia producing organisms, reducing the amount of N in the final product due to the volatization of ammonia.
- One of the oversights of this study pertains to recording the mass of the end product. For future studies, the mass at the end of the observed composting process needs to be determined. This would enable better interpretation of the chemical composition of the composts.
- The study did not include any microbial biomass data. Such data would have resulted in a larger scope on the microbial diversity dynamics. It would therefore be recommended that any future study pertaining to microbial diversity dynamics includes estimations of biomass.
- Culture based techniques are biased in their selection of organisms and may thus not be suitable for microbial diversity and dynamics studies. However, the implementation of culture based techniques could aid in determining the survival of pathogenic organisms.
- The use of PCR-DGGE analysis was successful for this study, however it is time consuming and the cost per identification ratio is high. With new technology becoming more accessible, the use of next-generation sequencing (NGS) would be a more cost effective use for studying microbial diversity in environmental samples. For NGS analysis the cost per sample is quite high. However, the huge amount of sequences (several thousand) that are being produced brings down the cost per sequence tremendously.
- The optimization of the C:N ratio is crucial to the successful composting of broiler litter. A carbon source should be added to increase the initial substrate C:N ratio. This will increase windrow internal temperatures, resulting in a compost that is free of pathogenic organisms and weed seeds.

- Constant monitoring of temperature, moisture and chemical composition would help ensure a stabilized end-product that is free from phytotoxic effects, weed seeds and pathogenic organisms. Ensuring that the compost remains aerobic, with a corrected C:N ratio and sufficient moisture would result in a greater microbial diversity. This in turn would aid as a buffer, as greater microbial diversity results in a higher redundancy should environmental factors suddenly change.
- Results presented in this study has clearly demonstrated that the commissioning company should seriously consider the last two recommendations to ensure that they produce compost of a sustainable quality standard.

The study has demonstrated that composting broiler litter without a carbon-amendment results in an immature and uncomposted end-product, which could possibly have negative effects on the environment. Microbial community succession during composting was successfully studied using PCR-DGGE analysis. Thus the aim was achieved by the successful execution of the five objectives.

REFERENCES

ABOLNIK, C. 2007. Molecular epidemiology of Newcastle disease and avian influenza in South Africa. Pretoria: UP (Dissertation – Ph. D. Zoology)

ADAMS, J.D.W. & FROSTICK, L.E. 2009. Analysis of bacterial activity, biomass and diversity during windrow composting. *Waste Management,* 29:598-605.

ADELI, A., SISTANI, K.R., ROWE, D.E. & TEWOLDE, H. 2005. Effects of broiler litter on soybean production and soil nitrogen and phosphorus concentrations. *Agronomy Journal,* 97:314-321.

ANON. 2012. Zeolite Structure.
http://www.grace.com/EngineeredMaterials/MaterialSciences/Zeolites/ZeoliteStructure.aspx
Date of access: 3 Aug. 2012.

AOSHIMA, H., KIMURA, A., SHIBUTANI, A., OKADA, C., MATSUMIYA, Y., & KUBO, M. 2006. Evaluation of soil bacterial biomass using environmental DNA extracted by slow-stirring method. *Applied Microbial Biotechnology,* 71:875-880.

ASH, C., PRIEST, F.G., & COLLINS, M.D. 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie van Leeuwenhoek*, 64(3-4):253-260.

ATKINSON, C.F., JONES, D.D., & GAUTHIER, J.J. 1996. Putative anaerobic activity in aerated composts. *Journal of Industrial Microbiology,* 16:182-188.

AURY, K., LE BOUQUIN, S., TOQUIN, M.T., HUNEAU-SALAüN, A., LE NôTRE, Y., ALLAIN, V., PETETIN, I., FRAVALO, P., & CHEMALY, M. 2011. Risk factors for *Listeria monocytogenes* contamination in French laying hens and broiler flocks. *Preventive Veterinary Medicine*, 98(4):271-278.

AZIZI-SHOTORKHOFT, A., ROUZBEHAN, Y. & FAZAELI, H. 2012. The influence of the different carbohydrate sources on utilization efficiency of processed broiler litter in sheep. *Livestock Science*, 148:249-254.

BAFFI, C., DELL'ABATE, M.T., NASSISI, A., SILVA, S., BENEDETTI, A., GENEVINI, P.L., & ADANI, F. 2007. Determination of biological stability in compost: A comparison of methodologies. *Soil Biology & Biochemistry,* 39:1284-1293.

BARRINGTON, S., CHOINIèRE, D., TRIGUI, M., & KNIGHT, W. 2002. Effect of carbon source on compost nitrogen and carbon losses. *Bioresource Technology*, 83(3):189-194.

BERNAL, M.P., LOPEZ-REAL, J.M., & SCOTT, K.M. 1993. Application of natural zeolites for the reduction of ammonia emissions during the composting of organic wastes in a laboratory composting simulator. *Bioresource Technology*, 43(1):35-39.

BERNAL, M.P., ALBURQUERQUE, J.A., & MORAL, R. 2009. Composting of animal manures and chemical criteria for compost maturity assessment. A review. *Bioresource Technology*, 100:5444-5453.

BHATTACHARYA, A.N., & FONTENOT, J.P. 1965. Utilization of different levels of poultry litter nitrogen by sheep. *Journal of Animal Science*, 24:1174:1178.

BHATTACHARYA, A.N., & FONTENOT, J.P. 1966. Protein and energy value of peanut hull and wood shaving poultry litters. *Journal of Animal Science*, 25(2):367-371.

BIOGAS ENERGY INC. 2008. Key elements of biogas energy anaerobic digesters. www.biogas-energy.com/site/BiogasEnergy.pdf Date of access: 5 Aug. 2012

BAKSHI, M.P.S. & FONTENOT, J.P. 1998. Processing and nutritive evaluation of broiler litter as livestock feed. *Animal Feed Science and Technology*, 74:337-345.

BEFFA, T., BLANC, M., MARILLEY, L., LOTT-FISCHER, J., & LYON, P.-F. 1995. Taxonomic and metabolic microbial diversity during composting. (*In* de Betoldi, M., Sequi, P., Lemmes, B., & Papi, T., eds. The science of composting. Glasgow:Blackies academic and Professional, vol. 1:149-161).

BEFFA, T. 2002. The composting biotechnology: A microbial aerobic solid substrate fermentation complex process.
http://www.compag.ch/pdf/THE%20COMPOSTING%20BIOTECHNOLOGY%20COMPLETE.pdf Date of access: 16 Nov. 2012.

BRINTON, W.F. 2000. Final Report: Compost Quality Standards & Guidelines. http://www.woodsend.org/pdf-files/nysar-ne.pdf Date of access: 20 Jan. 2010.

BRODIE, H.L., CARR, L.E., & CONDON, P. 2000. Poultry litter composting comparisons. *Biocycle*, 41:36-40.

BOLAN, N., A. SZOHI, A., CHUSAVAVATHI, T., SESHADRI, B., ROTHROCK, M., & PANNEERSELVAM, P. 2010. Uses and management of poultry litter. *World's Poultry Science Journal*, 66:673-698.

BüRGMANN, H., PESARO, M., WIDMER, F., & ZEYER, J. 2001. A strategy for optimizing quality and quantity of DNA extracted from soil. *Journal of Microbiological Methods*, 45(1):7-20.

BUSCA, G., & PISTARINO, C. 2003. Abatement of ammonia and amines from waste gases: a summary. *Journal of loss prevention in the process industries*, 16:157-163.

CASEY, W.R., FAIRCHILD, B.D. & LACY, M.P. 2005. Litter quality and broiler performance. www.thepoultrysite.com/articles/388/litter-quality-and-broiler-performance Date of access: 16 Jul. 2012.

CAMBARDELLA, C.A., RICHARD, T.L., & RUSSELL, A. 2003. Compost mineralization in soil as a function of composting process conditions. *European Journal of Soil Biology*, 39(3):117-127.

CAMBRIDGE ONLINE DICTIONARY. 2012. Broiler. http://dictionary.cambridge.org/dictionary/british/broiler?q=broiler Date of access: 08 May 2012.

CARDENAS, E., & TIEDJE, J.M. 2008. New tools for discovering and characterizing microbial diversity. *Current Opinion in Biotechnology*, 19(6):544-549.

COMBET-BLANC, Y., OLLIVIER, B., STREICHER, C., PATEL, B.K.C., DWIVEDI, P.P., POT, B., PRENSIER, G., & GARCIA, J.-L. 1995. *Bacillus thermoamylovorans* sp. nov., a moderately thermophilic and amylolytic bacterium. *International Journal of Systematic and Evolutionary Microbiology*, 45(1):9-16.

CRESSMAN, M.D., YU, Z., NELSON, M.C., MOELLER, S.J., LILBURN, M.S., & ZERBY, H.N. 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. *Applied and Environmental Microbiology,* 76 (19): 6572-6582.

COONEY, E.L., BOOKER, N.A., SHALLCROSS, D.C., & STEVENS, G.W. 1999. Ammonia removal from wastewaters using natural Australian zeolite. II. Pilot-Scale study using continuous packed column process. *Separation Science and Technology*, 34(14):2741-2769.

CORNELL UNIVERSITY. 1996. Compost Chemistry. http://compost.css.cornell.edu/chemistry.html Date of access: 15 Nov. 2012

DÁVALOS, J.Z., ROUX, M.V., & JIMéNEZ, P. 2002. Evaluation of poultry litter as a feasible fuel. *Thermochimica Acta*, 394:261-266.

DAVIS, M.E. & LOBO, R.F. 1992. Zeolite and molecular sieve synthesis. *Chemistry of Materials,* 4(4):756-768.

DELAUNE, P.B., MOORE, P.A., DANIEL, T.C., & LEMUNYON, J.L. 2004. Effect of chemical and microbial amendments on ammonia volatilization from composting poultry litter. *Journal of Environmental Quality*, 33:728-734.

DEMHARTER, W., & HENSEL, R. 1989. *Bacillus thermocloaceae sp. nov.,* a new thermophilic species from sewage sludge. *Systematic and Applied Microbiology*, 11(3):272-276.

DEVI, S., SHARMAY, C.R., & SINGH, K. 2012. Microbiological biodiversity in poultry and paddy straw wastes in composting systems. *Brazilian journal of microbiology*, (2012):288-296.

DICE, L.R. 1945. Measures on the amount of ecologic association between species. *Ecology*, 26:297-302.

DONG, X., & TOLLNER, E.W. 2003. Evaluation of Anammox and denitrification during anaerobic digestion of poultry manure. *Bioresource Technology*, 86: 139-145.

DWAF (DEPARTMENT OF WATER AFFAIRS). 2005. A guide to clearing invasive alien plants. www.dwaf.gov.za/wfw/weedbuster/clearingbrochure07.pdf Date of access: 14 May 2012.

ECN. (EUROPEAN COMPOST NETWORK). 2012. ECN info paper no. 01/2012. http://www.compostnetwork.info/wordpress/wp-content/uploads/2010/08/20120503_ECN_Info-paper_ECN_QAS_2012.pdf Date of access: 6 Nov. 2012.

EICHNER, C.A., ERB, R.W., TIMMIS, K.N., & WAGNER-DöBLER, I. 1999. Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge microbial community. *Applied and Environmental Microbiology*, 65(1):102-109.

EILAND, F., KLAMER, M., LIND, A.M., LETH, M., BååTH, E. 2001. Influence of initial C/N ratio on chemical and microbial composition during long term composting of straw. *Microbial Ecology*, 41(3):272-280.

ELEROĞLU, H. & YALÇIN, H. 2005. Use of natural zeolite-supplemented litter increased broiler production. *South African Journal of Animal Science*, 35 (2) : 90-97.

ELWELL, D.L., KEENER, H.M., CAREY, D.S., & SCHLAK, P.P. 1998. Composting unamended chicken manure. *Compost Science and Utilization*. Spring:22-35

EPRL. 2012. Energy for tomorrow's generation. www.eprl.co.uk Date of access: 10 Oct. 2012.

ENTICKNAP, J.J., NONOGAKI, H., PLACE, A.R., & HILL, R.T. 2006. Microbial diversity associated with odor modification for production of fertilizers from chicken litter. *Applied Environmental Microbiology*, 72(6):4105-4114.

ETTENAUER, J.D., PIñAR, G., LOPANDIC, K., SPANGL, B., ELLERSDORFER, G., VOITL, C. & STERFLINGER, K. 2012. Microbes on building materials – Evaluation of DNA extraction protocols as common basis for molecular analysis. *Science of The Total Environment*, 439:44-53.

FAO (FOOD AND AGRICULTURE ORGANIZATION). 1968. Non-protein nitrogen in the nutrition of ruminants. http://www.fao.org/docrep/004/AC149E/AC149E00.HTM Date of access: 08 May 2012

FELSKE, A., ENGELEN, B., NUBEL, U., & BACKHAUS, H. 1996. Direct ribosomal isolation from soil to extract bacterial rRNA for community analysis. *Applied and Environmental Microbiology*, 62: 4162-4167.

FELSKE, A.D., TZENEVA, V., HEYRMAN, J., LANGEVELD, M.A., AKKERMANS, A.D., & DE VOS, P. 2004. Isolation and biodiversity of hitherto undescribed soil bacteria related to *Bacillus niacini*. *Microbial Ecology,* 48(1):111-119.

FNB (FIRST NATIONAL BANK). 2012. 2012 Agriculture Weekly Newsletters. https://www.fnb.co.za/business-banking/industry-focused-area/agriculture/weekly-newsletters.html Date of access: 08 May 2012.

FIBROPHOS. 2012. Homepage. http://www.fibrophos.co.uk/ Date of access: 5 Aug. 2012

FISCHER, S.G. & LERMAN, L.S. 1983. DNA fragments differing by single-basepair substitutions are separated in denaturing gradient gel: Correspondence with melting theory. *Proceedings of the National Academy of Sciences,* 80:1579-1583.

FURTULA, V., FARELL, E.G., DIARRASSOUBA, F., REMPEL, H., PRITCHARD, J., & DIARRA, M.S. 2010. Veterinary pharmaceuticals and antibiotic resistance of *Escherichia coli* isolates in poultry litter from commercial farms and controlled feeding trials. *Poultry Science*, 89(1):180-188.

GAJALAKSHMI, S., & ABBASI, S.A. 2008. Solid waste management by composting: State of the Art. *Critical reviews in Environmental Science and Technology*, 38(5):311-400.

GAMZE-TURAN, N. 2007. The effects of natural zeolite on salinity level of poultry litter compost. *Bioresource Technology*, 99(7):2097-2101.

GAO, M., LI, B., YU, A., LIANG, F., YANG, L., & SUN, Y. 2010. The effect of aeration rate on forced-aeration composting of chicken manure and sawdust. *Bioresource Technology,* 101:1899-1903.

GCIS (GOVERNMENT COMMUNICATIONS AND INFORMATION SYSTEM). 2010. Pocket Guide To South Africa 2010/11 AGRICULTURE, FORESTRY AND FISHERIES http://www.gcis.gov.za/resource_centre/sa_info/pocketguide/2010/014_agriculture_forestry_and_fisheries.pdf Date of access: 08 May 2012.

GELEGENIS, J., GEORGAKAKIS, D., ANGELIDAK, I., & MARRIS, V. 2007. Optimization of biogas production by co-digesting whey with diluted poultry manure. *Renewable Energy*, 32:2147-2160.

GIRAFFA, G., & NEVIANI, E. 2001. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *International Journal of Food Microbiology,* 67(1-2):19-34.

GOLUEKE, C.G., CARD, B.J., & McGAUHEY, P.H. 1953. A critical evaluation of inoculums in composting. *Applied and Environmental Microbiology,* 2(1):45-53.

GONG, C.M., KOICHI, I., SHUNJI, I., & TAKASHI, S. 2005. Survival of pathogenic bacteria in compost with special reference to *Escherichia coli. Journal of Environmental Science (China)*, 17 (5):770-774 (Abstract in English).

GOYAL, S., DHULL, S.K., & KAPOOR, K.K. 2005. Chemical and biological changes during composting of different organic wastes and assessment of compost maturity. *Bioresource Technology,* 96:1584-1591.

GRIFFITHS, R.I., WHITELEY, A.S., O'DONNELL, A.G., & BAILEY, M.J. 2003. Physiological and community responses of established grassland bacterial populations to water stress. *Applied and Environmental Microbiology,* 69(12):6961-6968.

HALL, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acid Symposium Series* 41: 95-98

HASSEN, A., BELGUITH, K., JEDIDI, N., CHERIF, A., CHERIF, M., & BOUDABOUS, A. 2001. Microbial characterization during composting of municipal solid waste. *Bioresource Technology,* 80:217-225.

HEUER, H., SMALLA, K. 1997. Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. (*In* Van Elsas, J.D., Trevors, J.T., Wellington, E.M.H. (eds.). Modern soil microbiology. New York:Marcel Dekker. p. 353-373).

HEYRMAN, J., VANPARYS, B., LOGAN, N.A., BALCAEN, A., RODRIQUEZ-DIAZ, M., FELSKE, A., & DE VOS, P. 2004. *Bacillus novalis* sp. nov*., Bacillus vireti* sp. nov*., Bacillus soli* sp. nov*., Bacillus bataviensis* sp. nov*.,* and *Bacillus drentensis* sp. nov*.,* from the Drentse A grasslands. *International Journal of Systematic and Evolutionary Microbiology*, 54(1):47-57.

HIRSCH, P.R., MAUCHLINE, T.H., & CLARK, I.M. 2010. Culture-independent molecular techniques for soil microbial ecology. *Soil Biology and Biochemistry*, 42(6):878-887.

HOLM, D. 2011. Hoendermis as aanvullende voer vir beeste.
http://www.landbou.com/kundiges/vra-vir-faffa/hoendermis-as-aanvullende-voer-vir-beeste
Date of access: 5 Aug. 2012

HOON, J.H., JONKER, M.L., HERSELMAN, M.J., & VAN ZYL, C.B. 2011. The utilisation of broiler litter as winter supplement for beef cattle on tarchnonanthus veld. http://gadi.agric.za/articles/Hoon_JH/broiler.php?PHPSESSID=f63aa18abac04a2315c19a666c95933b Date of access: 05 Aug. 2012.

HORI, T., HARUTA, S., UENO, Y., ISHII, M., & IGARASHI, Y. 2006. Direct comparison of single-stranded conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) to characterize a microbial community on the basis of 16S rRNA gene fragments. *Journal of Microbiological Methods*, 66(1):165-169.

HUANG, G.F., WONG, J.C.W., WU, Q.T., & NAGAR, B.B. 2004. Effect of C/N on composting of pig manure with sawdust. *Waste Management,* 24(8):805-813.

HURT, R.A., QIU, X., WU, L., ROH, Y., PALUMBO, A.V., TIEDJIE, J.M., & ZHOU, J. 2001. Simultaneous recovery of RNA and DNA from soils and sediments. *Applied and Environmental Microbiology*, 67(10):4495-4503.

ICHIDA, J.M., KRIZOVA, L., LEFEVRE, C.A., KEENER, H.M., ELWELL, D.L. & BURTT, E.H. 2001. Bacterial inoculum enhances keratin degradation and biofilm formation in poultry compost. *Journal of Microbiological Methods,* 47:199-208.

JANSSEN, P.H., YATES, P.S., GRINTON, B.E., TAYLOR, P.M. & SAIT, M. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria,Actinobacteria, Proteobacteria,* and *Veruucomicrobia*. *Applied and Environmental Microbiology*, 68(5):2391-2396.

JANDA, J.M. & ABBOTT, S.L. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils and pitfalls. *Journal of Clinical Microbiology,* 45(9):2761-2764.

JONES, D.L., HEALEY, J.R., WILLETT, V.B., FARRAR, J.F., & HODGE, A. 2005. Dissolved organic nitrogen uptake by plants – an important N uptake pathway? *Soil Biology and Biochemistry,* 37(3):413-423.

JONGMAN, R. H. G., TER BRAAK, C. J. F. & VAN TONGEREN, O. F. R., eds (1995). Data analysis in community and landscape ecology. Cambridge:Cambridge University Press.

JORDAAN, J.D. 2004. The influence of bedding material and collecting period on the feeding value of broiler and layer litter. Bloemfontein:UOFS. (Dissertation – M.Sc. Agric)

JUNG, W.-J., JIN, Y.-L., KIM, Y.-C., KIM, K.-Y., PARK, R.-D., & KIM, T.-H. 2004. Inoculation of *Paenibacillus illinoisensis* alleviates root mortality, activates of lignification-related enzymes, and induction of the isozymes in pepper plants infected by *Phytophthora capsici. Biological Control,* 30(3):645-652.

KALEMELAWA, F., NISHIHARA, E., ENDO, T., AHMAD, Z., YEASMIN, R., TENYWA, M.M. & YAMAMOTO, S. 2012. An evaluation of aerobic and anaerobic composting of banana peels treated with different inoculums for soil nutrient replenishment. *Bioresource Technology* Article in Press.

KARAMANLIS, X., FORTOMARIS, P., ARSENOS, G., DOSIS, I., PAPAIOANNOU, D., BATZIOS, C., & KAMARIANOS, A. 2008. The effect of natural zeolite *(Clinoptilolite)* on the performance of broiler chickens and the quality of their litter. *The Asian-Australasian Association Of Animal-Production Societies*, 21(11):1642-1650.

KATO, K., & MIURA, N. 2008. Effect of matured compost as a bulking and inoculating agent on the microbial community and maturity of cattle manure compost. *Bioresource Technology*, 99:3372-3380.

KELLEHER, B.P., LEAHY, J.J., HENIHAN, A.M., O'DWYER, T.F., SUTTON, D. & LEAHY, M.J. 2002. Advances in poultry litter disposal technology – a review. *Bioresource Technology*, (83):27-36.

KELLEY, T.R., PANCORBO, O.C., MERKA, W.C. THOMPSON, S.A., CABRERA, M.L., & BARNHART, H.M. 1998. Accumulation of elements in fractionated broiler litter during re-utilization. *Journal of Applied Poultry Research,* 7:27-34.

KPOMBLEKOU-A, K., ANKUMAH, R.O., & AJWA, H.A. 2002. Trace and nontrace element contents of broiler litter. *Communications in Soil Science and Plant Analysis,* 33(11-12):1799-1811.

KRIEL, G. 2012. Pasop vir hoendermis as beesvoer. *Landbouweekblad.* 27 July 2012.

KWAK, W.S., HUH, J.W., & McCASKEY, T.A. 2005. Effect of processing time on enteric bacteria survival and on temperature and chemical composition of broiler poultry litter processed by two methods. *Bioresource Technology,* 96:1592-1536.

LAMONTAGNE, M.G., MICHEL F.C., HOLDEN, P.A., & REDYY, C.E. 2002. Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *Journal of Microbiological Methods*, 49:255-264.

LANE, D.J. 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Editors). New York:Wiley. p. 115-175.

LAZAROVITS, G. 2001. Management of soil-borne plant pathogens with organic soil amendments: a disease control strategy salvaged from the past. *Canadian Journal of Plant Pathology*, 23:1-7.

LEE, J.-C., & YOON, K.-H. 2008. *Paenibacillus woosongensis* sp. nov., a *xylanolytic* bacterium isolated from forest soil. *International Journal Of Systematic And Evolutionary Microbiology*, 88:612-616.

LICHTENBERG, E., PARKER, D., & LYNCH, L. 2002. Economic value of poultry litter supplies in alternative uses. http://www.naturalresources.umd.edu/Publications/PDFs/Other/PoultryLitter.pdf Date of access: 8 Apr. 2012.

LOVANH, N., COOK, K.L., ROTHROCK, M.J., MILES, D.M., & SISTANI, K. 2007. Spatial shifts in microbial population structure within poultry litter associated with physicochemical properties. *Poultry Science*, 86:1840-1849.

LU, J., SANCHEZ, S., HOFACRE, CH., MAURER, J.J., HARMON, B.G., & LEE, M.D. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. *Applied and Environmental Microbiology*, 69 (2): 901-908.

MANUNGUFALA, T.E., CHIMUKA, L., & MASWANGANYI, B.X. 2008. Evaluating the quality of communities made compost manure in South Africa: A case study of content and sources of metals in compost manure from Thelamela Municipality, Limpopo province. *Bioresource Technology*, 99:1491-1496.

MARSHALL, M.N., COCOLIN, L., MILLS, D.A., & VAN DER GHEYNST, J.S. 2003. Evaluation of PCR primers for denaturing gradient gel electrophoresis analysis of fungal communities in compost. *Journal of Applied Microbiology*, 95:934-948.

MATERECHERA, S.A., & MKHABELA, T.S. 2002. The effectiveness of lime, chicken manure and leaf litter ash in ameliorating acidity in a soil previously under black wattle *(Acacia mearnsii)* plantation. *Bioresource Technology*, 85(1):9-16.

MATSUO, Y., KATSUTA, A., MATSUDA, S., SHIZURI, Y., YOKOTA, A. & KASAI, H. 2006. *Mechercharimyces mesophilus* gen. nov., sp. nov. and *Mechercharimyces asporophorigenens* sp. nov., antitumour substance-producing marine bacteria, and description of *Thermoactinomycetaceae* fam. nov. *International Journal of Systematic and Evolutionary Microbiology,* 56:2837-2842.

MAVIMBELA, D.T., VAN RYSSEN, J.B.J., & LAST, R. 1997. The effect of high broiler litter diets as survival ration on the health of sheep. *Journal of the South African Veterinary Association*, 68(4):121-124.

MAVIMBELA, D.T. 1999. The nutritional value of broiler litter as a feed source for sheep during periods of feed shortage. Pretoria:UP (Thesis - Ph.D.Agric).

MACGREGOR, S.T., MILLER, F.C., PSARIANOS, K.M., & FINSTEIN, M.S. 1981. Composting process control based on interaction between microbial heat output and temperature. *Applied and Environmental Microbiology*, 41(6):1321-1330.

MEFANE, M.A. 2009. Compost Standards [email]. 2 Dec. 2009.

MIGNARD, S., & FLANDROIS, J.P. 2006. 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. *Journal of Microbiological Methods,* 67: 574-581.

MILES, D.M., ROWE, D.E. & OWENS, P.R. 2008. Winter Broiler Litter Gases and Nitrogen Compounds: Temporal and Spatial Trends. *Atmospheric Environment*, (42):3351-3363.

MILLIGAN, E.E., TEMPLE, W.D., & BOMKE, A.A. 2008. Factsheet: Poultry Litter Composting http;//www.certifiedorganic.bc.ca/programs/osdp/l-050 Compost Factsheet.pdf Date of access: 16 Feb. 2012.

MITCHELL, C.C & TU, S. 2005. Long term evaluation of poultry manure as a source of nitrogen for cotton and corn. *Agronomy Journal*, 97:399-407.

MKHABELA, T.S. 2004. Substitution of fertiliser with poultry manure: Is it economically viable? *Agrekon*, 43 (3):347-356.

MONTEFUSCO, A., NAKAMURA, L.K., & LABEDA, D.P. 1993. *Bacillus peoriae* sp. nov. *International Journal of Systematic Bacteriology,* 43 (2):388-390.

MORAL, R., PAREDES, C., BUSTAMANTE, M.A., MARHUENDA-EGEA, F., & BERNAL, M.P. 2009. Utilisation of manure composts by high-value crops: Safety and environmental challenges. *Bioresource Technology*, 100:5454-5460.

MOSS, B.R., REEVES, D.W., LIN, J.C., TORBERT, H.A., McELHENNEY, W.H., MASK, P. & KEZAR, W. 2001. Yield and quality of three corn hybrids as affected by broiler litter fertilization and crop maturity. *Animal Feed Science and Technology,* 94:43-56.

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

MUYZER, G., & SMALLA, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek,* 73:127-141.

MYERS, R.M., FISCHER, S.G., LERMAN, L.S., & MANIATIS, T. 1985. Nearly all single base substitutions in DNA fragments joined to a GC clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Research*, 13: 3131-3145.

NAGEL, M., & ANDREESEN, J.R. 1991. *Bacillus niacini* sp. nov., Nicotinate-Metabolizing mesophile isolated from soil. *International Journal Of Systematic Bacteriology*, 41(1):134-139.

NAKASAKI, K., NAG, K., & SHUICHI, K. 2005. Microbial Succession associated with organic matter decomposition during thermophilic composting of organic waste. *Waste Management & Research,* 23 (1):48-56.

NDA (NATIONAL DEPARTMENT OF AGRICULTURE). 2000. The broiler industry. http://www.nda.agric.za/docs/genpub/9broilerseggs.pdf Date of access 17 Jun. 2012.

NICHOLSON, F.A., CHAMBERS, B.J., & SMITH, K.A. 1996. Nutrient composition of poultry manures in England and Wales. *Bioresource Technology*, 58: 279-284.

NICHOLSON, F.A., GROVES, S.J., & CHAMBERS, B.J. 2005. Pathogen survival during livestock manure storage and following land application. *Bioresource Technology*, 96 (2):135-143.

NOLAND, P.R., FORD, B.F., & RAY, M.L. 1955. The use of ground chicken litter as a source of nitrogen for gestating-lactating ewes and fattening steers. *Journal of Animal Science*, 14:860-865.

NOLL, M., & JIRJIS, R. 2012. Microbial communities in large-scale wood piles and their effects on wood quality and the environment. *Applied Microbiological Biotechnology*, 95:551-563.

NOVINSCAK, A., DECOSTE, N., SURETTE, C., & FILION, M. 2009. Characterization of bacterial and fungal communities in composted biosolids over a two year period using denaturing gradient gel electrophoresis. *Canadian Journal of Microbiology*, 55(4):375-387

OGUNWANDA, G.A., OSUNADE, J.A, ADEKALU, K.O. & OGUNJIMI, L.A.O. 2008. Nitrogen loss in chicken litter compost as affected by carbons to nitrogen ratio and turning frequency. *Bioresource Technology*, (99):7495-7503.

OHTAKI, A., AKAKURA, N, & NAKASAKI, K. 1997. Effects of temperature and inoculums on the degradability of poly-Ɛ-caprolactone during composting. *Polymer Degradation and Stability*, 62:279-284.

PANG, H., ZHANG, P., DUAN, C, MO, X., TANG, J., & FENG, J. 2009. Identification of Cellulase Genes from the Metagenomes of Compost Soils and Functional Characterization of One Novel Endoglucanase. *Current Microbiology*, 58:404-408.

PARR, J.F., HORNICK, S.B., & KAUFMAN, D.D. 1994. Use of microbial inoculants and organic fertilizers in agricultural production. www.agnet.org/library.php?func=view&id=20110722114739&type_id=4 Date of access: 15 Oct. 2012.

PARTANEN, P., HULTMAN, J., PAULIN, L., AUVINEN, P., & ROMANTSCHUK, M. 2010. Bacterial diversity at different stages of the composting process. BMC Microbiology 10:94 1-11.

PETERS, S., KOSCHINSKY, S., SCHWIEGER, F., & TEBBE, C.C. 2000. Succession of microbial communities during hot composting as detected by PCR-Single-Strand-Conformation polymorphism-based genetic profiles of Small-subunit rRNA genes. *Applied and Environmental Microbiology*, 66 (3):930-936.

PETRIC, I., SESTAN, A., & SESTAN, I. 2009. Influence of initial moisture content on the composting of poultry manure with wheat straw. *Biosystems Engineering*, 104(1):125-134.

PETTI, C.A. 2007. Detection and identification of microorganisms by gene amplification and sequencing. *Clinical Infectious Disease,* 44:1108-1114

RAMASWAMY, J., PRASHER, S.O., PATEL, R.M., HUSSAIN, S.A., & BARRINGTON, S.F. 2010. The effect of composting on the degradation of a veterinary pharmaceutical. *Bioresource Technology*, 101:2294-2299.

RAMESH, K., & REDDY, D.D. 2011. Zeolites and their potential uses in agriculture. *Advances in Agronomy*, 113:219-241.

RAZA, W., YANG, W., & SHEN, Q-R. 2008. *Paenibacillus polymyxa:* antibiotics, hydrolytic enzymes and hazard assessment. *Journal of Plant Pathology*, 90(3):419-430.

RIBEIRO, S.S., VASCONCELOS, J.T., MORAIS, M.G., ITAVO, C.B.C.F. & FRANCO, G.I. 2011. Effect of ruminal infusion of a slow-release polymer-coated urea or conventional urea on apparent nutrient digestibility, in situ degradability, and rumen parameters in cattle fed low-quality hay. *Animal Feed Science and Technology*, 164:53-61.

RICHARDSON, S.J. 1993. Evaluation of poultry litter ash as a fertilizer. http://www.fibrophos.co.uk/trialreports/TR1%20Fibrophos%20as%20a%20Fertiliser.pdf Date of access: 13 Nov. 2012.

RISSE, L.M., CABRERA, M.L., FRANZLUEBBERS, A.J., GASKIN, J.W., GILLEY, J.E., KILLORN, R., RADCLIFFE, D.E., TOLLNER, W.E., & ZHANG, H. 2001. Land application of manure for beneficial reuse.
http://www.cals.ncsu.edu/waste_mgt/natlcenter/whitepapersummaries/landapplication.pdf Date of access: 16 Nov. 2012.

REUTER, T., XU, W., ALEXANDER, T.W., STANFORD, K., XU, Y., & McALLISTER. 2009. Purification of polymerase chain reaction (PCR)-amplifiable DNA from compost piles containing bovine mortalities. *Bioresource Technology*, 100: 3343-3349.

RODRIGUEZ, R.A., PEPPER, I.L., & GERBA, C.P. 2009. Application of PCR-based methods to assess the infectivity of enteric viruses in environmental samples. *Applied and Environmental Microbiology,* 75(2):297-307.

RUBEIZ, I.G., KHANSA, M & FREIWAT, M.M. 1998. Evaluation of layer litter rates as a fertilizer for greenhouse strawberry and lettuce. *Communications In Soil Science And Plant Analysis,* 29:1-2, 161-167.

RYCKEBOER, J., MERGAERT, J., VAES, K., KLAMMER, S., DE CLERCQ, D., COOSEMANS, J., INSAM, H., & SWINGS, J. 2003. A survey of bacteria and fungi occurring during composting and self-heating processes. *Annals of Microbiology,* 53 (4) 349-410.

SANBORN, J.R. 1925. Physiological studies of accessory and stimulating factors in certain media. *Journal of Bacteriology*, XII (1):1-11.

SAPA see South African Poultry Association

SHANNON, C.E., & WEAVER, W. 1963. The mathematical theory of communication. Urbana:University of Illinois Press.

SCHIMEL, J.P., GULLEDGE, J.M., CLEIN-CURLEY, J.S., LINDSTROM, J.E., & BRADDOCK, J.F. 1999. Moisture effects on microbial activity and community structure in decomposing birch litter in the Alaskan taiga. *Soil Biology & Biochemistry*, 31:831-838.

SHEFFIELD, V.C., COX, D.R., & MYERS, R.M. 1989. Attachment of a 40-bp G+C rich sequence (GC-clamp) to genomic DNA fragments by polymerase chain reaction results in improved detection of single-base changes. *Proceedings of National Academy of Science USA*, 86: 232-236.

SHIDA, O., TAKAGI, H., KADOWAKI, K., NAKAMURA, L.K., & KOMAGATA, K. 1997. Emended description of *Paenibacillus amylolyticus* and description of *Paenibacillus illinoisensis* sp. nov. and *Paenibacillus chibensis* sp. nov. *International Journal Of Systematic Bacteriology*, Apr :299-306

SHLOSBERG, A., HARMELIN, A., PERL, S., PANO, G., DAVIDSON, M., ORGAD, U., KALI, U., BOR, A., VAN HAM, M., HOIDA, G., YAKOBSON, B., AVIDAR, Y., ISRAELI, B.A. & BOGIN, E. 1992. Cardiomyopathy in cattle induced by residues of the coccidiostat maduramicin in poultry litter given as a feedstuff. *Veterinary Research Communications*, 16: 45-58

SILVA, M.E., LEMOS, L.T., CUNHA-QUEDA, A.C., & NUNES, O.C. 2009. Co-composting of poultry manure with low quantities of carbon-rich materials. *Waste Management & Research*, 27:119-128.

SIMPSON, E.H. 1949. Measurement of diversity. *Nature,* 163:688.

SISTANI, K.R., ROWE, D.E., JOHNSON, J. & TEWOLDE, H. 2004 Supplemental nitrogen effect on broiler-litter-fertilized cotton. *Agronomy Journal*, 96 (3) 806-811.

SISTANI, K.R., ADELI, A., MCGOWEN, S.L., TEWOLDE, H. & BRING, G.E. 2008. Laboratory and field evaluation of broiler litter nitrogen mineralization. *Bioresource Technology,* 99(7):2603-2611.

SMART, J.L., JONES, T.O., CLEGG, F.G. & McMURTRY, M.J. 1987. Poultry waste associated type C botulism in cattle. *Epidemiology and Infection*, 98:73-79.

SMIT, E., LEEFLANG, P., GLANDORF, B., VAN ELSAS, J.D. & WERNARS, K. 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied and Environmental Microbiology*, 65 (6): 2614-2621.

SOUTH AFRICA. 2012. The nine provinces of South Africa. www.southafrica.info/about/geography/provinces.htm#.UH0-p2--rNI Date of access 15 Mar. 2012.

SOUTH AFRICAN POULTRY ASSOCIATION. 2010. Overview of the South African Poultry Industry From 2000 to 2010. http://sapoultry.co.za/pdf/2011%2002%20OVERVIEW%20OF%20THE%20SOUTH%20AFRICAN%20PULTRY%20INDUSTRY%20FROM%202000%20TO%202010.pdf Date of access: 08 May 2012

SOUTH AFRICAN POULTRY ASSOCIATION. 2011. Report on highly pathogenic notifiable avian influenza (HPNAI) surveillance monitoring by SAPA for the surveillance period January 2011 to June 2011. www.sapoultry.co.za/information_Nai_surveillance_monitor.php. Date of access: 23 Jul. 2012

SOUTH AFRICAN POULTRY ASSOCIATION. 2012a. Home page. sapoultry.co.za Date of access: 08 May 2012.

SOUTH AFRICAN POULTRY ASSOCIATION. 2012b. Disease outbreak kills 170 chickens. http://www.iol.co.za/news/south-africa/north-west/disease-outbreak-kills-170-chickens-1.1252897#.ULWqW--_dYI Date of access: 8 May 2012.

SOUTH AFRICAN POULTRY ASSOCIATION. 2012c. South African Poultry Association code of practice 2012. http://www.sapoultry.co.za/information_code_of_practice_broilers.php Date of access 05 Aug. 2012

STACKEBRANDT, E., & GOEBEL, B.M. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology*, 44 (4): 846-849.

STATISTICS SOUTH AFRICA. 2009. Census of Commercial Agriculture 2007 (Preliminary). http://www.statssa.gov.za/agriculture/Documents/2008docs/P1102%20-%20Publication_statistical%20release_Final(16th-02)%20.doc Date of access: 08 May 2012.

STEGER, K., EKLIND,Y., OLSSON, J., & SUNDH, I. 2005. Microbial community growth and utilization of carbon constituents during thermophilic compostinig at different oxygen levels. *Microbial Ecology*, 50:163-171.

SUN, X. 2006. Nitrogen transformation in food-waste composting. Saskatchewan:University of Regina (Dissertation: Master of Applied Science in Environmental Systems Engineering)

SUTTLE, N.F. & PRICE, J. 1976. The potential toxicity of copper-rich animal excreta to sheep. *Animal Production*, 23 (2): 233-241 [Abstract].

SWEETEN, J.M. & MATHERS, A.C. 1985. Improving soils with livestock manure. *Journal of soil and water conservation,* 40(2):206-210.

SZÉKELY, A.J., SIPOS, R., BRIGITTA BERTA, B., VAJNA, B., HAJDÚ, C., & MÁRIALIGETI, K. 2009. DGGE and T-RFLP Analysis of Bacterial Succession during Mushroom Compost Production and Sequence-aided T-RFLP Profile of Mature Compost. *Environmental Microbiology*, 57:522-533.

SZOGI, A.A., & VANOTTI, M.B. 2009. Prospects for phosphorus recovery from poultry litter. *Bioresource Technology*, 100:5461-5465.

TEWOLDE,H., BUEHRING, N., ADELI, A., SISTANI, K.R., ROWE, D.E. & PRATT R.G. 2009. Cotton response to chicken litter in rotation with corn in clayey soil. *Agronomy Journal,* 101:626-634.

THOMAS, L.V., & WIMPENNY J.W.T. 1993. Method for investigation of competition between bacteria as a function of three environmental factors varied simultaneously. *Applied and Environmental Microbiology*, 59(6):1991-1997.

THORMAN, R.E., CHADWICK, D.R., BOYLES, L.O., MATTHEWS, R., SAGOO, E. & HARRISON, R. 2006. Nitrous Oxide Emissions During Storage of Broiler Litter and Following Application to Arable Land. *International Congress Series* (1293):355-358

TIQUIA, S.M., TAM, N.F.Y., & HODGKISS, I.J. 1996. Microbial activities during the composting of spent pig-manure sawdust litter at different moisture contents. *Bioresource Technology,* 55(3):201-206

TIQUIA, S.M. & TAM, N.F.Y. 2000. Fate of nitrogen during composting of chicken litter. *Environmental Pollution*, 110:535-541.

TOHGYANI, M., GHEISARI, A., MODARESI, M., TABEIDIAN, S.A. & TOGHYANI, M. 2010. Effect of different litter material on performance and behaviour of broiler chickens. *Applied Animal Behaviour Science*, 122: 48-52.

TOMPKINS, D.K., CHAW, D., & ABIOLA, A.T. 1998. Effect of windrow composting on weed seed germination and viability. *Compost Science & Utilization*, Winter: 30-34.

TRAUTMAN, N., & OLYNCIW, E. 1996. Compost microorganisms. compost.css.cornell.edu/microorg.html Date of access: 14 Oct. 2012

TUOMELA, M., WIKMAN, M., HATAKKA, A. & ITÄVAARA, M. 2000. Biodegradation of lignin in a compost environment: a review. *Bioresource Technology* 72:169-183

UNITED STATES POULTRY & EGG ASSOCIATION. 2012. Poultry Industry Frequently Asked Questions Provided by the U.S. Poultry & Egg Association. www.uspoultry.org/faq/docs/industryFAQ.pdf Date of access: 08 May 2012.

USPEA see UNITED STATES POULTRY & EGG ASSOCIATION

VAN DAMME, P., POT, B., GILLIS, M., DE VOS, P., KERSTER, K., & SWINGS, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics*., Microbiological Reviews*, 60 (2):407-438.

VAN RYSSEN, J.B.J. 2001. Poultry litter as a feedstuff for ruminants: A South African scene. Available online: www.sassas.co.za/sites/sassas.co.za/files/Van RyssenPoultry litterReg.pdf Date of access: 17 Jul. 2012.

VAN RYSSEN, J.B.J., VAN MALSEN, P.S.M., & VERBEEK, A.A. 1993. The mineral composition of poultry manure in South Africa with reference to the Farm Feed Act*. South African Journal of Animal Science*, 23, 54-57.

VARGAS-GARCIA, M.C., SUÁREZ-ESTRELLA, F., LÓPEZ, M.J., & MORENO, J. 2006. Influence of microbial inoculation and co-composting material on the evolution of humic-like substances during composting of horticultural wastes*. Process Biochemistry*, 41: 1438-1443.

VARGAS-GARCIA, M.C., SUÁREZ-ESTRELLA, F., LÓPEZ, M.J., & MORENO, J. 2007. Effect of inoculation in composting processes: Modifications in lignocellulosic fraction*. Waste Management,* 27:1099-1107.

VENGLOVSKY, J., SASKOVA, N., VARGOVA, M., PACAJOVA, Z., PLACHA, I., PETROVSKY, M., & HARICHOVA, D. 2005. Evolution of temperature and chemical parameters during composting of the pig slurry solid fraction amended with natural zeolite*. Bioresource Technology*, 96 (2):181-189.

VON DER WEID, I., ALVIANO, D.S., SANTOS, A.L.S., SOARES, R.M.A., ALVIANO, C.S., & SELDIN, L. 2003. Antimicrobial activity of *Paenibacillus peoriae* strain NRRL DB-62 against a broad spectrum of phytopathogenic bacteria and fungi*. Journal of Applied Microbiology*, 95:1143-1151.

WAKASE, S., SASAKI, H., ITOH, K., OTAWA, K., KITAZUME, O., NONAKA, J., SATOH, M., SASAKI, T. & NAKAI, Y. 2008. Investigation of the microbial community in a microbiological additive used in a manure composting process. *Bioresource Technology,* 99(7):2687-2693.

WARD, J.H. 1963. Hierarchical grouping to optimize an objective function. *Journal of American Statistical Association*, 58:236–244.

WHITEHOUSE, C.A., & HOTTEL, H.E. 2007. Comparison of five commercial DNA extraction kits for the recovery of *Francisella tularensis* DNA from spiked soil samples. *Molecular and Cellular Probes*, 21:92-96.

WHITELEY, A.S., GRIFFITHS, R.I., & BAILEY, M.J. 2003. Analysis for the microbial functional diversity within water-stressed soil communities by flow cytometric analysis and CTC+ cell sorting. *Journal of Microbiological Methods*, 54:257-267.

WICHUK, K.M., & MCCARTNEY, D. 2007. A review of the effectiveness of current time-temperature regulations on pathogen inactivation during composting. *Journal of Environmental Engineering and Science,* 6:573-586.

WILLIAMS, Z.T. 2012. Effects of management on the bacterial community present in poultry litter. Alabama:Auburn University (Thesis – Ph. D)

WITTER, E., & LOPEZ-REAL, J. 1988. Nitrogen losses during the composting of sewage sludge, and the effectiveness of clay soil, zeolite, and compost in adsorbing the volatilized ammonia. *Biological Wastes*, 23 (4):279-294

XUE, Y., VENTOSA, A., WANG, X., REN, P., ZHOU, P., & MA, Y. 2008. *Bacillus aidingensis* sp. nov., a moderately halphilic bacterium isolated from Ai-Ding salt lake in China. *International Journal of Systematic and Evolutionary Microbiology*, 58(12):2828-2832.

YAMAMOTO, N., OTAWA, K., & NAKAI, Y. 2009. Bacterial communities developing during composting processes in animal manure treatment facilities. *Asian-Australian Journal of Animal Science,* 22(6):900-905.

YOON, J.H., KIM,I.G., KANG, K.H., OH, T.K., & PARK, Y.H. 2004. *Bacillus hwajinpoensis sp. nov. and an unnamed Bacillus genomospecies, novel members of Bacillus rRNA group 6 isolated from sea water of the East Sea and the Yellow Sea in Korea*. *International Journal of Systematic Evolutionary Microbiology*, 54(Pt3):803-808.

YOON, J.-H., KANG, S., -J., PARK, S. & OH, T, -K. 2007. *Pedobacter lentus* sp. nov. and *Pedobacter terricola* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology,* 87:2089-2095

YU, S., CLARK, O.G., & LEONARD, J.J. 2008. A statistical method for the analysis of nonlinear temperature time series from compost. *Bioresource Technology*, 99:1886-1895.

YUN, H., LIM, S., SONG, H., KIM, B., CHUNG, B., & KIM,D. 2007. Reduction of pathogenic bacteria in organic compost using gamma irradiation. *Radiation Physics and Chemistry*. 76:1843-1846.

ZHOU, J., BRUNS, M.A., & TIEDJE, J.M. 1996. DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology*, 62(2):316-322.

APPENDICES:

APPENDIX A – TECHNICAL DATA SHEET FOR TYPICAL CHEMICAL COMPOSITION OF MINED ZEOLITE

TECHNICAL DATA SHEET

Product Group: ZEOLITE
Product: ECCALITE M

DESCRIPTION

A powder (milled) natural Zeolite from the Clinoptilolite mineral group. It has high cationic exchange capacity and absorption properties. Acts as mycotoxin binder and absorbs heavy metals which lower digestive stress and need for antibiotics. Absorbs moisture and traps ammonia in stalls to reduce odour. Increases retention of nitrogen compounds in soils.

MINERALOGICAL CONSTITUENTS (Approximate)

Clinoptilolite: >90%
Quartz: <5%

TYPICAL CHEMICAL ANALYSIS

SiO_2	Al_2O_3	Fe_2O_3	TiO_2	CaO	MgO	K_2O	Na_2O	L.O.I.
64.3	12.7	1.3	0.1	1.2	1.3	1,7	2,3	8.4

PHYSICAL PROPERTIES (Typical)

Colour (crude):	White to grey
Odour:	None
Appearance:	Powder (<150microns)
Moisture:	<15%
Specific gravity:	2,51 g/cm^3
Bulk Density:	1,2 g/cm^3
Viscosity Marsh Funnel:	Immediate: 21 seconds
Particle size distribution:	20% retained on 150 µm.
PH (30g in 60ml water):	8-9
Cationic Exchange Capacity:	20 mg/kg
Water Absorption;	400 (on sinter plate)
Surface Area: BET Method	> $50m^2/g$
Average Pore Volume:	>0.1cm^3/g
Average Pore Size:	73 Angstrom or 7 nm

APPENDIX B - NITROGEN CONTRIBUTIONS OF DIFFERENT SUBSTRATES DURING THE COMPOSTING PROCESS.

	Broiler litter - N %	Previously composted material (PCM) - N %	Woodchips - N %	Zeolite - N %	Contribution of N - Broiler Litter (kg)	Contribution of N - PCM (kg)	Contribution of N - Woodchips (kg)	Contribution of N - Zeolite (kg)	Total N at Start (kg)	Mass at start (kg)	Percentage N at the start of composting	Percentage N at the end of composting	Difference in N content %
Windrow 1	**4.87**	0	**0**	**0**	243.5	0	0	0	243.5	5000	4.87	2.56	2.31
Windrow 2	**4.87**	**2.56**	**0**	**0**	243.5	12.8	0	0	256.3	5500	4.66	2.28	2.38
Windrow 3	**4.87**	**2.56**	**0.33**	**0**	121.75	12.8	8.25	0	142.8	5500	2.60	1.69	0.91
Windrow 4	**4.87**	**2.56**	**0.33**	**ND**	121.75	12.8	8.25	ND	142.8	6000	2.38	1.1	1.28

ND- No Data