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Investigation of Plant Growth and Associated Soil Microbial Stimulation by Digestate Fertilisers

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Declaration

No element of the work and research described in this thesis has been previously submitted aiming a degree at this or any other institution. The work in this thesis has been performed entirely by the author in collaboration with his supervisors.

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Abstract: The main aim of this PhD thesis was to investigate how different types of liquid anaerobic digestates fertilisers affect plant growth responses, and if these plant growth responses can be associated with a microbial stimulation of the soil due to repeated applications of these biofertilisers. Recent field and laboratory trials have indicated that anaerobic digestates may stimulate the soil/plant interaction in a different way to other fertilisers, with growth enhancement effects sometimes being higher than expected for the amount of nutrients supplied, especially for grass species. The mechanism for this stimulation of plant growth is not fully clear, but it is thought that digestate may stimulate complex interactions between the plant, soil, and soil microorganisms. The thesis is subdivided into five research chapters, where, in two of them described the characterisation of the physical-chemical and microbial properties of different types of anaerobic digestates. The second part is divided into three chapters, based on the results of a one-season fertilisation trial in a glasshouse using different types of plants combination with perennial ryegrass (*Lolium perenne* L.) and white-clover (*Trifolium repens* L.), and a two-season fertilisation trial performed in field conditions using a ryegrass sward. Effects of the digestates on plant growth and soil physical-chemical and microbial properties were investigated. Different types of liquid anaerobic digestates exhibited significant differences for most of the physical and chemical traits evaluated, with higher variability found for dry matter (DM) and K (CV= 17.2 and 16.8 respectively), and lower variation for pH and P (CV= 1.78 and 3.55 respectively). Anaerobic digestates exhibited varied quantities and fertiliser potential in terms of plant macro and micronutrients. Most of the anaerobic digestates met the recommendations of Irish standards on the quantity of pathogen indicators and potentially toxic elements. Bacterial and fungal colony-forming units (CFU) ranged widely in liquid anaerobic digestates (10^5 to 10^{10}; 0 to 10^5 g^{-1} DW, respectively). Bacterial, archaeal and fungal gene copies numbers (GCN) showed narrower ranges

than CFU (10^8 to 10^{10}; 10^7 to 10^9; 10^4 to 10^6 g^{-1} dry weight (DW), respectively) between different commercial anaerobic digestates. Microorganisms with agronomic importance were detected in all anaerobic digestates, including N-fixing bacteria, plant-growth-promoting bacteria (PGPB), nitrifying and denitrifying bacteria, arbuscular mycorrhizal fungi (AMF), cellulolytic microbes, methanogens and saprotrophic organisms; however, most of them were found in very low abundances. Digestates with different chemical composition, when equally balanced in terms of dry matter, drove comparable forage yield responses in ryegrass and mixed ryegrass/white clover pots. In the glasshouse trial, the soil bacterial (16S) GCN responded to the interaction between fertiliser/vegetation ($p<0.05$), while archaeal (16S) and fungal (18S) GCN only to the type of vegetation ($p<0.05$). No detectable effect of the digestates on soil GCN was observed. In the field trial, different digestates, when balanced in terms of dry matter, also drove comparable forage yield responses in ryegrass. Plant growth responses were strongly associated with the amounts of NPK supplied. In the field trial no detectable effect of the repeated applications of anaerobic digestates on soil microbial abundance and diversity could be observed. The dominant microbial community from the biofertilisers failed to replace the native microbial populations of the soil, possibly due to niche incompatibilities and competitiveness of indigenous soil microbes. In conclusion, most of the plant-growth effects associated with anaerobic digestate application were due to nutrients supplied, especially NPK; no evident biostimulation of the soil could be confirmed.

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List of abbreviations

ABP: Animal by Product

AD: Anaerobic Digestion

ADF: Acid Detergent Fibre

AMF: Arbuscular Mycorrhizal Fungi

ANOVA: Analysis of Variance

AOA: Ammonia-oxidising Archaea

AOB: Ammonia-oxidizing Bacteria

BLAST: Basic Local Alignment Search Tool

BSA: Bovine Serum Albumin

C/N: Carbon/Nitrogen ratio

CAN: Calcium Ammonium Nitrate

CFU: Colony Formation Units

CH_4: Methane

CP: Crude Protein

CV: Coefficient of Variation

DAFM: Department of Agriculture, Food and the Marine

DGGE: Denaturing Gradient Gel Electrophoresis

DHPLG: Department of Housing Planning and Local Government

DM: Dry Matter

DNA: Deoxyribonucleic Acid

DW: Dry Weight

EC: Electrical Conductivity

EU: European Union

FW: Fresh Weight

GATC: Eurofins Genomics Company

GCN: Gene Copies Numbers

GHG: Greenhouse Gases

GWh: Gigawatts hours

Ha: Hectare

HRT: Hydraulic Retention Time

HSD: Honestly Significant Difference

ICP:OES: Inductively Coupled Plasma-Optical Emission Spectrometry

IrBEA: The Irish Bioenergy Association

ITS: Internal Transcribed Spacer

KCL: Potassium Chloride

LAI: Leaf Area Index

LSMEANS: Least squares Means

MPN: Most Probable Number

N_2: Dinitrogen

N_2O: Nitrous Oxide

NCBI: National Center for Biotechnology Information, U.S. National Library of Medicine

NDF: Neutral Detergent Fibre

$NH_{3:}$ Ammonia

NH_4^+: Ammonium

NH_4:N: Ammonium Derived N

NMDS: Non-metric Multidimensional Scaling

NO: Nitric Oxide

NO_2^- Nitrite

NO_3^- Nitrate

NOB: Nitrite-oxidising Bacteria

ODM: Organic Dry Matter

OM: Organic Matter

OTU: Operational Taxonomic Unit

PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar`

PDIFF: Probability of the Difference

PGPB: Plant-growth Promoting Bacteria

PRIMER v6: Plymouth Routines in Multivariate Ecological Research Statistical Package

PROC MIXED: The Mixed Procedure from SAS 9.3

PTE: Potentially Toxic Elements

qPCR: Quantitative Polymerase Chain Reaction

rRNA 16S/18S: Ribosomal Ribonucleic Acid

SAS: Statistical Analysis System

SEAI: Sustainable Energy Authority of Ireland

SEM: Standard Error of the Mean

SOC: Soil Organic Carbon

SPSS: Statistical Package for the Social Sciences

RDP: Ribosomal Database Project

TEAGASC: Irish Agriculture and Food Development Authority

TKN: Total Kjeldahl Nitrogen

TOC: Total Organic Carbon

TRFLP: Terminal Restriction Fragment Length Polymorphism

TSA: Tryptic Soy Agar

XLD: Xylose Lysine Deoxycholate

XLSTAT: Statistical Software & Data Analysis Add on for Excel

Chapter 1 : General introduction

1.1 Ireland: grasslands and demand for biofertilisers

Recent figures suggest that approximately 80% (3.36 million ha) of the agricultural area in Ireland is dedicated to grassland (silage, hay and pasture), with a further 11% (0.46 million ha) in rough grazing and the remaining percentage in crop production (Chiodi et al. 2016). This large area devoted to grassland in Ireland provides evidence of the importance of this type of crop for the country, which sustains the livestock production industry, mostly beef/dairy cattle and sheep (Department of Agriculture, Food and the Marine, DAFM, 2018). In recent years, the Irish Government has encouraged improvement of grass-based agriculture by launching Food Wise 2025 (DAFM, 2015), a ten year plan for the development of the agriculture and food sector in Ireland, which among its main actions for these sectors mentions the development of strategies to improve soil fertility/health and nutrient recovery (O'Sullivan et al. 2018; Thomas et al. 2018).

One of the keys to increasing livestock productivity is the efficient management of crop nutrition (Van Vuuren et al. 2010; Breen et al. 2012; Oenema et al. 2014; Simpson et al. 2014; Rumpel et al. 2015). In this scenario, a natural demand for synthetic fertilisers is expected (Bouwman et al. 2013). However, although commonly used, artificial fertilisers are becoming more expensive as the raw minerals (e.g. phosphate rock and sylvite (potassium chloride)), used in their production are becoming depleted worldwide (Van Vuuren et al. 2010; Dawson and Hilton, 2011). The use of these finite resources (particularly phosphorous) means that synthetic fertilisers are unsustainable in the long-term. In addition, their use also has negative impacts on the environment, including greenhouse gas emissions, eutrophication and nutrient imbalances (Crosson et al. 2011; Lesschen et al. 2011). Indeed, the process of producing synthetic nitrogen fertilizers is extremely fossil fuel-intense, bringing with it inevitable environmental concerns (Townsend and Howarth, 2010). An alternative to synthetic fertilisers could be traditional

biofertilisers (e.g. animal manure) and the new generation of biofertilisers (e.g. anaerobically digested fertilisers from biogas production).

1.2 Biogas and anaerobic digestate fertilisers

1.2.1 Biogas production status in Europe and Ireland

Worldwide, there is an increase in biogas production as an alternative source of renewable energy and for treatment of organic wastes/residues. This technology provides two valuable products: biogas (methane (CH_4)) and anaerobic digestate fertilisers (Holm-Nielsen et al. 2009). Biogas technology is considered an excellent alternative to treat organic residues (farming, food/beverage industry, households and municipal wastes) as it can reduce the environmental impacts associated with greenhouse gas (GHG) emissions, improve carbon sequestration, eliminate/reduce pathogens in wastes, recycle nutrients and reduces demand for inorganic fertilisers (Albuquerque et al. 2012; Nkoa, 2014; Coelho et al. 2018; García-González et al. 2019). In the European Union (EU), there is an incentive for the implementation of this technology; some countries like Germany provided state funding and considerable government financial aid, and it is considered a leader on the use of this technology (Figure 1.1), with an estimated 62% of the total EU biogas plants (Torrijos, 2016). The current potential for electricity production via biogas in the EU is estimated to be around 73,518 Gigawatt hours (GWh); for Ireland, this potential is expected to be 2886 GWh (Scarlat et al. 2018). In 2030, the world market for anaerobic digestion (AD) is predicted to reach over 50 billion USD$ (Yousuf et al. 2017). In Ireland, the agriculture sector is estimated to be responsible for approximately 33% of the country's GHG emissions, according to estimates from (EPA, 2019) (Figure 1.2), the highest percentage of any sector.

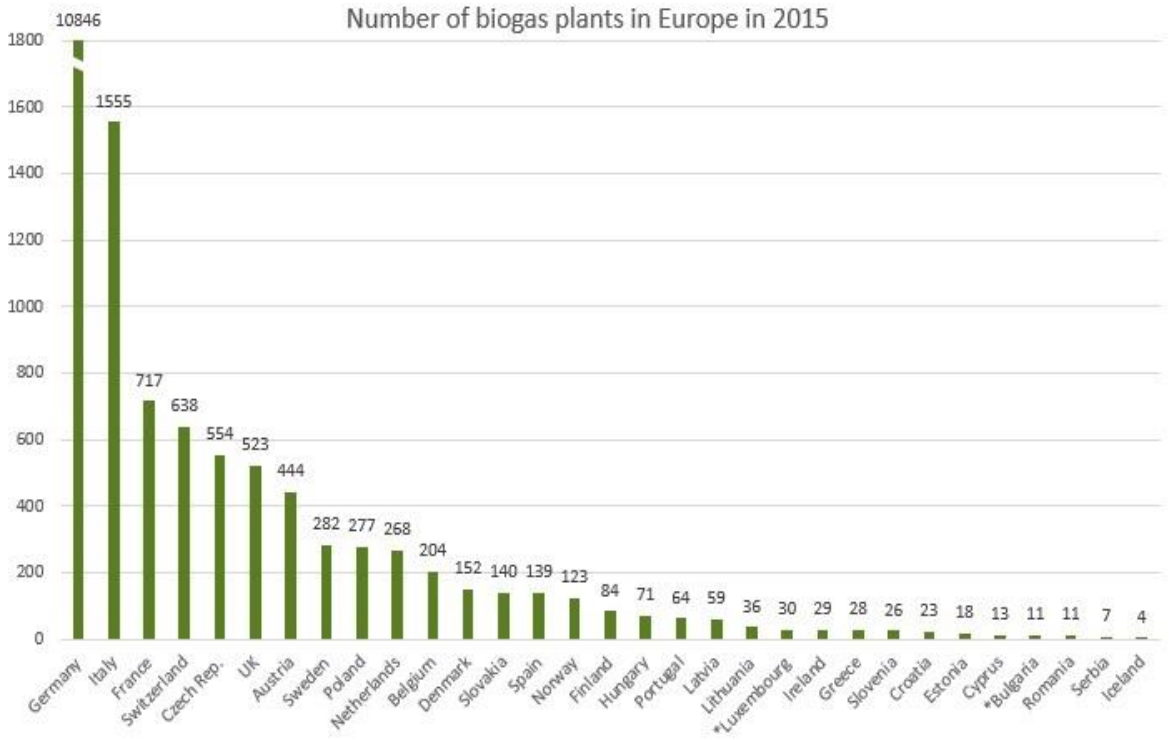

Figure 1.1. Number of biogas plants in Europe 2015.
Source: http://biomassmagazine.com/articles/14141/european-biogas-association-reports-17-376-biogas-plants-in-eu

In Ireland, biogas production was performed by around 30 registered biogas plants in 2015 (Figure 1.1). From these plants, 17 facilities were devoted exclusively to AD biogas production, and the remaining facilities were related to recovering landfill gas (Štambaský, 2016). O'Shea et al. (2016) reported that biomethane production in Ireland has the potential to supply up to 26% of the industrial gas used in the country. However, Auer et al. (2017) reported that despite Ireland having a large livestock sector producing large amounts of agricultural waste, the potential utilisation of biogas technology is still considered unexplored compared to other EU countries. Štambaský (2016) reported that three types of organic feedstock streams have great potential to be explored in Ireland: the organic fraction of municipal solid wastes and food wastes,

manures/slurries from animal production, and fresh grass from permanent grasslands or garden wastes. This vast potential needs to be explored, but the impetus to increase the biogas industry is still dependent on fiscal incentives for investors. According to the Sustainable Energy Authority of Ireland-(SEAI) (2016), Ireland is only exploring 2% of its potential for biogas; this institution estimated that 900 AD plants in Ireland could be projected by 2050 if proper government incentives for the sector were offered. Based on this estimate, the potential increase of AD plants in Ireland would consequently increase the amount of anaerobically digested fertilisers available.

Biogas is produced by a process called anaerobic digestion, which is a biochemical process in which organic matter is broken down by anaerobic microorganisms in the absence of oxygen. The raw feedstock can range from purpose-grown crops such as maize to wastes such as animal slurries and food industry wastes (Ward et al. 2008). These raw feedstocks are used as substrates for the microbial community to decompose anaerobically inside a sealed digestion vessel (Figure 1.3). Decomposition of organic material in this way causes the removal of carbon from the digesting material in the form of methane gas and carbon dioxide gas, which are the two main components of biogas. This biogas can be used as a renewable energy source through the combustion performed in a power unit (Appels et al. 2011). In addition to biogas, AD produces liquid and solid by-products known as anaerobic digestates; these contain water, minerals and other nutrients, and can be used as fertilisers or soil conditioners (Lukehurst et al. 2010). The feedstock composition influences the physical-chemical composition of the digestate at the end of the process (Albuquerque et al. 2012; Coelho et al. 2018).

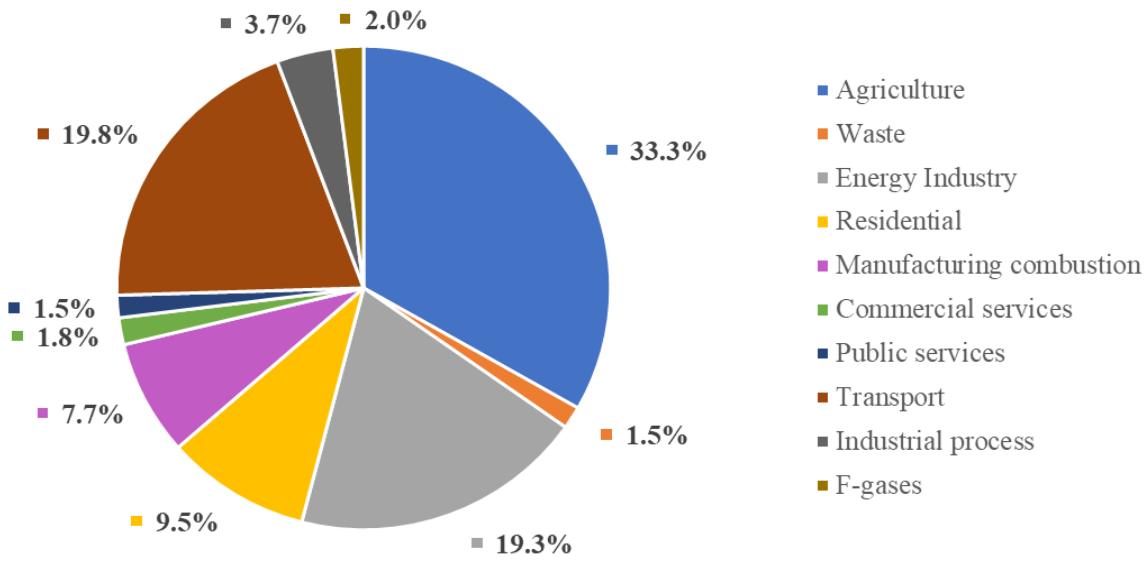

Figure 1.2. Greenhouse gas emissions in Ireland by sectors according to the Environmental Protection Agency (2019).
Source:https://www.epa.ie/climate/communicatingclimatescience/whatisclimatechange/whatareirelandsgreenhousegasemissionslike/

1.2.2 Anaerobic digestion: feedstocks and operational parameters

1.2.2.1 Biogas plants

Biogas plants are mostly classified into two types: large scale joint co-digestion plants, and farm-scale plants (Horváth et al. 2016). Large-scale joint biogas plants generally operate with different types of organic substrate sources available from the surrounding geographical region, such as farm, food and beverage industry wastes (Figure 1.3). On a farm scale, biogas is generally produced from animal slurries and/or purpose-grown crops from the farm. In integrated joint co-digestion within farms, animal slurries and fresh manure are collected and stored in tanks at the farms, and later transported to large AD plants, where they are generally digested mixed with other organic residues such as food/industry wastes (Holm-Nielsen et al. 2009). The digested fertiliser

produced is usually returned to the farms to be used as fertiliser (Figure 1.3). The use of digestate was noted by Gebrezgabher et al. (2010) as an excellent alternative to overcome some of the problems associated with the regulations for protecting the environment from issues relating to the application of animal manure in the soil, such as: GHG emissions, odours generated by decomposition, and landspreading of pathogenic organisms (Alburquerque et al. 2012; Möller and Müller, 2012; Nkoa, 2014; Coelho et al. 2018)

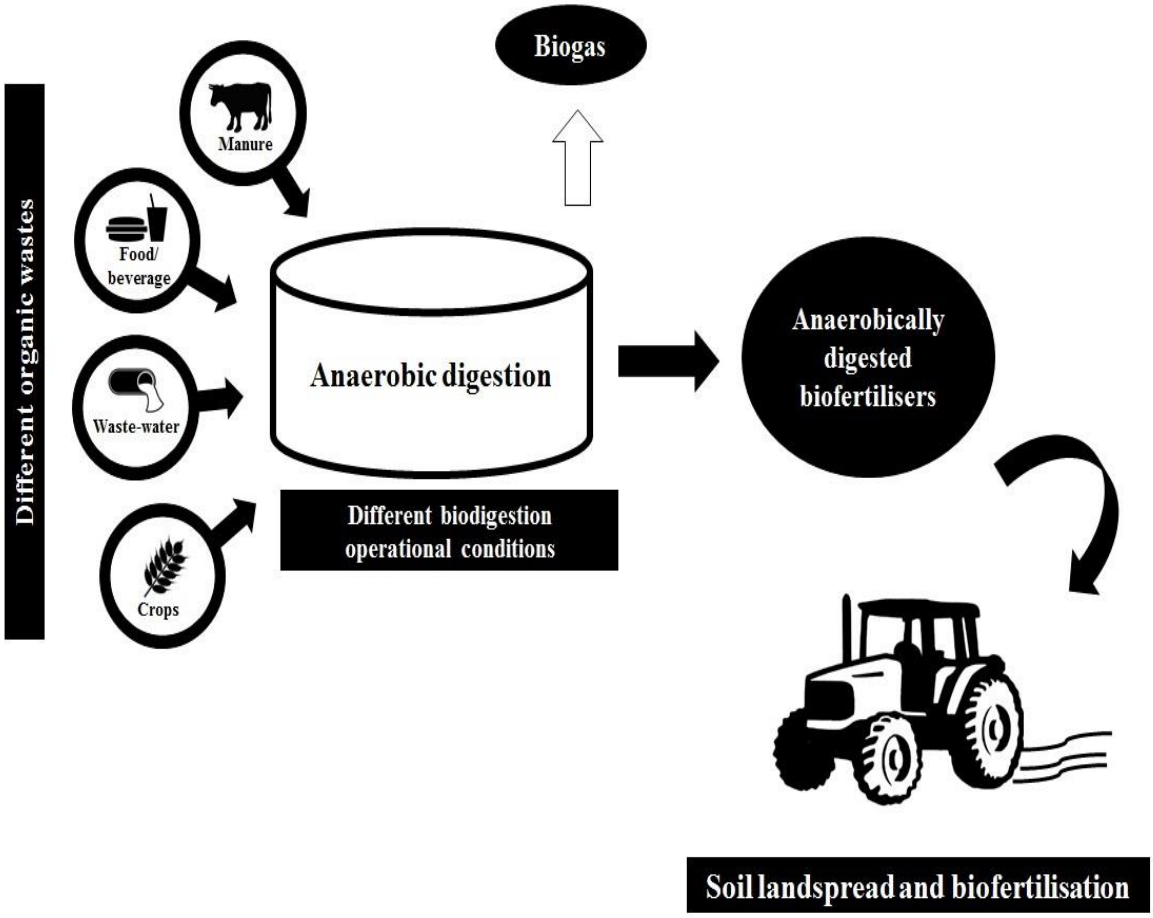

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Figure 1.3. Diagram of anaerobic digestion: biogas and anaerobically-digested biofertiliser production.

1.2.2.2 Anaerobic digestion general features

AD can be performed in many different types of anaerobic reactors with various capacities. Generally, these anaerobic reactors are run using batch or continuous organic matter flow. In the batch type, the organic material to be digested is fully loaded in one compartment and remains there until the digestion is completed. In continuous type, the organic material is loaded continuously in the tank with constant flow, mostly passing through multiple containers with different digestion features and/or purposes. At the end of the process, the digested material is generally kept in storage tanks. The pH of the AD process is usually maintained between 6.8-7.4 (Mao et al. 2015). Another important feature during the AD process is the Hydraulic Retention Time (HRT), which is the time required to complete the decomposition of the organic materials. According to Mao et al. (2015), HRT is dependent on the growth rate of the microorganisms present, temperature, organic loading rate and the composition of the substrates. In terms of temperature, AD can be performed under psychrophilic (0-20°C), mesophilic (20-45°C), or thermophilic (55-70°C) conditions (Lettinga et al. 2001). Higher temperatures are generally associated with faster digestions and higher organic load capacities (Mao et al. 2015). Technology such as the use of chemical and biological additives or pre-treatment of the feedstock can be implemented to enhance biogas production.

During the AD process, the decomposition of the organic material is performed by the microorganisms growing within the anaerobic reactor and in the substrate (Figure 1.4). Two microbial domains (bacteria and archaea) are considered the key microbes involved during the AD process (Nelson et al. 2011; Regueiro et al. 2012; Vanwonterghem et al. 2014; Conversando et al. 2015; Maynaud et al. 2016). However, there is evidence that fungi also have a role in the

decomposition of organic matter within the biogas tank (Procházka et al. 2012; Dollhofer et al. 2015; Young et al. 2018).

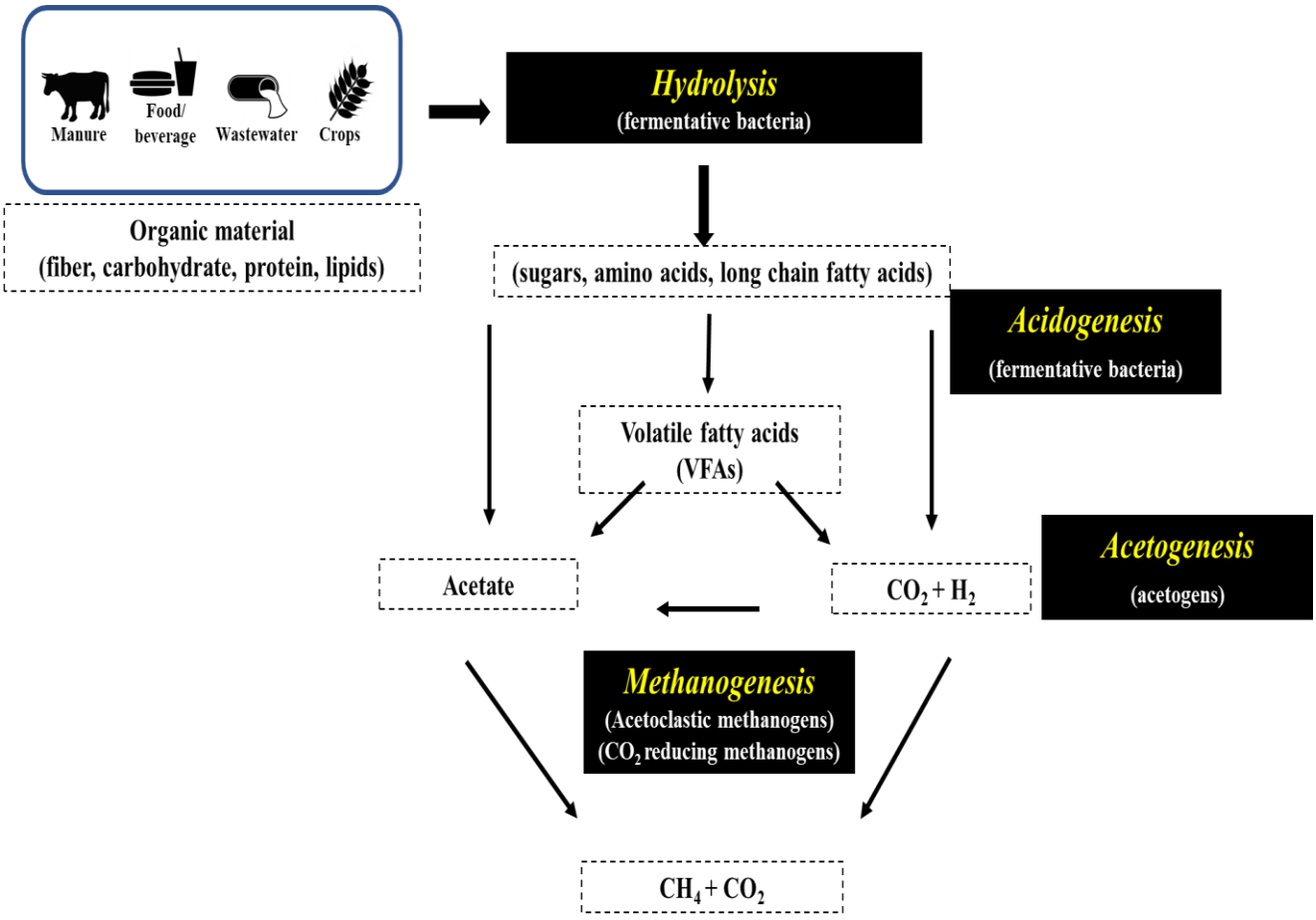

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Figure 1.4. Anaerobic digestion process phases.

Four phases are evident during the AD process: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Angelidaki et al. 2011; Meegoda et al. 2018). During hydrolysis, complex organic compounds are broken down into carbohydrates, fats, cellulose and proteins; this step is performed by hydrolytic bacteria. The second phase is acidogenesis where carbohydrates, fats, cellulose and proteins are used by acidogenic bacteria to produce Volatile Fat Acids (VFA) such

as acetates, propionate and butyrate. In the third stage, the VFA and ethanol produced during the fermentative stages are converted by hydrogen-utilising acetogens producing hydrogen, CO_2 and acetic acid. The final step of the AD process is methanogenesis, where methanogenic archaea convert the free hydrogen and acetic acid into methane (CH_4) and carbon dioxide (Figure 1.4).

1.3 Anaerobic digestate: biofertiliser

1.3.1 Fertiliser composition of digestates

Although manures/organic wastes can be used as biofertilisers without being digested, digestion improves the fertiliser value of manures by making their nutrients more readily available for crops to absorb (Möller and Müller, 2012). The chemical composition of the digestate depends on the feedstock used and the process of digestion. Generally, there are great variations in terms of the amount of nutrients contained in digestate if it comes from AD plants that use different feedstock sources (Tambone et al. 2010; Alburquerque et al. 2012; Möller and Müller, 2012; Nkoa, 2014; Coelho et al. 2018). In cases where the feedstock used is under strict control in terms of amounts and origin of each feedstock, it may be possible to predict the nutrient content of the digested fertiliser. A range for primary plant macronutrients of digestates from many different countries and types of feedstocks was reviewed by Möller and Müller (2012): dry matter (DM) 1-13%; Organic DM 64–75%; Total N (% DM) 3–14%; NH_4^+-N (44-88%); Total P content (% DM) 0.6–1.7; Total K (% DM) 1.9–4.3; pH 7.3–9.0. Beyond the primary macronutrients, digestates can have considerable amounts of secondary macronutrients (Ca, Mg, S) and micronutrients (Fe, Mn, B, Cu, Zn, Cl, Ni, Co, Na) (Tambone et al. 2010; Möller and Müller 2012; Albuquerque et al. 2012; Nkoa 2014; Coelho et al. 2018). These secondary macro and micronutrients can enhance fertiliser effects or might affect plant growth negatively via phytotoxicity (Nkoa 2014; Di Maria

et al. 2014). Practitioners and farmers must be aware that differences in anaerobic digestate composition might drive different fertiliser performances and impacts on the environment.

1.3.2 Policy and regulation for digestate use and land spread

In Europe, different regulations and guidelines for using anaerobic digestate can be found (Holm-Nielsen et al. 2009). Most countries develop guidelines based on the environmental policy established by governmental authorities. In Ireland, the Irish Bioenergy Association (IrBEA), in consultation with industry and current environmental policies from the Irish Government, has developed a draft standard for anaerobic digestate use (IrBEA 2013), based on reviews of standards and quality assurance throughout Europe. These standards deal with environmental impacts, health risks and waste management practices. According to the IrBEA (2013) document, the limits for the Potentially Toxic Elements (PTEs) in anaerobic digestates should be: Pb=149; Zn=397; Cu=149; Cr=92; Cd=1.3; Ni=56; Hg=0.4 mg kg^{-1} DW, and for pathogen indicator detection: *Salmonella* spp. = not detected in 25 g; and *Escherichia coli* = <1000 CFU g^{-1} fresh mass. Biogas plant operators, farmers and practitioners must be aware of the current legislation of their country for use and land spreading of anaerobic digestates.

The use of anaerobic digestates can have negative impacts on the environment, including greenhouse gas emissions, especially nitrous oxide (N_2O) (Nkoa, 2014), as well as inadequate practices of storage and landspread leading to gaseous nitrogen release. Leaching and runoff of nutrients can contaminate water bodies and cause eutrophication of aquatic systems, and cause nutrient imbalances (Crosson et al. 2011; Lesschen et al. 2011). Increasing crop fertilisation efficiency and avoiding nutrient losses to the environment is considered one of the essential keys for the sustainability of agriculture in Ireland. Efficiency in the use of biofertilisers such as

anaerobic digestates must consider the current legislation and actions designed to protect the environment from agricultural pollution due to the use of fertilisers and biofertilisers. Regulations and actions such as the European Water Framework Directive (Statutory Instrument S.I. No. 605) and the Irish Nitrate Directives (DAFM/DHPLG, 2017), emphasise regulations, guidelines and measurements that must be taken to reduce the impacts of agriculture activities on water bodies (e.g. rivers, lakes, groundwaters). Among actions that can mitigate detrimental environmental impacts arising from anaerobic digestate use are: maximising the efficiency of N and P fertilisation rates in crop and pasture systems, limiting of the amounts of livestock manure landspread per year, appropriate collection/storage of manure/biofertiliser before landspreading, and/or prohibited periods for landspreading of slurry and organic fertilisers (e.g. during winter and when heavy rain is forecast).

1.3.3 Fertiliser performance and effects on plant growth

Recent field and laboratory trials have indicated that digestates from anaerobic digestion of animal wastes may stimulate the soil/plant interaction in a different way to other traditional organic fertilisers. Digestate has been found to positively affect crop growth yields, with increases in plant growth due to digestate application either matching or exceeding those from equivalent amounts of mineral fertilisers (Alburquerque et al. 2012; Bougnom et al. 2012; Möller and Müller 2012; Walsh et al. 2012a; Andruschkewitsch et al. 2013, Walsh et al. 2018). Some studies have found the growth-enhancing effects of digestate to be plant-specific, with plants (e.g., grasses) having a more significant response to digestate than other plants (e.g., cereal crops) (Heslop and McCabe, 2012). Digestate has been found to increase grass yields in both field (Bougnom et al. 2012) and pot trials (Walsh et al. 2012a; Andruschkewitsch et al. 2013). This increase in crop

yields is usually credited mainly to anaerobic digestate's enhancement of plant-available nitrogen in the soil (Möller and Müller, 2012; Johansen et al. 2013). However, some studies have noted that digestate can enhance plant growth above and beyond what would be expected from an increase in nutrients (Heslop and McCabe, 2012), with one study noting digestate's "phytohormone-like effects" on plant growth (Alburquerque et al. 2012).

The mechanism for this stimulation of plant growth is not entirely clear, but it is thought that digestate may stimulate complex interactions between the plant, the soil, and soil microorganisms. The effect of digestate on soil microbes has been found to vary, with some studies finding digestate increased microbial biomass and activity in soils (Odlare et al. 2008), while others found few changes in soil microbiology after digestate application (Andruschkewitsch et al. 2013; Johansen et al. 2013). A pot study of ryegrass and clover treated with digestate found that it stimulated bacterial, but not fungal, communities in the soil (Walsh et al. 2012b). The lack of consistency in these findings indicates a need for more research on how digestate works to improve grass production, be it entirely through the supply of available nutrients, or also due to a biostimulant effect. The effects of biofertilisers on plant growth are mostly credited to direct interactions between the microbial community from the biofertilisers with the plant rhizosphere and/or by the solubilisation of soil nutrients (Mohammadi and Sohrabi, 2012). Biostimulant effects may include improving root development or the colonisation and activity of plants with beneficial microorganisms such as mycorrhizal fungi and clover/N-fixing bacteria.

Anaerobic digestates can carry enormous quantities of different types of microorganisms (Nelson et al. 2011; Regueiro et al. 2012; Vanwonterghem et al. 2014; Insam et al. 2015; Guo et al. 2015; Chen et al. 2016; Treu et al. 2016). Many of these microbes that can be found in anaerobic

biofertilisers might have an influence on plant growth and soil nutrient cycles, such as plant-growth-promoting bacteria (PGPB) (e.g. *Bacillus and Pseudomonas*) (Qi et al. 2018; Iwasaki et al. 2018); arbuscular mycorrhizal fungi (AMF) (e.g. *Glomeromycota*) (Wang et al. 2018); nitrogen-fixing bacteria (e.g. *Bradyrhizobium*) (Guo et al. 2015), denitrifying and nitrifying bacteria (e.g. *Achromobacter denitrificans* and *Thiobacillus denitrificans*, *Nitrosomonas*, respectively) (Sarkar et al. 2016), soil methanogens (e.g. *Methanosaeta* and *Methanosarcina*) (Guo et al. 2015), *Actinomyces* (Franke-Whittle et al. 2009; Guo et al. 2015), and saprophytic fungi (Santi et al. 2015), among others.

The fact that there are different types of anaerobic digestates being produced creates a challenge for their use and recommendation, as anaerobic digestates with different physical-chemical and microbiological properties might drive different plant growth responses. From this point, it becomes necessary to understand how different types of anaerobic digestates can affect the same kind of crop.

1.4 Methodologies for measuring the microbes of soil/slurry/digestate

The analysis of the microbial community from agricultural and/or environmental samples plays a crucial role in the understanding of essential processes in organic matter transformations, nutrient cycling and plant nutrition (Mocali et al.2010; Paul, 2014). The measurement of these types of environmental samples (e.g. soil, slurry/anaerobic digestate) can be considered a challenge since they are highly diverse in terms of their microbial communities and features. Many different methodologies/approaches are available for measuring these microbial communities, including: culture-based techniques, physiological/enzymatic approaches and molecular (DNA/RNA based)

methods. In the following sections, some of the possible methodologies available for measuring microbial communities from environmental samples are briefly described, with a focus on the techniques that were used during this PhD work.

1.4.1 Culture-dependent methods

Culture-dependent methods were the first techniques used to detect and quantify microorganisms in environmental samples; however, despite still being used and useful, these are now considered limited compared to newer molecular methodologies, as it is estimated that more than 99% of the microorganisms existent were never or could be cultured in laboratory (Madgan et al. 2010). They are based on the induced growth and development of microorganisms using selective growing mediums under controlled conditions. According to Carraro et al. (2011), the use of culture-dependent methods essentially requires knowledge of the physiologic and metabolic requirements of the microorganisms cultivated. This limits the accuracy of these techniques in investigating microbial communities, especially in complex matrixes such as soil/slurry, as selective media only allow a specific fraction of the whole microbial community to grow.

Many groups of microorganisms present in environmental samples, such as soil, cannot be detected by traditional culture-dependent methods (Hill et al. 2000, Anderson and Cairney, 2004, He et al. 2008). Despite the importance of these culture-dependent methods, they are prone to bias in their evaluation of microbial numbers/diversity because they only measure the growth of a limited number of organisms adapted to the growing media (Rastogi and Sani, 2011). Even though they are important in microbial ecology research, culture-dependent methods, when compared to molecular ones, have been shown to be inefficient in providing a profile of microbial communities

(Leung and Topp, 2001; Sanz and Kochling, 2007). Ecological evaluation of microbial communities nowadays has increasingly relied on culture-independent methods, based on molecular approaches (Smith and Osborn, 2008).

1.4.1.1 Most probable number (MPN)

This methodology is based on the concentration of viable microbes present in a given sample that is diluted 10-fold and inoculated in a specific broth medium. According to Sutton (2010), bacterial cells follow Poisson statistics, where one single viable cell can generate turbidity in the test media under specific growth conditions. The accuracy of this methodology is generally increased by comparing higher dilutions of the inoculum against lower dilutions, because, in more concentrated samples, more dilution will be required to reach a concentration where no growth can be detected (Tortora et al. 2015). The ratio between growth and non-growth of replicate samples provides information to estimate the MPN of a given sample (Figure 1.5). This methodology has been widely used to estimate microbial populations, especially pathogens, in organic samples and water matrixes (Madgan et al. 2010).

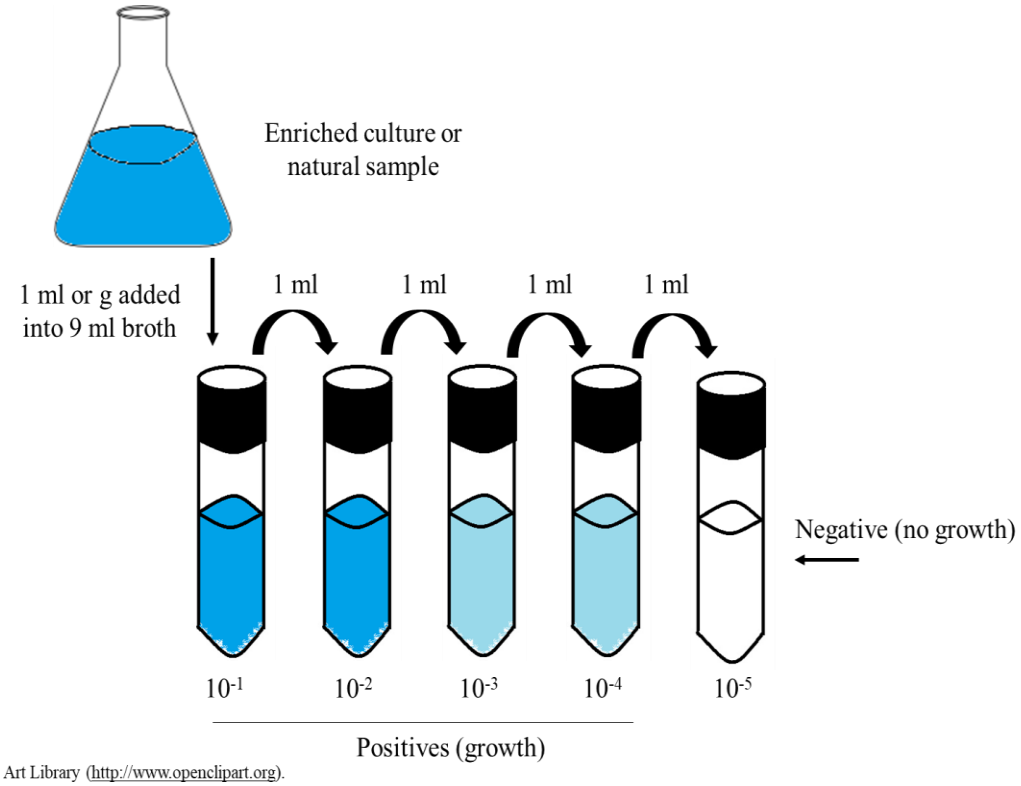

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Figure 1.5. Most probable number technique example

In this PhD, two types of pathogen indicators, with importance in relation to human and animal health, were quantified in anaerobic digestates using the MPN principle (*Salmonella* spp. and *Escherichia coli*) (Chapter 2.). Other pathogen indicators such as *Clostridium perfringens*, *Yersinia enterocolitica*, and *Listeria monocytogenes* are also commonly tested in anaerobic digestates/slurries (Sahlström, 2003). Among the advantages of this technique are that is a rapid method for detecting viable microbes and can routinely be performed in a microbial laboratory. One of the disadvantages is that that method is not 100% precise according to Tortora et al. (2015), the MPN only indicate a 95% chance that the microbial quantity of a given sample will be in a certain range.

1.4.1.2 Colony-forming units (CFU) (Pour plates and spread plates)

This method is based on the principle of counting the number of colonies forming units (CFU) present in a given diluted sample. Generally, a known sample is diluted 1:10 and then one aliquot of this diluted sample (e.g. 1 ml) is inoculated in sterile diluent and serially diluted (e.g. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}). The dilution selected to be plated is defined by the optimal range of growth that allows for reasonable counting. For example, for bacterial CFU it is suggested to plate dilutions which will give CFU numbers between 30-300 CFU after overnight growth. The serially-diluted sample can be poured or spread (Figure 1.6) in agar plates with specific media for selective growth of a determined group of microbial (e.g. bacteria, fungi). In the pour plate technique, the aliquot of the sample is placed in the centre of a petri dish, and then agar media is added, followed by incubation at specific temperatures for optimal growth. For spread plates, the agar is already condensed in the Petri dish, and the sample is spread over the surface of the medium and incubated at a specific temperature for optimal growth of the target microbial organism. Generally, for bacterial CFU, overnight growth is enough to provide reliable results; fungi take three to five days to show optimal growth ranges for counting. To calculate the CFU of the original sample, the number of CFU in each replicate plate is averaged and corrected for the dilution factor used. In the present PhD research, the CFU technique was used to quantify the number of bacteria (pour) and fungi (spread) in anaerobic digestates/slurry and soil samples (e.g. Chapter 3.3.2).

Pour and spread plates techniques to quantify culturable microbes were used widely in microbial ecology in the past; however, with the advance of molecular-based analyses their use has been reduced. The advantage of using these techniques is that they can be easily performed in a microbial laboratory to quantify known culturable organisms. The main disadvantage of their

use in the analysis of environmental samples is that only a small portion of the microorganisms will grow in the growing media, underestimating the actual microbial numbers.

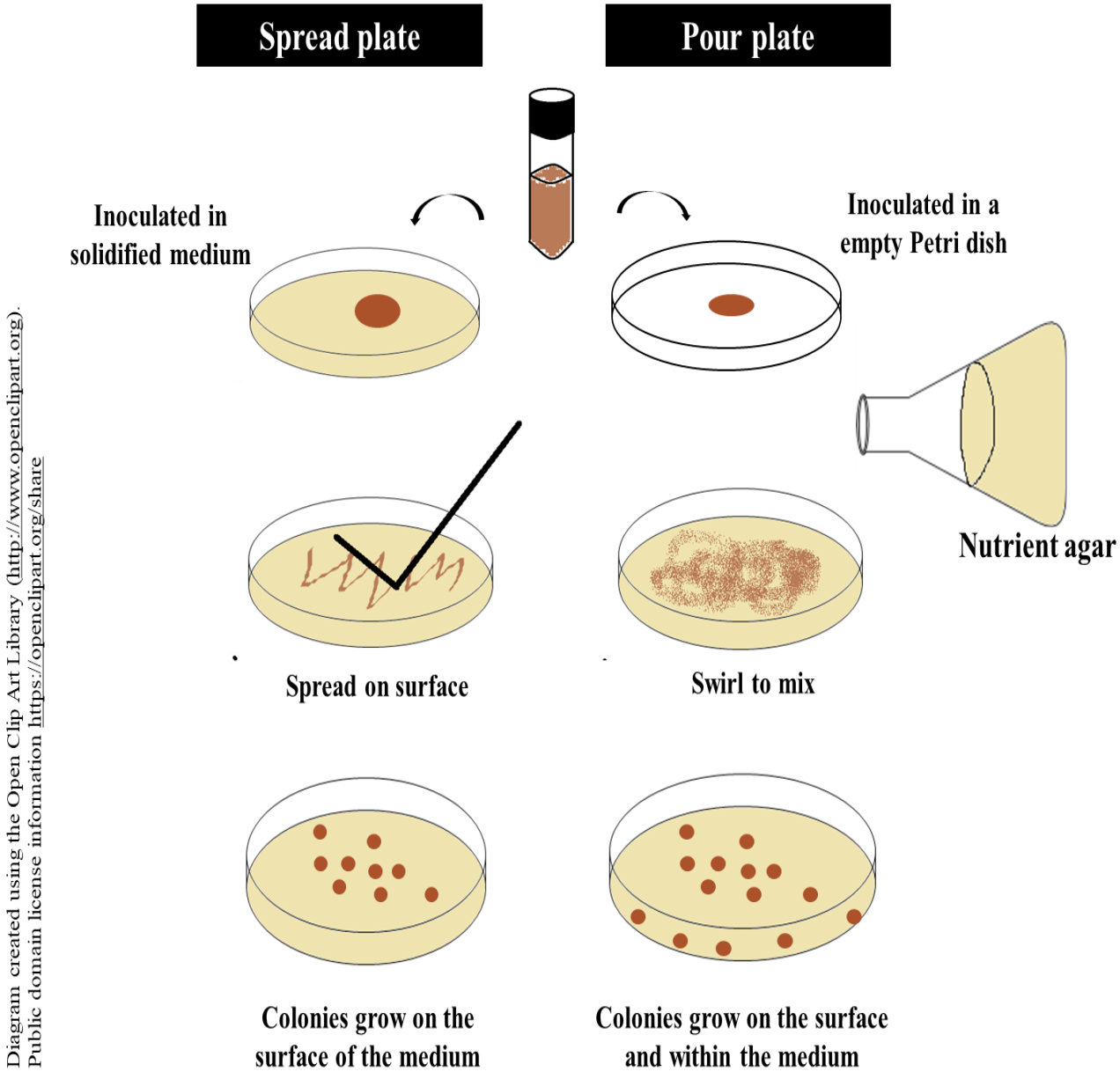

Figure 1.6. Spread and pour plates techniques.

1.4.2 Enzymatic/metabolic/rRNA activity analyses

Other possible types of measurements used to access the viable microbial populations in environmental samples are based on enzymatic or metabolic activity. The growth of microorganisms can be generally associated with increases in the production/consumption of metabolites/substrates (e.g. acid production, nutrient depletion, oxygen consumption, production of CO_2). The basis for these types of analyses is that the microbial activity which occurred during a specific period can be contrasted with the production/consumption of metabolites/substrates observed. These types of methods have been widely used in the measurement of the microbial activity/biomass in soils, and some examples of these types of analyses can be cited: substrate-induced respiration (Ananyeva et al. 2011; Kaur et al. 2015) (Figure 1.7Figure 1.7), fungal biomass based on the analysis of ergosterol (Parsi and Górecki, 2006), and fluorescein diacetate (FDA) hydrolysis (Adam and Duncan, 2001).

Another type of analysis for measuring microbial growth is based on the concentration of rRNA. In this type of analysis, it is assumed that a specific microbial species/group can have its growth rate estimated based on the increase/concentration of a specific target rRNA. Blazewicz et al. (2013) reviewed the use and limitations of this type of analysis and emphasize that the measurement can be biased since not all rRNA detected is from growing organisms, as dormant and/or active but not growing cells can contribute to a substantial percent of the rRNA measured. Also, the relationship between growth rate and the concentration of rRNA can vary between different microbial groups, which can lead to biased analyses.

In summary, the various methodologies available for measuring the microbial activity and growth always have advantages/disadvantages; the choice will depend on the target of the analysis,

costs and availability of resources, apparatus necessary, validation of the methodology and suitability of the method for the type of environmental samples being investigated.

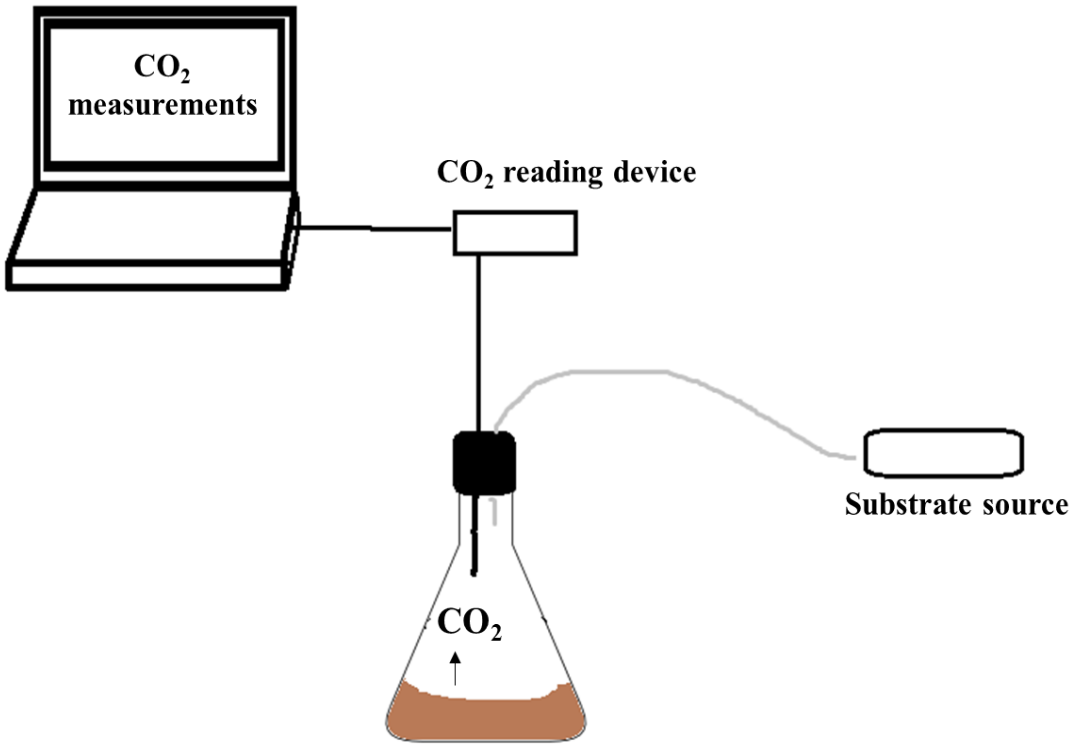

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Figure 1.7. Example of a system for measurement of soil microbial activity via substrate-induced respiration.

1.4.3 Nucleic acid-based molecular analyses

The use of molecular techniques to study microbial ecology has been increasing in recent years since only a small portion of microorganisms in environmental samples can be accessed/analysed via culturable methods (Schmeisser et al. 2007; Madgan et al. 2010). Also, culture-dependent methodologies are often more time consuming and inaccurate compared to

molecular approaches. The use of molecular techniques allows not only one specific group of microbes in environmental samples to be targeted, but also the analysis of organisms that cannot be cultivated or that are not well-known (Christen, 2008; Deng et al. 2008; Smith and Osborn, 2008; Ansorg, 2009; Claesson and O'Toole, 2010; Hirsch et al. 2010; Kircher and Kelso, 2010; Gao and Tao, 2012). Most molecular techniques are based on the analysis of the DNA and/or RNA of the microorganisms. Also, mRNA can be used instead of DNA. It is first converted into complementary DNA (cDNA) using reverse transcription, then treated in the same way as direct DNA extracts. The advantages are that it gives a better indication of the active microbial population (since mRNA is much more short-lived than DNA and can only really be extracted from a metabolically-active cell) (Saleh-Lakha et al. 2005; McGrath et al. 2008; Mettel et al. 2010). However, mRNA extraction and reverse transcription are technically difficult and add time and expense to analysis, so DNA is much more commonly used.

Molecular approaches presented in this PhD study include quantification of DNA via Real-Time Polymerase Chain Reaction (Real-time PCR) of 16S (bacteria/archaea) and 18S (fungi), and a diversity analyses based on high-throughput DNA sequencing of 16S rRNA (for bacteria and archaea) and Internal Transcriber Spacer (ITS) (fungi). Other molecular techniques also available for the microbial study of environmental samples, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP), will be briefly cited in the following section; however, their use has been largely replaced by modern next-generation sequencing (NGS) (Roh et al. 2010; Simon and Daniel et al. 2011; Bokulich et al. 2012). In the following section, a brief description of the methodologies used in the present PhD work is presented.

1.4.3.1 Polymerase chain reaction (PCR)

The PCR is a molecular technique that was initially proposed and developed by the Nobel Prize winner Dr Kary Mullis in the early ’80s. The basis of the process is the replication of the DNA in vitro. In the PCR, a single copy of a DNA/RNA segment or target region can be amplified into billionfold of copies. The process relies on the denaturation of the DNA by heating and the amplification of specified target regions using a specific set of DNA templates called primers (artificially synthesised oligonucleotides) (Madigan et al. 2014; Tortora et al. 2015). The primers’ nucleotide sequences align with the DNA sequences being targeted to amplify, flanking the target single strands of the denatured DNA, doubling the amount in each cycle of the process. The PCR is basically performed in three stages: denaturation of the DNA double-strand, annealing of the denatured DNA strands with target primers, and final extension of DNA (Figure 1.8). The essential components for a successful PCR are DNA containing the target sequence to be amplified, a heat-stable DNA polymerase (e.g. Taq polymerase), DNA nucleotide bases (dNTPs) A-T-C-G, PCR buffers, short sequences known as primers which are complementary to the ends of the target DNA/RNA, and a thermocycler machine. During the first step of the PCR, a sample containing extracted DNA is heated by raising the temperature to denature the DNA (e.g. 94-98 °C). This leads to the breakdown of hydrogen bonds of DNA, causing its denaturation, and consequently separation of the double strands. At the second phase of PCR, also known as the annealing phase, the temperature is decreased (e.g. 55-65 °C), and the primers (forward and reverse) perform a hybridisation to the flanking regions of the target sequence or gene of interest. The last step of the PCR is the elongation/extension, where the temperature is raised (e.g. 72°C, ideal temperature for Taq polymerase), then the polymerase enzyme incorporates the free dNTPs to the DNA template in the 5'-to-3' direction, producing a new copy of the target region. PCR is an exponential cycle;

in the first cycle each DNA containing the target region is multiplied by two, and in a conventional PCR with 35-40 cycles, billions of copies of the target region can be made (Madigan et al. 2014).

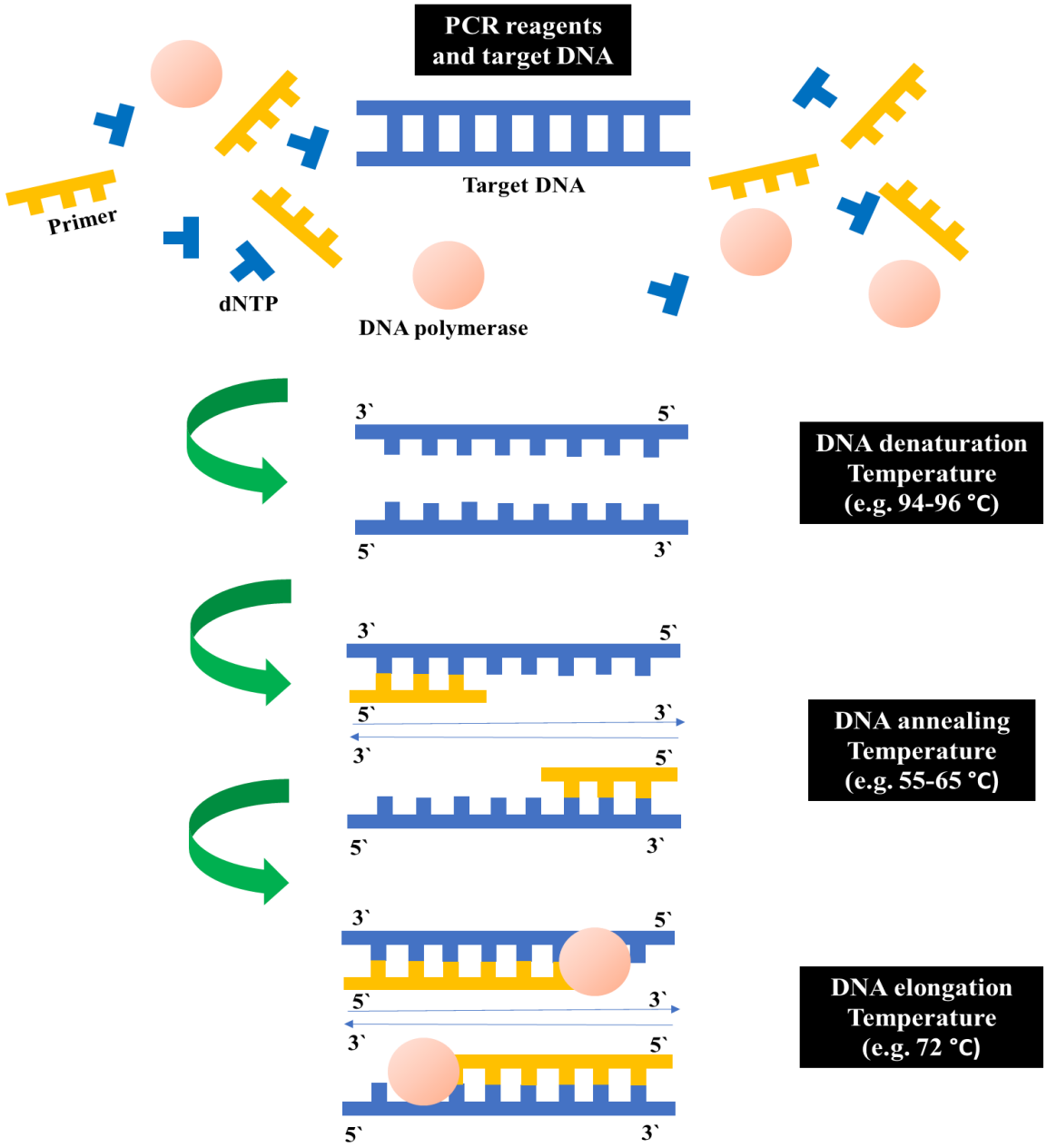

Figure 1.8. Polymerase chain reaction.

In microbial ecology studies, samples are quite complex, and a single environmental sample can have more than 10,000 different microorganisms (Wooley et al. 2010.). The proper selection of primers that can match a broad range of microbes plays a fundamental role in the success of the studies. One of the first tasks during the selection of primers is to verify their potential for high coverage rates of the targeted microbial groups (e.g. archaea, bacteria and fungi) already identified, sequenced and classified in public databases (Wang and Qian, 2009; Toju et al. 2012). Once primers with high coverage are selected, which means primers that cover a broad range of microbial groups are identified in the databases, PCR analysis of environmental samples has a better chance of amplifying from as many individuals of the target microbial groups as possible, decreasing the bias of the analysis. End-point PCR is a very good method for a quick detection of specific microbial/gene in a given environmental sample (e.g. detection of a pathogen), as it can be performed routinely and quickly in a microbial laboratory; however, one of the main limitations of this method, in terms of microbial quantification, is that it needs a post-PCR analysis (e.g. analysis of DNA bands in agarose gel).

1.4.3.2 Denaturing gradient gel electrophoresis (DGGE)

One of the post-PCR analyses that can be used to profile microbial DNA is Denaturing Gradient Gel Electrophoresis (DGGE). This technique is based on the molecular differences between base-pairings: adenine-thymine (two hydrogen bonds) and guanine-cytosine (three hydrogen bonds) (Madigan et al. 2014; Green et al. 2010; Strathdee and Free, 2013). DGGE analysis is used to separate PCR-generated DNA products. During PCR, primers target specific regions of the target DNA; after amplification, the amplified PCR products are subjected to the

electrophoresis process. In DGGE analysis the DNA is denatured with formamide/urea, and the mobility of single-strand molecules is slow compared to the double-stranded molecule, allowing the separation of DNA fragments that differ in their sequences. As the double-strand DNA moves in the gel, the molecules will be denatured. This denaturation is dependent on the %GC content. After the analysis, the pattern displayed by the DNA bands in the gel can be used to analyse the microbial diversity of the samples. According to Green et al. (2010), DGGE analysis is considered a rapid fingerprinting methodology for the analysis of microbial community composition and diversity, allowing multiple samples to be analysed at the same time. Green et al. (2010) reported that one of the limitations of this method is that DGGE only represents predominant phylotypes present in the microbial community. According to Madigan et al. (2014), the numbers of bands observed in a gel of a DGGE analysis can be an indicator of the diversity; however, sequencing analysis is still required for identification. Compared to modern tools such as high-throughput sequencing, DGGE is quite limited.

1.4.3.3 Terminal Restriction Fragment Length Polymorphism (TRFLP)

Terminal Restriction Fragment Length Polymorphism (TRFLP) is a post-PCR analysis based on the amplification of DNA by using fluorescently labelled primers followed by digestion with restriction enzymes, which allow the identification of polymorphisms in the sequences, resulting in fragments of different lengths (Madigan et al. 2014; Walker et al. 2015). After digestion, the amplified DNA bands are separated and visualised as peaks by capillary or polyacrylamide electrophoresis using a DNA sequencer. The results can then be compared to databases of known species available in microbial repositories. TRFLP has been used as a tool in the study of bacterial and fungal communities in environmental samples such as soil, slurry and anaerobic digestates due to its ability to identify multiple microbial groups (Liu et al. 1997;

Blackwood et al. 2003; Hoppe and Schnittler, 2015). However, this type of analysis is prone to many sources of bias including: random and erroneous matches, shared peaks between different microbial groups, and multiple peaks within the same microbial group (e.g. genus) analysed (Dickie and FitzJohn, 2007). According to Madigan et al. (2014), TRFLP generally underestimates the microbial diversity due to the fact that many closely related sequences might not be differentiated in this analysis.

1.4.3.4 Real-time PCR (Quantitative PCR)

A useful technique to quantify the number of DNA copies that amplified during the PCR process is real-time/quantitative PCR (qPCR). In contrast to other techniques such as DGGE or TRFLP (previously discussed), which use the final products of the amplified DNA during a conventional PCR to analyse bands on a gel, this technique is based on the amplification process itself. In qPCR, the quantification of DNA is calculated through the fluorescence intensity produced by the amplified sequences/target genes (Heid et al. 1996; Rebrikov et al. 2006; Biosystems, 2010; Life Technologies, 2012) (Figure 1.9). This quantification analysis can be processed during two phases of DNA amplification (exponential phase). For qPCR, as the gene-targeted increases its copy numbers, the fluorescence is increased, being recorded and calculated through image analysis. The fluorescence is detected during the exponential phase of DNA amplification, with increases in numbers of amplicons over each qPCR cycle. The numbers of amplicons detected are then exponentially correlated with the estimated initial amounts of a target sequence or gene present in the sample DNA analysed. The detection of fluorescence is possible

by using chemical reagents; the most used are SYBR Green and hydrolysis probes (e.g. PerfectProbe and TaqMan®) (Figure 1.9).

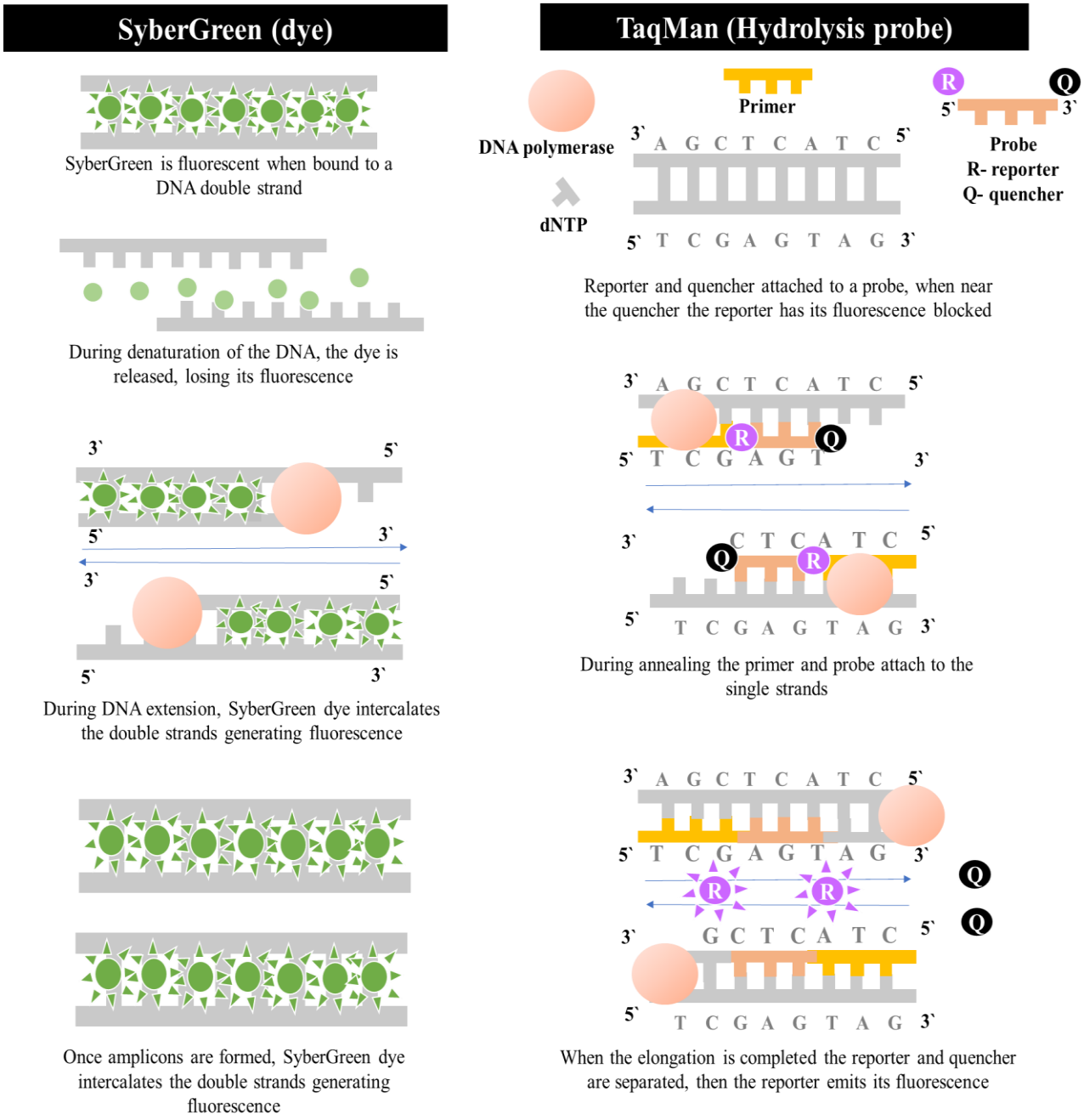

Figure 1.9. Comparison of SyberGreen and TaqMan technologies for performing qPCR.

SYBR Green fluorescence occurs when the dye molecules intercalate with the strands of DNA amplified (Figure 1.9). This dye is naturally fluorescent, but when it binds into DNA strands, it turns more fluorescent. Smith and Osborn (2008) cautioned that as SYBR Green attaches to all double-stranded DNA amplified, primers with high specificity to the target sequences must be used, avoiding bias or overestimation of genes or sequences amplified. It is also recommended to run a post-PCR dissociation curve, to assure that the fluorescence was generated by the target sequence amplified. The process is based on heating the amplified DNA, creating a reverse curve with the denaturation of the DNA. Among the advantages of SyberGreen is the fact that once the primers and reactions of the assay are well designed, there is no need to use a probe. However, SyberGreen has a disadvantage in that it can bind nonspecific target DNA sequences due to lower specificity than hydrolysis probes.

Hydrolysis probes are DNA oligonucleotides that bind in the up and downstream of DNA. The one that binds in the downstream 3' is called a quencher molecule, and on the opposite strand upstream 5' is the reporter molecule, which is naturally fluorescent (Figure 1.9). The reporter molecule is prone to emit photons, but when it is close to the quencher dye, its fluorescence is blocked. This occurs at the initial stages of the qPCR solution, when primers, DNA and reagents are all mixed. Along with gene amplification, when the extension of the primer is performed by Taq polymerase, the probe dye is cleaved. This releases the reporter dye, responsible for the emission of the fluorescence; during each qPCR cycle more and more reporters are cleaved, and fluorescence increases in the reaction. The advantages of TaqMan include the fact that the probes can be labelled with different types of reporter dyes, increasing the specificity of assays compared to SyberGreen; however, the synthesis of different probes is necessary for different sequences.

Real-time PCR is a method that allows better control over the reactions that occur during gene amplification. In real-time PCR it is possible to identify the reactions that had been successful and the ones that had failed. Applications of real-time PCR in microbial ecology studies have been spread worldwide. Smith and Osborn (2008) pointed out the advantages of the use of real-time PCR in studies related to microbial ecology, such as high reproducibility of experiments and proper monitoring abundance and expression of genes in environmental samples. Other advantages of qPCR in comparison to traditional PCR methods include: data on the DNA amplification if collected during the exponential phase of the amplification, no post-PCR gel analysis, highly increased detection level compared to traditional PCR, and confirmation using melting curve observations increases the accuracy and reliability of the results.

For the analysis of environmental samples such as slurry/anaerobic digestates and soil, as analysed in this PhD research, qPCR has been frequently used. For example, in soils, qPCR has been used to target specific microbial groups such as ammonia-oxidising bacteria (Hermansson and Lindgren, 2011; Yamamoto et al. 2010), quantification of specific genes involved in N cycle (Wallenstein and Vilgalys, 2005), quantification and comparison of different microbial domains (Siles and Margesin, 2016); for slurry/anaerobic digestates it has been used for quantification of pathogens (Jiang et al. 2013; Maynaud et al. 2016) and the quantification of methanogens during anaerobic digestion (Traversi et al. 2012; Williams et al. 2013), among other applications. qPCR has been used to calculate the gene copy numbers of environmental samples. Tatti et al. 2016 provided useful information related to the calculation of gene copies numbers where: (6.023×10^{23} (copies mol^{-1}) x concentration of target DNA (g μl^{-1})) / target molecular weight (g mol^{-1}). The use of gene copy numbers has been used for the quantification of microbial groups in environmental samples (Rousk et al. 2010; Humbert et al. 2012; Siles and Margesin, 2016).

1.4.3.4.1 16S and 18S rRNA (qPCR)

These molecular methods are based on the analysis of the DNA sequences that code for ribosomal RNA (rRNA). According to Devereux and Wilkinson (2004), rRNA is one of the most conserved cellular molecules, and because of this, it keeps close genetic similarities among phylogenetically-related organisms. Also, rRNA keeps enough variability to allow genetic comparisons among different groups and individuals. This fact makes rRNA genes suitable for phylogeny studies. Through analysis of the DNA sequences that code for rRNA, comparisons among different microbial communities are possible. Two DNA gene sections that code rRNA which has been widely used in molecular studies in microbial ecology are 16S (prokaryotes) and 18S (eukaryotes).

The 16S rRNA genes are present in all bacterial and archaeal groups. 16S rRNA genes are composed of approximately 1500 nucleotide base pairs (Gutell, 2016). In contrast with other parts of the genome, the 16 rRNA genes that code rRNA do not undergo many evolutionary changes, which allows their use to trace relationships among phylogenetically-close prokaryotes. The 16S rRNA, together with ribosomal proteins, make up the 30S small subunit (SSU) of the ribosome. 16S rRNA analysis of bacteria and archaea has relied on the use of selected primers that have high specificity to general gene region sequences among target organisms studied (Devereux and Wikinson, 2004).

Target sequences used in the analysis of bacterial 16S rRNA are within the hypervariable regions (V1-V9) (Chakravorty et al. 2007). Sequences within hypervariable regions are useful targets because it is possible to find enough variability to differentiate between different

bacterial/archaeal groups. Hypervariable regions (D-loop), located within the nuclear DNA, are sections of DNA where a double-strand is separated by another strand which has a base pair sequence that matches one of the double-strands; they are flanked by strongly conserved regions (C1-C9) (Baker et al. 2003). Primers are designed to match and bind to these conserved regions flanking the hypervariable regions, and then they amplify the V1-V9 targeted sequences. Wang and Qian (2009) pointed out that with the increases in the detection of new polymorphisms in the conserved regions, the coverage rate of known primers tends to decline.

The 18S rRNA gene is a region of eukaryotic DNA. It is homologous to the 16S in prokaryotes. 18S rRNA is also a component of the small subunit of the ribosome and is considered a high genetically conserved region. The small subunits in eukaryotic ribosomes are formed by 40 subunits. 18 rDNA gene sequences have been used in fungal identification and quantification in environmental samples such (e.g. soil/slurry/digestate) (Oros-Sichler et al. 2006, Castro et al. 2008; Hoshino and Morimoto, 2008; Yildirim et al. 2017; Duan et al. 2018). Anderson et al. (2003) reported that the use of PCR techniques to amplify 18S rRNA genes in environmental samples faces a problem because some of the primers utilized do not have high specificity for fungal DNA. This leads to biased analysis because other, non-fungal DNA may be amplified by PCR.

Hunt et al. (2004) reported that molecular evaluations of fungi have also relied on the analysis of the internally transcribed spacer (ITS) regions of DNA. These are regions (ITS 1 and 2) of ribosomal DNA (rDNA), located between the coding genes for the small and large subunits. rDNA is the sequence responsible for the genetic codification of the rRNA. Schoch et al. (2012) reported that the use of ITS regions to analyse fungal communities leads to higher probabilities of

successful identification of different groups of fungi. These authors reported that the genes for the small subunits of the rRNA have a low level of resolution for fungal species. They suggested the use of ITS regions as the first fungal barcode marker. Liu et al. (2015) compared ITS and 18S rDNA on their performance analysing fungal diversity in soil and water samples and concluded that ITS regions presented more precision and potential than 18S rDNA for use in microbial studies of fungal communities.

The selection of adequate primers for qPCR analysis targeting genes or sequences for 16S and 18S rRNA is one of the most important steps to acquire reliable results in microbial ecology studies (Borneman and Hartin, 2000; Klindworth et al. 2012). Wang and Qian (2009) reported that qPCR failures could occur if the primers do not match the target sequences properly. This is not an easy task when the analysis subject is an environmental sample such as soil/slurry. Although considerable efforts have been made to identify more and more different fungal, bacterial and archaeal organisms, just a small fraction of some of these groups are considered well-known. To have an idea of how quickly the estimated number of species in different microorganism groups change, Blackwell et al. (2011) reported that for around twenty years it was accepted that total fungal diversity in the world was 1.5 million species. But with the advances provided by molecular analysis techniques, this estimate has been increased to around 3.5 to 5.1 million fungal species.

1.4.4 DNA Next-Generation Sequencing (NGS)

DNA sequencing-based methodologies applied in the study of microorganisms in environmental samples are also known as metagenomics. They are considered one of the best available approaches for evaluating the microbial diversity of complex samples. Metagenomic

analysis can identify and analyse microbes that cannot be cultured or accessed by other methods, or that are not even known yet (Tringe and Rubin, 2005; Hugenholtz and Tyson, 2008; De Mandal, 2015). In relation specifically to the analysis of soil and slurry/anaerobic digestates which are the major focus of this PhD research, sequencing analyses have been demonstrated to be a valuable tool for analysing the diversity of agriculture soils and the effect of agricultural practices on soil microorganisms (Nielsen et al. 2014; Sapp et al. 2015; Chen et al. 2016; Fernandez et al. 2016). In terms of anaerobic digestates, sequencing analyses have been widely used, especially for understanding the microbial dynamics of anaerobic reactors (Rivière et al. 2009; Vanwonterghem et al. 2014; Fontana et al. 2016; Fitamo et al. 2017); also, for studies related to the digestive tract of farming animals (Kim et al. 2015; Peng et al. 2015; Zeng et al. 2017). In the analysis of complex samples such as environmental ones, it is common for the sequencing analysis to not identify all the microbial organisms present in a given sample since many microorganisms are not sequenced or properly identified in the genetic banks, which is considered a current limitation of these types of technologies. However, sequencing is one of the best approaches to describe the microbial diversity of environmental samples.

Sequencing analyses investigate the genome of the microbes in terms of the ordination of their DNA nucleotide bases: adenine, thymine, cytosine and guanine (ATCG) (Madigan et al. 2014). The principle relies on the fact that each microbial individual/group has a specific nucleotide ordination that characterises it as unique. With DNA sequencing approaches, it is possible to have an accurate phylogenetic classification of the microorganisms present in environmental samples. The sequencing of the DNA is categorised into two main groups: individual sequencing reactions (Sanger method) and Next-Generation Sequencing (NGS) (second, third and fourth) with multiple parallel sequencings (e.g. Illumina, ABI SOLiD, Roche

454 and Heliscope, Ion torrent). The NGS methods are mostly based on the methodologies that use amplification of the DNA and fluorescence analysis of the nucleotide sequences. Madigan et al. (2014) provided a characterisation of the basic features of the sequencing methods and their classification according to generation (Table 1.1).

Table 1.1. DNA sequencing methods. Source: adapted from Madigan et al. (2014).

Generation	Method	Reading length
First	**Sanger dideoxy method** (radioactivity or fluorescence; DNA amplification)	700–900 bases
Second	**454 Pyrosequencing** (fluorescence; DNA amplification; massively parallel)	400–500 bases
	Illumina/Solexa method (fluorescence; DNA amplification; massively parallel)	50–100 bases
	SOLiD method (fluorescence; DNA amplification; massively parallel)	50–100 bases
Third	**HeliScope Single Molecule Sequencer** (fluorescence; single molecule)	up to 55 bases
	Pacific Biosciences SMRT (fluorescence; single molecule; zero mode waveguide)	2500–3000 bases
Fourth	**Ion torrent** (electronic—pH; DNA amplification)	100–200 bases
	Oxford nanopore (electronic—current; single-molecule; real-time)	Thousands of bases Portable MinION unit is approximately the size of a flash drive

In the classical sequencing Sanger method, named after the Nobel Prize winner Dr Fred Sanger, the concept is that a copy of the target original DNA single-strand is produced using DNA polymerase (Madigan et al. 2014). Unlike a regular DNA amplification, in this method chain terminator inhibitors, known as dideoxynucleotides (ddNTPs), are added instead of dNTPs; these

altered nucleotides are absent of one hydroxyl at the third carbon, and terminate the elongation of the DNA at specific points (Kircher and Kelso, 2010) (Figure 1.10). In this method, the target DNA is amplified, and then the DNA is subjected to a denaturation, producing single-strands which are used in the sequencing. Primers are added to flank the target regions of the DNA; in this step, the target DNA (single-strand and with a primer), is equally distributed among four reactions tubes. Each reaction tube receives DNA polymerase, dNTPs and ddNTPs (one type of each for each tube). The DNA being extended is subjected to the mixture of dNTPs and ddNTPs; once a ddNTP is added to the complementary strand, the sequence is terminated since the ddNTPs is absent of a hydroxyl group at the 3' carbon, thus produce many different copies of the DNA with restrictions on various points. This generates target sequences with varying lengths, and after cleaning (PCR reagents), sequences are read by electrophoresis-based procedures. In the Sanger method, the single sequences range from 500 to 1000 base pairs. Nowadays, most microbial ecological studies preferentially use NGS, due to the possibility of running massively parallel sequencing of DNA, instead of methodologies based on the Sanger method which is quite limited (only one forward and reverse sequence per analysis), cannot detect rare microorganisms, has lower sensitivity to detected low-frequency variants and can be cost and depending of the size of the project (De Mandal et al. 2015).

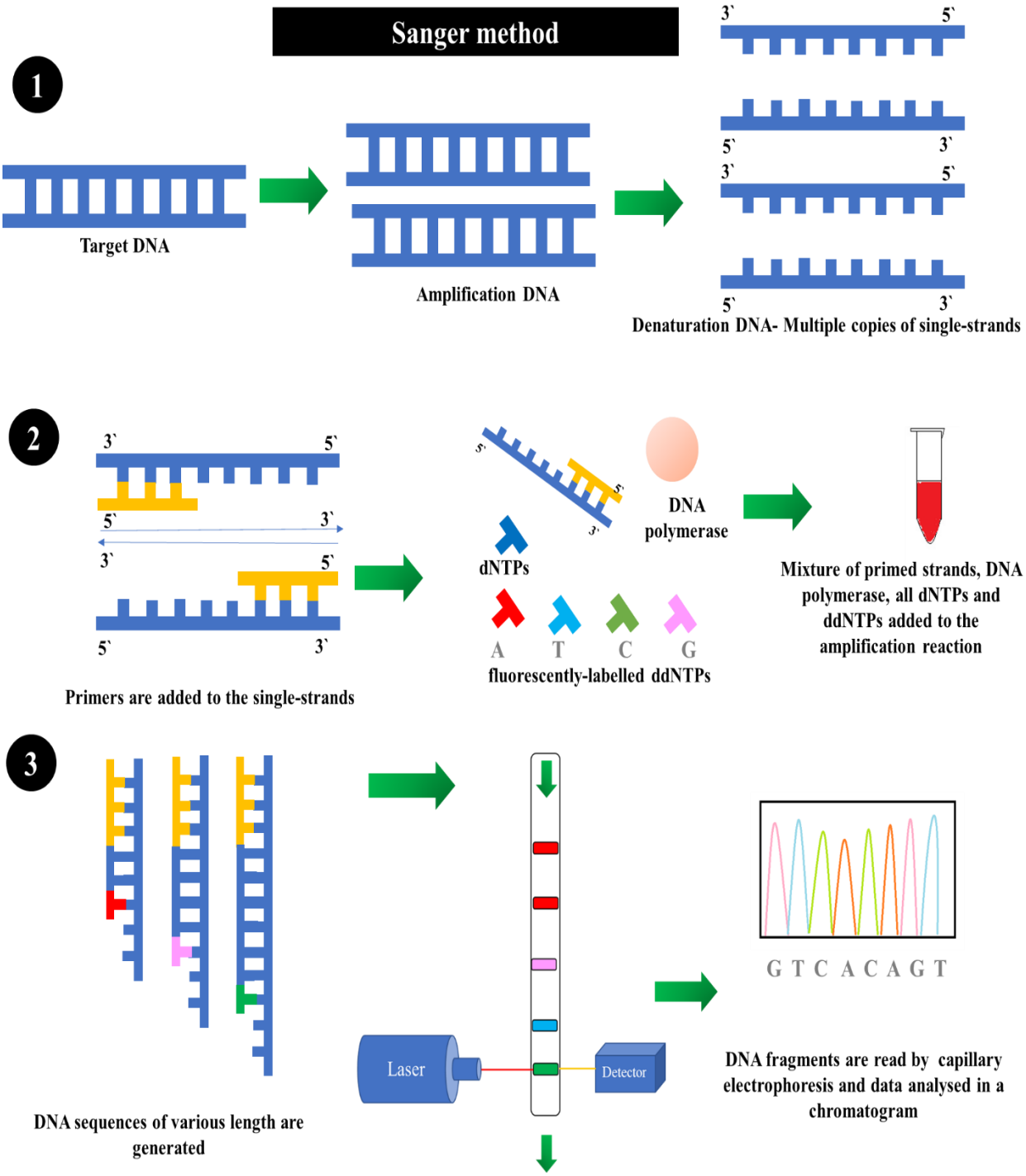

Figure 1.10. A simplified example of the Sanger sequencing method.

Among the NGS methods available, in this PhD work, the Illumina Genome Analyser (MiSeq sequencing platform) was used and will be briefly described here. In NGS by Illumina

methodology, four basic steps are performed: library preparation, cluster generation, sequencing and data analysis (Figure 1.11). According to Madigan et al. (2014), the Illumina method resembles the Sanger Method in the fact that it uses DNA synthesis and chain terminators. Nevertheless, the chain terminators used by the Illumina system are dNTPs instead of ddNTPs. In the Illumina system, the dNTPs are labelled with fluorescent tags. This fluorescent tag blocks the 3'-OH, terminating the chain. In the first step (library preparation), the DNA is subjected to a tagmentation reaction, in which transposons are cleaved and tagged to every single strand of DNA (5' and 3') by using forked adapters (Kircher and Kelso, 2010). In the second step, cluster amplification, the DNA library fragments created are added into a flow cell (glass slide with lanes), where the transposons added to the DNA are captured and bonded to complementary surface-bound oligos in the lanes. Then the fragments attached to the surface-bound oligos are subjected to amplification and cloned in clusters by bridge amplification in the flow cell. In the sequencing step, all the reserve strands cloned are washed out from the oligo cells, then fluorescently labelled nucleotides are attached to the forward strand in lanes. The oligo flow cells are then analysed by image and the fluorescence emitted is recorded. The emission record is then used to identify the nucleotide bases added to the forward strand. Once these readings are finished, which can take many cycles, the last step of data analysis takes place, where the nucleotide sequence readings are aligned to a bioinformatic genetic bank. One of the most used genetic banks is GenBank, which is an open-access online repository, for recording, annotating and collecting of all types of public available DNA/RNA nucleotide sequences. This database is controlled by the U.S. National Center for Biotechnology Information (NCBI).

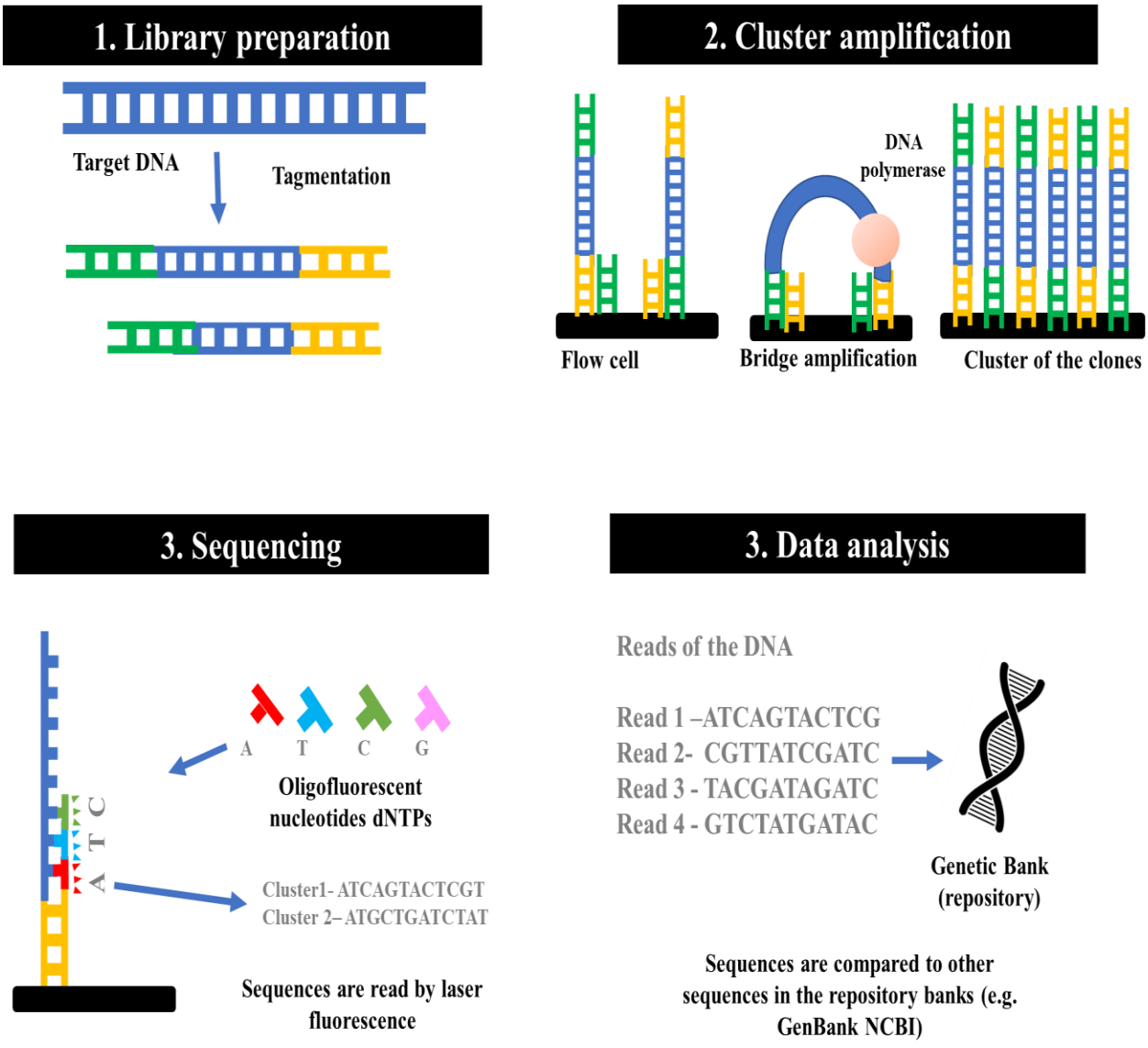

Figure 1.11. Simplified scheme for the steps of Illumina sequencing analysis.

Lately, the Illumina sequencing has been widely adopted by microbial ecologists to investigate metagenomics, such as the ones investigated in our trial (soil, slurry and anaerobic digestates) (Nielsen et al. 2014; Sapp et al. 2015; Chen et al. 2016; Fernandez et al. 2016; Guo et al. 2015; Kim et al. 2015; Peng et al. 2015; Zeng et al. 2017). Among the advantages of using Illumina sequencing are that this methodology, in comparison with traditional Sanger sequencing,

is a more sensible method for detecting sequences with lower frequencies, has higher genomic coverage, and can perform parallel sequencing. De Mandal et al. (2015) also cited the high throughput and low cost as advantages and as disadvantages short reads and long time for running the analysis. Quail et al. (2012) reported lower error rates in Illumina sequencing compared to other NGS analyses; however, some Illumina instruments have a high cost of acquisition compared to other NGS types such as Ion Torrent.

1.5 Outline of the thesis

In the following chapters, the work performed in this PhD research 'Investigation of plant growth and associated soil microbial stimulation by digestate fertilisers', will be detailed. In the first two research chapters (Chapters 2 and 3), an investigation related to the physical-chemical and microbial characterisation of different types of commercial anaerobic digestates will show the main characteristics of these biofertilisers and display their importance in terms of use and fertilisation. Issues related to the use and security of anaerobic digestates will also be discussed. The aim of these two initial research chapters was to address the main fertiliser properties of the anaerobic digestates, seeking to find a range for specific nutrients, as well as to understand the range of their microbial abundance. The next chapters test the same concept: the fertiliser performance of different types of anaerobic digestates, cattle slurry and inorganic N-fertiliser. The main aim of these research chapters was to analyse if different types of anaerobic digestates could drive different plant-growth responses, and if these plant-growth responses were associated only with the nutrients supplied or with a combination of nutrients and microbial stimulation of the soil. In Chapter 4, different types of plants (ryegrass and white clover) were tested in a glasshouse trial.

In this chapter, plant growth characteristics, soil primary plant macronutrients and microbial abundance were evaluated. In Chapters 5 and 6, a similar fertiliser trial with the same types of fertiliser treatments as in the glasshouse trial (excluding one anaerobic digestate) was performed in a field trial dominated by ryegrass. In Chapter 5, plant growth responses and nutritional quality were evaluated in response to the types of fertiliser used, as well as their effect on soil chemistry. In Chapter 6, the microbial abundance and diversity of the biofertilisers used in the field trial were evaluated, as well as their effect on soil microbial communities. In Chapter 7, a general conclusion based on the findings of all previous chapters is presented.

Chapter 2 : Physical-chemical traits, phytotoxicity and pathogen detection in liquid anaerobic digestates

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2.1 Abstract

Anaerobic digestates, which are co-products from biogas production, have been recognised as potential biofertilisers for their benefits in nutrient recovery and recycling of different types of organic wastes. Due to the increasing number of different types of organic wastes being used to produce biogas, it is necessary to identify how different types of anaerobic digestates vary in their physical-chemical traits, and how these can impact upon their use as fertilisers. In addition, safe land spreading of anaerobic digestates must be within recommended limits for potentially toxic elements (PTEs) and pathogens. This study analysed physical-chemical traits, phytotoxicity, PTEs and indicator pathogens in a set of eleven different commercial liquid anaerobic digestates from Ireland and the UK and compared them to the Irish draft standard for digestate. The liquid anaerobic digestates analysed originated from biogas plants processing feedstocks including: animal slurries, food/beverage industry, farm wastes, garden wastes, municipal wastes, whole grass and wastewater. Liquid anaerobic digestates exhibited significant differences ($P<0.001$) for most of the physical and chemical traits evaluated, with higher variability found for dry matter (DM) and K (CV= 17.2 and 16.8 respectively), and lower variation for pH and P (coefficient of variation (CV)= 1.78 and 3.55 respectively). PTE concentrations were in general within recommended limits; nevertheless, some digestates showed higher concentrations than the recommended limits for Pb, Zn and Cu. Digestate from wastewater treatment feedstock was shown to be high in PTEs. Anaerobic digestates were found to negatively affect early stages of seed germination, but phytotoxicity effects were decreased by dilution in water. Levels of *Salmonella* spp. and *E. coli* were within recommended limits for most of the anaerobic digestates analysed.

2.2 Introduction

The use of renewable energy derived from biogas has risen all over the world, stimulated by benefits such as the generation of green energy, low-cost treatment for organic wastes from households, industry and agriculture, reduction of GHG emissions from organic waste degradation, associated methane capture from biological systems, and co-production of potential biofertilisers (Mao et al. 2015). These resulting residues, known as anaerobic digestates, are rich in nutrients and have been recognised as potential sustainable alternatives to conventional inorganic and other undigested organic fertilisers (Tambone et al. 2010; Albuquerque et al. 2012; Möller and Müller 2012; Walsh et al. 2012a).

The utilisation of anaerobic digestates as fertiliser still faces many challenges in terms of uses such as land spreading, due to a broad range of physical-chemical compositions (Albuquerque et al. 2012; Möller and Müller 2012; Nkoa 2014), which make it difficult to establish standard management practices such as fertilisation rates. Physical-chemical and microbiological traits of anaerobic digestate depend on several factors; however, most can be attributed to the type of feedstock utilised (Amani et al. 2010), pre-treatment of the feedstock (Appels et al. 2008), the effect of physical-chemical traits of the feedstocks used for digestion on the activity of microbial community within the reactor (Dai et al. 2016), and post-treatment and storage after digestion (Pell Frichmann consultants 2012). Differences between anaerobic digestates directly impact the management practices related to them.

In Europe, many different regulations and guidelines for anaerobic digestate production and use can be found (Holm-Nielsen et al. 2009). In the United Kingdom (UK), the utilisation of anaerobic digestates is subjected to environmental permitting or licenses (BSI, 2010). In Ireland, the Irish Bioenergy Association, in consultation with industry, has developed a draft standard for

anaerobic digestate use (IrBEA 2012), based on reviews of standards and quality assurance throughout Europe. These standards deal with environmental impacts, health risks and waste management practices. In order to develop useful standards, it is necessary to better understand how different types of anaerobic digestates vary in their physical-chemical and microbiological composition. Such information can lead to improvements in the regulations about their use and land spreading and improve agriculture management practices related to them.

The aim of this study was to analyse physical-chemical traits, total nutrients, PTEs, phytotoxicity and indicator pathogens in a set of eleven different types of commercial liquid anaerobic digestates and compare them to the concentrations recommended in the draft Irish digestate standards.

2.3 Material and methods

2.3.1 Digestate sampling

Liquid anaerobic digestate samples from eleven different types of biogas plants were collected from Ireland and the United Kingdom in October 2015 (Table 2.1). For microbial analysis, all anaerobic digestate samples were kept refrigerated at 4 ºC and analysed a maximum of one week from sampling. Samples for elemental composition and PTEs were kept at -20ºC. Samples were analysed according to the methods outlined in the draft IrBEA industry standard for digestate (IrBEA 2013). They were prepared in accordance with European standard EN 16179 (2012). For elemental analysis, samples were air-dried at 40°C until constant weight.

Table 2.1. Feedstock composition and operational aspects of biogas plants supplying the set of anaerobic digestates evaluated.

Digestate	**Feedstock**	**Operation**	**Temperature (ºC)**	**HRT (days)**	**Volume (m^3)**	**Pasteurisation**
AD1	Food waste (dairy industry)	Continuous	Mesophilic	70	1200	Pre-digestion
AD2	Food waste, pig slurry	Continuous	40	90	2000	Post-digestion
AD3	Food waste (farm and food)	Continuous	38	54	600	No
AD4	Food waste, municipal sludge	Continuous	37-42	60	1850	Post-digestion
AD5	Waste water treatment	Batch	Mesophilic	14	1700	Pre-digestion
AD6	Food waste, garden waste	Continuous	Mesophilic	26	5200	Pre-digestion
AD7	Whole cattle slurry	Continuous	27	22	220	No
AD8	Whole grass	Continuous	40	70	0.2	No
AD9	Cattle slurry, chicken manure, food	Continuous	Mesophilic	40	265	No
AD10	Whole cattle slurry	Continuous	Mesophilic	40-50	870	No
AD11	Food waste (kitchen), garden waste	Continuous	Mesophilic	70	0.2	No

HRT= Hydraulic retention time.

Operation = type of organic matter loading

Pasteurisation= treatment of feedstock (pre-digestion), treatment of anaerobic digestates (post-digestion).

2.3.2 Physical-chemical, elemental composition and PTEs

Three replicates samples were used for all analysis. For Dry Matter (DM) analysis, samples were oven-dried (drying oven model) at 105°C according to European standard EN 13040 (2007). The Organic Dry Matter (ODM) was determined by loss on ignition according to European standard EN 15935 (2012). Total Organic Carbon (TOC) was calculated based on the OM analysis, estimating that TOC was approximately 58% of the Organic Matter (OM) (Bernal et al. 1998). Total Kjeldahl Nitrogen (TKN) was measured using a Buchi Kjeldahl apparatus, distillation Unit K-350 and Speed Digester K-425 according to European standard EN 16169 (2012). The C/N ratio was calculated using the ratio of the TOC and the TKN. For pH and Electrical Conductivity (EC), samples were extracted with deionised water at a ratio of 1:5 (v/v) according to European standard EN 15933 (2012). pH was measured with by probe (Mettler Toledo, Switzerland). After pH measurement, samples were centrifuged at 4500 rpm for 10 min in a centrifuge Universal 320 (Hettich), then the supernatant was filtered and measured for EC using a probe (CON-700, EUTECH), according to CEN/TS 15937 (2013). Total concentrations of the following chemical elements (P, Ca, K, Mg, Na, Mn, B, Co, Se, Al, Fe) and PTEs (Cd, Cr, Cu, Pb, Ni, Zn) were analysed using ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry) VARIAN model 710-ES, according to guidelines of CEN/TS 16170 (2012). The extracts analysed were produced after total digestion of dried anaerobic digestates (Section 2.1) in aqua regia (6 ml HCl + 2 ml HNO_3) using a microwave digester (Mars 240/50, CEM) in accordance with the guidelines described in European standard EN 16174 (2012). For Hg, samples were sent to an external laboratory and analysed using ICP-MS.

2.3.3 Phytotoxicity

Seed germination assays were carried out by adapting the methodology described by Albuquerque et al. (2012). Seed germination tests were performed in square Petri dishes, where two filter papers moistened with 1 ml of solution (digestate or water) and served as an environment for seed germination. Ten cresses (*Lepidium sativum*) seeds were sown in between filter papers. The dishes were sealed with parafilm and incubated in darkness at 23°C for 72 hours. Anaerobic digestates were diluted with deionised water to solution concentrations of 10%, 25%, 50%, and 100%. After incubation, the number of germinated seeds was noted, and germination was calculated as a percentage of the control (deionised water). The phytotoxicity assay was performed in triplicates for each digestate concentration.

2.3.4 Detection of indicator pathogens

Salmonella spp. were enumerated in digestate samples by enrichment in selenite-cystine broth, where three flasks of 90 ml containing selenite-cystine broth were inoculated with 10 ml of digestates, three tubes of 10 ml containing selenite-cystine broth with 1 ml of digestates and three tubes of 10 ml containing selenite-cystine broth with 0.1 ml of digestates. Samples were incubated at 36ºC for 24 hours. Then, 0.1 ml of pre-enrichment culture was aseptically transferred to Rappaport-Vassiliadis broth and confirmed by streaking on Xylose Lysine Deoxycholate (XLD), a specific selective media for *Salmonella and Shigella* species, and confirmed in Rambach agar (specific for *Salmonella*), incubated at 42ºC for 24 hours. All procedures and most-probable-number (MPN) calculations were performed in accordance with CEN/TR 15215-2 (2006). *Escherichia coli* were enumerated following methods described in CEN/TR 16193 (2013), where a sample of 20 g of digestates/slurry was added to 180 ml sterile sodium chloride solution and

shaken at 150 rpm for 20h 5 ºC. Then, 1 ml of the suspension was used to prepare serial dilutions until 10^{-7} (1 ml sample + sterile sodium chloride). Each dilution step was inoculated in Fluorocult lauryl sulphate broth (1:9 solution) and incubated at Fluorocult at 44ºC for 40 h. Tubes were observed for gas formation, and positive results were confirmed by adding 0.5 ml Kovac's reagent, then the MPN was calculated. Triplicates of inoculated (positive) and autoclaved (negative) samples were run in parallel as controls, for both *Salmonella* spp. and *Escherichia coli* tests.

2.3.5 Statistical analysis

Physical-chemical trait data were tested for normality and equal variance (Levene's test) and analysed using one-way ANOVA. Seed germination data were analysed by descriptive statistics. Phytotoxicity was correlated with physical-chemical traits of anaerobic digestates using Pearson's correlation test ($P>0.05$ and $P>0.01$) in SPSS. Magnitudes of correlation follow: if $|r| < 0.20$, non-existent correlation; $0.20 < |r| < 0.40$, weak correlation; $0.40 < |r| < 0.60$, moderate correlation; $0.60 < |r| < 0.80$ strong correlation; if $|r| > 0.80$ very strong correlation. Relationships among physical-chemical characteristics were analysed by principal component analysis (PCA) using XLSTAT, Microsoft Excel® extension software.

2.4 Results and discussion

2.4.1 Physical-chemical traits

All traits related to organic matter (DM, ODM, N, C, TOC, C/N) exhibited significant differences between anaerobic digestates ($P<0.001$). Liquid anaerobic digestates were found to have DM contents varying from 1.50 to 7.75% (Table 2.2), with an average of 3.61%. The ODM average was 57.8%, corresponding to an average of approximately 40% of mineral content in the

DM in liquid anaerobic digestates. Total nitrogen in liquid anaerobic digestates varied between 6.58 to 24.11%, with an average of 11.69% and total organic carbon (TOC) from 18.3 to 41.9 %, with an average of 32.22%. The C/N ratio average was 3.49. Among these organic matter traits, the lowest coefficient of variation was found for ODM (*CV*= 6.28%), while the highest detected was for DM (*CV*=17.2%). The low *CV* found for ODM indicated that despite differences in DM content among liquid anaerobic digestates, there was a tendency for lower variability in the ratio between organic compounds and mineral content, although the range varied from 31.46 to 72.09%.

In a literature review, Nkoa (2014) reported that ODM in liquid anaerobic digestates varied between 38.6 to 75.4%, similar results to the ones found in the present study. In agriculture, DM is sometimes used a means of standardising application rates between different types of digestates (i.e., the volume of each digestate will be varied so that the same amount of DM of each is spread). However, since there is high variability in ODM content, applying the same amount of total DM of different digestates can result in varying amounts of ODM being provided. These differences in ODM can have a substantial impact on nutrient supply and environmental issues, especially PTE accumulation. It is recommended that anaerobic digestates should have their ODM contents analysed before fertilisation, especially when fertilisation rates were established based on DM contents. Variations in dry/organic matter in liquid anaerobic digestates are generally due to a combination of factors, such as: feedstock used for biogas production, initial C/N ratios, pre-and post-treatment, and/or efficiency of the anaerobic digestion process (microbial activity, HRT and temperature) (Yadvika et al. 2004). Another factor that causes variability in anaerobic digestates is the fact that biogas plants use different types of feedstocks available in the moment, generally using mixtures of different feedstocks. Common combinations that can be found in biogas plants are mixtures of animal slurries and food wastes (Nkoa 2014). In the present study, most of the

anaerobic digestates evaluated were from mixtures of animal slurry and food wastes (Table 2.1), which might have contributed to the variability found in many of the traits evaluated.

pH values in liquid anaerobic digestate had an average of 8.23 and had low variability among different types of liquid anaerobic digestates (CV= 1.78), although significant differences were detected ($P<0.0001$). The pH of anaerobic digestates in this study varied from slightly (7.73) to moderate alkaline (8.49). Due to this alkaline nature, the land spreading of anaerobic digestates has been associated with increases in soil pH (Makádi et al. 2012; Voelkener et al. 2015). Nevertheless, the presence of acid compounds in liquid anaerobic digestates might also cause pH decreases due to organic acid condensation, physical-chemical transformations, and connections to other organic and inorganic colloids (Makádi et al. 2012). The process of nitrification of NH4-N from anaerobic digestate after land spreading can also result in the release of considerable amounts of H+ in the soil, as anaerobic digestates are generally composed of 40 to 70% NH4-N (Albuquerque et al. 2012).

Table 2.2. Physical-chemical characterisation of liquid anaerobic digestates.

Sample	DM ±%	ODM %	TKN%	TOC%	C/N	pH	EC µS cm^{-1}
AD1	2.82	64.17	16.54	37.4	2.37	8.49	442.3
SE	±0.06	±1.66	±1.37	±0.50	±0.09	±0.27	±8.5
AD2	5.08	61.18	10.62	35.6	3.35	8.25	559.3
SE	±0.07	±5.77	±0.44	±3.21	±0.19	±0.06	±95.8
AD3	3.27	53.78	7.47	31.27	4.18	8.13	227.2
SE	±0.35	±3.72	±0.25	±7.21	±1.02	±0.10	±31.7
AD4	7.75	68.05	10.41	41.1	3.96	8.28	595.7
SE	±0.08	±3.58	±0.20	±0.10	±0.85	±0.10	±8.3
AD5	1.92	55.45	10.37	32.2	3.11	7.73	152.7
SE	±0.30	±1.51	±0.21	±0.85	±0.13	±0.03	±6.7
AD6	3.62	47.48	10.36	23.2	2.30	8.17	529.3
SE	±0.08	±0.45	±2.31	±0.01	±0.51	±0.01	±21.7
AD7	2.36	62.82	10.10	35.8	3.54	7.88	205.3
SE	±0.85	±3.06	±0.44	±1.69	±0.06	±0.05	±8.5
AD8	1.50	31.46	13.60	18.3	1.24	8.33	296.7
SE	±0.08	±4.67	±0.49	±2.62	±0.10	±0.04	±21.9
AD9	1.73	48.99	24.11	28.5	1.16	8.85	425.3
SE	±0.14	±4.35	±0.33	±2.49	±0.14	±0.27	±47.5
AD10	4.78	72.09	6.58	41.9	6.40	8.33	384.0
SE	±0.20	±3.34	±0.42	±1.96	±0.72	±0.23	±14.9
AD11	4.89	70.68	8.39	41.1	4.93	8.07	412.0
SE	±1.83	±4.31	±0.61	±2.51	±0.61	±0.01	±59.5
Average	3.61	57.83	11.69	32.22	3.49	8.23	384.5
ANOVA	***	***	***	***	***	***	***
CV	17.2	6.28	7.00	9.06	14.89	1.78	10.35

Means (n=3) are followed by standard errors SE (±). ANOVA: ***: significant at probability level $P < 0.001$. DM= Dry matter; ODM= Organic dry matter; TKN= Total Kjeldahl N%; TOC= Total organic carbon; C: N= Carbon/nitrogen ratio; EC= Electrical conductivity; *CV*= Coefficient of variation.

The EC of liquid anaerobic digestates ranged from 152.7 to 595.7µS cm^{-1}, averaging 384.5 µS cm^{-1}, with significant differences among values ($P<0.001$). The EC variability can be mostly explained by differences in the number of free ions in the solution, salinity level and physical

properties of the liquid digestates. EC of anaerobic digestates should be considered when using them as fertiliser because their land spreading might affect directly soil electrical properties. Voelkener et al. (2015) reported that fertilisation with anaerobic digestates was associated with increases in electrical conductivity in loamy and sandy soils, while Albuquerque et al. (2012) cautioned that special attention should be given when using excessive doses or continuous applications of anaerobic digestates, especially when salt concentrations are high. In the literature, considerable variability of results can be found for EC in liquid anaerobic digestates. For example, Voelkener et al. (2015) reported EC of liquid anaerobic digestates ranging from 77 to 91 μS cm^{-1}, while in the work of Albuquerque et al. (2012), the results ranged from 5,200 to 30,800 μS cm^{-1}. Considering the variability of EC found for anaerobic digestates in different studies, the results found in the present trial were closest to the ones reported in four recent European studies (Bougnom et al. 2012; Walsh et al. 2012a; Walsh et al. 2012b; Pokój et al. 2015).

Elemental analysis of the liquid anaerobic digestates showed that nutrients with highest concentrations were K, Ca, Na, P and Fe (61.53, 32.84, 27.27, 17.39 and 10.60 g kg^{-1} DM respectively) (Table 2.3). K had the highest variability among the elements (*CV*= 16.82), due to the large range of differences in K concentrations (7.49 to 173.48 g kg^{-1} DM). In contrast, P had lower variability (*CV*= 3.55), with concentrations ranging from 8.10 to 32.80 g kg^{-1} DM. Significant differences ($P<0.001$) were found between the digestate samples for all elements analysed, except for the elements that were below the detection limit such as B and Co.

Anaerobic digestion has been recognised as an excellent option for recycling and recovering essential nutrients from a variety of organic wastes, and these recycled elements, especially plant macronutrients (N, P and K), can contribute to reducing agriculture costs by decreasing artificial fertiliser use (Albuquerque 2012; Möller and Müller 2012; Nkoa 2014).

Nevertheless, the use of anaerobic digestates as fertiliser faces many issues related to high variability in their chemical composition. The results found in the present study indicated that high variability in essential macro and micronutrients in liquid anaerobic digestates can lead to different supplementation requirements to meet the specific needs of different types of agriculture crops (Sheets et al. 2015). For example, the ratios of N, P and K varied widely, with some having almost the same concentrations of P and K, while others had more P than K or vice-versa. Farmers must keep this variability in mind and perform an analysis of the macronutrients such as N, P and K present prior to using digestate as fertiliser. The levels of N in the anaerobic digestates varied widely, and this can have an impact on the fertiliser value of some of the digestates as N is the primary plant-growth macronutrient. The amount of N in digestates has been demonstrated to have the strongest effect on plant growth (e.g. grass species) (Gunnarsson et al. 2010; Walsh et al. 2012a; Andruschkewitsch et al. 2013; Walsh et al. 2018). Several different fertilisation trials have reported the need for supplementation of nutrients when using anaerobic digestates as fertilisers. Liedl et al. (2006) reported anaerobic digestate was an incomplete fertiliser for the set of crops evaluated, and supplementation of nutrients was necessary to meet specific crop growth requirements, while Svensson et al. (2004) reported that P was the main supplementation requirement when anaerobic digestates were used as fertilisers. One of the main challenges for the use of anaerobic digestate as fertiliser is to produce standard fertilisation rates for different crops, which depends on research trials aiming to address crop growth responses to different types of anaerobic digestates.

Table 2.3. Elemental composition (g kg^{-1} dry weight) of liquid anaerobic digestates.

	P	K	Ca	Mg	Na	Fe	Al	Mn	B	Co	Se
						±g kg^{-1} DW					
AD1	12.06	45.15	36.55	3.10	61.08	6.32	1.51	0.12	<0.0006	<0.0006	<0.0008
SE	±0.72	±23.65	±39.97	±0.35	±32.49	±0.41	±0.09	±0.006	±0	±0	±0
AD2	29.71	58.37	33.90	12.09	39.33	2.28	8.26	0.24	<0.0006	<0.0006	0.0083
SE	±10.06	±13.06	±0.57	±0.53	±11.73	±0.12	±0.21	±0.004	±0	±0	±0.0014
AD3	32.80	32.69	22.34	12.69	7.28	16.01	20.87	0.26	<0.0006	<0.0006	<0.0008
SE	±0.67	±31.32	±63.98	±0.93	±11.37	±0.46	±0.78	±0.003	±0	±0	±0
AD4	23.80	7.49	24.04	3.57	8.06	15.94	21.80	0.28	<0.0006	<0.0006	<0.0008
SE	±0.42	±16.94	±0.41	±0.39	±54.47	±0.90	±10.17	±0.016	±0	±0	±0
AD5	21.65	8.09	32.42	9.37	5.62	13.14	12.40	0.28	0.0028	<0.0006	0.0039
SE	±0.35	±0.63	±18.81	±0.26	±0.31	±0.44	±0.48	±0.006	±0.003	±0	±0.006
AD6	20.55	75.84	48.68	7.92	72.70	5.17	2.29	0.41	<0.0006	0.00160	0.0039
SE	±15.23	±24.24	±52.48	±11.78	±35.84	±0.38	±0.16	±0.007	±0	±0.0004	±0.006
AD7	9.78	38.18	24.62	11.98	7.55	2.57	1.76	0.29	<0.0006	<0.0006	0.0014
SE	±0.32	±44.38	±0.65	±0.31	±12.47	±0.02	±0.07	±0.006	±0	±0	±0.002
AD8	9.21	173.48	30.81	3.22	25.07	23.39	6.87	0.25	<0.0006	<0.0006	0.0216
SE	±0.04	±43.04	±0.21	±0.18	±0.62	±0.14	±0.08	±0.002	±0	±0	±0.03
AD9	11.51	119.90	25.67	2.34	47.42	22.30	19.47	0.25	<0.0006	<0.0006	0.0115
SE	±0.007	±13.08	±0.17	±0.08	±0.55	±0.12	±0.10	±0.001	±0	±0	±0.001
AD10	8.10	63.93	28.50	5.48	7.86	3.05	1.50	0.19	<0.0006	<0.0006	0.0100
SE	±0.41	±63.85	±11.27	±0.41	±0.80	±0.17	±0.27	±0.004	±0	±0	±0.001
AD11	12.11	53.75	53.77	3.14	18.01	6.48	1.45	0.34	<0.0006	<0.0006	0.0130
SE	±0.15	±267.2	±23.52	±0.86	±94.29	±0.43	±0.12	±0.029	±0	±0	±0.001
Average	17.39	61.53	32.84	6.81	27.27	10.60	8.92	0.26	0.0003	0.00015	0.0067
ANOVA	***	***	***	***	***	***	***	***	N/A	N/A	***
CV	3.55	16.82	9.16	8.65	15.31	3.84	4.87	4.24	N/A	N/A	94.99

ANOVA (n=3): ***: significant at probability level $P < 0.001$. *CV*= Coefficient of variation; (<)= under limit of detection; NA= Not analysed

2.4.2 Potentially toxic elements

Although PTE averages were generally below or close to the recommended limits suggested by Irish agencies (Table 2.4), some anaerobic digestates did exceed the limits for Zn and Pb. For Zn, four anaerobic digestates showed higher concentrations than recommended: AD1, AD4, AD5 and AD11 (434.03, 515.63, 1155.23 and 445.73 mg kg^{-1}, respectively). For Pb, average concentrations were in general lower than the recommended limits in most of the anaerobic digestates analysed. However, one sample (AD5) stood out from the other anaerobic digestates due to its high Pb concentration (1959.83 mg kg^{-1}). This digestate was from a wastewater treatment plant. Wastewater treatment sludge is known for being a source of concentrated heavy metals (Fu and Wang 2011; Barakat 2011). Total concentrations of heavy metals in this study were similar to other recently published findings that evaluated different types of anaerobic digestates (Albuquerque et al. 2012; Kupper et al. 2014), where most of the anaerobic digestates evaluated had PTE concentrations below or close to the recommended limits cited in Table 2.4.

Safe environmental application of anaerobic digestates, in terms of PTEs, depends upon the chemical composition and availability of these elements in the anaerobic digestate. Although most of the anaerobic digestates investigated were within the recommended limits for PTE, the total concentration is only an indication of the potential for toxicity. Many other factors and interactions between anaerobic digestates, soil, and plants can influence the level of heavy metal bioavailability, and therefore toxicity (Tchounwou et al. 2012). According to Zhu et al. (2014), only when heavy metals are in their ionic form or in the exchangeable fraction of the soil do, they migrate and accumulate in plants and other living organisms; therefore, heavy metals in the water-soluble fraction of the anaerobic digestates (Cu, Zn, Mn, Ni and Cd) deserve more attention, due to direct toxicity to the environment. Another factor to consider about PTEs in anaerobic digestates

is leaching and accumulation in agricultural soils due to constant application of anaerobic digestates, which can result in their accumulation in plant/crops tissues and waterbodies (Bonten et al. 2008; Möller and Müller 2012; Nkoa 2014).

Table 2.4. Potentially toxic elements (PTEs) (mg kg^{-1} DM) in liquid anaerobic digestates.

Sample	Pb	Zn	Cu	Cr	Cd	Ni	Hg
				±mg kg^{-1} DW			
AD1	5.87	434.03	54.70	16.00	<0.00002	8.6	0.0002
SE	±0.46	±38.45	±4.78	±0	±0	±0	±0
AD2	1.37	359.90	60.93	11.80	<0.00002	5.93	<0.000001
SE	±2.36	±12.75	±1.55	±0	±0	±3.25	±0
AD3	9.47	300.97	112.97	68.77	<0.00002	27.63	0.0002
SE	±2.13	±5.82	±1.13	±0	±0	±0	±0
AD4	17.23	515.63	306.77	20.77	<0.00002	13.7	0.0004
SE	±1.69	±37.11	±28.40	±0	±0	±0	±0
AD5	1959.83	1155.23	223.97	46.83	<0.00002	27.93	0.0006
SE	±93.15	±13.90	±1.34	±0	±0	±0	±0
AD6	15.67	755.00	209.57	15.20	<0.00002	20.23	<0.000001
SE	±3.63	±7.10	±2.47	±0	±0	±0	±0
AD7	0.60	319.07	47.70	4.70	<0.00002	2.37	<0.000001
SE	±1.03	±20.11	±1.90	±0	±0	±0	±0
AD8	19.03	237.93	91.10	4.47	<0.00002	6.87	<0.000001
SE	±1.22	±2.10	±1.77	±0	±0	±0	±0
AD9	7.17	251.40	70.37	9.83	<0.00002	10.60	<0.000001
SE	±0.83	±2.00	±0.90	±0	±0	±0	±0
AD10	1.80	153.47	42.27	8.43	<0.00002	4.27	<0.000001
SE	±0.45	±15.01	±3.68	±0	±0	±0	±0
AD11	48.43	445.73	281.10	6.37	<0.00002	33.20	0.0002
SE	±1.85	±87.60	±54.75	±0	±0	±0	±0
Average	189.7	448.0	136.5	19.4	0	14.7	0.0001
ANOVA	***	***	***	***	N/A	***	N/A
CV	14.83	7.25	13.72	3.35	N/A	40.4	N/A
Limits for PTEs Irish Bioenergy Association ±IrBEA, 2013.	**149**	**397***	**149***	**92**	**1.3**	**56**	**0.4**

***Note**: Copper and Zinc are plant micronutrients and limit values are not absolute. Should these values be exceeded, specific labelling/provision of information to the end-user is required. Absolute levels must not exceed 30% above limit values.

ANOVA: ***: significant at probability level $P < 0.001$. *CV*= Coefficient of variation; ±<= under the limit of detection. Hg and Cd were not analysed statistically since most values were below detection limits.

2.4.3 Relationships among physical-chemical traits

The principal component analysis was conducted in order to determine relationships between digestates and their physical-chemical characteristics (Figure 2.1). DM content was correlated with the C/N ratio, indicating that the anaerobic digestates with lower dry matter content tended to have higher values of total N. Both DM and C:N are related to the type of feedstock used in the anaerobic digestion process. Digestates AD1 and AD9 were most highly correlated with total N. AD1 originated from a biogas plant that processes dairy industry wastes, mostly composed of whey, and AD9 was produced from a mixture of animal slurries, including chicken manure. Whey and chicken manure are known for their considerable concentrations of N and low C/N ratios compared to other organic wastes commonly used in anaerobic digestates (Wang et al. 2012; Carlini et al. 2015). The total N concentration in liquid digestates was also correlated with K and pH. Albuquerque et al. (2012) reported high correlation coefficients (r=0.90) between total N and K in liquid anaerobic digestates. The positive correlations between N and pH can be explained by the fact that during anaerobic digestion of organic feedstocks, the pH is increased by the production of ammonia (Melamane et al. 2007; Tambone et al. 2009). Ammonia concentration in liquid anaerobic digestates is associated with the total N content or low C/N ratios (Albuquerque et al. 2012; Wang et al. 2012). In relation to the PTEs, it was observed that they were mostly correlated among themselves. The anaerobic digestate most strongly correlated with PTE concentrations was (AD5), produced from wastewater treatment. As discussed above, wastewater treatment sludge is known to be high in heavy metals (Fu and Wang 2011; Barakat 2011).

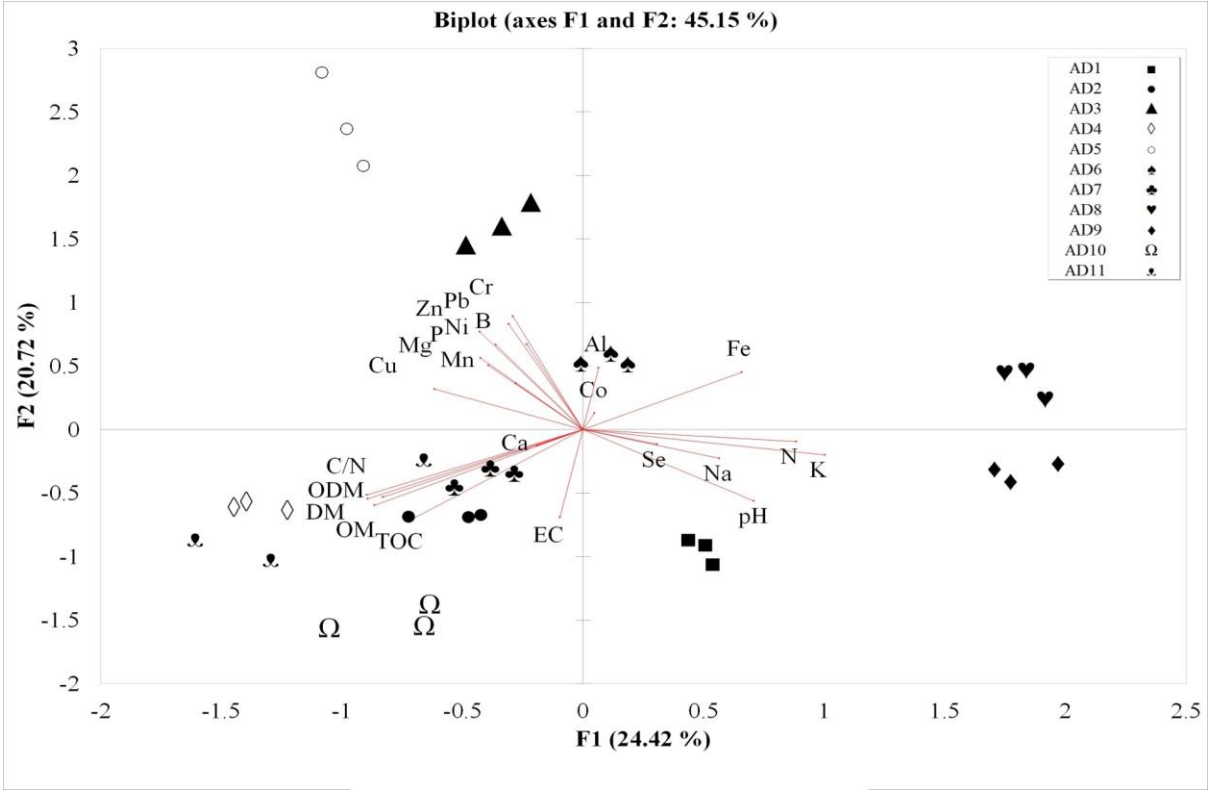

Figure 2.1. Principal component analysis of physical-chemical traits in liquid anaerobic digestates. Physical-chemical traits as arrows and digestate samples (*n*=3) as symbols. AD= Anaerobic digestate; DM= Dry matter; OM= Organic matter; ODM= Organic dry matter; TKN= Total Kjeldahl N%; TOC= Total Organic Carbon; C:N= Carbon/nitrogen ratio; EC= Electrical conductivity.

2.4.4 Phytotoxicity

Phytotoxicity results showed that in general, concentrations of liquid anaerobic digestates greater than 50% completely suppressed cress seed germination. The anaerobic digestate AD7 showed the lowest phytotoxicity effects, exhibiting high germination at concentrations of 50 and 100% (Figure 2.2). Many factors, such as electrical conductivity, can influence seed germination when in contact with anaerobic digestates. Correlation analysis showed that EC was the only variable that had a significant ($P<0.05$) strong positive correlation with phytotoxicity ($r= 0.76$), although moderate positive correlations were also detected for DM ($r=0.46$), pH ($r=0.50$), and Na

(r=0.44). The relationship between EC and phytotoxicity was seen in AD7, which had one of the lowest values for EC (20.53 mS cm^{-1}), and the highest germination rate. Three digestates (AD4, AD9 and AD11) suppressed germination completely at all dilutions tested; these digestates also had high EC μS cm^{-1} (595.7, 425.3 and 412, respectively). Similar results have been reported in the literature, with Albuquerque et al. (2012) finding that germination of cress and lettuce seeds was inversely correlated with electrical conductivity, and McLachlan et al. (2004) reporting a negative correlation between the germination index of cress and radish seeds and electrical conductivity of anaerobic digestates. Abdullahi et al. (2008) found that seed germination can be increased by diluting anaerobic digestates, which was also observed for the anaerobic digestates in this study; according to Möller and Müller (2012), once anaerobic digestates are spread on a field site, the possible risks and negative effects of phytotoxicity can quickly decrease.

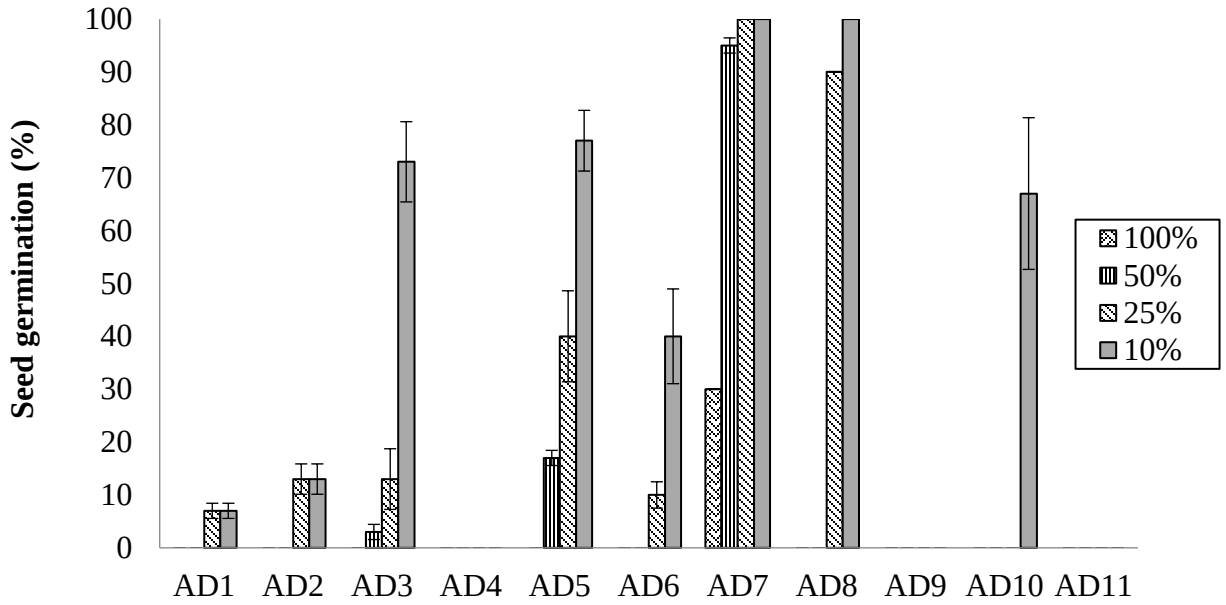

Figure 2.2. Anaerobic digestate phytotoxicity under different concentrations (100, 50, 25 and 10%) of anaerobic digestate, calculated based on the germination (%) of cress seeds (Lepidium sativum) in relation to the control (distilled water). (n=3).

2.4.5 Pathogen detection

Salmonella spp. was not detected in most of the anaerobic digestates (Table 2.5); only one sample (AD1) contained a low level (7 CFU 10 g^{-1} fresh mass). Low detection of *Salmonella* spp, meets current legislation for Animal By-Product (ABP) handling and processing (IrBEA 2013). *Salmonella* is one of the most common pathogens that can be spread in the environment through animal slurries and sewage sludge (Sahlström 2003). *Salmonella* strains that can be harmful to humans are mostly originated from livestock such as pigs, cattle and poultry (Kagambèga et al. 2013). As most of the anaerobic digestates evaluated in this study were from animal slurry feedstocks, the low detection of *Salmonella* spp. indicated that despite differences in operational parameters, temperature and HRT, the inactivation of this pathogen has been achieved by the biogas plants. Thermophilic conditions, combined with longer HRT and pre/post pasteurisation, are the main components in *Salmonella* spp. inactivation in biogas tanks. Additionally, volatile fatty acids play an important role in the inactivation of *Salmonella* spp, with high concentrations of organic acids such as acetic, propionic and butyric acid produced during the AD process directly reducing this pathogen (Salsali et al. 2006).

The concentration of *E. coli* varied from <0.3 (not detected), to 2400 CFU g^{-1} fresh mass (Table 2.5). Except for one digestate (AD3), all digestates met Irish recommended limits (IrBEA, 2013) for *E. coli* detection in anaerobic digestates. AD3 was not pasteurised pre- or post-digestion, which may have contributed to its relatively high levels of *E. coli*. Anaerobic digestion, in general, is known to reduce or inactivate *E. coli* (Aitken et al. 2007; Massé et al. 2011; Pandey and Soupir 2011); however, this effect seems to vary according to digestion temperature. Massé et al. (2011) reported that *E. coli* concentrations in pig slurry were decreased to undetectable levels by psychrophilic anaerobic digestion in sequential batch reactors operated at 7 and 14 days. Pandey

and Soupir (2011) demonstrated that batch anaerobic digestion of dairy cattle manure affected *E. coli* in different ways according to the temperature level, with higher temperatures requiring shorter times for inactivation. All anaerobic digestates tested in the present study were produced under mesophilic conditions with varied HRTs ranging from 14-70 days; the majority were carried out for over 40 days, which should encourage inactivation of *E. coli*.

Table 2.5. Detection of *Salmonella* spp. (MPN CFU 10 g^{-1} FW) and *Escherichia coli* (MPN CFU g^{-1} FW) in liquid anaerobic digestates.

Sample	***Salmonella* spp. (MPN CFU 10 g^{-1} FW)**	***Escherichia coli* (MPN CFU g^{-1} FW)**
AD1	7	<0.3 (not detected)
AD2	<10 (not detected)	8
AD3	<10 (not detected)	2400
AD4	<10 (not detected)	<0.3 (not detected)
AD5	<10 (not detected)	460
AD6	<10 (not detected)	<0.3 (not detected)
AD7	<10 (not detected)	15
AD8	<10 (not detected)	9
AD9	<10 (not detected)	<0.3 (not detected)
AD10	<10 (not detected)	<0.3 (not detected)
AD11	<10 (not detected)	23
Draft digestate standard limits **Irish Bioenergy Association (IrBEA, 2013).**	Not detected in 25 g	< 1000 CFU g^{-1} fresh mass

FW= fresh weight. (n=3)

2.5 Conclusion

The anaerobic digestates analysed in this study were shown to be potentially useful biofertilisers due to their concentrations of plant essential nutrients such as N (6.6 to 24.1%, average 11.7%), P (8.1 to 32.8 g kg^{-1} DW, average 17.4), and K (8.1 to 173.5 g kg^{-1} DW, average 61.5). However, the proportions of N-P-K in each digestate were widely variable. All anaerobic digestates analysed were below recommended limits for the concentrations of the following potentially toxic

elements: Cr, Cd, Ni, and Hg (limits 92, 1.3, 56, 0.4 mg kg^{-1} DW, respectively). However, three PTEs were over-limit in some of the digestates analysed: Pb (limit 149 mg kg^{-1} DW; AD5=1959); Zn (limit 397 mg kg^{-1} DW; AD1=434, AD4=516, AD5=1155, AD6=755, AD11=456); and Cu (limit 149 mg kg^{-1} DW; AD4=307, AD5=224, AD6=210, AD11=281). AD5 was derived from wastewater treatment feedstock, which may be responsible for its higher concentrations of PTEs. Phytotoxicity was associated with EC and decreased with anaerobic digestate dilution. Levels of *Salmonella* spp. and *E. coli* in the anaerobic digestates analysed were within the suggested limits recommended.

In conclusion, the liquid anaerobic digestates evaluated showed substantial differences in terms of nutrients and physical-chemical characteristics. Due to the complexity of anaerobic digestates and especially their widely variable composition, it may be difficult to produce standard fertilisation rates for different digestates. Therefore, it is strongly recommended that their land spreading should be preceded by a physical-chemical and nutrient analysis.

Chapter 3 : Determination of microbial numbers in liquid anaerobic digestates

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3.1 Abstract:

This study aimed to quantify total numbers of bacteria, fungi and archaea in different types of commercial liquid anaerobic digestates, and to identify common patterns in their microbial numbers post-digestion and possible implications of their use as biofertiliser. Relationships between microbial numbers and physical-chemical traits of the digestates were investigated. Quantification was performed using culturable and molecular (quantitative PCR) approaches. Bacterial and fungal CFUs ranged up to five orders of magnitude (10^5 to 10^{10}; 0 to 10^5 g^{-1} DW, respectively) between different types of anaerobic digestates. Bacterial, archaeal and fungal gene copy numbers (GCN) varied by two orders of magnitude (10^8 to 10^{10}; 10^7 to 10^9; 10^4 to 10^6 g^{-1} DW, respectively) between digestates. All microbial variables analysed showed significant differences between the different types of anaerobic digestate investigated ($p<0.05$). Culturable microbial numbers for fungi (6.43 x 10^4 CFU g^{-1} DW) were much lower than for bacteria (2.23 x 10^9 CFU g^{-1} DW). Gene copy numbers were highest for bacteria (16S) (1.09 x 10^{10} g^{-1} DW), followed by archaea (16S) (5.87 x 10^8 g^{-1} DW), and fungi (18S) (1.77 x 10^6 g^{-1} DW). Liquid anaerobic digestates were predominantly dominated by bacteria, followed by archaeal and fungal populations. At 50% similarity level, the microbial profiles of the eleven anaerobic digestates tested separated into just two groups, indicating a broad relative degree of similarity in terms of microbial numbers. Higher bacterial (16S) GCN was associated with low OM and C/N ratio in digestates.

3.2 Introduction

The utilisation of anaerobic digestate as fertiliser has increased all around the world following the intensification of biogas production (Alburquerque et al. 2012; Mao et al. 2015). Digestates are recognized for their readily available plant macronutrients, especially N (NH_3/NH_4) and K^+, and for carrying most of the other plant-essential macro and micronutrients, e.g. P, Ca, Mg, Fe, Mn, Cu, and Zn (Trambone et al. 2010; Möller and Müller, 2012; Nkoa, 2014; Coelho et al. 2018). Anaerobic digestates generally have a broad range of physical-chemical compositions, which is associated with the different types of organic wastes used as feedstock, and differences in digestion conditions such as temperature, hydraulic retention time (HRT), batch flow, and pre- and post-treatment e.g. pasteurisation (Trambone et al. 2010; Alburquerque et al. 2012; Möller and Müller, 2012; Nkoa, 2014, Coelho et al. 2018). Apart from the known variability in physical-chemical traits, anaerobic digestates also can contain many different quantities and types of microorganisms. These microorganisms in digestates can impact upon many management strategies related to digestate use, especially storage/shelf-life, biosecurity, and environmental impacts of their landspread.

When spread on land, digestates are potential vectors for dispersion of large amounts of microbes to the environment. Generally, the first public concern about microorganisms in digestates is the presence and viability of pathogenic organisms, especially the ones that can affect human and animal health. Pathogenic strains of *Escherichia coli*, *Salmonella* spp., and *Clostridium* spp., for example, have been quantified in anaerobic digestates in order to understand their inactivation, detection, viability and risks before digestate landspreading (Sahlström, 2003; Massé et al. 2011; Maynaud et al. 2016; Coelho et al. 2018). Apart from pathogenic microorganisms, other kinds of beneficial and harmful microbes can be found in anaerobic digestates (Conversando

et al. 2015; Insam et al. 2015). Considering the variety of anaerobic digestates produced, it is useful to quantify and estimate the total amounts of bacteria, fungi and archaea present in them, in order to provide information on the possible numbers of microbes that will be applied once the digestate is spread on land.

Many different methods can be used to quantify microorganisms in digestates, including measurement of metabolites, presence of ergosterol for fungi (Wentzel et al. 2016a), colony-forming units (Voća et al. 2005), and real time-PCR (Kim et al. 2013), which can be used to measure the gene copy numbers among others. Recently, microbial communities in digestates have been efficiently DNA sequenced and described by advanced molecular techniques (Nelson et al. 2011; Regueiro et al. 2012; Vanwonterghem et al. 2014; Conversando et al. 2015; Maynaud et al. 2016). The microbial communities described and sequenced during anaerobic digestion possibly predict much of the microbial profile that can be found in the material post-digestion. However, quantification using nucleic acid techniques also includes extracellular DNA from dead microorganisms, overestimating microbial numbers in digestates (Wolffs et al. 2005; Cangelosi and Meschke, 2014). Removal of extracellular/dead DNA from environmental samples such as sludge is quite complex and not sufficiently efficient to recommend its use (Wagner et al. 2008; Albertsen et al. 2015). Therefore, combining different methods, such as culturing and molecular approaches, can provide a useful approach to determining microbial amounts in anaerobic digestates.

Another question that arises relating to microbial quantification in digestates is whether there are evident associations between microbial numbers and the physical-chemical composition of digestates. Nutrient concentrations have an influence on microbial numbers and activity within anaerobic reactors, including cell growth, toxicity, and activation of microbial enzymes (Gerardi,

2003; Chen et al. 2008; Rajagopal et al. 2013, Wintsche et al. 2016). As digestates are highly variable in their physical-chemical compositions, detection of possible associations between microbial numbers and physical-chemical traits would be useful for predicting microbial profiles based on digestate composition.

This study aimed to quantify total bacteria, fungi and archaea from eleven different types of commercial liquid anaerobic digestates produced in Ireland and the United Kingdom, using two different quantification methods: culturable (fungi and bacteria) and molecular (quantitative PCR of bacterial, fungal, and archaeal rRNA gene copy numbers). Also, associative patterns between microbial numbers and physical-chemical traits of anaerobic digestates were studied.

3.3 Material and methods

3.3.1 Sampling

Eleven different types of liquid anaerobic digestates from Ireland and the United Kingdom were collected in triplicate from different biogas plants in October of 2015. Feedstock and general operational conditions of the biogas plants can be found in (Table 2.1.). Samples were refrigerated at 4 ºC for bacterial and fungal colony-forming units (CFU) analyses. For molecular analysis, samples were kept frozen at -20 ºC, before and after DNA extraction. Samples were processed following the guidelines in European standard EN 16179 (2012). Detailed physicochemical characterisations of the anaerobic digestates evaluated,(Chapter 2).

3.3.2 Bacterial and fungal colony-forming units

Bacterial and fungal CFU were determined using a serial dilution plating technique in selective media. Serial dilutions were prepared in sterile quarter-strength Ringer's solution from 5

g (fresh mass) of digestate. For bacteria, pour plates using tryptic soy agar (TSA) supplemented with 50 µg ml^{-1} cycloheximide were prepared in triplicate using 1 ml of the diluted sample. Different dilutions were tested, ranging from 10^{-1} to 10^{-7}. Samples were incubated at 28°C. Colony formation units were analysed by visualisation using a manual colony counter (Stuart®). Bacterial CFU was counted at the optimal time for most of the digestates (CFU between 30-300) of 38 hours of growth. For fungi, spread plates using potato dextrose agar (PDA) supplemented with 100 µg ml^{-1} streptomycin, and 50 µg ml^{-1} novobiocin were prepared in triplicate using 0.1 ml of diluted sample. Dilutions tested ranged from 10^{-1} to 10^{-4}. Plates were incubated at 28°C. CFUs were counted at the optimal time of 132 hours (the majority of anaerobic digestates showed fungal CFU ranging between 30-300 at approximately five days of growth). CFU results were corrected to dry matter (DM) of digestate.

3.3.3 DNA extraction and treatment

Between 50-100 ml of each anaerobic digestate samples were centrifuged at 3200 rpm for 15 minutes to produce a semi-solid pellet of around 10 ml, then the supernatant was discarded. Centrifuged samples were analysed for DM by being oven-dried at 105°C according to European standard EN 13040 (2007). DNA was extracted using the PowerSoil® DNA Isolation kit (Qiagen®) from an aliquot of 0.25 g fresh weight (FW) of the centrifuged samples, and eluted in 100 µl. 1 µl of DNA was used to quantify (ng $µl^{-1}$) and absorbance ratios (260/280 nm and 260/230 nm) using the spectrophotometer NanoDrop™ 1000 Thermo Scientific. Due to detection of PCR inhibitors in most digestates (see an example of 260/230 ratio in Table 3.1), all genomic DNA extracted was cleaned using DNA Clean & Concentrator (Zymo Research®) and re-quantified using Qubit 4 Fluorometer (Invitrogen®). All DNA samples were brought to a concentration of 2

ng μl^{-1}prior to real-time PCR analysis, as some of the digestates showed this concentration after DNA extraction and cleaning. Also, PCR inhibitors were noticed to be reduced by diluting digestates samples to a lower concentration. No real-time PCR amplification problems were noticed due to a DNA concentration of 2 ng μl^{-1}.

Table 3.1. Evaluation of the ratios 260/280 and 260/230 and DNA yields using Thermo Scientific NanoDrop™ 1000 Spectrophotometer in digestates, slurry and soil samples tested. AD= Anaerobic digestate.

Sample	260/280	260/230	ng μl^{-1}
AD1	1.61	1.04	30.1
AD1	1.55	0.55	9.9
AD2	1.8	1.48	57.9
AD2	1.82	1.23	47.9
AD3	1.79	1.51	64.5
AD3	1.82	1.67	81.8
AD4	1.77	0.94	41.8
AD4	1.58	0.85	39.3
AD5	1.81	1.77	91.9
AD5	1.8	1.6	49.8
AD6	1.81	1.82	84.6
AD6	1.8	1.68	110.1
Cattle slurry	1.81	1.7	64.6
Cattle slurry	1.85	0.4	6.1
Soil	1.9	1.6	67.9
Soil	1.91	1.65	65.1

3.3.4 Real-time PCR

3.3.4.1 Cultures used as standards and external control

Bacterial genomic DNA was extracted from isolated pure cultures of *Escherichia coli* and *Serratia marcescens* using Nucleic Acid MiniPrep Kit (Anachem®). Archaeal genomic DNA was extracted from pure cultures of *Haloferax denitrificans* and *Halobacterium salinarum* using Nucleic Acid MiniPrep Kit (Anachem®). For fungi, genomic DNA was extracted from pure

cultures of *Fusarium* sp. and *Aspergillus niger* using Quick-DNA Fungal/Bacterial Kit (Zymo Research®). After DNA extraction, the genomic DNA was amplified using PCR. The procedure of DNA amplification via conventional PCR was performed using a reaction of 15 µl, where: 7.5 µl Promega GoTaq Master Mix Green, 1 µl DNA template, 0.45 µl (100 pmol) forward primer and 0.45 µl (100 pmol) reverse primer, and 5.6 µl ultra-pure sterilised water. DNA amplification was confirmed by gel electrophoresis, and measurements of the ratios 260/280 and 260/230 and yields were made using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer. The PCR primers, thermocycles and identification parameters are described (Table 3.2). After amplification of the genomic DNA, gel electrophoresis tests were performed (Figure 3.1). To identify cultures, the PCR products of the genomic DNA of 16S (bacteria/archaea) and 18S (fungi) rRNA gene regions amplified by conventional PCR, were cleaned using the DNA Clean & Concentrator (Zymo Research®) kit. Following the PCR products were sent for sequencing at GATC Biotech (Konstanz, Germany). Sequencing results were analysed using Chromas 2.6 software and their FASTA file can be found in (Appendix 8.1 and 2.). Microbial identification was performed through Basic Local Alignment Search Tool (BLAST) from the NCBI, U.S. National Library of Medicine.

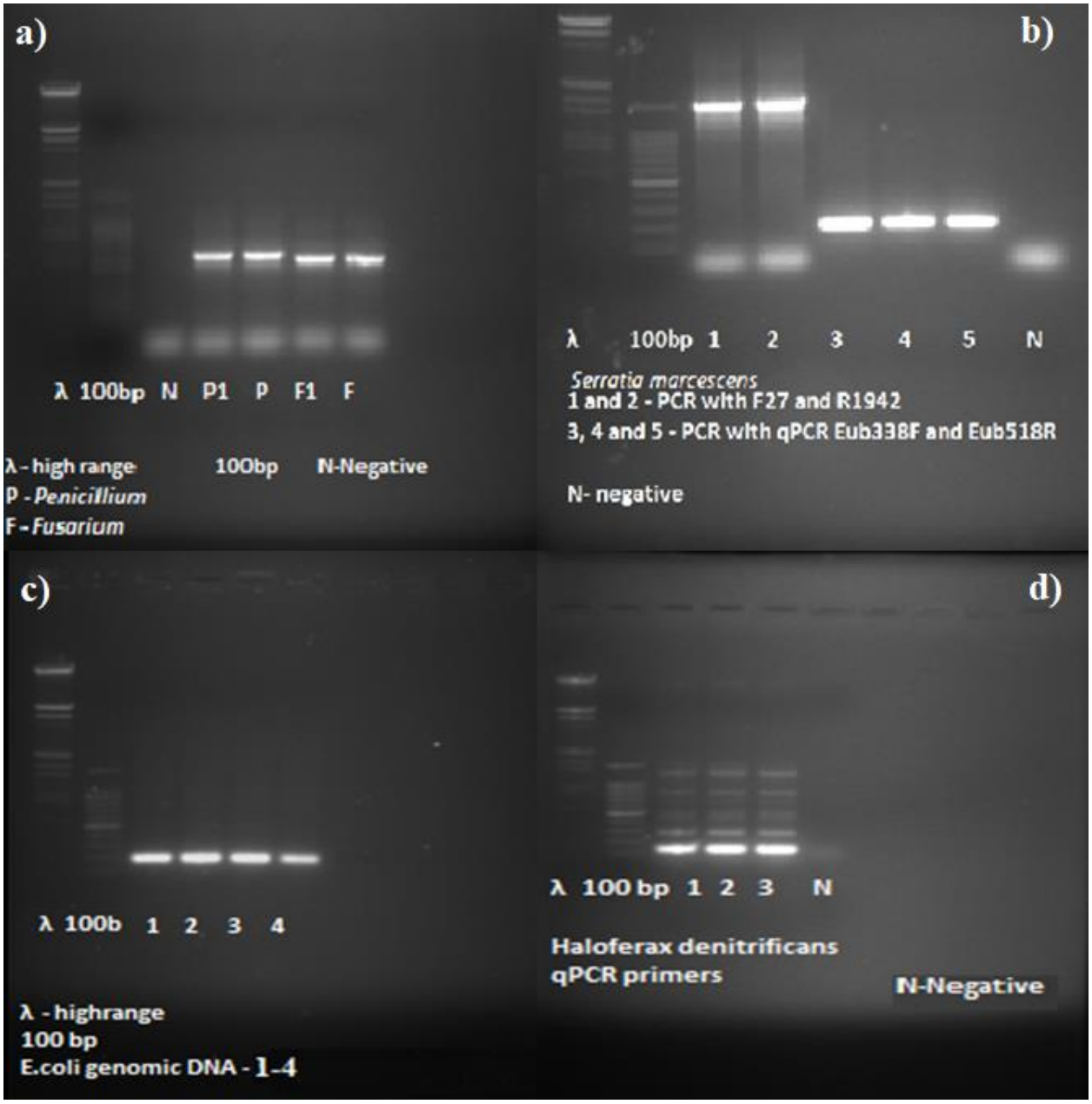

Figure 3.1 Examples of the gel electrophoresis of the PCR-amplified genomic DNA from some of the cultures used as standard/or external controls. In all gels, first well contains high-range DNA size marker, second 100 bp size marker, and N = negative control (no template). a) – Fungal ITS amplicons from *Penicillium* sp. (P, P1) and *Fusarium* sp. (F, F1) genomic DNA. b) – Bacterial 16S amplicons from *Serratia marcescens* genomic DNA using conventional (Lanes 1 and 2) and qPCR (Lanes 3, 4, and 5) primers. c) - Bacterial 16S amplicons from *Escherichia coli* genomic DNA using qPCR primers (Lanes 1-4). d) – Archaeal 16S amplicons from *Haloferax denitrificans* genomic DNA using qPCR primers (Lanes 1-3).

For fungi, cloned 18S genes were used as standard controls. A portion of the 18S gene was amplified from extracted pure culture genomic DNA using qPCR primers FF390/FR1 (Table 3.3). Each reaction of 15 µl contained: 7.5 µl Promega GoTaq® Master Mix Green, 0.3 µl bovine serum albumin (BSA) Thermo Fisher Scientific® (10 mg μl^{-1}), 0.45 µl (100 pmol) of each primer, and 5.3 µl of molecular grade water. PCR products were cleaned with DNA Clean & Concentrator (Zymo Research®) and subjected to a Poly A reaction to improve the ligation reaction: 1 µl 10x buffer (Amresco®), 0.6 µl $MgCl^{+2}$ (25 mm), 1 µl Taq Polymerase, 0.2 µl dNTP (10 mm) and 7.2 µl PCR template. PCR products were reacted using p-GEM-T Easy Vector (Promega®) and then inserted in Nova Blue Giga Singles TM Competent Cells (Novagen®). Clones were grown in LB agar plates with ampicillin (100 µg μl^{-1})/IPTG (0.1M)/X-Gal (50 mg ml^{-1}). Plasmid DNA was extracted using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich®) and quantified (ng μl^{-1}) using Qubit 4 Fluorometer (Invitrogen®). Plasmid DNA was diluted to an initial concentration of 3 x 10^9 copies of the target gene; then, serial dilutions down to 10^{-9} were created.

3.3.4.2 Generation of standard curves

Standard curves for bacteria and archaea were generated using genomic DNA of pure cultures (Table 3.2) and followed calculations proposed by Tatti et al. (2016), where: (6.023 x 10^{23} (copies mol^{-1}) x concentration of target DNA (g μl^{-1})) / target molecular weight (g mol^{-1}). All DNA extracts were quantified (ng μl^{-1}) using Qubit 4 Fluorometer (Invitrogen®). Pure culture DNA was diluted to an initial concentration of 3 x 10^7 copies of the target gene; then, serial dilutions down to 10^{-7} were created. All standard curves aimed to have R^2>0.98 and amplification efficiencies between 90-110%, for both the standards and external controls in each assay; however, assays were run if only the standard met the criteria to generate the curve (Figure 3.2 a-c), and in cases

where a combination of the aimed R^2 and an efficiency value near the target range, were confirmed by a good correspondence between the GCN expected in the results of the qPCR from the external controls (Figure 3.3). The qPCR efficiency was calculated using the formula $E= 10(^{-1/slope})-1$. The standard curve for fungi curve was generated from a highly concentrated sample of the plasmid of 18S (10^9); some dilutions below 10^3 presenting error in some of the analysis were excluded. The use of more concentrated standards for fungi led to some early Ct values, therefore the entire curve was not within the ideal linear range. For future studies using this set of fungal primers for analysing digestates/soil samples, it is recommended to test intermediate ranges of dilutions, for example, 1:2 or 1:5 rather than 1:10 dilutions, which possibly could provide better efficiency and r^2 values than the ones found in this study. Nevertheless, most of the samples analysed were within the accepted range of between 10^3 and 10^5. In addition to testing the qPCR assays by generating standard curves using two species per assay, the species selected as standards were validated by the external controls by running known quantities of external control DNA and using the standard curve to calculate the GCN present. These data were regressed, and good congruence was observed for all three assays ($r^2<0.97$) (Figure 3.3).

Table 3.2. Primers, thermocycling conditions, and identification parameters for the standards and external controls used for bacterial (16S), archaeal (16S) and fungal (18S) GCN.

	Bacteria		**Archaea**		**Fungi**	
Primer set	F27 (5-′AGAGTTTGATCMTGGCTCAG-3′) R1492 (5'-TACGGYTACCTTGTTACGACTT-3') Lane, D.J. 1991		Arch967F (5'-AATTGGCGGGGGAGCAG-3') Arch1060R (5'-GGCCATGCACCWCCTCTC-3') Amann et al. 1990		ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') White et al. 1990 ITS4 (5'-TCCTCCGCTTATTGATATGC-3') Gardes and Bruns, 1993	
Thermocycle	Initial denaturation 94 ºC (3 min), followed by 26 cycles of 94 ºC (1 min), annealing 53 ºC (2 min), and elongation 72 ºC (2 min). Final step 72 ºC (10 min)		Initial denaturation 94 ºC (3 min), followed by 26 cycles of 94 ºC (1 min), annealing 53 ºC (2 min), and elongation 72 ºC (2 min). Final step 72 ºC (10 min)		Initial denaturation 94 ºC (3 min), followed by 26 cycles of 94 ºC (1 min), annealing 53 ºC (2 min), and elongation 72 ºC (2 min). Final step 72 ºC (10 min)	
Amplicon (bp)	1465		140		420 to 825	
	Standard	Control	Standard	Control	Standard	Control
Species	*Escherichia coli*	*Serratia marcescens*	*Haloferax denitrificans**	*Halobacterium salinarum**	*Fusarium sp.*	*Aspergillus niger*
Query cover	100%	100%	96%	94%	100%	100%
E-value	0	0	3e-17	2e-42	0	0
Identity %	99%	100%	96%	97%	99%	97%
Genome size (Mb)	4.6	5.1	3.9	2.5	41.9	36.1
Copies per genome (16S /18S)	2	7	1	1	1 plasmid cloned	1 plasmid cloned
Source of culture	Waterford Institute of Technology	Waterford Institute of Technology	Deutsche Sammlung von Mikroorganismen und Zellkulturen	Carolina®	Waterford Institute of Technology	Waterford Institute of Technology

. *For archaea identification only a short portion of the 16S was sequenced (qPCR primers were used) as cultures were purchased from external culture banks

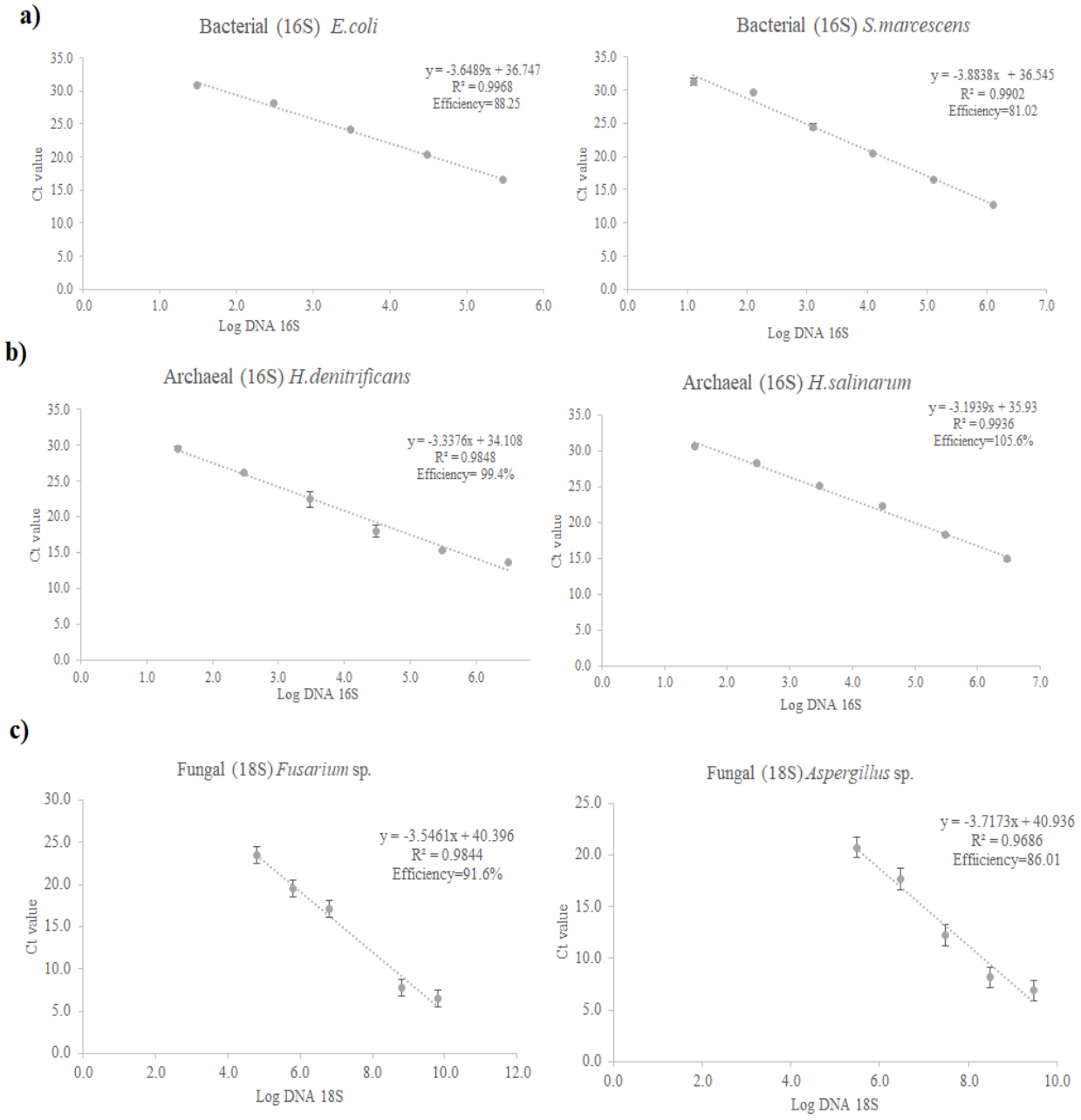

Figure 3.2. Examples of standard curves from the qPCR analysis of bacterial 16S (a), archaeal 16S (b) and fungal 18S (c). Ct=cycle threshold. Error bars are standard deviation (n=2); when not visible, the error bar is beneath the data point. The curves on the left-hand side (*Escherichia coli*, *Haloferax denitrificans* and *Fusarium* sp.) were those used in calculating GCN of unknowns; those on the right-hand side (*Serratia marcescens*, *Halobacterium salinarum*, and *Aspergillus* sp.) were used as external controls to validate the qPCR assays. Analysed in 7300 Real-Time PCR System (Applied Biosystems®). Data in Appendix 3.

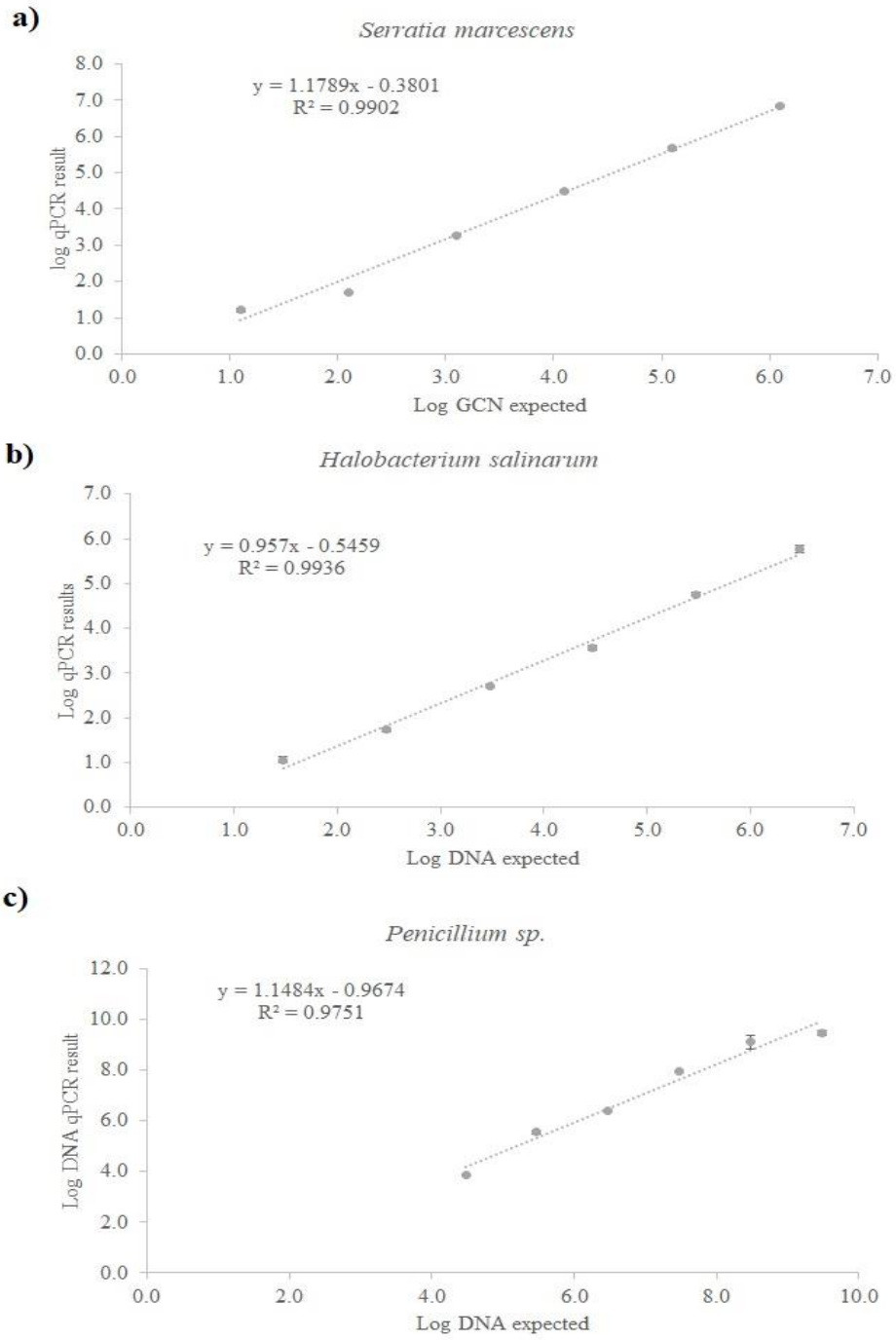

Figure 3.3. Expected GCN and actual qPCR values of external control DNA were assayed using standard curves for bacterial 16S (a), archaeal 16S (b) and fungal 18S (c). Analysed in 7300 Real-Time PCR System (Applied Biosystems®). *Values displayed in log scale. Error bars are standard deviation (n=2); when not visible, the error bar is beneath the data point.

3.3.4.3 Real-time PCR: reagents, primers and thermocycle conditions

Microbial abundance (gene copy numbers - GCN) of bacterial (16S), archaeal (16S) and fungal (18S) were analysed using a reaction of 15 µl: 7.5 µl Sybr® Green qPCR (Sigma-Aldrich), 0.3 µl (10 pmol μl^{-1}) forward primer and 0.30 µl (10 pmol μl^{-1}) reverse primer, 5.9 µl molecular biology grade water and 1 µl DNA template. Thermocycle conditions and primers are shown in Table 3.3. For the bacterial primers, Fierer et al. (2005) tested the pair Eub338F/Eub518R for qPCR assays of soil samples. These primers showed high specificity for the sequences of the target group (soil bacteria), these authors used the Ribosomal Database Project (RDP) to classify their results. Nakano (2018) analysing environmental samples reported Ct values between 15 to 21 for this set of bacterial primers. In relation to the archaeal primers Arch 967F/Arch1060R, Cadillo-Quiroz et al. (2006) used these primers for the qPCR of archaeal communities in soil due to their high coverage for important archaeal phyla, with no matches for bacterial and eukaryotic sequences. In relation to the fungal primers used (FF390/FR1), Vainio and Hantula (2000) originally developed these primers in an assay with environmental samples based on regular PCR and DGGE. They reported an amplicon size of 390 bp, but this set of primers has been well accepted for use in qPCR (Prevost-Boure et al. 2011; Siles and Margesin, 2016). In a study for validation and application of fungal PCR primers in qPCR analysis, Prevost-Boure et al. (2011) validated and classified the pair FF390/FR1 as the best set of primers for the analysis of soil fungi in qPCR, due to their high specificity, coverage and amplicon length.

Real-time PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems®). The standards and external controls were run in duplicates. DNA concentrations used for bacteria 16S, archaea 16S and fungi 18S were 3 to 3 x 10^5; 3 x 10^1 to 3 x 10^5;10^4 to 10^9,

respectively. Digestate samples, as well as soil in their respective chapters, were run in duplicates of each replicate (n=3) of the DNA. Negative template controls NTC (n=3) (molecular-grade water) were run in each plate analysed. The GCN g DW^{-1} was calculated based on the data generated by the qPCR in relation to the total DW of the DNA sample extracted: (GCN in 1 µl (qPCR loaded amount) x total dilution of DNA extracted)/ total DW of sample used in the DNA extraction (Tatti et al. 2016). Siles and Margesin (2016), analysing soil samples with the same set of primers used in this PhD study, reported very similar ranges for the GCN of the soil bacteria (10^8 to 10^9) fungi (10^8 to 10^9) and archaea (10^5 to 10^6), to the ones found in our study (Figure 6.5).

Table 3.3. Primers and thermocycles used in qPCR analyses of bacterial (16S), archaeal (16S) and fungal (18S) GCN.

	Primer Set	Approximate Amplicon (bp)	Primer reference	Thermocycle
Bacteria	Eub338F (5'-ACTCCTACGGGAGGCAG-3') Eub518R (5'- ATTACCGCGGCTGCTGG-3')	200	Fierer et al. 2005	Initial denaturation 95 ºC (3 min), followed by 39 cycles of 95 ºC (15 s), annealing 53 ºC (15 s), and elongation 72 ºC (1 min). Dissociation
Archaea	Arch967F (5'AATTGGCGGGGGAGCAG-3') Arch1060R (5' GGCCATGCACCWCCTCTC-3')	140	Cadillo-Quiroz et al. 2006	Initial denaturation cycle 95 ºC (7 min), followed by 40 cycles of 95 ºC (15 s), annealing 55 ºC (30 s) and elongation 70 ºC (1 min). Dissociation stage*
Fungi	FF390 (5'-CGATAACGAACGAGACC-3') FR1 (5'-A[I]CCATTCAATCGGTA[I]T-3')	Varied: 280 to 390	Vainio and Hantula (2000) Prevost-Boure et al. (2011)	

*A dissociation stage was added to the end of the amplification cycles: 95 ºC (15 s), 60 ºC (30 s) and 95 ºC (15 s).

3.3.5 Statistical analysis

All the molecular results after conversion for their equivalent DM data were tested for normality (Kolmogorov-Smirnov, Shapiro-Wilk) and homoscedasticity (Levene's test). The one-way ANOVA on Kruskal-Wallis H Test compared bacterial, fungal and archaeal numbers ($p<0.05$), using the software SPSS 24 (IBM®), due to non-homogeneous variance between different digestates/slurry samples. Nonmetric multidimensional scaling (NMDS) was conducted to evaluate relationships between microbial communities from different anaerobic digestates using the software PRIMER v6 (Clarke and Gorley, 2006). Samples were root square transformed, and their resemblance was measured using the Bray-Curtis similarity matrix (0 to 1 level). NMDS graphs were overlaid with cluster analysis at 50 and 75% degrees of similarity. Associative patterns between microbial numbers and physical-chemical traits were analysed by the best-fit of regression models: linear, quadratic, cubic, logarithmic, exponential and power.

3.4 Results and discussion

3.4.1 Microbial quantification

Bacterial CFU ranged widely, from 5×10^5 to 2×10^{10} CFU g^{-1} dry weight (DW), a difference of five orders of magnitude (Table 3.4). There was a group of six digestates (AD1, AD2, AD6, AD7, AD8 and AD9) with high bacterial CFU; however, only AD1 differed significantly from other digestates (AD3, AD4, AD5, AD10, AD11) that showed lower bacterial CFU ($P<0.05$). Bacterial (16S) GCN varied from 5.03×10^8 to 4.67×10^{10} g^{-1} DW, showing a difference of two orders of magnitude, a narrower range between digestates than bacterial CFU. Six digestates showed higher bacterial (16S) GCN (AD1, AD5, AD6, AD7, AD8 and AD9). AD9 had the highest bacterial (16S) GCN (4.67×10^{10} g^{-1} DW) and was the only digestate that differed significantly

from digestates with lower numbers ($p<0.05$). Bacterial CFU and GCN analysis were not correlated ($p>0.05$).

Fungal CFU ranged from 0 (not detected) to 2.68×10^5 g^{-1} DW, a difference of five orders of magnitude. AD 10 and AD11 had the highest numbers of fungal CFU, significantly higher ($p<0.05$) than AD1, AD4, AD5 and AD6, which all had lower numbers. As seen with bacteria, fungal (18S) GCN showed a narrower range compared to CFU, from 4.87×10^4 to 7.52×10^6 g^{-1} DW, a difference of only two orders of magnitude (Table 3.4). Six digestates (AD2, AD3, AD7, AD8, AD10 and AD11) had higher fungal (18S) GCN, and like CFU the digestates AD10 and AD11 were the only that differed significantly from the digestates with lower fungal GCN ($p<0.05$). Despite substantial differences regarding orders of magnitude, fungal CFU and (18S) GCN showed similar trends, with a positive correlation ($r=0.63$; $P<0.01$), indicating good agreement between the two methods.

Archaeal (16S) GCN varied from 1.63×10^7 to 4.20×10^9 g^{-1} DW, a difference of two orders of magnitude (Table 3.4). Four digestates (AD3, AD5, AD6 and AD7) showed higher archaeal (16S) GCN, and from this group, only AD6 differed significantly from the other digestates with low archaeal (16S) GCN ($P<0.05$).

Table 3.4. Quantification of bacterial and fungal colony-forming units (CFU) (g^{-1} DW), and bacterial (16S), archaeal (16S) and fungal (18S) gene copy numbers (GCN) (g^{-1} DW) in liquid anaerobic digestates (AD).

	Bacterial		**Fungal**		**Bacterial 16S**		**Fungal 18S**		**Archaeal 16S**	
Sample	**(CFU g^{-1} DW)**				**GCN (g^{-1} DW)**					
AD1	2.03×10^{10}	a	0*	c	1.25×10^{10}	abc	1.61×10^{5}	bcd	7.57×10^{7}	cd
AD2	8.47×10^{8}	abc	8.52×10^{3}	b	9.93×10^{8}	cd	5.39×10^{5}	abc	2.93×10^{7}	d
AD3	1.07×10^{8}	def	2.05×10^{4}	ab	7.93×10^{9}	abcd	6.64×10^{5}	abc	8.03×10^{8}	ab
AD4	5.00×10^{5}	f	0*	c	5.03×10^{8}	d	2.22×10^{5}	bcd	7.87×10^{7}	bcd
AD5	4.60×10^{7}	ef	1.40×10^{4}	b	8.03×10^{9}	abcd	9.39×10^{4}	cd	3.67×10^{8}	abc
AD6	1.67×10^{9}	ab	9.30×10^{2}	b	1.73×10^{10}	ab	4.87×10^{4}	d	4.20×10^{9}	a
AD7	4.60×10^{8}	abcd	2.39×10^{4}	ab	1.23×10^{10}	abc	5.37×10^{5}	abcd	6.30×10^{8}	abc
AD8	3.30×10^{8}	abcde	6.87×10^{4}	ab	8.97×10^{9}	abcd	3.70×10^{6}	ab	1.48×10^{8}	bcd
AD9	3.37×10^{8}	abcde	5.32×10^{4}	ab	4.67×10^{10}	a	1.38×10^{5}	cd	1.63×10^{7}	d
AD10	1.73×10^{8}	cdef	2.68×10^{5}	a	4.63×10^{9}	bcd	5.83×10^{6}	a	7.77×10^{7}	bcd
AD11	2.07×10^{8}	bcdef	2.42×10^{5}	a	6.00×10^{8}	d	7.52×10^{6}	a	3.13×10^{7}	d
SE	1.07×10^{9}		1.76×10^{4}		2.39×10^{9}		5.61×10^{5}		2.16×10^{8}	

Means followed by different letters differed in the Independent-Samples Kruskal-Wallis Test ($p<0.05$).
* no visual detection of colony growth. SE= standard error (n=3)
AD1- Food waste (dairy industry), AD2- Food waste, pig slurry, AD3- Food waste (farm and food), AD4- Food waste, municipal sludge, AD5 - Wastewater treatment, AD6- Food waste, garden waste, AD7 - Whole cattle slurry, AD8 - Whole grass, AD9 - Cattle slurry, chicken manure, food waste, AD10 - Whole cattle slurry, AD11 - Food waste (kitchen), garden waste. *Note:* AD1, AD5 and AD6 were pasteurised pre-digestion, AD2 and AD4 post-digestion; other digestates did not undergo any pasteurisation.

The broad range and differences between bacterial, archaeal and fungal numbers in liquid anaerobic digestates can be attributed to many sources of variability, such as feedstock used, as well as differences in the conditions under which each anaerobic digestate was produced (Rivière et al. 2009; Ziganshin et al. 2013; Vanwonterghem et al. 2014). In relation to the AD technology used, Abendroth et al. (2015) evaluated the microbial profile of bacteria and archaea in seven different types of mesophilic anaerobic reactors and concluded that the type of facility used had a strong correlation with the bacterial profile of the anaerobic reactors. Guo et al. (2015), comparing the microbial communities in mesophilic and thermophilic anaerobic reactors, pointed out

substantial differences in the microbial profile, including microbial dominance of bacterial and archaeal communities, in relation to the temperature used for the AD.

The range of microbial GCN found in our present study can give an idea of expected numbers and proportions of bacteria, fungi, and archaea. Microbial communities described by culturable methods showed that fungal CFU (6.43×10^4 g^{-1} DW) were much lower than bacterial CFU (2.23×10^9 g^{-1} DW) (Figure 3.4.a) ($p>0.05$), a difference of five orders of magnitude. Regarding microbial abundance, bacterial (16S) GCN were highest with an average of 1.09×10^{10} g^{-1} DW, followed by archaeal GCN (16S) (5.87×10^8 g^{-1} DW), and fungal (18S) GCN (1.77×10^6 g^{-1} DW) (Figure 3.4 b).

Lower fungal numbers compared to bacterial and archaeal can be related to the anaerobic digestion process. As fungi comprise both yeasts and moulds, and most of the moulds are strict aerobes, it is expected that the anaerobic digestion process would decrease or suppress fungal growth, consequently lowering their numbers. Anaerobic digestion is performed by a combination of bacteria and archaea acting in a consortium, which explains their dominance in digestates. In the initial steps (hydrolysis, acidogenesis and acetogenesis) bacterial groups play the primary roles, with methanogenesis then being conducted by archaeal groups (Ziganshin et al. 2013). Nevertheless, Schnürer and Schnürer (2006) found few effects of anaerobic digestion on reducing total fungal numbers compared to the initial numbers in the feedstock. They reported that the microbial community at post-digestion is mostly influenced by the fungal numbers found in the substrate.

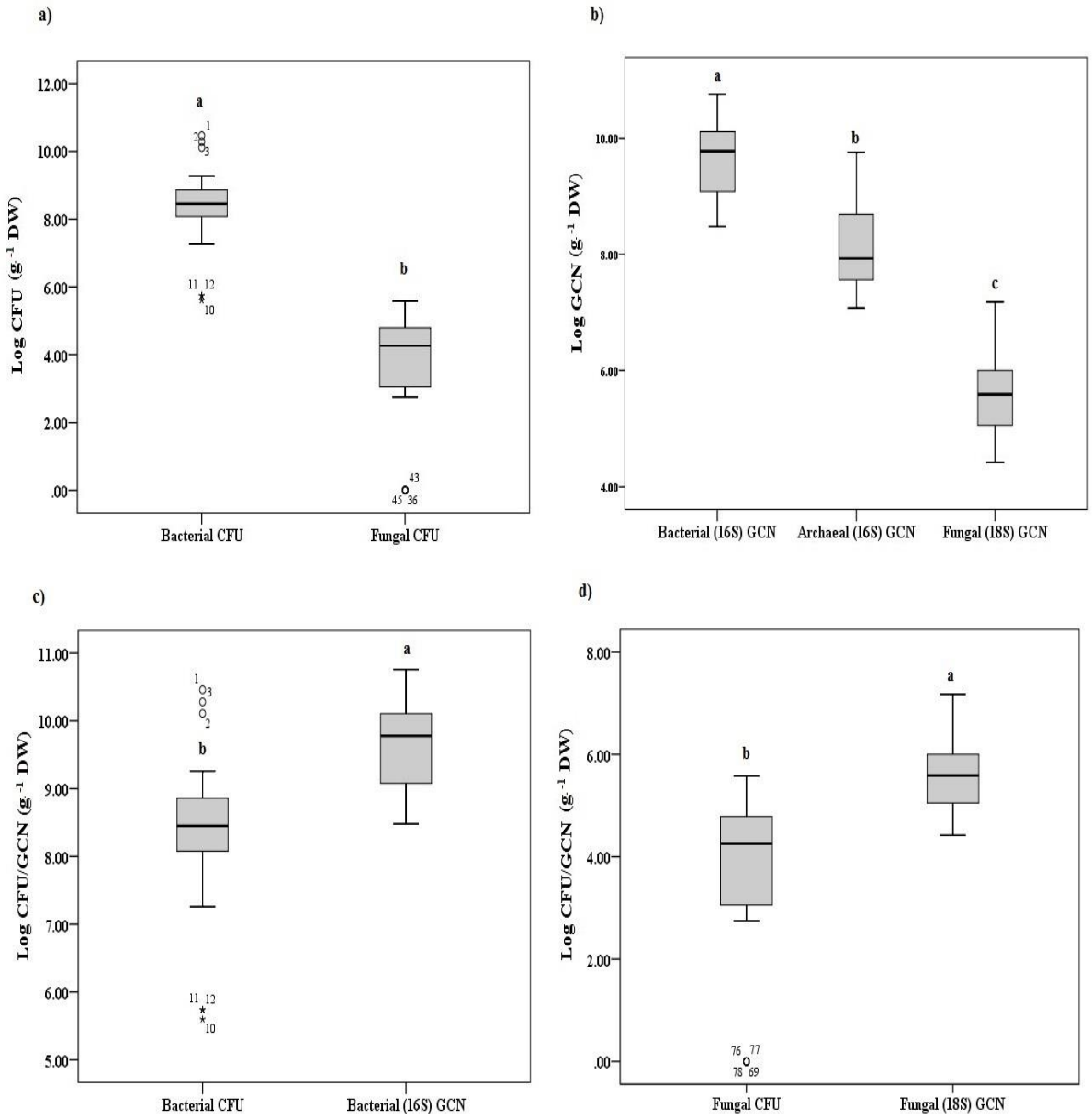

Figure 3.4. Microbial quantification (bacteria and fungi) by selective media (CFU g^{-1} DW) (a), gene abundance (bacteria, archaea 16S, fungi 18S) (GCN g^{-1} DW) (b), and quantification method comparison for bacteria (c) and fungi (d), for a range of different types of post-digestion liquid anaerobic digestates.

Box-plots with different letters differed in the Independent-Samples Kruskal-Wallis Test ($p<0.05$). CFU (colony-forming unit); GCN (gene copy number). ○ outliers, *extreme outliers. Data were log scale transformed.

One of the anaerobic digestates (AD10) which showed the highest average of fungal numbers for CFU and (18S) GCN was produced from whole cattle slurry feedstock, which possibly had the presence of strict and facultative anaerobic fungal groups from the ruminant tract. These types of anaerobic rumen fungi probably are capable of surviving and even reproducing during the anaerobic digestion process and can increase methane production in certain conditions (Procházka et al. 2012; Yıldırım et al. 2017). Rumen fungal species are known to produce many specific enzymes that aid in organic matter breakdown, such as carbohydrate hydrolysing, cellulolytic, hemicellulolytic, glycolytic and proteolytic enzymes. Kazda et al. (2014) reported evidence of active contributions of facultative anaerobic fungi species during anaerobic digestion.

Regarding microbial quantification by culture-dependent and molecular methods, the average bacterial number for all digestates described by culturing (2.23×10^9 g^{-1} CFU DW) was one order of magnitude lower than bacterial (16S) GCN (1.09×10^{10} g^{-1} DW) (Figure 3.4 c), while the fungal CFU average (6.36×10^4 g^{-1} DW), was two orders of magnitude lower than fungal (18S) GCN (1.77×10^6 g^{-1} DW) (Figure 3.4 d). For both bacteria and fungi, GCN had lower variability between different digestates than CFU. It must be considered that GCN may have also quantified dead microorganisms and fragmented extracellular DNA (Wolffs et al. 2005; Cangelosi and Meschke, 2014); possibly many organisms that did not survive anaerobic digestion contributed to the total GCN.

3.4.2 Microbial similarities between digestates

At a similarity level of 50%, nonmetric multidimensional scaling and cluster analysis revealed two main clusters formed between the anaerobic digestates evaluated (Figure 3.5). Vectors indicating the relative importance of the different microbial quantification methods in

discriminating between the digestate samples revealed a pronounced influence of fungal CFU and GCN and bacterial 16S. At a higher similarity level (75%), eight groups of digestate samples were formed, with clusters being mostly composed of isolated replicates of the same digestate sample. Three of the eight clusters were formed by two types of digestate clustering together. A cluster with a single isolated replicate (AD1) was observed; this was possibly associated with sampling or analytical error, as most of the replicates of the same digestate were within the same cluster at 75% similarity.

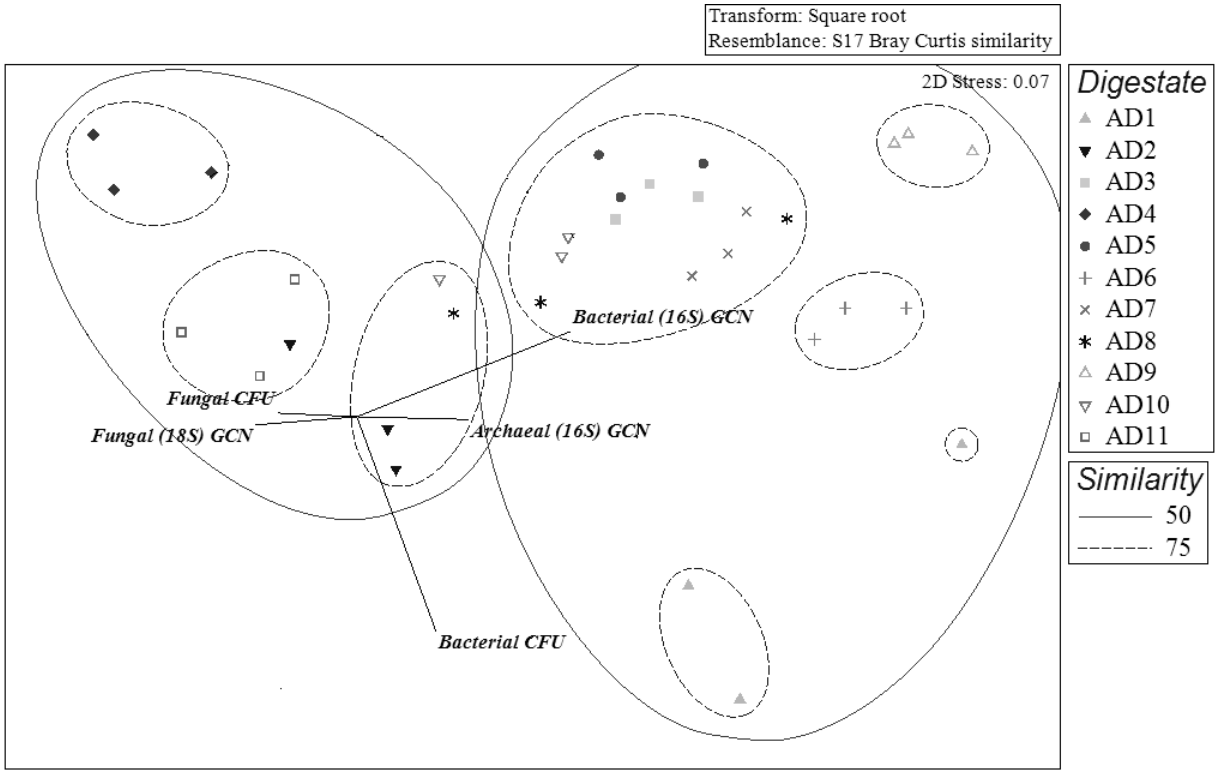

Figure 3.5. Nonmetric multidimensional scaling representation of quantified microbial communities in different types of liquid anaerobic digestates. Cluster analysis Bray-Curtis similarity (50% —, 75% ----). CFU (colony-forming unit); GCN (gene copy number).
AD1- Food waste (dairy industry), AD2- Food waste, pig slurry, AD3- Food waste (farm and food), AD4- Food waste, municipal sludge, AD5 - Wastewater treatment, AD6- Food waste, garden waste, AD7 - Whole cattle slurry, AD8 - Whole grass, AD9 - Cattle slurry, chicken manure, food waste, AD10 - Whole cattle slurry, AD11 - Food waste (kitchen), garden waste. *Note:* AD1, AD5 and AD6 were pasteurised pre-digestion, AD2 and AD4 post-digestion, others digestates did not undergo pasteurisation.

Between the two main clusters formed at 50% similarity, it was observed that each of them had at least four different types of feedstock: food waste, mixed food waste + animal slurry, wastewater, whole slurry and grass. No direct associations between the feedstock type and cluster were detected. However, the influence of the feedstock over microbial profiles could be expected, as feedstock has been reported as a factor that affects microbial communities in anaerobic reactors (Rivière et al. 2009; Ziganshin et al. 2013; Vanwonterghem et al. 2014). It is possible to speculate that as most of the anaerobic digestates used in the present trial were produced in commercial biogas plants with an expected lower control of the feedstock conditions and dynamics than a small-scale lab-controlled trial, higher variability and differences were seen in digestates with feedstocks classified in the same category.

The cluster associated with higher bacterial (16S) GCN comprised mostly digestates that underwent pre-digestion pasteurisation or no pasteurisation. Lower bacterial numbers were expected in digestates that underwent post-digestion pasteurisation. Post-digestion pasteurisation of liquid anaerobic digestates has demonstrated efficacy in reducing or eliminating certain types of bacteria (e.g. pathogenic bacteria) (Astals et al. 2012; Coultry et al. 2015). Nevertheless, Schnürer and Schnürer (2006) pointed out that some spore-forming microbes can be resistant to high temperatures, surviving common thermal treatments used to eliminate or reduce undesirable microbes during the anaerobic digestion process. The effects of post-digestion pasteurisation on the whole microbial community still need to be better understood, in order to assist in creating management strategies related to digestate storage, use as biofertiliser, and landspreading.

3.4.3 Microbial GCN relations with physical-chemical traits

Higher bacterial (16S) GCN was associated with lower OM and C/N ratio (Figure 3.6 a.b). These associations were possibly related to the amount of degradation that the organic matter

underwent during the anaerobic digestion process. Efficient organic matter degradation can contribute directly to higher availability of nutrients for microbial growth, especially N. As organic matter breakdown occurs, available forms of N such as NH_4-N are released in the digestate solution (Möller and Müller, 2012). Generation of new bacterial cells ($C_2H_7O_2N$) necessarily requires a source of available N. During anaerobic digestion of organic substrates, particularly in the initial stages, bacterial numbers rapidly increase in response to the degradation of the organic matter, as observed in batch reactors (Shin et al. 2010; Yi et al. 2014). For continuous anaerobic reactors, Jang et al. (2014) reported that bacterial numbers tend to increase in response to the availability of organic substrates.

Digestates with a higher C/N ratio showed a tendency to have higher fungal (18S) GCN (Figure 3.7), in contrast to the negative relationship seen between bacterial GCN and C/N ratio. A higher C/N ratio in digestates would be a consequence of low efficiency during anaerobic digestion. Possibly, higher efficiency of digestion would create an environment suitable for anaerobes, especially bacterial and archaeal groups, limiting fungal communities. The majority of fungal species are considered aerobes (Lee, 2014). Fungal numbers have been reported to be associated with organic materials with higher C/N ratio (Bossuyt et al. 2001; Six et al. 2006). The digestate that showed the highest fungal (18S) GCN (AD11) had the lowest bacterial and archaeal GCN (Table 3.4). However, fungal growth and survival during the anaerobic digestion process are complex, depending on a series of factors related to feedstock, digestion conditions and physiology of the fungal species evaluated. Schnürer and Schnürer (2006) reported a low efficiency of mesophilic anaerobic digestion in reducing fungal numbers. On the other hand, Bandte et al. (2013) found that mesophilic anaerobic digestion reduced or fully inactivated plant pathogenic fungal species. It should be considered that certain fungal species, such as facultative and obligate

anaerobes, can survive or benefit from an anaerobic environment (Schnurer and Schnurer, 2006; Procházka et al. 2012; Kazda et al. 2014; Yıldırım et al. 2017).

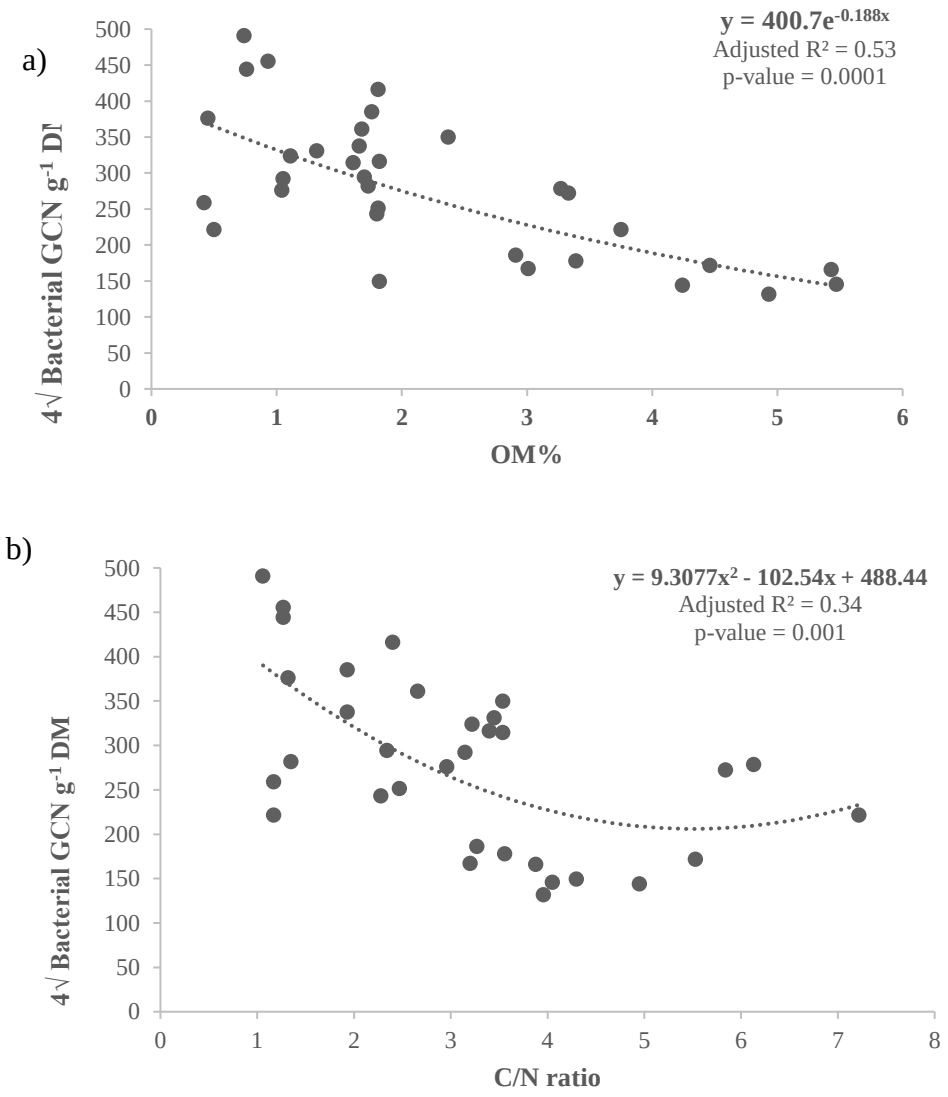

Figure 3.6. Bacterial (16S) GCN relations with OM% (a) and C/N ratio (b). *GCN $^{4}\sqrt{}$ root square transformed.

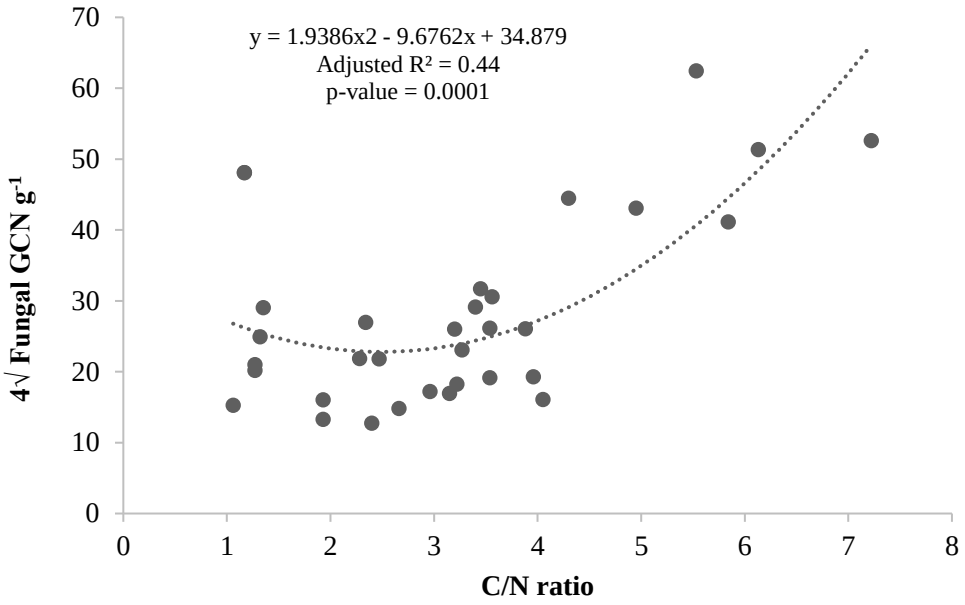

Figure 3.7. Fungal (18S) GCN relation with C/N ratio. * GCN $^{4}\sqrt{}$ root square transformed.

3.5 Conclusions

Bacterial and fungal CFU ranged widely in liquid anaerobic digestates (10^5 to 10^{10}; 0 to 10^5 g^{-1} DW, respectively). Bacterial, archaeal and fungal GCN showed narrower ranges than CFU (10^8 to 10^{10}; 10^7 to 10^9; 10^4 to 10^6 g^{-1} DW, respectively) between different commercial digestates. Liquid anaerobic digestates were predominantly dominated by bacteria, over archaeal and fungi populations. Bacterial CFU (2.23 x 10^9 g^{-1} DW) was five orders of magnitude higher than fungal CFU (6.43 x 10^4 g^{-1} DW). Similar patterns were observed for gene quantification, where bacterial (16S) GCN showed higher abundance (1.09 x 10^{10} g^{-1} DW), followed by archaeal GCN (16S) (5.87 x 10^8 g^{-1} DW), and fungal (18S) GCN (1.77 x 10^6 g^{-1} DW). Higher bacterial GCN were associated with lower OM and C/N ratio.

Chapter 4 : Biofertilisation of ryegrass and white-clover with anaerobic digestates, and associated soil microbial numbers

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4.1 Abstract

This study compared the fertiliser effects of different types of liquid anaerobic digestates on the growth of perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) and on soil NPK and microbial numbers. A factorial design with eight types of fertilisers and four plant types was tested in triplicate (microcosm pots n=96). Fertilisers included: five types of liquid anaerobic digestates, undigested cattle slurry, calcium ammonium nitrate (CAN) 27% N, and no fertiliser. Plants tested were: monocultures of perennial ryegrass and white clover, a mix of both, and bare soil. Plant (forage yield, leaf area index (LAI), canopy height and root mass) and soil (concentrations of total N, Morgan's extracted P and K) responses were measured, as well as soil bacterial/archaeal (16S) and fungal (18S) gene copy numbers (GCN) using real-time PCR. Digestates with different chemical compositions, when balanced for dry matter amounts applied, showed comparable forage yield responses ($p>0.05$), with higher yields observed in ryegrass and mixed vegetation ($p<0.05$), while white clover showed low response to digestates. The presence of white clover contributed to higher soil N ($p<0.05$). Soil available P was influenced by the type of fertiliser and vegetation ($p<0.05$) individually, while soil available K by the interaction ($p<0.05$). Soil bacterial (16S) GCN responded to the interaction between fertiliser/vegetation ($p<0.05$), while archaeal (16S) and fungal (18S) GCN only to the type of vegetation ($p<0.05$). The application of anaerobic digestates showed low direct impacts on the microbial GCN of the soil, mostly influenced by type of vegetation.

4.2 Introduction

Anaerobic digestates have been recognised as potential biofertilisers, and their utilisation is increasing in parallel with the intensification of biogas production (Alburquerque et al. 2012; Mao et al. 2015). Recent findings reported that anaerobic digestates positively affect forage crop yields, especially grasses, with increases in plant growth either matching or exceeding those from equivalent amounts of traditional inorganic fertilisers and/or undigested slurry (Gunnarsson et al. 2010; Bougnom et al. 2012; Walsh et al. 2012a; Andruschkewitsch et al. 2013; Walsh et al. 2018). The positive effect of anaerobic digestates on crop yields is mostly credited to their macronutrient concentrations, especially total N and its plant-available form ammonium (NH_4^+), as well as total concentrations of other primary macronutrients such as K and P (Albuquerque et al. 2012; Möller and Müller, 2012; Johansen et al. 2013). Due to the chemical variability that can be found between different types of anaerobic digestate (Alburquerque et al. 2012; Möller and Müller, 2012; Nkoa, 2014; Coelho et al. 2018), it is necessary to understand how these differences in their composition can affect their general performance as fertilisers.

The establishment of fertilisation rates for the use of anaerobic digestates can be complicated due to the variability in their compositions. For example, enormous variation can be found in the concentrations of plant primary macronutrients such as NPK. This variation is mostly associated with the type of feedstock used and with differences in operational conditions during biogas production (Tambone et al. 2010; Alburquerque et al. 2012; Möller and Müller, 2012; Nkoa, 2014; Coelho et al. 2018). Apart from the plant primary macronutrients, digestates can also exhibit considerable variation in their amounts of secondary plant macronutrients (Ca, Mg, S) and micronutrients (Fe, Mn, B, Cu, Zn, Cl, Ni, Co, Na) (Tambone et al. 2010; Möller and Müller, 2012; Albuquerque et al. 2012; Nkoa, 2014; Coelho et al. 2018). Secondary macro and micronutrients

might also account for enhancing fertiliser effects, or affect plant growth negatively via phytotoxicity (Nkoa, 2014; Di Maria et al. 2014). Additionally, the chemical availability of these plant nutrients can be an issue. For example, Möller and Müller (2012) estimated that NH_4^+ can vary between 44 and 81% of the total N in anaerobic digestates. These authors reported that the complexity of biochemical interactions found in different types of anaerobic digestates affects the bioavailability of their nutrients.

Another question that is not fully understood is how anaerobic digestate application can influence soil microbial communities and how it interacts with the type of vegetation. Anaerobic digestates carry different amounts and types of microorganisms (Nelson et al. 2011; Regueiro et al. 2012; Vanwonterghem et al. 2014; Conversano et al. 2015; Insam et al. 2015). Previous research showed that the effects of anaerobic digestate application on soil microbes varied, with some studies stating that digestate applications can increase microbial biomass and activity in soils (Odlare et al. 2008), while others only detected minor or not significant soil microbial changes (Andruschkewitsch et al. 2013; Johansen et al. 2013). Walsh et al. (2012b) reported that the application of anaerobic digestate stimulated soil bacterial, but not fungal, activity. The lack of consistency between trials makes more studies necessary to understand the impacts of different types of anaerobic digestate on soil microbial communities.

This study aimed to evaluate the fertiliser effects of different types of liquid anaerobic digestates on the growth of perennial ryegrass (*Lolium perenne* L.), white clover (*Trifolium repens* L.) and mixed ryegrass-clover, using a microcosm pot approach. Also, this trial investigated the soil pools of total N, available P and K, and microbial gene copy numbers of bacteria, archaea and

fungi in response to different types of biofertiliser application and their interaction with soil vegetation.

4.3 Material and methods

4.3.1 Treatments and experimental conditions

The experiment was conducted in a glasshouse located at Waterford Institute of Technology (WIT), Waterford, Ireland. The soil for the experiment was collected from a ryegrass field, located at WIT campus Carriganore, Waterford, Ireland. The soil was air-dried to constant weight, sieved (3.15 mm) and homogenised before establishing the treatments. Microcosm pots had a total area of 113.1 cm^2, and a volume of 1358 cm^3. Soil texture was classified as loam: sand (46 ± 2.1%), silt (29 ± 2.0%) and clay (26 ± 2.4%). Soil original pH-water was 5.32±0.4. Lime was applied (7.5 ton ha^{-1}) in one dose to bring soil pH to a target of pH 6.5-7 throughout the trial; at the end of the trial, the soil pH was 7.6 ± 0.2 (slightly alkaline). Soil chemical-elemental composition had the following features: soil organic carbon (SOC) = 43.1 g kg^{-1} (7.4% organic matter); electrical conductivity (EC) = 30.6 mS m^{-1}; Ca = 1.7, K = 2.1, Mg = 2.5, Na = 0.8, P = 0.7, Al = 17.3 Fe = 17.9 g kg^{-1}; Mn =373.1, B = 10.4, Co = <0.0006, Se = 9.2, Pb = 22.0, Zn = 39.4, Cu = 11.0, Cr = 15.4, Cd = <0.00002, Ni = 7.7 mg kg^{-1}. Ryegrass was sown at a rate of 30 kg ha^{-1}, and white clover 5 kg ha^{-1} (Schils et al. 2000), with half of each rate mentioned used in the establishment of the mixed vegetation pots. Treatments were randomly assigned to the pots approximately one month after sowing and establishment of the vegetation.

The experiment was conducted in a factorial design with three replicates each of eight fertiliser treatments and four types of vegetation. Fertiliser treatments included: five different types of commercial liquid anaerobic digestate, undigested cattle slurry, a nitrogen control with 100 kg

ha^{-1} of calcium ammonium nitrate (CAN) containing 27% N ($5Ca(NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$) (27 kg N ha^{-1}), and a no fertiliser control (Table 4.1).

Table 4.1. Experimental set-up of pots in the glasshouse trial.

Block 1	**Block 2**	**Block 3**
No fertiliser	CAN	AD4
Cattle Slurry	AD2	AD5
AD3	AD4	Cattle Slurry
AD1	AD5	Mineral
Mineral	AD3	AD2
Cattle Slurry	No fertiliser	AD3
AD5	AD1	No fertiliser
AD4	AD2	AD1

Note: This set-up was identical for the four types of vegetation tested.

The chemical and microbiological composition of the anaerobic digestate and cattle slurry are described in Table 4.2. The plant species tested were: perennial ryegrass (*Lolium perenne* L.) (monoculture), white clover (*Trifolium repens* L.) (monoculture), mixed perennial ryegrass + white clover, and no vegetation (bare soil). The experiment was conducted during the spring-summer season of 2016, totalling approximately six months. Three consecutive fertilisation/harvesting cycles of 45 days were performed, simulating typical management for silage cut from ryegrass swards in Ireland. The application of anaerobic digestates and undigested cattle slurry were based on a rate of 33 m^3 fresh weight ha^{-1} per fertilisation, a value typically recommended for cattle slurry applications in Irish grasslands (Brennan et al. 2014). Digestates and undigested cattle slurry were balanced in terms of dry matter applied (3%). Each fertilisation cycle provided an equivalent amount of 825 kg DM ha^{-1}. Total NPK quantities applied from each biofertiliser after dry matter balance can be found in Table 4.3. During the fertilisation cycles, all treatments except no fertiliser control received an additional amount of 27 kg N ha^{-1} via CAN per

fertilisation, aiming to minimise the effects of nitrogen imbalance between the biofertilisers and ensure that N was not a limiting factor in the trial.

Table 4.2. Chemical composition and microbial gene copy numbers (GCN) of anaerobic digestates and cattle slurry used in the fertilisation trial

Fertiliser Source	**AD1** Food waste (dairy industry)	**AD2** Food waste, pig slurry	**AD3** Food waste (farm and food)	**AD4** Food waste, municipal sludge	**AD5** Wastewater treatment	**Slurry** Beef cattle
DM%	**3.1** ±0.01	**6.0** ±0.10	**3.8** ±0.01	**7.3** ±0.31	**2.3** ±0.02	**11.2** ±0.05
pH	**8.8** ±0.02	**8.9** ±0.02	**8.8** ±0.01	**8.8** ±0.01	**7.9** ±0.02	**7.6** ±0.03
EC (mS m^{-1})	**35.7** ±1.58	**55.0** ±1.01	**19.2** ±0.27	**30.1** ±0.15	**10.5** ±0.21	**15.8** ±0.20
	(g kg^{-1} DW)					
TKN	**181** ±0.67	**137** ±0.71	**151** ±1.45	**104** ±1.53	**104** ±1.25	**40** ±1.86
NH_4^+	**112.9** ±3.20	**62.2** ±1.65	**47.2** ±0.37	**55.2** ±0.87	**48.8** ±2.01	**20.1** ±0.29
P	**11.2** ±0.13	**33.8** ±3.71	**22.6** ±0.17	**22.1** ±4.73	**26.7** ±0.25	**7.2** ±0.27
K	**43.0** ±0.22	**66.8** ±2.45	**67.0** ±0.29	**9.7** ±1.94	**12.1** ±1.00	**17.3** ±0.71
Ca	**11.5** ±0.77	**15.9** ±0.98	**22.0** ±0.32	**31.0** ±1.12	**35.4** ±0.17	**16.8** ±1.14
Mg	**2.4** ±0.03	**5.0** ±0.41	**6.2** ±0.03	**8.1** ±1.66	**7.4** ±0.12	**10.8** ±0.54
Na	**24.3** ±0.19	**24.0** ±1.23	**13.6** ±0.04	**16.0** ±3.22	**12.8** ±0.83	**3.8** ±0.18
Al	**1.77** ±0.66	**11.5** ±0.14	**14.7** ±0.18	**25.9** ±0.35	**7.9** ±0.52	**3.8** ±0.08
Fe	**6.68** ±0.11	**3.63** ±0.05	**11.7** ±0.05	**17.1** ±0.10	**8.6** ±0.09	**7.5** ±0.47
	(mg kg^{-1} DW)					
Mn	**160.0** ±1.22	**226.0** ±23.97	**184.4** ±0.43	**333.9** ±2.92	**117.9** ±0.55	**115.0** ±3.29
B	**32.2** ±0.84	**37.4** ±0.83	**29.5** ±0.22	**<0.0006**	**<0.0006**	**<0.0006**
Co	**0.5** ±0.11	**1.1** ±0.14	**<0.0006**	**<0.0006**	**1.3** ±0.37	**<0.0006**
Se	**<0.0008**	**6.4** ±5.51	**3.3** ±3.35	**24.4** ±6.73	**11.5** ±8.71	**2.9** ±2.89
Pb	**2.6** ± 1.63	**1.0** ± 0.97	**2.5** ±1.28	**24.8** ±0.24	**3253** ± 47.8	**1.0** ±0.99
Zn	**299.6** ±3.82	**317.0** ±38.76	**203.1** ±1.69	**458.9** ±4.59	**1143** ±2.44	**239.6** ±10.98
Cu	**62.7** ±1.71	**67.9** ±8.80	**102.5** ±0.96	**229.3** ±2.61	**233.9** ±1.86	**70.6** ±4.17
Cr	**<0.00004**	**0.9** ±0.85	**16.1** ±0.24	**26.6** ±0.36	**39.6** ±1.23	**2.1** ±0.11
Cd	**0.2** ±0.11	**<0.00002**	**0.4** ±0.04	**<0.00002**	**<0.00002**	**<0.00002**
Ni	**11.8** ±0.10	**18.3** ±1.77	**16.9** ±0.89	**16.7** ±0.10	**25.2** ±0.83	**0.8** ±0.19
	GCN (g^{-1} DW)					
Bacterial (16S)	**2.7×10^9** ±3.3×10^8	**6.7×10^9** ±1.2×10^9	**1.6×10^{10}** ±7.2×10^9	**1.7×10^{10}** ±3.3×10^9	**8.7×10^{10}** ±3.3×10^9	**3.3×10^9** ±1.5×10^9
Archaeal (16S)	**3.7×10^7** ±8.8×10^6	**8.7×10^7** ±6.7×10^6	**1.8×10^9** ±7.6×10^8	**3.7×10^9** ±3.3×10^8	**1.2×10^{10}** ±4.2×10^9	**1.2×10^8** ±4.2×10^7
Fungal (18S)	**5.0×10^5** ±6.0×10^4	**1.1×10^7** ±2.5×10^6	**2.3×10^6** ±1.1×10^6	**7.5×10^5** ±6.9×10^4	**1.9×10^5** ±2.5×10^4	**6.1×10^7** ±1.7×10^7

± = standard error of the mean (SEM n=3). The averages were from samples collected in 2016.
(<) = under the detection limit of ICP-OES.
DM= dry matter; EC= electrical conductivity; TKN= total Kjeldahl nitrogen.

Table 4.3. Average stoichiometry and amounts of NPK applied by each biofertiliser per fertilisation cycle.

Fertiliser	*Amounts applied per fertilisation cycle (kg ha^{-1} DW)			
	Total N	**Total P**	**Total K**	**Total N + CAN**
AD1 Food waste (dairy industry)	149	9	35	176
AD2 Food waste, pig slurry	113	28	55	140
AD3 Food waste (farm and food)	125	19	55	152
AD4 Food waste, municipal sludge	86	18	8	113
AD5 Wastewater treatment	86	22	10	113
Slurry Beef cattle	33	6	14	60

*****Digestates/slurry were balanced for 3% DM contents, based on the density of the biofertiliser. The fertilisation rate used per cycle was 33 m^3 ha^{-1} of fresh weight, with total amounts of dry matter inputs by biofertiliser 825 kg ha^{-1}/per cycle. Each biofertiliser when applied also had an additional complementary amount of 27 kg N ha^{-1} via calcium ammonium nitrate (CAN) to reduce N imbalance between the biofertilisers.

4.3.2 Physical-chemical analyses

Liquid anaerobic digestate samples were collected from Irish commercial biogas plants during the fertilisation trial over the spring-summer 2016 period. Samples were processed following the guidelines in European standard EN 16179 (2012). Soil samples were collected after the third harvesting (end of fertilisation trial). Both digestate and soil samples were kept refrigerated at 4 ºC or frozen at -20 ºC (for molecular analyses). Digestates were analysed according to the methodologies described in (Chapter 2.0 Physical-chemical, elemental composition and PTEs). For soils collected at the end of the trial, available P and K were analysed by extraction using Morgan's extractant (Daly and Casey 2005) and analysed using ICP-OES, following guidelines of CEN/TS 16170 (2012). Soil N (Total Kjeldahl) was analysed according to EN 16169 (2012). Samples were analysed in triplicate.

4.3.3 Plant growth analyses

Plant growth was measured based on four variables: forage yield, leaf area index (LAI), canopy height (cm), and total root mass. Canopy height, LAI and forage yield were measured during three harvesting cycles of approximately 45 days each, simulating silage-cut performed during spring-summer in Ireland. Forage herbage was cut at approximately 5 cm, and DM (105 °C) content was measured. LAI was estimated using a light meter device based on indirect measurements of the light interception (AccuPAR PAR/LAI Ceptometer Model LP-80 Decagon®). Root mass was measured after the third harvesting (end of the trial), approximately six months from sowing the plants. Total root mass was determined by washing in running water using four sieves (between 1 to 3.15 mm) and dried at 105 °C for DM.

4.3.4 DNA extraction and treatment: soil and biofertiliser

For soil samples, the DNA was extracted from an aliquot of 0.25 g fresh weight (FW) and corrected to the DM weight of each sample. DNA extraction and treatment methods were described in (Chapter 3.3.3).

4.3.5 Real-time PCR

The methods used in the real-time PCR were described in (Chapter 3.3.4Real-time PCR). Samples were run in duplicates of each replicate (n=3) of the DNA.

4.3.6 Statistical analysis

All data were tested for normality (Shapiro-Wilk, Kolmogorov-Smirnov) and equal variance (Levene's test) using the software SPSS 24 (IBM®). Data with non-homogeneous variance were transformed prior to analysis of variance (ANOVA). Plant growth (forage yield, LAI, canopy height, root mass) and microbial GCN were compared by factorial analysis using as fixed effects: fertiliser treatment (eight fertilisers) and vegetation type (ryegrass, white clover, mixed vegetation and bare soil). Factorial ANOVA was performed followed by the Bonferroni post-hoc test ($p<0.05$) using the software SPSS 24 (IBM®). Plant growth responses to fertilisation were analysed by principal component analysis (PCA), based on Pearson's correlation. Regressions between forage yield and NPK supplied were tested. PCA and regressions were performed in XLSTAT®, Microsoft Excel® extension software.

4.4 Results and discussion

4.4.1 Plant growth and yields

All plant growth variables evaluated (forage yield, LAI, canopy height and root mass) were significantly affected by the interaction between the type of fertiliser and vegetation ($p<0.05$) (Figure 4.1 a-d). It was observed that fertilisation with anaerobic digestates and cattle slurry contributed positively to higher yields and LAI in ryegrass and mixed vegetation pots compared to white clover (Figure 4.1 a-b). For the plants under the control treatment with no fertiliser, no significant differences in the yields and LAI between ryegrass, mixed vegetation, and white clover were observed ($p>0.05$). Higher forage yields were observed in ryegrass and mixed plants fertilised with the anaerobic digestates AD1, AD2, AD3 and AD4 ($p<0.05$). However, AD3 and AD4

showed intermediate results, as they did not differ from AD5, cattle slurry, CAN and no fertiliser ($p>0.05$), which showed lower forage yields. For mixed ryegrass-clover, it was observed that the pots were dominated by ryegrass, however, the percentage was not measured. At the third fertilisation cycle, most of the white clover in mixed ryegrass-clover pots had disappeared.

Canopy height in ryegrass and mixed vegetation pots showed no significant differences between different anaerobic digestates, cattle slurry and CAN ($p>0.05$) (Figure 4.1 c). Ryegrass and mixed pots had higher canopy height than white clover ($p<0.05$). Higher root mass was found in ryegrass and mixed vegetation pots that received the anaerobic digestates AD1, AD2, AD3, AD4 and cattle slurry (Figure 4.1 d) ($p<0.05$). However, cattle slurry and AD4 showed no difference compared to no fertiliser ($p>0.05$). In relation to white clover, there was no significant difference between most of the anaerobic digestates, cattle slurry, and the controls with CAN and no fertiliser for most of the growth variables evaluated (Figure 4.1 a-d). As digestates varied widely in their composition and stoichiometry (Table 4.2 and Table 4.3), it would be expected that this variation could have driven pronounced differences in plant growth responses between different types of anaerobic digestate. However, most of the plant growth variables analysed showed a similar trend between different types of anaerobic digestates applied, indicating that although digestates varied in their chemical composition, the plant response was much the same.

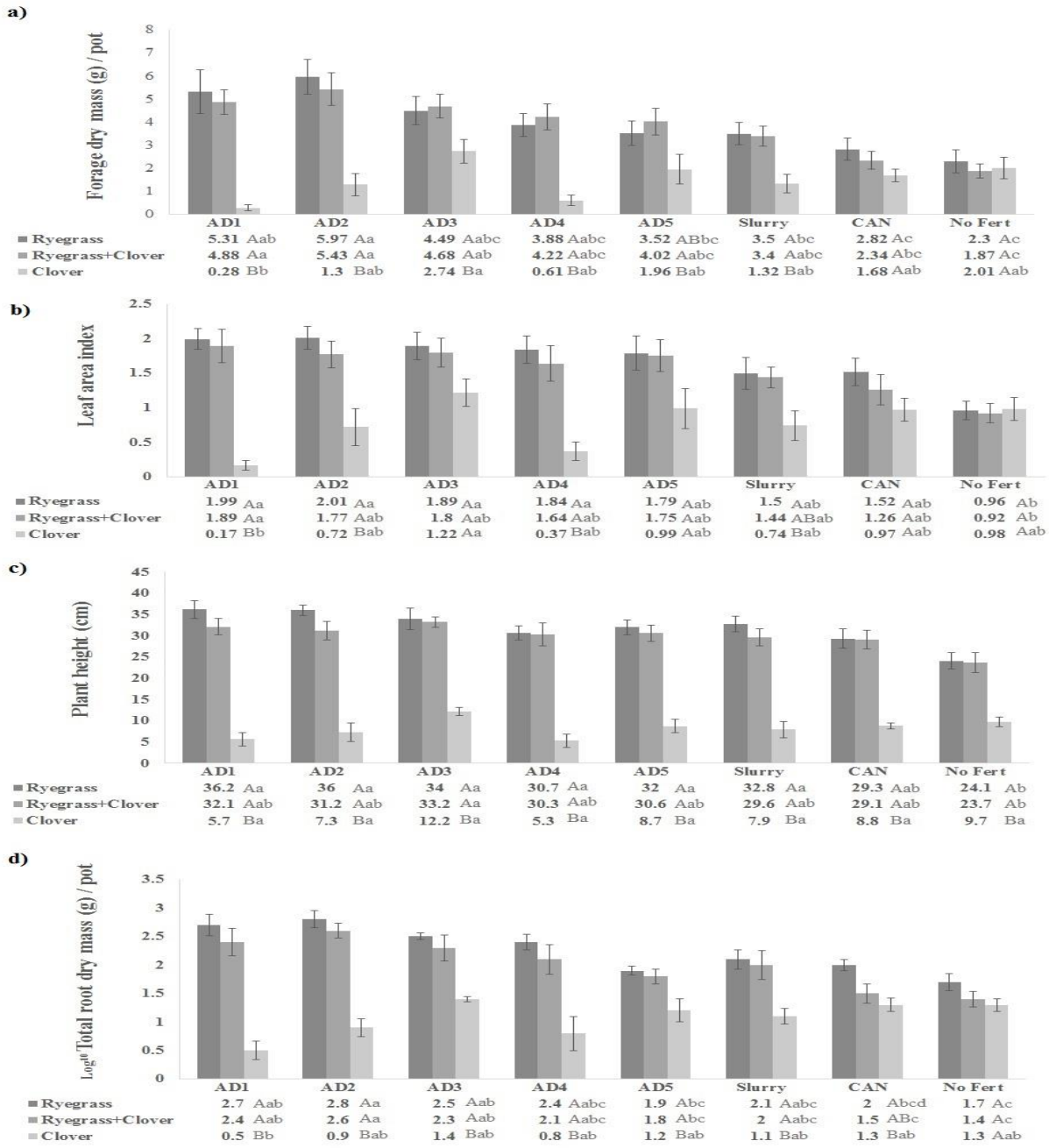


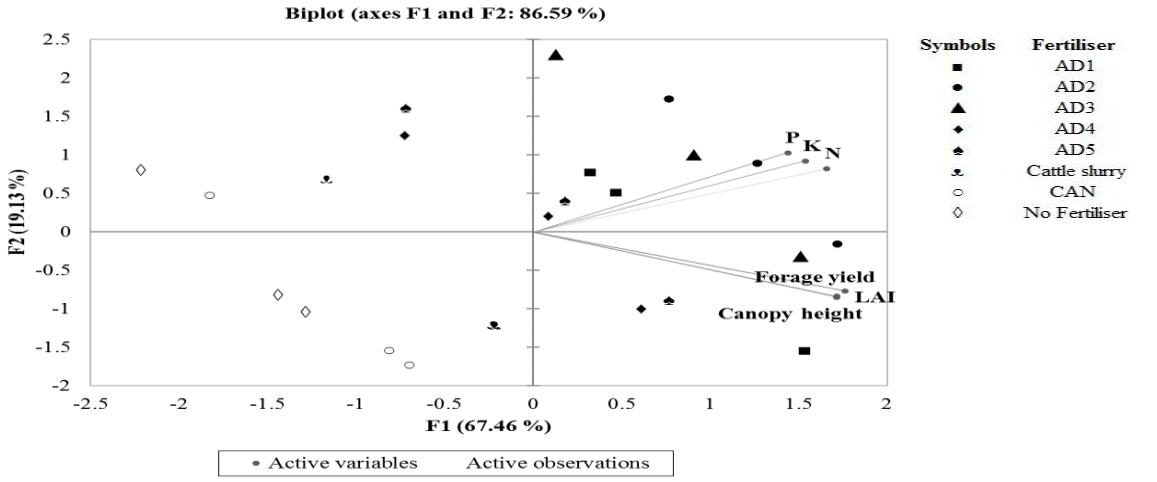
Figure 4.1. Plant responses measured as (a) average forage mass (g DM) per pot, (b) leaf area index (LAI), (c) canopy height (cm) and (d) root mass (log g^{-1} DM), of ryegrass (*Lolium perenne* L.), white-clover (*Trifolium repens* L.) and mixed ryegrass-clover, fertilised with different types of anaerobic digestate, cattle slurry, calcium ammonium nitrate (CAN) and no fertiliser, from three harvesting cycles over the spring-summer. Root mass was measured once, at the end of the growing season.

Error bars = SEM (n=9). Means followed by uppercase letters in the column and lowercase in lines differed significantly (Bonferroni post-hoc test, $p<0.05$).

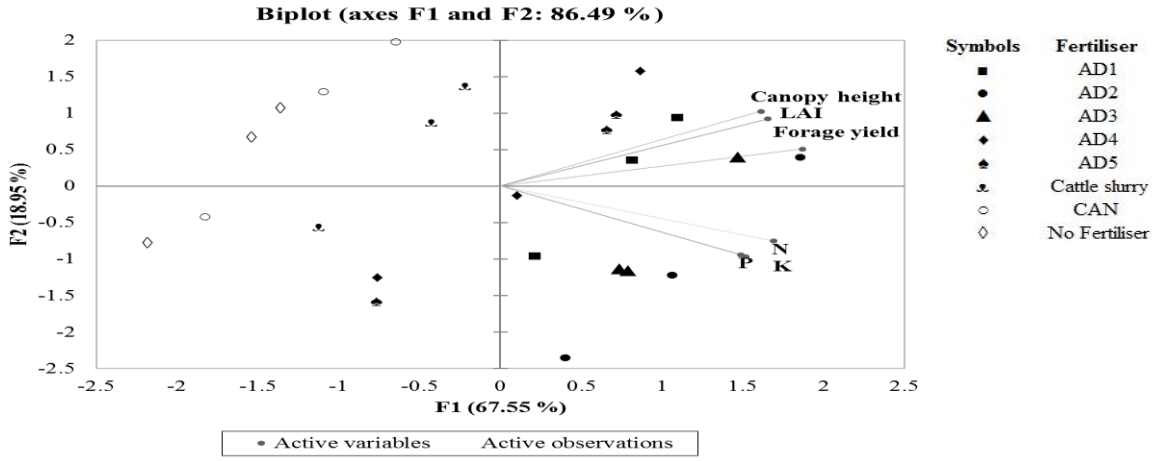
The PCA analyses showed correlated responses between plant growth characteristics of ryegrass and mixed vegetation (Figure 4.2 a-b) and the inputs of NPK from the biofertilisers. The convergent arrows in the biplot F1 demonstrated this positive correlative response. Most of the anaerobic digestates were plotted on the same side of the biplot where plant growth arrows pointed in ryegrass and mixed-vegetation pots (Figure 4.2 a-b). Cattle slurry, CAN and no fertiliser treatments were plotted on the opposite side of the plant growth arrows (Figure 4.2 a-b). In white clover pots, a low correlation between the NPK provided by the anaerobic digestates and growth responses was found (Figure 4.2 c), indicated by arrows in opposite directions. The individual analysis of forage yield responses in ryegrass and mixed vegetation pots to total inputs of N, P and K applied showed that forage yield was exponentially related to total amounts of N (biofertiliser + 27 kg N ha^{-1} CAN/ per fertilisation) supplied (Figure 4.3 a). The total P supplied and forage yield had a cubic relationship (Figure 4.3 b), while total K and forage yield had a quadratic relationship (Figure 4.3 c).

Regarding other secondary macro and micronutrients provided by the biofertilisers, the anaerobic digestates used in this trial showed a broad range and diversity of compositions (Table 4.2). Nevertheless, linear correlation tests performed between forage yield and total amounts of Ca, Mg, Na, Mn, B, Co, Se, Al, Fe, Cd, Cr, Cu, Pb, Ni, and Zn, did not detect any evident positive or negative patterns between the concentrations of these nutrients in the digestates and forage yields for the three types of plants evaluated. Correlation results did not find any significant relationship between the elemental concentrations of Ca, Mg, Na, Mn, Co, Se, Al, Fe, Cd, Cr, Cu, Pb, Ni, or Zn in the biofertilisers with forage yield responses.

a)



b)



c)

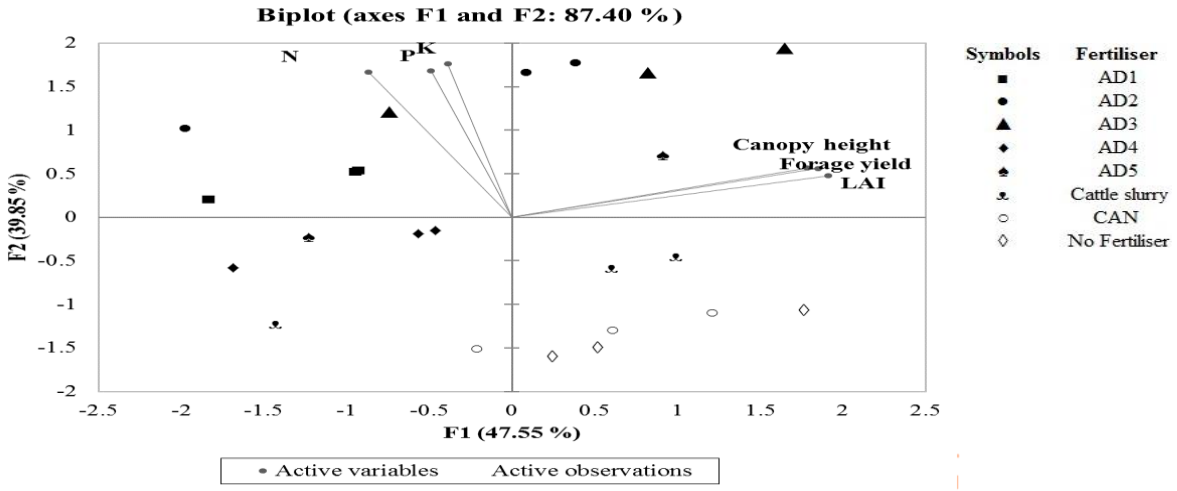
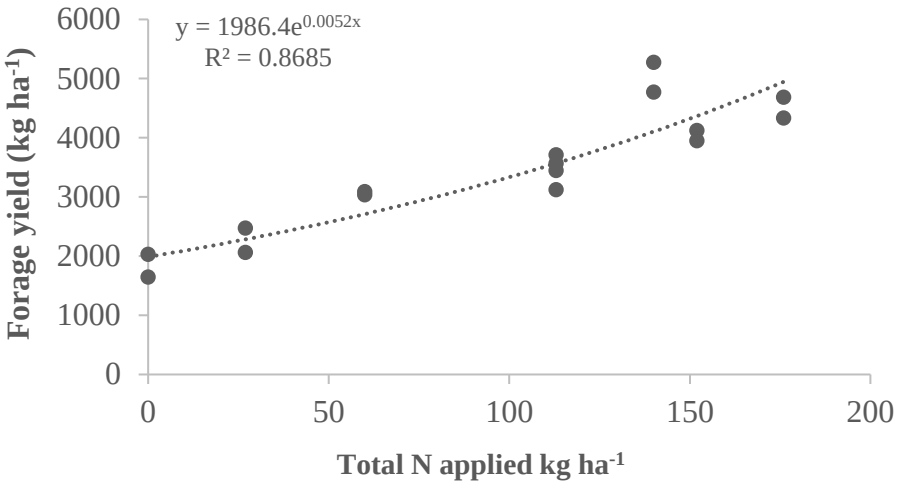
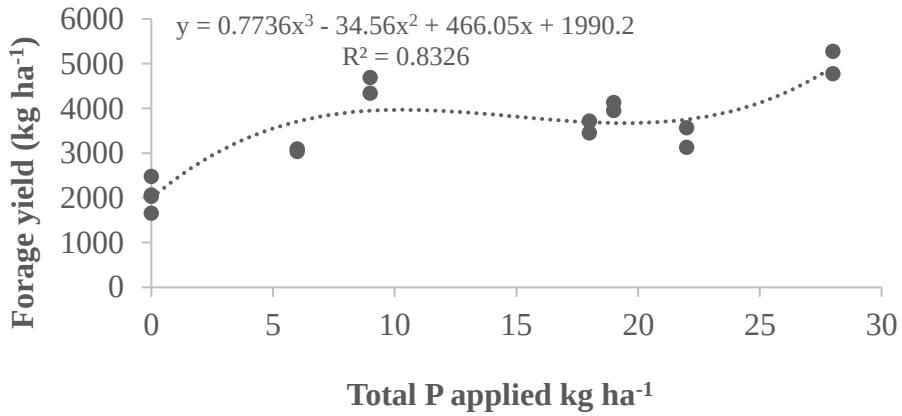


Figure 4.2. Principal component analysis (PCA) between NPK applied and forage yield, leaf area index (LAI) and canopy height in ryegrass (a), mixed ryegrass-clover (b), and white clover (c) pots

a)



b)



c)

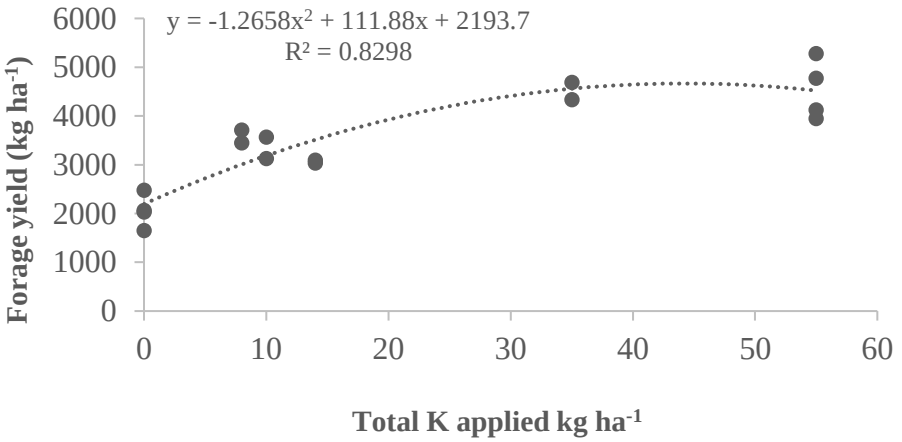


Figure 4.3. Estimated average forage yield per harvest (kg ha^{-1}) of ryegrass and mixed ryegrass-clover in response to total amounts of N (a) P (b) K (c) applied (kg ha^{-1}/per fertilisation). Note: ryegrass was dominant in mixed vegetation along the fertilisation trial.

The present trial found that anaerobic digestates with different chemical compositions, when balanced in terms of dry matter applied, displayed a similar trend in terms of forage production in ryegrass and mixed vegetation ryegrass/clover. In the literature, the fertiliser performance of liquid anaerobic digestates has been reported to vary, mostly due to the influence of their composition variability, availability of nutrients and secondary interactions that can occur between the soil, plants and the environment. Positive effects of liquid anaerobic digestate fertilisations on the production of ryegrass have been reported to match equivalent yields of forage production provided by inorganic fertilisers with equivalent amounts of NPK (Gunnarsson et al. 2010; Walsh et al. 2012a; Walsh et al. 2018). As detected in the trial, an exponential positive response of plant yield of ryegrass was associated with the amount of N supplied by the anaerobic digestates. Ryegrass yield responses to anaerobic digestate applications have been mostly associated with the amounts of N available (Gunnarsson et al. 2010; Andruschkewitsch et al. 2013).

Low productive responses of white clover to the fertilisation with anaerobic digestates may have been associated with suppressive effects of high N inputs. The digestates evaluated provided total N amounts between 86 to 149 kg N ha^{-1} (per cycle), plus the additional amount of 27 kg N ha^{-1} via CAN. Applications of high amounts of N via organic or inorganic fertilisers are reported to have detrimental effects on clover performance, especially under mixed grass-clover swards, where grass is favoured by N availability (Burchill et al. 2014; Enriquez-Hidalgo et al. 2016; Egan et al. 2017; McDonagh et al. 2017, Walsh et al. 2018). The fertilisation of white clover with anaerobic digestates high in N must be carefully analysed, as digestates might reduce clover growth and biological nitrogen fixation.

4.4.2 Soil NPK

Soil total N was affected only by the type of vegetation ($p>0.05$) (Figure 4.4). Higher levels of total N were detected in bare soils (8.65±0.18 g kg^{-1} DM), followed by the soils with white clover and mixed plants (7.78±0.13 and 7.27±0.17 g kg^{-1} DM, respectively). A lower concentration of total N was detected in soils with ryegrass (5.93±0.16 g kg^{-1} DM). Higher N levels found in bare soils likely occurred because there was no uptake by vegetation. The lowest levels of soil N found in ryegrass compared to mixed ryegrass-clover and to white clover may be because the N applied via biofertilisers/CAN was the only direct input of N for ryegrass. For white clover and mixed plants, apart from the N applied via biofertilisers/CAN, possible additional amounts of N derived from biological nitrogen fixation via symbiotic association legume-rhizobia could have occurred. Legume species are widely known for contributing additional amounts of N to the soil-plant systems via symbiotic fixation (Fustec et al. 2010; Rasmussen et al. 2011; Lüscher et al. 2014).

In terms of soil available P (Morgan's extraction), there were significant effects of the type of fertiliser ($p<0.05$) (Figure 4.5 a) and vegetation ($p<0.05$) (Figure 4.5 b), but not their interaction ($p>0.05$). High available P was found in the soils that received applications of AD2 (25.2±3.09 mg l^{-1}) ($p<0.05$), followed by the other anaerobic digestates that had no significant differences between them (14.8 to 16.7 mg l^{-1}). Lower available P was found in the soils that received CAN and no fertiliser (9.0±0.51 and 9.76±0.51 mg l^{-1}, respectively). Soil available P was higher in mixed ryegrass-clover and white clover (17.3±1.27 and 18.3±1.98 mg l^{-1}, respectively) than in ryegrass and bare soils (12.5±1.08 and 12.1±1.11 mg l^{-1}, respectively) ($p<0.05$).

The levels of available P found in the soils were associated with the P inputs applied via biofertiliser. Soil available P can be associated with the balance between P inputs via fertiliser and plant uptake (Oehl et al. 2002; McLaughlin et al. 2011). Nevertheless, lower levels of available P were found in bare soils and ryegrass. For ryegrass, a higher rate of production compared to white clover would explain the P depletion. For bare soil, it is possible that the lower levels of available P found might be linked with lower soil biological activity, as available P in soils is associated with biological and microbial activity. Fungal and archaeal numbers were lower in bare soil (Figure 4.6 a.b). Soil microorganisms from bacterial to fungal groups can play important roles in the solubilisation of P in soils (Richardson et al. 2011; Richardson and Simpson, 2011; Sharma et al. 2013; Bhat et al. 2017).

Table 4.4). Among the main patterns observed, higher concentrations of available K were found in bare soils and white clover soils that received anaerobic digestates, while lower levels were found in ryegrass and mixed pots, especially the ones that received no or low K supply via biofertilisation. The highest K concentration was observed in bare soils that received applications of AD2 and AD3 (519.5±89.2 and 375.5±31.2 mg l^{-1}, respectively). Lower levels of soil available K in ryegrass and mixed -vegetation pots were probably linked to plant uptake and biomass production. The fact that bare soils showed generally higher soil available K may be due to the balance between K inputs and plant uptake. K availability in soils is directly linked to higher forage yields (Pant et al. 2004; Kohmann et al. 2017). The higher range of soil available P and K reported in this study than usually reported for Morgan's extraction in Irish soils (Tunney et al. 2010) may be due at least in part to the use of ICP-OES, a very precise method, for measuring the

extracts. He et al. (2013) reported an increase of 35% in soil Morgan's P results when substituting the traditional colourimetric method for an ICP-based analysis.

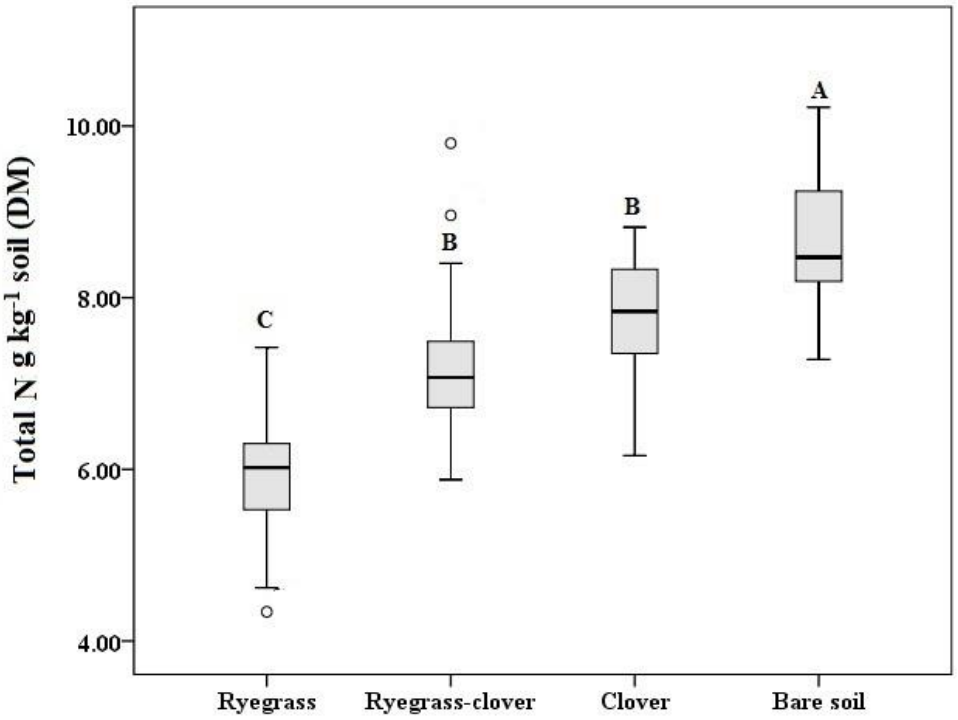


Figure 4.4. Total soil N (g kg^{-1} DM) after three fertilisation cycles with different types of anaerobic digestate, cattle slurry, CAN and no fertiliser in soils with ryegrass, mixed ryegrass-clover, white clover, and bare soil control. Box-plots with different letters differed significantly (Bonferroni post hoc test, $p<0.05$). SEM (n= 24). ○ outlier samples.

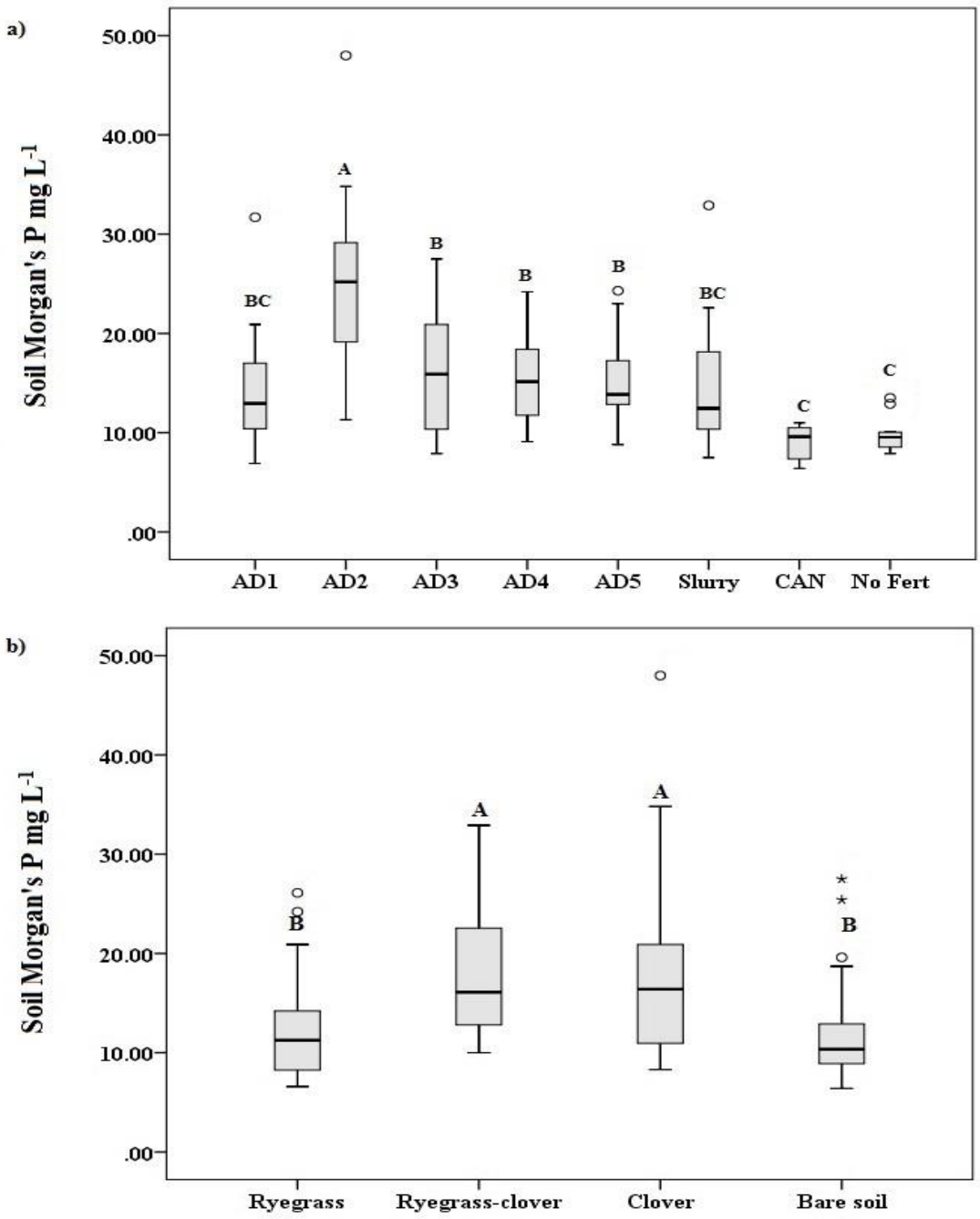


Figure 4.5. Soil Morgan's P (mg l^{-1}) analysed by ICP, after three fertilisation cycles with different types of anaerobic digestate, cattle slurry, CAN and no fertiliser in soils with ryegrass, mixed ryegrass-clover, white clover, and bare soil control. Box-plots with different letters differed significantly (Bonferroni post hoc test, $p<0.05$). SEM Fig. a (n=12), Fig. b (n=24). ○ outliers, *extreme outliers.

Table 4.4. Soil Morgan's K (mg l^{-1}) ICP-analysed, after three fertilisation cycles with different types of anaerobic digestates, cattle slurry, calcium ammonium nitrate (CAN) and no fertiliser, in soils with vegetations of ryegrass, mixed ryegrass-clover, white-clover swards and bare soil control.

	Morgan's K mg l^{-1}			
	Ryegrass	**Ryegrass-clover**	**Clover**	**Bare soil**
AD1	139.1 Ba	88.2 Bab	275.3 Aab	232.6 ABb
AD2	105.23 Bab	165.6 Ba	359.6 Aa	519.5 Aa
AD3	70.9 Cab	85.8 BCab	131.63 Bcd	375.5 Aa
AD4	84.0 BCab	70.8 Cb	142.8 ABbc	177.2 Ab
AD5	53.8 Cb	64.6 BCb	115.4 ABcd	162.7 Abc
Cattle slurry	95.4 Bab	107.5 ABab	159.13 Abc	159.5 Abc
CAN	51.8 Bb	50.3 Bb	67.4 ABd	119.2 Abc
No fertiliser	56.9 Ab	61.6 Ab	88.0 Acd	87.8 Ac
SEM (n=96)	8.36	9.33	19.9	30.5

Means followed by different uppercase letters in lines and lowercase in the columns differed significantly (Bonferroni post hoc test, $p<0.05$). SEM = standard error of the mean

4.4.3 Soil microbial abundance

Soil archaeal and fungal GCN were only affected by the type of vegetation ($p<0.05$) (Figure 4.6 a.b), and not by the kind of fertiliser or their interaction with the type of plant ($p>0.05$). For archaea, lower soil GCN were found in bare soils ($p<0.05$). Higher soil fungal GCN were observed in mixed vegetation and ryegrass ($p<0.05$), and lower in white clover and bare soils. Soil bacterial GCN were significantly affected by the interaction between the type of fertiliser and vegetation ($p<0.05$) (Table 4.5). In general, no specific pattern for the soil bacterial GCN could be detected from the interactions between the type of fertiliser and vegetation. The most pronounced interaction effect was seen in bare soils that received AD1 and AD2 having lower bacterial GCN compared to the soils with mixed vegetation receiving the same digestates ($p<0.05$). Soil bacterial GCN in the other three types of vegetation tested (ryegrass, white clover and bare soils), showed no difference between anaerobic digestates, cattle slurry, CAN and no fertiliser. In mixed ryegrass-

clover, soils treated with the anaerobic digestates AD1 and AD2 showed significantly higher soil bacterial GCN than those treated with AD5, cattle slurry and CAN ($p<0.05$).

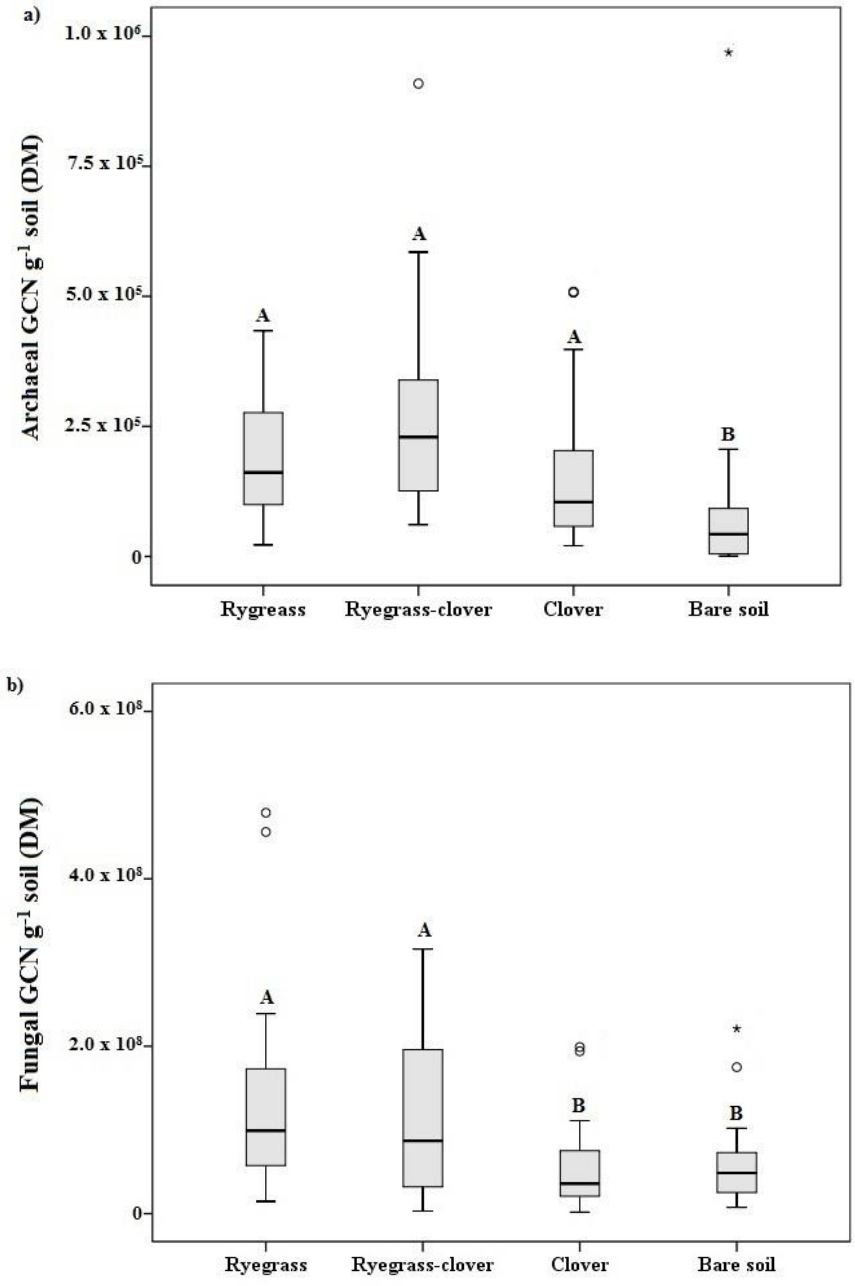


Figure 4.6. Archaeal (a) and fungal (b) gene copy numbers (GCN) g^{-1} soil (DM), after three fertilisation cycles with different types of anaerobic digestates, cattle slurry, calcium ammonium nitrate (CAN) and no fertiliser, in soils with ryegrass, mixed ryegrass-clover, white clover, and bare soil control. Box-plots with different letters differed significantly (Bonferroni post hoc test, $p<0.05$). SEM Fig. a.b (n= 24), ○ outliers, *extreme outliers.

Table 4.5. Bacterial gene copy numbers (GCN) g^{-1} soil (DM), after three fertilisation cycles with different types of anaerobic digestates, cattle slurry, calcium ammonium nitrate (CAN) and no fertiliser, in soils with ryegrass, mixed ryegrass-clover, white clover, and bare soil control.

Bacterial GCN g^{-1} soil (DM)

	Ryegrass	Ryegrass-clover	Clover	Bare soil
AD1	8.92×10^8 ABa	1.65×10^9 Aa	3.04×10^8 Ba	4.95×10^8 Ba
AD2	6.38×10^8 ABa	1.78×10^9 Aa	7.51×10^8 ABa	4.43×10^8 Ba
AD3	6.73×10^8 Aa	5.62×10^8 Aabc	1.01×10^9 Aa	2.03×10^8 Aa
AD4	9.06×10^8 Aa	4.21×10^8 Aabc	7.14×10^8 Aa	3.60×10^8 Aa
AD5	1.75×10^8 Aa	3.46×10^8 Abc	5.38×10^8 Aa	6.98×10^7 Aa
Cattle slurry	7.07×10^8 Aa	2.79×10^8 Abc	4.00×10^8 Aa	3.46×10^8 Aa
CAN	8.46×10^8 Aa	9.94×10^7 Bc	1.00×10^9 Aa	2.10×10^8 ABa
No fertiliser	6.65×10^8 Aa	9.30×10^8 Aab	6.59×10^8 Aa	5.66×10^8 Aa
SEM (n=96)	7.6×10^7	1.58×10^8	8.02×10^7	5.79×10^7

Means followed by different uppercase letters in lines and lowercase in the columns differed significantly (Bonferroni post hoc test, $p<0.05$). SEM = standard error of the mean

Among the three microbial domains evaluated in this present trial, only soil bacterial GCN had significant responses to the type of fertiliser applied. Digestate effects on soil microbial populations generally depend on a series of factors and interactions such as the type and volume of anaerobic digestate used, soil nutrient availability, vegetation type, fertiliser management, experimental and environmental conditions. Some studies reported that applications of anaerobic digestate increased or stimulated soil microbial activity and/or biomass (Odlare et al. 2008, Abubaker et al. 2012; Walsh et al. 2012b; Carraciolo et al. 2015). However, other studies found minor or no effects of anaerobic digestate applications in stimulating soil microbial changes in terms of quantity, biomass or activity (Andruschkewitsch et al. 2013; Johansen et al. 2013; Juárez et al. 2015), and Wentzel and Joergensen (2016b) detected deleterious effects of anaerobic digestate application on soil fungi. Our results corroborate the results found by Walsh et al. (2012b), where the application of anaerobic digestates stimulated soil bacteria but not fungi.

All domains evaluated (bacterial, archaeal and fungi) had their soil GCN affected by the type of vegetation, especially fungal and archaeal; neither of these were significantly affected by the kind of fertiliser or by the fertiliser/plant interaction. The absence of vegetation in bare soils was likely the main factor for lower soil GCN detected in archaeal and fungal domains. The existence of plants is an essential factor for atmospheric carbon fixation and its availability in the soil (Kuzyakov and Domanski, 2000), and consequently the availability of organic compounds for microbial growth (Millard and Singh, 2010; Blagodatskaya et al. 2014; Kuzyakov and Blagodatskaya, 2015). Among the plants evaluated in this trial, ryegrass and mixed pots showed generally higher microbial GCN, especially fungal GCN. Higher forage and root yields were observed in ryegrass and mixed vegetation, and this may have contributed to higher organic matter and carbon availability supply in these soils via litter deposition and root death, providing substrates for decomposers. It is widely known that plant material decomposition is strongly associated with soil fungal activity, especially saprotrophs (Millard and Singh, 2010; Voříšková and Baldrian, 2013; Eichlerová et al. 2015; Purahong et al. 2016).

The results outlined in this paper suggest little influence of the applications of different types of anaerobic digestates on soil bacterial, fungal and archaeal GCN in one growing season. The type of vegetation had more of an influence on the number of these microorganisms in the soil.

4.5 Conclusion

Digestates with different chemical composition, when equally balanced in terms of dry matter, can drive comparable forage yield responses in ryegrass and mixed ryegrass/white clover

pots. Ryegrass and mixed vegetation showed similar yields, with positive correlations to the amounts of NPK supplied by digestates. White clover alone showed a low response to digestate application and to the amounts of NPK supplied by the biofertilisers. The type of vegetation had a significant influence on the concentrations of soil total N, with white clover and mixed ryegrass-clover showing higher total soil N concentrations than monocultures of ryegrass. Higher inputs of total P and K via biofertilisers were associated with higher availability of these nutrients in the soil. Soil bacterial GCN were influenced by the interaction between the type of fertiliser and the plant. However, no pattern from this interaction could be concluded. Soil archaeal and fungal GCN were only influenced by the type of plant, and their quantity in soil was associated with plants with higher forage yields.

Chapter 5 : Effects of different types of anaerobic digestates on productive and nutritional traits of perennial ryegrass (*Lolium perenne* L.)

5.1. Abstract

This study evaluated the effects of repeated applications of four different types of anaerobic digestates and undigested cattle slurry on the growth responses and nutritional aspects of a ryegrass sward (*Lolium perenne* L.) in a two-season fertilisation trial. Fertiliser treatments included: four different types of commercial anaerobic digestate, undigested cattle slurry, a nitrogen control (N-control) with 100 kg ha^{-1} of calcium ammonium nitrate (CAN) (27 kg N ha^{-1}), and a no fertiliser control. Treatments were distributed in a randomised block design with three replicates. Digestate and slurry applications were based on a rate of 33 m^3 fresh weight (FW) ha^{-1} and balanced in 825 kg ha^{-1} of total DM applied per each fertilisation. Forage daily growth was influenced by the type of fertiliser ($p<0.05$), with the biofertilisers showing a comparable average forage daily growth varying between 65 to 79 kg ha^{-1} day^{-1} ($p>0.05$), but higher than the controls with CAN and no fertiliser, which averaged 49 and 34 kg ha^{-1} day^{-1}, respectively ($p<0.05$). LAI and canopy height were affected by the interaction between the type of fertiliser, cycle of growth and year ($p<0.05$). Higher LAI and canopy height were observed in ryegrass swards that received anaerobic digestates and cattle slurry ($p<0.05$), with few significant differences noted between the different types of digestates or cattle slurry. Crude protein, neutral or acid detergent fibre of the forage were not influenced by any type of fertiliser ($p>0.05$). Most of the anaerobic digestates led to increases in the level of soil available P and K ($p<0.05$). The results of this trial indicate that although different types of anaerobic digestates vary in composition, when equally balanced in terms of dry matter inputs they can drive comparable forage grass growth responses and nutritional quality.

5.2 Introduction

Anaerobic digestate, a co-product of biogas production, has been recognised as a potential type of biofertiliser, as it is a source of recycled macro and micronutrients for plant growth (Tambone, et al. 2010; Albuquerque, et al. 2012; Möller and Müller, 2012; Nkoa, 2014; Coelho, et al. 2018). For certain types of crops (e.g. grasses), the plant-growth effects of anaerobic digestates have been reported to match or even exceed the performance of other traditional inorganic and organic fertilisers (Gunnarsson, et al. 2010; Heslop and McCabe, 2012; Bougnom, et al. 2012; Walsh, et al. 2012; Andruschkewitsch, et al. 2013; Walsh, et al. 2018), which has attracted interest in their use. The main fertiliser effects of anaerobic digestates are generally reported to be due to their concentrations of high amount of N and its plant-available form of ammonium (NH_4^+), as well as the concentrations of other plant nutrients (Albuquerque, et al. 2012; Möller and Müller, 2012; Johansen, et al. 2013, Nkoa, 2014). Nevertheless, since there is much variability between physical-chemical compositions in the different types of anaerobic digestates being produced (Coelho et al. 2018), there is a need to understand how these differences between their compositions influence their fertiliser performance.

Predictions of crop yield responses to the application of fertilisers are generally based on the stoichiometry and quantities of the fertiliser applied, especially plant primary macronutrients NPK (Zhang, et al. 2007; Zhang, et al. 2016). Considering that complex organic fertilisers such as anaerobic digestates also contain other plant essential nutrients (e.g. Ca, Mg, S, Fe, Mn, B, Cu, Zn, Ni, Co, Na) (Tambone, et al. 2010; Möller and Müller, 2012; Albuquerque, et al. 2012; Nkoa, 2014; Coelho, et al. 2018), the predictions of plant growth may be not be precise, and comparisons between different types of anaerobic digestates are difficult to make. The biofertiliser effects observed for one type of anaerobic digestate in a certain crop might not be the same if another

anaerobic digestate is applied, as substantial chemical differences between these biofertilisers can possibly drive significant variances in the crop growth responses and might also affect qualitative nutritional traits.

Some anaerobic digestates might also carry elevated levels of potentially toxic elements such as high concentrations of salts (causing high conductivity), which might drive phytotoxicity or negative effects on plant growth (Alburquerque et al. 2012; Nkoa, 2014; Di Maria, et al. 2014, Coelho, et al. 2018). The repeated applications of anaerobic digestates can also impact soil physical-chemical characteristics, and this might affect the long-term sustainability of crop systems. The chemical impacts on the soil can include the growth of plant nutrients, nutrient overloading, pollution, and soil contamination with heavy metals (Albuquerque, et al. 2012; Möller and Müller, 2012; Nkoa, 2014). Due to this complexity involved in the application of anaerobic digestates, it is necessary to carry out comparative studies testing different categories of anaerobic digestates applied to the same type of crop; such tests can help to establish general best practices and recommendations for their use.

This study tested if different types of commercial anaerobic digestates and undigested cattle slurry drove different fertiliser effects on the growth responses and nutritional aspects of ryegrass (*Lolium perenne* L.) swards over a two-year fertilisation trial and if such effects differed from both traditional inorganic fertilisation and an unfertilised control.

5.3 Material and methods

5.3.1 Fertilisation trial

The experiment was conducted in grassland dominated by perennial ryegrass (*Lolium perenne* L.), located at Carriganore Campus at Waterford Institute of Technology, Waterford, Ireland (52°25′35″N 7°18′84″W) (Figure 5.1). Waterford has an oceanic climate (Cfb) according to the Köppen climatic classification. The monthly average temperature and rainfall during the experimental period in 2016 and 2017 can be seen in Figure 5.2. The soils of the region are mostly composed of Brown Earth, Gley and Regosol (Diamond and Sill, 2011). The texture of the soil of the experimental area was classified as loam: sand (46±2.1%), silt (29±2.0%) and clay (26±2.4%).

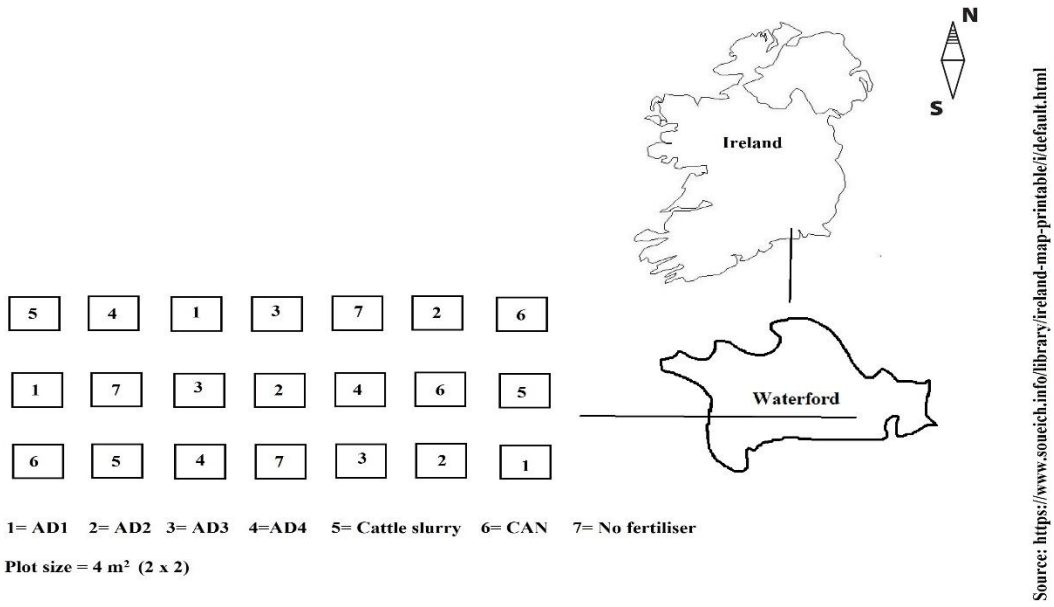
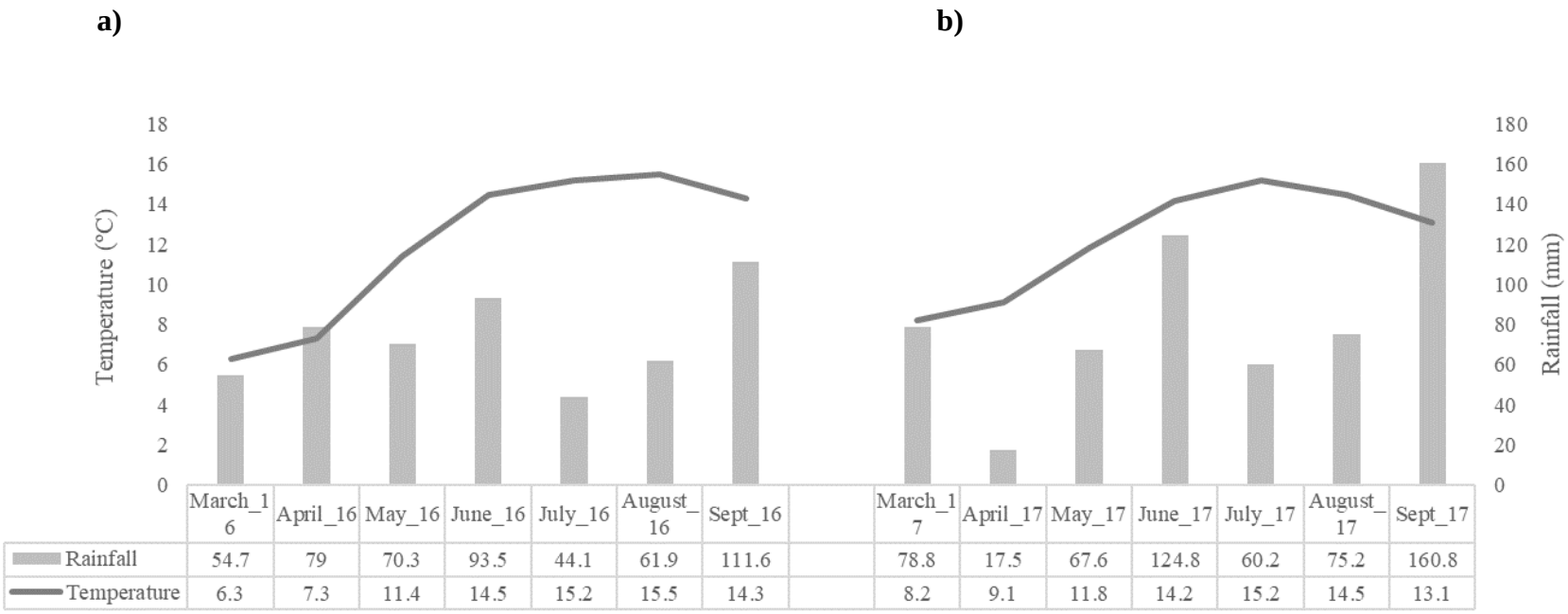


Figure 5.1.Location and plots set of the ryegrass-dominated field. Carriganore Campus at Waterford Institute of Technology, Waterford.



	March_16	April_16	May_16	June_16	July_16	August_16	Sept_16	March_17	April_17	May_17	June_17	July_17	August_17	Sept_17
Rainfall	54.7	79	70.3	93.5	44.1	61.9	111.6	78.8	17.5	67.6	124.8	60.2	75.2	160.8
Temperature	6.3	7.3	11.4	14.5	15.2	15.5	14.3	8.2	9.1	11.8	14.2	15.2	14.5	13.1

Figure 5.2. Average temperature and rainfall during the experimental period March-September 2016 (a) and 2017 (b). Data: Met Eireann, Johnstown Castle Monitoring Station, Co. Wexford.

The experiment was conducted during the spring-summer seasons of 2016 and 2017. Fertiliser treatments included four different types of anaerobic digestates (Table 5.1), fresh undigested cattle slurry, a nitrogen control (N-control) with 100 kg ha^{-1} of calcium ammonium nitrate (CAN) ($5Ca(NO_3)_2 \bullet NH_4NO_3 \bullet 10H_2O$) (27 kg N ha^{-1}), and a no fertiliser control. Treatments were randomised in blocks with three replicates, totalling 21 plots (4 m^2 each). Digestate and slurry applications were based on a rate of 33 m^3 fresh weight (FW) ha^{-1} per fertilisation, the typical rate recommended for slurry applications in Ireland (Brennan et al. 2014). Before the land-spread of fertilisers, the anaerobic digestates and undigested cattle slurry were balanced regarding the amounts of dry matter (DM) applied (3%). The balancing was based on the density of the biofertilisers. NPK inputs and stoichiometry after balancing can be observed in Table 5.2. During the fertilisation cycles, all treatments (except the no fertiliser control) received an additional amount of 27 kg N ha^{-1} (CAN) per fertilisation, aiming to minimise the effects of nitrogen imbalance between the different types of biofertilisers evaluated and to ensure that nitrogen was not a limiting factor in the experiment. Three consecutive fertilisation/harvesting cycles of 45 days were performed in each year (2016/2017), which simulated the typical management for silage cut from ryegrass swards in Ireland.

Table 5.1. Chemical composition and microbial gene copy numbers 16S/18S (GCN) of anaerobic digestates and cattle slurry used in the fertilisation trial during the fertilisation trial in 2016 and 2017.

Fertiliser Source	**AD1** Food waste (dairy industry)			**AD2** Food waste, pig slurry			**AD3** Food waste (farm and food)			**AD4** Food waste, municipal sludge			**Slurry** Beef cattle		
	2016	**2017**	**p-value**	**2016**	**2017**	**p-value**	**2016**	**2017**	**p-value**	**2016**	**2017**	**p-value**	**2016**	**2017**	**p-value**
DM%	**3.1** ±0.01	**2.7** ±0.01	**<0.05**	**6.0** ±0.10	**5.6** ±0.08	**<0.05**	**3.8** ±0.01	**3.4** ±0.01	**<0.05**	**7.3** ±0.31	**4.9** ±0.01	**<0.05**	**11.2** ±0.05	**11.5** ±0.09	**<0.05**
pH	**8.8** ±0.02	**8.9** ±0.01	**<0.05**	**8.9** ±0.02	**8.5** ±0.04	**<0.05**	**8.8** ±0.01	**9.0** ±0.02	**<0.05**	**8.8** ±0.01	**8.9** ±0.04	**<0.05**	**7.6** ±0.03	7.0 ±0.01	**<0.05**
EC (mS m^{-1})	**35.7** ±1.58	**32.2** ±2.10	**ns**	**55.0** ±1.01	**41.9** ±0.90	**<0.05**	**19.2**±0.27	**19.3** ±0.62	**ns**	**30.1** ±0.15	**34.4** ±1.16	**<0.05**	**15.8** ±0.20	**24.2** ±0.18	**<0.05**
(g kg^{-1} DW)															
TKN	**181** ±0.67	**198** ±1.45	**<0.05**	**137** ±0.71	**145** ±1.45	**<0.05**	**151** ±1.45	**112** ±9.74	**<0.05**	**104** ±1.53	**136** ±3.79	**<0.05**	**40** ±1.86	**40** ±1.33	**ns**
NH_4^+	**112.9** ±3.20	**92.3** ±2.68	**<0.05**	**62.2** ±1.65	**66.5** ±0.45	**ns**	**47.2** ±0.37	**49.9** ±0.50	**<0.05**	**55.2** ±0.87	**75.9** ±3.71	**<0.05**	**20.1** ±0.29	**29.6** ±0.27	**<0.05**
P	**11.2** ±0.13	**17.7** ±0.40	**<0.05**	**33.8** ±3.71	**20.9** ±0.81	**<0.05**	**22.6** ±0.17	**20.9** ±1.73	**ns**	**22.1** ±4.73	**34.3** ±2.19	**ns**	**7.2** ±0.27	**5.6** ±0.69	**ns**
K	**43.0** ±0.22	**56.1** ±0.57	**<0.05**	**66.8** ±2.45	**51.5** ±1.04	**<0.05**	**67.0** ±0.29	**49.5** ±2.02	**<0.05**	**9.7** ±1.94	**29.8** ±1.09	**<0.05**	**17.3** ±0.71	**36.5** ±2.14	**<0.05**
Ca	**11.5** ±0.77	**28.5** ±0.90	**<0.05**	**15.9** ±0.98	**24.6** ±0.53	**<0.05**	**22.0** ±0.32	**17.3** ±0.65	**<0.05**	**31.0** ±1.12	**36.5** ±1.00	**<0.05**	**16.8** ±1.14	**16.3** ±0.13	**ns**
Mg	**2.4** ±0.03	**3.4** ±0.08	**<0.05**	**5.0** ±0.41	**3.1** ±0.11	**<0.05**	**6.2** ±0.03	**5.2** ±0.28	**<0.05**	**8.1** ±1.66	**4.5** ±0.24	**ns**	**10.8** ±0.54	**4.3** ±0.44	**<0.05**
Na	**24.3** ±0.19	**24.6** ±0.29	**ns**	**24.0** ±1.23	**16.2** ±0.41	**<0.05**	**13.6** ±0.04	**12.3** ±0.68	**ns**	**16.0** ±3.22	**19.1** ±0.74	**ns**	**3.8** ±0.18	**4.0** ±0.38	**ns**
Al	**1.77** ±0.66	**1.43** ±0.01	**<0.05**	**11.5** ±0.14	**14.4** ±0.25	**<0.05**	**14.7** ±0.18	**22.7** ±0.15	**<0.05**	**25.9** ±0.35	**39.2** ±0.28	**<0.05**	**3.8** ±0.08	**<0.0002**	**<0.05**
Fe	**6.68** ±0.11	**7.51** ±0.04	**<0.05**	**3.63** ±0.05	**5.36** ±0.08	**<0.05**	**11.7** ±0.05	**11.5** ±0.02	**<0.05**	**17.1** ±0.10	**19.2** ±0.18	**<0.05**	**7.5** ±0.47	0.62 ±0.01	**<0.05**
(mg kg^{-1} DW)															
Mn	**160.0** ±1.22	**133.5** ±2.80	**<0.05**	**226.0** ±23.97	**197.1** ±8.83	**ns**	**184.4** ±0.43	**152.1** ±11.4	**ns**	**333.9** ±2.92	**249.9** ±16.0	**<0.05**	**115.0** ±3.29	**124.0** ±6.40	**ns**
B	**32.2** ±0.84	**27.1** ±1.08	**<0.05**	**37.4** ±0.83	**25.6** ±0.71	**<0.05**	**29.5** ±0.22	**19.4** ±1.29	**<0.05**	**<0.0006**	**43.3** ±1.70	**<0.05**	**<0.0006**	**18.3** ±0.57	**<0.05**
Co	**0.5** ±0.11	**0.1** ±0.08	**<0.05**	**1.1** ±0.14	**0.7** ±0.07	**ns**	**<0.0006**	**1.1** ±0.16	**<0.05**	**<0.0006**	**<0.0006**	**N/A**	**<0.0006**	**3.2** ±0.09	**<0.05**
Se	**<0.0008**	**1.7** ±1.52	**ns**	**6.4** ±5.51	**18.9** ±9.81	**ns**	**3.3** ±3.35	**<0.0008**	**ns**	**24.4** ±6.73	**2.6** ±2.59	**<0.05**	**2.9** ±2.89	**<0.0008**	**ns**
Pb	**2.6** ± 1.63	**0.7** ± 1.63	**ns**	**1.0** ± 0.97	**<0.0003**	**ns**	**2.5** ±1.28	**14.5** ±3.37	**<0.05**	**24.8** ±0.24	**15.6** ±2.02	**<0.05**	**1.0** ±0.99	**<0.0003**	**ns**
Zn	**299.6** ±3.82	**177.8** ±6.15	**<0.05**	**317.0** ±38.76	**207.5** ±11.1	**ns**	**203.1** ±1.69	**216.7** ±23.3	**ns**	**458.9** ±4.59	**316.1** ±22.7	**<0.05**	**239.6** ±10.98	**131.1** ±20.7	**<0.05**
Cu	**62.7** ±1.71	**45.5** ±1.56	**<0.05**	**67.9** ±8.80	**51.4** ±2.90	**ns**	**102.5** ±0.96	**140.5** ±14.9	**ns**	**229.3** ±2.61	**237.9** ±18.4	**ns**	**70.6** ±4.17	**146.4** ±22.6	**ns**
Cr	**<0.00004**	**<0.00004**	**N/A**	**0.9** ±0.85	**1.55** ±1.06	**ns**	**16.1** ±0.24	**41.9** ±6.59	**<0.05**	**26.6** ±0.36	**2.88** ±1.47	**<0.05**	**2.1** ±0.11	**<0.00004**	**<0.05**
Cd	**0.2** ±0.11	**<0.00002**	**ns**	**<0.00002**	**<0.00002**	**N/A**	**0.4** ±0.04	**1.4** ±0.28	**<0.05**	**<0.00002**	**0.3** ±0.14	**ns**	**<0.00002**	**<0.00002**	**N/A**
Ni	**11.8** ±0.10	**13.5** ±0.74	**ns**	**18.3** ±1.77	**11.1** ±0.42	**<0.05**	**16.9** ±0.89	**27.4** ±2.59	**<0.05**	**16.7** ±0.10	**16** ±0.80	**ns**	**0.8** ±0.19	**2.8** ±0.24	**<0.05**
GCN (16S) and (18S) g^{-1} DW															
Bacterial	**2.7×10^9**	**1.3×10^9**	**<0.05**	**6.7×10^9**	**2.7×10^9**	**<0.05**	**1.6×10^{10}**	**1.3×10^{10}**	**ns**	**1.7×10^{10}**	**5.0×10^9**	**<0.05**	**3.3×10^9**	**2.3×10^{10}**	**<0.05**
Archaeal	**3.7×10^7**	**1.7×10^7**	**ns**	**8.7×10^7**	**5.3×10^7**	**ns**	**1.8×10^9**	**4.3×10^9**	**ns**	**3.7×10^9**	**5.0×10^7**	**<0.05**	**1.2×10^8**	**3.3×10^8**	**<0.05**
Fungal	**5.0×10^5**	**4.5×10^6**	**<0.05**	**1.1×10^7**	**5.2×10^5**	**<0.05**	**2.3×10^6**	**3.9×10^5**	**ns**	**7.5×10^5**	**4.1×10^6**	**<0.05**	**6.1×10^7**	**1.8×10^7**	**ns**

± = standard error of the mean (SEM n=3). N/A = not analysed ns= not significant Independent sample T-test (p>0.05) Shaded p-values= p<0.05
(<) = under the detection limit of ICP-OES. DM= dry matter; EC= electrical conductivity; TKN= total Kjeldahl nitrogen.

Table 5.2. Average stoichiometry and amounts of NPK applied from each biofertiliser per fertilisation cycle (2016 and 2017).

Fertiliser Source	**AD1** Food waste (dairy industry)		**AD2** Food waste, pig slurry		**AD3** Food waste (farm and food)		**AD4** Food waste, municipal sludge		**Slurry** Beef cattle	
	2016	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**
						(kg ha^{1})				
Total N	149	163	113	120	125	92	86	112	33	33
Total N+ CAN	176	190	140	147	152	119	113	139	60	60
Total P	9	15	28	17	19	17	18	28	6	5
Total K	35	46	55	42	55	41	8	25	14	30

*****Digestates/slurry were balanced for 3% DM content, based on the density of the biofertiliser. The fertilisation rate used per cycle was 33 m^3 ha^{-1} of fresh weight, with total amounts of dry matter inputs by biofertiliser 825 kg ha^{-1} per cycle. Each biofertiliser when applied also had a complementary amount of 27 kg N ha^{-1} added via calcium ammonium nitrate (CAN) to reduce N imbalance between the biofertilisers.

5.3.2 Biofertiliser and soil: sampling and chemical analyses

Samples of liquid anaerobic digestate were collected from four Irish commercial biogas plants during spring-summer of 2016 and 2017, with an average of three collections per year. The commercial biogas plants chosen had different types of feedstock substrate (food waste (dairy industry); food waste + pig slurry; farm and food industry wastes; food waste + municipal wastes). All biogas plants were operating under continuous organic matter loading and digesting at mesophilic temperatures. Operational features can be found in Table 5.3. Cattle slurry was collected from an Irish beef cattle farm where animals are in a typical grazing system and housed during the winter. Anaerobic digestates and cattle slurry samples collected were processed following the guidelines specified in European standard EN 16179 (2012). Samples were stored refrigerated at 4 ºC (chemical analyses) or frozen at -20 ºC. All samples of biofertilisers used in the fertilisation trial were analysed in triplicates of mixed samples each year.

Table 5.3. Feedstock substrates and operational conditions of biogas plants of the anaerobic digestates analysed.

Digestate	Feedstock	Operation	Temperature (ºC)	HRT (days)	Volume (m^3)	Pasteurisation
AD1	Food waste (dairy industry)	Continuous	Mesophilic	70	1200	Pre-digestion
AD2	Food waste, pig slurry	Continuous	40	90	2000	Post-digestion
AD3	Food waste (farm and food)	Continuous	38	54	600	No
AD4	Food waste, municipal sludge	Continuous	37-42	60	1850	Post-digestion

HRT= Hydraulic retention time

Soil core samples for chemical analyses were collected four months prior to the beginning of the experiment and at 18 months from the first fertilisation (end of fertilisation trial). Soil samples were collected at 10 cm depth from random points within the plots. In average 100-150 g of wet soil were sampled, homogenised, dried (40 ºC) and sieved prior to analyses. As the soils at the beginning of the experiment showed an average pH of 5.32±0.4, granulated lime was applied one month before the trial, in a single dose of 7.5 tonne ha^{-1} targeting a soil pH throughout the trial of between 6.5-7.

Anaerobic digestates and soils had their physical-chemical traits analysis performed methodologies described in (Chapter 2.0 Physical-chemical, elemental composition and PTEs). Soil available P and K were analysed by extraction using Morgan's extractant (Daly and Casey

2005) and analysed using ICP-OES, following guidelines of CEN/TS 16170 (2012). Soil N (Total Kjeldahl) was analysed according to EN 16169 (2012).

5.3.3 Plant growth and nutritional analyses

Forage growth was measured based on three variables: canopy height (cm), leaf area index (LAI) and herbage growth (kg ha^{-1} day^{-1}). Measurements of plant growth variables occurred in an interval cycle of 45 days. Canopy height was measured based on the average of the higher leaf inflexion point of the sward. LAI was measured using a light-meter device based on indirect measurements of light interception (AccuPAR PAR/LAI Ceptometer Model LP-80 Decagon®). LAI and canopy height were measured together at the same points (three) in each plot. Herbage growth (kg DM ha^{-1} day^{-1}) was measured by the harvesting of the total herbage mass within an area of 0.25 m^2.

After harvesting, the forage herbage was separated from weeds, when they were present, and dried at 55ºC for 72 hours. Samples were then milled in a Willey type mill with a sieve of 1 mm, before laboratory analyses. The forage DM content was analysed by overnight drying at 105 ºC. Crude protein (CP) was analysed via estimation of Total Kjeldahl nitrogen (TKN) using a Buchi Kjeldahl apparatus according to European standard EN 16169 (2012). Neutral and acid detergent fibre (NDF/ADF) were analysed by digestion in an autoclave, based on procedures described in Detmann et al. (2012).

5.3.4 Statistical analysis

All data were tested for normality: Shapiro-Wilk, Kolmogorov-Smirnov and for equal variance (Levene's test) using the software SPSS 24 (IBM®). Statistical analyses of plant growth variables (canopy height, LAI, herbage growth) were based on a repeated measurements model. The model considered fertiliser treatment, cycle and year as fixed effects. Means were estimated using the procedure LSMEANS in PROC MIXED of statistical package SAS 9.3 (Statistical Analysis System) and compared using the probability of the difference (PDIFF) using T-test ($p<0.05$). Forage nutritional composition (CP, NDF, ADF) was compared using ANOVA followed by Tukey HSD test ($P<0.05$) and combined the results of two harvesting cycles from the year 2016. Plant growth responses to anaerobic digestate NPK inputs were analysed by principal component analysis (PCA). Regressions between forage yield and N, P and K were tested individually. PCA and regressions were performed in XLSTAT®, Microsoft Excel® extension software.

5.4 Results and discussion

5.4.1 Plant growth responses

Forage daily growth was influenced by the type of fertiliser and the cycle ($p<0.05$), however, no interaction between these effects or influence of year was detected ($p>0.05$) (Figure 5.3). There was no significant difference between any of the types of anaerobic digestates and cattle slurry in terms of herbage daily growth ($p>0.05$). The biofertilisers tested showed comparable averages for forage daily growth; cattle slurry was lowest at 65 kg ha^{-1} day^{-1} and AD1 highest at 79 kg ha^{-1} day^{-1}. All biofertilisers had higher forage growth rates than the controls with CAN and no fertiliser ($p<0.05$). The controls with no fertiliser and CAN did not differ from each

other ($p>0.05$); their daily forage growth varied between 34 to 49 kg ha^{-1} day^{-1} respectively. The growth cycle which showed the highest forage growth in both years was the second one, with an average of 72 kg ha^{-1} day ($p<0.05$). This cycle occurred in early July in the first year, and in the middle of June in the second year; this period coincided with increases in temperature (Figure 5.2 a.b.). The first and third growth cycle did not differ significantly ($p>0.05$), ranging from 56 and 59 kg ha^{-1} day^{-1} respectively.

For LAI and canopy height, there was an interaction between the type of fertiliser, cycle and year ($p<0.05$) (Figure 5.4 and Figure 5.5). Higher LAI values were observed in the ryegrass swards that received anaerobic digestates and cattle slurry. Nevertheless, during some cycles, some anaerobic digestates did not show any significant difference compared to the nitrogen control treatment with CAN ($p>0.05$). In most of the cycles, different types of anaerobic digestates and cattle slurry drove a similar LAI response in the ryegrass swards ($p>0.05$). The forage canopy height had a similar response as LAI, with higher canopies observed in the swards receiving biofertilisers in comparison to the controls with CAN and no fertiliser (Figure 5.5).

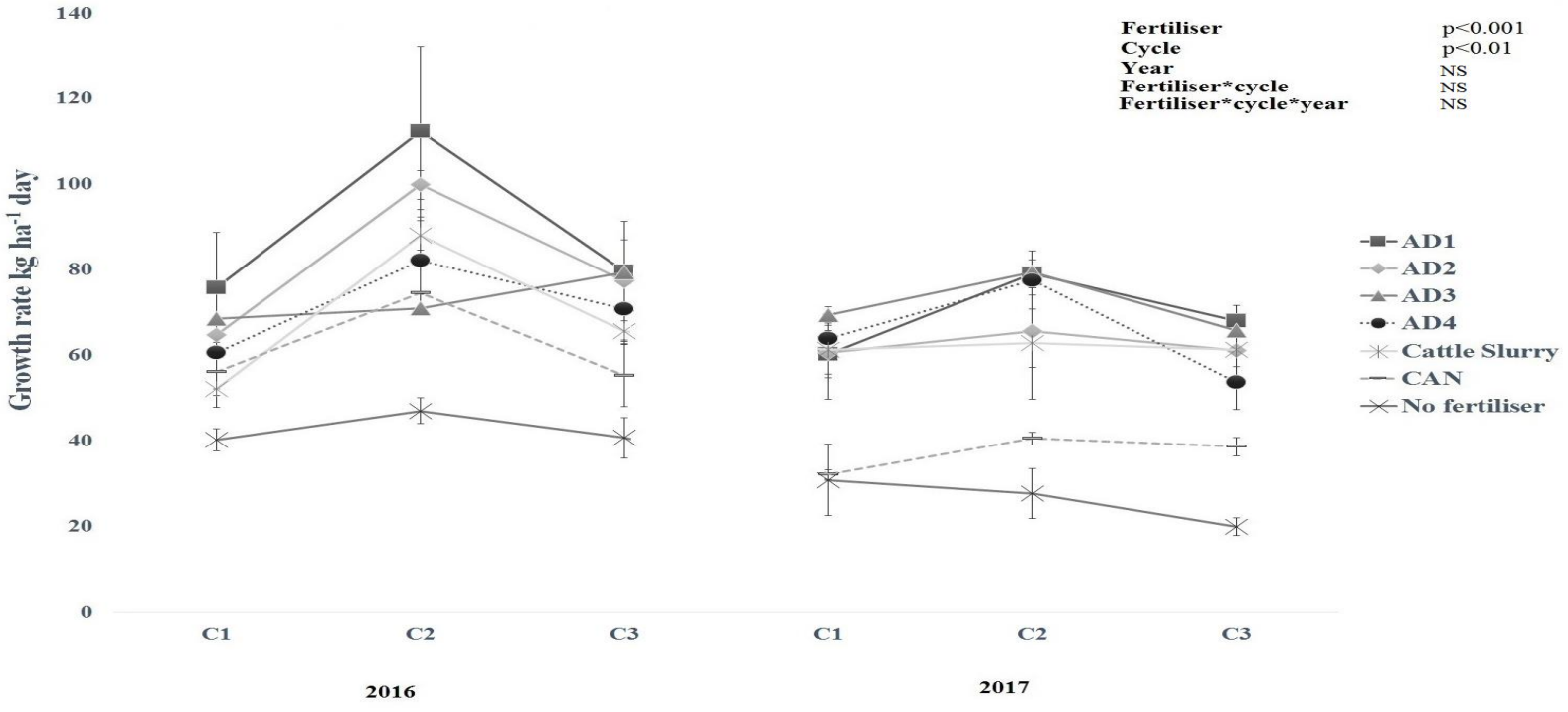
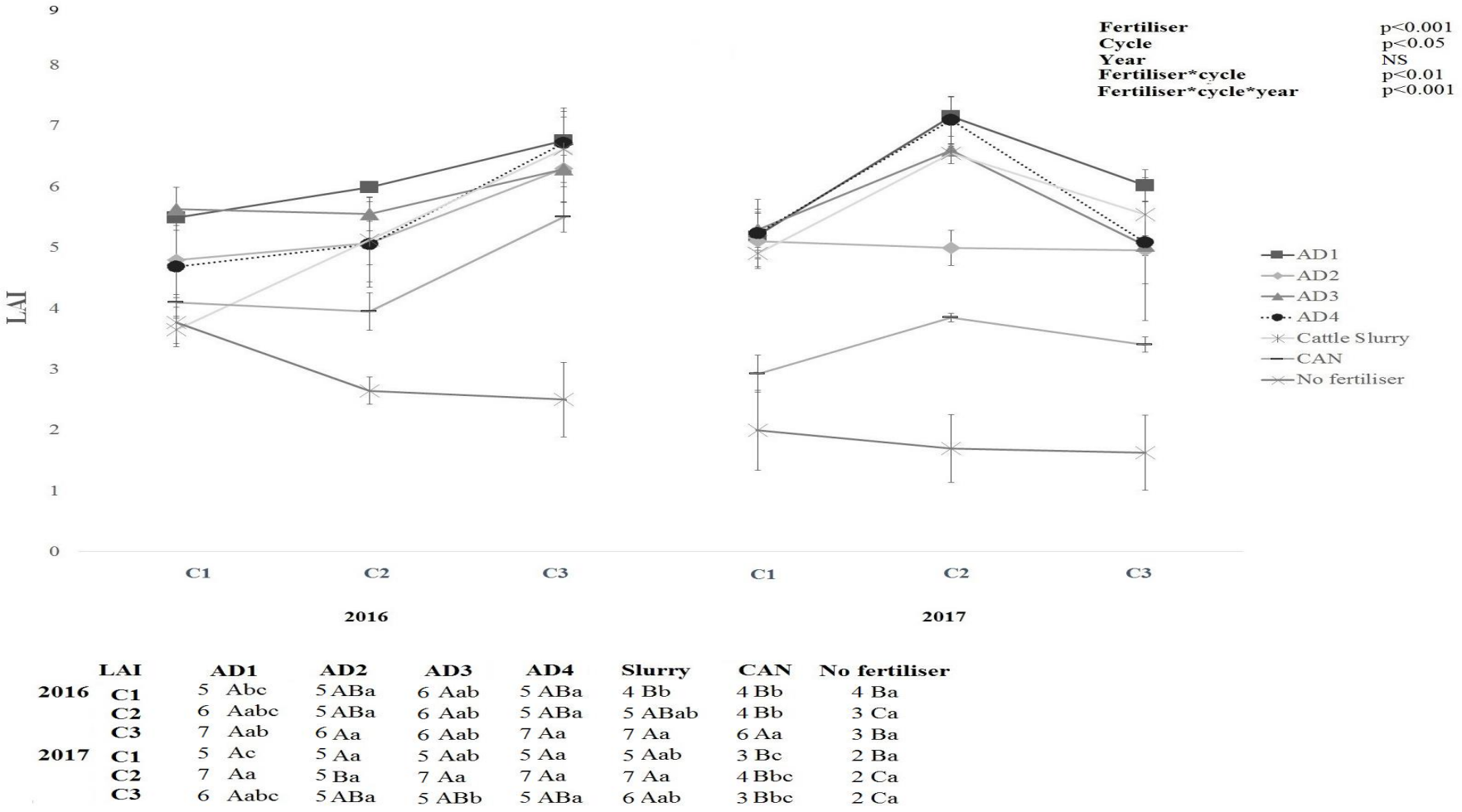
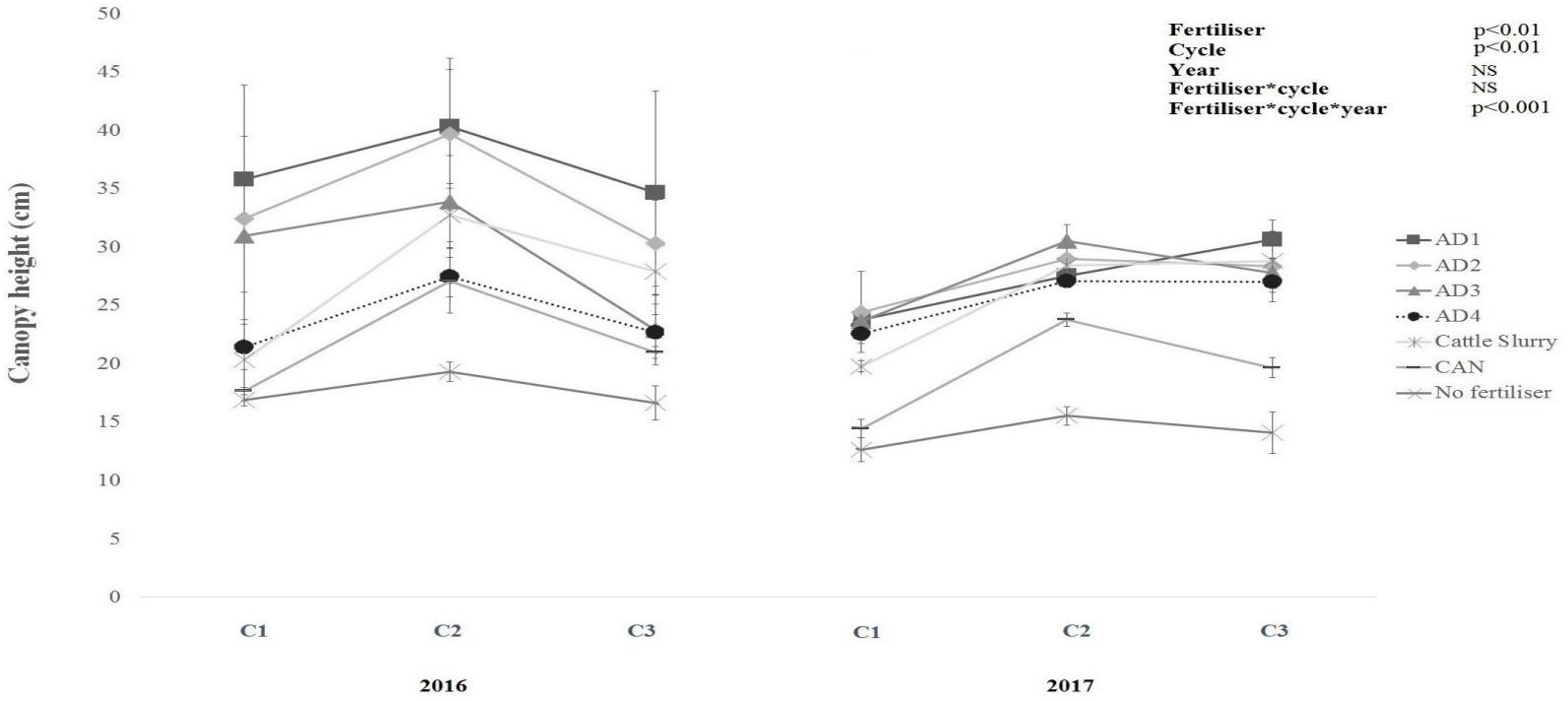


Figure 5.3. Average daily growth rate kg ha^{-1} of forage in ryegrass (*Lolium perenne* L.) swards after fertilisation with anaerobic digestates (AD), cattle slurry, CAN and no fertiliser, over the two growth periods of the fertilisation trial. Means followed by different letters differed in the pdiff/T-test (P<0.05). As there was no effect of year or interactions between factors, treatments and cycles are displayed based on all their averages (cycles/year).



	LAI	AD1	AD2	AD3	AD4	Slurry	CAN	No fertiliser
2016	C1	5 Abc	5 ABa	6 Aab	5 ABa	4 Bb	4 Bb	4 Ba
	C2	6 Aabc	5 ABa	6 Aab	5 ABa	5 ABab	4 Bb	3 Ca
	C3	7 Aab	6 Aa	6 Aab	7 Aa	7 Aa	6 Aa	3 Ba
2017	C1	5 Ac	5 Aa	5 Aab	5 Aa	5 Aab	3 Bc	2 Ba
	C2	7 Aa	5 Ba	7 Aa	7 Aa	7 Aa	4 Bbc	2 Ca
	C3	6 Aabc	5 ABa	5 ABb	5 ABa	6 Aab	3 Bbc	2 Ca

Figure 5.4. Leaf area index (LAI) of forage in ryegrass (*Lolium perenne* L.) swards after fertilisation with anaerobic digestates (AD), cattle slurry, CAN and no fertiliser, over the two growth periods of the fertilisation trial. Means followed by different uppercase letters in the line and lowercase in the column within each growth cycle differed in the pdiff/T-test (P<0.05). As there were interactions between treatment and cycle and year, all averages of cycles are displayed.



	LAI	AD1	AD2	AD3	AD4	Slurry	CAN	No fertiliser
2016	C1	36 Aa	32 Aa	31 Aa	21 Ba	20 Bb	18 Bcd	17 Bab
	C2	40 Aa	40 Aa	34 ABa	27 BCa	33 ABa	27 BCa	19 Ca
	C3	35 Aa	30 ABa	23 BCDa	23 BCDa	28 ABCab	21 CDbc	17 Dab
2017	C1	24 Aa	24 Aa	24 Aa	23 ABa	20 ABb	14 BCc	13 Cb
	C2	28 Aa	29 Aa	31 Aa	27 Aa	28 Aab	24 ABab	16 Bab
	C3	31 Aa	28 Aa	28 ABa	27 ABa	29 Aab	20 BCabc	14 Cab

Figure 5.5. Canopy height (cm) of forage in ryegrass (*Lolium perenne* L.) swards after fertilisation with anaerobic digestates (AD), cattle slurry, CAN and no fertiliser, over the two growth periods of the fertilisation trial. Means followed by different uppercase letters in the line and lowercase in the column within each growth cycle differed in the pdiff/T-test (P<0.05). As there was an interaction between treatment and cycle and year, all averages of cycles are displayed.

Although the biofertilisers were equally balanced in terms of dry matter (3%), they had substantial differences in their chemical compositions (Table 1.1). Consequently, nutrient inputs were varied (Table 5.2). Even for the same type of anaerobic digestate, there were a lot of nutrient variations between both years of the trial. Most of the nutrients changed significantly ($p<0.05$), increasing or decreasing, with no specific pattern obeyed (Table 5.1). This was expected to occur, as the anaerobic digestates used in this trial were collected at different times from commercial biogas plants operating with continuous organic matter loading, mostly using mixed types of feedstock. Inputs of the primary macronutrients (NPK) varied substantially between the biofertilisers tested (Table 5.2), especially when comparing the anaerobic digestates with cattle slurry, which showed lower concentrations of these nutrients. For example, AD1 provided three times more N than cattle slurry throughout the trial. Interestingly, despite these substantial differences in the chemical composition and nutrient inputs between the biofertilisers (including cattle slurry), plant growth responses were similar between them.

Evaluating the combined influence of total NPK inputs on forage growth responses, it could be observed in the PCA analysis (Figure 5.6) that the growth responses (forage yield, LAI and canopy height) were strongly associated with the NPK inputs provided by the biofertilisers. This association is highlighted by the arrows in the main biplot (F1=74.9%). The individual analysis of forage growth responses to N, P and K showed that grass yields were quadratically associated with the input of each of these nutrients (Figure 5.7 a.b.c, respectively).

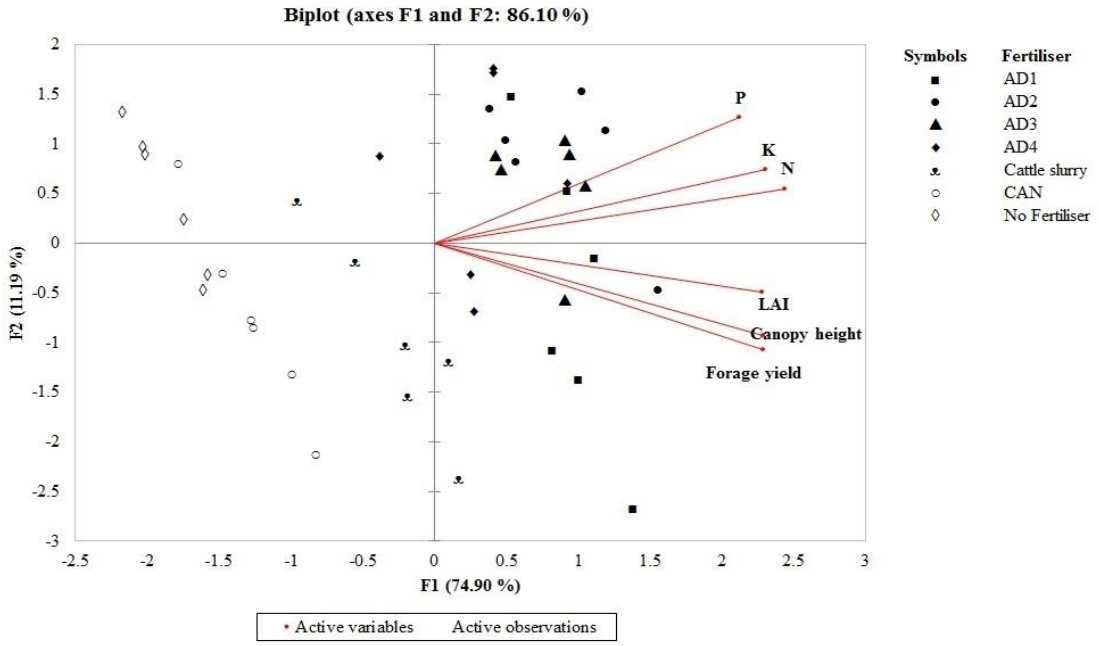


Figure 5.6. Principal component analysis (PCA) of NPK applied and forage yield, leaf area index (LAI) and canopy height in ryegrass swards. The averages of all cycles/treatments in both years 2016/2017 were included in the calculations.

Linear Pearson's correlations tested could not identify any significant associations between grass yields and the concentrations of Ca, Mg, Mn, B, Co, Se, Al, Fe, Cd, Cu, Pb, Ni, or Zn present in the biofertilisers ($p>0.05$). Only Na and Cr showed significant correlation ($p<0.05$), but they were only moderate ($r=0.4$). No evident toxic effect of the anaerobic digestates tested was noticed on grass throughout the trial. In general, plant growth characteristics measured showed good agreement, with linear regressions observed between the forage growth rate and LAI/canopy height (Figure 5.8 a.b.). Between LAI and canopy height a cubic effect was detected (Figure 5.8 c), where swards above 30 cm height displayed a tendency to stop increasing or even decrease their LAI values, possibly due to ryegrass reaching its mature and bloom stages.

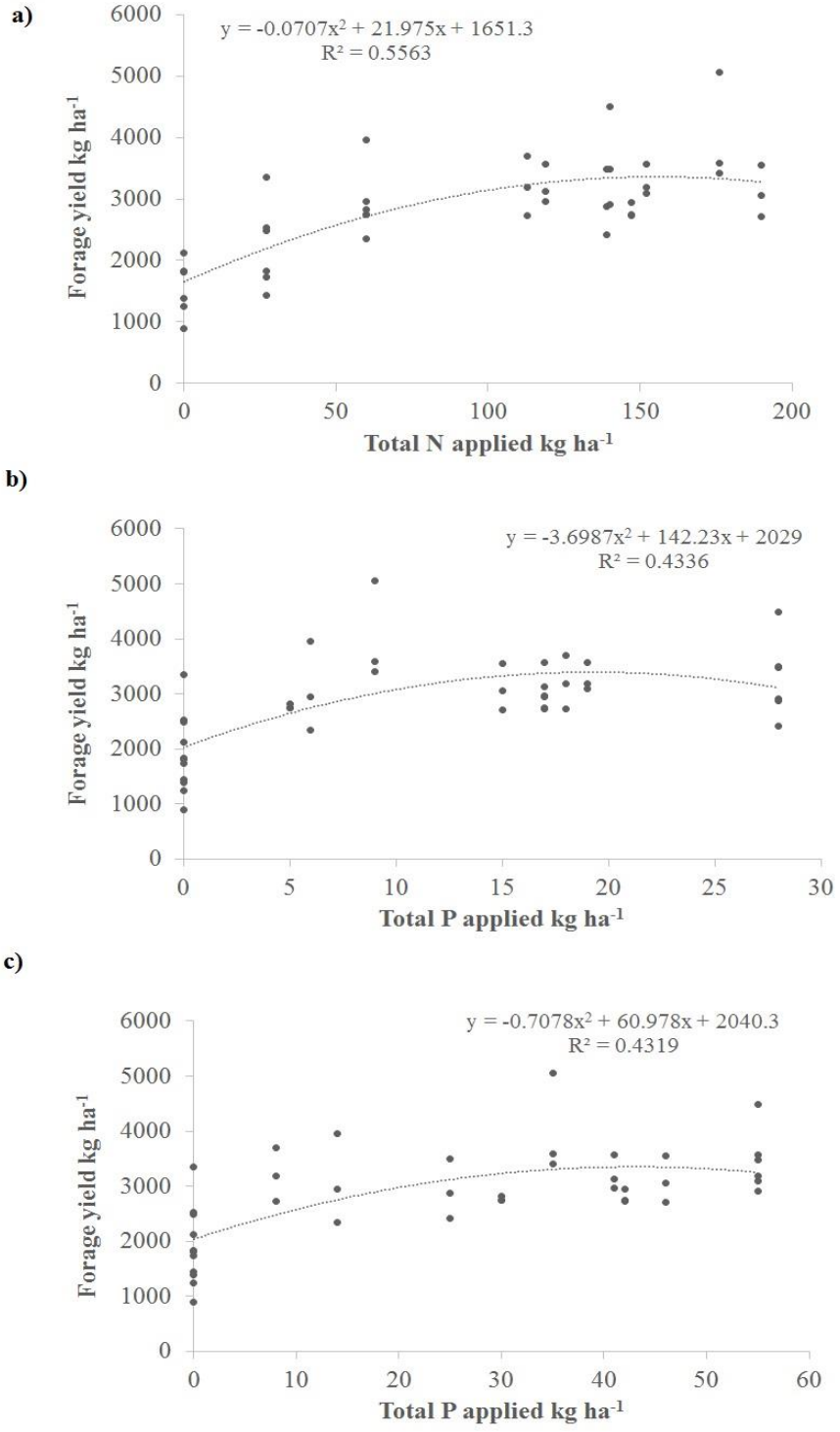


Figure 5.7. Estimated average forage yields in ryegrass swards per harvest (kg ha^{-1}) in response to total amounts of N (a) P (b) and K (c) applied (kg ha^{-1} per fertilisation). The averages of all treatments/cycles in both years 2016/2017 were included in the calculations.

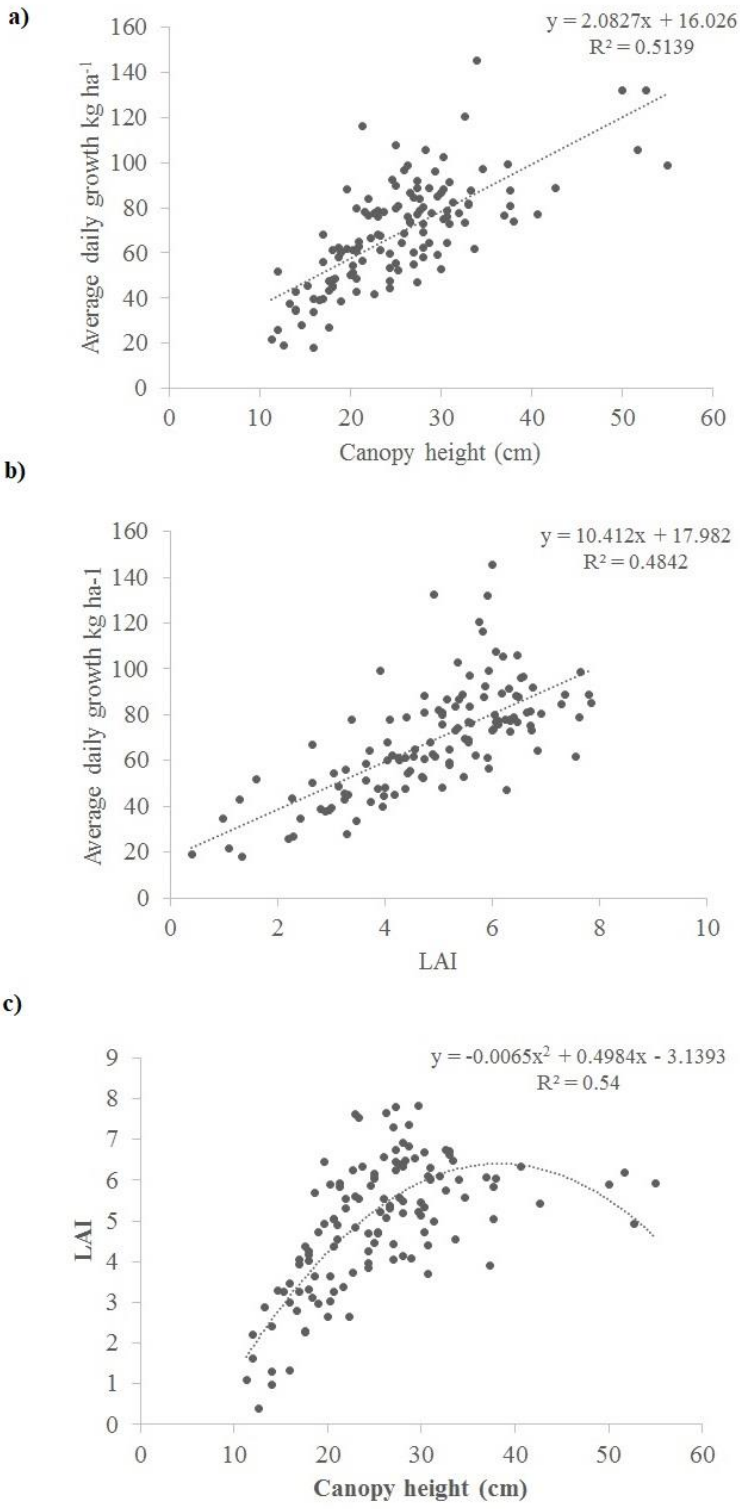


Figure 5.8. Relationship between average forage daily growth in relation to leaf area index (LAI) (a) and canopy height (cm) (b), and between LAI and canopy height (c) in ryegrass swards. The averages of all cycles and treatments in both years 2016/2017 were included in the calculations.

The fact that anaerobic digestates and cattle slurry with different chemical composition, when balanced in terms of dry matter, had similar effects on the growth of forage grass might be due to various effects and interactions relating to the bioavailability of the macro and micronutrients in the biofertilisers, and their interactions with environmental conditions (e.g. volatilisation, leaching of nutrients, decomposition, adsorption in soil particles, immobilisation by microorganisms) (Albuquerque et al. 2012; De la Fuente et al. 2013; Johansen et al. 2013; Möller and Müller, 2012; Nkoa, 2014, Insam et al. 2015). These complex interactions can create uncertainty related to the performance of anaerobic digestates on crop growth. When compared to traditional inorganic fertilisers, predicting the performance of anaerobic digestates is more challenging.

In the literature, the fertilisation effect of anaerobic digestates on grass growth has been reported to have a strong link with the total amount of N applied and its availability (NH_4^+) in the digested solution (Gunnarsson et al. 2010; Andruschkewitsch et al. 2013, Fouda et al. 2013; Walsh et al. 2018); indeed, grass growth responses are in general are also strongly associated with N amount and availability (Bryant et al. 2012; Foito et al. 2013). Fouda et al. (2013) verified that fertilisation with anaerobic digestates from different biogas plants resulted in a higher N offtake by ryegrass when compared to cattle slurry. In this present trial, digestates provided three to five times more N than cattle slurry, which was not reflected in differences in growth performance. It is possible that anaerobically digested materials with a higher N availability than undigested slurry/sludge can also have higher N loss rates (Pagans et al. 2006; Möller and Müller, 2012; Nkoa, 2014). The application of anaerobic digestates at the exponential phase of the crop growth could possibly contribute to better performance of these fertilisers and avoid N losses by volatilisation or leaching (Trindade et al. 2009; Delin and Engström, 2010; Suter et al. 2016).

The higher variability of in the values of ryegrass LAI and canopy height observed between cycles and years can be partially attributed to variations that occurred in the chemical composition of the anaerobic digestates tested (e.g. NPK) but could also relate to a time factor connected to repeated harvestings that can modify structural traits of the sward (Tuñon et al. 2014). The variation in N supplied, for example, can have a direct impact on the LAI, as N is an essential element for building amino acids and chlorophyll, compounds directly associated with cell division and with the photosynthetic process. In ryegrass, the N levels supplied are reported to have a direct impact on leaf expansion (Daepp et al. 2001; Akmal and Janssens, 2004). The additional N inputs that each biofertiliser received aimed to minimise N imbalances between the biofertilisers; despite this, some anaerobic digestates supplied two to three times more N than cattle slurry. Potassium levels also have a direct influence on the LAI of grass species, as K is an essential element for the photosynthetic process by regulating stomata opening (Wang et al. 2013). Jordan-Meille and Pellerin (2004) reported that deficiency of K could contribute to reductions of LAI and leaf elongation rates in crops. K can also influence plant height, as it contributes to the strength of plant stems (Wang et al. 2013). In relation to P, Ghannoum et al. (2008) reported that CO_2 assimilation rates in C_3 grasses are linearly associated with the contents of inorganic phosphate in the leaves; P is an essential element of DNA structure.

5.4.2 Forage nutritional analysis

Grass CP, NDF and ADF did not differ between the treatments that received anaerobic digestates, cattle slurry, CAN and no fertiliser ($P>0.05$) (Table 5.4), for two harvestings measured in the first year. Despite high N inputs via fertilisation with anaerobic digestates compared to zero inputs in no fertilised swards, no changes in crude protein content could be observed. In the literature the effect of N input levels on the CP or N content of ryegrass is varied. In a fertiliser

trial with anaerobic digestates, Walsh et al. (2018) reported higher N content in the ryegrass forage from swards treated with anaerobic digestates and/or inorganic N fertilisers compared to unfertilised swards. Peyraud and Astigarraga (1998) reported that N applications could influence the content of CP. On the other hand, Bryant et al. (2012) reported that N fertilisation has little influence on the chemical composition of ryegrass swards.

Table 5.4. Nutritional traits of ryegrass swards fertilised with anaerobic digestates (AD), cattle slurry, ammonium nitrate and no fertiliser.

	Crude protein (%)	**Neutral detergent fibre (%)**	**Acid detergent fibre (%)**
AD1	20.3 a	60.8 a	27.5 a
AD2	17.2 a	60.6 a	27.6 a
AD3	18.8 a	60.1 a	27.0 a
AD4	18.9 a	60.0 a	26.7 a
Cattle Slurry	17.1 a	59.8 a	26.9 a
CAN	18.9 a	61.8 a	27.5 a
No fertiliser	18.0 a	59.8 a	25.9 a

Means followed by different letters in column differed in Tukey HSD test ($P<0.05$).

In relation to NDF and ADF, despite the higher growth rates observed in the ryegrass swards treated with biofertilisers, these fibrous traits were not influenced significantly. Some influence of higher growth rates on the forage quality could have been expected since forage with higher growth rates tends to accumulate fibrous material more quickly. Salaun et al. (1999) reported that NDF results for ryegrass were not affected by N application. Walsh et al. (2018) reported that ryegrass swards treated with anaerobic digestates/inorganic fertilisers showed no difference in terms of digestibility compared to unfertilised swards. Considering the results of the trial and the literature, it seems that the nutritional value of ryegrass in terms of CP, NDF and ADF is minimally affected by N inputs via fertilisers.

5.4.3 Soil

At the end of the fertilisation trial, the application of anaerobic digestates impacted significantly on the concentrations of total soil N, and available P and K ($p<0.05$) (Table 5.5). All the other soil chemical nutrients were not significantly affected by the type of fertilisation ($p>0.05$). In terms of soil N, most of the soils that received the anaerobic digestates/cattle slurry and solo applications of CAN showed higher soil N (3.7 to 4 g N kg^{-1}) than the control treatment with no fertiliser (3.1 g N kg^{-1}) ($p<0.05$). Only the soils that received the anaerobic digestate AD3 showed no difference ($p>0.05$) compared to the unfertilised control. In relation to available P, the soils that received AD2 and AD3 showed higher available P (4.6 and 4.4 P mg l^{-1}, respectively) than the soils that received AD1, cattle slurry, CAN, or the control with no fertiliser (2.0, 1.5, 1.5 and 1.9 mg P l^{-1}, respectively) ($p<0.05$). AD4 showed intermediate values between lower and higher averages (3.6 mg P l^{-1}). In relation to available K, the soils which received the anaerobic digestates AD1, AD2, AD3 (155.3, 147.7 and 171.0 mg K l^{-1}, respectively) had higher concentrations than the controls with CAN and no fertiliser (68.0 and 84.7 mg K l^{-1}, respectively) ($p<0.05$). Cattle slurry and AD4 had intermediate K concentrations, between the controls and the soils that received biofertilisers. No significant differences in terms of available P and K between CAN and no fertiliser soils were observed ($p>0.05$). However, the soils which received no fertiliser showed no difference to some of the biofertilisers (e.g. AD4) ($p>0.05$) (Table 5.5). The average concentrations of total P and K (aqua regia total digestion) remained unaltered in the soils (Table 5.5), with no effect of the biofertilisation on their concentrations ($p>0.05$).

Table 5.5. Soil physical-chemical characteristics before (2015) and after the fertilisation trial.

Treat	DM%	OM%	pH	Conductivity	TKN	NH_4^+	K	P	Ca	K	Mg	Na	P	Al	Fe	Cu	Cr	Mn	Zn	Pb	B	Co	Se	Cd	Ni
2015 Prior	(%)			(mS cm^1)	g kg^{-1}		Morgan's mg l^{-1}		g kg^{-1}							mg kg^{-1}									
AD1	65.7	8.9	5.2	305.7	3.3	<0.1	109.0	9.9	1.9	2.5	2.6	1.0	0.7	15.9	16.7	11.5	16.9	325.9	40.2	22.4	12.2	<0.0006	25.5	<0.00002	8.1
AD2	71.2	8.5	5.3	335.3	2.8	<0.1	103.7	6.2	1.6	2.0	2.3	0.7	0.7	16.9	15.7	10.4	15.0	334.3	38.2	20.9	10.4	<0.0006	6.7	<0.00002	7.7
AD3	68.2	8.6	5.6	243.7	3.5	<0.1	112.3	7.3	1.5	2.3	2.7	0.7	0.7	16.9	20.1	13.0	16.2	492.8	40.5	24.3	16.0	<0.0006	0.0	<0.00002	7.9
AD4	68.1	8.2	5.8	215.0	3.0	<0.1	110.7	8.4	1.5	1.8	2.6	0.6	0.6	17.3	17.5	8.9	15.0	340.9	40.4	24.0	5.7	<0.0006	3.7	<0.00002	7.6
Slurry	64.9	8.8	5.1	223.3	3.2	<0.1	93.7	7.1	1.9	2.0	2.4	0.9	0.7	17.8	17.3	11.2	15.5	338.9	38.6	21.0	8.5	<0.0006	0.0	<0.00002	7.4
CAN	63.8	9.8	4.9	250.3	3.3	<0.1	87.3	5.7	1.8	1.9	2.7	0.8	0.6	20.7	19.8	11.5	13.3	409.5	34.3	18.7	11.9	<0.0006	18.0	<0.00002	6.3
Control	67.7	8.8	5.4	346.7	3.0	<0.1	95.7	7.3	1.5	2.0	2.5	0.6	0.7	15.5	18.4	10.7	16.1	369.8	43.5	22.8	8.4	<0.0006	10.2	<0.00002	8.9
Average	67.1	8.8	5.3	274.3	3.2	<0.1	101.8	7.4	1.7	2.1	2.5	0.8	0 .7	17.3	17.9	11.0	15.4	373.1	39.4	22.0	10.4	0.0	9.2	0.0	7.7
ANOVA P-value	NS	NS	NS	NS	NS	NA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NA	NS	NA	<0.05
2017																									
AD1	72.7	9.2	6.5	98.0	4.0 a	<0.1	155.3 a	2.0 bc	1.9	2.5	2.6	1.0	0.7	15.9	16.7	2.6	0.7	281.9	21.3	28.2	18.7	<0.0006	0.6	0.1	11.7
AD2	71.9	9.0	6.8	105.2	3.8 a	<0.1	147.7 a	4.6 a	1.6	2.0	2.3	0.7	0.7	16.9	15.7	2.1	0.0	266.5	16.5	21.9	17.1	<0.0006	0.0	0.0	10.8
AD3	73.0	9.1	7.1	85.0	3.6 ab	<0.1	171.0 a	4.4 a	1.5	2.3	2.7	0.7	0.7	16.9	20.1	1.5	0.0	260.1	13.0	23.1	17.3	<0.0006	0.0	0.2	11.1
AD4	72.8	8.8	7.0	97.2	3.7 a	<0.1	81.3 bc	3.6 ab	1.5	1.8	2.6	0.6	0.6	17.3	17.5	1.3	0.0	245.1	10.7	21.7	16.2	<0.0006	11.0	0.1	8.5
Slurry	71.2	9.2	7.1	74.1	3.8 a	<0.1	133.7 ab	1.5 c	1.9	2.0	2.4	0.9	0.7	17.8	17.3	1.4	1.4	245.8	11.1	20.2	19.7	<0.0006	0.0	0.2	9.2
CAN	72.5	8.2	6.7	72.3	3.7 a	<0.1	68.0 c	1.5 c	1.8	1.9	2.7	0.8	0.6	20.7	19.8	1.6	1.6	263.6	13.6	20.1	22.6	<0.0006	6.6	0.0	10.5
Control	71.3	8.4	7.1	42.9	3.1 b	<0.1	84.7 bc	1.9 bc	1.5	2.0	2.5	0.6	0.7	15.5	18.4	8.1	0.0	358.0	20.4	24.4	20.2	<0.0006	6.2	0.1	10.6
Average	72.2	8.8	6.9	82.1	3.7	<0.1	120.2	2.8	1.7	2.1	2.5	0.8	0.7	17.3	17.9	2.7	0.5	274.4	15.2	22.8	18.8	<0.0006	3.5	0.1	10.3
ANOVA P-value	NS	NS	NS	NS	<0.05	NA	<0.05	<0.05	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Means followed by different letters differed in Tukey HSD test (P<0.05). The ANOVA compared means between the plots before application of treatments (2015), and after (2017). NA= not analysed TKN= Total Kjeldahl Nitrogen (<) = under the detection limit.

The higher N found in the soils that received the biofertilisers and CAN in comparison to unfertilised soil is a natural consequence of the repeated N inputs. However, differences in the concentrations of soil N would be expected between anaerobic digestates and the soils that only received CAN. Some digestates, for example, AD1, supplied at least five times more N than CAN in each fertilisation cycle. Several factors possibly contributed to this, such as higher grass yields in biofertilised soils, and possibly higher N losses from the anaerobic digestates, mostly high in free NH_4^+ (Table 5.1). The supply of other plant macro and micronutrients (e.g. P and K) by the biofertilisers in comparison to solo applications of CAN could possibly have stimulated higher grass growth rates (Figure 5.7 b.c) and consequently higher N uptake from soil. Kim et al. (2003) reported higher N uptake from the soil by ryegrass plants supplied with a P source compared to plants deprived of this element. In terms of N losses, Chantigny et al. (2007) reported three to six times higher ammonia volatilisation rates in organic fertilisers compared to ammonium nitrate (NH_4NO_3). Anaerobic digestates are reported to have a high potential for N loss via volatilisation, leaching and by denitrification after the conversion of NH_4^+ to NO_3^- (Möller and Müller, 2012). In the present trial the biofertilisers were spread at the soil surface and not incorporated into the soil, which may have resulted in more N losses (Nkoa, 2014).

Higher levels of available P and K found in soils treated with the biofertilisers compared to the controls with CAN and no fertiliser can be considered a natural consequence of the repeated applications of these nutrients via biofertilisation (Table 5.2). The quantities of available soil P are associated with P inputs via fertilisation (Oehl et al. 2002; McLaughlin et al. 2011). Before the trial, the average soil available P for all field was 7.4 mg l^{-1}, while after the trial the general average was 2.8 mg l^{-1}. However, the application of biofertilisers minimised the levels of depletion of available P compared to the controls. Available K in soils treated with biofertiliser showed increases after the

trial, and this possibly contributed to the higher forage growth observed in the ryegrass swards (Figure 5.3, Figure 5.4, Figure 5.5). Higher forage yields are linked to the availability of K in soils (Pant et al. 2004; Kohmann et al. 2017).

5.5 Conclusion

Anaerobic digestates and cattle slurry with different chemical compositions, when equally balanced in terms of dry matter amounts applied, drove comparable forage grass yield responses. In terms of nutritional value, no effect of the type of fertiliser was observed in the CP, NDF or ADF of ryegrass. The application of anaerobic digestate, cattle slurry, and CAN all drove in an increase of N in the soil; however, there were no differences between them, despite considerable differences in terms of N inputs. Available P and K in soils were depleted throughout the trial; however, the application of most of the anaerobic digestates minimised these losses in comparison to the controls with CAN and no fertiliser. The results of the trial indicate that when using anaerobic digestates for fertilising ryegrass swards, standardising in terms of dry matter amounts results in comparable forage yield and nutritional quality.

Chapter 6 : Effect of the repeated applications of different types of anaerobic digestate on soil microbial numbers and diversity

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Biofertilisation with anaerobic digestates: effects on soil microbial abundance and diversity

6.1 Abstract

This study evaluated the effects of repeated applications of different types of anaerobic digestates on soil microbial numbers and diversity, in order to see if soil microbial stimulation might account for the plant growth results reported in the previous chapter for perennial ryegrass (*Lolium perenne* L.)-dominated swards. Microbial communities in the biofertiliser and soil were quantified by gene copy numbers (GCN) (16S/18S qPCR) and DNA NGS (Illumina). Soil microbial numbers were also analysed by colony forming units (CFU). The fertilisation trial was conducted over two years in a ryegrass-dominated grassland. Fertiliser treatments included four different types of anaerobic digestate, undigested cattle slurry, a nitrogen control with calcium ammonium nitrate (CAN) 27% N, and a no-fertilisation control. Treatments were randomised in blocks with three replicates. Bacteria had the highest GCN in the anaerobic digestates, followed by archaea; fungi had the lowest. Genes from microorganisms with agronomic/environmental importance were detected in the anaerobic digestates, including N-fixing bacteria, plant-growth promoting bacteria (PGPB), nitrifying and denitrifying bacteria, arbuscular mycorrhizal fungi (AMF), cellulolytic microbes, methanogens and saprotrophic organisms; however, most of them were found in very low abundances. AMF (*Acaulospora*) and methanogens were found in considerably higher abundances than other microbes with recognised soil-plant effects or functions. Soil bacterial, fungal and archaeal GCN were not significantly influenced by the type of fertiliser ($p>0.05$), and only temporary effects of the application of anaerobic digestates were noted on the soil bacterial and fungal CFU populations ($p<0.05$). The application of anaerobic digestates had no detectable impact on the soil microbial diversity. The dominant microbial community from the biofertilisers failed to replace the native microbial populations of the soil, possibly due to niche incompatibilities and competitiveness of indigenous soil microbes.

6.2 Introduction

Liquid anaerobic digestates have been recognised and used as valuable biofertilisers. They are a co-product of the anaerobic digestion, and their use as fertilisers is rising with the expansion of biogas production (Alburquerque et al. 2012; Mao et al. 2015). They have a variety of chemical compositions and carry considerable amounts of plant-essential macro and micronutrients (Trambone et al. 2010; Alburquerque et al. 2012; Möller and Müller, 2012; Johansen et al. 2013; Nkoa, 2014; Coelho et al. 2018). In addition, these biofertilisers also carry enormous quantities of different types of microorganisms (Nelson et al. 2011; Regueiro et al. 2012; Vanwonterghem et al. 2014; Insam et al. 2015; Guo et al. 2015; Chen et al. 2016; Treu et al. 2016). Their microbial diversity is mostly associated with the type of feedstock used and operational conditions during the biogas production (Li et al. 2013; Niu et al. 2015; Fontana et al. 2016; Satpathy et al. 2016; Fitamo et al. 2017). The microbial community in anaerobic digestates is generally not adequately credited and studied in terms of its potential for adding value to these types of biofertilisers. Mohammadi and Sohrabi (2012) reported that the active microbial community in the biofertiliers can significantly impact crop nutrition, via direct interactions with plant rhizosphere and/or indirectly by solubilising plant nutrients in the soil.

Generally, the main concern related to the land spreading of microorganisms in anaerobic digestates is biosecurity, such as the presence of pathogenic microbes (Sahlström, 2003; Maynaud et al. 2016). However, apart from pathogenic organisms, many other different microorganisms with potential for agronomical benefit or environmental impact can be found in anaerobic digestates, for example: nitrogen-fixing bacteria (e.g. *Bradyrhizobium*) (Guo et al. 2015), denitrifying and

nitrifying bacteria (e.g. *Achromobacter denitrificans* and *Thiobacillus denitrificans*, *Nitrosomonas*, respectively) (Sarkar et al. 2016), soil methanogens (e.g. *Methanosaeta* and *Methanosarcina*) (Guo et al. 2015), plant growth promoting bacteria (PGPB) (e.g. *Bacillus and Pseudomonas*) (Qi et al. 2017; Iwasaki et al. 2018), *Actinomyces* (Franke-Whittle et al. 2009; Guo et al. 2015), saprophytic fungi (Santi et al. 2015), and arbuscular mycorrhizal fungi (AMF) (e.g. *Glomeromycota*) (Wang et al. 2018), among others. In the literature, fewer studies have focused on the biofertiliser potential of the microbial community in anaerobic digestates; most of the microbes cited previously were from studies which were focusing on biogas production. Many questions still need to be appropriately answered, for example, what are the main microorganisms in anaerobic digestates with potential agronomic/biofertiliser use? Are they present in sufficient quantities to impact the soil and plant systems?

Currently, the land spreading of anaerobic digestates has been reported to have a variety of effects on the recipient soils' microbial communities. Some findings suggest that the application of anaerobic digestate can increase microbial biomass and activity in soils (Odlare et al. 2008; Abubaker et al. 2012; Carraciolo et al. 2015), while others reported little effects (Andruschkewitsch et al. 2013; Johansen et al. 2013, Juárez et al. 2015). Walsh et al. (2012) found digestate applications positively influenced the growth of soil bacteria but had minor effects on fungal populations. Similarly, Sapp et al. (2015) reported a positive influence of anaerobic digestate applications on soil bacterial diversity, while Wentzel and Joergensen (2016b) detected a negative impact of digestate application on the soil fungal community. The sort of effects reported raise some issues that still need to be adequately understood, such as, do repeated applications of anaerobic digestate drive changes in soil microbial communities? Does it vary according to the type of anaerobic digestate applied? Do the microbial communities most abundant in anaerobic digestates have the potential to

survive in the soil? Could biostimulation of soil microbial communities account for the plant-growth-promoting properties of digestate?

This study aimed to evaluate the effects of repeated applications of different types of anaerobic digestates, undigested cattle slurry, calcium ammonium nitrate (CAN), and no fertilisation on the quantities and diversity of soil bacteria, archaea and fungi in ryegrass (*Lolium perenne* L.)-dominated grassland. These fertilisation treatments had been found to have effects on plant growth in the previous chapter, and it was hypothesised that there may have been concurrent shifts in the soil microbial community that might indicate microbial stimulation. Additionally, this study quantified and profiled the bacterial, archaeal and fungal communities from different types of biofertilisers, noting organisms with the potential to impact on plant growth and soil nutrient cycles.

6.3 Material and methods

6.3.1 Biofertiliser sampling and characterisation

Described in (Chapter 5.3.2 Biofertiliser and soil: sampling and chemical analyses).

6.3.2 Fertilisation trial

Described in (Chapter. 5.3.1 Fertilisation trial).

6.3.3 Soil sampling and chemical composition

Described in (Chapter. 5.3.2 Biofertiliser and soil: sampling and chemical analyses).

6.3.4 Physical-chemical analyses: biofertiliser and soil

Anaerobic digestates and soils had their physical-chemical traits analysis performed methodologies described in (Chapter. 2.3.2 Physical-chemical, elemental composition and PTEs). Soil available P and K were analysed by extraction using Morgan's extractant (Daly and Casey 2005) and analysed using ICP-OES, following guidelines of CEN/TS 16170 (2012). Soil N (Total Kjeldahl) was analysed according to EN 16169 (2012).

6.3.5 Plant growth

In the results and discussion section, there are mentions to the results of the fertiliser trial related to the growth and yield of ryegrass growth described in Chapter 5.

6.3.6 Molecular and microbial analyses: biofertilisers and soil

6.3.6.1 DNA extraction and preparation

Described in (Chapter.3.3.3 DNA extraction and treatment).

6.3.6.2 Real-time PCR: standards and external control

6.3.6.2.1 Cultures used and confirmation of identity

Described in (Chapter.3.3.4.1 Cultures used).

6.3.6.2.2 Generation of standard curves

Described in (Chapter. **Error! Reference source not found. Error! Reference source not fo und.**).

6.3.6.2.3 Real-time PCR thermocycle

Described in (Chapter.3.3.4.3 Real-time PCR: reagents, primers and thermocycle).

6.3.6.3 High-throughput sequencing of 16S rRNA gene and Internal Transcribed Spacer (ITS) regions

One composite sample (n=1) (pooled from 6 extractions) for each biofertiliser, combining the genomic DNA of all collections/fertilisations for both years 2016 and 2017, was prepared for high-throughput sequencing analyses of bacterial, archaeal and fungal communities. For soil samples, a composite sample (n=1) of the DNA (pooled from 3 extractions) from the three replicates of each fertilisation treatment was prepared for the year 2015 (before the beginning of the trial) and 2017 (after the end of fertilisation trial). For this analysis, the DNA was extracted and cleaned, but not diluted to a standard concentration.

Bacterial and archaeal communities were analysed through amplification of a fragment of the V4 region of the 16S rRNA gene, using the universal primers 515f (5'-GTGCCAGCMGCCGCGGTAA) and 806r (5'-GGACTACHVHHHTWTCTAAT). For fungal communities, DNA samples were analysed by amplification of the internal transcribed spacer (ITS) region using primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (CTGCGTTCTTCATCGATGC). The DNA was amplified using HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following thermocycle conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute. And a final elongation step at 72°C for 5 minutes was performed. After PCR, products were checked on a 2% agarose gel to determine the amplification success and the relative intensity of the bands. The PCR products were pooled according to their molecular weight and DNA concentrations. PCR products were

purified using calibrated Ampure XP beads. The purified PCR products were used to prepare Illumina Truseq DNA libraries. Sequencing paired-end (2x300bp) was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq sequencing platform (Illumina, San Diego, USA) (15-20,000 reads/assay), following the manufacturer's guidelines.

Sequenced raw data readings were gathered using the pipelines for 16S rRNA and ITS1 libraries of MR DNA (MR DNA, Shallowater, TX, USA). Sequences were joined and depleted of barcodes, and sequences <150 bp or with ambiguous base call removed. The sequences were then denoised, and operational taxonomic units (OTUs) generated. Chimeric sequences, single DNA sequences/genes originating from different and/or multiple parental sequences, which can appear during PCR or by overlapped spots during DNA sequencing (Gloor et al. 2010), were removed. The OTUs were defined by clustering at 3% divergence level (97% similarity). The taxonomic classification of the final OTUs was performed using BLASTn and compared with the database from The Ribosomal Database Project RDP-II (http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov). In terms of numbers of OTUs generated by the analysis, soil and digestates/slurry samples showed, respectively (bacteria= 992 and 4436; archaea=52 and 38; fungi= 5051 and 3419).

6.3.7 Bacterial and fungal colony-forming units (CFU): soil

Soil samples were analysed for bacterial and fungal colony-forming units (CFU), by serial dilution followed by plate counts on selective media. The dilutions were made in sterile quarter-strength Ringer's solution from 1 g FW of soil. Samples were plated in triplicate, and results were later corrected to the corresponding dry matter of each soil sample. For bacterial CFU, 1 ml of the

10^{-5} dilution was pour plated in tryptic soy agar (TSA) supplemented with 50 µg ml^{-1} cycloheximide and incubated at 28°C. Bacterial CFU was counted after overnight incubation. For fungal CFU, 0.1 ml of the 10^{-4} dilution was spread plated onto potato dextrose agar (PDA) supplemented with 100 µg ml^{-1} streptomycin, and 50 µg ml^{-1} novobiocin. Fungal plates were incubated at 28°C and counted after five days. Colony-forming units were counted using a manual colony counter (Stuart®).

6.3.8 Statistical analyses

Microbial GCN data were verified for normality (Kolmogorov-Smirnov, Shapiro-Wilk) and homoscedasticity (Levene's test), and due to non-homogeneous variances, differences between biofertiliser GCN were analysed by one-way ANOVA using Kruskal-Wallis H Test ($p<0.05$), using the software SPSS 24 (IBM®). Soil microbial GCN and CFU data were converted to logarithmic base 10, to reduce the effects of unequal variance and to fit better the sphericity criteria requirements for ANOVA based on repeated measurements. Comparisons were performed using the procedure LSMEANS in PROC MIXED from the statistical package SAS 9.3 (Statistical Analysis System), followed by the probability of the difference (PDIFF) using T-test ($p<0.05$). Fertilisation and time were used as fixed factors. Relative abundances of the microbes in biofertilisers and soil were analysed by descriptive statistics at the taxonomic level of genus. Pie and bar charts were generated from the percentages of the genera present in each sample, based on the total number of each individual genus count from the sequencing results. For biofertilisers, genera with sequences below 1% were grouped as “others”; for soil, genera sequences below 2% were placed in the “others” grouping. However, in some points of the discussion, it was necessary to refer to phylum and species level, which were also analysed but not reported in the figures. Cluster analysis was also performed; prior to these analyses the data were root square transformed and their resemblance evaluated by

Bray-Curtis similarity matrix (0 to 1 level) using the software PRIMER v6 (Clarke and Gorley, 2006). Genera richness, evenness and diversity were calculated using the software PRIMER v6 by the following formulas: Genera richness (Margalef): d= (S-1) /log(N), Genera evenness (Pielou's) = J'=H'/log(S) and Shannon's diversity index H'= - Σ (P_i (lnP_i), where S= total of genera, N= the total number of individuals and P_i= the proportion of total number of individuals in the genus *i*.

6.4 Results and discussion

The following results and discussion section will be divided into two parts: first the microbial characterisation of the biofertilisers used in the fertilisation trial will be detailed, including the microbial quantities and diversity from three domains (bacteria, archaea and fungi), noting the presence of microorganisms with recognised roles for plant nutrition or soil nutrient cycling. The second part of the results and discussion analyses the effects of repeated applications of different types of anaerobic digestates and undigested cattle slurry on soil microbial quantities and diversity (bacteria, archaea and fungi) in a ryegrass-dominated grassland.

6.4.1 Microbial numbers and diversity in the biofertilisers

6.4.1.1 Bacteria

Bacterial GCN had the highest number of any of the microbial groups tested (bacteria/archaea/fungi), ranging between 10^9 to 10^{10} g^{-1} DM, and differed significantly among the biofertilisers ($p<0.05$) (Figure 6.1). Cattle slurry showed intermediate values of bacterial GCN compared to the anaerobic digestates. The similarity level based on Bray-Curtis analysis of the bacterial community diversity between different types of anaerobic digestates and cattle slurry was 44%, while the similar figure for just anaerobic digestates was 56% (Figure 6.2 a-c). This result indicates that the overall types and abundance of bacterial genera found in different anaerobic

digestates were more like each other than to the predominant genera in cattle slurry, which is reasonable as they came from a similar type of environment (anaerobic reactors for biogas production).

A total of 433 genera were detected in cattle slurry, while in the digestates, 629 different genera were found. On average each digestate had 410±70 genera. Eight genera comprised about 50% of the bacterial genes in cattle slurry: *Clostridium* (11%), *Bacteroides* (9%), *Petrimonas* (7%), *Proteiniphilum* (6%), *Bifidobacterium* (6%), *Paludibacter* (6%), *Pseudomonas* (5%) and *Cytophaga* (5%) (Figure 6.2 a). In the anaerobic digestates, more than 50% of the bacterial genes were dominated by five genera: *Clostridium* (14%), *Cloacimonas* (11%), *Bacteroides* (10%), *Acetevibrio* (10%), *Rikenella* (8%) (Figure 6.2 b). Nevertheless, the predominant bacterial genera were very variable between each digestate (Figure 6.2.c).

The values of richness and evenness indicate that digestates and cattle slurry showed a similar pattern in terms of average numbers of genera counted and that these number of genera were evenly distributed among different types of bacterial genera (Table 6.1). In descriptive terms cattle slurry showed a higher value for bacterial diversity than most of the anaerobic digestates, meaning that a given bacterial genus present in cattle slurry has a higher chance of having a similar abundance to other bacterial genera present in the DNA sample. Because of this higher diversity, it is expected that the bacterial community from cattle slurry can have a higher number of individuals displaying different functions and occupying ecological niches. The tendency of lower bacterial diversity in the digestates, in comparison to cattle slurry, might be linked to the fact that the anaerobic digestion process might select for specialised individuals more adapted to the anaerobic conditions of the bioreactors.

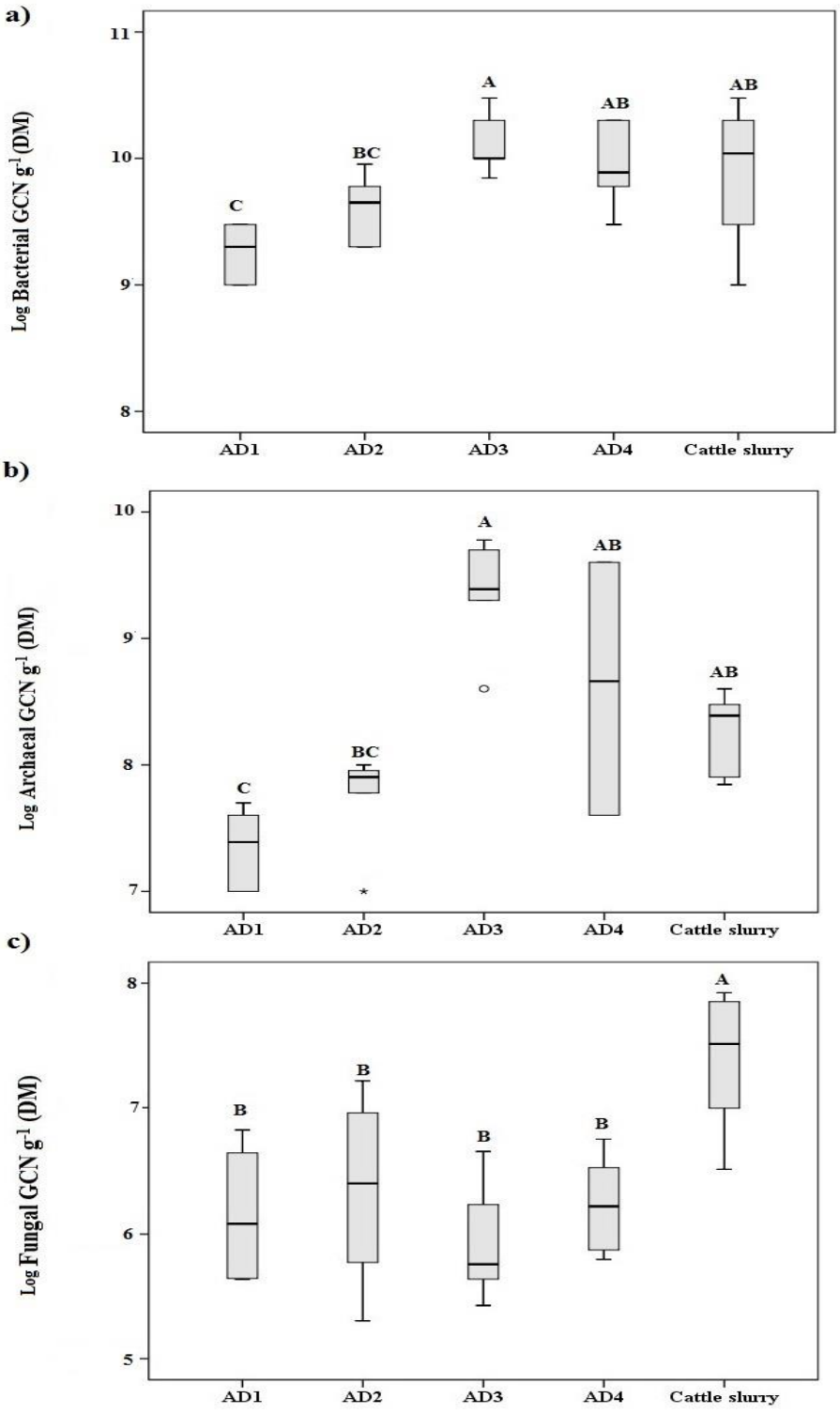


Figure 6.1. Microbial quantification of rRNA gene copy numbers (GCN g^{-1} DW) of bacteria (16S) (a), archaea (16S) (b) and fungi (18S) (c) in the biofertilisers used in the fertilisation trial. Box-plots with different letters differed significantly in Kruskal-Wallis H Test ($p<0.05$) (n= 6). ○ outliers, *extreme outliers.

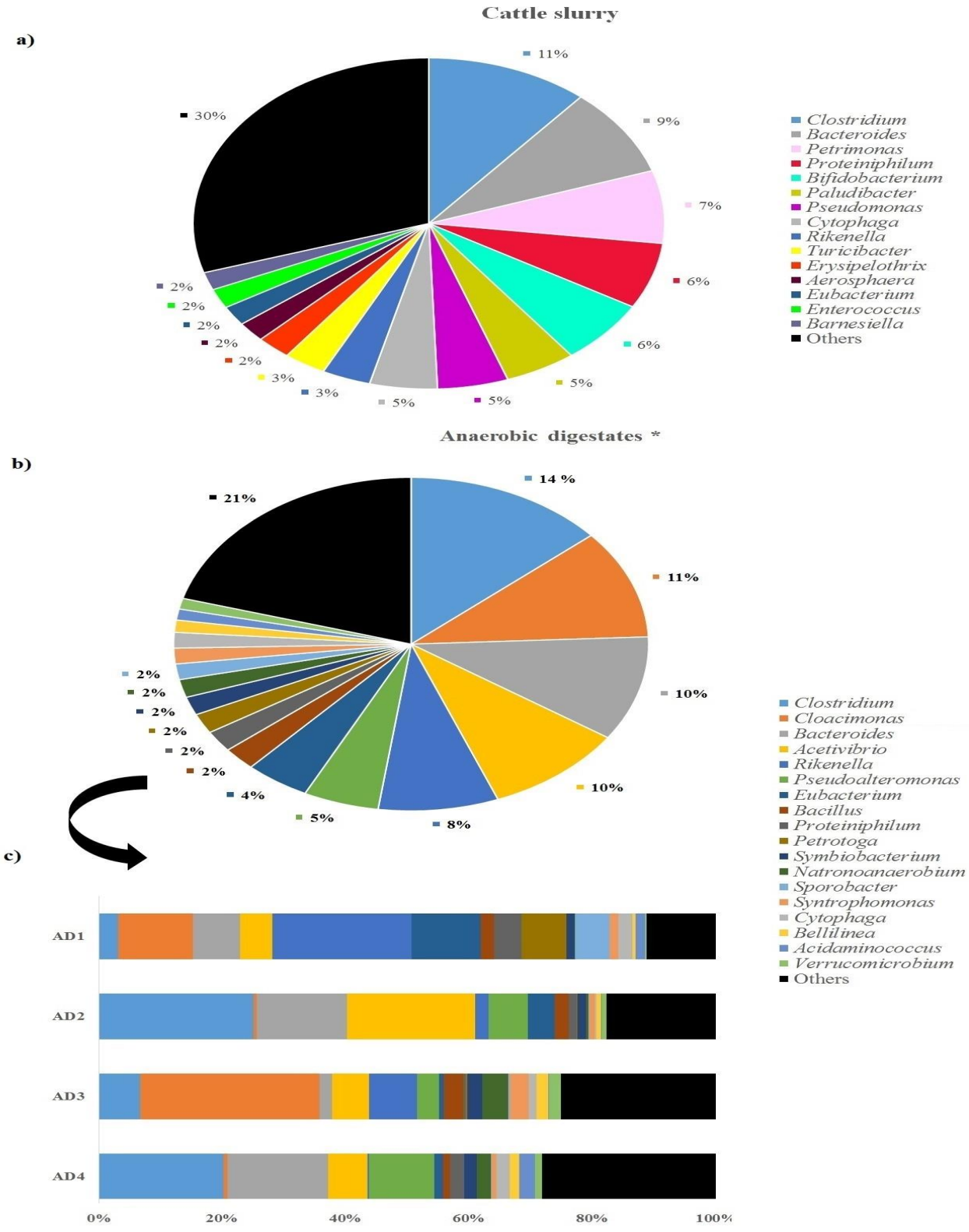


Figure 6.2. Diversity of bacterial genera in cattle slurry (a) and liquid anaerobic digestates (b and c), as determined by high-throughput sequencing (n=1). *Average of all four anaerobic digestates (AD1-AD4). *Others = microbial genera found with (< 1%) of the DNA sequences.

Table 6.1. The total number of genera, richness, evenness and diversity for bacteria, archaea and fungi in different anaerobic digestates (AD) and cattle slurry as determined by high-throughput sequencing (n=1).

	Number of genera	Richness (Margalef)	Evenness (Pielou's)	Diversity (Shannon)
Bacteria				
AD1	297	26.10	0.50	2.85
AD2	418	36.78	0.47	2.81
AD3	430	37.69	0.53	3.22
AD4	494	43.21	0.54	3.37
Digestates average	410	35.95	0.51	3.06
Cattle slurry	433	39.04	0.61	3.72
Archaea				
AD1	8	0.99	0.20	0.41
AD2	9	1.36	0.43	0.95
AD3	12	1.40	0.51	1.26
AD4	14	1.61	0.34	0.90
Digestates average	11	1.34	0.37	0.88
Cattle slurry	8	0.93	0.30	0.62
Fungi				
AD1	511	46.88	0.60	3.72
AD2	388	33.25	0.39	2.31
AD3	406	35.17	0.49	2.94
AD4	385	33.84	0.54	3.19
Digestates average	423	37.3	0.50	3.04
Cattle slurry	423	34.51	0.28	1.68

Variations of bacterial GCN and diversity in the different anaerobic digestates can be linked to type of feedstock and operational conditions in which the anaerobic digestates were produced (Rivière et al. 2009; Li et al. 2013; Ziganshin et al. 2013; Vanwonterghem et al. 2014; Niu et al. 2015; Fontana et al. 2016; Satpathy et al. 2016; Fitamo et al. 2017). Digestates used in this trial were from different biogas plants using different feedstock and operational conditions (Table 5.3), which may explain the variation. It is worth noting that the number of rRNA genes can vary from species to species, with some species containing multiple copies of the target gene per cell (Herrera et al.

2009); so, although analysis of GCN cannot provide a precise number of microbial cells present, it can be used as a general indication.

In relation to diversity, one of the most abundant genera found in anaerobic digestates and cattle slurry, *Clostridium* (Figure 6.2), has species which can perform important roles in soil nutrient cycles, especially N, with associated plant-growth effects. *Clostridium* is mostly composed of obligate anaerobic species, and some of them can fix N_2 in soil (Choudhury and Kennedy, 2004; Kennedy et al. 2004a). Beneficial plant-growth effects in different types of crops due to inoculation of *Clostridium* sp. were reported by Polyanskaya et al. (2002), with these effects attributed to the nitrogen-fixing activity from this bacterium. N supply to plants via *Clostridium* might also occur by endophytic species (Saito and Minamisawa, 2006). Another possible role of *Clostridium* in the N cycle is that some species/strains can reduce nitrate (NO_3^-) to ammonium (NH_4^+) (Van den Berg et al. 2017). According to Aislabie et al. (2013), *Clostridium* is diverse metabolically, with some species having the ability to ferment both less complex substrates such as sugars/starch, as well as complex ones such as pectin and cellulose. The decay of cellulolytic material in the soil impacts on the carbon cycle and on nutrient availability.

One of the dominant genera in cattle slurry, *Cytophaga* is composed of aerobic or anaerobic species (Kirchman et al. 2002) and has individual species specialised in digesting crystalline cellulose (Mayrberger, 2011; Zhu and McBride, 2017). It also has denitrifiers species (Topp, 2003; Kumar et al. 2010). Another predominant genus found in cattle slurry, *Pseudomonas,* is considered important in terms of biofertilisation, having some strains classified as PGPB (Qi et al. 2017; Iwasaki et al. 2018). According to Naiman et al. (2009), certain *Pseudomonas* species/strains can produce phytohormones such as cytokinins, and others can solubilise organic phosphorus. Similarly,

in anaerobic digestates, one of the dominant genera *Acetevibrio* has species/strains that can digest cellulose under anoxic conditions in soil (Zahar Haichar et al. 2007; Baldrian et al. 2010). For other dominant genera found in higher abundances in cattle slurry (*Petrimonas, Bacteroides, Proteiniphilum, Bifidobacterium, Paludibacter*) and in digestates (*Cloacimonas*, *Rikenella*, *Bacteroides*), no well-documented evidence of their roles in soil nutrient cycling or as PGPB were found, based on current literature.

From other types of bacterial genera with recognised roles in soil nutrient cycles and plant growth, the genus *Bacillus*, with species/strains that are classified as PGPB (Souza et al. 2015; Qi et al. 2017; Iwasaki et al. 2018), was found in small abundances in anaerobic digestates (2±0.7%) and cattle slurry (0.5%). Other types of plant beneficial bacteria such as diazotrophic rhizobia (*Mesorhizobium, Bradyrhizobium, Rhizobium*), were detected in all anaerobic digestates and cattle slurry, although in very low abundances (<0.01 to 0.1%). These types of bacteria are well known for fixing atmospheric dinitrogen (N_2) to stable forms of N, e.g. ammonia (NH_3), via an endosymbiotic relationship within the cells of root-nodules of legumes (Souza et al. 2015; Hayat et al. 2010). The order of *Rhizobiales*, which comprises the majority of rhizobia types of bacteria, had only 0.45±0.5% of the sequences found between all biofertilisers evaluated. Some diazotrophic nitrogen-fixing bacteria genera (e.g. *Azotobacter*, *Azospirillum*, *Beijerinckia*, *Derxia*, *Rhodopseudomonas*) were detected, but also in very low relative abundances (<0.05%). Some bacterial genera with recognised species/strains with a direct role in organic matter decay (e.g. *Actinomycetes*, *Bacillus*, *Cellulomonas*, *Streptomyces*, *Cytophaga*, *Cellvibrio* and *Pseudomonas*) (Ulrich et al. 2008) were detected in different levels in the biofertilisers analysed, most of them in very low abundance. Nitrifying (*Nitrosococcus*, *Nitrosovibrio, Nitrospira* and *Nitrosospira*) and

denitrifying (*Nitratireductor*, *Denitratisoma*, *Denitrobacterium* and *Alcaligenes*) bacterial genera were detected in all biofertilisers, but in very low abundances (<0.01 to 0.1%).

The analyses of bacterial diversity in anaerobic digestates and cattle slurry indicated that *Clostridium* species and other cellulolytic species were the predominant beneficial organisms present in the biofertilisers analysed. Although many different types of bacterial genera that can be involved in the N cycle, such as N-fixing bacteria, nitrifying, and denitrifying bacteria were detected, they were present in very low abundances, possibly reducing their potential for affecting soil/plant systems once applied.

6.4.1.2 Archaea

Archaeal GCN ranged between 10^7 to 10^9 g^{-1} DM, intermediate between high bacterial GCN and lower fungal GCN, and differed significantly among the biofertilisers ($p<0.05$) (Figure 6.1). Cattle slurry showed intermediate values of bacterial GCN compared to the anaerobic digestates. The similarity of archaeal genera between different types of anaerobic digestates and cattle slurry was 35%, and between anaerobic digestates exclusively was 43%. This result indicates that archaeal genera and their abundances found in digestates were more similar between themselves when compared to cattle slurry (Figure 6.3 a-c).

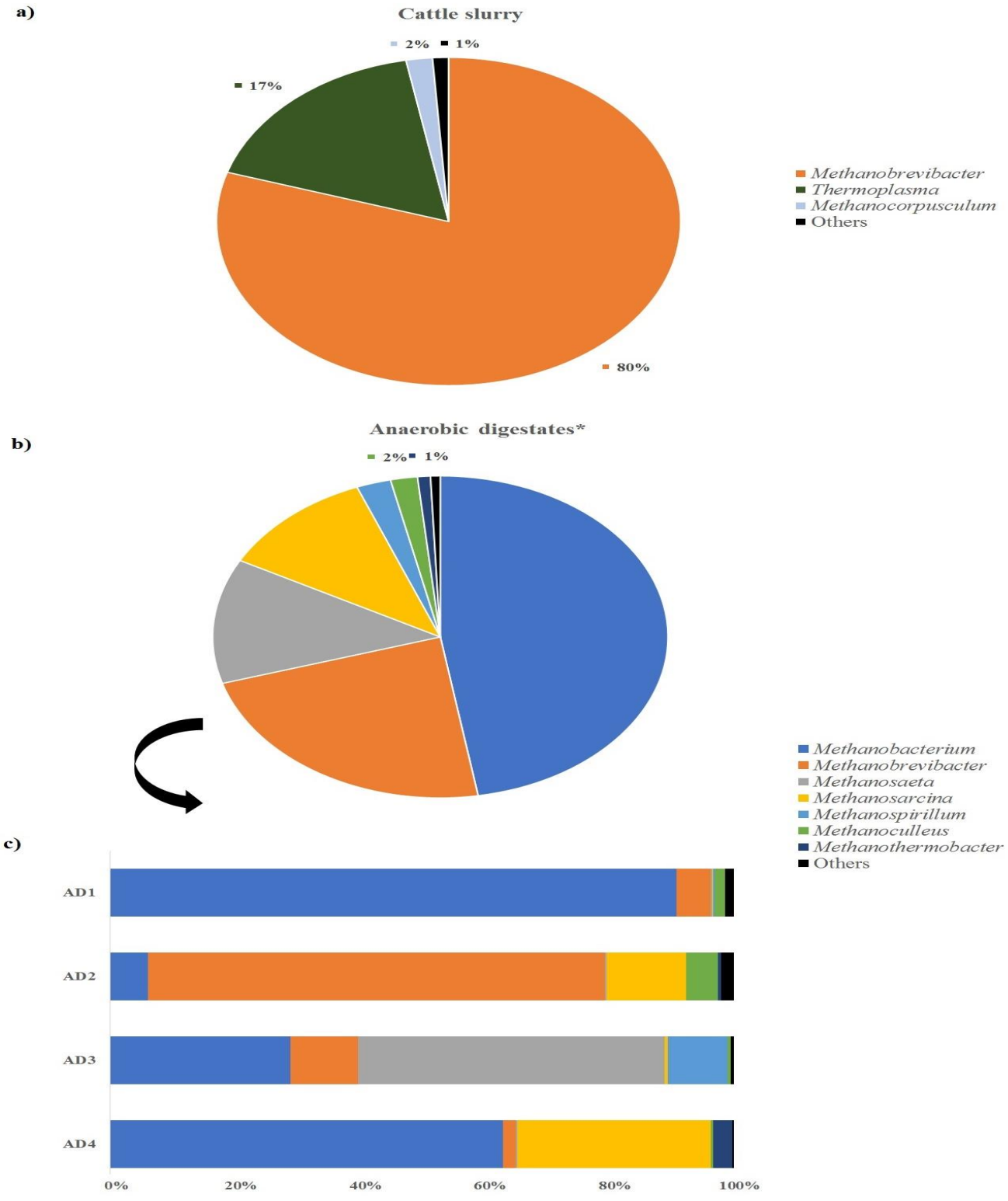


Figure 6.3. Diversity of archaeal genera in cattle slurry (a) and liquid anaerobic digestates (b and c), as determined by high-throughput sequencing (n=1). *Average of all four anaerobic digestates (AD1-AD4). *Others = microbial genera found with (< 1%) of the DNA sequences.

Archaeal abundance in cattle slurry was basically dominated by two genera, *Methanobrevibacter* (80%) and *Thermoplasma* (17%), and to a lesser extent *Methanocorpusculum* (2%) (Figure 6.3 a). Archaeal dominance varied between different digestates; on average, four genera showed high abundances: *Methanobacterium* (47%), *Methanobrevibacter* (23%), *Methanosaeta* (13%) *and Methanosarcina* (11%) (Figure 6.3 b). Two digestates (AD1 and AD4) were dominated by *Methanobacterium* species (91 and 63%, respectively), while AD2 was dominated by *Methanobrevibacter* (73%), and AD4 by *Methanosaeta* (49%) (Figure 6.3 c). On average, each digestate had 11 archaeal genera detected.

Most of the anaerobic digestates showed higher average genera richness, evenness and diversity values than cattle slurry (Table 6.1); as a consequence of that, an archaeal community with higher numbers of genera well distributed proportionally is expected. This indicates that the archaeal community from digestates was more diversified and balanced than cattle slurry, which can imply a higher number of archaea with different types of metabolic functions and ecological niches in the digestates.

Variations in archaeal GCN and diversity were found between different types of anaerobic digestates. As archaea together with bacteria are the most active microbes in the biogas production process, it is expected that differences in the feedstock and operational conditions during the production of each digestate would drive differences in their archaeal communities (Rivière et al. 2009; Li et al. 2013; Ziganshin et al. 2013; Vanwonterghem et al. 2014; Niu et al. 2015; Fontana et al. 2016; Satpathy et al. 2016; Fitamo et al. 2017).

The most dominant archaeal genus found in cattle slurry and one of the digestates, *Methanobrevibacter* is a strict anaerobic methanogen, with species being commonly found in the

digestive tract of ruminants and other animals, but also in anaerobic soils such as rice paddy fields (Miller et al. 2015). *Methanobrevibacter* in soils can contribute to methane emissions. However, as a strict anaerobe, the degree of anaerobiosis found in the soil will have a strong influence over its metabolism and activity (Angel et al. 2012). The second principal genus in cattle slurry, *Thermoplasma* (17%), is a facultative anaerobe and obligate thermoacidophile, whose growth is stimulated by the presence of sulphur, which it reduces to hydrogen sulphide (H_2S) (Langworth, 2015). Hu et al. (2013) reported the occurrence of *Thermoplasma* in upland soils and paddy fields, with lower predominance in paddy soils.

In anaerobic digestates, one of the genera that showed high abundances in two anaerobic digestates, *Methanobacterium*, is composed of species of strict anaerobes with hydrogenotrophic methanogenic metabolism (Maus et al. 2013). *Methanobacterium* species were reported to be found in anaerobic soils (e.g. paddy fields) and can contribute to methane emissions (Kitamura et al. 2011; Cadillo-Quiroz et al. 2014). Two other genera found in moderate dominance in some of the digestates, *Methanosaeta* and *Methanosarcina*, are both anaerobic, known for using acetate as the substrate for their methanogenesis. Acetoclastic methanogens are relevant to the rate of greenhouse gas emissions since large amounts of biological CH_4 are derived from acetate conversion (Smith and Smith, 2007; Pan et al. 2016). In flooded paddy soils, Feng et al. (2013) reported that acetoclastic genera such as *Methanosaeta* and *Methanosarcina* are the dominant archaeal groups. Ammonia-oxidising-archaea (AOA) is an essential group of microbes for the N cycle in the soil. These were in very low abundances in all biofertilisers. They belong to the phylum *Thaumarchaeota* (Hatzenpichler, 2012), which had only <0.1% of the sequences.

In conclusion, the dominant archaeal groups found in the anaerobic digestates were composed of different types of methanogens varying according to the type of anaerobic digestate. Different digestates showed substantial differences in terms of the dominant archaeal genera; many of these can play important roles in soil, especially related to methane production. Digestates showed a much more diversified archaeal community than cattle slurry.

6.4.1.3 Fungi

Fungal GCN had lower quantities than bacterial and archaeal, ranging from 10^6 and 10^7 g^{-1} DM, and differed significantly among the biofertilisers ($p<0.05$) (Figure 6.1). For fungal GCN, cattle slurry had a higher average (10^7 g^{-1} DM) ($p<0.05$) than all anaerobic digestates (ranges within 10^6 GCN g^{-1} DM). The similarity of anaerobic digestates and cattle slurry in terms of their fungal genera was 45%, and between digestates exclusively it was 47%. In contrast to the trend observed in bacterial and archaeal genera communities where the digestates were more similar between themselves in comparison to cattle slurry, for fungi, digestates' similarity levels were almost the same as comparing a digestate to cattle slurry (Figure 6.4 a-c). A total of 423 genera were found in the cattle slurry, with *Cyllamyces* the most abundant (67%) (Figure 6.4.a). Another six genera showed abundances of at least 1%: *Neocallimastix* (7%), *Piromyces* (4%), *Anaeromyces* (3%), *Penicillium* (3%), *Galactomyces* (3%) and *Kazachstania* (1%). 12% of the sequences were composed of genera with abundances lower than 1%. In anaerobic digestates, the genus *Acaulospora* (Figure 6.4.b), showed high abundances (22%) in most of the digestates. A total of 30 genera had at least 1% abundance. Of note were *Scedosporium* (6%), *Hanseniaspora* (6%), *Kazachstania* (6%), *Penicillium* (6%) and *Saccharomyces* (4%). A total of 615 different fungal genera were detected between the digestates; on average each digestate had 423 ± 52 genera (Table 6.1).

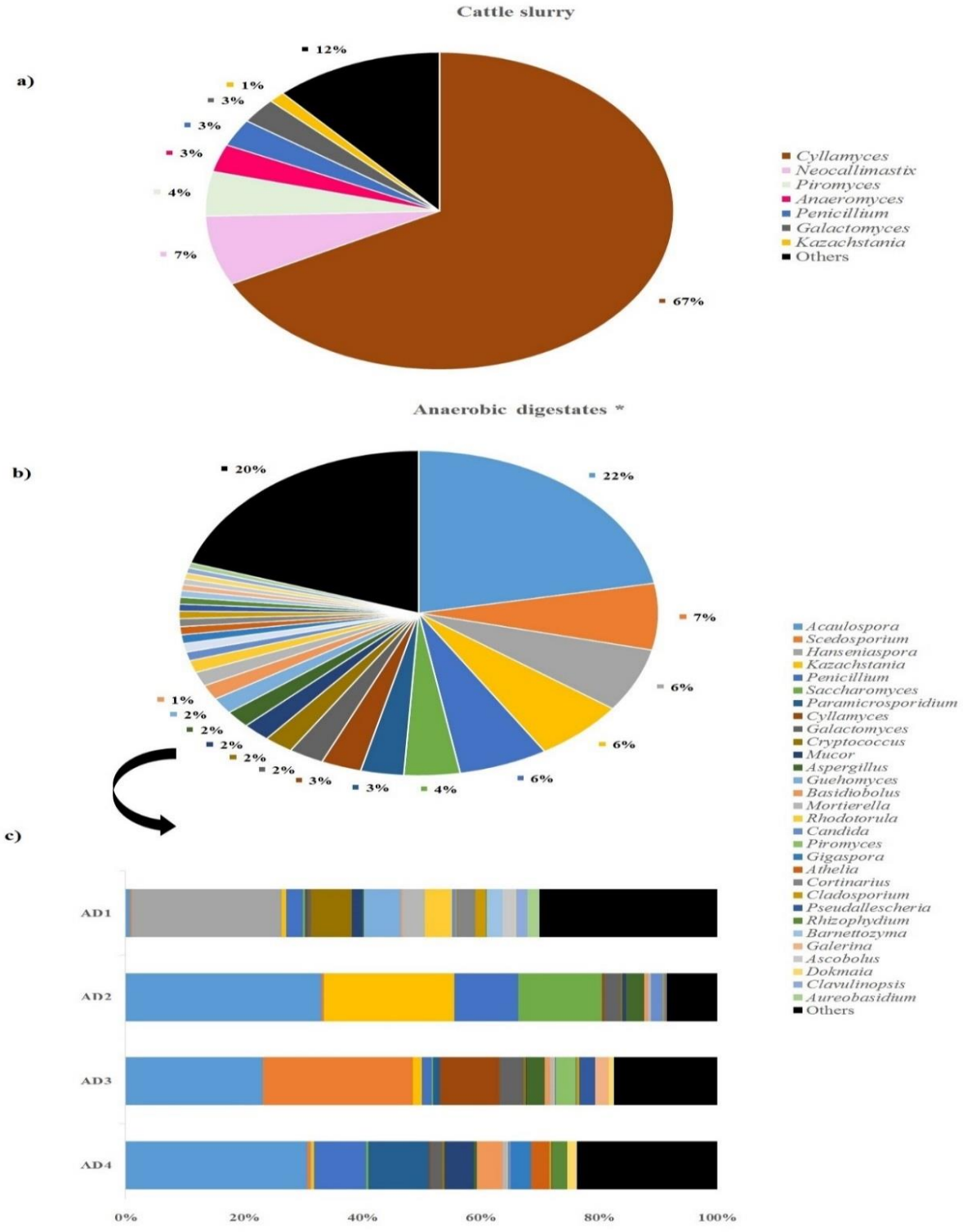


Figure 6.4. Diversity of fungal genera in cattle slurry (a) and liquid anaerobic digestates (b and c), as determined by high-throughput sequencing (n=1). *Average of all four anaerobic digestates (AD1-AD4). *Others = microbial genera found with (< 1%) of the DNA sequences.

The richness values of anaerobic digestates and cattle slurry were quite similar, indicating that the number of different types of fungal genera were proportional to the total number of fungal individuals found. However, most of the digestates showed a higher evenness than cattle slurry, indicating that the fungal genera in digestates were better distributed in their individual proportions. Average diversity of digestates was almost double compared to cattle slurry, which indicates a fungal community more proportionally balanced between the fungal genera, with different functions and ecological niches.

In relation to the lower GCN found in anaerobic digestates in comparison to cattle slurry, it is likely because the anaerobic environment in the biogas reactors reduced the populations of fungal groups, especially moulds. The majority of fungi are classified as aerobes (Lee, 2014), although Young et al. (2018) reported the detection of many aerobic fungal species from the phyla *Ascomycota*, *Basidiomycota* and *Zygomycota* in biogas reactors. Some anaerobic fungi, e.g. from the phylum *Neocallimastigomycota*, have also been reported to be found in anaerobic reactors with possible participation in the digestion of cellulolytic compounds (Dollhofer et al. 2015; Dolhofer et al. 2017).

The fungal community from cattle slurry was mostly dominated by genera from the phylum *Neocallimastigomycota* (82%) (e.g. *Cyllamyces*, *Neocallimastix, Piromyces* and *Anaeromyces*). *Neocallimastigomycota* is predominantly composed of anaerobic fungi adapted to live in the digestive tract of large ruminants and non-ruminant herbivores (Liggenstoffer et al. 2010; Griffith et al. 2010; Gruninger et al. 2014). The land spreading of cattle slurry on soil, especially under aerobic conditions (e.g. land spread at the soil surface), can pose a challenge to *Neocallimastigomycota* species to retain their metabolism and to survive. Gruninger et al. (2014)

reported that despite *Neocallimastigomycota* DNA being found in soils, their survival out of non-gut niches is limited to some viable aerotolerant propagules produced by some species.

In most of the anaerobic digestates, there was a considerable abundance of the genus *Acaulospora*. In the majority, this genus was composed of the species *Acaulospora kentinensis* (20%). The *Acaulospora* genus is well known for its role as AMF for many different types of plants (Oehl et al. 2010; Krüger et al. 2012). Some of the other most dominant fungal genera found in anaerobic digestates were from the phylum *Ascomycota*, which is one of the dominant fungal phyla found in soils (Lim et al. 2010; Detheridge et al. 2016), with organisms participating in a variety of processes such as formation of lichen symbiosis, litter decomposition, or acting as plant endophytes (Lim et al. 2010). *Ascomycota* has many individuals with abilities to degrade cellulose under aerobic conditions (Boer et al. 2005; Ma et al. 2013). A known *Ascomycota* abundant in the anaerobic digestates was *Penicillium*, which has species that can also solubilise phosphorus in the soil (Chai et al. 2011; Osińska-Jaroszuk et al. 2015). *Hanseniaspora* and *Kazachstania*, two other dominant *Ascomycota* genera found in some digestates, were reported by Yurkov et al. (2018) as sugar fermenters.

In conclusion, the fungal diversity analysis of the anaerobic digestates indicated a potential for plant AMF, with the genus *Acaulospora* found to be abundant in most of the digestates. Further studies are required to understand the association between AMF in anaerobic digestates and their effectiveness as a plant inoculum. This trial covered their abundance in soil, which can be observed in the section (Soil fungal numbers and diversity). Anaerobic digestates showed considerable abundances of fungi from the phylum *Ascomycota*. The fungal community from cattle slurry was primarily composed of genera from *Neocallimastigomycota*.

6.4.2 Fertilisation trial

6.4.2.1 Soil bacterial numbers and diversity

Soil bacterial (16S) GCN was not significantly affected by the type of fertiliser applied to the soil, or by the interaction between fertiliser and time ($p>0.05$) (Figure 6.5). However, time did have a significant effect ($p<0.05$). For soil bacterial GCN, there was no significant difference between GCN before the trial compared to GCN found in soil at the end ($p>0.05$); some variations were observed during the experiment (Figure 6.5). Soil bacterial (16S) GCN were higher, than fungal (18S) and archaeal (16S) (Figure 6.5) ($p<0.05$). The culturable numbers of bacteria were affected by the interaction between the type of fertiliser and time ($p<0.05$) (Figure 6.6.a). Five months after the beginning of fertilisation, higher bacterial CFU was noticed in the soils that received AD1 in comparison to the control treatments (CAN and no fertiliser) ($p<0.05$). At ten months, soils treated with AD2 and AD3 showed higher bacterial CFU than the controls ($p<0.05$). At 15 months, AD1, AD2 and AD3 showed higher bacterial CFU than the controls. At the end of the trial, however, no significant bacterial CFU differences based on the type of fertiliser applied were observed ($p>0.05$). Soils treated with CAN and no fertiliser showed no significant changes in their bacterial CFU during the trial ($p>0.05$). Soils treated with AD1 and AD3 increased their bacterial CFU from before the trial to the end ($p<0.05$), while the other anaerobic digestates and cattle slurry, apart from detectable increases in their bacterial CFU at some sampling times, showed no significant differences between the start and the end of the experiment ($p>0.05$).

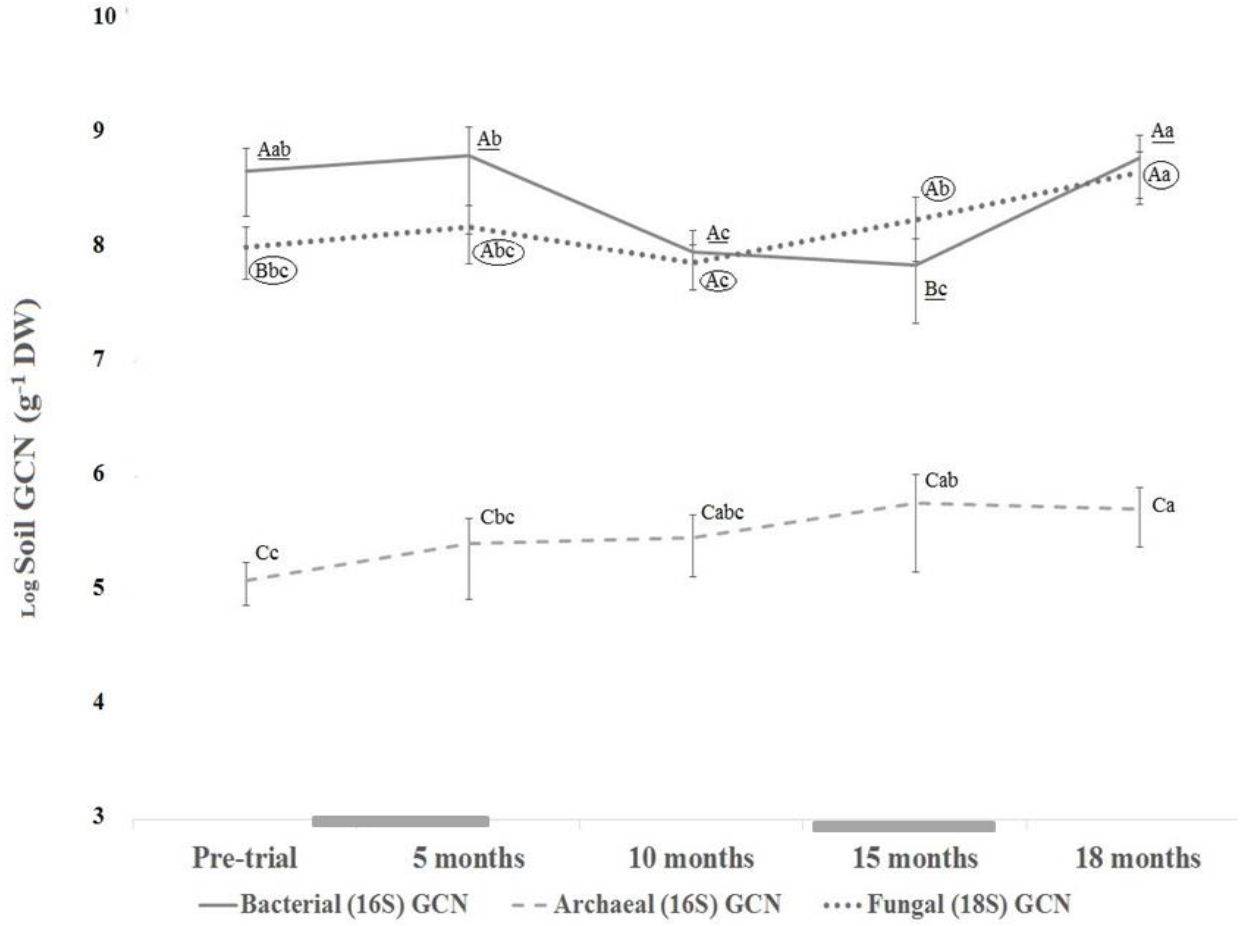
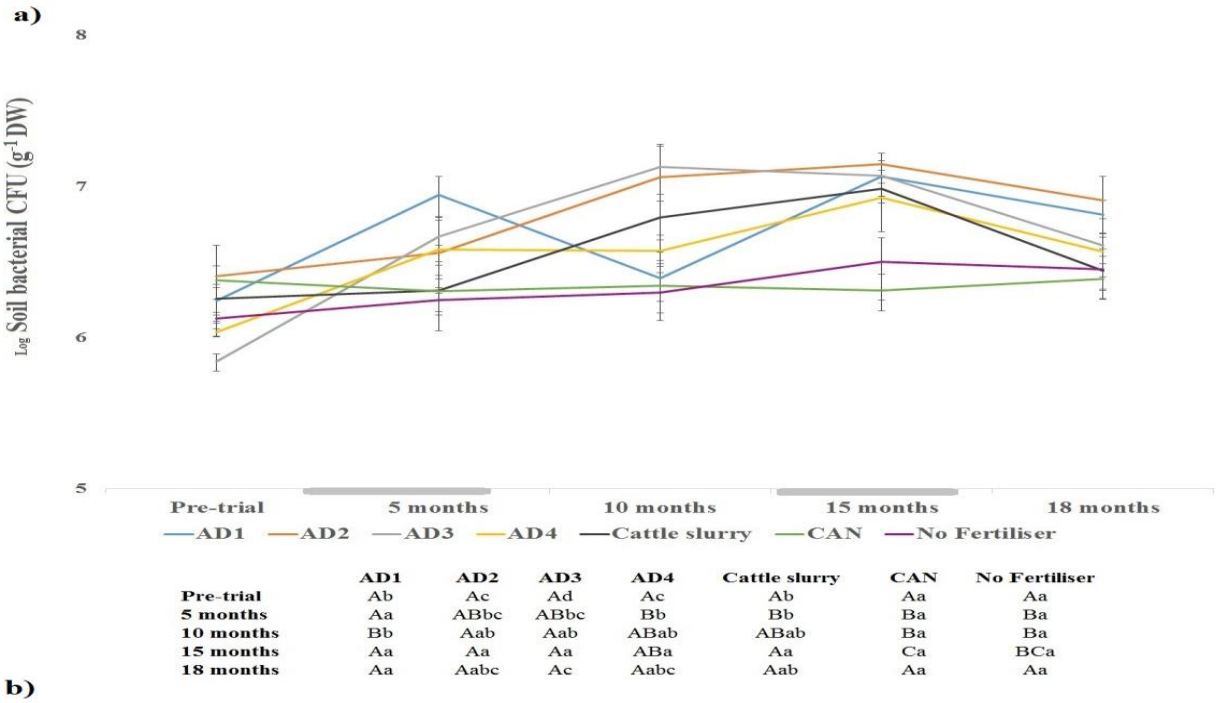
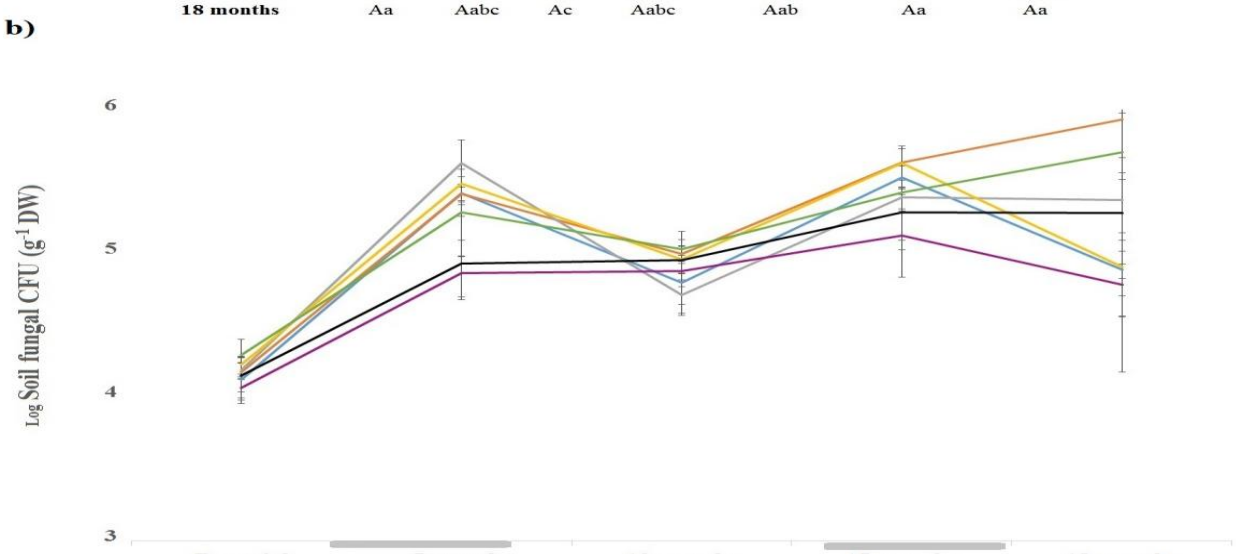


Figure 6.5. Soil bacterial (—), archaeal (- -) and fungal (····) gene copy numbers (GCN) g^{-1} DW, before (pre-trial) and during the fertilisation trial. Means followed by different uppercase letters in the Y-axis and lowercase in the X-axis differed significantly (PDIFF t-Test, $p<0.05$). Bars = standard error of the mean. ▬ Consecutive fertilisation cycles. Note: There was only a significant effect of time ($p<0.05$); therefore, all results from different types of fertilisers applied were plotted together. AD= anaerobic digestate, CAN = calcium ammonium nitrate DW=dry weight



	AD1	AD2	AD3	AD4	Cattle slurry	CAN	No Fertiliser
Pre-trial	Ab	Ac	Ad	Ac	Ab	Aa	Aa
5 months	Aa	ABbc	ABbc	Bb	Bb	Ba	Ba
10 months	Bb	Aab	Aab	ABab	ABab	Ba	Ba
15 months	Aa	Aa	Aa	ABa	Aa	Ca	BCa
18 months	Aa	Aabc	Ac	Aabc	Aab	Aa	Aa



	AD1	AD2	AD3	AD4	Cattle slurry	CAN	No Fertiliser
Pre-trial	Ab	Ab	Ac	Ac	Ab	Aa	Aa
5 months	Aa	ABb	Abab	Bbc	Bb	Ba	Ba
10 months	Bb	Aab	Aa	ABab	ABab	Ba	Ba
15 months	Aa	Aa	Aa	ABa	Aa	Ca	BCa
18 months	Aa	Aab	Ab	Aabc	Ab	Aa	Aa

Figure 6.6. Soil bacterial (a) and fungal (b) colony-forming units (CFU), before (pre-trial) and during the fertilisation trial. Means followed by different uppercase letters in columns and lowercase in lines differed significantly (PDIFF t-Test, $p<0.05$). ▬ Consecutive fertilisation cycles. Bars = standard error of the mean. AD= anaerobic digestate, CAN = calcium ammonium nitrate DW=dry weight

The application of anaerobic digestates to soil has been reported to produce a variety of results on soil microbial numbers and activity. Increases in soil microbial activity and biomass in response to digestate fertilisation have been reported in at least one type of microbial domain (Odlare et al. 2008; Abubaker et al. 2012; Carraciolo et al. 2015, Walsh et al. 2012). On the other hand, other trials only detected little and temporary responses (Andruschkewitsch et al. 2013; Johansen et al. 2013, Juárez et al. 2015). In the present trial, only soil bacterial and fungal CFU showed a small and temporary increase response to the repeated applications of anaerobic digestates. The higher CFU numbers were mostly detected in periods closer to the fertilisation times.

In terms of bacterial diversity, the soil bacterial community showed almost no changes in terms of the most abundant genera between the start and the end of the fertilisation trial (Figure 6.7.a.b, respectively). No detectable effect of the type of biofertilisers used, or of the controls with CAN and no fertiliser, on the dominant bacterial community of soil could be observed. The main noticeable change seen in all soils was a reduction in the abundances of *Arthrobacter* in favour of a slight increase in the abundances of other genera of the soil. Before and after the trial the average bacterial genera richness was slightly increased in the soils that received anaerobic digestates; however, the no fertilised control showed a similar pattern (Table 6.2). In relation to evenness and diversity, all treatments, including the controls, showed a relative increase after the trial; however. the general increases of these indices could not be attributed to any treatment. Higher values of richness, evenness and diversity indicate that after the trial all the soils tended to show an increase in and a more proportional distribution of the bacterial genera; this can be observed in Figure 6.7 a.b.

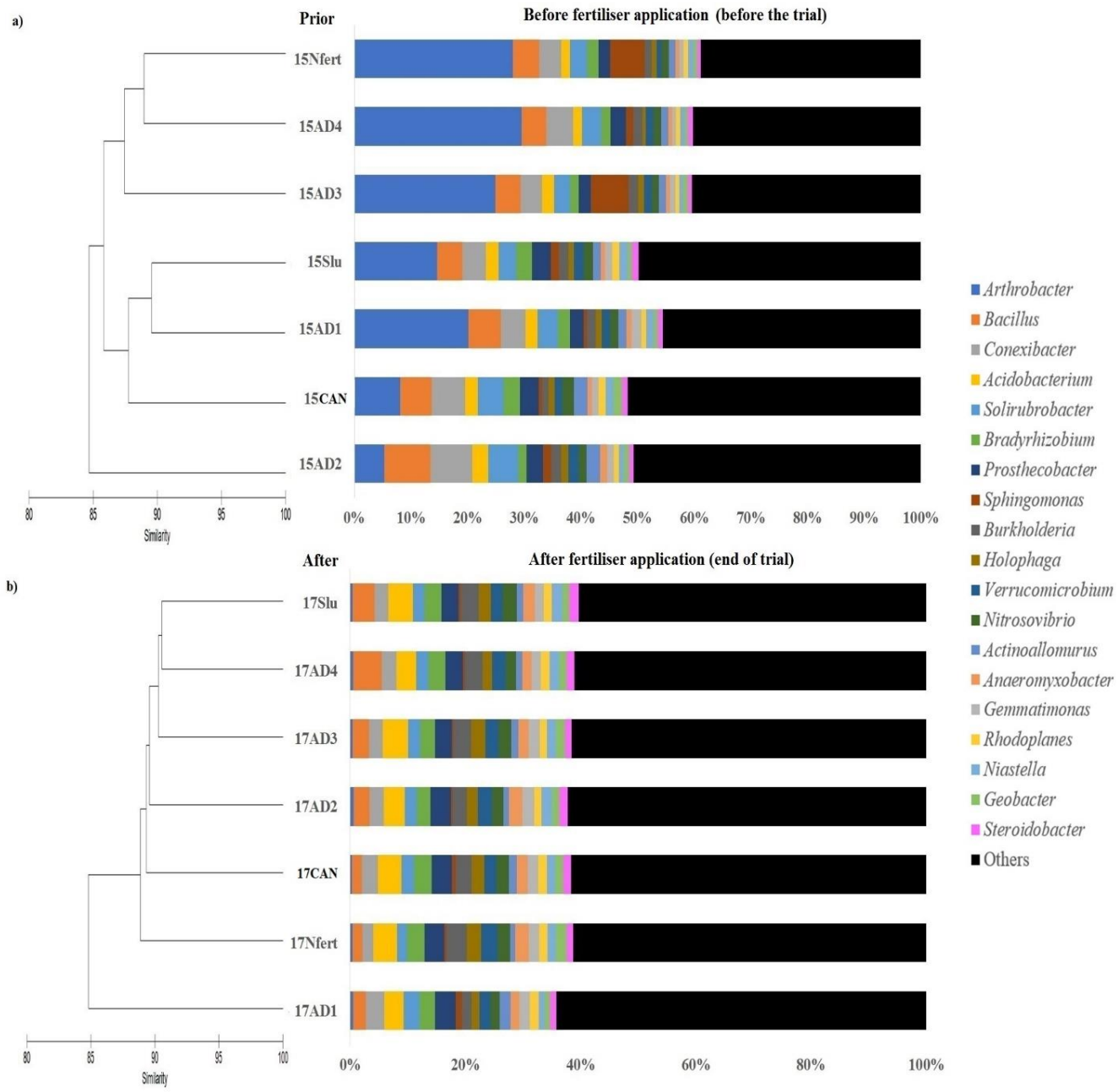


Figure 6.7. Soil bacterial genera diversity before (a) and after (b) the fertilisation trial. AD= anaerobic digestate, Slu= Cattle Slurry, CAN = calcium ammonium nitrate, Nfert= No fertilisation. 15 = before application of treatment. 17= after repeated application (two seasons) of treatment. Similarity based on Bray-Curtis similarity matrix. *Others = microbial genera found with (< 2%) of the DNA sequences.

Table 6.2. Total number of genera, richness, evenness and diversity of bacteria, archaea and fungi in the soils prior to and after fertilisation with anaerobic digestates (AD), undigested cattle slurry, calcium ammonium nitrate (CAN) 27% N, and no fertilisation, as determined by high-throughput sequencing (n=1).

	Number of genera	Richness (Margalef)	Evenness (Pielou's)	Diversity (Shannon)
Bacteria				
Prior_AD1	619	55.55	0.67	4.32
Prior_AD2	575	52.82	0.72	4.61
Prior_AD3	595	54.46	0.64	4.07
Prior_AD4	593	54.36	0.63	4.00
Prior_Cattle slurry	688	60.91	0.70	4.58
Prior_CAN	688	60.68	0.72	4.68
Prior_No fertiliser	632	56.64	0.62	3.97
Average	685	61.67	0.68	4.41
After_AD1	706	62.14	0.77	5.05
After_AD2	699	63.10	0.76	5.00
After_AD3	715	63.66	0.76	4.99
After_AD4	714	63.27	0.76	4.99
After_Cattle slurry	672	60.50	0.76	4.93
After_CAN	674	60.72	0.77	4.98
After_ No fertiliser	668	60.75	0.76	4.96
Average	756	67.67	0.76	5.03
Archaea				
Prior_AD1	12	2.42	0.63	1.55
Prior_AD2	13	2.21	0.38	0.97
Prior_AD3	11	2.22	0.69	1.65
Prior_AD4	10	2.26	0.86	1.99
Prior_Cattle slurry	13	2.33	0.79	2.03
Prior_CAN	13	2.27	0.84	2.17
Prior_No fertiliser	13	2.33	0.68	1.75
Average	14	2.62	0.69	1.83
After_AD1	15	2.11	0.30	0.82
After_AD2	11	1.69	0.39	0.95
After_AD3	15	2.47	0.52	1.40
After_AD4	13	2.26	0.57	1.47
After_Cattle slurry	14	2.50	0.47	1.25
After_CAN	10	1.66	0.38	0.87
After_ No fertiliser	14	2.74	0.72	1.91
Average	14	2.27	0.43	1.15
Fungi				
Prior_AD1	471	41.64	0.50	3.10
Prior_AD2	440	39.45	0.51	3.08
Prior_AD3	472	41.01	0.50	3.06
Prior_AD4	427	38.54	0.46	2.77
Prior_Cattle slurry	501	44.64	0.55	3.42
Prior_CAN	515	44.16	0.49	3.04
Prior_No fertiliser	453	40.67	0.47	2.90
Average	536	47.37	0.53	3.34
After_AD1	500	42.77	0.56	3.46
After_AD2	536	46.28	0.63	3.98
After_AD3	515	44.92	0.63	3.91
After_AD4	528	45.87	0.59	3.72
After_Cattle slurry	493	43.01	0.61	3.80
After_CAN	540	47.24	0.61	3.84
After_ No fertiliser	493	44.00	0.63	3.93
Average	601	52.34	0.64	4.12

The most abundant bacterial genera found in the anaerobic digestates (e.g. *Clostridium*, *Cloacimonas*, *Bacteroides*, *Acetivibrio* and *Rikenella*) (Figure 6.2.b.c) and in the cattle slurry (e.g. *Clostridium*, *Bacteroides*, *Petrimonas*, *Proteiniphilum* and *Bifidobacterium*) (Figure 6.2 a) applied to the soils were not detected in the most abundant (≥2%) bacterial genera in the soil by the end of the trial. This indicates that the bacterial communities from the biofertilisers did not establish significant populations in the soil.

In the literature, repeated applications of organic fertilisers (e.g. animal manure and anaerobic digestates) are reported to stimulate the increase of the soil bacterial diversity. In a long-term fertilisation trial, Van der Bom et al. (2018) reported that the application of animal slurry stimulated increases in the soil bacterial richness and diversity. Other long-term trials showed that the application of livestock manure contributed to reducing the negative impacts of the repeated applications of inorganic fertilisers on soil bacterial diversity (Sun et al. 2015; Ding et al. 2016). Relating to anaerobic digestates specifically, Sapp et al. (2015) in a short-term trial of months in a glasshouse reported that their application to soil attenuated the decrease of bacterial diversity due to inorganic fertilisation. Johansen et al. (2013), also in a short-term trial, reported that the applications of anaerobic digestates and cattle slurry only produced small, transient changes in the soil bacterial diversity. Positive effects of repeated application of biofertilisers on soil bacterial diversity seem to be more notable in long-term fertilisation trials. In the current study, which was conducted in a relatively short period of two years, no evident effect of the repeated applications of different types of biofertilisers could be detected on soil bacterial diversity.

The slight increase of the soil bacterial richness, evenness and diversity at the end of the trial for all soils, including unfertilised controls, might be associated with the only significant chemical

change that all the soils had in common: a pH increase due to the lime application. Before the trial, the soils were characterised as being acidic (pH=5.32 ± 0.4), and approximately one month prior to the first fertilisation, one single dose of lime (7.5 ton ha^{-1}) was applied, raising the pH to an average closer to neutral (6.89 ± 0.51). At the end of the trial when soil bacterial diversity was again assessed, the pH was still near neutrality (6.91±0.33). In fact, a much more pronounced effect of this pH change on the soil bacterial community might have been expected, as significant pH changes in the soil are generally reported to have a strong influence on the structure and diversity of the bacterial communities within the soil (Kennedy et al. 2004b; Lauber et al. 2009; Rousk et al. 2010; Zhalnina et al. 2015; Zhang et al. 2017).

Few of the bacterial genera abundant in the biofertilisers were found in the soil they were applied to, probably due to a combination of niche incompatibilities and higher adaptation/competitiveness of native bacterial communities in the soil. In terms of niche incompatibility, possibly the aerobic conditions of the soil surface and lower temperatures compared to the mesophilic anaerobic environment in digesters/slurry tanks made adaptation and survival difficult. The predominant bacterial communities from anaerobic digestates and animal slurry are generally obligate and facultative anaerobes, as seen from the microbial profiling done in this experiment and as reported in Gerardi (2003). The competitiveness of indigenous bacterial groups is generally observed in inoculation trials, where native bacterial strains often display many ecological advantages compared to inoculum from the same species (Bogino et al. 2008; Archana, 2010). Another contributing factor was that the biofertilisers were land spread on the soil surface, where oxygen exposure is generally much higher than in deeper levels of the soil. Oxygen exposure can have deleterious effects on the metabolism and survival of many different types of anaerobes in soil (Fenchel and Finlay, 2008; Schellenberger et al. 2011; Cederlof et al. 2013; Schellenberger et

al. 2013). In relation to temperature, most of the digestates were from mesophilic biogas plants operating between 37-42ºC (Table 5.3), much higher temperatures than the average found in the soils of the region (around 7-9ºC) (Met Éireann, 2019). Temperature is considered a strong selective factor for the growth and activity of microbial communities in soil (Bárcenas-Moreno et al. 2009; Taylor et al. 2017; Alster et al. 2018).

In conclusion, no noticeable effects of repeated applications of any biofertiliser on soil bacterial diversity were observed. The bacterial community from the biofertilisers appeared unable to succeed the native bacterial populations present in the soil, possibly due to niche incompatibilities (e.g. soil oxygen and temperature conditions) and to the expected higher competitiveness/adaptability of the indigenous soil populations.

6.4.2.2 Soil archaeal numbers and diversity

Soil archaeal (16S) GCN was not significantly affected by the type of fertiliser applied to the soil, or by the interaction between fertiliser and time ($p>0.05$) (Figure 6.5). However, time did have a significant effect ($p<0.05$) Soil archaeal GCN increased gradually over the trial, with significant differences ($p<0.05$) found between the soil pre-trial and at the end of the experiment (log 5.1 and 5.7, respectively) (10^5 GCN g^{-1}) (Figure 6.5). Archaeal (16S) GCN were considerably lower than bacterial (16S) and fungal (18S) GCN in all samples ($p<0.05$). As reported and discussed in the bacterial section, the application of anaerobic digestates has been reported to have a variety of results on soil microbial numbers, from no influence some stimulation.

The archaeal community of the soil showed almost no changes in terms of the most abundant genera found in the soil before and after the fertilisation trial (Figure 6.8 a. b). It was noticed that the application of fertilisers, in comparison to the no fertiliser control, stimulated increases in the abundance of *Candidatus Nitrosotalea*, which before the trial had an average in the soils of 47%, and at the end of the trial of 69% (Figure 6.8.b). Soil archaeal richness was not altered in most of the treatments. On the other hand, soil archaeal evenness and diversity were seen to decrease in most of the fertilised soils in comparison to the control with no fertiliser (Table 6.2). This can be attributed to the increases in the abundance of *Candidatus Nitrosotalea* in fertilised soils.

Other predominant archaeal genera in soil were *Methanobrevibacter*, *Candidatus Nitrosoarchaeum*, *Methanobacterium*, *Methanosaeta*, *Nitrososphaera*, *Haloferax*, *Candidatus nitrososphaera*, *Methanospirillum* and *Methanosarcina* (Figure 6.8.a). The archaeal genera found in high abundances in the biofertilisers were *Methanobacterium*, *Methanobrevibacter*, *Methanosaeta* and *Methanosarcina* (Figure 6.3.a.b.c). These genera were also abundant in the soil before the trial (Figure 6.8 a). However, no noticeable increase of these genera in soil was found in the soils fertilised with biofertilisers (Figure 6.8 b).

The predominant archaeal community of the soils was mainly composed of two functional archaeal groups, AOA and methanogens, with AOA in higher abundances and methanogens being the most prominent genera. Among all archaea, the AOA *Candidatus Nitrosotalea* was the most abundant; this genus is classified as an obligate acidophile AOA (Proser and Nicol, 2015), and plays an important role in the nitrification process in acidic agricultural soils (Herbold et al. 2017). However, despite being considered a strict acidophile, its dominance persisted during the pH change that occurred in the soils during the course of the trial. A recent finding suggested that *Candidatus*

Nitrosotalea has special mechanisms for cytoplasmatic pH regulation (Lehtovirta-Morley et al. 2016), which possibly allows it to adapt to a broader pH range in soils. Other AOA (*Candidatus Nitrosoarchaeum, Nitrososphaera, Candidatus Nitrososphaera*) were also found in the soils (Figure 6.8.a.b), but with smaller abundances compared to *Candidatus Nitrosotalea*. AOA together with AOB has a crucial role in the biogeochemical cycle of N, oxidising ammonium to nitrate at the first step of nitrification process (Leininger et al. 2006; Gubry-Rangin et al. 2017; Herbold et al. 2017).

The other dominant functional group of archaeal found in the soils were the methanogens, which in total had 11 genera detected. Methanogens were the major archaeal group in the biofertilisers applied (e.g. *Methanobrevibacter, Methanobacterium, Methanosaeta, Methanospirillum* and *Methanosarcina*); however, their abundances in soils had not increased by the end of the trial. This was possibly due to a combination of niche incompatibilities between species from the same genus, and to the high competitiveness of the AOA *Candidatus Nitrosotalea*. In terms of incompatibilities between species from the same genus found in digestates and soil, sequencing results could not identify differences at the species level; most archaeal species were identified by sp. or spp. Nonetheless, it would be expected that the methanogen archaeal community in the biofertilisers were mostly adapted to an environment with lower oxygen and higher temperatures than found in soil. Most methanogenic archaea are strict anaerobes and thrive under anoxic conditions (Angel et al. 2012), e.g. flooded areas such as paddy fields (Feng et al. 2013; Kitamura et al. 2011; Cadillo-Quiroz et al. 2014). Despite this, some of them can also be found in more aerated soils (Liu et al. 2015). In terms of temperature, archaeal communities may have struggled to adapt when going from mesophilic or thermophilic conditions in a digester or slurry tank to a psychrophilic soil (<15ºC). Conrad et al. (2009) reported that temperature changes have a strong impact on the structure, function and metabolism of methanogens in soils.

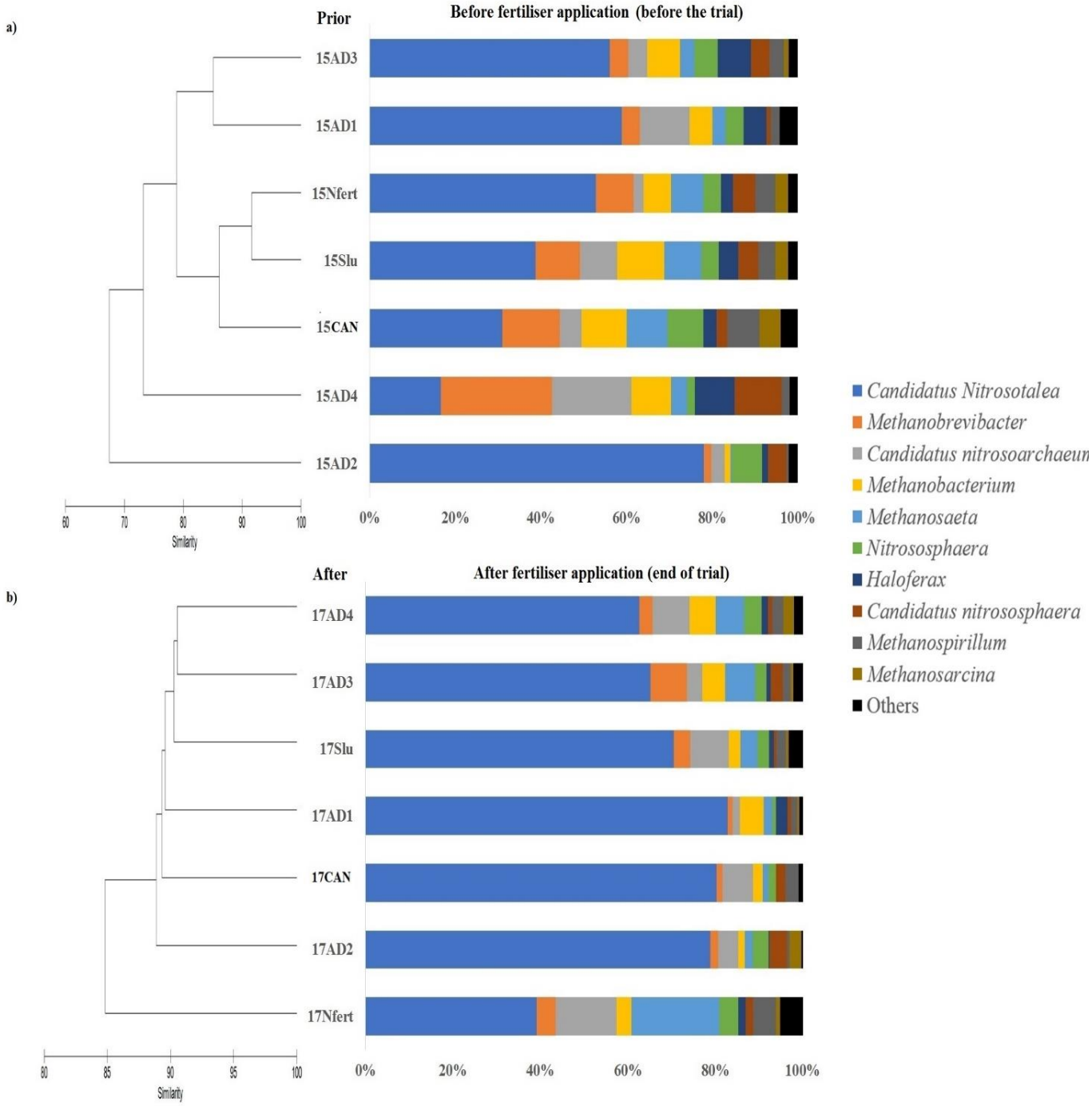


Figure 6.8. Soil archaeal genera diversity before (a) and after (b) the fertilisation trial. AD= anaerobic digestate, Slu= Cattle Slurry, CAN = calcium ammonium nitrate, Nfert= No fertilisation. 15 = before application of treatment. 17= after repeated application (two seasons) of treatment. Similarity based on Bray-Curtis similarity matrix. *Others = microbial genera found with (< 2%) of the DNA sequences.

In conclusion, the application of anaerobic digestates and CAN increased the amounts of *Candidatus Nitrosotalea* in the soil in comparison to the control. This acidophile AOA was the dominant archaeal genus in the soil studied. Archaeal genera found in higher abundances in the biofertilisers resulted in no detectable increase in their abundances in soils after the fertilisation trial, which possibly indicates niche incompatibilities and low potential to survive in the soil. Two archaeal functional groups dominated the grassland soil studied, AOA and methanogens, with AOA showing higher dominance and methanogens a higher number of genera.

6.4.2.3 Soil fungal numbers and diversity

Soil fungal (18S) GCN was not significantly affected by the type of fertiliser applied to the soil, or by the interaction between fertiliser and time ($p>0.05$). Soil fungal (18S) had comparable values to bacterial (16S) GCN in most of the samples and was consistently higher than archaeal (16S) GCN ($p<0.05$). However, time did have a significant effect ($p<0.05$). Soil fungal GCN showed a significant ($p<0.05$) increase from before the trial (10^7 GCN g^{-1}) to the end (10^8 GCN g^{-1}) (Figure 6.5). Soil fungal CFU in the soil was affected by the interaction between the type of fertiliser and time ($p<0.05$) (Figure 6.6 b). Higher soil fungal CFU were observed in soils treated with AD1 in comparison to the controls ($p<0.05$) (Figure 6.6 b), at five months. At ten months, higher fungal CFU was noticed in the soils treated with AD2 and AD3 ($p<0.05$). At 15 months, soils receiving biofertilisers showed higher fungal CFU than the controls treated with CAN and no fertiliser ($p<0.05$). Once again, however, at the end of the trial, no significant differences were detected between the treatments ($p>0.05$). As discussed in the bacterial section, the application of anaerobic digestates has been reported to have a variety of results on soil microbial numbers. In terms of fungi, Wentzel and Joergensen (2016b) observed negative effects of anaerobic digestates application on

soil fungi, while Walsh et al. (2012b) reported that application of anaerobic digestates did not influence fungi in soil.

In terms of diversity, the soil fungal dominance markedly changed between after the start and the end of the trial in all soils; however, no noticeable relationship with the type of fertilisation could be noticed, as unfertilised soils also showed the same fungal shift experienced by the soils treated with anaerobic digestates, cattle slurry and CAN (Figure 6.9 b). Before the trial, the soil was dominated by four genera: *Arthrinium* (22%), *Mrakiella* (12%), *Mortierella* (12%) and *Cryptococcus* (12%) (Figure 6.9 a). After the fertilisation trial, *Arthrinium* and *Mrakiella* showed very low abundance, 1.5% and 0.14%, respectively, disappearing from the list of genera that showed at least 2% of abundance. The genus *Cryptococcus* had its abundance reduced to 3%, with only *Mortierella* (19%) still in considerable abundance. A slight increase of the soil average richness, evenness and diversity was noticed in most of the treatments including the control with no fertiliser when comparing between before and after the trial (Table 6.2) . This increase of the diversity indices indicates that despite no influence of the type of fertilisation, most of the soils experienced an increase in the number of fungal genera associated with a better distribution of the abundance between them. As observed for bacterial and archaeal genera, fungal genera found in higher abundances in the biofertilisers did not tend to successfully survive in the soils after the fertilisation trial. Genera abundant in cattle slurry such as *Cyllamyces*, *Neocallimastix, Piromyces* and *Anaeromyces* (Figure 6.4 a) showed no predominance in the soils that received cattle slurry applications (Figure 6.9 b). The same pattern was observed for anaerobic digestates, where dominant genera such as *Acaulospora*, *Scedosporium*, *Hanseniaspora*, *Kazachstania, Penicillium* and *Saccharomyces* (Figure 6.4 b.c), had very low abundances in the soils that received anaerobic digestate applications (Figure 6.9 b).

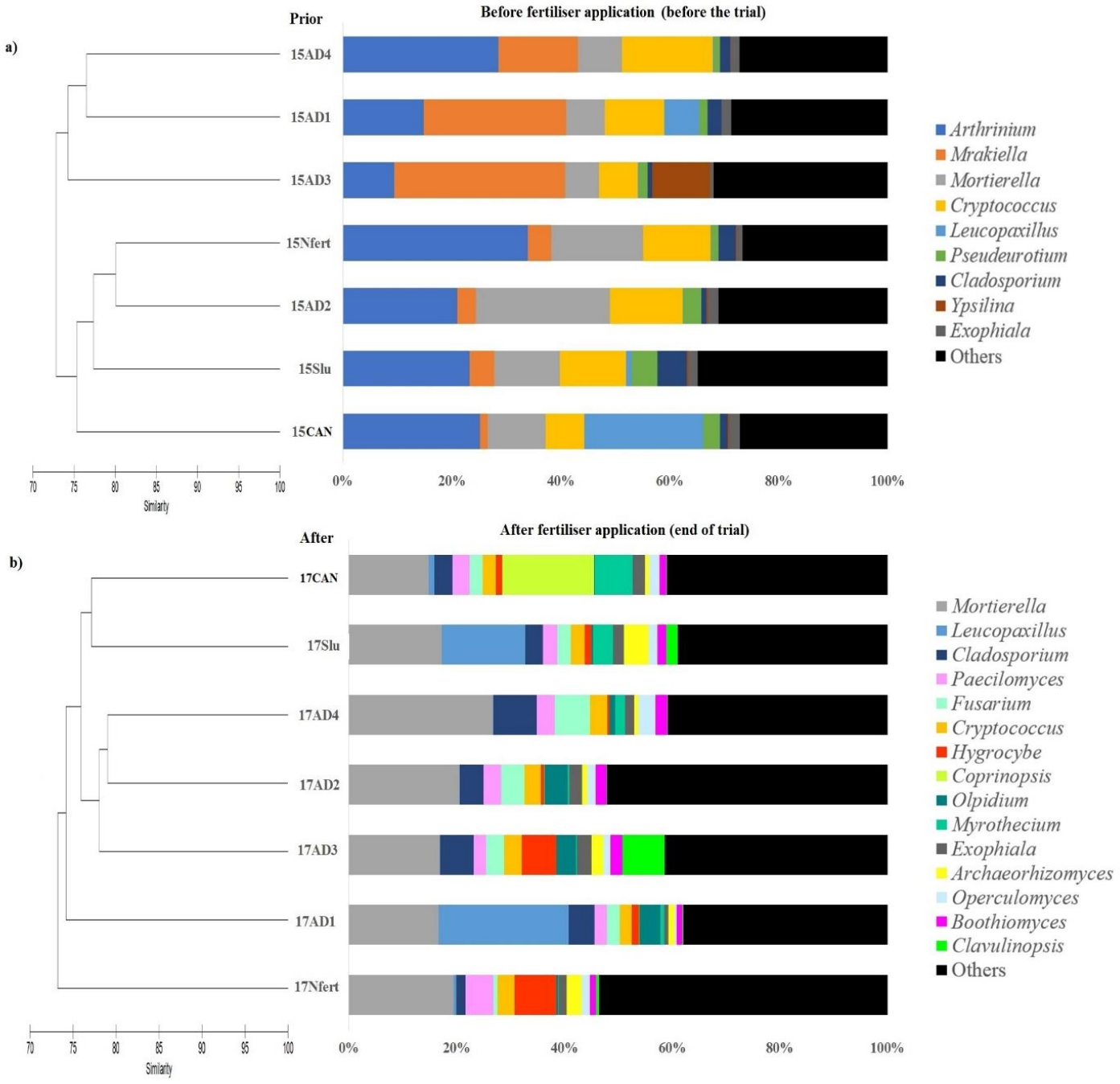


Figure 6.9. Soil fungal genera diversity before (a) and after (b) the fertilisation trial. AD= anaerobic digestate, Slu= Cattle Slurry, CAN = calcium ammonium nitrate, Nfert= No fertilisation. 15 = before application of treatment. 17= after repeated application (two seasons) of treatment. Similarity based on Bray-Curtis similarity matrix. *Others = microbial genera found with (< 2%) of the DNA sequences.

The soil fungal community, which had been dominated by four genera before the trial, changed to a more diverse fungal community after the trial; this change was possibly associated with the increase in soil pH due to the application of lime that all soils, including no fertiliser control, received (Table 5.5). Responses of soil fungal communities to the lime application or soil pH variations are generally reported to be less than normally observed for bacterial communities. Nevertheless, this pattern has been better observed when the microbial diversity of the soil is evaluated at higher taxonomic ranks (e.g. phylum), where variations are less detectable. Rousk et al. (2010) reported that soil fungal diversity, analysed at the class level, was moderately affected by soil pH differences (range 4 to 8). However, when the same fungal community was analysed at the order level, detectable effects of pH change on fungal diversity could be better observed. Cassman et al. (2016) reported that fungal phyla evenness and diversity from grassland soils were not influenced by lime application, and similar results found by Kennedy et al. (2005) reported that pH changes due to the application of lime in grassland had little effects on fungal community structure. Guo et al. (2012) reported that soil AMF species had their diversity reduced in response to liming.

The saprotrophic genus *Mortierella* was a dominant species before and after the trial. According to Frac et al. (2018), *Mortierella* is one of the most abundant fungal genera in fertilised soils. Detheridge et al. (2016) also reported that *Mortierella* is one of the most abundant fungal genera in temperate soils under different types of forage cultivation (e.g. grasses, clover). Li et al. (2018) reported the species *Mortierella elongata* as the most successful organism responding to long-term organic fertilisations and application of lime in soils. Results of this trial corroborate to these findings, indicating that *Mortierella* is a well-adapted soil fungus, possibly performing essential roles in nutrient cycles via organic matter breakdown, especially when the organic substrate availability is increased in the soil.

As already described for bacterial and archaeal communities, the fact that most of the fungal genera dominant in the biofertilisers failed in the soil was possibly linked to niche incompatibilities (e.g. aerobic conditions and lower temperatures of the soil), and to a possible higher adaptation and competitiveness of the soil native species. Regarding niche incompatibilities, the dominant fungal community from cattle slurry were strict anaerobes from the phylum *Neocallimastigomycota* (Figure 6.4 a). Species from this phylum have very low chances of surviving within non-gut niches (Gruninger et al. 2014). Regarding temperature, the psychrophilic temperature (<15ºC) of the soil could have been a stressor for a fungal community that came from a mesophilic environment where biofertilisers were produced. Another factor that should be considered is the differences between species from the same genus found in the biofertilisers and the soil. For example, in anaerobic digestates the genus *Acaulospora* was one of the most abundant genera 22 ± 13%; this genus was mainly composed of species of *Acaulospora kentinensis* (20 ± 13%). In soils after the trial, a total of sixteen different species of *Acaulospora* were found.

In conclusion, the application of anaerobic digestate showed no noticeable effects on the fungal diversity of the soil. Also, fungal genera with higher abundance in the biofertilisers showed no survival or establishment in soil. The application of lime drove pH increases, which possibly influenced the very noticeable changes in the whole fungal community in the soils evaluated. These changes observed included a whole change of the most dominant fungal genera in the soil and increases of diversity indexes.

6.4.3 Ryegrass sward growth and the microbiology of the soil

The application of anaerobic digestates and cattle slurry drove comparable grass growth performance and yields (Figure 5.3, Figure 5.4, Figure 5.5) with higher averages than the controls with CAN and no fertiliser. The input levels of NPK had a substantial role (Figure 5.6, Figure 5.7). In terms of microbial stimulation of the soil, the application of the biofertilisers did not seem to cause many changes in the soil microbial numbers and diversity in the ryegrass sward. In specific periods straight after application of the biofertilisers, bacterial and fungal CFU of soil were elevated (Figure 6.6). In terms of diversity, the application of biofertilisers and/or solo CAN (N inputs) seems to have stimulated increases of the abundance of the archaeal genera *Candidatus Nitrosotalea*, an AOA (Figure 6.8). Abundant bacterial, archaeal and fungal genera found in the biofertilisers showed no increases of their abundances in the soil by the end of the trial, evidencing a low capability to survive and compete once in soil. The results of soil/digestate GCN, accessed by qPCR, and diversity which was accessed by DNA NGS were complementary to each other, where a microbial profile in terms of quantification and diversity of the communities could be explored. In fact, both analyses of soil microbial communities showed no detectable effects of the biofertiliser repeated applications over microbial abundance and diversity after two-seasons trial.

In the literature, some specific types of live microorganisms present in the biofertilisers are reported to influence plant nutrition and growth, especially by the stimulation of the plant rhizosphere and by the solubilisation of essential nutrients immobilised on soil particles or in the organic matter (Mohammadi and Sohrabi, 2012). For example, Wu et al. (2005) reported that the application of biofertilisers containing AMF and different types of bacteria involved in N-fixation and solubilisation P and K in the soil stimulated the growth of maize. In this study, a potential AMF (*Acaulospora*) (Oehl et al. 2010; Krüger et al. 2012) that was found in most of the anaerobic

digestates applied (Figure 6.4 b.c), was not detectable in the soil after two years of repeated applications (Figure 6.9 b). The same pattern was seen with *Clostridium*; highly abundant in biofertilisers (Figure 6.2) and reported for having species with beneficial effects on plant growth (Polyanskaya et al. 2002), the genus was not seen to increase in soils treated with biofertilisers (Figure 6.7 b). *Bacillus* and *Pseudomonas*, both known PGPB (Qi et al. 2017; Iwasaki et al. 2018), also found in the biofertilisers (Figure 6.2), *Bacillus* with 2% in anaerobic digestates, and *Pseudomonas* with 5% in cattle slurry, showed no increase in their abundance in treated soils (Figure 6.7). *Bacillus* was already present in all the soils before the trial commenced (Figure 6.7 a), and its abundance remained almost unaltered (Figure 6.7 b).

Based on these results, this research trial concludes that there was no evidence for microbial biostimulation in the soil after repeated applications of anaerobic digestates as a factor that contributed to plant growth responses observed in the ryegrass swards. The main effects on ryegrass growth were partially associated with the inputs of plant macronutrients (NPK). Nevertheless, there is a high chance that some factors not measured, such as nutrient bioavailability of each digestate and their particular interactions with the environment (plant-soil-climate), contributed to the fact that different types of anaerobic digestates drove comparable plant growth responses in ryegrass, despite having differences in their chemical composition.

6.5 Conclusions

Liquid anaerobic digestates contained a microbial community with high number of bacterial 16S genes, fewer numbers of archaeal 16S genes, and much lower numbers of fungal 18S gene copies. Many types of microorganisms with potential for agronomic use were found in the anaerobic

digestates including cellulolytic organisms, N-fixing bacteria, PGPB, nitrifiers and denitrifiers, AMF, and saprotrophic organisms. However, few of them showed high abundances in these biofertilisers. Many dominant microbial populations in the biofertilisers, especially from archaeal communities, were strict anaerobes; their survival after land spreading possibly depended on whether the soil conditions were anoxic enough. Soil bacterial, fungal and archaeal GCN did not differ between biofertilisers and the control treatments. Soil bacterial and fungal CFU were temporally stimulated by the application of biofertilisers, however, these effects were minor. The application of anaerobic digestates showed few detectable impacts on soil microbial diversity, with no significant differences between different types of digestates and the controls. The microbial community found in higher abundances in the biofertilisers failed to establish in high numbers in soils, possibly unable to outcompete the native soil microbial populations ecologically. This may be due to a combination of niche incompatibilities (e.g. aerobic conditions and lower temperature of the soil), and to an expected higher adaptation and competitiveness of the indigenous species of the soil. There was little evidence that the plant growth response to biofertilisers (Chapter 6) was driven by microbial stimulation of bacterial or fungal communities of the soil. For archaea, the application of any fertiliser stimulated increases of *Candidatus Nitrosotalea*, an AOA that possibly plays an important role in the N cycle and availability in soil.

Chapter 7 : General conclusions

The findings of this PhD work indicate that all anaerobic digestates evaluated showed potential for being used as biofertilisers. They carry different amounts of essential plant macro and micronutrients (e.g. N, P, K, Ca, Mg, Fe, Mn, B, Cu, Zn, Ni, Co, Na). However, they can differ widely in their physical-chemical and microbial compositions, particularly commercial digestates, as these are collected from biogas plants often operating with different types of feedstocks. This is a possible issue when attempting to produce standard fertilisation rates for their use; therefore, it is highly recommended to farmers and practitioners to perform chemical analysis of anaerobic digestates before land spread and use anaerobic digestates as fertiliser. Access to historical average chemical compositions of the digestates over time should be requested, as this would help to improve the efficiency of their use. The use of on-farm biogas systems might provide less variation of the chemical composition of the anaerobic digestates, as less variation in feedstock and operational conditions is expected in comparison to commercial biogas plants.

In relation to the security and safety of the use and land spreading of anaerobic digestates, most of the anaerobic digestates analysed in this trial showed acceptable levels of potentially toxic elements, based on recommendation limits determined by government agencies from Ireland. However, anaerobic digestate from a wastewater treatment plant showed a tendency to display higher levels of potentially toxic elements, above recommended levels, and the use of such digestates must be carefully analysed. Another security issue related to the use of anaerobic digestates is the potential presence of pathogenic organisms. In our study, pathogen indicators (e.g. *Salmonella spp.* and *E. coli*) were within the recommended limits suggested by Irish agencies in most of the anaerobic digestates tested. Nevertheless, it is recommended to request and verify which practices the biogas plants perform to avoid the presence and survival of potentially pathogenic organisms (e.g. pre/post digestion pasteurisation, sterilisation). Another safety issue is the

phytotoxicity of anaerobic digestates. In our field and greenhouse trials, the use of anaerobic digestates did not show any signs of phytotoxicity on established plants/swards of perennial ryegrass and white clover; however, seed germination tests suggested that some types of anaerobic digestates can negatively impact on the germination of cress seeds. This phytotoxicity was linked to the electrical conductivity of the digestates and was reduced when they were diluted in water.

In terms of the agronomic potential of the microbial communities present in anaerobic digestates, it was observed that the majority of digestates were dominated by bacterial, followed by archaeal and in lower quantities fungal, organisms and/or genes. DNA sequences from microorganisms with agronomic importance were identified in the anaerobic digestates studied, including: N-fixing bacteria, plant-growth promoting bacteria (PGPB), nitrifying and denitrifying bacteria, arbuscular mycorrhizal fungi (AMF), cellulolytic microbes, methanogens and saprotrophic organisms; however, most of these were found in very low DNA abundances. One type of AMF genera (*Acaulospora*), was found in three anaerobic digestates AD2, AD3 and AD4 in high abundances. Methanogens were also found in considerable amounts in the digestates.

Plant growth trials indicated that anaerobic digestates with different chemical compositions, when equally balanced in terms of dry matter, could drive comparable forage yields responses in ryegrass, mixed ryegrass/white clover and white clover swards/plants. These results are significant in practical terms, as the establishment of fertilisation practices for this type of fertiliser might be based on the inputs of dry matter, combined with chemical analysis. Ryegrass plants showed positive growth responses to the application of anaerobic digestates. White clover plants under mixed consortia were suppressed by ryegrass under digestate application, while solo white clover plants fertilised with digestates showed no changes compared to the application of no fertiliser.

Despite comparable growth responses between digestates with different compositions, plant growth responses, especially in ryegrass-dominated swards, were associated with the inputs of plant macronutrients (NPK). In both field and glasshouse trials, the application of anaerobic digestates contributed to increase levels of, or reduce losses of, total N, available P, and available K in the soils. The pools of other soil nutrients were not affected by the repeated applications of anaerobic digestates. The results of these short-term experiments could not identify significant changes in soil microbial numbers and diversity due to the application of anaerobic digestates. However, it is expected that long-term experiments with repeated applications of anaerobic digestates might drive different responses in the soil microbiology. Also, other factors might influence these results such as the type of soil used, geographical/environmental conditions of the trial, type of digestate application (e.g. surface vs. injected), exposition to pesticides/herbicides, type of crops used, tillage practices, combined fertilisation practices (e.g. lime, use of inorganic fertiliser) among other possible environmental and management practices. Most of the dominant microbial genes found in the anaerobic digestates from the three domains (bacteria, archaea and fungi) could not replace the native microbial populations from the soil, possibly due to niche incompatibilities and/or competitiveness of indigenous soil microbes. In this study, no association between the application of anaerobic digestates and the microbial stimulation of the soil could be detected; consequently, no relationship between the microbial stimulation of the soil via application digestates and plant growth could be observed.

The results found in terms of plant growth, soil chemistry and for microbial communities (no detectable effects) indicate an expected pattern for the short-term effects of the application of different types of anaerobic digestates. However, is strongly recommended that more research be done testing different kinds of anaerobic digestates with specific crops and under different types of

environmental conditions and time, in order to gain more specific information on the effects of these types of biofertilisers. The use of different crop systems possibly can drive different interactions between these types of fertiliser and respective plant-growth responses, as well as dynamics of their nutrients in the soil and effects on the soil microorganisms. Environmental conditions such variations from a temperate and tropical climate, can possibly have a huge impact on the dynamic of the effects of digestates as biofertilisers; for example, some soils might have similar temperature conditions from the biogas reactors which the digestates were produced, which might increase the chances of survival of some microbes added via biofertilisation. It is highly recommended that more research be done with these types of fertilisers under the most diverse conditions possible to increase the understanding of their dynamics and general patterns. As biogas production is widely expanding worldwide, there will be an increase in the supply and use of anaerobic digestates, and more research will be required in terms of the use of these complex biofertilisers. At the public level, farmers and practitioners must be aware of following the guidelines for their use (e.g, fertilisation rates), and be aware of specific legislation/guidelines for avoiding issues related to pollution, nutrient overload and other environmental impacts.

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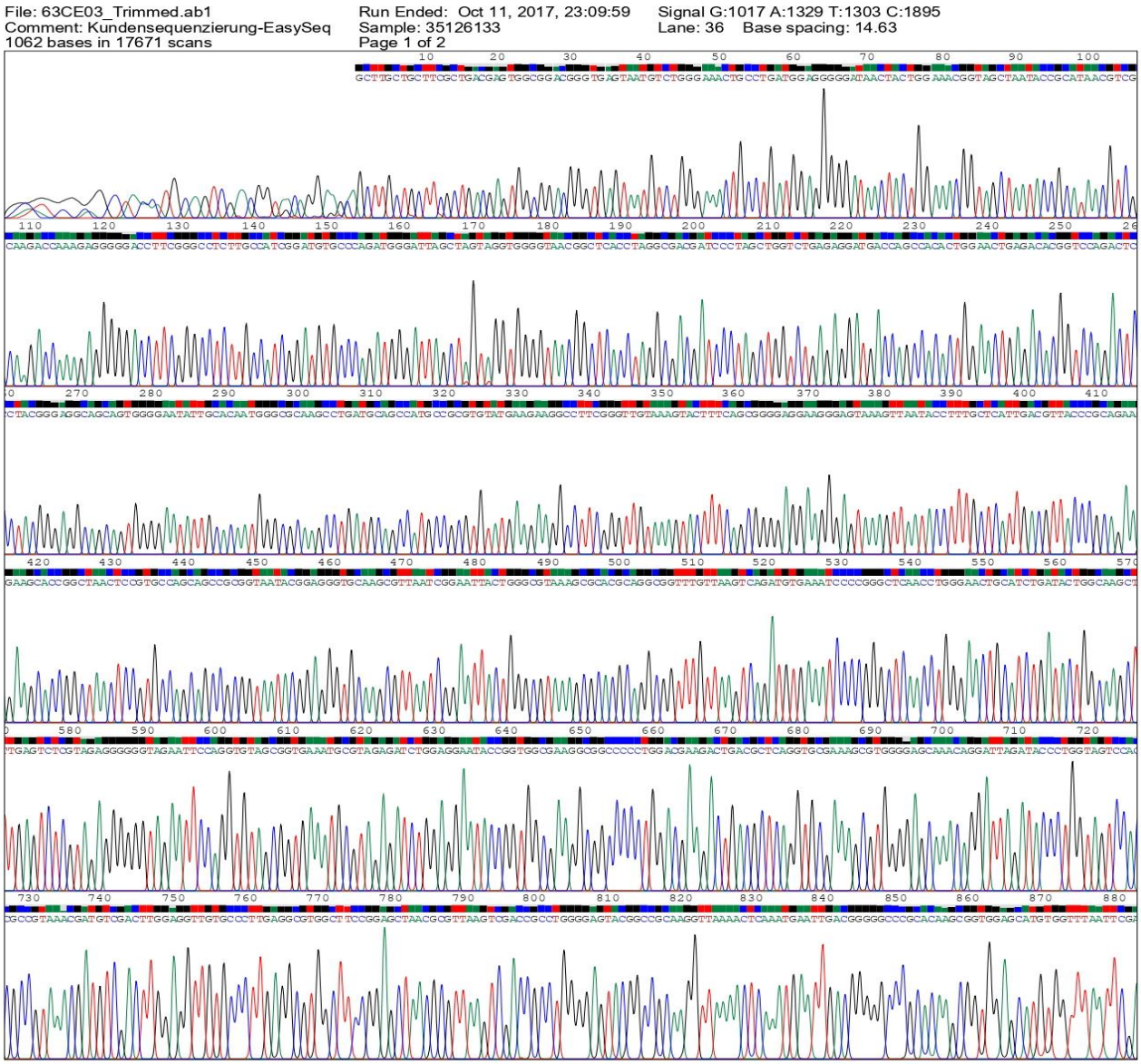
Appendix

8.1 PCR product sequencing of the standards and external controls. GATC Biotech (Konstanz, Germany).

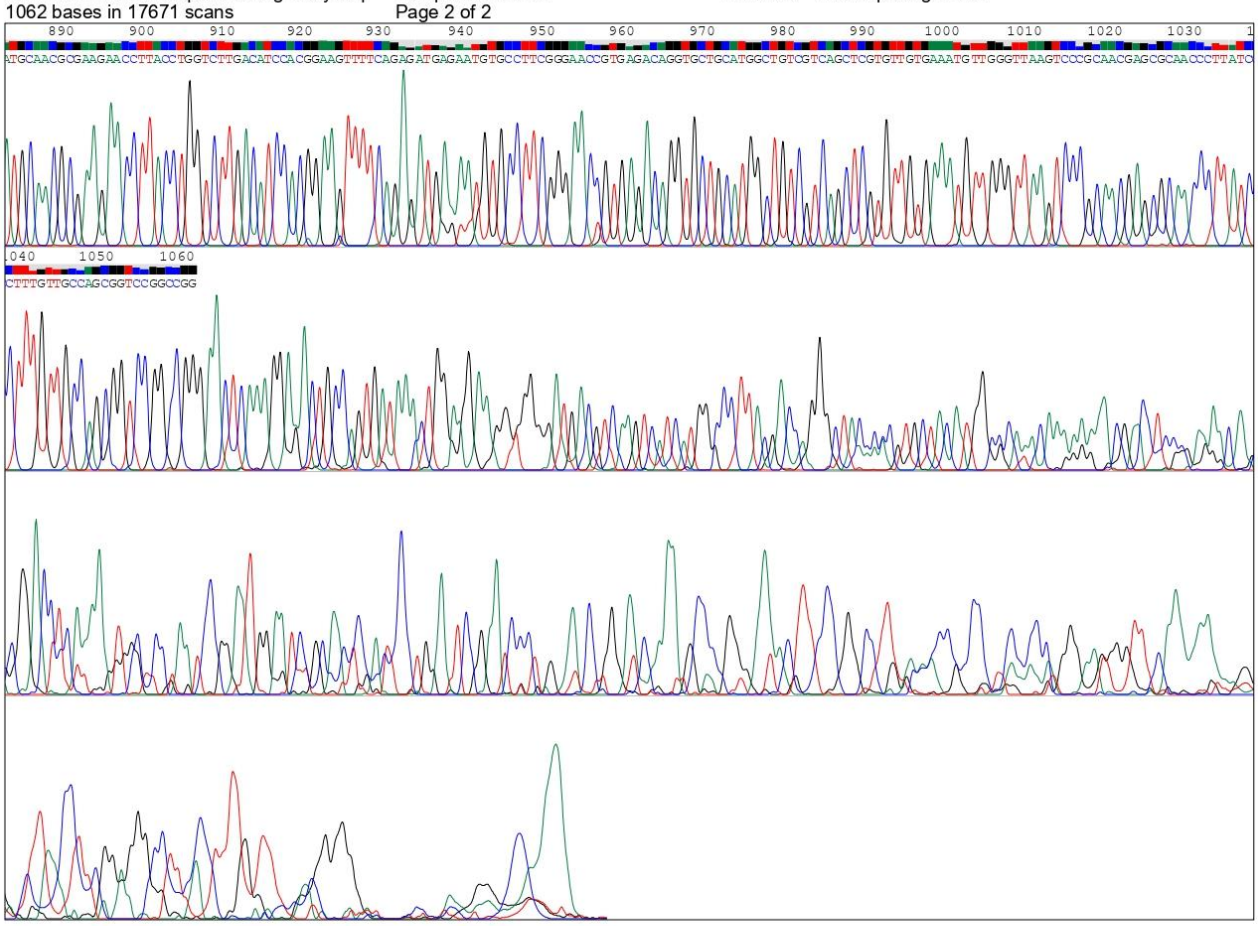
Escherichia coli



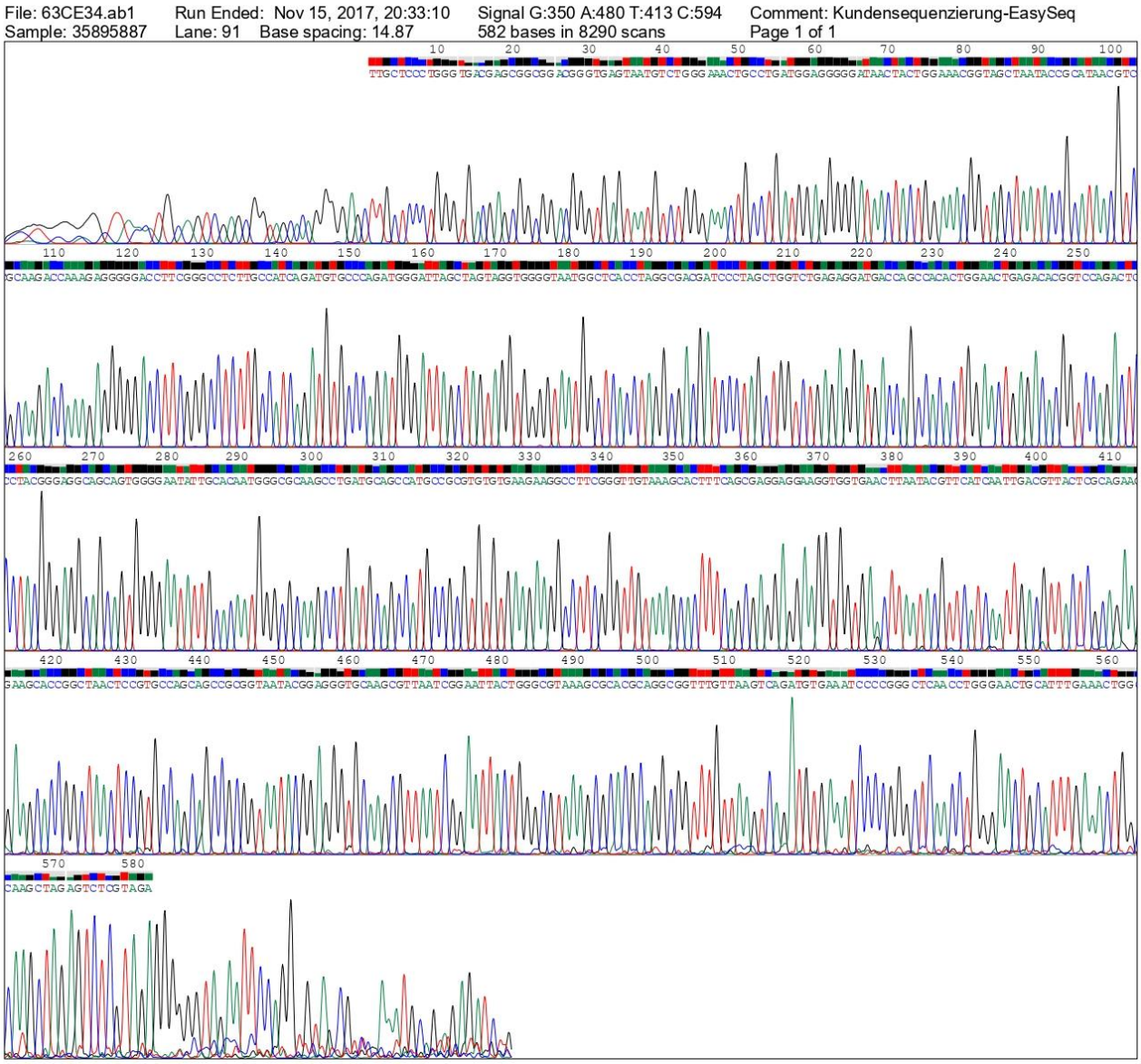
File: 63CE03_Trimmed.ab1
Comment: Kundensequenzierung-EasySeq
1062 bases in 17671 scans

Run Ended: Oct 11, 2017, 23:09:59
Sample: 35126133
Page 2 of 2

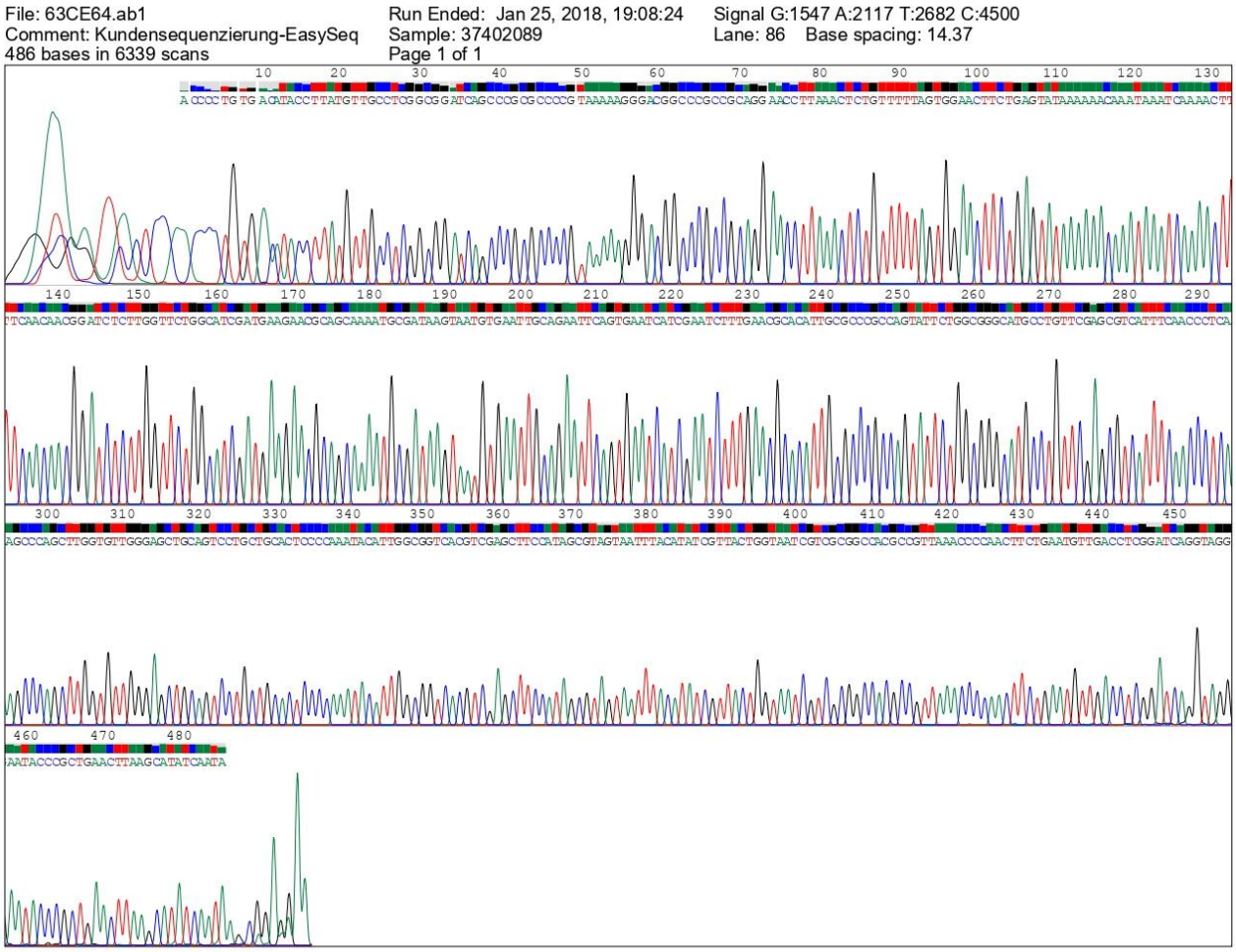
Signal G:1017 A:1329 T:1303 C:1895
Lane: 36 Base spacing: 14.63



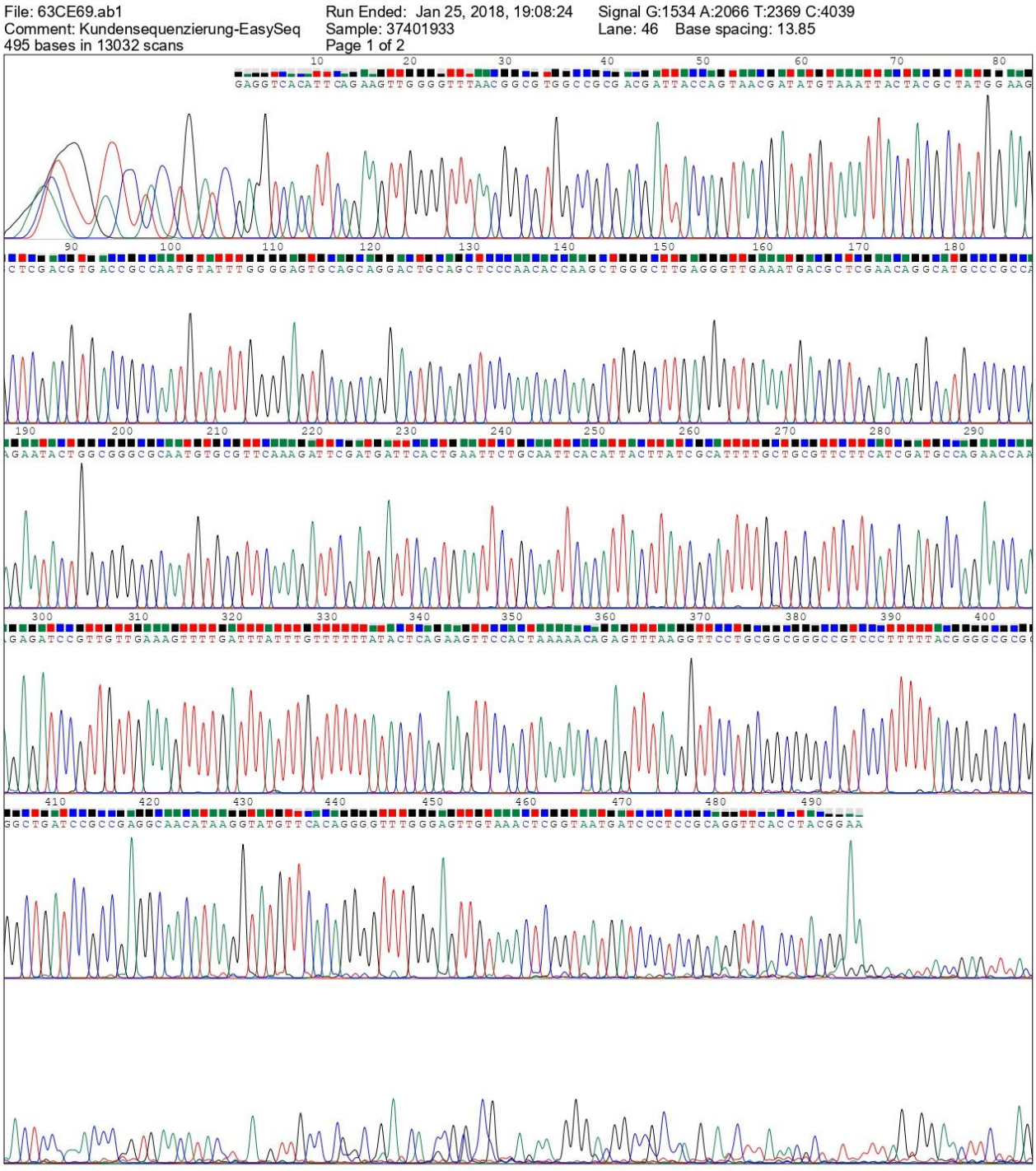
Serratia marcescens



Fusarium sp.


File: 63CE64.ab1
Comment: Kundensequenzierung-EasySeq
486 bases in 6339 scans
Run Ended: Jan 25, 2018, 19:08:24
Sample: 37402089
Page 1 of 1
Signal G:1547 A:2117 T:2682 C:4500
Lane: 86 Base spacing: 14.37

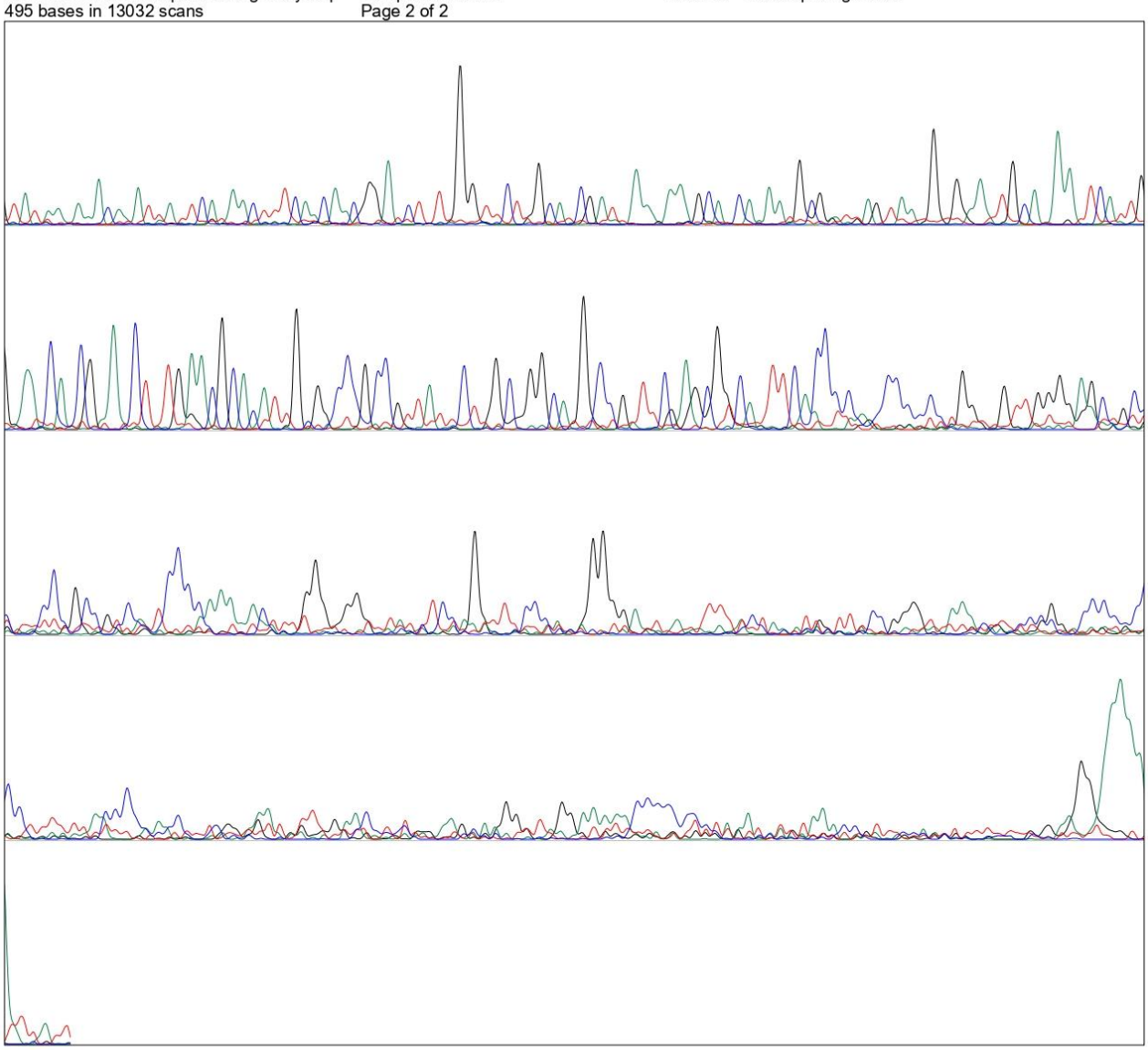
Penicillium sp.



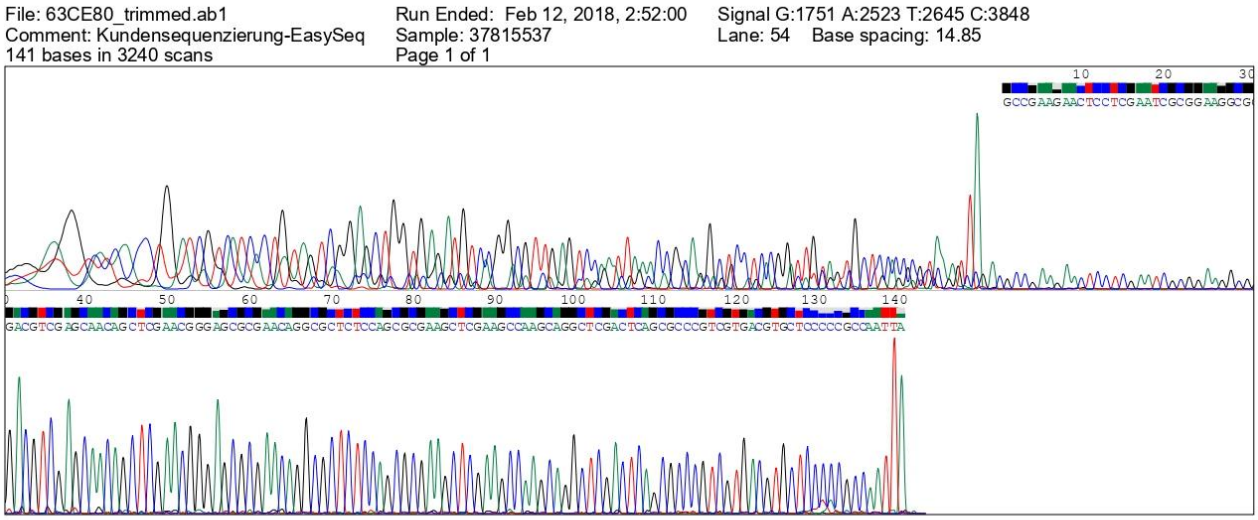
File: 63CE69.ab1
Comment: Kundensequenzierung-EasySeq
495 bases in 13032 scans

Run Ended: Jan 25, 2018, 19:08:24
Sample: 37401933
Page 2 of 2

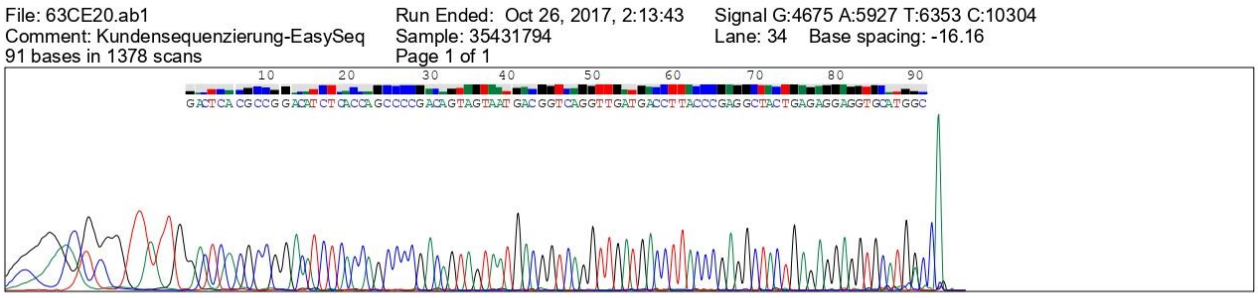
Signal G:1534 A:2066 T:2369 C:4039
Lane: 46 Base spacing: 13.85



Haloferax denitrificans



Halobacterium salinarum



8.2 FASTA file PCR products sequencing of the standards and external controls. GATC Biotech (Konstanz, Germany).

Escherichia coli

>35126133

GCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG
GATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGG
GCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCG
ACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATG
AAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCT
CATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
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ATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAG
AATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCT
GGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTA
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TCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTC
CGGCCGG

Serratia marcescens

>35895887

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TAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCC

TCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACG

ATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG

GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAG

AAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACGTTCATCAAT

TGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC

AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATC

CCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGA

Fusarium sp.

>37402089

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CCGCAGGAACCTTAAACTCTGTTTTTAGTGGAACTTCTGAGTATAAAAAACAAATAAATCAAAACTT

TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAA

TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCAT

GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGGGAGCTGCAGTCCTGCTGCACT

CCCCAAATACATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAATTTACATATCGTTACTGGTAAT

CGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCT

GAACTTAAGCATATCAATA

Penicillium sp.

>37402065

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CAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGAG

ATCGGGGGACGGGGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATG

CCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTTGCAATTC

ACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGT

TTTAAATAATTTATATTTTCACTCAGACTTCAATCTTCAGACAGAGTTCGAGGGTGTCTTCGGCGGG

CGCGGGCCCGGGGGCGTAAGCCCCCCGGCGGCCAGTTAAGGCGGGCCCGCCGAAGCAACAAGGTA

AAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCTT

Note: For archaea identification only a short portion of the 16S was sequenced (qPCR primers were used) as cultures were purchased from certified external culture banks

Haloferax denitrificans

>37815537

TGGATGTCTCACCTGATCGTCATCACTGTAGTCGGAGCTGGTGAGATGTCCGGCGTTGAGTCCAATT

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GGAAGGCGGACGTCGAGCAACAGCTCGAACGGGAGCGCGAACAGGCGCTCTCCAGCGCGAAGCTC

GAAGCCAAGCAGGCTCGACTCAGCGCCCGTCGTGACGTGCT

CCCCCGCCAATTA

Halobacterium salinarum

>35431797

ACCGGCAGGTATCACCTGACCGTCATTACTACTGTCGGGGCTGGTGAGATGTCCGGCGTTGAGTCCA

ATTAAACCGCAGGCTCCTCCGGTTGTAGTGCTCCCCCGCTAATTC

8.3 Example of the data of the qPCR standards curves generated.

Bacterial 16S

Well	16S	Task	Qty	DNAlog	Ct	Ct average	Ct Stddev	Tm
A1	E.coli	Standard	3.00E+05	5.48	16.5962	16.61475	0.01855	80
A2	E.coli	Standard	3.00E+05	5.48	16.6333	16.61475	0.01855	80
A3	E.coli	Standard	3.00E+04	4.48	20.2707	20.3396	0.0689	80.3
A4	E.coli	Standard	3.00E+04	4.48	20.4085	20.3396	0.0689	80.3
A5	E.coli	Standard	3.00E+03	3.48	24.2818	24.23	0.0518	80.3
A6	E.coli	Standard	3.00E+03	3.48	24.1782	24.23	0.0518	80.3
A7	E.coli	Standard	3.00E+02	2.48	28.6203	28.16425	0.45605	80
A8	E.coli	Standard	3.00E+02	2.48	27.7082	28.16425	0.45605	80.3
A9	E.coli	Standard	3.00E+01	1.48	31.0364	30.94695	0.08945	80
A10	E.coli	Standard	3.00E+01	1.48	30.8575	30.94695	0.08945	80
A11	E.coli	Standard	3.00E+00	0.48	32.0387	32.52405	0.48535	81.9
A12	E.coli	Standard	3.00E+00	0.48	33.0094	32.52405	0.48535	81.9
B1	Serratia	Standard2	1.27E+06	5.48	12.6684	12.76195	0.09355	80.7
B2	Serratia	Standard2	1.27E+06	5.48	12.8555	12.76195	0.09355	80.9
B3	Serratia	Standard2	1.27E+05	4.48	16.7349	16.57355	0.16135	80.9
B4	Serratia	Standard2	1.27E+05	4.48	16.4122	16.57355	0.16135	80.9
B5	Serratia	Standard2	1.27E+04	3.48	20.6249	20.5119	0.113	80.9
B6	Serratia	Standard2	1.27E+04	3.48	20.3989	20.5119	0.113	80.9
B7	Serratia	Standard2	1.27E+03	2.48	24.3913	24.47785	0.08655	80.9
B8	Serratia	Standard2	1.27E+03	2.48	24.5644	24.47785	0.08655	80.9
B9	Serratia	Standard2	1.27E+02	1.48	29.6798	29.66355	0.01625	80.9
B10	Serratia	Standard2	1.27E+02	1.48	29.6473	29.66355	0.01625	80.7
B11	Serratia	Standard2	1.27E+01	0.48	31.4816	31.30155	0.18005	80.7
B12	Serratia	Standard2	1.27E+01	0.48	31.1215	31.30155	0.18005	80.3
H10		NTC			33.071	33.509	0.43387238	82.2
H11		NTC			34.100	33.509	0.43387238	78.8
B12		NTC			33.356	33.509	0.43387238	79.7

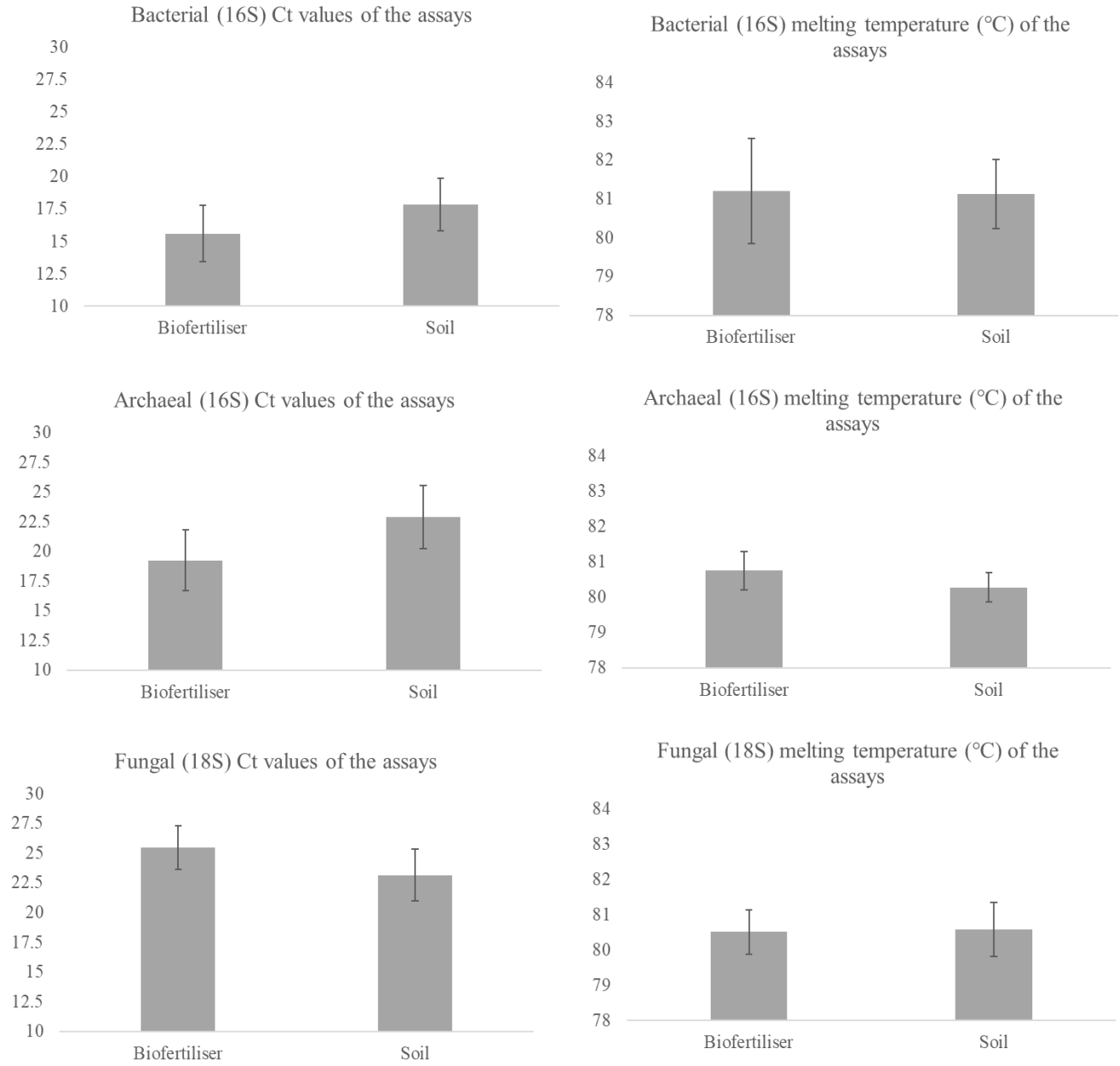
Archaeal 16S

Well	16S	Task	Qty	DNAlog	Ct	Ct average	Ct Stddev	Tm
A1	H.Deni	Standard	3.00E+06	6.47712	13.5832	13.58465	0.00145	79.3
A2	H.Deni	Standard	3.00E+06	6.47712	13.5861	13.58465	0.00145	79.3
A3	H.Deni	Standard	3.00E+05	5.47712	15.2173	15.27065	0.05335	79.6
A4	H.Deni	Standard	3.00E+05	5.47712	15.324	15.27065	0.05335	79.6
A5	H.Deni	Standard	3.00E+04	4.47712	18.8789	18.0427	0.8362	79.3
A6	H.Deni	Standard	3.00E+04	4.47712	17.2065	18.0427	0.8362	79.6
A7	H.Deni	Standard	3.00E+03	3.47712	23.5367	22.42315	1.11355	79.3
A8	H.Deni	Standard	3.00E+03	3.47712	21.3096	22.42315	1.11355	79.6
A9	H.Deni	Standard	300	2.47712	26.1268	26.12715	0.00035	79.3
A10	H.Deni	Standard	300	2.47712	26.1275	26.12715	0.00035	79.3
A11	H.Deni	Standard	30	1.47712	29.3214	29.55795	0.23655	78.7
A12	H.Deni	Standard	30	1.47712	29.7945	29.55795	0.23655	79
B1	Hallo	Standard	3.00E+06	6.47712	15.1527	14.8579	0.2948	80.2
B2	Hallo	Standard	3.00E+06	6.47712	14.5631	14.8579	0.2948	80.6
B3	Hallo	Standard	3.00E+05	5.47712	18.3726	18.22905	0.14355	80.6
B4	Hallo	Standard	3.00E+05	5.47712	18.0855	18.22905	0.14355	80.6
B5	Hallo	Standard	3.00E+04	4.47712	22.055	22.2535	0.1985	80.6
B6	Hallo	Standard	3.00E+04	4.47712	22.452	22.2535	0.1985	80.6
B7	Hallo	Standard	3.00E+03	3.47712	25.1237	25.1156	0.0081	80.6
B8	Hallo	Standard	3.00E+03	3.47712	25.1075	25.1156	0.0081	80.6
B9	Hallo	Standard	300	2.47712	28.4654	28.3216	0.1438	80.2
B10	Hallo	Standard	300	2.47712	28.1778	28.3216	0.1438	80.2
B11	Hallo	Standard	30	1.47712	30.8085	30.58745	0.22105	79.9
B12	Hallo	Standard	30	1.47712	30.3664	30.58745	0.22105	79
H9		NTC			31.3789	31.656	0.3168	79.3
H10		NTC			32.0992	31.656	0.3168	80.2
H12		NTC			31.4889	31.656	0.3168	79

Fungal 18S

Well	16S	Task	Qty	DNAlog	Ct	Ct average	Ct_StdDev	Tm
A1	Fusarium	Standard	6.350E+09	9.80277	6.13733	6.490735	0.353405	80.6
A2	Fusarium	Standard	6.350E+09	9.80277	6.84414	6.490735	0.353405	80.6
A3	Fusarium	Standard	6.350E+08	8.80277	7.5663	7.790775	0.224475	80.6
A4	Fusarium	Standard	6.350E+08	8.80277	8.01525	7.790775	0.224475	80.6
A5	Fusarium	Standard	6.350E+07	7.80277	10.8213	11.10895	0.287650	80.9
A6	Fusarium	Standard	6.350E+07	7.80277	11.3966	11.10895	0.287650	80.9
A7	Fusarium	Standard	6.350E+06	6.80277	17.938	17.0795	0.858500	80.6
A8	Fusarium	Standard	6.350E+06	6.80277	16.221	17.0795	0.858500	80.6
A9	Fusarium	Standard	6.350E+05	5.80277	19.5196	19.4806	0.039000	80.6
A10	Fusarium	Standard	6.350E+05	5.80277	19.4416	19.4806	0.039000	80.3
A11	Fusarium	Standard	6.350E+04	4.80277	23.3289	23.4289	0.100000	80.3
A12	Fusarium	Standard	6.350E+04	4.80277	23.5289	23.4289	0.100000	80.3
B1	Asperg	Standard	3.000E+09	9.47712	7.22203	6.897805	0.324225	82.8
B2	Asperg	Standard	3.000E+09	9.47712	6.57358	6.897805	0.324225	83.1
B3	Asperg	Standard	3.000E+08	8.47712	9.16128	8.157395	1.003885	83.1
B4	Asperg	Standard	3.000E+08	8.47712	7.15351	8.157395	1.003885	83.1
B5	Asperg	Standard	3.000E+07	7.47712	12.2784	12.2548	0.023600	83.1
B6	Asperg	Standard	3.000E+07	7.47712	12.2312	12.2548	0.023600	83.4
B7	Asperg	Standard	3.000E+06	6.47712	17.667	17.66445	0.002550	83.1
B8	Asperg	Standard	3.000E+06	6.47712	17.6619	17.66445	0.002550	83.1
B9	Asperg	Standard	3.000E+05	5.47712	20.9397	20.7308	0.208900	83.1
B10	Asperg	Standard	3.000E+05	5.47712	20.5219	20.7308	0.208900	82.8
B11	Asperg	Standard	3.000E+04	4.47712	26.7716	26.7777	0.006100	82.8
B12	Asperg	Standard	3.000E+04	4.47712	26.7838	26.7777	0.006100	82.5
		NTC			36.5589	36.065	0.48825	80.6
		NTC			35.4	36.065	0.48825	80.9
		NTC			36.2353	36.065	0.48825	80.9

8.4 Range for Ct values and melting temperature of the environmental samples tested for bacterial, archaea and fungi in the qPCR analyses.



Range for Ct values and melting temperature of the environmental samples of bacterial (16S), archaeal (16S) and fungal (18S) in the qPCR assays.
Note: This ranges includes all values obtained in assays that were used in Chapter 3, Chapter 4 and 6.
Errors bars= standard deviation of all assays/samples.

8.4 Calculation of the sample gene copies number (GCN) g ^{1} DW after the qPCR

$$\textbf{GCN g}^{1}\textbf{ DW} = \frac{\textit{Average gene copies (qPCR) x Dilution factor x volume of DNA sample (µl)}}{\textit{sample DW(g)}}$$

Average gene copies (qPCR) = average of the qPCR replicates results after standard curve calculation
Dilution factor = all genomic DNA extracted (biofertiliser and soil)) were diluted to 2 ng μl^{-1} prior to qPCR analysis
Volume of DNA sample (µl) =total volume of the DNA extracted elution
sample DW(g)= dry weight of the sample used for DNA extraction