Christina van Midden

Using high organic carbon materials to manipulate soil microbiology for improved nitrogen bioavailability from anaerobic digestate

School of Water Energy and Environment
PhD Environment and Agrifood

PhD
Academic Year: 2020 - 2024

Supervisor: Dr Mark Pawlett
Associate Supervisors: Prof Jim Harris
Prof Tom Sizmur (University of Reading)
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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Academic integrity declaration

I declare that:

- the thesis submitted has been written by me alone.
- the thesis submitted has not been previously submitted to this university or any other.
- that all content, including primary and/or secondary data, is true to the best of my knowledge.
- that all quotations and references have been duly acknowledged according to the requirements of academic research.

I understand that to knowingly submit work in violation of the above statement will be considered by examiners as academic misconduct.

Abstract

Anaerobic digestate is a by-product of biogas production, often used as a fertiliser due to its high nitrogen content. However, nitrogen losses from its application leads to environmental pollution. The aim of this PhD project was to add agronomic value to anaerobic digestate and reduce its environmental impact by understanding the microbial mechanisms associated with improving its nutrient use efficiency by crops. Digestate with a high organic carbon content is known to stimulate microbial growth and the immobilisation of nitrogen into soil microorganisms. However, after phase separation the liquid fraction contains large quantities of nitrogen in bioavailable forms but has reduced organic carbon. Soil incubation experiments were designed to determine the type (i.e. labile or recalcitrant) and rate of organic carbon required to stimulate microbial immobilisation of nitrogen from liquid digestate. A polytunnel pot experiment with spring barley and a field experiment with sugar beet tested the addition of two carbon additives (straw and glycerol) selected from the previous experiments on plant growth and nitrogen use efficiency. The addition of glycerol increased microbial biomass carbon within a month from application in both experiments, however there was no subsequent increase in crop yield or nitrogen uptake, nor were N_2O emissions and ammonia volatilisation affected. This indicates that either the carbon rate was too low to stimulate a nitrogen immobilisation that was significant enough to impact crop nitrogen uptake or that nitrogen remineralised too rapidly to be of benefit to later key nitrogen demanding crop growth stages. Future studies need to focus on determining the optimal amount of carbon to add with digestate to positively impact yield and reduce nitrogen losses. In conclusion this PhD thesis demonstrated a proof of concept that materials high in organic carbon content can be used to temporally immobilise digestate supplied nitrogen within the soil microbial biomass.

Keywords:

Biogas residue, crop production, organic fertiliser, nitrogen immobilisation, microbial community, glycerol, straw, nitrogen use efficiency, PLFA

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List of Abbreviations

~	Approximately
°C	Degrees Celsius
µg	Micro-gram
µl	Micro-litre
µmol	Micro-mol
AE	Agronomic efficiency
AMF	Arbuscular mycorrhiza fungi
ANOVA	Analysis of Variance
B	Biochar
C	Carbon
CAN	Calcium ammonium nitrate
$CHCl_3$	Chloroform
CH_4	Methane
cm	Centimetre
CO_2	Carbon dioxide
DI	Distilled water
dm	Dry matter
dm^3	Cubic decimeter
F:B	Fungi:Bacteria
fw	Fresh weight
g	Gram
G	Glycerol
G+	Gram positive bacteria
G-	Gram negative
GC	Gas chromatograph
ha	Hectare
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HPLC	High performance liquid chromatograph
K	Potassium
K_2SO_4	Potassium sulphate
KCl	Potassium Chloride
K_{EC}	Extractable part of microbial biomass C after fumigation
K_{EN}	Extractable part of microbial biomass N after fumigation
LD	Liquid Digestate
kg	Kilogram
LD	Liquid Digestate
LDPE	Low density polyethylene
M^3	Meter cubed
MBC	Microbial biomass carbon
MBN	Microbial biomass nitrogen
mg	Milligrams
ml	Millilitre
mmol	Milli-mol
mol	Molarity
N	Nitrogen
N_2	Nitrogen gas
N_2O	Nitrous oxide
NH_4-N	Ammonium nitrogen
nm	Nanometre
NPS	Nitrogen, phosphorus, sulphur
NUE	Nitrogen use efficiency
OPA	Ortho-phthaldialdehyde
P	Phosphorus
PCA	Principal component analysis
PLFA	Phospholipid fatty acids
PVC	Polyvinyl Chloride
ppm	Parts per million
RE	Apparent crop recovery efficiency
S	Straw
SOC	Soil Organic Carbon
SOM	Soil Organic Matter

t	Tonne
TOC	Total organic carbon
TN	Total nitrogen
TON-N	Total oxides of nitrogen
v/v	Volume to volume
W	Woodchip
w/w	Weight for weight
x	Multiplication

1 Introduction

1.1 Background

The world population is predicted to grow from 8.2 to 10.2 billion by the end of the century, peaking in the mid-2080's then declining (UN, 2024). This growth will increase the pressure on the agricultural sector to provide food. This is turn puts pressure on the resources required to grow crops, including the soil in which the crop is grown. Soil is essential for food production, with 95% currently produced on land (FAO, 2015). Soil serves many functions, including the transformation of nutrients by its biological community into forms that are available for crops to uptake. With current practises of removing the crop biomass as food, energy production, animal forage or bedding, few nutrients are returned to the soil and its nutrient pool is depleted. The depleted nutrients are replenished through mineral fertiliser application by the farmer, the three macro-nutrients most commonly added as a fertiliser for crop growth being nitrogen, phosphorus and potassium. The application of synthetic nitrogen is responsible for feeding 50% of the global population (Erisman *et al.*, 2008), yet its production currently relies on fossil fuels (Smith, Hill and Torrente-Murciano, 2020). Considering that fossil fuels are a finite resource, there is a critical need to source fertilisers from renewable sources. Anaerobic digestate offers one such alternative source of nitrogen because it is produced from organic matter such as energy crops, animal waste, sewage sludge and the organic fraction of municipal wastes.

1.1.1 The process of producing quality anaerobic digestate

The fundamental process of anaerobic digestion to produce biogas involves creating an oxygen-free environment to induce methane and carbon dioxide production by anaerobic microbes during the decomposition of organic matter (Al Seadi *et al.,* 2008). The methane is usually then purified before either being fed directly into the gas grid or converted into electricity, accounting for 12% of the UK's electricity in 2020 (Ember, 2021). There are four successive phases to methane production (Figure 1.1; Anukam *et al.*, 2019). During the hydrolysis phase, microorganisms break down complex compounds in the feedstock into simple compounds, the speed of which is determined by the complexity of the compound. Lignin, a complex structure is highly resistant to decomposition whereas the simpler structured hemicellulose rapidly decompose (Molinuevo-Salces *et al.*, 2013). In the acidogenesis stage, the products of hydrolysis are absorbed into microbial cells and broken down to form acidic products. Crucially, the microbes carrying out the transformation use up the remaining oxygen. During the acetogenesis phase the products from the previous phase are transformed into the volatile fatty acids needed by methanogenic microbes to produce methane, which is carried out in phase four (methanogenesis).

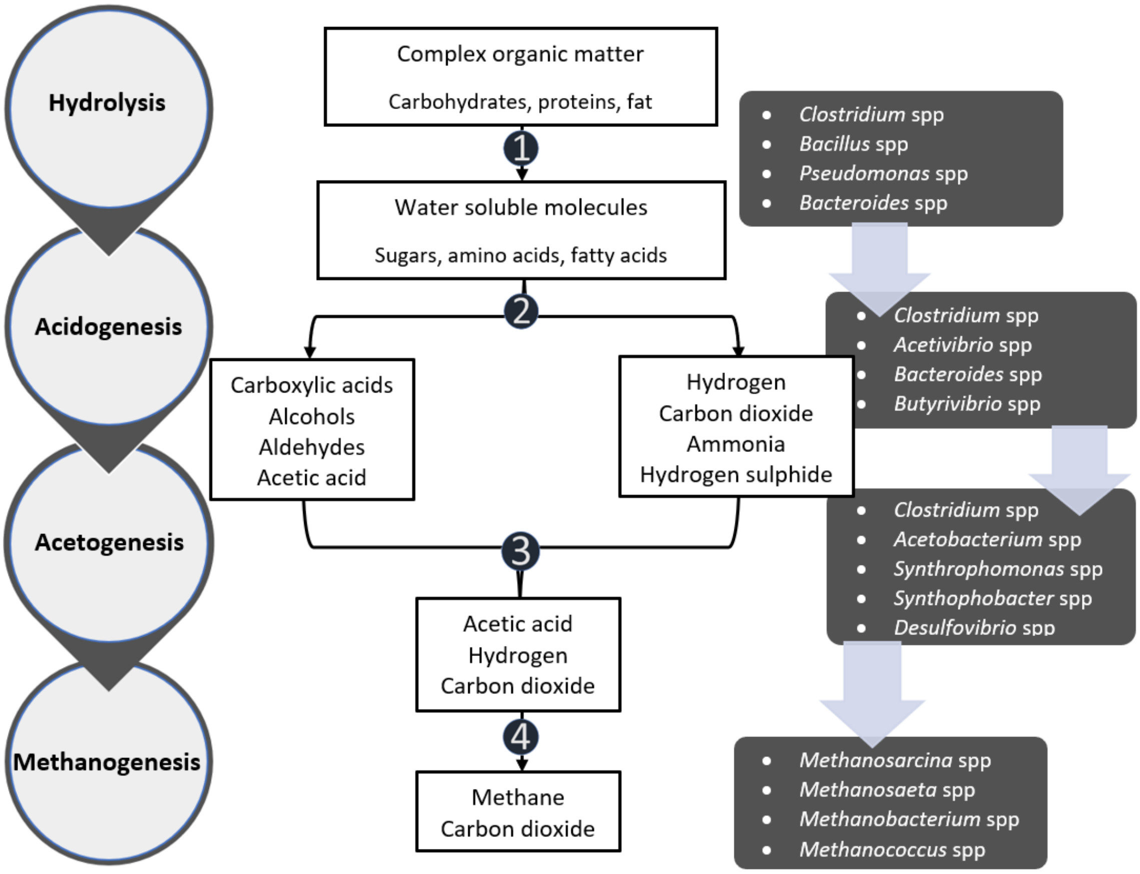

Figure 1-1 Flow diagram of the four stages of biogas production through anaerobic digestion and the microbial groups involved. This figure was created from information in a booklet provided by Future Biogas Ltd on the fundamentals of anaerobic digestion.

The conditions to achieve anaerobic digestion are flexible, so long as the unit is sealed. The temperature of the digestate can be mesophilic (35-40°C) or thermophilic (50-60°C). The latter has a higher potential to increase methane production, whereas mesophilic digestion is energetically cheaper because the liquid does not require as much heating (Gebreeyessus and Jenicek, 2016). Feedstock can be fed into the digestor either continually or in batches, which allows for the manipulation of moisture levels (Al Seadi *et al.,* 2008). Batch feeding reduces the amount of liquid in the digestate which reduces dewatering costs, but results in fluctuating methane production. Whereas continuous feeding reduces the costs of heating the digestate every time feedstock enters the digestate and results in steadier methane production but higher dewatering costs. The process of biogas production itself can be made more efficient if there are connected digestor tanks tailored with optimal conditions for the microorganisms carrying out the separate stages of methane production (De Gioannis *et al.*, 2017).

Once the biogas has been extracted, the digestate is ready for post-processing. Anaerobic digestate can be applied directly to fields in the form that it is in (whole digestate), so long as its feedstock was of a sanitary origin, such as agricultural wastes (Al Seadi and Lukehurst, 2012). This system works well for on-farm digestors, where the farmer can add their own animal and/or crop wastes, or purpose-grown energy crops such as maize or ryegrass, into the tank and spread the digestate on to their fields. For off-farm digestors, particularly those that handle food or sewage waste, the resulting digestate will likely need to meet quality assurance schemes set by governmental legislation, such as the EU's ECN-QAS or the UK's BSI PAS110 schemes. Satisfying the conditions of these standards usually involves post-processing of the digestate to remove, for example, biological contaminants such as pathogens by pasteurisation or reduce chemical and physical contaminants such as heavy metals and plastics, to acceptable levels prior to land application.

After removing contaminants to meet the standards required by quality assurance schemes, the digestate is still a liquid rich substance that is bulky to store and expensive to transport, costing £3-4 per tonne of feedstock digestated for a 50 mile round trip for land application (WRAP Cymru, 2014). To reduce transport costs, whole digestate is dewatered (usually with mechanical separation by screw press separators or decanter centrifuges) into solid and liquid fractions. The solid fraction can be stored in an open heap, whereas the liquid needs to be stored in lagoons, preferably with a gas tight seal to reduce greenhouse gas emissions and ammonia volatilisation during storage (Liebetrau *et al.*, 2013). The solid fraction can be transported in trailers whereas the liquid and whole digestate require specialised tankers.

The separation of digestate into the two fractions has consequences for the nutrient distribution. Between 70-80% of the nitrogen and potassium remains in the liquid fraction, while 55-65% of phosphorus remains in the solid fraction (Fuchs and Drosg, 2013). This is due to the chemical nature of the nutrients, as phosphorus often precipitates as struvite minerals during the digestor process and settles to become part of the solid fraction (Marti *et al.*, 2008), while nitrogen and potassium remain water soluble (Aguirre-Villegas, Larson and Sharara, 2019). The nutrient content of digestate, regardless of separation, is also influenced by feedstock. Food-based digestate contains more total nitrogen than manure based digestate (Taylor *et al.*, 2010). The variability and unpredictability of the nutrient content of digestate is a key challenge in utilising it as a fertiliser that represents a barrier that prevents its acceptance by farmers. However, this barrier can be mitigated by a comprehensive nutrient analysis of the digestate to help the farmer integrate it into their fertiliser management plan.

1.1.2 Using digestate as a nitrogen fertiliser

The use of liquid and whole digestate as either a partial or whole replacement for synthetic nitrogen has been shown to produce equivalent yields for:

- cereals (Šimon, Kunzová and Friedlová, 2015; Du et al., 2019; Zicker et al., 2020; Doyeni et al., 2021; Grillo et al., 2021; Pastorelli et al., 2021; Ran et al., 2022; Buligon et al., 2023)
- fodder or energy crops such as grasses and silage maize (Vaneeckhaute et al., 2013; Montemurro et al., 2015; Vanden Nest et al., 2015; Riva et al., 2016; Tilvikienė, Šlepetienė and Kadžiulienė, 2018; Walsh et al., 2018; Tsachidou et al., 2019; Luo et al., 2022; Samoraj et al., 2022; Brychkova et al., 2024).

- vegetable crops (Alburquerque et al., 2012; Stoknes et al., 2016; Elbashier et al., 2018; Barzee et al., 2019; Weimers et al., 2022; Yagüe and Lobo, 2024).

The reason why digestate produces these equivalent yields is that the majority of its nitrogen is in the form of ammonium (Czekała, 2022), which is plant available. As the rate of nitrogen fertilisation is based on total nitrogen content, the high ammonium content in digestate means that most of the nitrogen applied in digestate is immediately available for plant uptake.

Best practice when applying nitrogen is to time application to match crop demand. Crop plant growth can be divided into three development phases: (i) foundation, (ii) construction when they establish a canopy, and (iii) production when they develop storage units such as grains or tubers (Sylvester-Bradley et al., 2008). The construction phase is the most nitrogen demanding followed by the production phase (Sylvester-Bradley et al., 2008). However, it is difficult to apply fertiliser, especially digestate, to a fully established crop to supply nitrogen for the production phase. The recommended best practise for fertiliser application is to apply at seedbed and at the beginning of the construction phase (AHDB, 2023) on the basis of the optimal amount of nitrogen the plant needs for its entire growth. However, the problem with this method of directly feeding nitrogen to the crop means that the nitrogen applied is vulnerable to losses because the plant does not require all the nitrogen at once. These losses can result in environmental pollution such as eutrophication of water bodies, reduction in air quality, and emission of greenhouse gases (Nkoa, 2014). Therefore, it is important to better match nitrogen provision with crop demand, for which soil microorganism holds the potential solution.

Soil microorganisms use nitrogen in cellular development (Robertson and Groffman, 2007), transforming upon their death from living biomass into necromass (Kindler et al., 2006) and forming soil organic matter (Liang et al., 2019). Nitrogen in its organic form is less vulnerable to losses compared to inorganic ammonium and nitrate nitrogen. When the plants require nitrogen for growth, they release root exudates which prime microorganisms to decompose soil organic matter and transform the organic nitrogen into plant available forms (Meier, Finzi and Phillips, 2017). This provides plants with nitrogen when they need it, as opposed to farmers applying it based on their best estimate for when the plant requires the nitrogen.

As a source of readily available nitrogen, digestate is a material that feeds crops directly as opposed to feeding soil microorganisms, as it contains a low amount of carbon, typically between 0.43-3.4% (Risberg *et al.*, 2017). Carbon is an essential energy source for microbial growth and is generally considered to be the main growth limiting nutrient in soils (Coleman *et al.,* 2004; Demoling, Figueroa, & Baath, 2007; Hobbie and Hobbie 2013). As such, the addition of low carbon containing digestate does not alleviate the carbon limitation. Therefore, methods to utilise the mechanism of microbial immobilisation and remineralisation of nitrogen needs to be explored to increase the quantity of digestate supplied nitrogen that is taking up by the plant. Therefore, this PhD project focused on the execution of experiments to understand how organic carbon could be added to the nitrogen rich liquid fraction of anaerobic digestate to stimulate microbial uptake, using existing application technologies, so that the results of this project could be integrated into current practise. The liquid fraction of anaerobic digestate was chosen as it is commonly supplied by biogas plants for fertiliser use and most vulnerable to nitrogen losses due to its high ammonium content.

1.2 Aims and Objectives

The aim of this PhD project was to add agronomic value to anaerobic digestate and reduce its negative environmental impact by understanding the microbial mechanisms associated with improving the nutrient use efficiency by crops from anaerobic digestate application. The following objectives were addressed to achieve the aim:

Objective 1 – Conduct a literature review into the effects of digestate application on soil biota.

Objective 2 – identify carbon amendments that maximise immobilisation of nitrogen within microbial biomass and investigate subsequent nitrogen remineralisation dynamics.

Objective 3 – test selected amendments from incubation experiments on crop growth, yield, and nitrogen use efficiency, and quantify nitrogen loss pathways.

Objective 4 – test selected amendments from incubation experiments on crop growth, yield, and nitrogen uptake under field conditions.

The overarching hypothesis of this thesis was that the addition of materials with a large quantity of organic carbon into digestate would stimulate the immobilisation of nitrogen within microbial biomass and therefore increase the nitrogen use efficiency by crops. Additionally, each experimental chapter (chapters 3, 4, 5 and 6) have their own specific hypotheses relating to the aim and objectives of the individual experiment.

1.2.1 Thesis outline

This thesis has been written in paper format, an approved style for Cranfield University. As such each chapter contains an introduction, methods, results, and discussion sections, as relevant to its style. As a consequence, there is some repetition within the introduction and methods sections between experimental chapters, as many of the same analytical techniques were undertaken to measure key responses. The author contributions to each chapter that has been published, submitted or intended for publication in peer-reviewed journals is given in Table 1-1. An illustration of how the objectives, chapters and research aim interlink is given in Figure 1-2.

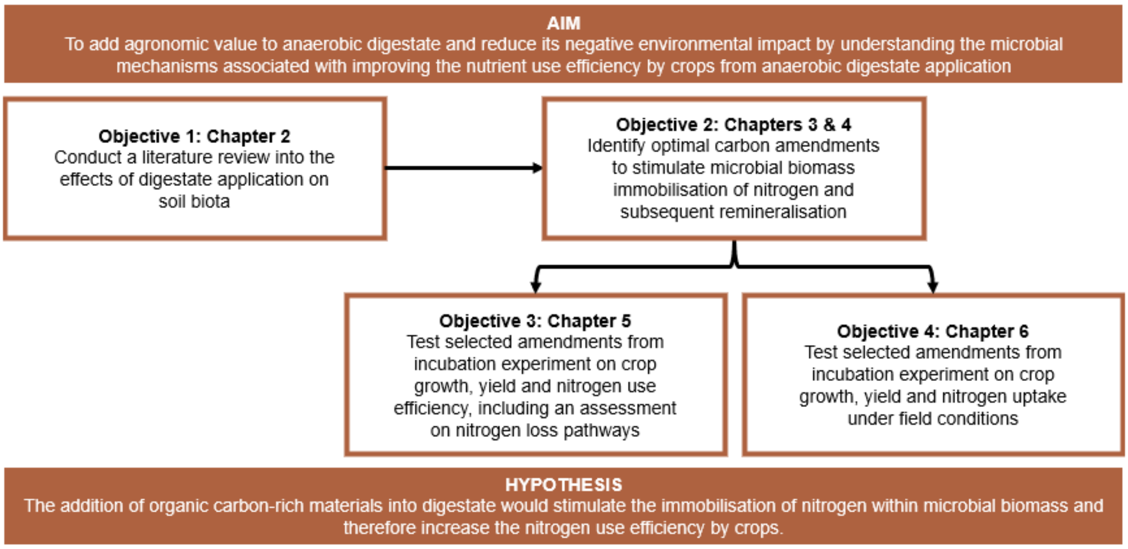

Figure 1-2 Research aim and objectives with associated chapters.

Chapter 2 provides a critical review of the literature concerning the impact of digestate on soil biota, and how the response is modified by the different fractions of digestate. It also identifies research gaps in our understanding of this topic and formalises the evidence to support the research gap that is addressed by this project. This chapter has been published in *Applied Soil Ecology* (2023) doi: 10.1016/j.apsoil.2023.105066

Chapter 3 reports a 5 month laboratory soil incubation experiment designed to identify the type of carbon, in terms of labile to recalcitrant, that is best suited to stimulate microbial immobilisation of nitrogen. This chapter has been published in *Frontiers in Sustainable Food Systems: Section Waste Management in Agroecosystems* (2024) doi: 10.3389/fsufs.2024.1356469.

Chapter 4 was a 50 day laboratory soil incubation experiment designed to identify the optimal rate of carbon (as glycerol) to add with digestate to stimulate microbial immobilisation of nitrogen. This chapter has been accepted for publication in *Waste and Biomass Valorisation*. doi: 10.1007/s12649-024-02876-8

Chapter 5 was a plant pot experiment run in a polytunnel to test the effects of the two high organic carbon additives that demonstrated the greatest potential for microbial immobilisation from the first two experiments on crop yield and nitrogen use efficiency, whilst also quantifying effects on nitrogen loss pathways of ammonia volatilisation, N_2O emissions and nitrate leaching.

Chapter 6 was a field experiment designed to test whether the positive effects measured in the lab would occur in field conditions as well as to understand the practicality of applying these treatments on farm.

Chapter 7 is the PhD synthesis. This chapter summarises the project findings, identifies implications for research and industry, discusses limitations and makes recommendations for future research.

Table 1-1 Author contributions to chapters already submitted or intended for publication in peer-reviewed academic journals

Author	Contribution
Christina van Midden	Conceptualisation, methodology development, investigation, data analysis, discussion, writing (all chapters)
Mark Pawlett - supervisor	Conceptualisation, guidance on method and structure, advice on data analysis, writing - review and editing, funding acquisition (all chapters)
Jim Harris Liz Shaw Tom Sizmur co-supervisors	Conceptualisation, guidance on method and structure, advice on data analysis, writing - review and editing (all chapters)
Hayden Morgan - case partner	Advice, funding acquisition, resources, writing – review and editing (chapters 3, 4, 5 and 6)

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2 The impact of anaerobic digestate on soil life: A review

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2.1 Abstract

Using organic amendments to fertilise crops is a crucial part in the sustainability of agricultural systems. The residual slurry remaining after biogas production (anaerobic digestate) contains a rich source of plant nutrients that provides an alternative to mineral fertilisers. The delivery of many nutrients to plants is facilitated by a healthy soil biota: free-living and symbiotic microflora (e.g. archaea, bacteria and fungi) mineralize, solubilize and facilitate plant uptake of nutrients and the soil fauna (e.g. protozoa, microarthopods and earthworms) influence nutrient cycling processes as higher-level consumers and litter transformers. The delivery of nutrients to plants via the activity of this soil food web is influenced by fertiliser inputs. Here we review the impact of anaerobic digestate on soil biota. The quantity and composition of the carbon in digestate has a large influence on soil heterotrophic microbial dynamics and their subsequent influence on nutrient bioavailability. The main points are (1) digestate low in carbon has little effect on soil microorganisms, whereas digestate higher in carbon increases soil microbial abundance and diversity; (2) labile carbon stimulates fast-growing bacteria, whereas recalcitrant carbon shifts the microbial community in favour of slower-growing fungi and Gram-positive bacteria; and (3) earthworms, springtails and nematodes dwelling in the soil surface layer can be negatively affected by digestate application due to toxicity when compounds such as ammonia are present in high concentrations. Generalized understanding of the effect by digestates on soil biota is made difficult by differences in digestate properties caused by varying feedstock and production methods and the inherent heterogeneity of soil. There is a lack of research investigating the impact of

repeated digestate application on soil biota and subsequently soil health. This information would give end users more confidence to substitute mineral fertilisers with digestate.

2.2 Introduction

Anaerobic digestion transforms organic matter into energy in a well-developed industrial process that generates biogas (Figure 2-1). During anaerobic digestion organic matter is broken down in oxygen-free conditions, producing CH_4 and CO_2 that are used to generate electricity and heat (Al Seadi *et al*., 2008; Figure 2-1). In 2009 the European Union set a mandatory target that, by 2020, 20% of all energy consumption should come from renewable sources (European Parliament and Council of European Union, 2009). This target resulted in numerous EU governments subsiding biogas plants installations (Edwards *et al*., 2015), with over 18,000 biogas plants being registered by end of 2018, an increase of 192% from 2009 (EBA, 2020). Anaerobic digestion to produce renewable energy has several advantages; biogas can be produced when needed, the produced biogas can supply the current natural gas grid, and energy is produced from organic wastes such as household, food and drink processing, agriculture, and sewage works.

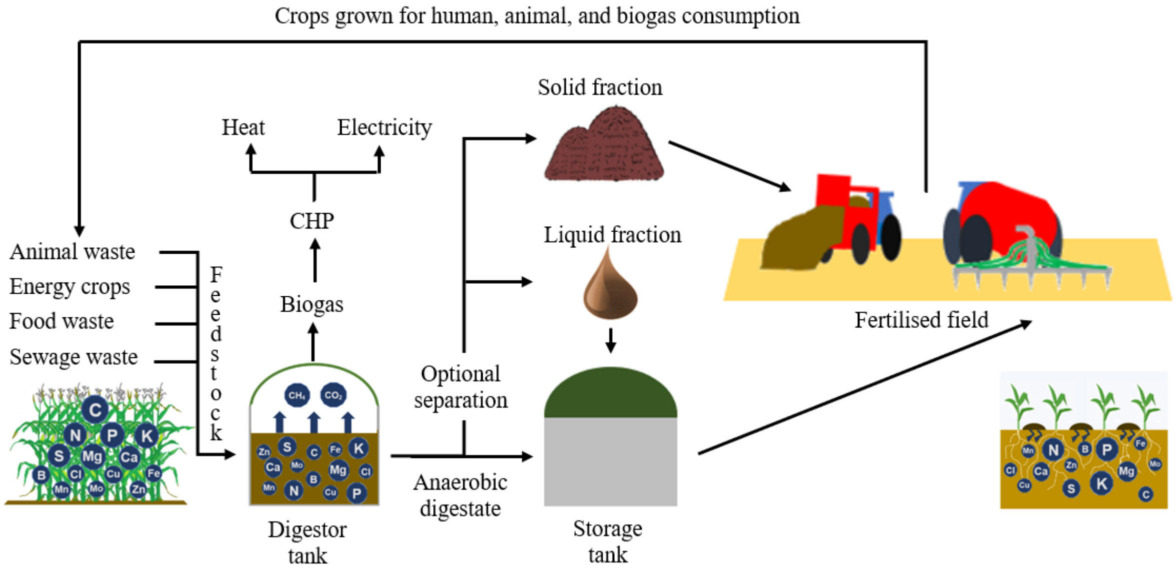

Figure 2-1 The process of anaerobic digestion and end-use of anaerobic digestate as a fertiliser

After biogas production the resulting slurry, known as anaerobic digestate, requires removal from the biogas plant. Originating from organic matter feedstock, and with only carbon and hydrogen removed as biogas (Möller, 2015), digestate contains the remaining nutrients from the digested feedstock (Figure 2-1). Digestate can be used as a fertiliser in agriculture and has been shown to support crop yields equivalent to mineral fertilisers (Šimon, Kunzová and Friedlová, 2015; Riva *et al.*, 2016; Ehmann, Thumm and Lewandowski, 2018; Walsh *et al.*, 2018; Barzee *et al.*, 2019; Zicker *et al.*, 2020). However, digestate has a low nutrient to volume content when compared to mineral fertilisers (Table 2-1), therefore the cost of transporting it from biogas plants to farms increases with distance and becomes uneconomical (Möller, Schulz and Müller, 2010). To address this limitation, digestate is often separated into a “liquid” and a more fibrous “solid” fraction to reduce the volume and therefore the cost of transporting (Al Seadi *et al*., 2012).

Table 2-1 Comparison of nutrient content between a 30:11:24 NPK compound fertiliser and the average values from four digestates used by Abubaker *et al*., (2012)

	Digestate kg t^{-1}	**NPK kg t^{-1}**
Total nitrogen	5.4	303
Ammonia nitrogen	3.6	148
Phosphorus	0.6	114
Potassium	2.3	245

The separation of digestate causes uneven nutrient distribution; between 65-75% of the total nitrogen and potassium remains in the liquid fraction, while 55-65% of phosphorus and 60-70% of the carbon remains in the solid fraction (Fuchs and Drosg, 2013). As liquid digestate contains the majority of the nitrogen, of which typically over 70% is in readily available form (Drosg *et al*., 2015) it has good potential as a fertiliser. The solid fraction contains a greater amount of phosphorus, but also carbon as organic matter (Table 2-2) and is considered both a source of nutrients (Al Seadi *et al*., 2012) and a soil conditioner to build soil organic matter (SOM) (Logan and Visvanathan 2019). Little work has been done to understand the influence of solid or liquid fractions of digestate on SOM, particularly the living component of SOM. Therefore, this review aims to address this knowledge gap by providing a greater understanding of the impact of digestate application on soil biota.

Table 2-2 Nutrient content per weight of the whole, solid and liquid fractions of digestate. Data from screw extractor and rotary screen separator experiments by Bauer *et al*., (2009).

	Liquid phase kg t^{-1}	Whole phase kg t^{-1}	Solid phase kg t^{-1}
Dry matter	45.0	73.1	193.1
Volatile solids	31.3	53.8	165.4
Total nitrogen	4.0	4.2	4.6
Ammonia nitrogen	2.6	2.7	3.0
Phosphorus	0.9	1.2	2.5
Potassium	3.5	3.6	3.4

2.3 The influence of anaerobic digestate on soil microorganisms

A significant proportion of SOM consists of living and dead microorganisms (Liang and Balser, 2011). Soil microorganisms consist of archaea, bacteria, fungi, and protozoa, though the majority of studies investigating the impact of digestate on soil microorganisms have focused on bacteria and fungi as dominant groups in terms of abundance and biomass. The focus on these two microbial groups is largely because they are considered the largest functional groups responsible for nutrient cycling in soil (Buerkert *et al.,* 2012).

2.3.1 Effect of digestate on soil microorganism activity and abundance

The application of the liquid fraction and non-separated whole digestate to soils rapidly stimulates microbial activity (Risberg *et al.*, 2017; Iocoli *et al.*, 2019; Meng, Ma and Petersen, 2022). Similar increases in the soil microbial biomass have been observed within hours after digestate application (Johansen *et al.*, 2013; Monard *et al.*, 2020), but both changes in activity, abundance and biomass are temporary and often subside within days of application (Alburquerque *et al*., 2012a; Galvez *et al*., 2012; Iocoli *et al*., 2019; Barduca *et al*., 2021) and are not detectable after a few weeks (Walsh *et al.*, 2012, 2018; de la Fuente *et al.*, 2013; Gómez-Brandón *et al.*, 2016; Viaene *et al.*, 2017; Mórtola *et al.*, 2019; Gebremikael *et al.*, 2020; Ren *et al.*, 2020; Różyło and Bohacz, 2020; Valentinuzzi *et al.*, 2020).

The majority of soil microorganisms are heterotrophic and use organic carbon as their energy source. As the anaerobic microbes in the biogas tank have already converted much of the readily available carbon in the feedstock into CH_4 and CO_2 (Thomsen *et al.*, 2013) there is less readily available carbon present for soil microbes to utilise, compared to undigested feedstock materials (Chen *et al*., 2012). In pot studies that added a high amount (10-50% w/w) of whole or liquid digestate to soil (García-Sánchez *et al.*, 2015; Muscolo *et al.*, 2017; Panuccio *et al.*, 2021) microbial biomass increased. Manfredini *et al*., (2021) altered the concentration of dissolved organic carbon in the digestate and observed that higher levels of dissolved organic carbon resulted in increased microbial biomass by the end of the study. These studies show that it is when carbon concentrations are increased beyond standard field application rates, that microbial activity and abundance increase for more than a few weeks. This indicates that typical liquid or whole digestate application rates do not supply enough available carbon for soil microorganisms to support sustained growth.

The application of solid digestate led to sustained increases in microbial biomass and activity (de la Fuente *et al.*, 2013; Badagliacca *et al.*, 2020; Cattin *et al.*, 2021) indicating that the solid fraction did not result in the carbon-limited microbial growth observed for whole or liquid digestate. Furthermore, de la Fuente *et al*., (2013) observed that solid digestate increased microbial biomass to a greater extent than any other form of digestate and reported a concurrent increase in nitrogen within the microbial biomass in solid digestate treated soils that was absent in others. Therefore, the authors reasoned that the high immobilisation of nitrogen in the microbial biomass receiving this treatment had an important influence over the growth and activity of the soil microorganisms. Although the digestate separation process removes most of the nitrogen in the liquid fraction (Tambone *et al.*, 2017), it is apparent that the solid fraction still contains sufficient nitrogen to support microbial growth.

The characteristics of the feedstock can influence the impact that digestate has on soil microorganisms. The rate of liquid and whole digestate applied to land is routinely based on its nitrogen content; subsequently digestate with a higher C:N ratio delivers more carbon to the soil, which influences its impact on soil microbial activity (Abubaker *et al.*, 2013; Iocoli *et al.*, 2019). Muscolo *et al*., (2017) observed that the biochemical nature of the carbon is important, since, when both liquid and solid digestate with a lower percentage of carbon as recalcitrant plant material was applied, a greater positive effect on microbial biomass growth was observed. Alburquerque *et al*., (2012a) and Risberg *et al.,* (2017) both reported significant differences in the effects of digestate on microbial activity due to the digestate feedstock type; digestates containing a greater amount of readily available carbon resulted in increased levels of microbial activity.

2.3.2 Effect of digestate on soil microbial community

Hupfauf *et al*., (2016) showed that microbial community level physiological profiles (using principal components analysis of MicroResp^{TM}) for soils receiving applications of solid digestate were distinct from those receiving liquid digestate, whilst whole digestate resulted in a community profile that lay between the two. The physiochemical characteristics of the digestate influence microbial community composition. The high ammonium and water content of whole and liquid digestate create favourable conditions for bacterial groups associated with the nitrogen transformation, with increases in the abundance of bacterial nitrifiers and denitrifiers being reported (Sawada and Toyota, 2015; Brenzinger *et al.*, 2018; Ogbonna, Stanley and Abu, 2018).

Another explanation for the differences in the physiological profiles of the microbial community may be due to changes in fungi:bacteria (F:B) ratios, as the two groups occupy different functional niches in the soil. Walsh *et al*., (2012) observed an increase in bacterial growth six months after applying liquid digestate, which reduced the F:B ratio. Similarly, Pezzolla *et al*., (2015) applied a digestate with a dry matter equivalent to liquid digestate, and observed an increase in Gram-negative bacteria, causing a decrease in the F:B ratio. These quick growing bacteria are better able than fungi to take advantage of the labile carbon supplied in the liquid digestate, whilst very little complex carbon is added that fungi can use. This may explain the negative effect of liquid digestate on fungi that Wentzel and Joergensen (2016), Elbashier *et al*., (2018) and Barduca *et al*., (2021) found. However, Coelho *et al*., (2019) and Gryń *et al*., (2020) observed negligible changes to both groups.

The application of whole digestate resulted in transient (Ren *et al.*, 2020; Różyło and Bohacz, 2020) or insignificant changes (Makádi *et al.*, 2016; Brenzinger *et al.*, 2018) to bacterial or fungal abundance and no changes to the F:B ratio were observed (Gebremikael *et al.*, 2020). In contrast, Chen *et al*., (2012) observed a shift in microbial community, as inferred from growth kinetic parameters, to one dominated by slower growing organisms under the application of whole digestate made from maize. This response was interpreted to be due to the presence of the recalcitrant plant fibres that the microbes in the anaerobic digestor did not break down, which support the relatively slower growing microbes, such as fungi and Gram-positive bacteria (Meidute, Demoling and Bååth, 2008; Bastian *et al.*, 2009). Chen *et al*., (2012) used a digestate made only from maize, and therefore a comparatively larger proportion of its organic carbon would be in a recalcitrant form compared to the digestates used in the other studies.

When digestate with a higher ratio of carbon to nitrogen is applied, an increase in fungal content (García-Sánchez *et al*. 2015; Barduca *et al*., 2021; Panuccio *et al*., 2021) and F:B ratio (Cattin *et al.*, 2021) were observed. The solid fraction of digestate contains a greater availability and variability of organic carbon, including a high quantity of recalcitrant organic matter that saprophytic fungi utilise (Meidute, Demoling and Bååth, 2008). Furthermore, Tambone *et al*., (2017) demonstrated that the nitrogen content in the solid fraction is high enough to consider it an organic fertiliser, consequently reducing direct competition between fungi and bacteria for nitrogen and thereby relieving the nitrogen limitation on fungal growth (Rousk and Bååth, 2007).

Not all fungi are decomposers and an important fungal group, the arbuscular mycorrhizal fungi (AMF), gain their carbon from a symbiotic relationship with plants. Despite having their carbon needs met by the plants, AMF are affected by digestate application and the fraction of digestate applied determines the direction of the effect. Solid digestate application has a positive effect on AMF colonization (Caruso *et al*., 2018). This effect may be due to the slow release of phosphorus from both the decay of its fibrous material (Gosling *et al.*, 2006) and the struvite minerals that precipitate during the anaerobic process (Marti *et al.*, 2008). This makes it beneficial for the host plant to maintain the symbiosis through supply of photosynthate for the purposes of improved phosphorus acquisition.

Unlike phosphorus, nitrogen addition has been shown to have positive effects on AMF stimulation (Nouri *et al.*, 2014; Johnson *et al.*, 2015) through increasing phosphorus demand by alleviation of nitrogen as the nutrient most limiting to plant growth. Although liquid and whole digestate are rich in nitrogen, positive effects on AMF colonisation were not seen (Wentzel and Joergensen, 2016; Caruso *et al.*, 2018; Dahlqvist, 2018; Ren *et al.*, 2020) though Ren *et al*., (2020) did measure an increase in hyphal length. Ren *et al*., (2020) observed a slight but significant decrease of 0.18 in soil pH as they increased digestate dosage rates. Since they applied digestate in its whole form, it will have contained a high concentration of ammonium N. Although ammonium N initially increases soil pH due to its alkaline nature, it reduces soil pH as it undergoes nitrification. Furthermore, as plants take up ammonium ions, they release protons into the soil around the roots to balance their internal pH (Smith and Read, 2008). These factors result in soil acidification, which has been shown by Pan *et al.*, (2020) as a cause for suppressing AMF colonization.

The physiochemical properties of digestate (such as carbon content and type, nutrients, and water volume) influence the physiological profile of the microbial community. However not all the aforementioned studies observed the same result for the same form of digestate. These differences are due to variability in digestate characteristics caused by different feedstock sources (Table 2-3;Table 2-4) Other factors contributing to different patterns observed in these studies include different soil properties, dose rates and analytical methodologies adopted by the researchers (Table 2-3;Table 2-4). These differences between disparate studies makes understanding the effects of digestate application on microbial community structure difficult to quantify. Currently the number of studies investigating the impact of digestate application on distinct groups of soil microorganisms are too low to generate consensus by reviewing only those using similar measurements and current trends identified should be taken with caution.

Table 2-3 The effects of the three forms of digestate on soil microbial biomass as measured by chloroform fumigation extraction method. In all experiments the comparison of effect by digestate is against a non-fertilised control. Only studies longer than 30 days were selected. In multi-year trials, only data from first year was considered. *The data in this table was used to generate Figure 2-2*.

AD form / fraction	Digestate Feedstock*	Application rate	Plant present	Sampling time post application	Effect	Authors
Liquid	M/OFMIW/EC	10-50% soil w/w	No	6 months	increase in biomass	Panuccio *et al.*, 2021
Liquid	M/OFMIW/EC	10-30% soil	No	3 months	increase in biomass	Muscolo *et al.*, 2017
Liquid	S/OFMIW/SS	64m^3/ha	Watermelon	152 days	increase in biomass	Alburquerque *et al.*, 2012b
Liquid	OFMIW/M/EC	10-30% soil	No	3 months	no change in biomass	Muscolo *et al.*, 2017
Liquid	M	180kg N/ha	No	7 weeks	no change in biomass	Valentinuzzi *et al.*, 2020
Liquid	S/EC	120kg N/ha	Ryegrass	70 days	no change in biomass	Wentzel and Joergensen 2016
Liquid	S	4.25L/m2	No	60 days	no change in biomass	Monard *et al.*, 2020
Liquid	S/M/EC	96m^3/ha	No	56 days	no change in biomass	de la Fuente *et al.*, 2013
Solid	M/OFMIW/EC	25-75% soil w/w	No	6 months	increase in biomass	Panuccio *et al.*, 2021
Solid	M/OFMIW/EC	20-75%	No	3 months	increase in biomass	Muscolo *et al.*, 2017
Solid	OFMIW/M/EC	20-75%	No	3 months	increase in biomass	Muscolo *et al.*, 2017
Solid	S/M/EC	48Mg/ha	No	56 days	increase in biomass	de la Fuente *et al.*, 2013
Whole	S/M/EC	96m^3/ha	No	56 days	increase biomass	de la Fuente *et al.*, 2013
Whole	S	20t/ha	No	30 days	increase in biomass	Galvez *et al.*, 2012

AD form / fraction	**Digestate Feedstock***	**Application rate**	**Plant present**	**Sampling time post application**	**Effect**	**Authors**
Whole	S/EC	120kg NH_4-N/ha	No	6 weeks	increase in biomass	Ernst *et al.*, 2008
Whole	S/EC/OFMIW	25-35t FW / ha	No	112 days	no change in biomass	Gebremikael *et al.*, 2020
Whole	M	70 & 210kg N/ha	Lettuce	34 days	no change in biomass	Mórtola *et al.*, 2019
Whole	M	80kg N/ha	No	60 days	no change in biomass	Gómez-Brandón *et al.*, 2016
Whole	S	1.4g/kg soil	No	90 days	no change in biomass	Pezzolla *et al.*, 2015

***S: animal slurry, M: animal manure, OFMIW: organic fraction of municipal and industrial waste, EC: energy crops, SS: sewage sludge**

Table 2-4 The effects of the three main forms of digestate on the soil bacterial, fungal and mycorrhizal fungal (MF) abundance. CFU=colony forming units. GCN=gene copy numbers. PLFA=phospholipid fatty acids. For digestate effects on MF, only colonisation measurement was accepted for comparison. For the effects of digestate on fungi and bacteria, different measurement techniques had to be accepted for enough studies to be selected to provide a pattern. *The data in this table was used to generate Figure 2-2*.

AD form / fraction	Digestate Feedstock*	Application rate	Plant present	Sampling time after application	Effect	Authors
BACTERIA						
Liquid	S	150kgN/ha	grass mix	16 weeks	increase in growth (leucine)	Walsh *et al.*, 2012
Liquid	S/C/OFMIW	170kgN/ha	no	150 days	no change in CFU	Gryń *et al.*, 2020
Liquid	OFMIW; SS	33m3 FW/ha	grass-sward mix	6 months	No change in GCN	Coelho *et al.*, 2019
Liquid	M	1100 L/ha	melon	<1 yr	no change in CFU	Elbashier *et al.*, 2018
Solid	OFMIW/C	10g/100g soil	wheat	60 days	increase in PLFAs	García-Sánchez *et al.*, 2015
Whole	S	340 kg ha	no	90 days	increase in PLFAs	Pezzolla *et al.*, 2015
Whole	C/OFMIW/M	3.4t DW/ha	no	6 months	no change in CFU	Różyło and Bohacz 2020
FUNGI						
Liquid	M	1100 L/ha	melon	<1 year	Decrease in CFU	Elbashier *et al.*, 2018
Liquid	S/C/OFMIW	170kgN/ha	no	150 days	no change in CFU	Gryń *et al.*, 2020
Liquid	M/C/OFMIW	170kgN/ha	sweetcorn	1st year	No change in CFU	Makádi *et al.*, 2016
Liquid	OFMIW; SS	33m3 FW/ha	grass mix	6 months	No change in GCN	Coelho *et al.*, 2019

AD form / fraction	**Digestate Feedstock***	**Application rate**	**Plant present**	**Sampling time after application**	**Effect**	**Authors**
FUNGI continued						
Liquid	S	150 kgN/ha	grass mix	16 weeks	no change in growth (ergosterol)	Walsh *et al.*, 2012
Liquid	S/C	120kgN/ha	ryegrass	70 days	decrease in ergosterol	Wentzel and Joergensen 2016
Solid	OFMIW/C	10g/100g soil	wheat	60 days	increase in PLFAs	García-Sánchez *et al.*, 2015
Whole	S	340 kg ha	No	90 days	no change in PLFAs	Pezzolla *et al.*, 2015
Whole	C/OFMIW/M	3.4t DW/ha	no	6 months	no change in CFU	Różyło and Bohacz 2020
MYCORRHIZA FUNGI						
Liquid	S/C	120kgN/ha	ryegrass	70 days	decreased colonisation	Wentzel and Joergensen 2016
Liquid		140kgN/ha	triticale	223 days	non-sig. decrease in colonisation	Caruso *et al.*, 2018
Solid		140kgN/ha	triticale	223 days	increased colonisation	Caruso *et al.*, 2018
Whole	OFMIW	100kgN/ha	spring wheat	10 weeks	no effect on colonisation	Dahlqvist 2018
Whole	OFMIW	25 and 50kgN/ha	ryegrass	75 days	no effect on colonisation	Ren *et al.*, 2020

***S: animal slurry, M: animal manure, OFMIW: organic fraction of municipal and industrial waste, EC: energy crops, SS: sewage sludge**

2.3.3 Effect of repeated digestate application on soil microbial community

The changes in microbial community previously discussed were observed in experiments that ran for a short time (< 1 year) and under controlled laboratory conditions. A two-year field experiment run by Coelho *et al*., (2020), showed no significant changes in soil bacteria and fungi abundance and diversity through repeated liquid digestate applications. Similarly, Makádi *et al*., (2016) saw no significant change in the microbial groups they studied over two years. Furthermore, no significant increases in microbial biomass were observed after three years of repeated liquid or whole digestate application (Johansen *et al.*, 2015; Šimon, Kunzová and Friedlová, 2015; Bhogal *et al.*, 2016; Pastorelli *et al.*, 2021), although Alburquerque *et al*., (2012b) did observe a positive effect after two years. This positive effect may have been due to the application of digestate twice per year in the rotation, whereas the others only applied once per year. Similarly, Odlare *et al*., (2008) observed a significant increase in microbial biomass after four annual digestate applications. It could be that fluctuating environmental conditions in the field masked the impact of the digestate influence on soil microbes. Indeed Pastorelli *et al*., (2021) observed that the season when soil was sampled had a greater influence on microbial community composition than the digestate treatment. Therefore, for sustained changes to be detected, more than three digestate applications are recommended.

The indirect effects of repeated digestate application on microorganisms due to changes in soil physiochemical properties are currently unknown. The most important soil property for determining microbial biomass and community diversity is soil pH (Fierer and Jackson, 2006; Hermans *et al.*, 2017; Ma *et al.*, 2019). Variability in soil pH influences many other soil properties, including the solubility of inorganic and organic compounds such as nutrients and metals (Veroney and Heck, 2015). A significant reduction or increase in pH leads to microbial community changes (Lauber *et al.*, 2009; Rousk, Brookes and Bååth, 2009). pH may also have a direct effect on microorganisms, many of which have intracellular pH levels close to neutral and a significant alteration in soil pH may exert a physiological stress that tolerant or extremophile taxa can grow better in (Hozzein, Ali and Ahmed, 2013; Quatrini and Johnson, 2018). Digestates tend to be slightly alkaline (Abubaker, Risberg and Pell, 2012; Wentzel and Joergensen, 2016; Prays *et al.*, 2018; Coelho *et al.*, 2019; Iocoli *et al.*, 2019). However digestate application can cause soil acidification depending on ammonia load that gets transformed into nitrate (Ren *et al.*, 2020) or the content of volatile fatty acids (Risberg *et al.*, 2017). Multi-year trials running between 2-4 years found no change in soil pH (Odlare *et al*., 2008; Alburquerque *et al*., 2012b; Bhogal *et al*., 2018; Elbashier *et al*., 2018; Barłóg *et al*., 2020) but after six years Zicker *et al*., (2020) observed a significant decrease in soil pH from digestate application, indicating that the effects of digestate application take time before they are noticeable. However, these effects can be remediated by liming, a common practice in agriculture.

Soil organic carbon (SOC) is a major determinant of soil microbial biomass (Hu *et al.*, 2014) and community composition (Drenovsky *et al.*, 2004) with low SOC concentrations favouring oligotrophic microbes (Semenov, 1991). Multi-year field trials have reported no change in SOC (Odlare, *et al*., 2008; Šimon, Kunzová and Friedlová, 2015; Barłóg, Hlisnikovský and Kunzová, 2020; Pastorelli *et al.*, 2021) or SOM (Bhogal *et al.*, 2018), of which SOC is a major component. It may be that these trials were too short in duration for changes to be seen, as Smith (2004) showed that it takes between 6-10 years for changes in SOC to be detected under various rates of carbon inputs, land uses and soil types.

There is some concern that digestate application may lead to the accumulation of heavy metals in soils, particularly as some studies have shown that digestate sourced from sewage, industrial, and urban waste contain levels of copper, cadmium, nickel, lead and zinc above those set by the relevant governing bodies as acceptable for land application (Govasmark *et al.*, 2011; Bonetta *et al.*, 2014; Coelho *et al.*, 2018). A high concentration of heavy metals in the soil can reduce enzyme function, inhibit respiration, and shift the composition of the microbial community to favour organisms that can tolerate the contamination (Giller, Witter and McGrath, 2009; Chu, 2018). Multiple studies have analysed digestates made from a range and mix of organic materials for their heavy metal content and found them to be below the advised threshold levels set by their nation or federation (Kuusik *et al.*, 2017; Coelho *et al.*, 2018; Mórtola *et al.*, 2019; Panuccio *et al.*, 2021) and so are considered safe to apply. However, the long-term cumulative effect of repeated digestate applications on heavy metal concentration in soils is unexplored, either due to direct accumulation in the soil or indirectly due to changes in metal solubility through an alteration of pH.

There are concerns about the presence of hazardous compounds in digestate based on animal, industrial or household waste, such as antibiotics (Widyasari-Mehta, Hartung and Kreuzig, 2016), hormones (Withey *et al.*, 2016; Congilosi and Aga, 2021), pesticides (Govasmark *et al.*, 2011), pharmaceuticals and personal care products (Narumiya *et al.*, 2013; Samaras *et al.*, 2014; Malmborg and Magnér, 2015), phenols (Levén, Nyberg and Schnürer, 2012; Limam *et al.*, 2013), salinity (Pawlett and Tibbett, 2015), microplastics (Weithmann *et al.*, 2018), and persistent organic compounds including PAHs, phthalates, and dioxin-like compounds (Govasmark *et al.*, 2011; Bhogal *et al.*, 2016). The presence of these compounds can have negative effects on microorganisms (Levén *et al.*, 2006; Chen *et al.*, 2013; Lipińska, Kucharski and Wyszkowska, 2014; Molaei *et al.*, 2017; Al-Ani *et al.*, 2019; Mahfouz *et al.*, 2020), but their influence on the soil microbiota due to digestate application is underexplored. Some studies have shown that pesticides and phthalates can stimulate microbial growth as the compounds provide an energy source to species able to utilize them (Iocoli *et al*., 2019; Osadebe, Maduabum and Okpokwasili, 2020; Zhang *et al*., 2020a). However, they can also inhibit the activity of other microorganisms (Baćmaga, Wyszkowska and Kucharski, 2018; Gao *et al.*, 2020) and therefore will alter the microbial community structure.

Digestate contains a consortium of microorganisms that are introduced to the soil when applied, which can be negative in the case of pathogens and altering the native microbial community composition. Pathogen transfer is a particular concern for biogas facilities that supply digestate to multiple farms. To do so, they must meet quality assurance schemes set by governmental legislation, such as the EU's ECN-QAS or the UK's BSI PAS110. The thermophilic conditions of the anaerobic digestion process reduce pathogen load (Jiang *et al*., 2020; Nag *et al*., 2019) compared to original feedstock and pre-or post- pasteurization further sanitize the digestate (Thwaites *et al*., 2013, Nag *et al*., 2019). Regarding digestate sourced microorganisms altering the soil microbial community, Coelho *et al*., (2020) observed that these microorganisms did not replace the native microbial populations and attributed this to two factors. Firstly, most digestate sourced microorganisms are obligate or facultative anaerobes and therefore cannot survive the aerobic conditions in the soil surface and secondly that digestors operate at higher temperatures than those found in soil, which impacts growth and activity. Fernández-Bayo *et al*., (2017) and Podmirseg *et al*., (2019) tested the establishment of digestate sourced microorganisms that can survive in the soil by applying digestate to sterilised and non-sterilised soil. They discovered that only in sterilised soils could the digestate-sourced microorganisms establish.

2.4 The influence of anaerobic digestate on soil meso-organisms

Very few studies have looked at how anaerobic digestate impacts soil meso-organisms. Meso-organisms contribute to the carbon and nitrogen cycles via herbivory on belowground plant and fungal structures (Zhao and Neher, 2014), predation (Murray *et al.*, 2009) and fragmentation of plant litter (Song *et al.*, 2020), such as recalcitrant plant fibres remining in digestate. These actions free up the carbon locked in complex plant, fungal and faunal bodies into smaller particles and compounds which microorganisms can utilise. Meso-organisms include a diverse faunal range including nematodes and small arthropods such as springtails and mites. However, of the studies found looking at meso-organisms in relation of digestate application to soil, only springtails, mites, and plant parasitic nematodes were investigated.

The application of digestate had either no effect on springtails (Alves, 2016; Pommeresche, Løes and Torp, 2017) or a positive effect on both springtails and mites (Platen and Glemnitz, 2016) over the course of multiple applications. Platen and Glemnitz (2016) observed a positive correlation between soil moisture and springtail abundance, with the liquid digestate providing more water to the soil than a mineral nitrogen control. Yet, Pommeresche, Løes and Torp (2017) observed a reduction in surface dwelling springtails shortly after liquid digestate application. This reduction may be due to elements or compounds in the digestate being toxic, as Renaud *et al*., (2017) observed depressive effects on springtail reproduction caused by cadmium and zinc. Digestate also contains a high concentration of ammonia (Möller and Müller, 2012) which Domene et al., (2010) showed was the main reason for springtail mortality after sewage sludge application. This mortality may be due to an increase in soil pH beyond levels that springtails could tolerate, as observed by Maccari *et al*., (2020) under high doses of ammonia rich poultry litter application. However, springtails produce multiple generations within a year (Badejo and Van Straalen, 1993), suggesting that populations may well recover a few months after application. This ability to recover could explain why Platen and Glemnitz (2016) observed a positive effect on springtails, as the temporary negative effects may have been negated by more beneficial changes in soil properties from digestate application, such as the addition of organic matter which, dependant on the species, either provides a direct food source or indirectly by increasing microbial growth upon which are a food source for springtails (Hopkin, 1997).

Several studies have investigated the use of digestate on suppressing plant parasitic nematodes, as these cause considerable damage to important crops. Laboratory studies demonstrated reductions in the number of root knot nematodes (Jothi *et al.*, 2003; Westphal, *et al*., 2016; Wang *et al.*, 2019; Das *et al.*, 2022), and eggs produced by soybean cyst nematodes (Xiao *et al.*, 2007) between digestate and non-digestate treated soils. Mechanisms proposed for the suppressive effects of digestate include: promoting populations of nematode suppressing bacteria (Westphal, *et al*., 2016), nematicidal compounds from plants in digestate mixtures (Wang *et al*., 2019) or elevated ammonium and organic acids content produced from the digestion process (Min *et al.*, 2007). Xiao *et al*., (2007) compared ammonia enriched digestate against volatile fatty acid enriched digestate and observed the latter being more effective at reducing egg counts. However, the suppressive effects declined over time, and after two

(Xiao *et al*., 2007; Wang *et al*., 2019) and 6 months (Westphal *et al*., 2016) from application no differences between treatments were found. This indicates that these nematodes are likely to produce multiple generations during their host plants' growing season, enabling their population to recover. Indeed, in an experiment growing mangolds, Westphal *et al*., (2016) observed an increase in nematode egg and cyst numbers in digestate treated soils compared to soils receiving no digestate after 5 months, despite a reduction early in the growth of the mangold.

There is great difficulty in directly attributing the effects of digestate applications to changes in meso-organism abundances due to too few studies having been conducted (Table 2-5). Whilst the research here indicates that meso-organisms living close to the soil surface are negatively impacted, they can recover due to quick generation times and even be positively impacted in the longer term due to changes in soil properties caused by the digestate. However, both the number of studies involving meso-organisms and the number of meso-organism groups studied, are too small to make a scientifically robust generalisation. Much more work in this area is needed to properly understand the effects of digestate application on meso-organisms. This is a challenge due to the immense diversity of meso-organisms, but necessary to do as they are a key link in carbon and nutrient cycling.

Table 2-5 The effects of whole digestate application on soil mesofauna (springtails and nematodes) and macrofauna (earthworms). ***The data in this table was used to generate Figure 2-2*.**

AD form / fraction	Application rate	Field site	Sampling time post application	Effect	Authors
SPRINGTAILS					
Whole	147kg N/ha	grassland	1.5 months	no change in abundance	Pommeresche, Løes and Torp, 2017
Whole	-	arable	4 months	no change in abundance	Alves, 2016
Whole	196kg N/ha	arable	1-6 months	increase in abundance	Platen and Glemnitz, 2016
NEMATODES		**Pot and plant**			
Whole	300 kg NH_4-N/ha	Pot – None	3 months	no change in abundance	Wang *et al*., 2019
Whole	120kg N/ha	Pot - Sugarbeet	6 months	no change in egg / cyst count	Westphal, *et al*., 2016
Whole	120kg N/ha	Pot - Mangold	5 months	increase in egg / cyst count	Westphal, *et al*., 2016
Whole NH_4^+ enriched	23.4-187.2 m^3/ha	Pot – Soybean	2 months	no change in egg count	Xiao *et al*., 2007
Whole VFA enriched	23.4-187.2 m^3/ha	Pot – Soybean	2 months	no change in egg count	Xiao *et al*., 2007

AD form / fraction	Application rate	Field site	Sampling time post application	Effect	Authors
EARTHWORMS					
Whole	140-167kg N/ha	arable	2 years	non-significant increase in abundance	Moinard *et al*., 2021
Whole	120-250kg N/ha	arable	3.5 years	no change in abundance	Rollett *et al*., 2020
Whole	120-250kg N/ha	grassland	3.5 years	decrease in abundance	Rollett *et al*., 2020
Whole	160kg N/ha	arable	4 weeks	non-significant increase in abundance	Koblenz *et al*., 2015
Whole	71.9kg N/ha	arable	6 weeks	non-significant increase in abundance	Clements, 2013
Whole	120kg N/ha	arable	1 month	non-significant increase in abundance	Bermejo, Ellmer and Krück, 2010

2.5 The influence of anaerobic digestate on soil macro-organisms

Earthworms are the most studied soil macro-fauna in relation to impacts of anaerobic digestate application. Earthworms are considered ecological engineers (Lavelle *et al.*, 1997); mixing of organic matter through the soil profile, aerating and improving soil fertility, increasing soil porosity, and breaking down organic matter into segments that other decomposers can utilise (Blouin *et al.*, 2013). As such they are candidate indicators of soil health (Fusaro *et al.*, 2018) and the reasons for selecting this group to determine the effect of digestate application on macro-organisms are logical. The majority of arable field experiments showed no significant change in earthworm abundance after whole digestate application (Bermejo, Ellmer and Krück, 2010; Clements, 2013; Frøseth *et al.*, 2014; Johansen *et al.*, 2015; Koblenz *et al.*, 2015; Rollett *et al.*, 2020; Moinard *et al.*, 2021). The overall lack of an effect may be due to the inherently low numbers of earthworms found in arable fields (Stroud, 2019) as Rollett *et al.,* (2020) observed a decrease in earthworms abundance in a densely populated perennial ley field after digestate application. To understand the reason for this decrease, looking at how digestate influences individual ecological groups of earthworms is necessary.

Earthworms can be broadly defined into three ecological groups: epigeic, endogeic and anecic (Bouché, 1977), though species are found corresponding to multiple categories (Bottinelli *et al.*, 2020). Epigeic (litter dwelling) earthworms actively avoided digestate amended soils where possible (Clements, 2013; Ross *et al.*, 2017). Whilst endogeic (topsoil dwelling) did not express such clear avoidance behaviour (Ross *et al.*, 2017), their biomass decreased after digestate application (Ernst *et al.*, 2008; Bhogal *et al.*, 2016). In contrast epi-anecic (subsoil dwelling who collect food from soil surface) earthworms responded positively to digestate application (Ernst *et al.*, 2008). Digestate is commonly applied either to the top of the soil surface or shallowly injected, and the negative effects caused by digestate indicate the presence of potentially toxic constituents, such as high ammonia and salt contents, which were both found to contribute to greater earthworm mortality (Bhogal *et al.*, 2016; Natalio *et al.*, 2021). Epi-anecic earthworms can avoid these toxic effects due to their deep burrowing nature, although a small number were found dead shortly after digestate application as a result of being present in the surface soil immediately after application (Moinard *et al.*, 2021). Overall, very few ecotoxicological tests have been done to understand the impact of digestate application on earthworms and there may be other factors involved.

Digestate is applied to a rate of total nitrogen per hectare, to match the nutrient requirements of the crop, which can require high volumes of digestate to be applied. At a volume of 50t t ha^{-1} to supply 170kg-N ha^{-1}, more dead earthworms were found compared to a lower volume of 25t/ha (Johansen *et al*., 2015). As such a method to mitigate earthworm mortality would be to reduce the application rate, which can be done by using a split application method where the crop is fertilised at two or more time periods during its growth. Another option is to alter the method by which digestate is applied, which is either broadcast, bandspread or injected. Investigation into the effects of these application methods on earthworm mortality has not been undertaken. Additionally, it would be worth investigating whether transforming the physiochemical properties of digestate would influence its toxicity.

2.6 Transforming anaerobic digestate

2.6.1 Composting

The high ammonium nitrogen content of digestate that makes it a good fertilizer has the negative effects on micro- to macro-organisms as discussed previously, and on the environment through leaching and volatilisation (Nkoa 2014). Transforming it through composting reduces these problems, with additional benefits such as reduction in pathogen contamination (Bustamante *et al*., 2012; Tambone *et al*., 2015; Subirats, Sharpe and Topp, 2022) and odours (Rincón *et al.*, 2019). In order to be composted effectively, additional materials such as woodchips, corn stalks, or oyster shells as bulking agents, and, in the case of organic materials, to increase the C:N ratio are required (Zeng, De Guardia and Dabert, 2016; Li *et al.*, 2020; Lu *et al.*, 2020). Usually, only the solid fraction is composted, but the liquid fraction can be used to water compost piles (Bustamante *et al.*, 2013; Vu *et al.*, 2015).

Applying composted digestate to soil had a positive and lasting effect on microbial abundance (de la Fuente *et al.*, 2013) but it reduced the peak of microbial activity, compared to when solid digestate was applied, and lowered the amount of carbon that was mineralised. This dampening of respiration is because compost contains a higher amount of carbon that is resistant to decomposition, as the readily and semi degradable carbon has already been decomposed during the anaerobic digestion and aerobic composting stages. Maynaud *et al*., (2017) demonstrated that the solid fraction of digestate still contained a substantial amount of the easily accessible carbon of the digestate. As a result, the microbial biomass did not increase as much under composted digestate application compared to the application of the solid digestate, yet was still higher than the biomass in whole digestate or liquid digestate treated soils. Adding composted digestate to degraded agricultural land had a positive influence on the soil microbial diversity (Caracciolo *et al.*, 2015; Manasa *et al.*, 2020).

2.6.2 Additives

Attention is being given to studying the effects of adding biochar into digestate to reduce environmental pollution risks from its application. Biochar is a material derived from the thermal decomposition of organic material in the absence of oxygen (pyrolysis), often using feedstock materials that are otherwise considered a waste product. Biochar is a high carbon and highly porous material and has been found to reduce N_2O emissions (Dicke *et al.*, 2015; Martin *et al.*, 2015) and nitrate leaching (Plaimart *et al.*, 2021) when applied with digestate.

Multi-year field trials running between 1.5-4 years showed that the co-application of digestate with biochar had a positive effect on soil microbial biomass compared to soil receiving digestate only (Hewage, 2016; Greenberg *et al.*, 2019). This increase could be due to a variety of reasons. The biochar provides a surface for bacteria to adhere to (Hill *et al.*, 2019), preventing them being leached by the liquid in the digestate. Similarly, nutrients may sorb to the surface of biochar due to its high cation exchange capacity, which steadies the supply of nutrients delivered from the digestate and thereby increases the availability of nutrients to microbes over time (Zhu *et al.*, 2017). The highly porous nature of biochar can increase the water holding capacity of sandy soils (Glaser, Lehmann and Zech, 2002), the soil texture used in both aforementioned studies, retaining moisture from sources such as the digestate and ensuring microorganisms have access to water during drier periods. The pH of biochar should also be considered. An alkaline biochar may offset soil acidification by digestate, thereby creating a more favourable environment for microorganisms, as Hewage (2016) observed that soils applied with digestate and a biochar of pH 8 had a higher soil pH than digestate treated soils by the end of their experiment.

2.6.3 Nutrient recovery

The recovery of nutrients from anaerobic digestate is of interest to the biogas industry as it reduces problems of storage and cost of transporting the bulky liquid material. Techniques are being investigated to remove nutrients which can then be applied to soils as a fertiliser. Methods can be physical, such as drying or filtering the digestate to concentrate the nutrients and clean the water for safe disposal or reuse (Knoop *et al*., 2018; Chiumenti *et al*., 2013). Chemical methods include ammonia stripping to recover nitrogen (Liu *et al*., 2015; Zarebska *et al*., 2015), the formation of struvite crystals to capture phosphorus and nitrogen (Zhang *et al*., 2020b; Muhmood *et al*., 2019), and the use of materials such as biochar or zeolites that have a high cation exchange capacity to absorb nutrients (Kocatürk-Schumacher *et al*., 2017; Shepherd, Sohi and Heal, 2016). Biological methods include reed beds and algae runways (Nielsen and Stefanakis, 2020; Díez-Montero *et al*., 2020).

These techniques are mostly in the early stages of development (Khoshnevisan *et al*., 2021; Shi *et al*., 2018; Logan and Visvanathan, 2019) and their effects on soil biota is not a primary research concern. However, a similarity with all these products is the zero to very low carbon content, or in the case of biochar highly recalcitrant carbon. Therefore, it can be conjectured that the application of these products will have indirect benefits to soil microbial community should they stimulate crop yield, with bigger crops equalling more roots for decomposition as well as triggering nutrient mining by plants through increased exudates. Sorbent materials such as zeolites and biochar positively influence microorganisms involved in nitrogen cycling (Costamanga *et al*., 2020; Karličić *et al*., 2017). Yet these benefits may be outweighed by any significant changes in the soil physiochemical status, particularly pH. Nitrogen based fertiliser has been shown to acidify soils (Pan *et al*., 2020). P-struvite crystals may increase levels of magnesium to above optimum, turning this essential metal toxic (Gell *et al*., 2011). Being of organic material origin there are also potential toxic elements in biochar based on its feedstock and pyrolysis process that can have subsequent negative effects on the soil biology (Godlewska *et al*., 2021).

Unlike chemical and physical nutrient recovery techniques, biological nutrient recovery methods may be most promising for benefiting soil microbes. Algae grown in a digestate substrate can then be processed and used as a biofertiliser (Hussain *et al*., 2021; Solovchenko *et al*., 2016). The application of algae as a fertiliser has been shown to have positive effects on the microbial biomass in the soil (Marks *et al*., 2019). When applied as necromass, the algae cells decompose and release nutrients and carbon into the soil, providing resources to support microbial growth. Living algae are also applied and can contribute to microbial biomass growth in multiple ways, which include the following. Firstly, some algae such as cyanobacteria can grow in the soil and directly add to the abundance (Perin *et al*., 2019). Secondly, algae produce extracellular polysaccharides, which provide a carbon source to other microbes (Marks *et al*., 2019). Thirdly they may be able to ameliorate soil pollution (Subashchandrabose *et al*., 2011) and improve conditions for soil microorganisms. The application of algae as a nutrient recovery technology is facing challenges for implementation, such as digestate turbidity and algae biomass processing (Xia and Murphy, 2016).

2.7 Conclusion and future research requirements

The addition of anaerobic digestate to soil has variable effects on the soil biota (Figure 2-2) and long-term research is needed to understand the cumulative effects of repeated digestate application on soil organisms. Digestate can be altered by physical separation to liquid and solid fractions. Evidence from the reviewed literature suggests that the solid fraction of digestate has positive effects on all groups of soil microorganisms. The liquid fraction only slightly benefits bacteria and negatively affects mycorrhizal and saprophytic fungi. Digestate in its whole form negatively affects litter surface dwelling springtails, nematodes and earthworms, though these effects are reduced for organisms that inhabit deeper layers of soil. The negative effects of digestate on soil organisms are due to a combination of factors including, but not limited to, (i) lack of carbon supplied to support growth, (ii) toxicity due to ammonia and contaminant content, and (iii) changes to habitat conditions caused by shifting soil pH.

The focus of biogas production should include optimisation of digestate quality for fertiliser use, without detrimentally affecting biogas production. Plant operators can separate the digestate for fertiliser use to reduce handling costs or add materials such as biochar to the digestate to improve the retention of nutrients in the soil following its application. Digestate can be stabilised by composting, reducing its toxicity and the negative environmental impacts such as nutrient losses at application, and positively benefiting soil microorganisms. In all cases research needs to be done to understand the long-term effects of these digestate products on soil organic matter, including the life within, which underpins all soil processes necessary for productive crop growing. By ensuring that anaerobic digestate promotes the development of soil organic matter and functioning of soil biota, biogas facilities can provide farmers with a sustainable alternative to mineral fertilisers.

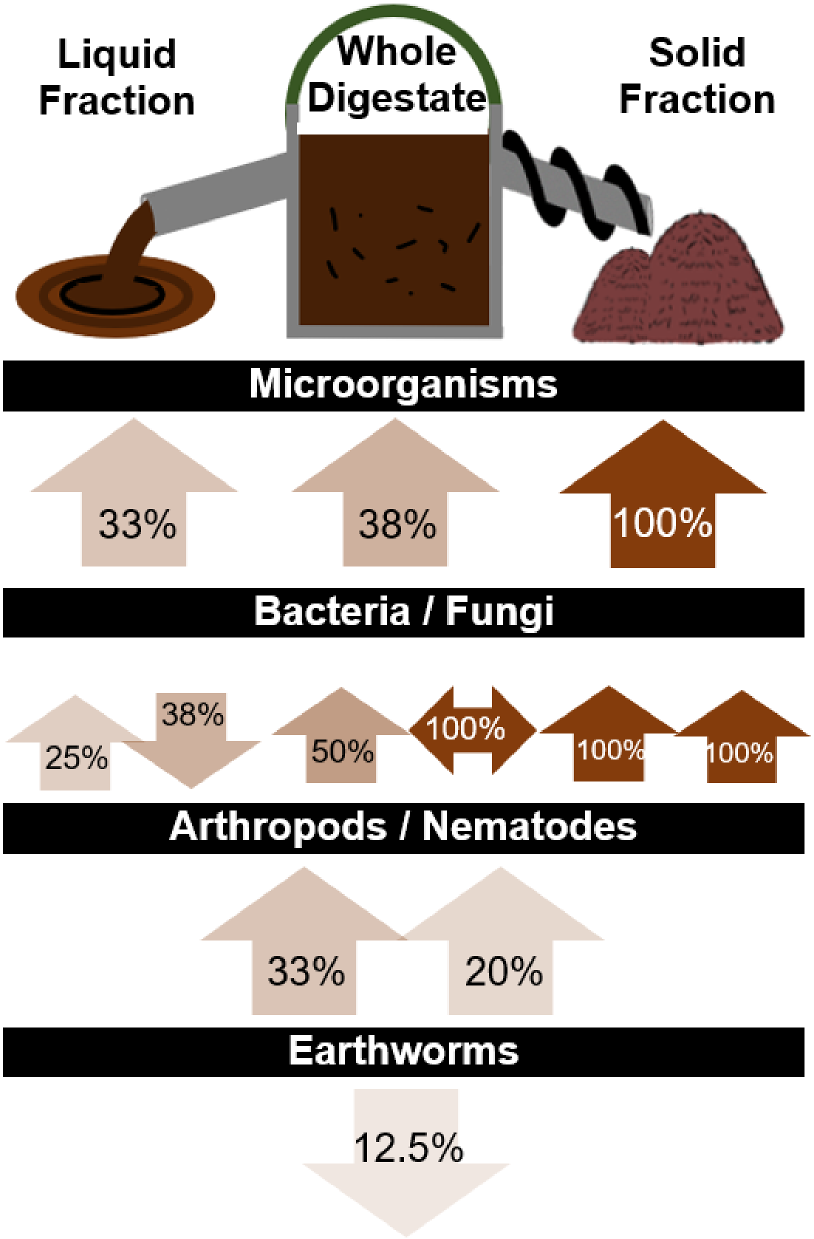

Figure 2-2 Effect of anaerobic digestate and its fractions on the abundance of soil biota groups, based on the percentage of studies indicating the effect out of all the studies that included this measurement. Studies used to generate this figure are in Tables 2-3, 2-4, 2-5.

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3 Immobilisation of anaerobic digestate supplied nitrogen into soil microbial biomass is dependent on lability of high organic carbon materials additives.

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3.1 Abstract

Anaerobic digestate is a nutrient rich slurry by-product derived from biogas production, often used as a fertiliser due to its high nitrogen content. However, nitrogen losses from its application can lead to environmental pollution. In a laboratory experiment, the addition of high organic carbon materials to digestate-amended soil as a potential means to stimulate microbial immobilisation of digestate supplied nitrogen was investigated. Soil was incubated in pots for five months with digestate (equivalent to 250 kg-N ha^{-1}). The impact of adding carbon into the digestate (equivalent to 540 kg-C ha^{-1}) as either glycerol, straw, woodchip, or biochar on soil microbial and chemical parameters was quantified. Glycerol amended soils had significantly higher microbial biomass compared to digestate alone during the first month and at 30 days after application had a 4x higher on average microbial N. The digestate+straw treatment resulted in a 2.5x greater nitrogen immobilisation compared to digestate alone after three months of incubation. The digestate+woodchip had a 2x higher mean microbial N after 5 months, whilst the biochar amendment did not stimulate significant nitrogen immobilisation at any time. These results suggest that mixing a labile to moderately labile organic carbon amendment, such as glycerol and straw, with digestate has the greatest potential to reduce nitrogen losses following digestate application through microbial immobilisation.

3.2 Introduction

Anaerobic digestate is a nutrient-dense slurry generated from biogas production. The process to create biogas requires the breakdown of a biodegradable feedstock such as animal waste, purpose grown energy crops, sewage waste, and the organic fraction of household, municipal and industrial wastes, in the absence of oxygen. During anaerobic digestion between 20-95% of the carbon in the feedstock is converted into methane and carbon dioxide (Möller, 2015) and collected as biogas. As the organic matter is digestated, between 15-82% of the organic nitrogen bound within is mineralised into ammonium, (Bareha *et al.*, 2018). As this form of nitrogen is readily available for plants to utilise, digestate can be applied to land as a fertiliser. However, due to its high liquid content it is expensive for the biogas industry to store and transport the digestate (Al-Seadi *et al.*, 2012). A solution to reduce costs is to separate the digestate into its fibrous (solid) and liquid fractions, which reduces the volume of the liquid by 20-30% (Lyons *et al.*, 2021).

The liquid fraction of the separated digestate has been extensively studied for its use as a fertiliser and been found to produce yields comparable to synthetic nitrogen (Šimon, Kunzová and Friedlová, 2015; Riva *et al.*, 2016; Walsh *et al.*, 2018; Barzee *et al.*, 2019). However, sustainable use of digestate as a fertiliser on agricultural land is dependent on good management practises. On average, over 80% of the nitrogen content of the liquid fraction is in the form of ammonium and nitrate (Czekała, 2022), which are the N forms most susceptible to loss processes by volatilisation, denitrification, nitrification and leaching. Therefore, land spreading digestate can result in groundwater and atmospheric pollution (Nkoa, 2014). These nitrogen losses from the soil reduce the nitrogen use efficiency of digestate fertilisers. Therefore, methods need to be developed to keep nitrogen supplied by the liquid digestate in the soil and available for crop uptake, while minimising losses and detrimental impacts on the environment.

Ammonia volatilisation losses represent a key nitrogen loss pathway when applying anaerobic digestate with 35-60% of total nitrogen applied lost (Tiwary *et al.*, 2015). However, these losses are reduced by 40-50% using precision slurry spreading techniques such as band spreading or injecting digestate directly into the soil compared to the traditional practise of broadcast spreading (Nicholson *et al.*, 2018). Similarly adding sulphuric acid into digestate to lower its pH can effectively reduce ammonia volatilisation with reductions ranging from 45% (Wagner *et al.*, 2021) to 95% (Sánchez-Rodríguez *et al.*, 2018) when compared to non-acidified digestate. Once in the soil, the ammonium nitrogen from digestate is converted by nitrifying and denitrifying microorganisms, resulting in nitrogen losses before plant uptake as nitrate leaching and N_2O emissions. Research is ongoing on the applicability of using nitrification inhibitors, which are chemical compounds added to a fertiliser to delay the conversion of ammonium into nitrate and therefore reduce these losses. Huf and Olfs (2020) and Giacometti *et al.*, (2020) observed that the addition of nitrification inhibitors to digestate reduced N_2O and leaching losses respectively within a month of application. Hegewald *et al.*, (2021) observed lower annual N_2O emissions by 36% over a three-year period. However, the cost of using nitrification inhibitors can be significant because factors such as climate, soil type and crop influence their effectiveness (Macleod *et al*., 2015; Drame *et al.*, 2023). Furthermore, these nitrification inhibitors are agrochemicals which cannot be used in organic farming systems. As such, it is necessary to investigate alternative methods of reducing nitrogen losses from the soil.

Soil microorganisms can immobilise nitrogen into their biomass, as they use nitrogen to build proteins, nucleic acids and other cellular components. This nitrogen forms part of the necromass when the microbes die, which is subsequently remineralised when the necromass is primed by plant root exudates (Meier *et al.,* 2017). This provides plants with a source of nitrogen when they need it as opposed to farmers applying synthetic nitrogen, which can result in a mismatch between time of input and plant demand. Most soil microorganisms are heterotrophic and require an external source of organic carbon as a precursor to synthesising their own molecules. Digestate has a low total carbon content, typically between 0.43-3.4% (Risberg *et al.*, 2017). The nutrient flush following digestate application causes temporary increases in microbial activity and abundance that subside within 24 hours of application (Alburquerque, de la

Fuente and Bernal, 2012; Iocoli *et al.*, 2019) and disappear within a few weeks (Walsh *et al.*, 2012; Johansen *et al.*, 2013; Mórtola *et al.*, 2019; Gebremikael *et al.*, 2020; Ren *et al.*, 2020; Różyło and Bohacz, 2020). When digestate is separated into its solid and liquid fractions and applied separately to land, the organic carbon richer solid fraction increases microbial growth and immobilised nitrogen into microbial biomass, which is not seen when liquid digestate is applied (de la Fuente *et al.*, 2013). This observation indicates that to stimulate microbial immobilisation of nitrogen in liquid digestate, more carbon is needed to satisfy microbial stoichiometry.

Organic materials high in carbon but low in nitrogen are known to immobilise nitrogen into soil microorganisms; as the microbes utilise the bioavailable carbon, they simultaneously use the material supplied nitrogen to meet their own, lower, carbon to nitrogen (C:N ratio) requirements (Robertson and Groffman, 2007). However, C:N ratio alone does not determine the rate of microbial growth, as the carbon in material can be of varying accessibility to microbes. For example, carbon that forms a simple monomer can be readily taken up through diffusion or active transport into microbial cells (da Silva, Mack and Contiero, 2009; Dobson, Gray and Rumbold, 2012). More complex carbon structures include plant polymers such as hemicellulose, cellulose and lignin. To break down these materials into assimilable molecules, microbes require a variety of externally excreted enzymes, with residues containing a high lignin content predominately decomposed by enzymes secreted by fungi and bacterial cells that form multicellular assemblages such as *Actinomycetes* (Mekonnen, 2021).

This research aimed to investigate the effects of adding different sources of organic carbon to liquid digestate on soil microbial biomass, microbial nitrogen immobilisation and microbial community dynamics. The objective was to determine which type of organic material, in terms of carbon accessibility, was most effective at inducing nitrogen immobilisation. It was hypothesised that adding organic carbon additives to liquid digestate would stimulate microbial immobilisation of nitrogen, and that the magnitude and the timing of the effect would be influenced by the accessibility of the carbon substrates to soil microorganisms. Since the additives vary in structural complexity, they are utilised by different microbial groups as a carbon source for growth and energy, it was predicted that the microbial community would shift to one with a higher fungi-to-bacteria ratio with the addition of complex carbon materials into the digestate. A pot experiment was established to test these hypotheses in the absence of confounding environmental variables, such as temperature.

3.3 Materials and Methods

3.3.1 Soil, digestate and high carbon organic materials

A sandy loam topsoil (69% sand, 20% silt, 11% clay) bought from Bourne Amenity Ltd, was used for the study. Prior to the experiment the soil was passed through a 2 mm sieve to remove any stones and large debris and thoroughly mixed to homogenise. Liquid anaerobic digestate was supplied by Future Biogas Ltd from a biogas plant, using a mixed feedstock of 85 tonnes maize silage, 7.5 tonnes cow manure, and 18 tonnes chicken manure. The plant is mesophilic, continuously fed, operating at 43°C with a retention time of 98 days. The liquid fraction was mechanically obtained from the whole digestate after screw press separation. The biochar applied in the experiment (CreChar™, supplied by Carbogenics Ltd) was produced from office waste in a kiln run at 700-800°C for 60 minutes. Wheat straw was obtained from a farmer (Bedfordshire UK) and woodchips from the Milton Keynes council parks department. Straw and woodchips were air-dried and chopped into smaller pieces (1-2 cm) to fit into the pots. Glycerol was bought from Sigma Aldrich. Details of material properties are in Table 3-1.

Table 3-1: The biophysiochemical properties of the materials used in this study. Data for the liquid digestate, biochar, glycerol and select soil properties (organic matter, phosphorus, potassium) were provided by the suppliers.

Properties	Soil	Liquid digestate	Biochar	Woodchips	Straw	Glycerol
Dry matter (%)	90.4	5.2	97.7	86.2	93.1	-
Organic matter (%)	5.3	3.9	-	-	-	-
pH	8.1	8.3	11.3	-	-	-
Total Carbon (g/kg fw)	37.5	20.6	628	546	479	391.9

Properties	Soil	Liquid digestate	Biochar	Woodchips	Straw	Glycerol
Nitrogen						-
Total (g/kg)	2.85	6.3	5.22	11.2	6.0	-
Ammonium (mg/kg)	-	4147	-	-	-	-
Nitrate (mg/kg)	-	<10	-	-	-	-
Phosphorus						
Total (mg/kg)		521	1.02	-	-	-
Available (mg/l)	49	-	-	-	-	-
Potassium						
Total (mg/kg)	-	4275	21.5	-	-	-
Available (mg/l)	755	-	-	-	-	-
C:N ratio	12.95	3.27	120.39	48.97	79.85	-
Microbial biomass Carbon (mg-C/kg fw)	392	-	-	-	-	-
Microbial Biomass Nitrogen (mg-N/kg fw)	98	-	-	-	-	-

fw = fresh weight basis.

3.3.2 Soil incubations

The incubation experiment was carried out in the dark at 20±4°C for 5 months. 250 g (dry weight basis) soil was added to 330 ml capacity PVC containers (top diameter 8 cm, bottom diameter 5 cm, height 12 cm) and pots were gently tapped on the worktop to ensure soil settled to a bulk density of 1 g cm^3. Before the start of the experiment the soils were adjusted to 40% water holding capacity and pre-incubated at 20±4°C in the dark under aerobic conditions for a week, to allow soil microbial activity to recover after being sieved. Water holding capacity was determined using a saturate and drain method modified from Harding and Ross (1964). 50 g of soil was added to a stoppered funnel and saturated with 100 ml of deionised water for 30 minutes. The stopper was then removed, and the water drained for 30 minutes. The volume of water retained in the soil was combined with the known moisture content of the soil to calculate the water holding capacity.

The experiment consisted of six treatments, arranged in a randomised block design with four replications: 1) liquid digestate control (LD); 2) liquid digestate with glycerol (LD-G); 3) liquid digestate with straw (LD-S); 4) liquid digestate with woodchip (LD-W); 5) liquid digestate with biochar (LD-B); and 6) unfertilised control (CONT). Sufficient sets were set-up to allow for destructive sampling for soil biochemical analysis on four occasions: 3 hours after application, then 30, 90 and 150 days after application. This gave a total of 96 experimental units (6 treatments x 4 replicates x 4 sampling times).

Digestate was applied at 23 ml per pot, a rate equivalent to 250 kg-N ha^{-1}, which supplied 0.59 mg-N g^{-1} dry soil. This rate of nitrogen was selected as it is the maximum amount farmers are allowed to apply in a 12 month period from organic sources in areas designated as Nitrate Vulnerable Zones under UK law (DEFRA and EP, 2018). Prior to application, the digestate was mixed with additives at a rate of 12 kg-C m^3 of digestate (equivalent to 540 kg-C ha^{-1}), resulting in a material with a C:N ratio of approximately 5:1. This equalled an addition to the pots of 0.7 ml glycerol and 0.68 g, 0.59 g or 0.51 g of straw, woodchip, and biochar respectively. The treatments were then mixed into the soil. A volume of water equal to the volume of digestate was added to the non-amended control pots and similarly mixed. The rationale for this amendment rate is given in the supporting information. The pots were loosely covered with lids to reduce moisture loss and weighed twice a week to check the moisture content and deionised water was added to maintain soils at 40% water holding capacity, which is optimal for microbial development (Gulledge and Schimel, 1998). At each sampling time, the pots were destructively sampled, and the soil passed through a 2 mm sieve to break down the aggregates that had formed when mixing treatments into the soil, in order to homogenise the sample ready for analysis.

3.3.3 Microbial analyses

Microbial biomass carbon and nitrogen (microbial C and N respectively) were determined following the fumigation-extraction method (Vance, Brooks and Jenkinson, 1987). After extraction, the extracts were analysed on a Shimadzu TOC with a TN module. The microbial C and N were calculated using K_{EC} and K_{EN} values of 0.45 and 0.54 respectively (Brooks *et al.*, 1985; Vance, Brooks and Jenkinson, 1987). PLFA profiles were determined using a modified method from Frostegård, Tunlid and Bååth (1991) by freeze-drying the soil after sieving. Lipids were extracted from 10 g freeze-dried soil using the Bligh and Dyer (1959) solvent ratio 1:2:0.8 v/v/v of chloroform, methanol, and a pH 4 citrate buffer, fractionated, and the phospholipids derivatised by mild alkaline methanolysis. The resultant fatty acid methyl esters were separated by gas chromatography (Agilent Technologies, Santa Clara, CA, U.S.A) using a HP-5 (Agilent Technologies) capillary column (30 m length, 0.32 mm ID, 0.25 μm film). The GC conditions were reported in Pawlett *et al.*, (2013). Resultant peak areas were integrated using G2070 ChemStation (Agilent Technologies) for gas chromatography and calculated as relative abundance (mol %). Bacteria were identified by the PLFA bioindicators 14:00, 15:0i, 15:0ai, 15:00, 16:0i, 16:1ω7c, 16:00, 17:0i, cyc17:0, ai17:0, 17:0br, 17:1ω8c, 17:1ω8t, 17:1ω7, 18:00, 18:0 (10Me), 18:1ω13 and 20:00 (Frostegård and Bååth, 1996; White, Stair and Ringelberg, 1996; Zelles, 1997, 1999; Bossio and Scow, 1998; Kourtev, Ehrenfeld and Haggblom, 2002). Fungi were identified by the biomarker 18:2ω6,9 (Frostegård and Bååth, 1996). The fungi-to-bacteria ratio was calculated by dividing the mol % of the fungal biomarker (18:2ω6,9) by the summed mol % of bacterial fatty acids (Frostegård and Bååth 1996).

3.3.4 Chemical analyses

Soil total nitrogen was determined by dry combustion according to the British Standard Institution (BS EN 13654-2:2001) and analysed using an elemental analyser (Elementar, Vario EL III). Available nitrogen as the sum of ammonia and total oxides of nitrogen was determined using the potassium chloride (KCl) extraction method (MAFF, 1986). 20 g of soil was eluted with 100 ml of 2 mol KCl solution and filtered, after which the extracts were analysed on an analytical segmented flow multi-chemistry analyser (Seal, AA3).

3.3.5 Statistics

Data were first tested for normality using Kolmogorov-Smirnov & Lilliefors test and homoscedasticity using Levene’s Test, following this the available N, microbial C and N datasets were Box-Cox transformed. As the independent experimental variable of interest was treatment, a one-way ANOVA was used to determine treatment effects at each sampling time on the soil parameters, whilst time as factor was not included due to unequal variance in data between sampling times. Significant differences between treatments for available and total N, and fungi:bateria (F:B) ratio were determined by Tukey’s post hoc test. The microbial

C and N datasets still failed homoscedasticity so a one-way Welch ANOVA was used as it can tolerate unequal variance (Wilcox, 2003) and significant differences between treatments were determined by Games-Howell's post hoc test. Principal Component Analysis was run on the PLFA profile data for each timepoint, which was normalised by measuring each biomarker as the relative abundance (%mol) to all the biomarkers. The resultant factor scores for each timepoint were analysed using a one-way ANOVA. All differences were considered statistically significant if $p<0.05$. All statistical analysis was carried out in Statistica version 14.

3.4 Results

3.4.1 Biological properties of the soil

The addition of digestate alone to the soil did not result in higher microbial biomass C than the soil only control treatment at any timepoint. At 3 hrs after application, the addition of glycerol to digestate resulted in a significantly ($p=0.004$) higher microbial biomass C by 344% compared to the digestate only control (Figure 3-1A). At 90 days the digestate with either straw or woodchip had a higher microbial biomass than soil only, but not the digestate control. At 150 days, the ANOVA recorded a significant effect due to treatment ($p=0.001$), but the post hoc test did not identify any significant differences between individual treatments.

Microbial N was not significantly ($p=0.95$) affected by treatments at 3 hrs after application (Figure 3-1B). At 30 days the LD+biochar had a significantly ($p=0.02$) lower concentration of microbial N than soil only control, but not the digestate only control. At 90 days after application digestate applied with straw significantly ($p=0.007$) immobilised 80 µg-N g^{-1} (dry weight soil), equivalent to 75 kg-N ha^{-1} (see supplementary information for calculation) compared to the digestate only control, a difference of 309%. After 90 days no further significant treatment effect was observed, however digestate with woodchip had a 2x higher on average microbial N content compared to digestate only treatment.

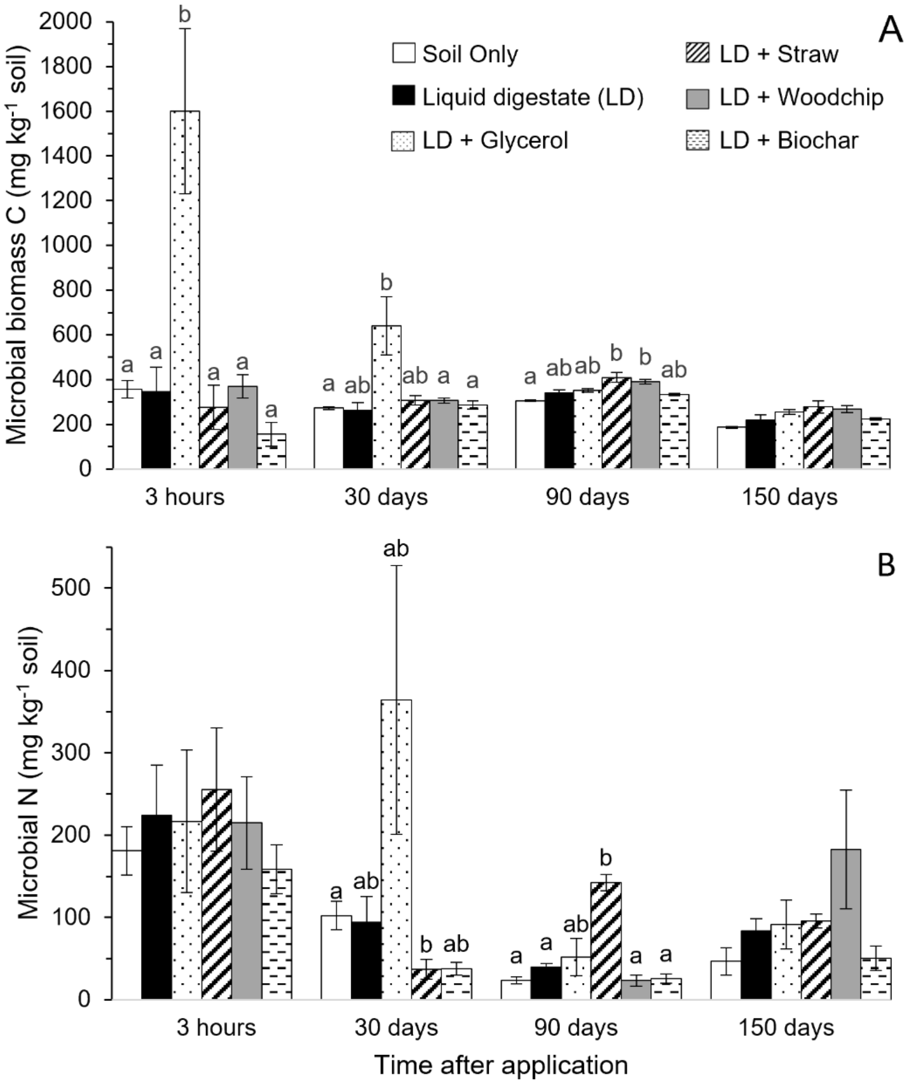


Figure 3-1 Changes in soil microbial biomass carbon (A) and microbial N (B). At each sampling time points denoting different lower-case letter have statistically different treatments effects according to Games-Howell's test at 5% probability on box-cox transformed data. Error bars denote the standard error of the mean, n = 4. In graph B, LD+woodchip at 30 days was not included into the analysis, due to only one replicate producing analysable data.

The first two principal components (PC) on each of the PLFA datasets accounted for ≥50% of the total variation (Figure 3-2), with significant ($p<0.05$) treatment effects on PC1 axis at every timepoint except 150 days after application. Fatty acid loadings that contributed the most (≥0.8 and ≤0.8) included 16:00, 16:1ω5, 17:0c, 18:2ω6,9 and 19:1ω6 for PC1 and 17:0br, 17:1ω8c and 18:1ω7t for PC2, for information on specific PLFAs for each PC axis see supplementary Table 1.

At both 3 hrs and 30 days after application the microbial communities between the digestate and the soil only controls were distinctly separate (Figure 3-2A and B; p=0.01) with a higher fungi:bacteria (F:B) ratio in the digestate control at day 30 (Table 3-2). At 30 days the addition of woodchips and biochar resulted in separate cluster with the soil only control, distinct from a group made of the glycerol additive and the digestate control (Figure 3-2B; p<0.001), with a lower F:B ratio in the former treatments compared to the latter (Table 3-2), whilst the straw treatment lay non-distinctly between the two groups. At 90 days the woodchip amended grouped separately from the glycerol and straw amended digestate treatments (Figure 3-2C; p=0.02), with a lower F:B ratio in the woodchip treatment compared to the straw and glycerol treatments (Table 3-2). By day 150 there were no distinct groupings between the digestate with and without additives (Figure 3-2D; p=0.06) and no difference in the F:B ratios (Table 3-2).

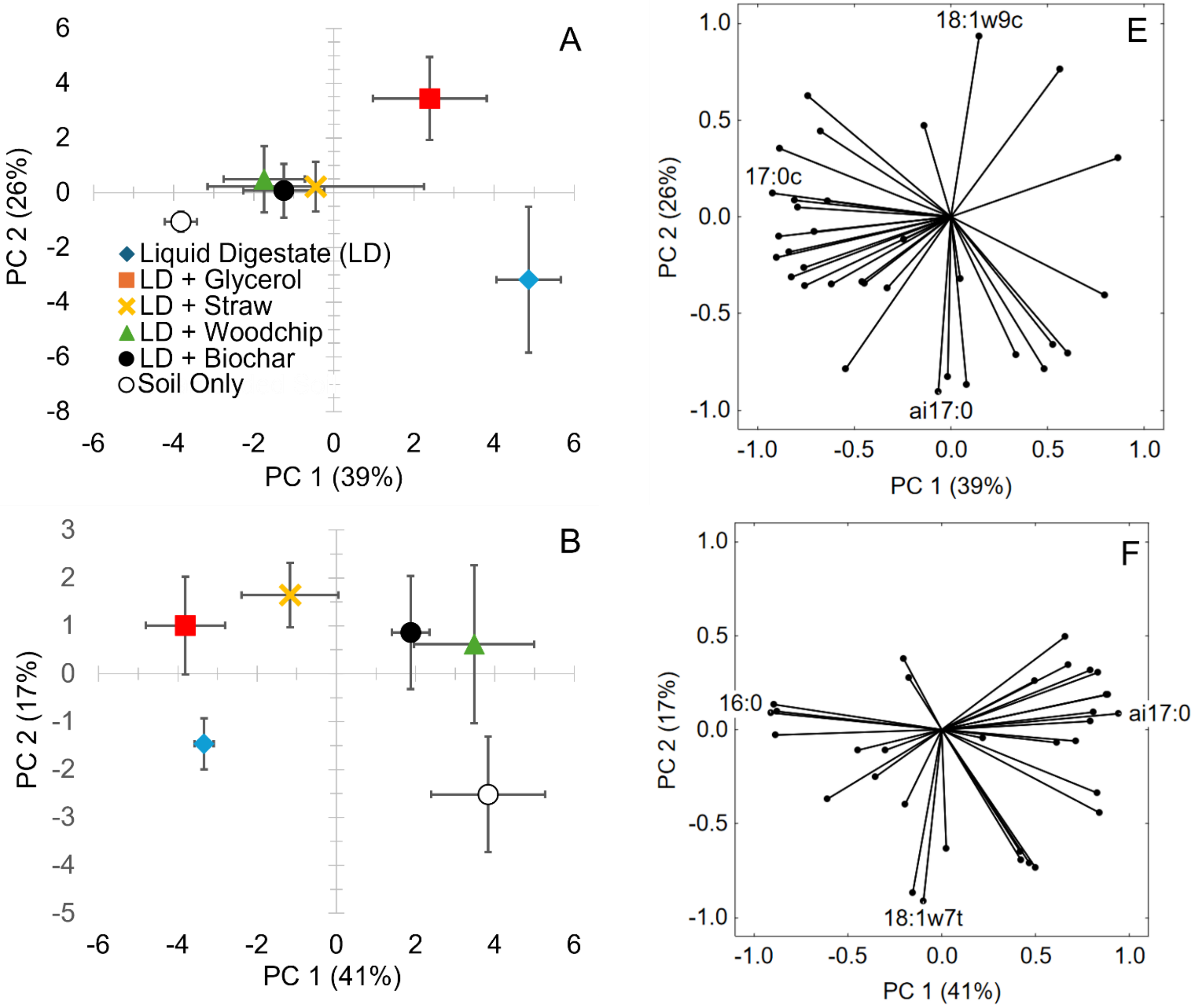


Figure 3-2 Principal component analysis on the PLFA profiles (mean ± SE, n=4) of the microbial community and their corresponding loadings plots labelling the top three contributing biomarkers as affected by the treatments at each sampling time: A = 3 hours, B = 30 days, C = 90 days and D = 150 days, after application. The loading plots E-H relate to A-D respectively. Error bars denote the standard error of the mean, n=4.

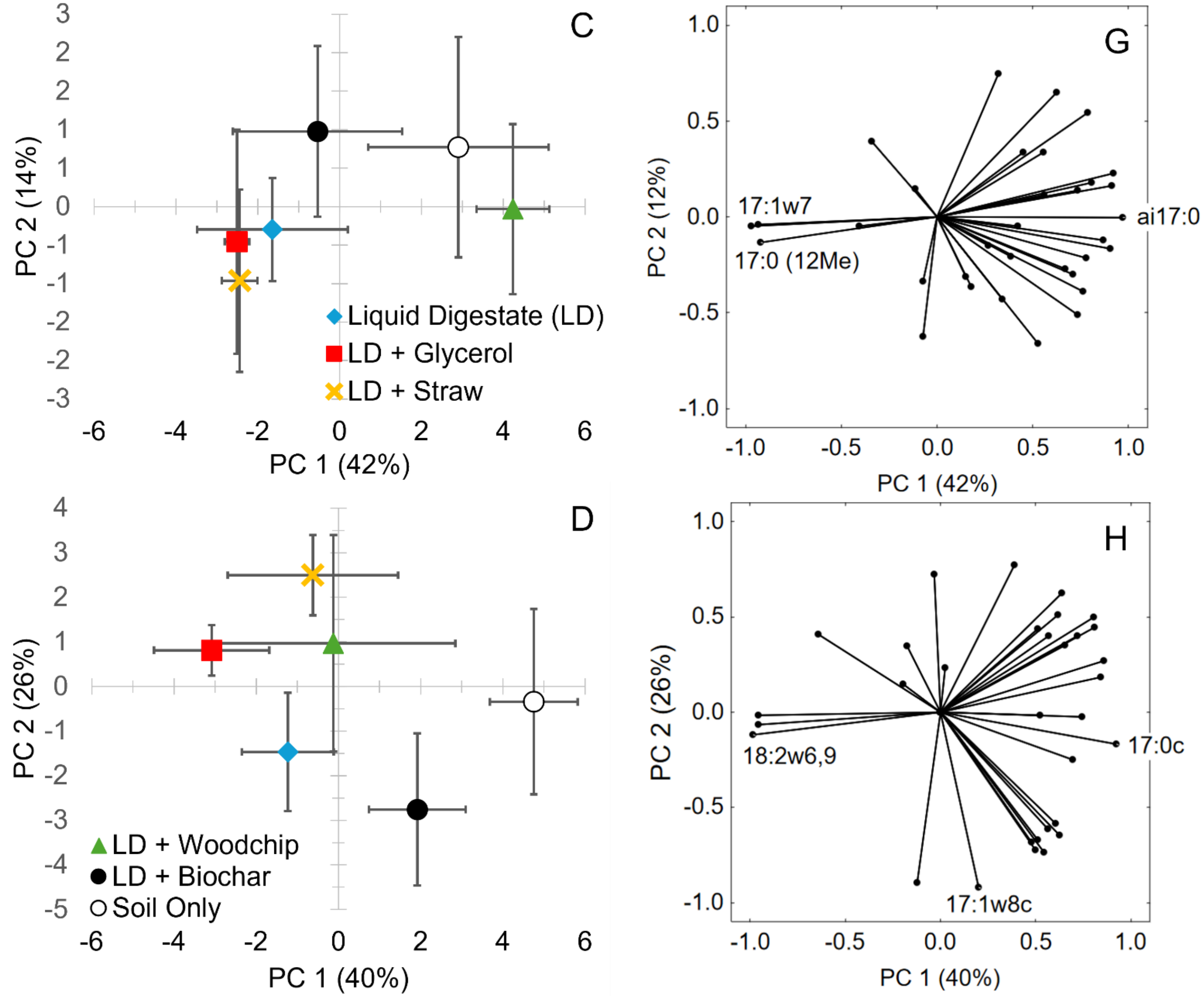


Figure 3-2 continued Principal component analysis on the PLFA profiles (mean ± SE, n=4) of the microbial community and the corresponding loadings plots labelling the top three contributing biomarkers as affected by the treatments at each sampling time: A = 3 hours, B = 30 days, C = 90 days and D = 150 days, after application. The loading plots E-H relate to A-D respectively. Error bars denote the standard error of the mean, n=4.

Table 3-2: Fungi:bacteria (F:B) ratio (mean ± SE) in the soil after treatment manipulations. Mean (n=4) values between treatments in a row (sampling time) denoted with a different lower-case letter are statistically different according to Tukey's test at the 5% probability level.

Parameter	Days after application	Soil only	Liquid digestate	LD+ Glycerol	LD+ Straw	LD+ Woodchip	LD+ Biochar
F:B ratio	0	0.118± 0.001	0.125± 0.017	0.173± 0.025	0.153± 0.026	0.139± 0.017	0.146± 0.009
	30	0.084± 0.005^{a}	0.202± 0.007^{c}	0.172± 0.019^{c}	0.154± 0.016^{bc}	0.091± 0.004^{a}	0.104± 0.009^{ab}
	90	0.161± 0.023^{ab}	0.215± 0.020^{ab}	0.218± 0.003^{a}	0.219± 0.005^{a}	0.142± 0.007^{b}	0.187± 0.024^{ab}
	150	0.124± 0.011	0.186± 0.013	0.208± 0.013	0.183± 0.024	0.174± 0.028	0.148± 0.010

3.4.2 Chemical properties of the soil

The liquid digestate resulted in a significantly ($p<0.005$) greater amount of total soil nitrogen compared to the soil only control from 30 days onwards (Table 3-3). Mixing additives into digestate did not result in any further significant differences to the total soil nitrogen content apart from at 90 days after application where there was a lower total nitrogen in the digestate with straw treatment compared to woodchip and biochar amended digestates.

Table 3-3: Total nitrogen content of soils (mean ± SE) following treatment incorporations. Mean (n=4) values between treatments in a row (sampling time) denoted with a different lower-case letter are statistically different according to Tukey's test at the 5% probability level.

Parameter	Days after application	Soil only	Liquid digestate	LD+ Glycerol	LD+ Straw	LD+ Wood chip	LD+ Biochar
Total N	0	2600± 115	2966± 33	2933± 120	2833± 120	2933± 120	3000± 100
mgN kg^{-1}	30	2575± 48^{a}	2950± 65^{b}	2725± 25^{ab}	2925± 25^{b}	2850± 87^{ab}	2975± 111^{b}
	90	2800± 41^{a}	3125± 103^{b}	3050± 96^{ab}	3000± 41^{a}	3125± 48^{b}	3300± 58^{b}
	150	2575± 25^{a}	3025± 48^{b}	3050± 29^{b}	2875± 25^{b}	3075± 75^{b}	3050± 29^{b}

3 hrs after treatment applications, the amount of total available nitrogen was significantly greater ($p<0.05$) in the digestate (LD) only treatment compared to the soil only control and remained so for the experimental duration (Figure 3-3). The addition of additives into digestate had no effect on total available N compared to digestate control at all timepoints, however at day 90 the digestate with glycerol had a lower content compared to the digestate mixed with either woodchip or biochar.

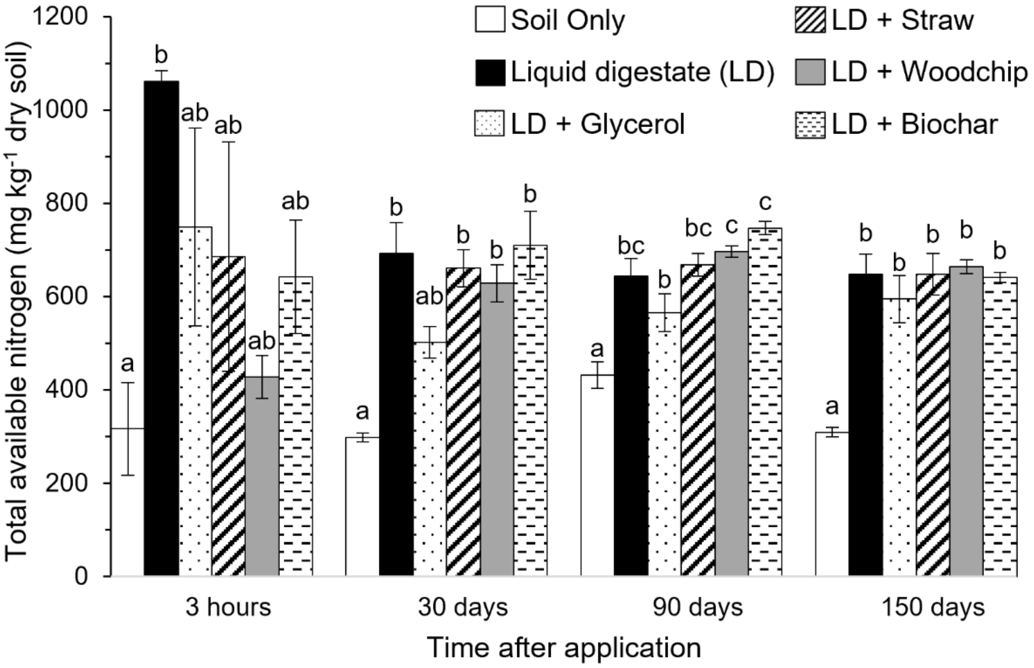


Figure 3-3 Changes in soil available nitrogen. At each sampling time point different lower-case letters between treatments have statistically different effects according to Tukey's test at 5% probability on box-cox transformed data. Error bars denote the standard error of the mean, n=4.

3.5 Discussion

The addition of straw to digestate elevated microbial N in the soil by 2.5x compared to digestate alone, by an amount equivalent to 75 kg-N ha^{-1}, demonstrating that straw stimulated the immobilisation of N into microorganisms. This immobilisation is in agreement with studies by Cao *et al.,* (2018) and Reichel *et al.,* (2018) who applied straw with synthetic nitrogen fertilisers, Chaves *et al.,* (2005) who added straw with nitrogen rich crop residue, and Wang *et al.,* (2019) who applied digestate with rice straw. However, in this study, microbial growth cannot explain the uptake of N in biomass as the microbial biomass carbon under straw+digestate was not significantly greater than the digestate treatment. Microbes do not only uptake N as they grow and reproduce, but their C:N stoichiometry can change to adapt to nutrient limitations, or alleviation thereof (Heuck, Weig and Spohn, 2015; Chen *et al.*, 2019). In fungi, Khan and Joergensen, (2019) suggested that N immobilisation can occur by storage of nitrogen-based compounds in vacuoles. Therefore, it is likely that a combination of both biomass incorporation and storage in fungal vacuoles caused the significant microbial N effect due to straw addition.

Contrary to the hypothesis, the addition of glycerol to digestate did not result in a significant microbial nitrogen immobilisation, despite the significantly greater growth in the first month from application. However, its addition did lead to a 4x higher on average microbial N at 30 days after application compared to digestate control. Significant results may be masked by experimental artifacts that can occur when comparing comparatively low amounts of extracted nitrogen between fumigated and non-fumigated soil relative to the large inputs of soluble N (Widmer, Brookes and Parry, 1989), as from digestate. Time of sampling could also have been an issue, as Alburquerque, de la Fuente and Bernal, (2012) observed that digestates made from a feedstock mix of cattle slurry with 4% and 6% glycerol stimulated a peak nitrogen immobilisation within a week of application. Since we did not sample between 3 hrs and 30 days after application, it is possible that the peak of N immobilisation was missed.

The addition of woodchips to digestate had no significant effect on microbial growth or microbial N compared to digestate alone, although at 150 days microbial N was 2x higher on average than digestate alone. Similarly, Tahboub, Lindemann and Murray, (2007) observed no effect on nitrogen immobilisation when adding woodchips with synthetic fertiliser. However Reichel *et al.* (2018) observed significant nitrogen immobilisation when adding sawdust with synthetic N, as did Chaves *et al.,* (2005) when adding sawdust with nitrogen rich celery leaves. These significant effects could be due to the smaller particle size of sawdust (~1 mm) compared to woodchips (majority between 2.5-5 cm) used by Tahboub, Lindemann and Murray, (2007) and the 1-2 cm woodchips used in our study. This meant that our samples had less surface area per volume exposed for microbial colonisation which reduces decomposition rates (Idler, Pecenka and Lenz, 2019). Furthermore, woodchips are mainly decomposed by fungi (Noll and Jirjis, 2012), yet the fungi to bacteria ratio in the woodchip amended soil of this study was never greater than digestate alone amended soil. This indicates that the fungal community in the soil did not include those that could effectively utilise the woodchips as carbon source. This could also have been a result of sampling bias, as the soil was sieved prior to PLFA analysis, removing pieces of woodchip greater than 2 mm and excluding any fungi colonising those woodchips from the analysis.

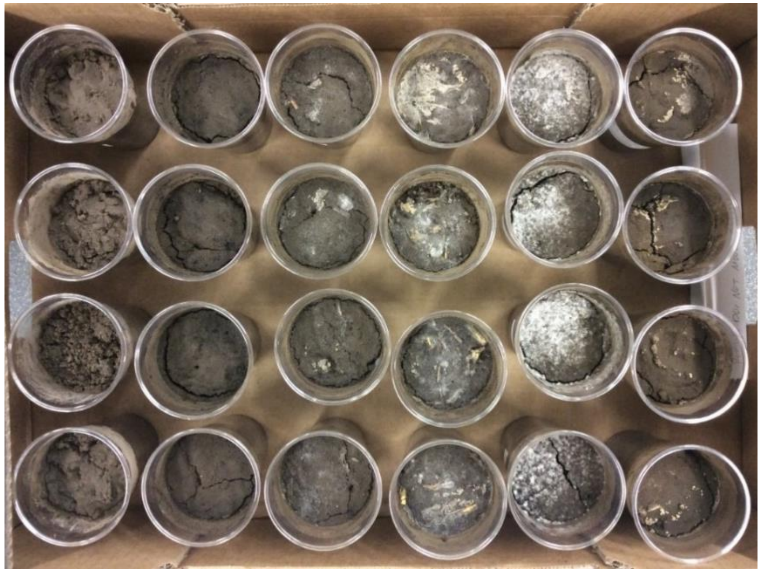
Mixing biochar into digestate did not have any effect on microbial biomass or nitrogen immobilisation. This corroborates results from Martin *et al.,* (2015) who also observed no effect of adding biochar with digestate on either microbial C or N. In biochar the carbon is formed into complex aromatic structures (Schmidt, Noack and Osmond, 2000) that are extremely resistant to decomposition (Wang, Xiong and Kuzyakov, 2016). Additionally, the highly porous structure of biochar absorbs dissolved organic carbon, reducing the amount available to microbes to utilise (Mukherjee *et al.*, 2016). Yet, Holatko *et al.,* (2021) observed increased microbial biomass C from digestate amended with biochar after 6 weeks, with a correlated decrease in soil nitrogen that could indicate nitrogen immobilisation had occurred. It is possible that the differing effects of biochar addition on nitrogen immobilisation between the current study and Holatko *et al.,* (2021) may be due to differences in the amount and type of biochar used. Holakto *et al.,* (2021) applied twice the concentration of biochar (0.4 kg per litre of digestate compared to 0.2 kg per litre) and used agricultural grain-waste biochar pyrolysed at 600-650°C (cf 700-800°C office waste biochar used here). Biochar feedstock and processing temperatures, of which there are numerous options (Lehmann *et al.*, 2011; Wang, Xiong and Kuzyakov, 2016), determine the size of a small biochar-associated labile carbon pool. It is possible that the biochar used by Holatko *et* *al.,* (2021) had a greater concentration of labile carbon and therefore stimulated microbial growth.

The immobilisation of nitrogen observed when digestate was mixed with straw demonstrates that microorganisms can be used as a potential mechanism to reduce the nitrogen losses from anaerobic digestate application. However, the quantity of N immobilised should be reflected in a lower concentration of soil available nitrogen. Yet contrary to this, the greater microbial N under straw addition did not result in a lower soil available nitrogen. This could be due to the extent of change in microbial N being too small to make a noticeable difference in the soil available N pool. There are several studies that supplied greater quantities of carbon than the 0.5 t-C ha^{-1} in this study. For example, 1.3 t-C ha^{-1} was applied by Cao *et al.,* (2018) and 4.5 t-C ha^{-1} was applied by Reichel *et al.,* (2018) as either straw or sawdust with synthetic nitrogen fertilisers, and 2.5 t-C ha^{-1} was applied by Chaves *et al.,* (2005) who added straw and sawdust separately with nitrogen rich crop residue. All these studies recorded significant N immobilisation. Alburquerque, de la Fuente and Bernal, (2012) used digestates with a feedstock of 4% and 6% glycerol and saw N immobilisation, whereas our digestate mix contained 3% glycerol. However, for the amount of carbon added in this experiment (1400 mg-C kg^{-1}), the changes in microbial N and soil available N under the digestate and glycerol did meet expectations. Working on the assumption that microbes use 1 unit of N per 8 units of carbon to satisfy stoichiometry for growth (Sinsabaugh *et al.,* 2016), then the increase of 1200 mg-C kg^{-1} in microbial biomass due to glycerol addition at 3 hours after application would immobilise 175 mg-N kg^{-1} (see supplementary information for calculation). The measured average reduction in available N due to glycerol addition compared to the digestate alone was higher than the estimate at 195 mg-N kg^{-1}, due to variability in sample measurements between replicates. It appears that the high volume of nitrogen added by the digestate, requires a larger N uptake by microbes than what occurred in this study to significantly reduce the soil available N pool. This indicates that adding more carbon than we did into the digestate is necessary to increase the magnitude of microbial growth.

Whilst our system was carbon limited, we were still able to identify trends in the influence of carbon source on its bioavailability and subsequent impact on the magnitude and timing of soil microbial N uptake. Glycerol resulted in the greatest and quickest growth that occurred in the first month with a microbial N higher on average by 4x than digestate, followed by straw with a microbial N concentration greater by 2.5x than digestate. In an experiment comparing glycerol, straw and grass as N immobilisers, Redmile-Gordon *et al.,* (2014) observed that the addition of glycerol with nitrogen had a quicker and greater magnitude of immobilisation compared to the straw-N mix, attributable to the greater lability, and therefore bioavailability, of carbon in glycerol compared to straw. Soil incubation experiments by Reichel *et al.,* (2018) and Chaves *et al.,* (2005), who used both straw and sawdust as immobilisers of nitrogen-rich sources, observed that nitrogen immobilisation under sawdust was of lower magnitude and occurred later compared to straw. Similarly, our woodchip-digestate had a later and lower on-average microbial N peak compared to the straw addition. This is attributable to the higher lignin content in woody material, which slows decomposition rates and therefore carbon availability to microbes (Melillo *et al.*, 1982; Lehmann, Schroth and Zech, 1995; Rahn and Lillywhite, 2002). Biochar was the most recalcitrant material used in our study and its addition to digestate had no positive influence on microbial N, as was also observed by Manirakiza *et al.,* (2019) when they mixed biochar into biosolids. Alotaibi and Schoenau (2016) mixed biochar with urea to a C:N ratio of 20:1, higher than our digestate+biochar mix, and measured a reduction in microbial biomass after 4 months compared to urea alone. In contrast, the microbial biomass under their glycerol-urea mix was no different to urea control, which corroborates our results, confirming that glycerol has a quick and short-term influence. As such the hypothesis that the lability of carbon in the organic material influences the time and magnitude of microbial N uptake can be accepted.

The phenotypic (PLFA) profile of the soil community changed with the application of digestate, with the soil only treatment resulting in a group distinct from the digestate treated soil. A month after application two distinct groups emerged: digestate alone and with glycerol and straw, and digestate with woodchip and biochar and the unamended soil. A higher ratio of fungi to bacteria was present in the former group. This is contrary to the hypothesis, which expected that the additives containing more labile carbon would increase the relative abundance of bacteria compared to fungi. Approximately a third of the fungi in digestate are yeasts and moulds, which are quick growing (Coelho *et al.*, 2020). The labile carbon supplied by the digestate, glycerol and straw provided a carbon source for these quick growing fungi from both the digestate and the soil (Photo 3-1). Meanwhile biochar, being able to sorb carbon and nutrients (Mukherjee *et al.*, 2016; Ding *et al.*, 2020), may have reduced the availability of these compounds, which may be a reason that digestate with biochar did not result in a change in the community composition compared to the soil only control. The merging of groups after 3 months is consistent with decreases in abundance of quick growing microorganisms as they exhaust their labile carbon supply (Meidute *et al.*, 2008), shifting the community to a composition similar to the control soil.

There are practical implications for adding materials into digestate. In this study chopped straw was used, which resulted in significant immobilisation, however this is difficult in terms of mixing it into the digestate to ensure a homogeneous distribution followed by difficulties spreading it evenly onto a field, particularly with band or injection spreading equipment and systems where the digestate is transported to the applicator unit by an umbilical system, where straw addition may increase the chances of blockages occurring. An alternative would be to apply digestate and straw separately. Adding digestate to straw on the soil surface is problematic, as this reduces the infiltration rate of the digestate, resulting in increased ammonia volatilisation losses (Cao *et al.*, 2018). Whilst there are applicators that inject digestate straight into the soil through a straw layer to reduce ammonia losses, this results in spatial separation of the straw on the soil surface and the digestate below the surface. Therefore, microorganisms in the soil do not have access to the straw and cannot acquire the carbon they need to immobilise nitrogen from the digestate, as evidenced by Aita *et al.,* (2012) who observed increased decomposition rates when slurry and straw were incorporated into the soil as opposed to straw remaining on soil surface. This



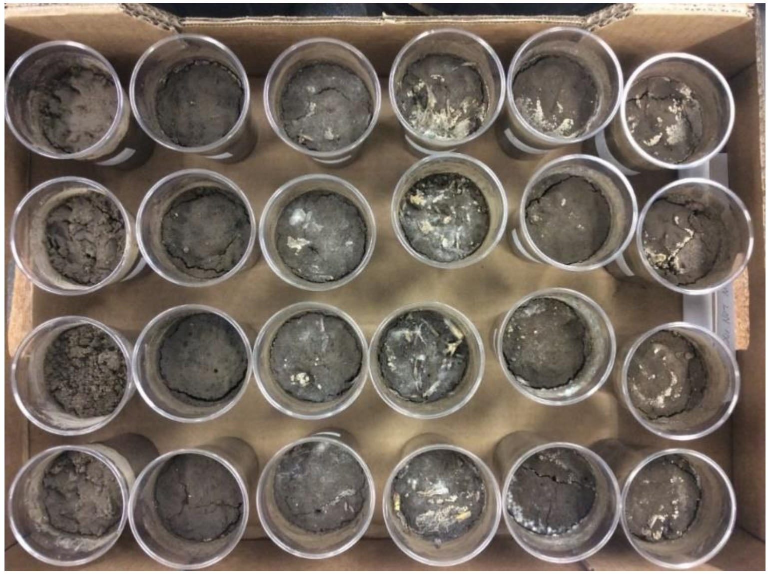


Photo 3-1 Pots at 14 (top left), 30 (top right) and 90 (bottom left) days after treatment application. Treatment order from left to right in each photo is: Soil only, LD + biochar, LD + woodchip, LD + Straw, LD + Glycerol and Liquid Digestate (LD*)*.

Pots were lined up by treatment for photo then returned to positions according to randomised block design.

would mean that the addition of straw to digestate is a mixture that is limited for use on crops that combine fertilisation with seedbed preparation.

This study demonstrated that the use of a high organic carbon material additives can result in microbial growth and uptake of digestate supplied nitrogen. To meet its potential as a mechanism to reduce nitrogen losses from the application of anaerobic digestate, more carbon is required than was applied in this study, however we have demonstrated that materials high in carbon, of which the majority is in an easily degradable form (ie low in lignin or char content) have the greatest potential to do this. Under field conditions the nitrogen immobilisation and remineralisation may be slower than found in this study, due to fluctuating temperatures and variable rainfall (Sun *et al.,* 2019). Potentially less nitrogen would be immobilised overall as leaching and volatilisation would remove some nitrogen from the soil alongside immobilisation. Therefore, our findings that high organic carbon materials immobilise digestate supplied nitrogen need to be verified under field conditions. Should it prove possible to control digestate supplied nitrogen using this approach, then further work will be required to determine agronomic effects.

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3.7 Supplementary Material

3.7.1 Determination of additive amount

Chambers *et al.*, (2007) state that a slurry with a content of 12% solids is the maximum before there are difficulties transporting and spreading due to blockages caused by the organic matter particles. Therefore, increasing total solid content of the liquid digestate to a maximum of 12% was selected. The lightest and bulkiest material (straw) was chosen as the material to provide information on the amount of carbon to add to the digestate. The process involved 3 steps:

1) Liquid digestate contained 5% total solid content, so to reach the 12% total solid content, an extra 7% consisting of straw was added.
2) To work out the weight of straw needed to bring total solid volume up to 12%, 93 ml of digestate was added to a 100 ml cylinder. Straw was then added until the digestate reached the 100 ml mark. The weight difference between the cylinder before and after the straw addition showed how much straw was needed.
3) The total carbon added was calculated from the straw dry weight and the % total carbon content of the straw. Based on this value a total of 0.28 g of carbon per 23 ml of digestate applied to each pot was calculated. From this, the amount of woodchip, biochar and glycerol needed to supply 0.28 gC per pot could be calculated using their respective carbon contents and dry weights.

3.7.2 Converting µg N/g soil to kg N/ha to find out amount of N immobilised by microbes due to treatment.

Step 1: determine how much nitrogen immobilised by additive+digestate compared to digestate treatment alone: mean LD+ straw has 142 µg-N g^{-1} soil and mean LD has 62 µg-N g^{-1} soil => 142-62 = 80. So, addition of straw resulted in 80µg of nitrogen per gram of soil immobilised in the microbial biomass.

Step 2: convert µg/g to mg/kg: 1µg/g = 1mg/kg, so 80µgN/g = 80mgN/kg.

Step 3: convert mg/kg to kg/ha need (i) area (ii) bulk density and (iii) layer depth

Area = 1 ha (10,000m^2)
Bulk density (of the soil in the pot) = 1.04 g/cm^3 (1040 kg/m^3)
Soil depth (the depth of the soil in the pot) = 9 cm (0.09 m)
Volume of soil layer is 10,000 x 0.09 = 900 m^3

Mass of soil layer is 900m^3 x 1040 kg/m^3 = 936,000 kg
Multiply by microbial N (mg/kg) content => 936,000 x 80 = 74,880,000 mg microbial N / ha
Convert mg to kg divide 74,880,000 by 1,000,000 = 74.88 kg N per ha at a depth of 9 cm.

So, the addition of straw resulted in 74.88 kg N per ha immobilised in microbial biomass.

3.7.3 Calculating expected amount of microbial nitrogen from a given microbial biomass carbon accounting for atomic mass of each element

- Atomic mass of carbon = 12.01 g / mol
- Atomic mass of nitrogen = 14,01 g / mol

Step 1: Convert carbon mass to moles

$$\text{Moles of carbon} = \frac{\text{Mass of carbon (mg)}}{\text{Atomic mass of carbon (mg/mol)}} \quad \frac{1200}{12.01} \quad = 99.92 \text{ moles}$$

Step 2: Use the C:N ratio to find moles of nitrogen

$$\text{Moles of nitrogen} = \frac{\text{Moles of carbon}}{8} \quad \frac{99.92}{8} \quad = 12.49 \text{ moles}$$

Step 3: Convert moles of nitrogen to mass

Mass of nitrogen (mg) = moles of nitrogen x atomic mass of nitrogen
Mass of nitrogen => 12.49 x 14.01 = 175 mg

Supplementary table 1: The fatty acid biomarkers that contributed the most (≥0.8 and ≤-0.8) to each PLFA PCA graph at 3hrs, 30 days, 90 days and 150 days after application (AA) are marked with an X

Fatty Acid Biomarker ID	**0 days AA**		**30 days AA**		**90 days AA**		**150 days AA**	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
15:0i	-	-	-	-	X	-	-	-
15:0	-	-	X	-	-	-	-	-
16:1ω11t	X	-	-	X	-	-	-	-
16:1ω5	X	-	-	-	X	-	X	-
16:0	X	-	X	-	X	-	X	-
Me17:0 isomer	X	-	-	-	X	-	-	-
Me17:0 isomer2	-	-	-	-	X	-	X	-
cyc17:0 isomer	-	X	X	-	-	-	-	-
ai17:0	-	X	X	-	X	-	-	-
17:0br	-	X	-	-	-	-	-	-
17:1ω8c	-	-	-	-	-	-	-	X
17:0c	X	-	X	-	X	-	X	-
17:1ω8t	X	-	X	-	-	-	-	-
17:1ω7	-	-	-	-	-	-	-	X
UK 29.68	-	-	X	-	-	-	-	-
18:2ω6,9	-	-	X	-	X	-	X	-
18:1ω9c	-	X	X	-	X	-	X	-
18:1ω7t	-	-	-	X	-	-	-	-
18:1ω13	X	-	-	-	-	-	-	-
19:1ω6	-	-	X	-	X	-	X	-
18:0 (10Me)	-	-	-	-	-	-	X	-
19:0c	X	-	-	-	X	-	-	-

4 Glycerol immobilises anaerobic digestate supplied nitrogen

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4.1 Abstract

Anaerobic digestate, a nutrient rich by-product of the biogas industry, is frequently applied to agricultural land as a fertiliser. However, nitrogen losses from its application negatively impact air and water quality. Therefore, methods are needed to reduce these losses. The aim of this study was to test the efficacy of applying digestate with glycerol, an organic carbon rich by-product of the biodiesel industry, on microbial nitrogen immobilisation and the soil microbial community. Soil was incubated with digestate, applied at a rate equivalent to 250 kg-N ha^{-1}, in a laboratory experiment over 50 days with glycerol additions at either 0, 12, 24 and 36 kg-C m^3 of digestate. The addition of glycerol resulted in significantly higher microbial biomass carbon and increased the relative abundance of Gram-negative bacteria. The 24 and 36 kg-C m^3 doses of glycerol resulted in similarly greater and longer lasting effect on microbial biomass carbon, indicating that beyond 24 kg-C m^3 digestate that nitrogen (or other essential nutrients) became the limiting factor for microbial growth instead of carbon. Soil available nitrogen decreased throughout the study and remained at lower concentrations in glycerol treatments than the digestate only treatment by the end of the study. These results demonstrate that glycerol has the potential to reduce nitrogen losses from digestate application by immobilising nitrogen in the microbial biomass. Therefore, the co-application of digestate and glycerol to soil is a potential mechanism for the biogas and biofuel industries to valorise their respective by-products. Further research is needed to verify that this method is viable under field conditions.

4.2 Introduction

The United Nations Sustainable Development Goals (SDGs) were agreed by 193 Member States in 2015, establishing targets to guide countries to a sustainable future (UN General Assembly, 2015). One of these goals, SDG 7, involves the need for a substantially greater amount of energy to be produced from renewable sources. Energy can be renewably produced from biomass (e.g. biogas, biodiesel), but bioenergy industries create by-products that also require sustainable management, both from an environmental and economic viewpoint. The anaerobic digestion of organic waste is an expanding industry, producing energy from biologically generated gases. These biogas plants produce a nutrient rich by-product, known as anaerobic digestate, of which over 90% produced in UK is applied to agricultural land as an alternative to mineral forms of nitrogen (WRAP, 2020). However, there are environmental impacts of digestate application, such as ammonia and nitrous oxide (N_2O) emissions and nitrate leaching (Nkoa, 2014; Möller, 2015). Therefore, it is imperative to find mechanisms to keep the nitrogen supplied by anaerobic digestate within the rooting zone of the soil, where it is beneficial for crop production. Biological immobilisation of nitrogen could provide a sustainable pathway to achieve this objective.

Most soil microorganisms are heterotrophic and require a source of carbon to utilise for energy and growth. Soil microorganisms immobilise nitrogen within their biomass which forms part of the soil organic matter (SOM). Upon microbial death, nitrogen is subsequently remineralised from the SOM. Plants release root exudates that stimulate microbial SOM decomposition, thereby enabling plants to acquire the nitrogen necessary for growth (Meier, Finzi and Phillips, 2017). Digestate has a low total carbon content; typically between 0.43-3.4% with C:N ratios between 1.4-6.3 (Risberg *et al.*, 2017). At these low C:N ratios, microbes are carbon limited and use digestate carbon for metabolism but digestate nitrogen that is in excess of microbial demand is excreted to soil. Therefore, an additional source of carbon is needed to promote microbial growth and nitrogen immobilization. Studies on mixing carbon substrates with synthetic nitrogen, which would otherwise supply no carbon to the soil, resulted in positive nitrogen immobilisation (Romero *et al.*, 2015; Pittaway *et al.*, 2018). When coating urea with poly-γ-glutamic acid, Xu *et al.*, (2013) observed a short-term increase in microbial biomass nitrogen, followed by an improved crop yield, evidencing that microbes assisted in ensuring plants were supplied with nitrogen throughout their growing period via nitrogen immobilisation and remineralision.

Glycerol, a by-product of the biodiesel industry, is a labile hydrocarbon which many microorganisms can utilise as a carbon source (da Silva, Mack and Contiero, 2009). Glycerol is created during the transformation process of vegetable oils and fats into biodiesel and makes up around 10% w/w per unit of biodiesel produced (Yang, Hanna and Sun, 2012), resulting in considerable volumes that require disposal. This is restricting the economic growth of the industry as traditional markets for glycerol, which include pharmaceuticals and personal care products, become saturated (Chilakamarry *et al.*, 2021). Therefore, new ways of utilising glycerol are needed. Several studies have looked at applying glycerol on agricultural soil to immobilise nitrogen, either for reducing environmental pollution (Redmile-Gordon *et al.*, 2014; De, Sawyer and McDaniel, 2022) or to improve the nitrogen use efficiency of fertilisers (Qian, Schoenau and Urton, 2011) by stimulating microbial growth and immobilising nitrogen.

For the co-application of digestate and glycerol to function successfully as a fertiliser, the nitrogen locked within the necromass, resulting from the glycerol stimulated microbial growth, needs to be mineralised by the native soil microbial community into plant available forms. A review by Karimi *et al.*, (2022) on the effects of digestate on the soil microbial community, found that the majority of studies observed no significant difference when compared to synthetic nitrogen addition. There is evidence that microbial utilisation of glycerol produces several intermediate products, such as antimicrobial compounds (Axelsson *et al.*, 1989), but how it influences soil microbial community is little researched. Therefore, it is important to understand the potential effects of co-applying digestate and glycerol on soil microbial communities, to ensure its continual provision of nutrient cycling services.

This study aimed to investigate the effects of adding different rates of glycerol into liquid digestate on soil microbial biomass and nitrogen immobilisation. Liquid digestate was selected as it contains higher proportion of nitrogen following separation into solid and liquid fractions (Fuchs and Drosg, 2013), a procedure which biogas companies often do due to storage and transport constraints (Al-Seadi *et al*., 2012). The objectives were to determine which rate of glycerol would be most effective at inducing nitrogen immobilisation and what impact it would have on the soil microbial community. It was hypothesised that increasing glycerol rates would result in higher levels of microbial nitrogen immobilisation by increasing the availability of carbon to microorganisms. It was also hypothesised that the microbial community composition would change due to the addition of glycerol to digestate. A pot experiment was established to test these hypotheses in the absence of confounding environmental variables, such as temperature and moisture content.

4.3 Method

4.3.1 Soil, digestate and glycerol

A sandy loam topsoil (69% sand, 20% silt, 11% clay) bought from Bourne Amenity Ltd, was used for the study. Liquid digestate was supplied by Future Biogas Ltd from a biogas plant using a mixed feedstock of 85 tonnes maize silage, 7.5 tones cow manure and 18 tonnes chicken manure. The plant is mesophilic operating at 43°C with a retention time of 98 days. Post digestion, the digestate is pasteurised. The liquid fraction was collected fresh after separation from the whole digestate by a screw press. Glycerol comprising of 39.2% carbon was bought from Sigma Aldrich. Details on material properties are in Table 4-1.

Table 4-1 Characterisation of the materials used in the incubation

Properties	**Soil**	**Liquid digestate**	**Glycerol**
Dry matter (%)	84.5	4.8	-
pH	8.0	8.1	-
Total Carbon (g kg^{-1})	32.4	35.1	391.9
Nitrogen			
Total (g kg^{-})	2.8	5.1	-
Ammonium (mg kg^{-1})	0.9	3234	-
Nitrate (mg kg^{-1})	264	<10	-
Phosphorus			
Total (mg kg^{-1})	-	316	-
Available (mg l^{-1})	49	-	-

Properties	**Soil**	**Liquid digestate**	**Glycerol**
Potassium			
Total (mg kg^{-1})	-	4360	-
Available (mg l^{-1})	755	-	-
C:N ratio	11.57	3.27	-
Microbial biomass C (mg-C kg^{-1})	251	-	-
Microbial Biomass N (mg-N kg^{-1})	45	-	-

4.3.2 Soil incubations

Soil was air dried and sieved to 2 mm to remove any stones and large debris. 150 g (dry weight basis) of soil was added to 330 ml plastic containers (top diameter 8 cm, bottom diameter 5 cm, height 12 cm) without drainage holes. The soil water holding capacity was determined using a saturate and drain method modified from Harding and Ross (1964). Potted soil was then adjusted to 40% water holding capacity, which is optimal for microbial development (Gulledge and Schimel, 1998). The pots were pre-incubated at 20±4°C in the dark under aerobic conditions for two weeks, to allow soil microbial population to acclimatise after being disturbed and rewetted.

The incubation experiment consisted of five treatments, arranged in a randomised block design with five replications: 1) soil only control (CONT); 2) liquid digestate control (LD-CONT); 3) liquid digestate with glycerol at 3% v/v (LD+3%G); 4) liquid digestate with glycerol at 6% v/v (LD+6%G); and 5) liquid digestate with glycerol at 9% v/v (LD+9%G). Five sets were set-up to allow for destructive sampling on five occasions: 3 hours after application, then 7, 14, 30, and 50 days after application. This gave a total of 125 experimental units (5 treatments x 5 replicates x 5 sampling dates).

Digestate was applied at 14 ml per pot, a rate equivalent to 250 kg-N ha^{-1}. Before application, the digestate receiving glycerol was mixed with 0.42 ml, 0.84 ml or 1.26 ml glycerol to make mixes of 3%, 6% and 9% glycerol to digestate volume to volume (v/v), adding an extra 12.4, 24.8 and 37.2 kg-C per m^{-3} of digestate, equivalent to 614, 1228 and 1842 kg-C ha^{-1}. Water was added to treatments 1-4 to ensure they all received the same total amount of liquid as treatment 5. The amendments were then mixed into the soil. Pots were loosely covered to reduce moisture losses and incubated in the dark at 20±4°C until sampled. To maintain water holding capacity at 40%, the pots were weighed twice weekly to check moisture content and deionised water was added as required.

4.3.3 Microbial analysis

Microbial biomass carbon and nitrogen (MBC and MBN respectively) were determined following the fumigation-extraction method (Vance, Brooks and Jenkinson, 1987). Two weighed subsamples were taken from each soil sample, one subsample was fumigated for 24 hrs at 20°C with $CHCl_3$, then extracted with 50 ml of a 0.5 mol/l K_2SO_4 solution and filtered. The second portion was equally processed, but without the $CHCl_3$ fumigation step. The organic C and N were determined with an automatic analyser for liquid samples (Shimadzu TOC-V with a TN module). MBC and MBN were calculated as the difference between the C and N extracted from the fumigated samples and those extracted from the non-fumigated samples, multiplied using K_{EC} and K_{EN} values of 0.45 and 0.54 respectively (Brooks *et al.*, 1985; Vance, Brooks and Jenkinson, 1987).

Phospholipid fatty acid analysis (PLFA) was used to determine the phenotypic structure of the microbial community, based on the method modified from Frostegård, Tunlid and Bååth, (1993) at 3 timepoints: 3 hours, then at days 14 and 50 from application. Microbial lipids were extracted using a Bligh & Dyer solvent (1:2:0.8 (v/v/v) chloroform: methanol: citrate buffer). Lipids were then fractionated using solid phase extraction cartridges (SPE), the polar lipids then methylated and resulting fatty acid methyl esters (FAMES) extracted. The FAMES were analysed by gas chromatography (6890N Agilent Technologies) following the same procedure as Pawlett *et al.*, (2013). The relative abundance (%mol) of all PLFAs present in the sample, including the non-specified ones, were used for the analysis of the PLFA patterns. Bioindicator fatty acids used to identify microbial groups were: the sum of i15:0, ai15:0, i16:0, i17:0, ai17:0, 10me18:0 for Gram-positive (G+) bacteria (Frostegård and Bååth, 1996; Zelles, 1999), the sum of 16:1ω7c, 17:1ω7, 18:1ω7t, 18:1ω13 for Gram-negative (G-) bacteria

(Frostegård and Bååth, 1996; Zelles, 1999), 18:2ω6,9 for ectomycorrhizal and saprophytic fungi (Frostegård and Bååth, 1996). Total bacteria were calculated as the sum of G+ and G- bacteria. The fungi:bacteria (F:B) ratio was calculated as the fungal biomarker 18:2ω6,9 divided by the sum of the bacterial biomarkers (Frostegård and Bååth, 1996).

4.3.4 Chemical analyses

Total soil available nitrogen as the sum of ammonia (NH_4-N) and total oxides of nitrogen (TON-N), which is the sum of nitrite and nitrate, was determined using the potassium chloride extraction method (MAFF, 1986). 20 g of soil was eluted with 100 ml of 2 mol/l KCl solution, filtered and stored at -20°C until analysed on an analytical segmented flow multi-chemistry analyser (Seal, AA3). Soil pH was measured based on the British Standard BS ISO 10390:2005 method. 10 ml of air-dried soil was mixed with 50 ml 1 M KCl solution and pH was measured using a Jenway 3520 pH meter.

4.3.5 Statistics

Statistical analysis was carried out in Statistica version 14. Data was first tested for normality and homoscedasticity. The available nitrogen and microbial biomass carbon and nitrogen datasets failed to meet the assumptions for equal variance and were transformed using the Box-Cox function. The differences between treatments were analysed by a one-way analysis of variance (ANOVA) to determine treatment effects at each time point. Significant differences between glycerol rates were determined by Tukey's post hoc test. All differences were considered statistically significant if $p < 0.05$. Principal Component Analysis was run on the PLFA profile data, which was normalised by measuring each biomarker as the relative abundance (%mol) to all the biomarkers.

4.4 Results

4.4.1 Impact of digestate and glycerol application on microbial growth

The addition of only digestate to soil (without glycerol) did not result in significantly higher microbial biomass C at any timepoint (Figure 4-1A). Microbial biomass carbon was generally higher with increasing glycerol rates, with significantly highest concentrations found at days 7 and 14 under the LD+6%G and LD+9%G treatments. At 30 days, only LD+9%G had a significantly higher biomass C than digestate alone.

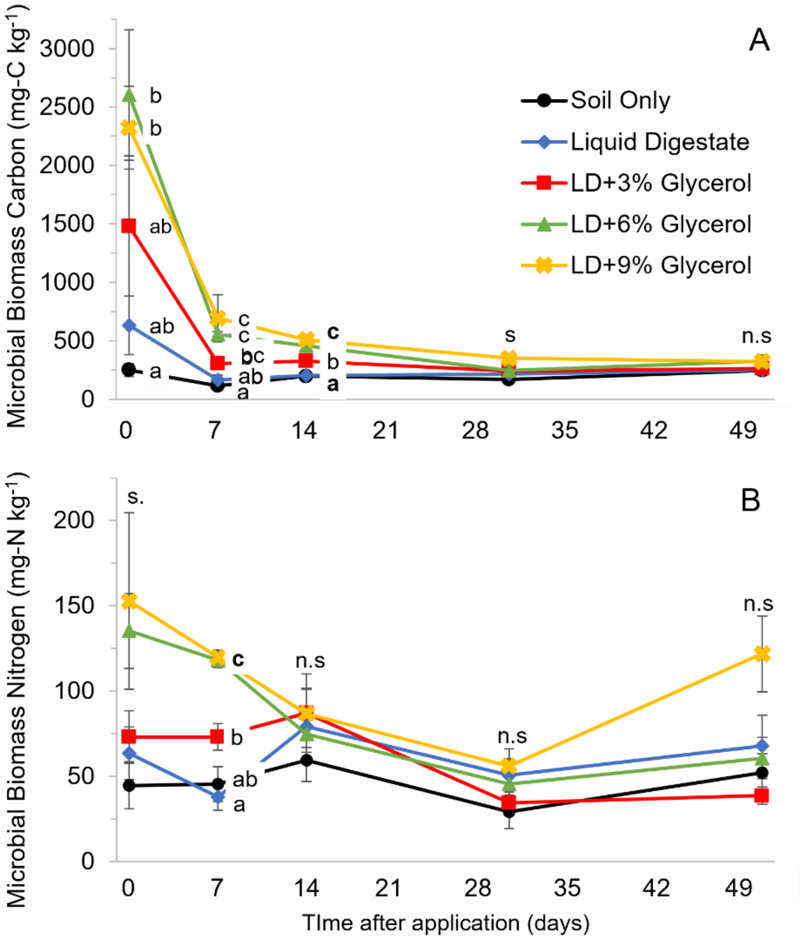

Figure 4-1 Changes in soil microbial biomass carbon (A) and nitrogen (B) during the incubation. Points denoting different lowercase letter have statistically different treatments effects according to Tukey's test at 5% probability. s = significant at p<0.05, n.s = non-significant at p≥0.05. Error bars denote the standard error of the mean, n = 5.

4.4.2 Impact of digestate and glycerol application nitrogen dynamics

Digestate supplied soil with NH_4-N, resulting in significantly ($p<0.001$) higher total soil available nitrogen by 488 mg-N kg^{-1} (Table 4-2) compared to the soil only control. During the first week of incubation concentrations of NH_4-N declined by 90% (Figure 4-2), but total soil available N remained significantly higher than the soil only treatment for the rest of the incubation (Table 4-2). From 7 days onwards, the majority of total available N comprised of nitrite and nitrate (TON-N). Microbial N was not significantly affected by the addition of digestate to soil at any timepoint (Figure 4-1B).

Table 4-2 Changes in total soil available nitrogen (mean ± SE) as sum of ammonium, nitrite and nitrate following treatment incorporations. Mean (n=5) values between treatments in a row (sampling time) denoted with a different lower-case letter are statistically different according to Tukey's test at the 5% probability level.

Sampling time after application	Treatment				
	Soil Only	Liquid Digestate (LD)	LD+3% Glycerol	LD+6% Glycerol	LD+9% Glycerol
3 hours	$332±34^{a}$	$821±47^{b}$	$754±57^{b}$	$658±63^{b}$	$577±77^{b}$
7 days	$227±8^{abc}$	$319±42^{a}$	$305±23^{ab}$	$176±46^{bc}$	$119±29^{c}$
14 days	$189±10^{a}$	$335±9^{b}$	$227±37^{a}$	$182±35^{a}$	$144±11^{a}$
30 days	$171±10^{a}$	$291±17^{b}$	$143±9^{a}$	$127±19^{a}$	$148±18^{a}$
50 days	$215±9^{a}$	$358±15^{b}$	$205±15^{a}$	$172±18^{a}$	$172±7^{a}$

A week after application, glycerol addition at LD+6%G and LD+9%G had significantly lower total soil available N of 129 and 186 mg-N kg^{-1} compared to digestate without glycerol (Table 4-2). Microbial N under LD+6%G and LD+9%G was significantly higher than digestate alone by 94 and 97 mg-N kg^{-1} (Figure 4-1B) respectively. Whilst LD+3%G did not significantly reduce total available N compared to digestate alone ($p=0.99$), it did have a significantly ($p=0.02$)higher microbial N concentration of 40 mg-N kg^{-1}. From day 14 onwards glycerol at all rates consistently had similarly lower total available nitrogen concentrations that was significantly different compared to digestate alone (Table 4-2). By the end of the 50-day incubation total soil available nitrogen remained lower than the digestate control by 43-52% (Figure 4-2).

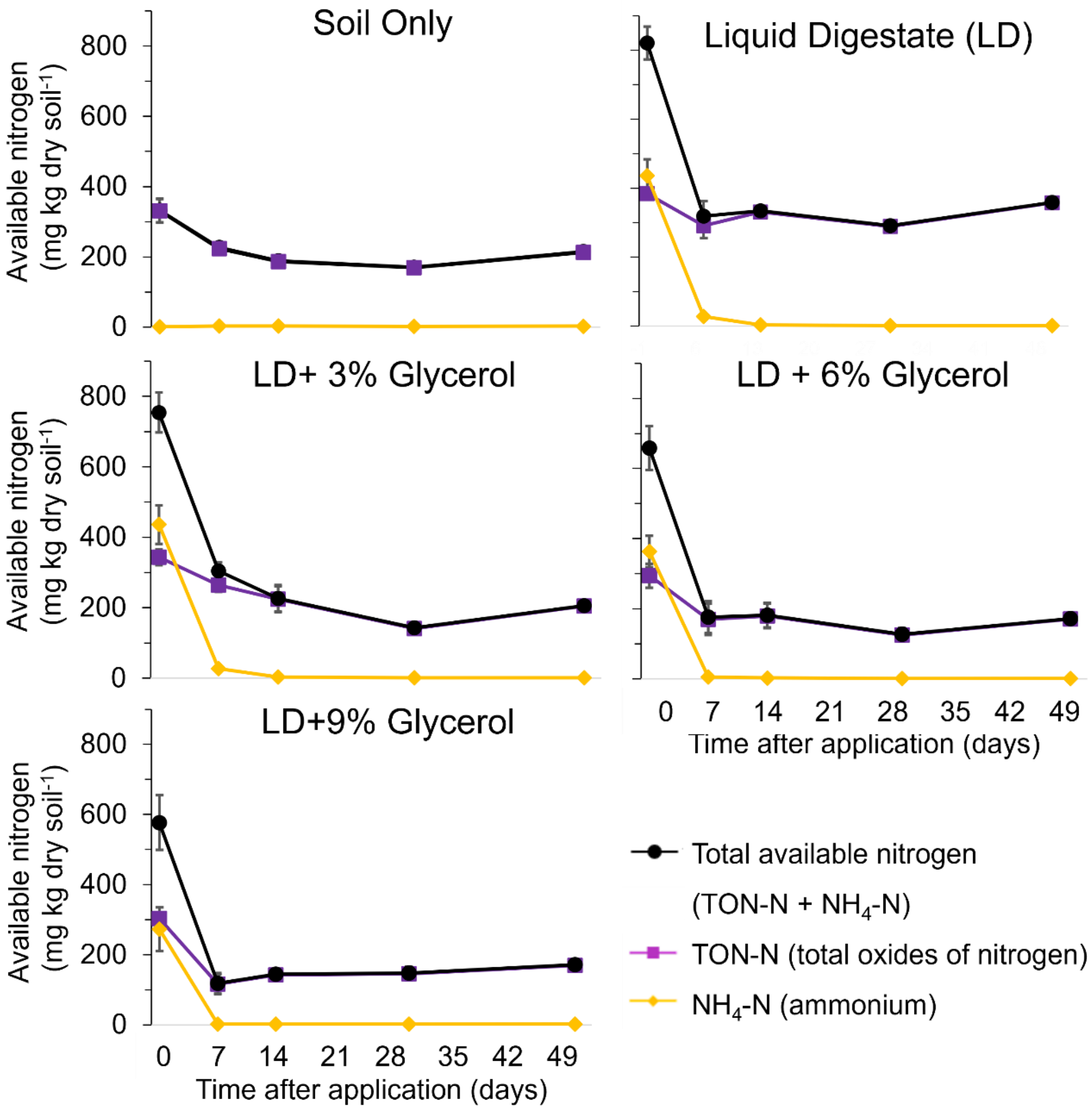

Figure 4-2 Changes in total soil available nitrogen as the sum of nitrite, nitrate (TON-N) and ammonium (NH_4-N) during incubation. Error bars denote the standard error of the mean, n=5.

4.4.3 Impact of glycerol and digestate application on soil microbial community

The first two principal components (PC) of the PLFA data accounted for over 50% of the total variation at each timepoint (Figure 4-3), with significant ($p<0.01$) effects on the PC2 axis 3 hours after application, on the PC1 axis at day 14 and both PC axes at 50 days. 3 hours after application the soi only control was distinctly separate from a cluster formed by the LD+0-6% G whilst the LD+9%G lay between the two. Fatty acid loadings (≥0.8 and ≤-0.8) that contributed the most were 16:1ω11t, 16:1ω5, 16:0, Me17:0 isomer1, Me17:0 isomer2, 17:0c, 18:1ω9c, 19:1ω6 and 19:0c, Gram-positive bacteria biomarkers 15:0i, 15:0ai, ai17:0 and 18:0 (10Me), Gram-negative bacteria biomarker 18:1ω13 and fungi biomarker 18:2ω6,9 on PC1 and Gram-positive bacterial biomarker 16:0i on PC2. At 14 days the unamended and digestate controls were distinctly separate and further separations were clear between two groups of glycerol additions: LD+0-3G% and LD+6-9%G. Fatty acid loadings (≥0.8 and ≤-0.8) that contributed the most at day 14 were 16:1ω5, Me17:0 isomer2, 17:0c, 17:1ω8t, 17:0 (12Me), 19:0c, 19:1ω6, 20:4 and 20:5ω3, Gram-positive bacterial biomarkers 16:0i, ai17:0 and 18:0 (10Me), Gram-negative bacterial biomarkers 16:1ω7c for PC1 and 16:00 on PC2. At day 50 the unamended and digestate controls continued to be distinctly separate, whilst glycerol addition resulted in distinct separations between 0% and a group of LD+6-9%G whilst LD+3%G lay between the two. Fatty acid loadings (≥0.8 and ≤-0.8) that contributed the most were 16:0, 18:1ω7t, Gram-positive bacterial biomarker 16:0i, and fungal biomarker 18:2ω6,9 on PC 1 and 17:1ω8c on PC 2.

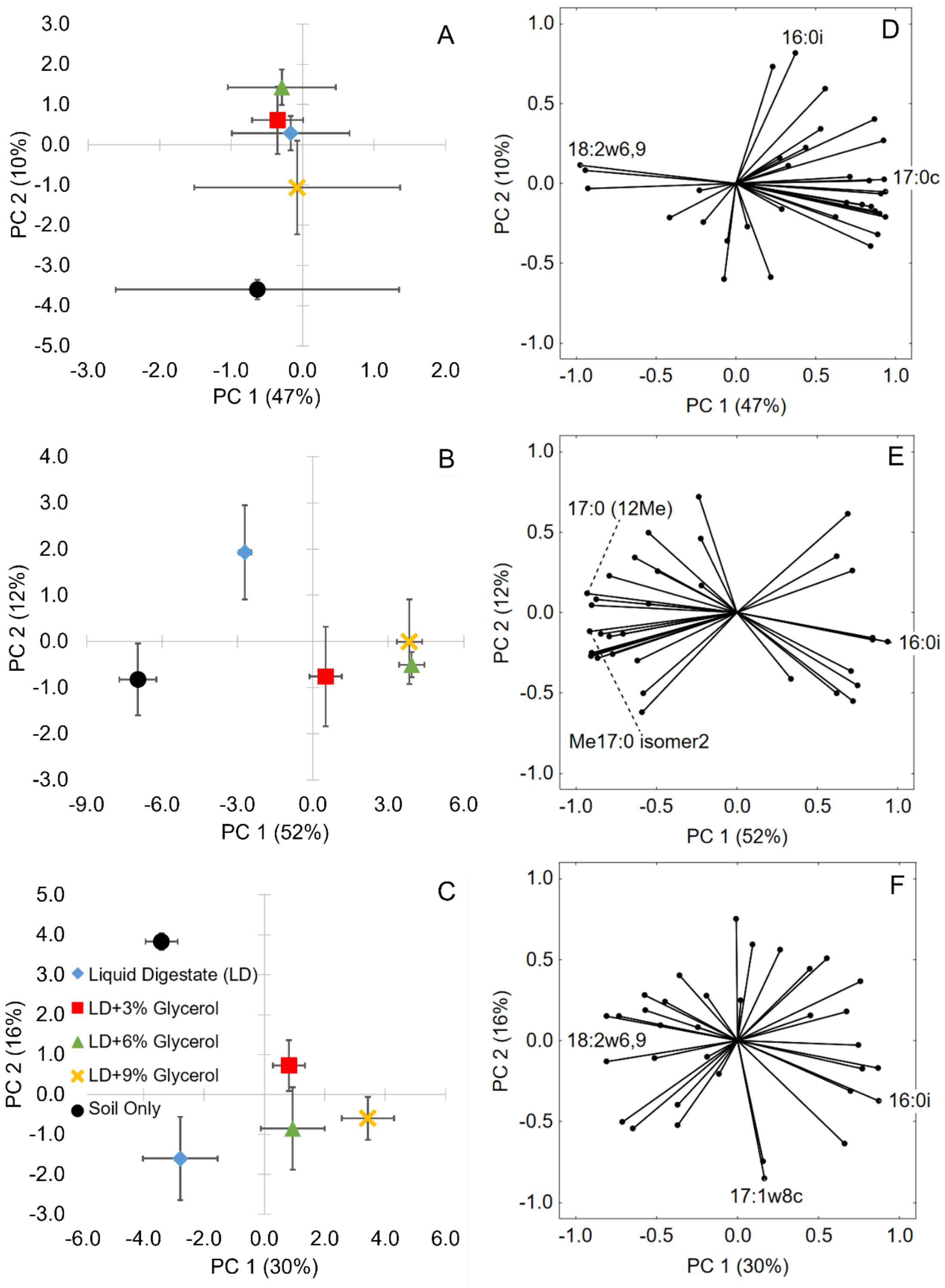

Figure 4-3 Treatment response of the microbial community over time identified by Principal Component Analysis of the PLFA data (PC mean ± SE, n=5) at 3 hours (A), 14 days (B) and 50 days (C) after application and the corresponding loading plots labelling the top three contributing biomarkers at 3 hours (D), 14 days (E) and 50 days (F). Error bars denote the standard error of the mean, n=5.

The addition of only digestate (without glycerol) to soil lead to no significant effects (p>0.05) on the F:B or G+:G- ratios at any timepoint compared to unamended soil (Figure 4-4). At day 50, LD+9%G addition had a lower F:B ratio by 13% than digestate without glycerol. LD+6%G resulted in a 17% lower G+:G- ratio at day 14 digestate without glycerol. LD+6-9%G had lower G+:G- ratios of 14% and 18% respectively at day 50 compared to digestate control (Figure 4-4 B).

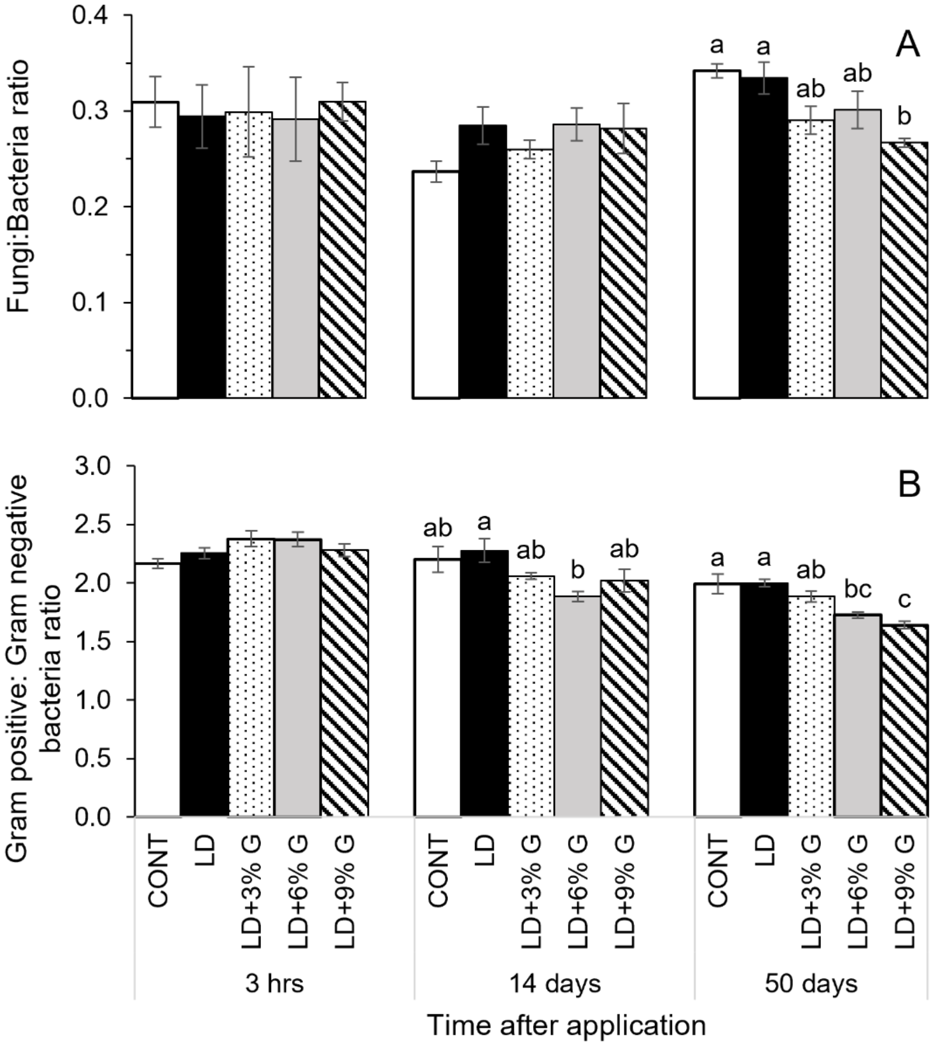

Figure 4-4 Fungal/Bacteria ratio (A) and Gram-positive/Gram-negative ratios (B) calculated using PLFA biomarkers on soil samples collected 3 hours, 14 days and 50 days after digestate application. Error bars denote the standard error of the mean, n = 5. Cont = soil only; LD = liquid digestate; LD+ 3 or 6 or 9 %G = liquid digestate + 3 or 6 or 9 %v/v glycerol.

4.4.4 Impact of digestate and glycerol application on soil pH

Soil pH was significantly (p=0.005) higher in digestate only treated soil compared to soil only at 7 days after application, whereafter there was no significant difference (Table 4-3). The addition of 3% glycerol to digestate resulted in a significantly (p=0.02) higher pH at 30 days after application compared to digestate without glycerol. Glycerol addition at 6 and 9% v/v did not result in higher pH values compared to the digestate treatment at any time.

Table 4-3: Changes in soil pH (mean ± SE) following treatment incorporations. Mean (n=5) values between treatments in a row (sampling time) denoted with a different lower-case letter are statistically different according to Tukey's test at the 5% probability level.

Time after application	Soil only	Liquid Digestate (LD)	LD + Glycerol at 3% v/v	LD + Glycerol at 6% v/v	LD + Glycerol at 9% v/v
3 hrs	7.98±0.02	8.01±0.01	8.01±0.01	8.02±0.02	8.01±0.01
7 days	8.02±0.01^{a}	8.08±0.01^{b}	8.05±0.01^{ab}	8.04±0.01^{ab}	8.04±0.01^{ab}
14 days	8.03±0.01^{a}	8.04±0.01^{ab}	8.07±0.02^{ab}	8.07±0.01^{ab}	8.08±0.01^{b}
30 days	8.02±0.01^{a}	8.02±0.02^{a}	8.08±0.01^{b}	8.07±0.01^{ab}	8.05±0.01^{ab}
50 days	7.99±0.01	8.00±0.01	8.02±0.01	8.01±0.01	8.01±0.01

4.5 Discussion

4.5.1 Effect of digestate on soil N immobilisation and availability

Digestate supplied soil with NH_4-N, leading to increased soil available nitrogen, which rapidly declined within the first week. Similarly, Alburquerque, de la Fuente and Bernal, (2012) and Rigby and Smith, (2013) measured a rapid decrease in soil NH_4-N after the initial input of NH_4-N from the application of either liquid digestate or whole digestate. As NH_4-N concentrations decreased, both studies measured a simultaneous increase in the nitrite and nitrate concentration from microbes oxidising NH_4-N to obtain energy. This conversion of digestate supplied ammonium nitrogen into oxidised nitrogen kept soil total available nitrogen concentrations higher in digestate treated soils compared to soil only controls, which was also observed in our study. A reason for available nitrogen remaining higher in soil applied with digestate is the lack of nitrogen uptake by the microbial biomass. The addition of only digestate (without glycerol) in our study did not significantly stimulate microbial biomass production compared to the soil only treatment, which is in agreement with de la Fuente *et al.*, (2013, Wentzel and Joergensen (2016) and Valentinuzzi *et al.*, (2020) when they applied liquid digestate to soil. This can be attributed to the low amount of carbon supplied in the liquid fraction, which was 3.5% in the digestate used in this study. The low amount of carbon in the liquid fraction is a result of two steps. Firstly, during anaerobic digestion, the easily degradable carbon in the feedstock is utilised by microbes, decreasing the total carbon content of the digestate compared to its feedstock (Tambone *et al.*, 2009) and concurrently increasing the proportion of carbon which is recalcitrant (Tambone *et al*., 2013) and harder for microbes to metabolise. Secondly post digestion separation, which is routinely done by biogas plants to reduce the volume of digestate for storage and transportation, removes a further 60-70% of the remaining carbon into the solid fraction (Fuchs and Drosg, 2013), therefore the liquid digestate fraction contains low amounts of carbon for soil microbes to utilise.

4.5.2 Effect of glycerol amended digestate on soil N immobilisation and availability.

The addition of glycerol to the digestate resulted in lower soil available nitrogen concentrations than the digestate control from 7 days onwards. The difference observed at day 7 corresponded with higher microbial biomass, with the highest microbial biomass and lowest soil available nitrogen concentrations resulting from the 6% v/v and 9% v/v glycerol rates. This supports the first hypothesis that adding glycerol to digestate would increase microbial biomass, and nitrogen immobilisation therein. Yet the 9% v/v addition had no greater effect on microbial growth than 6% v/v. This indicates that beyond 6% v/v glycerol addition, other nutrients may have become a limiting factor for microbial growth.

Our study is in agreement with other lab-based studies that showed increases in microbial N when glycerol and nitrogen were co-applied compared to nitrogen fertilisers alone (Alotaibi and Schoenau, 2011; Redmile-Gordon *et al.*, 2014; De, Sawyer and McDaniel, 2022). However, unlike Redmile-Gordon *et al.*, (2014) and De, Sawyer and McDaniel (2022) who observed nitrogen remineralisation after 4 and 7 days of incubation respectively, there was no noticeable N mineralisation after the microbial N peak. This may have been due to unmeasured nitrogen losses shortly after application. The addition of liquid digestate creates anaerobic microsites in the soil in which denitrifying activity takes place, which is further stimulated by the addition of labile carbon (Curtright and Tiemann 2023). Therefore, the addition of glycerol could have led to an early loss of digestate supplied nitrogen as N_2O and N_2. Soil moisture returned to pre-application levels of 40% water holding capacity after 7 days, which may have inhibited the rate of nitrogen mineralisation from organic matter due to low soil moisture conditions (Castellano *et al.,* 2012). Yet it should be noted that this experiment did not contain a plant, which play a key role in stimulating nitrogen remineralisation through the secretion of rhizodeposits (Henneron *et al.,* 2020).

4.5.3 Effect of digestate and glycerol on soil community structure

The addition of only digestate to soil (without glycerol) resulted in a change to soil community structure, but no effect on F:B or G+:G- ratios were observed. The changes may be due to a combination of factors such as the microorganisms added by the digestate (Fernández-Bayo *et al.*, 2017) and elevated soil pH, as changes in soil pH from by organic amendments applications have been found influence the microbial community composition (Cui *et al.*, 2023). The lack of change in F:B ratio was also observed by García-Sánchez *et al*., (2015) and Cattin *et al*., (2021), however Pezzolla *et al.*, (2015) observed an increase in gram-negative bacteria which drove a reduction in the F:B ratio. This difference may be due to the feedstock of the digestate, as Pezzolla *et al.*, (2015) used digestate made from pig slurry, whereas the digestate used in our study came from a feedstock of plant material and manure, similar to the digestates used by García-Sánchez *et al*., (2015) and Cattin *et al*., (2021). The carbon in digestates derived from pig slurry has a higher concentration of aliphatic forms, and is therefore more labile, compared to digestates derived from a feedstock mixture of plant and animal origin (Tambone *et al.*, 2013). Gram-negative bacteria are better able to quickly utilise labile carbon due to their rapid growth strategy when there is a resource flush, compared to the relatively slower growing Gram-positive bacteria and slower growing fungi group (Dungait *et al.*, 2013). However, assigning PLFA biomarkers to a specific microbial group should be done with caution, since some biomarkers are found across a range of organisms (Joergensen, 2022) and therefore changes in any one of these groups may be harder to compare between studies when different biomarkers are assigned to a particular group.

The addition of glycerol to digestate at 6 and 9% v/v resulted in a microbial group distinctly separate from the digestate alone from 14 days post-application onwards, supporting our hypothesis. At both 14 and 50 days after application, glycerol addition at either 6 or 9% v/v did significantly reduce the G+:G- ratio. It is likely that this reduction can be attributed to positive effects on Gram-negative bacteria growth from labile C inputs, considering the positive effects measured on microbial biomass at the same time. This finding is supported by Garcia-Pausas and Paterson, (2011) and Cui *et al*., (2020) who measured Gram-negative bacteria taking the majority of labile carbon (supplied as glucose in these studies), compared to other microbial groups. The increase of Gram-negative bacteria is likely the reason for the reduction in fungi:bacteria ratio at day 50 under 9% v/v glycerol addition. These observations have important consequences for the fate of nitrogen in the soil organic matter. As Gram-negative bacteria are usually associated with quick growing and short-lived life strategies (Chen *et al*., 2016), with a rapid turnover of living biomass into the soil microbial food web and non-living soil organic matter (Kindler *et al*., 2006), their necromass would increase the soil organic nitrogen pool more rapidly compared to the turnover from relatively slower growing Gram-positive bacteria and fungi. This in turn would ensure that nitrogen is available for plants to mineralise from the soil organic matter during their growing season.

4.5.4 Further considerations

There are several further steps that need to be taken to evaluate the practicality of digestate and glycerol co-application and to validate that this mechanism of using soil microbes to temporally immobilise digestate supplied nitrogen works in non-laboratory conditions. Firstly, to be effective as a fertiliser the nitrogen needs to remineralise whilst the crop is growing. As nitrogen remineralisation was not seen in this study, no assumptions can be made on the timing of nitrogen release. Two factors are proposed for the lack of remineralisation. Firstly, no plants were included in the incubation to prime soil organic matter decomposition, as such including plants to determine whether the addition of carbon additives to digestate does improve plant growth, nitrogen use efficiency and yield needs to be explored. Secondly no gaseous measurements were taken, which may have been a considerable nitrogen loss pathway. The influence of glycerol addition on N_2O emissions is a major concern, as it is a potent greenhouse gas, as such quantifying the effect of glycerol addition to digestate on N_2O emissions is important to determine the environmental sustainability of co-applying digestate and glycerol. Further studies using stable isotope probing are recommended to more accurately determine the pathways of digestate supplied nitrogen utilisation into the soil and its rate of transfer into the various nitrogen pools, and subsequent uptake in plants. Lastly the effects of glycerol addition on the soil community are unknown, although a start on understanding glycerol addition on soil microbial community is made in this experiment, further work using DNA sequencing to determine the effect of glycerol addition on taxonomically distinct groups or functional gene expressions would contribute to a greater understanding of how the microbial community composition and functioning is affected.

4.6 Conclusion

There is a growing body of research into the applicability of using labile carbon to immobilise nitrogen in the microbial biomass, and thereby reduce pollution from nitrogen rich sources. Our study contributes to this knowledge base by demonstrating for the first time that the addition of glycerol immobilises a significant proportion of the ammonium nitrogen supplied by anaerobic digestate into microbial biomass. This laboratory experiment was a proof of concept demonstrating the potential for both the biogas and biofuel industries to increase the value of their respective by-products by co-applying digestate and glycerol to soil to create a slow-release fertiliser, thereby mitigating the negative environmental impacts of applying digestate alone. Further experiments are necessary to evaluate the impacts on crop nitrogen use efficiency and yield, greenhouse gas emissions, and nitrate leaching. Although we have shown that glycerol addition stimulates the immobilisation of digestate nitrogen through its utilisation as a carbon source for microbial growth and biomass production, microorganisms also use glycerol to produce extracellular products, such as antibiotics (Spinler et al., 2008) and extracellular polymeric substances (Redmile-Gordon et al., 2015). Therefore, glycerol amendments could have broader applications for soil health, including improvements in soil structure and the potential for probiotic interventions to influence nutrient transformation and pathogen loads. Therefore, future studies should also investigate the effects of glycerol beyond its role as a growth substrate, focusing on microbial community composition and functioning. Additionally, field experiments will be necessary to understand how these parameters are affected by real-world conditions and to determine the practical viability for farmers to co-apply digestate and glycerol.

4.7 References

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5 Nitrogen use efficiency of digestate applied with high organic carbon materials on the growth and yield of spring barley.

5.1 Abstract

Anaerobic digestate is produced during the anaerobic digestion of organic materials for biogas generation and can be used as a fertiliser. However, nitrogen is lost following the application of digestate to soil, resulting in environmental pollution and a low proportion of nitrogen reaching the crop. This research aimed to improve the nitrogen use efficiency of digestate by combining it with materials containing a high content of organic carbon to stimulate soil microbial growth and nitrogen immobilisation. Liquid digestate was applied (140 kg-N ha^{-1}) in a pot-scale experiment with either glycerol, straw, or glycerol and straw combined (each treatment at 24 kg-C m^3 digestate). Controls comprised of digestate (without additional carbon) and soil only. All plots were planted with spring barley. After 7 days the glycerol and glycerol with straw treatments had greater microbial biomass and immobilised 120 mg-N kg^{-1} of soil more, compared to the digestate control, the majority of which had remineralised by 30 days after treatment application. Despite the statistically significant ($p=0.002$) immobilisation, barley nitrogen use efficiency and yield were unaffected by treatments. The addition of straw, glycerol, and both in combination with digestate had no effect on N_2O emissions, ammonia volatilisation, or post-harvest leaching, compared to the digestate control. However, the rapid nitrogen immobilisation caused by the addition of glycerol to digestate has the potential to reduce nitrate leaching from rainfall events occurring in the first month after digestate application.

5.2 Introduction

Biogas is a renewable energy product generated from the breakdown of organic matter, such as animal and human waste, energy crops, and the organic fraction of municipal waste under anaerobic conditions. A by-product of this process is anaerobic digestate, a slurry containing nutrients which can be used as a fertiliser, producing yields equivalent to synthetic fertilisers (Šimon, Kunzová and

Friedlová, 2015; Riva *et al*., 2016; Walsh *et al*., 2018; Barzee *et al*., 2019; Luo *et al*., 2022; Brychkova *et al*., 2024; Yagüe and Lobo, 2024). After biogas extraction, the digestate is often separated into liquid and solid fractions to reduce its volume and therefore reduce the cost of transporting and storing it (Al-Seadi *et al*., 2012). During separation the liquid fraction receives 65-75% of the total nitrogen and 70-80% of ammonium nitrogen (Fuchs and Drosg, 2013), which is readily available to plants. However, the high content of available nitrogen in the digestate can cause environmental pollution when it is applied to land due to ammonia volatilisation, N_2O emissions, nitrate leaching and digestate run-off (Nkoa, 2014).

Ammonia volatilisation accounts for up to 60% loss of digestate-supplied nitrogen (Nicholson *et al*., 2017). The use of low-emission slurry spreading techniques such as acidifying the digestate, or using precision application like bandspreading and injection, have been shown to reduce emissions by 45 to 95% (Smith *et al*., 2000; Nicholson *et al*., 2018; Sánchez-Rodríguez *et al*., 2018; Wagner *et al*., 2021). However, there are concerns that acidification could increase nitrate leaching and N_2O emissions (Fangueiro, Hjorth and Gioelli, 2015). Injecting digestate directly into the soil is more effective at reducing ammonia volatilisation compared to surface band application (Nicholson *et al*., 2018), but it can result in greater leaching and N_2O losses (Cameira *et al*., 2019). Nitrate leaching results in eutrophication of water courses, whilst N_2O is a potent greenhouse gas with a global warming potential of 273 times greater than that of CO_2 (IPCC, 2021). Therefore, it is necessary to look at methods to reduce nitrogen losses once the digestate is in the soil.

De Neve *et al*., (2004) and Reichel *et al*., (2018) demonstrated nitrogen immobilisation from nitrogen rich sources through the addition of materials with a high organic carbon content, such as straw, due to the carbon promoting microbial growth which simultaneously takes up nitrogen to support cell growth and maintain elemental stoichiometry. When adding glycerol or straw to digestate, we measured significant microbial growth and nitrogen immobilisation compared to digestate without the addition of either of these high organic carbon materials (van Midden *et al.*, 2024). However, that study was conducted in the absence of plants. The aim of this study was to identify whether the incorporation of the liquid fraction of digestate with high organic carbon material would stimulate microbial growth and nitrogen uptake, and understand how it would influence crop growth and nitrogen loss pathways. We hypothesised that the addition of a high carbon organic material to digestate would increase the nitrogen use efficiency and yield of spring barley due to microbial immobilisation of digestate-supplied nitrogen and subsequent remineralisation later in its growing season.

5.3 Method

5.3.1 Soil, digestate and high organic carbon materials

A sandy loam topsoil (Table 5-1) sieved to 5 mm was bought from Bourne Amenity Ltd (Newenden, UK), for use in this study. Liquid digestate was supplied by Future Biogas Ltd from a biogas plant using a mixed feedstock of 85 tonne maize silage, 7.5 tonne cow manure and 18 tonne chicken manure. The plant is mesophilic operating at 43°C with a retention time of 98 days and post digestor digestate is pasteurised at 70°C for 1 hour. The liquid fraction (Table 5-1) was collected fresh after separation from the whole digestate by a screw press. Glycerol (Sigma Aldrich) containing 39.2% carbon and chopped barley straw (Pillow Wad, UK) with a carbon content of 37% were used as the high organic carbon material additives.

Table 5-1 Properties of soil and liquid digestate

Parameter	Soil	Liquid Digestate
Dry Matter (%)	83.4	2.5
Organic Matter (% w/w)	8.3	1.5
pH	7.7	8.3
Total Carbon (% w/w)	2.4	2.8
Total Nitrogen (% w/w)	0.2	0.4
Available Nitrogen (mg kg^{-1})	68.6	2330
Total Phosphorus (mg kg^{-1})	412	242
Total Potassium (mg kg^{-1})	1022	3868
Cation exchange capacity (meq 100 g)	15.5	-
Microbial Biomass C (mg kg^{-1})	85.7	-
Microbial Biomass N (mg kg^{-1})	20.2	-

5.3.2 Experimental design

A polytunnel experiment was established using spring barley, *Hordeum vulgare,* (variety Laureate) grown in 10 litre pots (height 22 cm, top diameter 28 cm, bottom diameter 22 cm), filled with 10 kg of soil to a bulk density of 1 g cm^{-1}. The experiment consisted of five treatments, arranged in a randomised block design, with four replications: 1) liquid digestate control; 2) liquid digestate with glycerol; 3) liquid digestate with straw; 4) liquid digestate with glycerol and straw; and 5) soil only control. Treatments 2 and 4 received 13.4 ml and 6.7 ml of glycerol respectively, which was added into the digestate directly before it was applied to the soil. Treatments 3 and 4 received 14 g and 7 g of straw chopped to 10 cm, which was applied to the soil surface prior to digestate application. The organic carbon additives were applied to add 24 kg of C per m^3 of digestate, a rate equivalent to 875 kg-C ha^{-1}. Digestate was applied at 120 ml per pot, equivalent to 140 kg-N ha^{-1}, and then mixed into the top 10 cm of the soil. Water was added to the soil only treatment pots at 120 ml, to mimic the volume of digestate added to the other pots and mixed into the soil. Soil moisture was measured using a ThetaKit moisture meter HH2 with a SM150T moisture sensor probe (DeltaT, UK) three times a week, or daily during hot periods. Pots were watered to adjust soil to 60% water holding capacity, which was calculated following a method from Harding and Ross (1964). Seeds were sown (Photo 5-1) seven days after treatment application, to avoid phytotoxicity effects of digestate (Lencioni *et al*., 2016). 25 seeds were sown into the pot at a rate equivalent to 400 seeds per m^2, which was the supplier’s (Syngenta) recommended sowing rate for late sown Laurette. Soil samples were taken before treatment application and then at 7, 30, 75 and 110 days after application alongside plant sampling.

Photo 5-1 Seed sowing. Tape around a spoon handle was used to ensure consistent sowing depth of 2 cm and a pot saucer was used to guide the sowing circle.

5.3.3 Plant analysis

At stem extension (BBCH growth stage 31), the pots were thinned to 15 plants per pot. At grain fill (BBCH growth stage 71) a further 5 plants were removed for total nitrogen analysis. At harvest the remaining 10 plants were collected, and the ears separated for grain yield analysis. At each sampling time plants were measured for height, and then oven dried at 65°C until no further weight loss was recorded. The dry weights were then adjusted to a per plant basis. The plant samples were then finely ground and passed through a 0.75 mm sieve. The ground sample was measured for total nitrogen by dry combustion and analysed using an elemental analyser (Elementar, Vario EL III).

5.3.4 Soil analysis: microbial

Microbial biomass carbon and nitrogen was determined by the chloroform fumigation-extraction procedure (Vance, Brooks and Jenkinson, 1987) with soil moist samples equivalent to 12.5 g dry weight. Samples were extracted with 50 ml of 0.5 M K_2SO_4. The organic C and N concentration in the extracts was then determined by a TOC-L with a TN module (Shimadzu). The microbial biomass C and N was calculated based on the difference between the organic C and N extracted from the fumigated soil and that from the unfumigated soil, using K_{EC} and K_{EN} values of 0.45 and 0.54 respectively (Brooks *et al*., 1985; Vance, Brooks and Jenkinson, 1987).

The necromass analysis was based on the amino sugar extraction method by Appuhn *et al.*, (2004) with minor modifications based on Mou *et al.,* (2020). 1 g of field moist, sieved (2 mm) soil was hydrolysed in 10 ml of 6 M HCl at 105°C for 6 hours. Once cooled to room temperature the extracts were passed through a 0.45 µm filter and 0.5 ml was transferred to a clean vial and evaporated at 40°C to dryness under nitrogen. The sample was redissolved in DI water and evaporated to dryness again to remove any residual HCl. The residue was then redissolved in 1 ml of DI water and centrifuged at 5,000x*g*. The supernatant was transferred to vials and stored at -20°C until analysis on high performance liquid chromatograph (HPLC). The amino sugars were quantified according to standard solutions containing a mix of the four amino sugars; glucosamine, galactosamine, mannosamine and muramic acid. These four amino sugars were measured according to Appuhn *et al.*, (2004) and Indorf *et al.*, (2011) following pre-column derivatisation with ortho-phthaldialdehyde (OPA) reagent. The standard solutions and reagents were prepared as in Indorf *et al*., (2011).

The HPLC analysis was carried out on an Agilent 1200 Series with an autosampler (G1329A). For derivatisation, 10 µl of OPA reagent and 5 µl of sample were mixed before injection into the column (Phenomenex Hypersil 5u ODS C18 250 mm length x 4.6 mm diameter) carried on a gradient of mobile phases to facilitate chromatographic peak separation and column cleaning after each run. Mobile phase A was a 97.8/0.7/1.5 (v/v/v) mixture of a water phase, methanol and tetrahydrofuran, with the water phase containing 52 mmol sodium citrate and 4 mmol sodium acetate, adjusted to pH 5.3 with 1 M hydrochloric acid. Mobile B consisted of 50/50 (v/v) water and methanol. The mobile phase A and B entered the column at a flow rate of 1 ml min^{-1} with a volume ratio of 70/30 for the first 25 minutes. From 30 minutes the gradient changed to 10/90 for 3 minutes before returning to 70/30 until the end of the run to precondition the column for the next run. Fluorometric emission of amino sugar derivatives was measured at a wavelength of 445 nm with excitation wavelength of 340 nm (Agilent FLD Detector G1321AI).

5.3.5 Soil analysis: chemical

Total soil available nitrogen as the sum of ammonia and total oxides of nitrogen was determined using the potassium chloride extraction method (MAFF, 1986). 20 g of soil was eluted with 100 ml of 2 M KCl solution, filtered and stored at -20°C until analysed on an analytical segmented flow multi-chemistry analyser (Seal, AA3). Soil pH was measured based on the British Standard BS ISO 10390:2005 method. 10 ml of air-dried soil was mixed with 50 ml 1 M KCl solution, shaken for 60 minutes, left to stand for a further 60 minutes and then pH was measured using a Jenway 3520 pH meter.

5.3.6 Gas analysis: Ammonia volatilisation

A static chamber method based on Nommik, (1976) and Alexander *et al*., (2021) as modified by Wang *et al*., (2011) to make a vented chamber, was used to measure ammonia volatilisation. PVC tubes (30 cm in height and 28 cm in diameter) were inserted into the pots. Prior to use, polyurethane discs (density 26-29 kgm^3), brought from RGH Rubber and Plastics Ltd, were saturated with 1 M H_2SO_4 solution. The discs were then rinsed with water and squeezed to remove the water three times before the washed discs were oven dried at 35°C to remove any residual inorganic nitrogen. For ammonia absorption the discs were wetted with 120 ml of 1 M H_2SO_4 and 4% (v/v) glycerol solution. This volume of solution was chosen to dampen the foam without risking any solution dripping on to the soil. Two discs were used per chamber, the first 5 cm above the soil to capture volatised ammonia and the second disc 10 cm from the top to capture any atmospheric ammonia to prevent mixing with soil emitted ammonia. Discs were replaced after 24 hours, then 2, 3 and 5 days after application. After deployment the bottom discs were placed into ziplock LDPE bags containing 120 ml of deionised (DI) water. They were squeezed and refrigerated overnight, then leached and squeezed with a further 4 successive portions of 120 ml DI water. The leachates were then filtered through Whatmann 595.5 filter paper and the extract frozen at -20°C until analysis. Extracts were analysed using a 1:5 ratio of extract to Nessler's reagent to solution and read on a spectrophotometer (Hach Lange DR3900) at a wavelength of 450 nm.

5.3.7 Gas analysis: N_2O flux

Gas samples for determining N_2O flux were taken from midmorning, as this best represents the average emission rates from a 24 hour period (Charteris *et al*., 2020). Samples were taken 2 hours after application, then 1, 2, 4, 7, 10, 17, 28 and 44 days after application. Pots and their saucers were placed inside larger saucers filled with water. Pots were enclosed in a clear acrylic extruded chamber (Plastock, UK) with an inner diameter of 29 cm and height of 66 cm, so that the bottom of the chamber rested inside the water filled saucer to create an airtight seal. Gas samples were taken through a rubber septum inserted in the chamber top. Headspace gas was taken by an air-tight syringe and needle at 0, 15, 30 and 45 minutes after enclosure, to give four gas samples per pot, as recommended by Venterea *et al*., (2020). Gases was mixed before extraction by pumping the syringe 3 times, then 20 ml of gas was collected and injected into 12 ml evacuated exetainer vials (Labco, UK) prior to the analyses. The gas analyses were performed using a gas chromatograph (Agilent 6890), fitted with electron capture detector (ECD) for N_2O analysis using N_2 as a carrier gas.

Concentrations of N_2O in ppm were calculated by comparing peak areas against a standard curve created from six different concentrations 0.31, 0.63, 1.25, 2.5, 5 and 10 ppm which were prepared from a certified gas standard mixture (BOC, UK). The limit of detection was calculated from the mean of the blanks, made from N_2 gas, in the run plus 3 times its standard deviation The emission rate of each gas was calculated by the linear regression from all four time points. All emissions with a $R^2 < 0.70$ were discarded (Brenzinger *et al*., 2018). The gas data was then converted to flux (mass per volume per unit of time) using the molecular mass of each gas (1 mol of N_2O = 44.01 g) and the ideal gas law $PV = nRT$, where P = atmospheric pressure; V = volume of headspace in dm^3; n = number of moles (mol); R = universal Gas Constant law (8.314 $J\ K^{-1}\ mol^{-1}$); and T = temperature in kelvin (K) 273 + the ambient air temperature in degrees Celsius at time of sampling.

Photo 5-2 Gas sampling in progress. In foreground are the chambers and discs for capturing ammonia. The discs were replaced whilst the N_2O chambers were in operation on a row-to-row basis. The clear chambers in last row are for measuring N_2O along with collection vials.

5.3.8 Post harvest leaching

To evaluate the risk of nitrogen leaching after harvest, pots were watered to reach 100% water holding capacity, then leached with a total of 750 ml in 3 doses over an hour (Sogn *et al*., 2018), after which the leachate was collected and filtered for available analysis The extracts were frozen at -20°C until analysed using an analytical segmented flow multi-chemistry analyser (Seal, AA3).

5.3.9 Statistical analysis

Nitrogen use efficiency was calculated by the formulas for apparent crop recovery efficiency (RE) of applied nitrogen and agronomic efficiency (AE) to determine the contribution of fertiliser nitrogen towards yield compared to a non-fertilised treatment (Congreves *et al*., 2021).

$$\text{RE (kgN kgN}^{-1}\text{)} = \frac{\text{Grain N from the treatment} - \text{Grain N from the control}}{\text{Total applied N of fertiliser in the treatment}}$$

$$\text{AE (kg kgN}^{-1}\text{)} = \frac{\text{Grain yield from treatment} - \text{Grain yield from control}}{\text{Total applied N of fertiliser in treatment}}$$

Cumulative emissions for N_2O were estimated from the area under the curve (Keshavarz Afshar *et al*., 2018) by trapezoid integration, assuming linearity between sampling times. Data collected over several sampling times (microbial biomass, soil available N, plant height and nitrogen uptake) were analysed using repeated measures ANOVA, whereas single point data (grain yield, leached nitrogen and cumulative gaseous emissions) were analysed using a one-way ANOVA. All datasets were first tested for normality using Kolmogorov-Smirnov & Lilliefors test and homoscedasticity using Levene’s Test. Significant differences were determined using Tukey’s post-hoc test with significance determined at $p<0.05$. All statistical analysis was performed in Statistica Tibco version 14.

5.4 Results

5.4.1 Soil dynamics

The addition of glycerol, with or without straw, to digestate significantly increased microbial biomass C ($p<0.001$) and N ($p=0.002$) and significantly decreased available N ($p<0.001$), compared to digestate control treatment. Specifically, at 7 days after application, microbial biomass carbon in both the digestate with glycerol treatment and the glycerol+straw treatment was significantly higher (by

65% an increase of 156 mg-C kg^{-1} soil) than the digestate alone treatment (Figure 5-1A), whilst microbial nitrogen was not significantly different between the same treatments (Figure 5-1B). Soil available nitrogen was significantly lower (by 95%, a decrease of 96 mg-N kg^{-1} soil) between digestate and glycerol with or without straw treatments, compared to digestate only treatment (Figure 5-1C). 30 days after application, the digestate treatments with straw, glycerol, or both, had higher microbial biomass carbon than unamended soil treatment, but were not significantly greater than digestate control treatment. No significant differences in microbial biomass nitrogen were observed between any treatments after 30 days. Available nitrogen in soils treated with digestate and glycerol, or digestate, glycerol and straw, increased between days 7-30 post application, but remained lower (by 48%, a difference of 71 mg-N kg^{-1} soil) than the digestate only soils. From 75 days onwards there were no further significant differences between treatments, but at 75 days after application, microbial biomass carbon was significantly higher ($p<0.001$) compared to any other timepoint, whilst soil available nitrogen was at its lowest concentration compared to any other timepoint. Microbial biomass nitrogen was higher at 75 days after application, compared to 30 and 110 days after application.

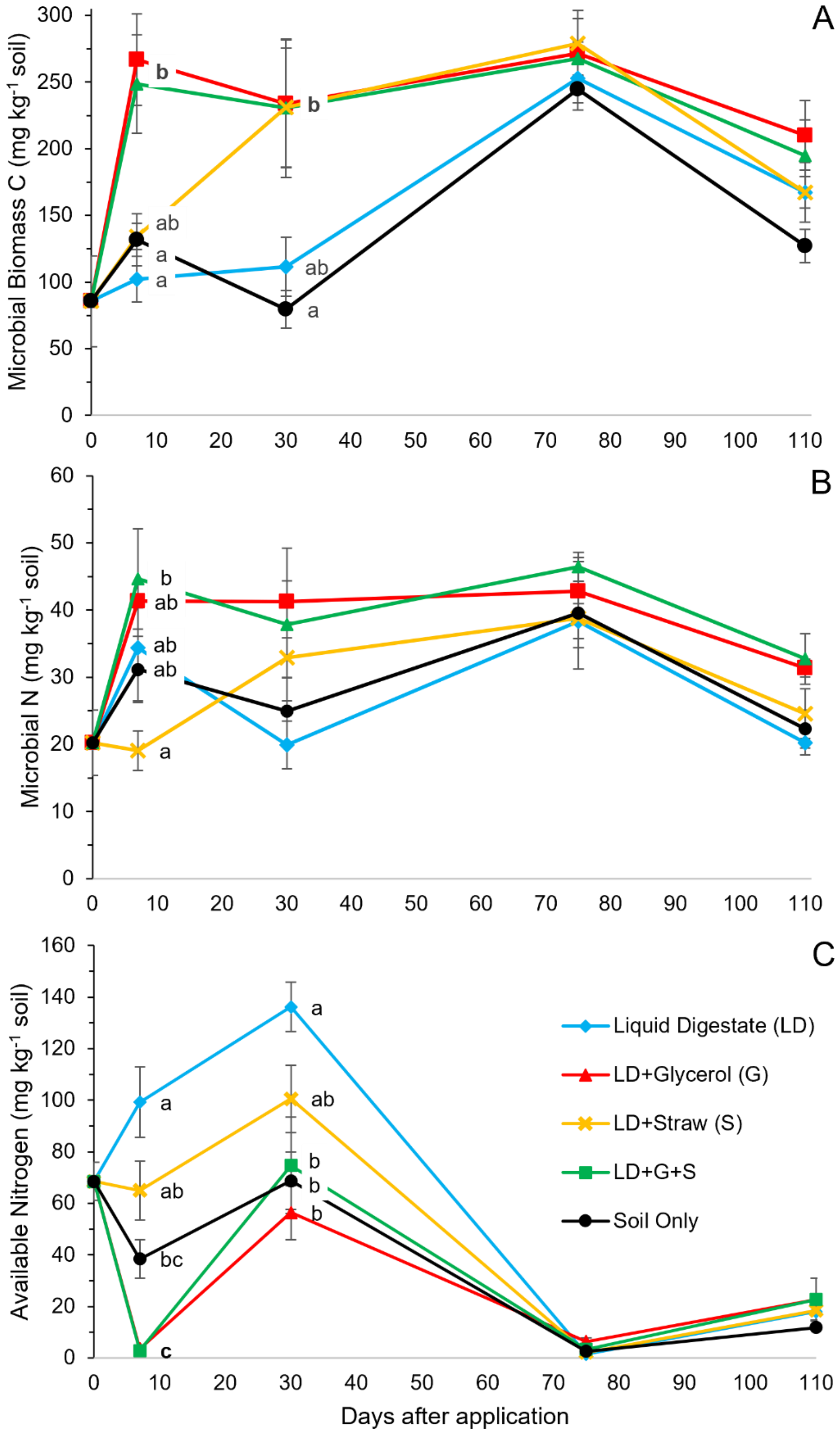

Figure 5-1 Changes in soil microbial carbon (A), soil microbial nitrogen (B) and soil available nitrogen (C) in soils over time on a fresh weight basis. At each sampling time treatments with different lower-case letter have statistically different effects according to Tukey's post-hoc test at 5% probability. Error bars denote the standard error of the mean, n=4.

Out of the four amino sugars measured the in the soil, galactosamine was found in the highest concentration in all samples, followed by glucosamine and then muramic acid, whereas mannosamine was not detected. Only muramic acid was significantly (p=0.02) affected by treatment. At seven days after application, muramic acid in both the digestate with glycerol, and digestate with glycerol and straw, treatments were higher by 20% compared to the digestate only treatment (Figure 5-2C). The concentration of each amino sugar detected significantly (p<0.001) changed with time. Both glucosamine and galactosamine were highest at days 7 and 110 after application and lowest at 30 days, whereas muramic acid was lowest at both 30 and 75 days after application and highest at 7 and 110 days. Soil pH was not influenced by the addition of digestate with or without additives compared to unamended soil, however soil pH increased by 0.1 unit between 7 and 30 days Table 5-2.

Table 5-2 Change in soil pH (mean ± SE, n=4) over time as influenced by treatments. Different upper-case letters indicate significant difference between sampling times according to Tukey's post-hoc test at 5% probability.

	Treatment				
Soil pH	Liquid Digestate (LD)	LD+Glycerol (G)	LD+Straw (S)	LD+G+S	Soil only
7 days ^{A}	7.66±0.03	7.74±0.01	7.75±0.01	7.42±0.19	7.50±0.12
30 days ^{B}	7.74±0.04	7.70±0.03	7.73±0.03	7.70±0.03	7.75±0.01
75 days ^{B}	7.76±0.02	7.72±0.02	7.78±0.01	7.73±0.02	7.73±0.01
110 days ^{B}	7.80±0.02	7.79±0.02	7.73±0.05	7.76±0.02	7.81±0.01

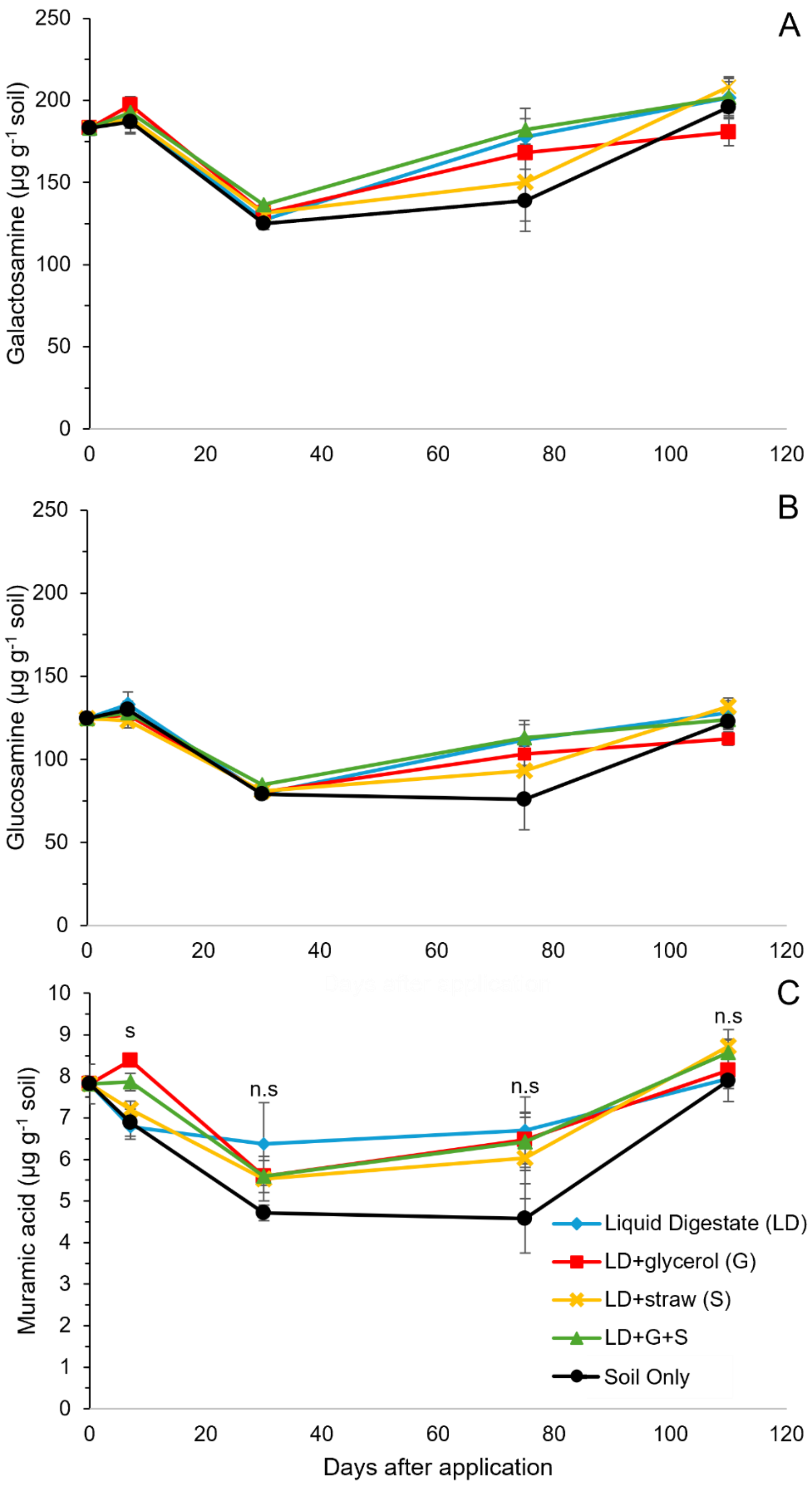

Figure 5-2: Changes in soil amino sugars over time; galactosamine (A), glucosamine (B) and muramic acid (C). At each sampling time lower-case letters denote either a significant (s) or non-significant (n.s) treatment effect according to Tukey's post-hoc test at 5% probability. Error bars denote the standard error of the mean, n=4.

5.4.2 Plant growth and yield

There was no treatment effect on the total nitrogen content in above-ground plant nitrogen content (p=0.2), plant weight (p=0.2) or plant height (p=0.3) at any sampling time. However, there was a time effect as they all significantly changed between 30 days and 75 days after application (p<0.001), with a decrease in total plant N content and an increase in both weight and height (Table 5-3). At harvest there was no significant treatment effect on grain yield, grain nitrogen content, nor either of the nitrogen use efficiency indices (Table 5-4).

Table 5-3 Effects of treatments on plant nitrogen content, weight and height over time (mean ± SE, n=4). Different upper-case letters indicate significant difference between sampling times according to Tukey's post-hoc test at 5% probability.

	Treatment				
Parameter	Liquid Digestate (LD)	LD+ Glycerol (G)	LD+ Straw (S)	LD+G+S	Soil Only
Plant N (mg g^{-1} dry biomass)					
30 days ^{A}	47.6 ± 0.9	44.4 ± 1.5	44.4 ± 2.0	45.2 ± 2.1	45.0 ± 2.2
75 days ^{B}	14.2 ± 0.6	11.9 ± 0.1	12.4 ± 0.5	12.5 ± 0.5	12.3 ± 0.3
110 days ^{B}	12.1 ± 1.0	11.5 ± 0.2	10.4 ± 0.6	11.6 ± 0.2	11.6 ± 0.3
Plant wt (g dry biomass)					
30 days ^{A}	0.17 ± 0.01	0.19 ± 0.01	0.19 ± 0.03	0.17 ± 0.02	0.18 ± 0.02
75 days ^{B}	3.4 ± 0.2	3.2 ± 0.1	3.0 ± 0.1	3.0 ± 0.2	3.1 ± 0.3
110 days ^{B}	3.4 ± 0.1	3.0 ± 0.2	3.2 ± 0.3	3.0 ± 0.2	2.6 ± 0.2
Plant height (cm)					
30 days ^{A}	30.0 ± 0.6	32.0 ± 0.6	30.5 ± 0.7	29.9 ± 0.9	31.1 ± 0.7
75 days ^{B}	63.1 ± 0.7	63.8 ± 0.7	65.2 ± 0.8	63.6 ± 0.5	65.0 ± 0.8
110 days ^{B}	63.6 ± 0.6	65.6 ± 0.5	66.4 ± 0.5	64.2 ± 0.8	65.2 ± 0.7

Table 5-4 Effect of treatments on grain yield, nitrogen content and two nitrogen use efficiency indices (mean ± SE, n=4) at harvest. RE = apparent crop recovery efficiency of applied nutrient (kg increase in N uptake per kg N applied), AE = agronomic efficiency of applied nutrient (kg yield increase per kg nutrient applied).

Parameter	**Treatment**				
	Liquid Digestate (LD)	LD+ Glycerol (G)	LD+ Straw (S)	LD+G+S	Soil Only
Grain Yield (g plant^{-1})	2.3 ± 0.1	2.1 ± 0.1	2.3 ± 0.2	2.1 ± 0.1	2.0 ± 0.0
Grain N Mg g^{-1}	15.1 ± 1.8	14.9 ± 0.6	12.6 ± 0.2	14.2 ± 1.0	14.2 ± 0.3
RE Kg-N Nkg^{-1}	0.07 ± 0.04	0.03 ± 0.02	0.01 ± 0.02	0.01 ± 0.02	n/a
AE kg Nkg^{-1}	4.1 ± 1.8	1.3 ± 1.1	3.4 ± 1.5	1.0 ± 0.7	n/a

5.4.3 Nitrogen losses

There were no significant (p=0.70) treatment differences in the cumulative N_2O emission (Figure 5-3A), which were calculated from the period between 2 hours and 17 days after treatment application, as beyond this time point N_2O concentration in samples were below the detection limit of the GC. Cumulative NH_3 emissions over the 5-day period after application were significantly affected by treatment (p=0.02), with the liquid digestate and straw treatment resulting in higher emissions than unamended soil (Figure 5-3B). Post-harvest leachate had no detectable ammonium-N and no significant (p=0.72) difference was measured in the nitrate-nitrite concentrations between treatments (Figure 5-3C).

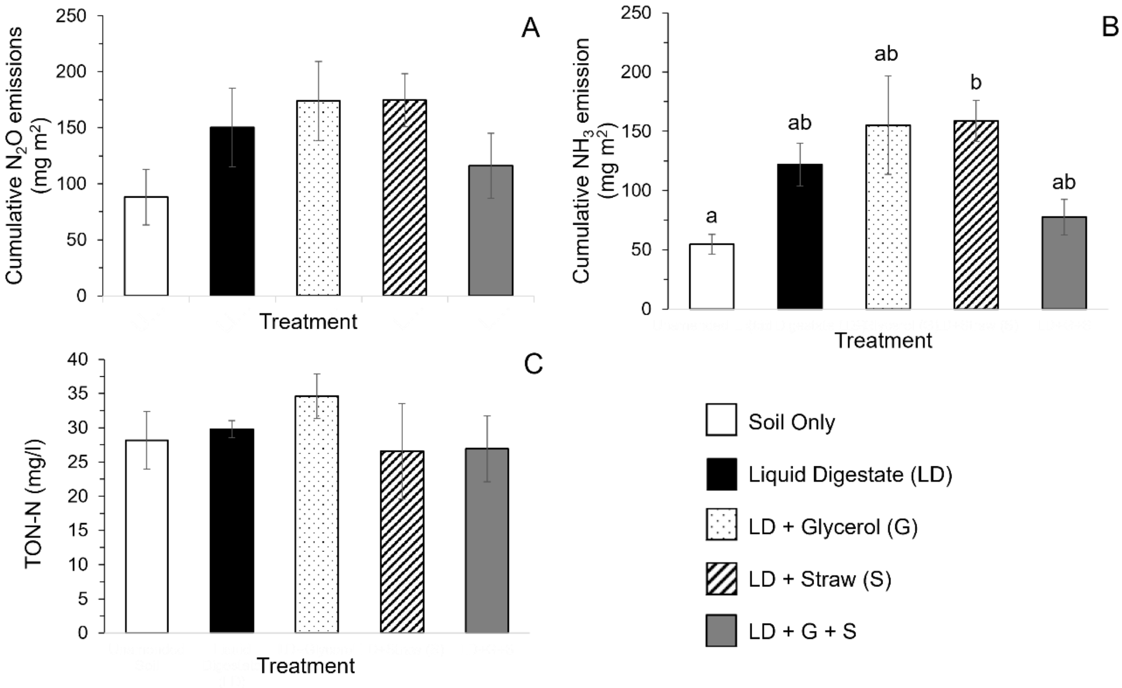

Figure 5-3: Effect of treatments on cumulative N_2O emissions over 17 days (A), cumulative NH_3 emissions over 5 days (B) after treatment application and total oxides of nitrate-nitrite (TON-N) in post-harvest leachate (C). Bars with different lower-case letter have statistically different effects according to Tukey's post-hoc test at 5% probability. Error bars denote the standard error of the mean, n=4.

5.5 Discussion

5.5.1 Soil microbial dynamics

The addition of glycerol to digestate, with or without straw, increased microbial biomass carbon, compared to digestate alone, a week after digestate application. Concurrently there was a decrease in soil available nitrogen. Yet, the decrease in available nitrogen of 95 mg-N kg^{-1} cannot be fully explained by the increase in microbial nitrogen by 20 mg-N kg^{-1}. As no leaching occurred during crop growth, nitrate losses via this pathway can be excluded, whilst cumulative ammonia volatilisation and N_2O emissions were not different between the digestate with or without organic carbon material additives treatments. However, the muramic acid, which is a component of microbial necromass (Joergensen, 2018), had increased indicating that digestate-supplied nitrogen transformed into soil organic nitrogen via microbial turnover from biomass into necromass. This is in agreement with results from He *et al*., (2011) and Engelking, Flessa and Joergensen (2007) who also measured similarly rapid increases in muramic acid after adding organic carbon materials as glucose or sucrose to soil with a nitrogen source. As muramic acid is bacterial in origin (Glaser, Turrio, and Alef 2004), it demonstrates that glycerol utilisation is dominated by bacteria. This finding is supported by the work in the previous chapter that showed the addition of glycerol and digestate to soil promoted an increase in the relative abundance of Gram-negative bacteria compared to digestate only soils.

The addition of straw and glycerol into digestate was expected to prime straw decomposition, as Qiu *et al*., (2022) observed that addition of labile carbon (glucose) resulted in faster mineralisation of straw-derived carbon compared to straw without glucose. Therefore, we expected that the duration of nitrogen immobilisation with glycerol and straw would be longer than glycerol alone, but this was not observed. This lack of an effect could be due to straw particle size. Both Angers and Recous, (1997) and Tarafdar, Meena and Kathju, (2001) found that straw particle size significantly influenced decomposition rates. Tarafdar, Meena and Kathju, (2001) recorded greatest microbial activity in soils applied with straw smaller than 0.9 cm compared to larger straw lengths between 2.9-4.4 cm. They attributed this to the grinding breaking residue structure which increases the surface area and thereby made it both more accessible for enzyme breakdown and for microbial colonisation. Additionally, the smaller particles mixed more evenly with the soil and increased decomposition sites. As our straw mimicked field size of 10 cm (Angers and Recous, 1997), the carbon within was less accessible for microbial utilisation compared to ground or finely chopped straw, or the 5 mm chopped straw used by Qiu *et al*., (2022) in their glucose-straw mineralisation experiment.

The effect of straw particle size on decomposition rate could also explain why no effect was observed in our study on nitrogen immobilisation from the addition of straw with digestate, which is in line with Hu *et al*., (2023) who had also chopped straw to ~10 cm and measured no effect on microbial biomass when co-applying straw with urea nitrogen, compared to urea nitrogen fertilisation alone. In contrast, studies that mixed crushed straw at <2mm sized particles with ammonium sulphate recorded significant microbial growth (Reichel *et al*., 2018), reduction of soil available nitrogen (Liang *et al.*, 2022), and greater soil necromass (Ding *et al.,* 2010). This indicates that to make the carbon in the straw more accessible to microorganisms in order to stimulate nitrogen immobilisation from the digestate, the straw would need to be ground. However, this would add additional processing steps and challenges to spreading the straw in field settings as, for example, light straw powder would be hard to mix into a digestate spreading tanker. Yet, it is worth noting that the soil used in our study did not come from a site subjected to historic straw return, whereas Zhao *et al*., (2019) demonstrated that adding straw to soil with a 25-year history of straw return had higher straw decomposition rates compared to soil with no history of straw return. This suggests that the microbial community needs time to adjust to effectively utilise new resources such as straw and this may be a contributing factor leading to the lack of microbial nitrogen immobilisation observed in our study between digestate with straw and digestate without straw.

5.5.2 Plant growth and yield

Despite the positive effect of glycerol addition on microbial biomass immobilisation, there was no subsequent effect on nitrogen content in spring barley during its growing season, nor its yield. This is contrary to our hypothesis as we expected that the immobilisation and remineralisation of nitrogen from adding carbon into digestate would increase the overall nitrogen use efficiency of the plant, as defined by an increase in production per unit of nitrogen applied (Meena *et al*., 2017). Spring barley has a high demand for nitrogen during grain fill, which is satisfied by a combination of remobilisation of nitrogen from vegetative parts into the grain and uptake of nitrogen from the soil (Bingham and Garzon, 2023). However, in our study, the majority of immobilised nitrogen in pots receiving digestate and glycerol (with or without straw) had remineralised by the time barley had started to extend its stem (30 days after application), which is the beginning of an earlier nitrogen demanding growth period lasting until heading (Delogu *et al*., 1998). Therefore, the nitrogen that remineralised in the soil treated with glycerol was taken up by the plant before it could contribute to the nitrogen requirements at the later grain fill stage and improve the nitrogen use efficiency of the crop compared to digestate alone.

Nitrogen use efficiency, as calculated by apparent recovery, was very low in our study, when compared against the expected range of 0.3-0.5 kg-N per kg-N applied for cereals (Dobermann, 2007), which indicates that factors other than nitrogen were limiting crop growth. This inference is supported by the lack of difference in plant height between pots receiving digestate with and without additives compared to the soil only control, as increases in nitrogen supply usually results in taller barely plants (Aghdam and Samadiyan, 2014). One potential growth limiting factor could be low supply of another necessary nutrient, such as another macro- or a micro- nutrient. In order to correct a nutrient deficit, plants release root exudates to stimulate mineralisation of organic matter, which occurred in the first month of the experiment as evidenced by the decrease in necromass between 7 and 30 days after application. By allocating more photosynthetic carbon to prime mineralisation, there is less available for above ground growth and consequently plant nitrogen requirement is reduced. Another factor that could explain the lack of significant difference in growth between treatments is heat stress. Temperatures in the polytunnel reached 40°C during the hotter periods, whilst the barley variety selected for this experiment was suited for cooler UK outdoor conditions. Heat stress negatively affects growth and physiological processes, such as reducing photosynthesis, and consequently reduce nutrient uptake (Mishra *et al.*, 2023). It is likely that a combination of factors such as these that occurred to prevent the crop from effectively utilising digestate supplied nitrogen and reaching its full yield potential.

5.5.3 Nitrogen losses

Nitrogen losses from digestate application to soil occur by pathways including ammonia volatilisation, nitrate leaching and N_2O emissions, which influences the nitrogen use efficiency of crops. In our study there was no difference in cumulative ammonia emissions between soil only and digestate only treated plots, supporting previous research that showed rapid incorporation of digestate is an effective means of preventing volatilisation losses of nitrogen (Tiwary *et al*., 2015). The addition of the organic carbon materials to digestate did not affect ammonia emissions compared to digestate alone, which supports the fact that volatilisation is primarily affected by abiotic factors such as temperature, moisture and soil pH (Clain *et al.,* 2020), which were the same across treatments.

N_2O emissions from soils depend on soil nitrate as the substrate for denitrifying bacteria (Robertson and Groffman, 2014). Concentrations of available nitrogen (sum of ammonium and nitrite-nitrate) was reduced in our study under the glycerol addition (with and without straw) to digestate in the first week after application, making less nitrate available to denitrifiers. However, there was no difference in the cumulative N_2O emissions measured between digestate with or without glycerol. Similarly, Reichel *et al*., (2018) also did not measure a significant difference in N_2O emissions over 21 days between the application of straw with ammonium-N and the N fertiliser alone. Both studies maintained soil at 60% water holding capacity, whereas denitrification activity is correlated with increasing water filled pore space (Wang *et al*., 2021), and it could be that our soil moisture conditions did not favour conditions for denitrifiers.

Post harvest nitrite-nitrate concentrations were not different between treatments, which is in agreement with Reichel *et al*., (2018) who measured no significant difference in soil nitrite and nitrate concentrations between nitrogen fertiliser with and without straw 113 days after application. This demonstrates that glycerol addition does not result in nitrogen remineralisation outside of growing period, although the same cannot be said for straw as no immobilisation was measured. The rapid reduction in soil available nitrogen concentrations under digestate mixed with glycerol (with or without straw) within a week of application demonstrates the potential for glycerol addition to reduce leaching losses. The period shortly after application is a particularly high risk for nitrate leaching caused by heavy rainfall (Jaynes *et al* 2001), as spring sown crops need to germinate and establish, during which nitrogen uptake is low (Delogu *et al*., 1998).

5.6 Conclusion

The aim of this study was to assess the effects of adding organic carbon materials into digestate on plant nitrogen use efficiency. Whilst the addition of glycerol to digestate did result in nitrogen immobilisation, the period of immobilisation was too short for there to be any measurable effect on barley growth and nitrogen use efficiency. Yet the increase in biomass and turnover rate into necromass within a week of application demonstrates the effectiveness of transforming digestate supplied nitrogen into more stable organic nitrogen. This rapid immobilisation could reduce nitrate leaching from rainfall events occurring in the month after digestate application, which in turn could increase nitrogen supply to crops. Meanwhile the addition of straw, a more complex form of carbon compared to glycerol, with digestate did not stimulate microbial uptake of nitrogen. This work has expanded our understanding of how to use organic carbon amendments to influence the nitrogen use efficiency by crops of a nitrogen source, specifically digestate, as well as the microbial groups and mechanisms driving this process. However, further plant pot studies are needed to optimise the type and rate of high organic carbon amendments to add with digestate to improve yield and nitrogen use efficiency of plants, whilst accounting for different crop physiologies to ensure that sufficient nitrogen is remineralised at the right time and amount to match plant nitrogen uptake requirement. These experiments would need to incorporate different soil types to understand how soil type affect the dynamics of microbial growth and turnover of digestate supplied nitrogen, whilst also testing different soil moisture levels to better understand how N_2O and nitrate leaching are affected. The outcomes of these experiments would need to be verified by field experiments. By thoroughly testing these parameters, a substantial step can be taken towards determining the viability of adding organic carbon materials with digestate to improve the environmental sustainability and productively of using digestate as a fertiliser.

5.7 References

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6 Addition of high organic carbon materials to improve crop uptake of anaerobic digestate supplied nitrogen influences soil microbial biomass in a field experiment

6.1 Abstract

Global food production relies on inorganic nitrogen to meet population demands. However, synthetic fertiliser production relies heavily on fossil fuels. Anaerobic digestate offers an alternative source of nutrients because it is produced from organic matter such as food waste or energy crops. Yet digestate application results in similar problems of environmental pollution that synthetic fertiliser use incurs, such as nitrate leaching and N_2O emissions. Co-amendment of digestate with a high carbon amendment may result in higher nitrogen use efficiency and fewer losses by adjusting the C:N ratio to encourage microbial assimilation of nitrogen. Therefore, we conducted a field plot experiment to understand the effects of adding carbon with digestate on the nitrogen uptake, yield, and quality of sugar beet grown on a calcareous sandy-loam soil. Liquid digestate was applied at 100 kg-N per ha, with carbon added as either glycerol or straw at 24 kg-C m^{-3} and compared against farm standard application of inorganic nitrogen. The addition of glycerol resulted in higher microbial biomass carbon than digestate alone in the first month after application. However, the treatments did not influence soil nitrogen availability, nitrogen uptake in sugar beet, nor improve sugar beet yield and quality. Potential leachable nitrogen at harvest showed no difference between treatments. Sugar beet yield and quality under liquid digestate application performed comparatively with synthetic fertiliser, indicating that digestate can be used to fertilise sugar beet. No further benefit to crop or nitrogen retention in soil was recorded by adding carbon to digestate, which could be due to the low carbon addition rate. Future studies need to focus on determining the optimal amount of carbon to add with digestate to positively impact yield and reduce nitrogen losses.

6.2 Introduction

It is estimated that approximately half of the global population is fed due to the use of synthesised inorganic nitrogen fertilisers on growing crops (Erisman *et al.*, 2008). However, there are negative consequences to its intensive use. The Haber-Bosch process that syntheses ammonium nitrogen by fixation of atmospheric dinitrogen is an energy intensive process, currently reliant largely on fossil fuels (Smith, Hill and Torrente-Murciano, 2020). Furthermore, environmental pollution occurs to watercourses from applying synthetic fertilisers to fields through leaching and runoff, whilst pollution of the atmosphere occurs due to ammonia volatilisation and N_2O emissions, the latter of which contributes 2% of global greenhouse gas emissions (Menegat, Ledo and Tirado, 2022). Using materials rich in already fixed nitrogen, such as anaerobic digestate, can reduce our reliance on fossil fuels, because digestate is a by-product of the biogas industry. Digestate is produced from the anaerobic decomposition of organic matter such as food waste, animal wastes and energy crops. Digestate is commonly separated into two fractions, to reduce cost of transport and storage (Al-Seadi and Lukehurst, 2012), with the liquid fraction receiving the majority of the nitrogen (Fuchs and Drosg, 2013). Both the unseparated digestate and the liquid fraction have been found to produce yields comparable to inorganic nitrogen fertilisers through extensive field trials on cereals, such as maize, wheat and rice (Šimon, Kunzová and Friedlová, 2015; Du *et al.*, 2019; Zicker *et al.*, 2020; Doyeni *et al.,* 2021; Grillo *et al.*, 2021; Pastorelli *et al.*, 2021; Ran *et al.*, 2022; Buligon *et al.*, 2023), and fodder or energy crops such as grasses and maize (Saunders *et al.*, 2012; Vaneeckhaute *et al.*, 2013; Montemurro *et al.*, 2015; Vanden Nest *et al.*, 2015; Tilvikienė, Šlepetienė and Kadžiulienė, 2018; Walsh *et al.*, 2018; Tsachidou *et al.*, 2019; Głowacka, Szostak and Klebaniuk, 2020).

Whilst anaerobic digestate is a suitable substitute for inorganic nitrogen, the concerns of nitrogen pollution as a result of ammonia volatilisation, nitrate leaching and N_2O emissions remain (Nkoa, 2014; O’Connor *et al.*, 2022). Ammonia losses can be reduced between 45-95% by changing spreading techniques to precision applicators such as injectors or band spreaders

(Nicholson *et al.*, 2018), or acidifying the digestate by adding sulfuric acid (Sánchez-Rodríguez *et al.*, 2018; Wagner *et al.*, 2021) or through plasma treatment (Graves *et al.*, 2019). To reduce N_2O emissions, the use of nitrification inhibitors is being explored and is demonstrating positive reductions (Hegewald *et al.*, 2021). Changing application time from autumn to spring reduces nitrate leaching by ~50% as an actively growing crop in spring takes up the supplied nitrogen (Schwager *et al.*, 2016). However, applying nitrogen in one bulk application has been shown to have lower nitrogen use efficiency compared to a split application of multiple smaller doses to match the nitrogen demanding stages of growth (Hu *et al.*, 2021). Yet, applying digestate in split applications is difficult as Wilson *et al.*, (2021) recorded mechanical damage to maize at later crop growing stages from an application system using an umbilical cord. As such it is useful to identify strategies that could slow the rate of nitrogen released from digestate once it is in the soil and therefore reduce nitrogen pollution.

Previous research (Pittaway *et al.*, 2018; Reichel *et al.*, 2018) suggests that adding high organic carbon additives to inorganic nitrogen fertilisers could be a method to reduce nitrogen losses in a microbial driven process. As soil microorganisms assimilate carbon for population growth, they also immobilise nitrogen for cellular development (Robertson and Groffman, 2007). After microbial cell death, a proportion of the immobilised nitrogen is held as necromass (Kindler *et al.*, 2006), which is subsequently remineralised due to necromass turnover. In planted systems, nitrogen mineralisation from necromass may be enhanced by priming effects induced by plant root exudates (Meier, Finzi and Phillips, 2017). To test whether immobilisation of digestate supplied nitrogen can be stimulated via addition of organic carbon additives, we added different high organic carbon materials to the liquid fraction of digestate (van Midden *et al.*, (2024). We found that the addition of glycerol and straw resulted in greater microbial biomass nitrogen. However, this work was done in the absence of a plant in a soil incubation experiment set at constant conditions (20°C and soil moisture at 40% of the water holding capacity) to promote microbial growth.

The aim of this experiment was, therefore, to evaluate how the addition of carbon additives to the liquid fraction of anaerobic digestate influences microbial nitrogen immobilisation. We aimed to assess both the direct effect of adding an energy source to stimulate microbial growth, and the indirect influence on soil properties such as compaction and pH which affect the accessibility of nitrogen to microbes (Nawaz, Bourrié and Trolard, 2013; Truog, 1947), as well as the subsequent effects on crop uptake of nitrogen and yield. This experiment was undertaken in field conditions and compared to the standard practice of using inorganic nitrogen fertilisation on sugar beet as a tuber producing crop. Sugar beet was selected as it is a high value UK cash crop, providing over 55% of sugar produced to meet the UK's total sugar demand (Short *et al.*, 2014) and it has not been studied in relation to fertilisation with liquid digestate. We hypothesised that the combination of a high carbon organic material with liquid digestate would increase the yield and quality of the sugar beet crop by improved nitrogen use efficiency due to microbial immobilisation and remineralisation of digestate supplied nitrogen. Since the additives vary in physical and chemical composition, we also hypothesised that the soil bulk density and pH would be reduced by the addition of straw and glycerol respectively.

6.3 Method

6.3.1 Experimental site, materials and treatments

A field experiment was established growing sugar beet (variety KWS Rixta) in 2023 on a commercial farm near Feltwell, UK (52° 29' 59.68'' N, 0° 32' 20.74'' E) on a calcareous sandy loam soil over chalk (Table 6-1). Average daily air temperature and precipitation data were collected from an on-farm weather station between February and November 2023 (Figure 6-1). The digestate (Table 6-1). used was generated by Future Biogas Ltd in a plant that utilised a mixture of maize and rye silage, with seasonal addition of sugar beet pulp and horse manure as its feedstock. The digestor has 2 primary and 1 secondary digestor tanks and ran at a temperature of 47°C with a retention time of 82 days. Post digestion, the digestate was separated by screw press into liquid and solid fractions. Wheat straw (40% total carbon content), sourced on-farm, was used and glycerol (38% total carbon content) was bought from Monarch Chemicals Ltd (UK).

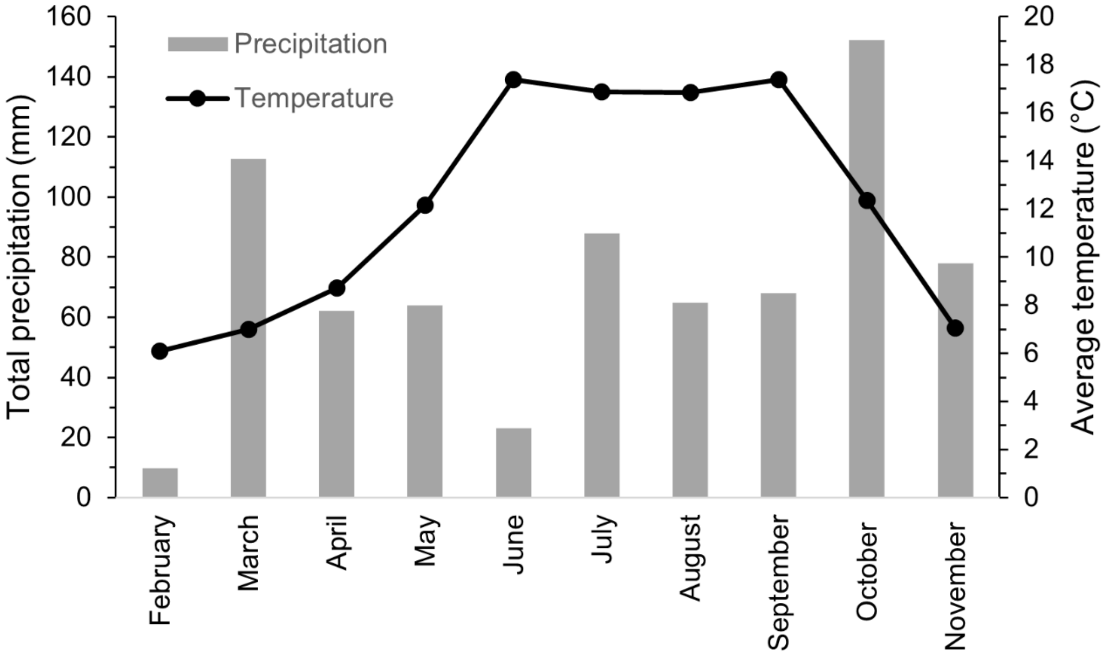

Figure 6-1 Monthly total rainfall and average temperature during the experiment

Table 6-1 Soil and liquid digestate characterisation.

Parameter	Soil	Liquid Digestate
Dry Matter (%)	84	6.4
Organic Matter (% $^{\text{d.w. soil}}_{\text{f.w. digestate}}$)	3.7	4.8
pH	8.5	8.1
Total Carbon (% $^{\text{d.w. soil}}_{\text{f.w. digestate}}$)	5.5	2.7
Nitrogen		
Total Nitrogen (% $^{\text{d.w. soil}}_{\text{f.w. digestate}}$)	0.2	0.53
Available Nitrogen (mg N kg^{-1})	6.9	3106
Phosphorus		
Total (mg P kg^{-1})	-	564
Available (mg P kg^{-1})	48.6	-
Potassium		
Total (mg K kg^{-1})		4759
Available (mg K kg^{-1})	561	-
Cation exchange capacity (meq 100 g^{-1})	14.1	-
Bulk Density (g cm^{-3})	1.5	-
Microbial Biomass C (mg C kg^{-1})	324	-
Microbial Biomass N (mg N kg^{-1})	48	-

dw= dry weight basis, fw = fresh weight basis.

The experiment was laid out in a randomised complete block design with 3 replicates. The four treatments were; (1) digestate, (2) digestate with glycerol at 24 kg-C per m^3 of digestate, (3) digestate with straw at 24 kg-C per m^3 of digestate, and (4) inorganic nitrogen. This gave a total of 12 experimental plots, each 26 m long and 32 m wide to fit within the tramlines. A combination of historic yield map, soil map provided by Landis.org.uk and normalised difference vegetation index (NDVI) images were used to identify the area within the field with the most homogenous conditions for siting the experimental area (Supplementary Material).

Photo 6-1 Marking out complete. Use of cross-sight ranging pole (York Survey Supply, UK) to ensure plots corners were 90° angled. Plot markers consisted of coloured plastic bags attached to 1.5 m sized plastic rigid tubes (outside diameter 12.5 mm, wall thickness 1.5 mm from MKM Extrusions, UK) that had enough flex to bend under the sprayer boom during pesticide applications.

The field had received a base fertiliser (from Law fertilisers Ltd) supplying 75 kg-potassium ha^{-1} 200 kg-sodium ha^{-1} and 80 kg-magnesium ha^{-1} in November 2022 to maintain the soil at target index for growing sugar beet (British Beet and Research Organisation, 2017) and an application of farmyard manure at 40 t ha^{-1} supplying approximately 24 kg-N ha^{-1}. In February 2023, preceding seedbed preparation, the straw was chopped and applied to plots of treatment 3 using a straw blower at 1.1 tonne ha^{-1}. The whole field was deep tilled (30-40 cm), disced, and rolled with a Cousins Patriot subsoiler in February, followed by shallow tillage (top 5 cm of soil) using a Dalbo Rollermaximum directly before sowing to prepare the seedbed. Liquid digestate (without or with glycerol) was applied to the plots of treatments 1, 2, and 3 by A&R Cramphorns (Rutland, UK) using a band-spreader at a rate equivalent to 100 kg-N ha^{-1} on March 30th 2023. The field was sown on April 4th at a rate of 115,000 seeds (variety KWS Rixta) per ha. Inorganic nitrogen fertiliser (Law Fertilisers Ltd), was applied to the plots of treatment 4 according to farm standard practices with a split dose of NPS (nitrogen, phosphorus and sulphur) fertiliser from at 50 kg-N ha^{-1} on April 18th and calcium ammonium nitrate at 52 kg-N ha^{-1} on 3rd May. All other necessary crop husbandry was done following farm standard practises equally across all plots.

6.3.2 Plant and Soil Sampling

Soil sampling was carried out six times throughout the year. The first occurred before the straw was added by way of pre-experimental characterisation (31st January 2023). The following five sampling events occurred between April and November to coincide with sugar beet emergence, the start of canopy growth, 85% canopy cover, beginning of senescence, and harvest. To measure nitrogen uptake in the sugar beet, a total of 20 youngest and fully expanded leaves were collected from 5 points per plot following a “W” pattern across each plot. At the same locations topsoil samples (0-15 cm) were collected using an auger and combined to make a 250 g bulk sample. At three points per plot along the “W”, undisturbed soil samples were collected using stainless steel cylindrical cores (57 mm internal diameter and 45 mm in height), oven dried at 105°C for 48 hrs and bulk density calculated as g per cm^3 using the formula of core volume multiplied by soil dry weight. Bulk density cores were not collected in July and September due to the hardness of the soil surface after a prolonged dry period.

Photo 6-2 Sugar beet at emergence in April (left photo), between 5-7 true leaves unfolded in May (middle photo) and canopy closure in July (right photo).

Soil was analysed for microbial biomass carbon and nitrogen following the chloroform-fumigation method (Vance, Brooks and Jenkinson, 1987). In brief, soil portions weighing 12.50 g dry weight equivalent were fumigated for 24 hrs and extracted using 0.5 M potassium sulphate. The extracts were filtered then analysed on Shimadzu TOC with a TN module and the microbial biomass carbon and nitrogen contents calculated from the difference between the fumigated and non-fumigated samples and converted using K_{EC} and K_{EN} values of 0.45 and 0.54 respectively (Brooks *et al.*, 1985; Vance, Brooks and Jenkinson, 1987). Three analytical replicates per plot were processed to account for inherent within-plot variation. Soil pH was measured in a 1:5 v/v air dry soil samples to 1 M KCl solution following the British Standard BS ISO 10390:2005 method. Soil available nitrogen was analysed at the last two timepoints in samples taken at 30-50 cm deep to estimate the amount of potentially leachable nitrogen. Although the depth of sugar beet roots can exceed 2 m (Windt and Märländer, 1994), samples were taken at this depth due to reaching the chalk layer underlying the soil. Soil samples were extracted with a 2 M KCl solution (20 g soil and 100 ml of solution) and analysed on a segmented flow analyser (Seal AA3) for total oxides of nitrate-nitrite (TON-N) and ammonium N following MAFF (1986).

Sugar beet was harvested on the 1st of November 2023: 20 sugar beet tubers per plot were collected from three rows in the plot, the leafy tops were removed, and the tubers stored in breathable hessian sacks. Upon return to the laboratory, five tubers were cleaned, chopped, weighed, and put into an oven at 80°C for 2 weeks until no further weight loss was recorded to determine dry matter content. The remaining 15 tubers were sent to the British Beet Research Organisation (Bexwell, UK) for analysis of sugar content, amino nitrogen, potassium and sodium analysis. They were analysed using techniques from the International Commission for Uniform Methods of Sugar Analysis (ICUMSA 1994, 2007), which are standard to the UK sugar industry. The sugar beet leaves collected over the experimental duration were dried at 80°C for 48 hours when no further weigh loss was recorded (Malnou, Jaggard and Sparkes, 2008), then finely milled and passed through a 0.75 mm sieve to be analysed for total nitrogen content by dry combustion using an elemental analyser (Elementar, Vario EL III).

6.3.3 Statistical analysis

A repeated measures ANOVA was used to analyse data from measurements taken at multiple time points and a one-way ANOVA was used for harvest data. In the case of the microbial biomass and available nitrogen datasets where three analytical replicates were conducted per plot, the average value was used in the ANOVA model. Data was first tested for normality using Kolmogorov–Smirnov & Lilliefors test and homoscedasticity using Levene’s Test. Significant differences were determined using Tukey’s post-hoc test with significance determined at $p<0.05$. All statistical analysis was performed on Statistica Tibco version 14.

6.4 Results

6.4.1 Plant analysis

Very few seedlings had emerged at the first post-application sampling, so the first leaf sampling for total N occurred in the 2nd post-application sampling time. There was no treatment effect ($p=0.75$) on the total nitrogen content in sugar beet leaves at any timepoint (Figure 6-2), however nitrogen concentration had significantly ($p<0.001$) decreased by 7%, 209 days after sowing compared to May and July sampling dates. The sugar beet tubers were harvested 210 days after planting. There were no treatment effects on yield, sugar content or impurities (Table 6-2).

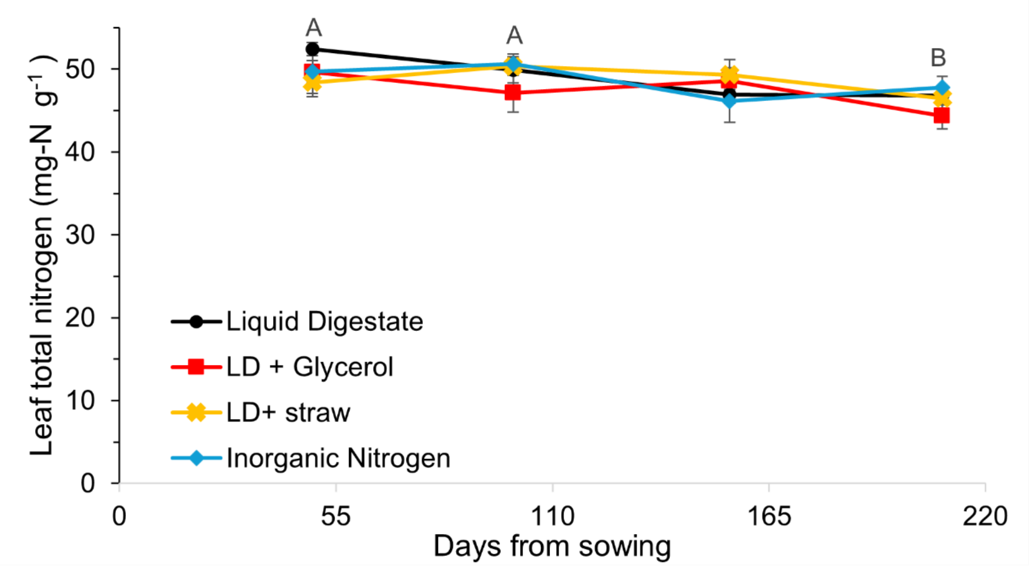

Figure 6-2 Changes in the nitrogen content of leaves over time. Different capital letters between sampling times indicate significant differences according to Tukey's test at 5% probability. There were no significant differences between treatments. Error bars denote the standard error of the mean, n = 3.

Table 6-2 Effect of treatments on various sugar beet tuber parameters (mean ± SE, n=3) at harvest.

Parameter	**Treatments**			
	Liquid Digestate (LD)	LD+Glyercol	LD+Straw	Synthetic nitrogen
Individual beet mass (kg)	0.89±0.11	0.97±0.1	0.91±0.11	0.95±0.13
Beet yield (t/ha)	77.8±3.6	84.9±6.1	79.5±1.8	83.0±2.8
Sugar content (%)	17.4±0.2	17.1±0.3	17.2±0.0	17.4±0.2
Dry matter (%)	21.0±0.3	21.3±0.3	22.0±0.3	21.4±0.3
Amino N (mg 100 g beet)	26.7±0.3	29.0±3.1	26.3±0.9	28.3±0.3
Sodium (mg 100 g beet)	9.9±0.4	10.2±0.8	9.3±0.5	10.0±0.4
Potassium (mg 100 g beet)	151±10	159±10	156±7	163±8

6.4.2 Soil analysis

In the first month following application, soils from the LD+Glycerol treatment had a higher (p=0.04) microbial C than digestate alone, but no treatment effect on microbial N (p=0.06) nor MBC:N ratio (p=0.85) was measured. Thereafter, no significant treatment effects on either microbial C or N were observed (Figure 6-3). Time of sampling significantly (p<0.001) affected both microbial C and N similarly, with lower concentrations in samples collected in the 16 and 155 days after sowing compared to 209 days. MBC:N ratio was also significantly affected by time (p=0.01) where MBC:N ratio was significantly higher at 100 days after application compared to 49 and 209 days (Table 6-3).

Table 6-3 Changes in soil microbial carbon:nitrogen ratio (mean ± SE, n=3) at different sampling times. Different upper-case letters indicate significant difference between sampling times according to Tukey's post-hoc test at 5% probability. Treatment effects were non-significant.

Days after sowing	**Treatment**			
	Liquid Digestate (LD)	LD + Glycerol	LD + Straw	Inorganic Nitrogen
16^{AB}	6.4±0.2	6.6±0.0	6.6±0.1	6.8±0.2
49^{A}	6.3±0.1	6.1±0.1	6.6±0.2	6.2±0.1
100^{B}	7.2±0.2	6.4±0.2	6.8±0.2	6.7±0.1
155^{AB}	6.7±0.0	6.7±0.3	6.3±0.1	6.4±0.2
209^{A}	6.0±0.2	6.9±0.2	6.0±0.2	6.5±0.4

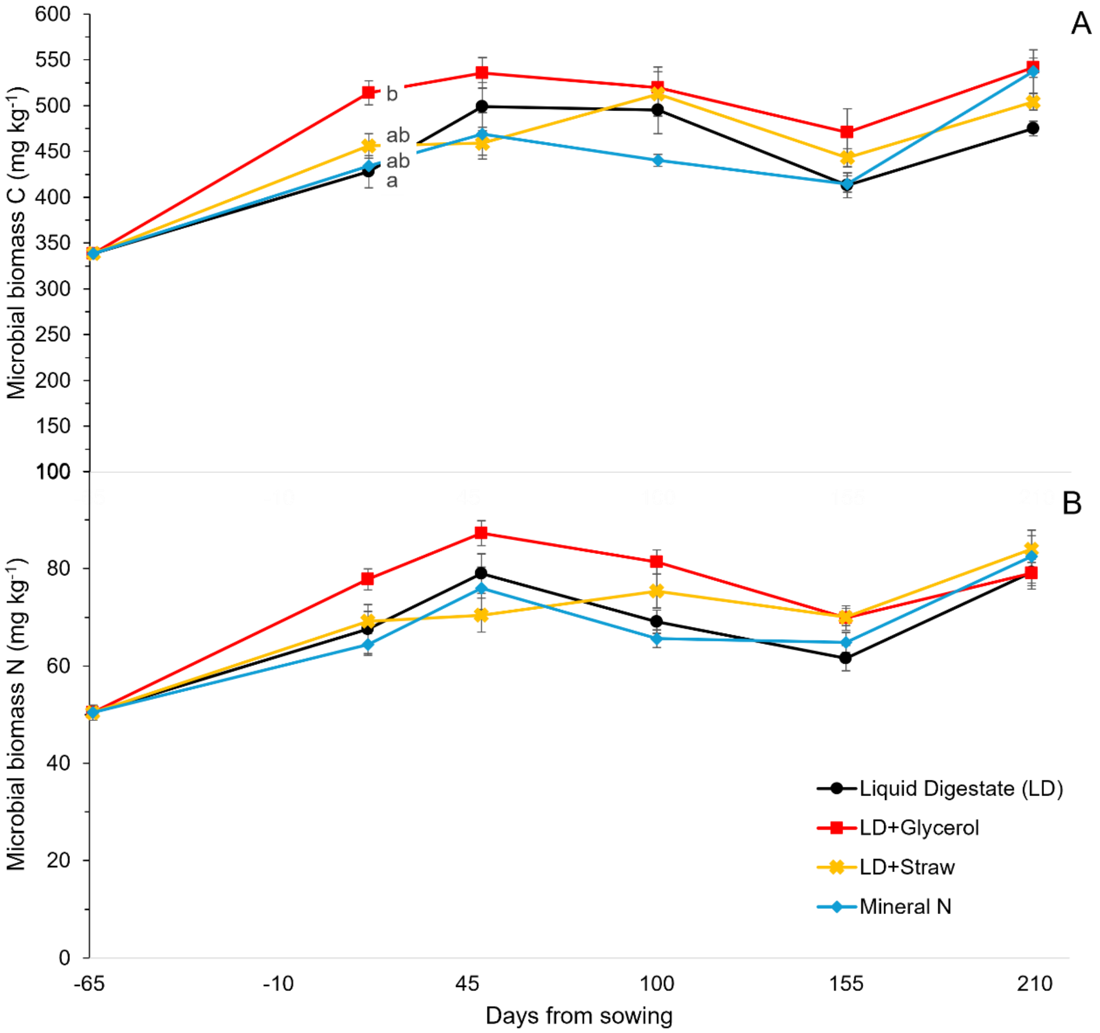

Figure 6-3 Changes in microbial biomass carbon (A) and microbial biomass nitrogen (B) in soils on a fresh weight basis over time. Different letters at each sampling time indicate significant treatment differences according to Tukey's test at 5% probability. Error bars denote the standard error of the mean, n = 3.

Soil available nitrogen was not significantly different between treatments (p=0.47) at either 155 or 209 days after sowing, however there was a significant decrease (p<0.001) in soil available nitrogen by 61% between the two sampling dates (Figure 6-4). Neither soil pH or bulk density were affected by treatments (p=0.10 and 0.41 respectively) at any timepoint (Table 6-4). However, both significantly (p<0.001) increased over time irrespective of treatment, with a rise in soil pH of 0.1 unit between units and increase in bulk density by 85% from 0.76 g cm^3 to 1.4g cm^3 between 16 and 209 days after sowing.

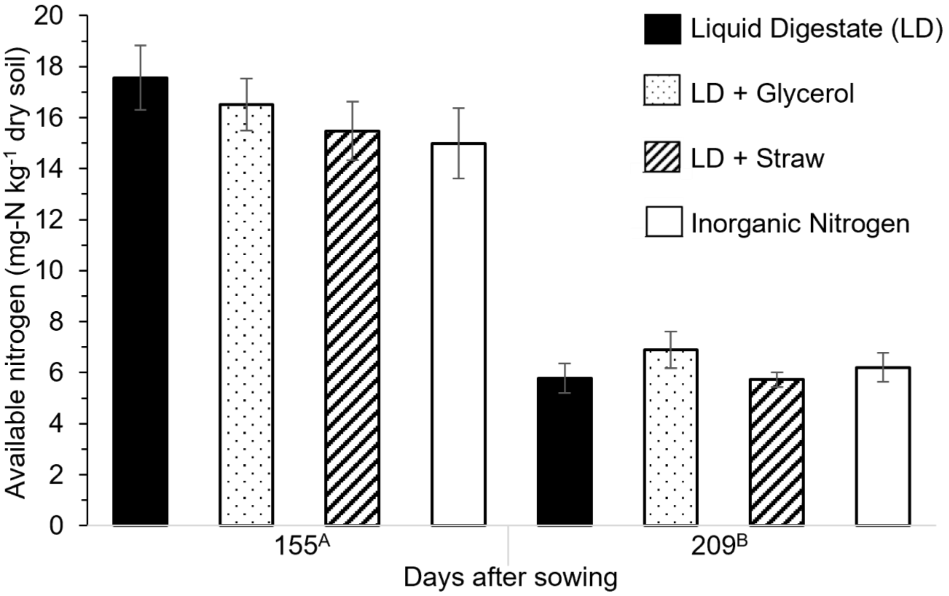

Figure 6-4 changes in soil available nitrogen between September and October. Capital letters indicate significant differences between sampling dates according to Tukey's test at 5% probability. Error bars denote the standard error of the mean, n = 3.

Table 6-4 Changes of soil pH and bulk density (mean ± SE, n=3) at different sampling times. Different upper-case letters indicate significant difference between sampling times according to Tukey's post-hoc test at 5% probability. Treatment effects were non-significant.

Parameter	**Days after sowing**	**Treatments**			
		Liquid Digestate (LD)	LD + glycerol	LD + Straw	Synthetic N
Soil pH	16^{A}	7.98±0.01	8.02±0.03	8.02±0.02	7.98±0.01
	49^{A}	7.98±0.02	8.06±0.03	8.00±0.01	7.96±0.02
	100^{B}	7.91±0.03	7.99±0.02	7.96±0.01	7.91±0.04
	155^{B}	7.92±0.01	7.98±0.04	7.96±0.02	7.92±0.02
	209^{C}	8.08±0.01	8.11±0.04	8.12±0.01	8.09±0.02
Bulk Density (g cm^{3})	16^{A}	0.79±0.02	0.74±0.03	0.74±0.02	0.77±0.02
	100^{B}	1.25±0.03	1.26±0.03	1.32±0.03	1.31±0.03
	209^{C}	1.41±0.04	1.35±0.04	1.45±0.03	1.45±0.05

6.5 Discussion

For crops to reach their yield potential, it is important that both the amount and timing of nitrogen fertiliser applied matches plant demand. Sugar beet requires an early dose of nitrogen to help it to quickly establish a full canopy, which is vital for it to develop high sugar yielding tubers (Draycott and Christenson, 2003). Inorganic nitrogen fertilisers and anaerobic digestate contain a high proportion of readily plant-available nitrogen and, in our study, these treatments produced similar sugar beet yields and sugar content, with no difference in nitrogen uptake in the leaves at any time. Additionally, the concentrations of the impurities (α-amino nitrogen, sodium and potassium) which affect the amount and purity of the sugar that can be extracted from the sugar beet were also not different between these two fertiliser sources (Table 6-2). Similarly Baryga, Połeć and Klasa, (2020) observed no difference in yield over a 3-year period between synthetic nitrogen and whole digestate. Therefore, our study corroborates their work to demonstrate that inorganic nitrogen can be substituted by anaerobic digestate, in our case the liquid fraction, without impacting either the yield, sugar content, or quality of the sugar beet. However, it is important to acknowledge that the nutrient content in digestate is variable, and because it is usually applied to meet crop nitrogen requirement, it can result in a deficit of other nutrients, such as phosphorus, potassium, magnesium and boron, which then need to be met by additional inputs (Baryga, Połeć and Klasa, 2021).

Once sugar beet reaches a full canopy it begins to develop its energy storing tuber which requires nitrogen. To meet this nitrogen demand it redistributes nitrogen taken up in the leaves from early growth into its root (Draycott and Christenson, 2003) as well as taking up nitrogen from the soil (Armstrong and Milford, 1985). A key source for soil available nitrogen is from the mineralisation of soil organic matter. As the combination of high carbon materials with a nitrogen rich source is known to immobilise nitrogen into soil organic matter via soil microbial uptake, and therefore reduce nitrogen losses that occur in the first few weeks of applying digestate, we expected to see a higher sugar beet yield in the digestate with either glycerol or straw treatments. As this was not the case, we must reject our hypothesis and consider reasons why.

The rate and magnitude of microbial nitrogen immobilisation is influenced by the bioavailability of the carbon in the amendment; as Redmile-Gordon *et al.*, (2014) and van Midden *et al.*, (2024) observed that the bioavailable glycerol-derived carbon had a quicker and greater effect on microbial uptake of nitrogen than the addition of the more recalcitrant straw derived carbon. The addition of glycerol to digestate in our study did significantly increase microbial biomass carbon by 18% compared to digestate alone. However, microbial biomass nitrogen which, whilst on average was higher by 10 mg-N kg^{-1} under glycerol addition compared to without, was not statistically significantly different. This may have been that the magnitude of change was not great enough to be statistically detectable above background microbial N, as the microbial C:N ratio did not change, indicating that nitrogen immobilisation did occur. As a consequence of this marginal difference in microbial nitrogen, there was no significant amount of extra nitrogen for the sugar beet to take up, which is reflected in the lack of difference in leaf total nitrogen content of sugar beet between treatments at any timepoint.

Compared to glycerol, we had expected that nitrogen immobilisation and remineralisation from the addition of straw with digestate would occur over a longer period, as the depolymerisation of lignin, cellulose and hemicellulose structures in the straw into monomers needs to occur for its carbon to become bioavailable for microbial uptake and metabolism. However, in our study the addition of straw to digestate did not significantly increase either microbial biomass carbon or nitrogen compared to digestate alone. This could be that because, unlike glycerol, not all the carbon in the straw was available at the time of digestate application for microbes to utilise. Another reason for the lack of effect could be that straw was applied a month and a half before fertiliser application. Gao *et al.*, (2016) measured a 37% loss of straw mass within the first month of burial, suggesting that the easily accessible carbon in the straw may have already been utilised before the digestate was applied. However, the straw and digestate could not be applied at the same time in the field, as straw needed to be incorporated into the soil for increased contact with microorganisms before digestate could be applied, and wet weather conditions delayed sowing and fertiliser application following this cultivation.

Two soil physiochemical parameters, bulk density and pH, that influence nitrogen mobility in the soil (Nawaz, Bourrié and Trolard, 2013; Truog, 1947) were measured in this study, but neither were significantly affected by treatment, although both did change over time. Changes in soil pH varied by 0.1 unit, which is within the seasonal range around the mean reported by Wuest, (2015) in a three year study. Soil bulk density was lower in spring following cultivation and increased back to pre-cultivation level as the growing season progressed. It was thought that straw addition would reduce bulk density, as recorded by Guo and Wang, (2013), Wang *et al.*, (2018) Han *et al.*, (2020) and Cui *et al.*, (2022). However, this may be due to the difference in application rates as we applied straw at 1.5 t ha^{-1} compared to the 9 t ha^{-1} applied by Cui *et al.*, (2022).

Potential leachable nitrogen, as inferred from soil available nitrogen taken at 30-50 cm soil depth, was not significantly different between treatments. This lack of effect is aligned with no difference observed in the microbial biomass carbon and nitrogen, indicating no extra nitrogen from the digestate with either straw or glycerol was being held in the microbial biomass compared to digestate alone in the autumn. Petraityte, Arlauskiene and Ceseviciene, (2022) measured lower concentration of soil nitrate taken from 30-60 cm soil depth in digestate treated plots compared to synthetic fertiliser following a dry spring, whereas under normal conditions they observed no difference. Similarly, when comparing digestate with synthetic nitrogen at comparative rates of nitrogen addition, neither Du *et al.*, (2019) or Zilio *et al.*, (2022) recorded significant differences in potential leaching as inferred from residual soil nitrate at harvest. Therefore, in combination with our results research suggests that there is no additional risk of residual nitrate leaching from digestate application versus synthetic fertiliser. However, the effect of adding carbon into digestate on potential leachable nitrogen cannot be inferred from our results, as no measurable microbial nitrogen immobilisation occurred earlier in the season. Yet the greater microbial biomass measured under the digestate with glycerol treatment compared to digestate alone does indicate the potential of this treatment to reduce leaching losses in the early months following application, a period of time when nitrogen is vulnerable to leaching (Nicholson *et al.*, 2017).

6.6 Conclusion

This experiment demonstrated that the addition of glycerol, but not straw, with digestate did increase microbial biomass carbon under field conditions, with no detrimental effect to the crop. Yet the increase in microbial biomass did not result in significantly greater quantities of nitrogen immobilised in the microbial biomass, which in turn did not affect sugar beet nitrogen uptake, yield or quality. To stimulate significant microbial nitrogen immobilisation, more bioavailable carbon is needed, however care needs to be taken to ensure that the carbon does not cause nitrogen immobilisation to the detriment of early crop growth, as recorded by Alotaibi and Schoenau, (2016) after they applied urea with glycerol (C:N ratio of 20:1) to canola. Therefore, this work contributes to developing the underlying principles of adding organic carbon materials to a nitrogen source to both reduce nitrogen losses and benefit crop growth. It demonstrated that the C:N ratio of 10:1 of the mix used in this study neither benefited nor adversely affected crops under field conditions. Although no statistically significant effect of adding glycerol with digestate on nitrogen immobilisation was measured, the increase of microbial biomass carbon due to glycerol addition is beneficial, as it is a precursor to increases in soil organic carbon concentrations (Brooks, 2001). Increases in soil organic carbon benefits soil health, the development of which is of interest to many farmers. Therefore, future studies are recommended to focus on two directions: firstly, to determine the amount of carbon to add to digestate to stimulate microbial nitrogen immobilisation to benefit crop nitrogen utilisation through a combination of pot experiments verified by field trials. The second direction is to explore both the direct and legacy effects of glycerol addition with fertiliser nitrogen on soil organic carbon, nitrogen and microbial community, which are fundamental to the nitrogen and carbon cycles in the soil . The impacts on the microbial community could be determined through methods such as molecular co-occurrence network analysis or phospholipid fatty acid analysis alongside respiration measurements to help understand potential longer-term shifts in the community and what this would mean for the dynamics of carbon and nitrogen cycling. Furthermore, it is necessary to understand how these effects are influenced by different soil type and crop nitrogen relationships such as variable timing of nitrogen demanding growth stages and differing acquisition strategies, including dependence on mycorrhizal associations. Answering these research gaps will provide confidence in the viability and sustainability of using organic carbon amended digestate to in the agricultural sector.

6.7 References

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6.8 Supplementary Material

6.8.1 yield map, NDVI and soil maps used to site the experimental site.

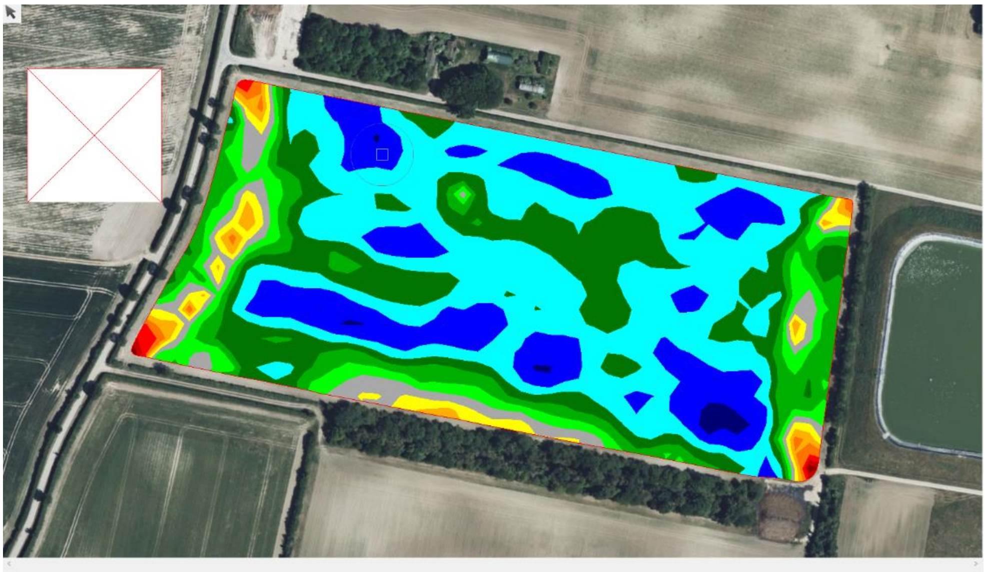


Figure S6-1 Yield Map of field from 2022 harvest

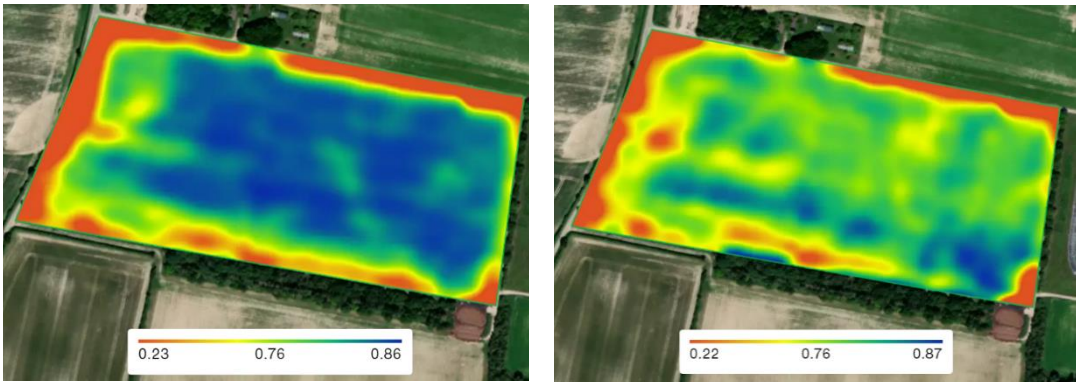


Figure S6-2 NDVI images of the field, left on 08/05/2022 and right on 22/06/2022

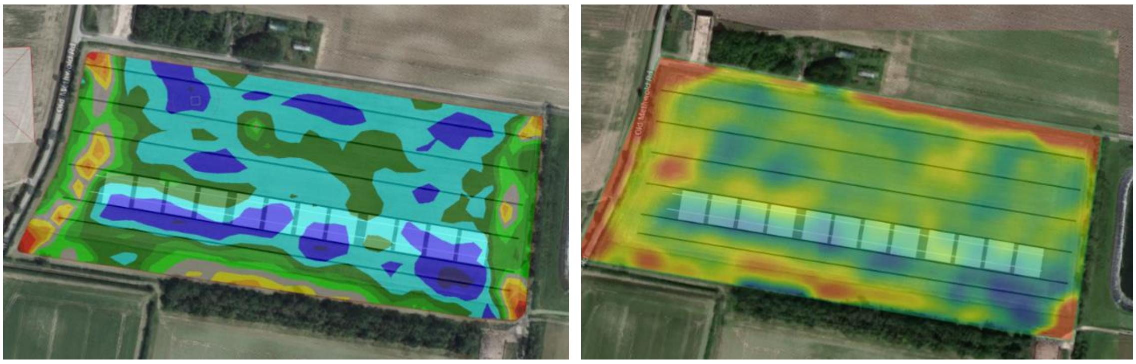
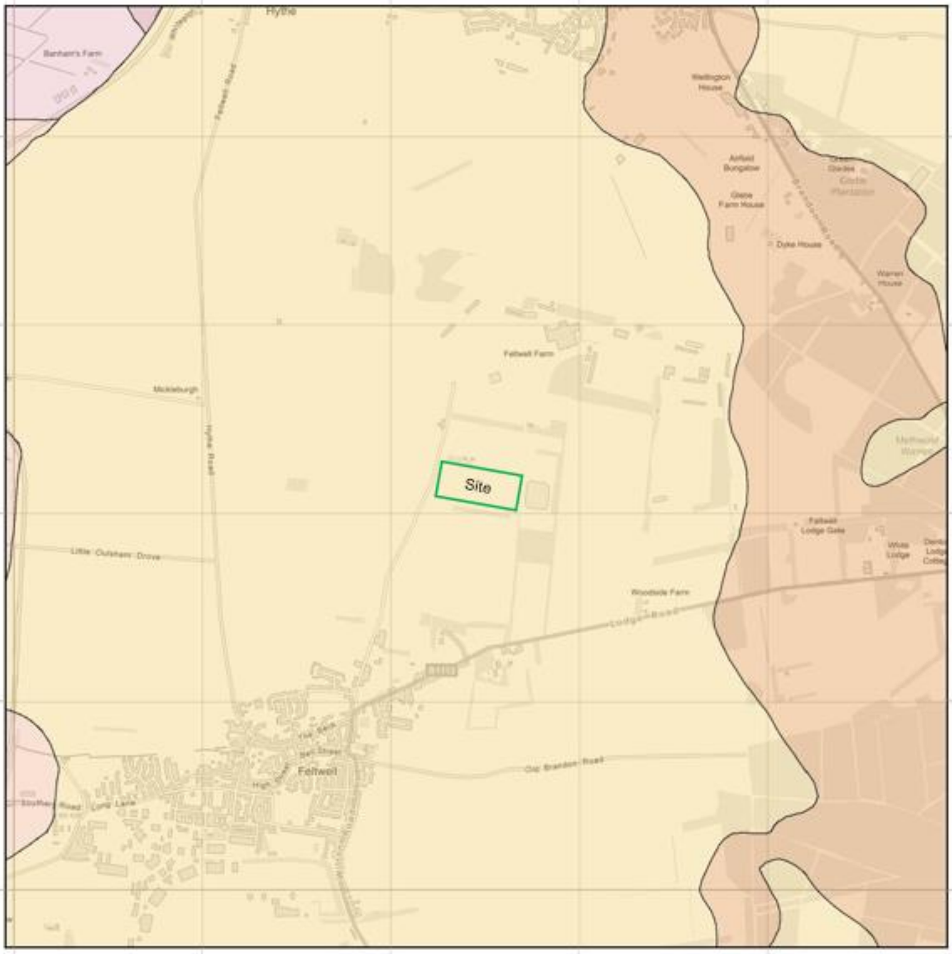


Figure S6-3: overlay of plots on NDVI (left) and yield map (right) images. Black lines on fields indicate tramlines and white lines are the tanker wheeling lines. The decision to site all blocks along one tramline rather than a block per tramline was a) each block could be sited within the most homogeneous area to reduce field condition variability between the plots within a block, and b) limit soil compaction from the digestate spreading tanker to one tramline.

1a Soils - Spatial Distribution



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7 General Discussion

7.1 Introductory remarks

The aim of this PhD project was to test the hypothesis that the addition of organic carbon-rich materials into digestate would stimulate the immobilisation of nitrogen within microbial biomass and therefore increase the nitrogen use efficiency by crops (Figure 7-1). The findings could have significant implications for enhancing the agronomic value of anaerobic digestate, specifically the liquid fraction, by understanding the microbial mechanisms associated with improved digestate nutrient use. Digestate is routinely separated into liquid and solid fractions by biogas plants to reduce storage and transport expenses (Al-Seadi and Lukehurst, 2012). During separation, most of the nitrogen goes into the liquid fraction (Fuchs and Drosg 2013), a high proportion of which is in plant available forms (Czekała, 2022). This makes it an effective fertiliser. However, the nitrogen is also susceptible to losses to the air and water, which need to be reduced (Nkoa, 2014). The proposed solution to the issue of nitrogen loss explored in this thesis was to investigate whether native soil microorganisms could be used to immobilise nitrogen within their biomass. This is due to their ubiquitous presence in the soil environment and their capability of immobilising nitrogen into their biomass when supplied with organic carbon that promotes growth (Chaves *et al.*, 2005). The organic carbon sources required to balance C:N stoichiometry can be sourced from industrial by-products, in this project represented by glycerol from biodiesel, straw from farm, woodchip from forestry and biochar made from organic waste materials. This use of by-products is important from a sustainability perspective with the need to most effectively use and reuse planetary resources.

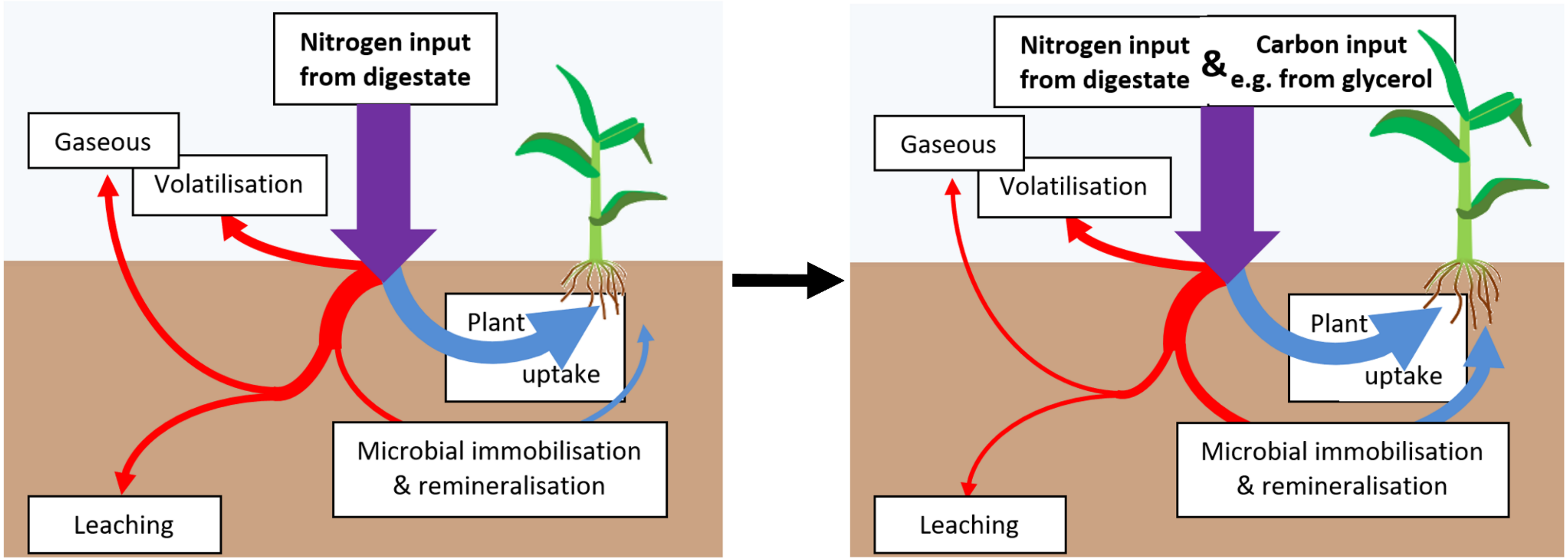

Figure 7-1 A schematic illustrating the influence of adding a high organic carbon material with an external nitrogen source on the dynamics of nitrogen in the soil and uptake by plants.

7.2 Summary of key findings

7.2.1 Effect of organic carbon material additives on microbial nitrogen immobilisation and community composition

The structural complexity, and therefore bioavailability, of carbon to microorganisms is an important consideration for inducing microbial immobilisation of nitrogen (Chaves *et al*., 2005). The first step in this project was to determine which type of carbon on a scale of labile to recalcitrant best stimulated microbial growth and nitrogen uptake. This was necessary as there was an absence of research into the effects of adding organic carbon materials on the dynamics of digestate-supplied nitrogen immobilisation. During a 5-month soil incubation study (Chapter 3), the addition of either glycerol and straw with the digestate stimulated significant microbial growth and nitrogen immobilisation, compared to digestate alone, whilst the more complex woodchip and biochar did not. However, the positive effects on microbial nitrogen concentration did not result in a corresponding reduction in the soil available nitrogen pool, indicating that the rate of carbon addition may be too low. Therefore, a follow-up experiment (Chapter 4) was carried out where glycerol was added at four rates to digestate (0, 3, 6 and 9% v/v). Both the 6% and 9% v/v had similar effects on microbial growth, indicating that above 6% that a different nutrient to nitrogen, such as phosphorus or potassium, had become the limiting factor.

The phenotypic profile of the microbial community composition, as determined by PLFA analysis, was also affected by the addition of these high organic carbon amendments. Glycerol added at 6 and 9% both resulted in a group separate to digestate with less or no glycerol, with similar differences in G+:G- ratios. The ratio of Gram-negative bacteria to Gram-positive increased under glycerol addition, which has important consequences for the fate of nitrogen in the soil organic matter. The increase in muramic acid, a bacterial residue biomarker, demonstrates the turnover of Gram-negative bacteria into necromass, thereby increasing the soil organic nitrogen pool more rapidly compared to the turnover from relatively slower growing Gram-positive bacteria and fungi. This in turn could ensure that nitrogen is available for plants to access from soil organic matter mineralisation during their growing season.

7.2.2 Effect of adding organic carbon materials into digestate on crop nitrogen use efficiency

Nitrogen use efficiency (NUE) is a metric used to measure how well a crop utilises fertiliser supplied nitrogen. An increase in crop NUE means it uses more of the supplied nitrogen, which is economically beneficial for the farmer because they need to supply less fertiliser for the crop to achieve the same yield. It is also environmentally beneficial since less nitrogen is lost leading to less pollution. The addition of either glycerol or straw, which were the two high organic carbon amendments found to stimulate microbial immobilisation from the first experiment, were added into digestate at 24kg-C per m^3 of digestate. This was the optimal rate identified from the second experiment. These treatments were applied to plants to determine how they influenced growth parameters. Spring barley was grown in pots in a polytunnel (Chapter 5) whilst sugar beet was grown in a field (Chapter 6).

Significantly greater microbial biomass and necromass concentrations occurred within the first month following digestate application when combined with glycerol (in combination with straw or not) compared to digestate alone in both experiments. Concurrently, in the spring barely experiment there was reduced soil available nitrogen. However, the levels in the field experiment were not analysed due to an instrument failure. Despite this, neither the spring barley nor the sugar beet showed any difference between treatments in nitrogen uptake into the above ground biomass throughout the growing season, which resulted in no difference in overall NUE. This is likely due to 2 factors. Firstly, the rate of immobilised nitrogen could have simply been too low to make a difference. In the field experiment microbial nitrogen was not higher in digestate with glycerol compared to digestate alone. This was despite significant difference in microbial biomass carbon, indicating that any change in microbial nitrogen was not of a great enough magnitude to be statistically detectable between the treatments. This indicates that there was no extra nitrogen to be remineralised and taken up by the sugar beet. The second factor that could explain the lack of effect on crop nitrogen uptake is the timing of nitrogen remineralisation relative to the growth stage of the crop. Crop development occurs in three phases; foundation, construction and production (Sylvester-Bradley *et al.*, 2008). The construction stage is the most nitrogen demanding as the crop grows rapidly to establish a canopy, followed by production. The addition of high organic materials into the digestate was hypothesised to remineralise the nitrogen in time for the production stage. The spring barley entered the construction phase 30 days after sowing, by which time most of the immobilised nitrogen had remineralised. The sugar beet began construction phase after approximately 50 days from sowing, at which time there was no difference in microbial biomass between digestate and digestate with glycerol, indicating the likelihood that the immobilised nitrogen had also remineralised. As such the immobilised nitrogen was utilised during construction phase instead of the production phase.

One important aspect of improving NUE is to reduce nitrogen losses. Most nitrogen losses from anaerobic digestate occur within the first month of application (Tiwary *et al.*, 2015). In the polytunnel experiment, neither cumulative ammonia volatilisation or N_2O emissions showed any difference between the digestate and digestate with additives. Post harvest leachate in the polytunnel experiment and potential leachable nitrogen in the field experiment were no different between treatments. This indicates that the nitrogen immobilised by the addition of glycerol did remineralise within the growing season. However, the same cannot be concluded for straw addition as no immobilisation in either experiment was measured. As there was no difference in the nitrogen loss pathways measured, this is another factor that explains the lack of impact of the high organic carbon additives on the NUE of digestate.

7.3 Research contributions and opportunities for industry

7.3.1 Novelty and contribution to knowledge

The mechanism of using high organic carbon materials to stimulate microorganisms into immobilising fertiliser supplied digestate has been tested with inorganic fertilisers (De Neve *et al.*, 2004; Pittaway *et al.*, 2018; Reichel *et* *al.*, 2018; De, Sawyer and McDaniel, 2022). However, there is a lack of research testing this mechanism on immobilising nitrogen from anaerobic digestate, a gap addressed by this PhD project. This project went beyond the soil incubation proof of concept stage of the aforementioned research and investigated treatment effects on plant nitrogen uptake and yield in both plant and field experiments. It demonstrated that a mixture with a C:N ratio of 10:1 had no detrimental effect on crop properties, contributing to our knowledge on the influence of the C:N ratio in applied organic materials on plant growth. This PhD project also studied the effects on the microbial community and necromass when mixing high organic carbon materials with a nitrogen source, which was a gap unaddressed by previous research. The increase in the necromass marker muramic acid indicated that bacteria were the main group responsible for microbial nitrogen immobilisation, whilst PLFA analysis identified that these were specifically Gram-negative bacteria. Furthermore, the effects seen on necromass confirm the postulation that this research is based on: that fertiliser nitrogen immobilised in microbial biomass is transformed into necromass (Kindler *et al*., 2006), which is subsequently mineralised when plants require nitrogen and prime microorganisms to break-down soil organic matter (Meier *et al*., 2017). This contributes to our fundamental knowledge regarding the pathway that fertiliser nitrogen undergoes from biomass immobilisation to eventual remineralisation and the main microbial group involved in the process.

7.3.2 Nitrogen management in agriculture

A key challenge facing agriculture is nitrogen management and how to improve the nitrogen use efficiency of fertilisers, including digestate. This is to ensure that farmers get the most out of the applied nitrogen and reduce the negative environmental consequences of applications, such as leaching and atmospheric pollution (Nkoa, 2014). This research in combination with other researchers (De Neve *et al.*, 2004; Pittaway *et al.*, 2018; Reichel *et al.*, 2018; De, Sawyer and McDaniel, 2022) demonstrates that it is possible to use soil microorganisms to retain fertiliser-applied nitrogen in the soil, and consequently reduce the potential for losses. Using microorganisms to retain fertiliser supplied nitrogen can be done by using industrial by-products that are high in organic carbon content, such as on-farm straw or biodiesel by-produced glycerol. This PhD project focused on retaining nitrogen from digestate application and measured significant nitrogen immobilisation from the addition of glycerol and straw. As using soil microorganisms requires materials to be in contact with the soil (Aita *et al.*, 2012), it is recommended to combine this with other digestate nitrogen loss reducing techniques, such as precision application or incorporation (Tiwary *et al.*, 2015; Nicholson *et al.*, 2018) to keep as much of the supplied nitrogen in the soil as possible for crop uptake.

7.3.3 Biogas industry guidance for digestate spreading

One question that arose during the planning of the field experiment was when to apply the digestate to sugar beet. According to best practise guidelines, synthetic nitrogen fertiliser is applied to sugar beet in two splits post-sowing (AHDB, 2023). However, there is a lack of consensus on how to best apply digestate. Concerns of spreading digestate include phytotoxicity (Lencioni *et al.*, 2016) and mechanical damage (Wilson *et al.*, 2021). The field experiment demonstrated that digestate with or without additives performed equally on plant growth parameters compared to synthetic nitrogen fertiliser. The benefit of using digestate with glycerol is its quick immobilisation which has the potential to prevent early on nitrate losses before the plant is ready to take up nitrogen during canopy establishment. Therefore, this mixture is an alternative to use in place of split applications of synthetic nitrogen fertiliser to achieve the same results on sugar beet with the additional benefit of saving fuel. It could be used for other spring sown vegetable crops such as carrots or potatoes. According to the fertiliser manual RB209 (AHDB, 2023) nitrogen application to vegetable crops is usually done at seedbed preparation. However, if the soil texture is light, and therefore susceptible to leaching losses, then a split application is recommended. As with sugar beet, there would be the same difficulties on how to apply digestate post seedling emergence, which would be negated by applying digestate with glycerol. As such, this work can contribute to best-spreading advice provided by biogas companies to encourage farmers to use their digestates for fertilising a greater range of crops.

7.3.4 Soil organic carbon management in agriculture

With growing awareness of the importance of sustainable soil management to the future of farming, there is a great deal of interest in how to do so on farm. A fundamental component to sustainable soil management is managing soil organic carbon (Powlson *et al.,* 2011). However, it can take over 6 years to identify whether the management techniques used to increase soil organic carbon contents are working (Smith, 2004). Soil microbial biomass changes occur in much shorter timescales and can be used as an early indicator for changes in SOM (Brooks, 2001). A microbial biomass increase under glycerol addition to digestate was measured in the field experiment. This research outcome provides a possible route by which farmers could improve soil organic carbon stocks by using glycerol together with digestate. By using nitrogen with glycerol derived carbon, the farmer benefits in two ways; by increasing NUE, and by building soil organic carbon. This provides a route for the both the biogas and biodiesel industry to valorise their respective by-products.

7.4 Limitations

7.4.1 Soil characteristics

The soil used in the experiments was a sandy loam texture with an alkaline pH (7.7 – 8.5) bought from Bourne Amenity Ltd for the pot trails whilst the field experiment site was selected based on the same soil type and pH range. The consistency in these soil properties used was to allow comparisons to be made between experiments, without the confounding effect of different soil properties. Nitrogen dynamics are affected by soil type, with clay soils increasing mineral associated fixation (Trehan, 1996) and physical protection of nitrogen from biological activity (Hassink, 1992), whilst also creating conditions more favourable for denitrification (Yu *et al.,* 2022), which would influence the amount available to microbes and plants to utilise. Soils that are alkaline have higher ammonia volatilisation rates compared to neutral and acidic soils (Zhenghu and

Honglang, 2000) whilst acidic soils have lower nitrification rates (Pietri and Brookes, 2008). These soil properties affect the dynamics of nitrogen cycling through the soil: greater ammonia volatilisation reduces concentrations available to microorganisms to utilise whilst lower nitrification rates reduce the susceptibility of available nitrogen to leaching and denitrification losses. This limits the generalisations about treatment responses that can be taken from this project, due to the challenges of extrapolating results to soils with different characteristics.

7.4.2 Challenges in quantifying nitrogen losses

A start was made in this project to quantify and determine the effects of the high organic carbon additives on nitrogen losses from the application of anaerobic digestate. N_2O emissions were accumulated from a period where fluxes were above the detection limit. However, the differences in flux at each sampling date could not be compared between treatments due to heterogenous variation in the samples. This may have been influenced by the storage time of the vials prior to analysis as the GC was non-operational during the duration of the experiment and was eventually sent back to the manufacturer to be fixed. Therefore, an external laboratory had to be used. This meant that samples collected at the start of the experiment spent ~4 months in storage before analysis. Both Laughlin and Stevens (2003) and Faust and Liebig (2018) measured negligible change in sample concentrations when stored for a month, but beyond this concentrations declined linearly with time (Laughlin and Stevens 2003).

The second nitrogen loss pathway that there were problems quantifying was nitrate leaching during the growing period of the crop. The rapid immobilisation of nitrogen measured when glycerol was added to digestate demonstrates the potential of this combination to reduce nitrate leaching. However, this was not quantified. In the pot experiment, soil moisture was kept at a constant level, to ensure that variable moisture levels were not affecting crop growth, consequently there was no leachate collected for analysis. Soil samples were collected from the field experiment and extracted for available nitrogen as a proxy for nitrate leaching. These extracts had to be discarded after the segmented flow analyser broke down whilst processing them. Backup extracts were sent to be analysed at an external lab. However, the concentrations were below detection limits of their equipment, which was probably due to the differences between the equipment settings and extract preparation.

7.4.3 Soil organic matter decomposition priming

An underlying assumption of this work is that the nitrogen immobilised in microbial biomass due to the addition of high organic carbon materials is from the digestate. However, digestate application to soil can stimulate soil organic matter mineralisation (Abubaker, Risberg and Pell, 2012), in response to the nitrogen "priming" microorganisms to decompose soil organic matter to get carbon for growth. Therefore, it may be that some of the nitrogen immobilised in microbial biomass did not come from the applied digestate. Stable isotope analysis, for example ^{15}N labelling, would better determine the quantities of nitrogen immobilised from digestate. ^{15}N labelling would also offer insights into the gross rates of nitrogen immobilisation and mineralisation, and enable the tracking of the fate of digestate N to plant uptake, leaching or gaseous emissions. However, this was not done in this PhD due time constraints from the need for an additional growing season to label plants, then anaerobically digest the plant material to have ^{15}N labelled digestate to use on soil with crop over another growing season.

7.4.4 Comprehensive microbial analysis

Microbial analysis such as PLFA and respiration in the polytunnel and field experiments would have been beneficial. In the polytunnel experiment, a PLFA analysis would have supported the conversion of amino sugars into necromass-carbon data, since the ratio of G+:G- bacteria is recommended for doing so (Joergensen, 2018). From this an estimate of the nitrogen quantity immobilised could have been calculated. The two microbial parameters measured in the field were biomass carbon and nitrogen, but a PLFA measurement would have allowed for a comparison with previous chapters, crucially it would have shown whether the treatment effects were changed, and how, by the difference in conditions (incubation vs field) or soil property (non-calcareous vs calcareous soil). This would increase our knowledge on how the microbial community is influenced by the addition of glycerol or straw with digestate to the soil. In depth microbial community analysis using 16S or 18S rRNA would have provided a more detailed insight into the effects of co-application of digestate with carbon additives on the microbial community, enabling inferences to be made about its functioning. This would be particularly useful to understand the treatment impact on the key microbial groups and species that facilitate the delivery of nitrogen to crops such as nitrifiers. Finally soil respiration measurements would also have provided useful insights, such as how treatments influence carbon use efficiency of the microbial community. This would allow for insights into the wider climatic implications of this research. However, whilst interesting, the key objectives of both the polytunnel and field experiment was to firstly measure how the treatments influenced plant growth, and secondly to measure effects nitrogen loss pathways. Neither of these core project parameters had been investigated yet, whereas effects on microbial community had been. Therefore, the limited project resources had to be allocated accordingly.

7.5 Future research directions

7.5.1 Determine quantity and quality of organic carbon materials to impact digestate nitrogen use efficiency

The central aim of this PhD project was to test the hypothesis that the addition of organic carbon-rich materials into digestate would stimulate the immobilisation of nitrogen within microbial biomass and therefore increase the nitrogen use efficiency by crops. However, the results did not demonstrate an improvement in nitrogen use efficiency, as no impact on either nitrogen uptake by the plant or nitrogen loss pathways were measured. Whilst positive effects on microbial immobilisation of nitrogen occurred, quantities were evidently too low to make a significant difference to the agronomic performance of the plant. Therefore, future work is necessary to determine the quantity of carbon required to add with digestate to make a difference, but without causing a detriment to early growth establishment.

An aspect that needs further investigation is the quality, in terms of complexity, of the organic carbon materials and their influence on the timescale of nitrogen immobilisation and remineralisation by the microorganisms. It is evident from this work that nitrogen remineralisation from the glycerol induced microbial immobilisation occurred before the crop plants entered their first high nitrogen demand stage during canopy establishment. Whilst this is beneficial in terms of keeping nitrogen in the soil during the first months from application, the speed of remineralisation meant that plants did not benefit from any additional nitrogen released during the later production growth stage, such as grain development in cereals. When applying nitrogen at the beginning of the production stage other researchers have recorded an increase in crop yield and quality (Chiluwal *et al.*, 2021; Deng *et al.*, 2023; Giordano, Sadras and Lollato, 2023). Therefore, a need remains to explore different sources of high organic carbon materials that would act quicker than straw to immobilise digestate supplied nitrogen, but that do not remineralise nitrogen as swiftly as glycerol. Mixing together two organic carbon materials to achieve this was tested in the spring barley experiment and showed no difference in immobilisation-remineralisation dynamics compared to the respective activities when mixed singly with the digestate. As such this mechanism of mixing labile and moderately labile materials can be eliminated as a mechanism to immobilise digestate supplied nitrogen within days and for longer than a period of a few weeks.

7.5.2 Microbial community

The microbial community is fundamental to the sustainability of digestate application. The literature review identified that those studies that did investigate the effects of digestate spreading on soil microbiota usually focused on biomass whilst less attention was given to diversity, activity and community composition changes. Incorporating these parameters are necessary to gain a deeper and more precise understanding of how microbiota respond, such as to determine community functioning and resilience. Whilst searching the literature for impacts of adding organic carbon materials with a nitrogen source on microbial communities, no papers were found that specifically analysed the impact of glycerol addition on soil microbial communities. In contrast, a few studies have examined the effects of straw (e.g. Wang *et al.*, 2021; Yang *et al.*, 2022), although these did not focus on combining straw with digestate as the N source. Therefore, future work on using the digestate with or without a carbon additive needs to include an assessment of how the microbial community is affected. This assessment should include specific microbial groups and species that drive the function of nitrogen cycling that is of interest, to help understand how crop nitrogen uptake is affected. The main crop plants in the UK have different nitrogen acquisition strategies. Legumes primarily rely on symbiotic relationships with rhizobia. The majority of crops, with the exception of the brassicas, form associations with mycorrhizal fungi, which contribute to nitrogen uptake. Brassicas, such as oil seed rape, in the absence of inorganic N fertilisation, rely on nitrogen supplied by the activities of the free-living microbes via mineralisation of soil organic nitrogen or inorganic nitrogen fertilisers. Whilst legumes such as peas and beans are not fertilised with external inputs of nitrogen (AHDB, 2023), the impact of digestate with or without organic carbon additives on either the free-living microbial community or mycorrhizal fungi need to be explored, including impacts on subsequent nitrogen uptake by crops.

7.5.3 Impact of glycerol addition with digestate (or other nitrogen sources) on soil organic carbon

The discovery in the field experiment that glycerol addition with digestate could improve soil organic carbon stocks needs further research. When Alotaibi and Schoenau (2016) applied glycerol with urea they measured no impact on microbial biomass a year after application, indicating that increases are short-term. This demonstrates that repeated applications are necessary to build up concentrations of organic carbon in the soil. Yet how these applications would shape the community and development of soil organic matter would need investigation, as well as to address concerns of negative effects caused by impurities, such as salts, in raw glycerol (Tan, Aziz and Aroua, 2013), or production of anti-microbial substances from *Lactobacilli reuteri* utilisation of glycerol (Axelsson *et al*., 1989). Possible mechanisms for soil organic carbon build up could be through a combination of necromass formation (Liang *et al.*, 2019) or extracellular polymeric substance (Redmile-Gordon *et al.*, 2015) binding soil aggregates together and protecting organic carbon from decomposers. An important factor to determine would be the ratio of glycerol carbon to fertiliser nitrogen. One reason that microbes produce extracellular polymeric substances is to facilitate enzyme activity, in order to break down organic matter and get nutrients required for growth (Flemming and Wingender, 2010). Without a nitrogen source Redmile-Gordon *et al.*, (2015) measured increased production of extracellular polymeric substances, which meant microbial biomass growth was lower than when glycerol and nitrogen were co-applied. Whereas in the case of supplying glycerol with nitrogen, the microbes could use more of the supplied carbon for growth as they did not require energy expenditure to obtain nitrogen to support growth. Therefore, supplying carbon and nitrogen together could be used to build soil organic carbon, but the optimal ratio of glycerol and nitrogen to achieve this needs to be determined without compromising crop development and yield. This would mean it would need to be tailored depending on the crop, as different growing rates require nitrogen at different times. In our work the digestate and glycerol was applied at a C:N ratio of 10:1 and showed increased microbial biomass carbon with neither a detrimental or beneficial effect on crop yield. In contrast, Alotaibi and Schoenau (2016) applied urea and glycerol at a C:N ratio of 20:1 and measured a positive effect on microbial biomass carbon but a negative effect on crop growth.

7.5.4 Long term studies

The results of the literature review highlighted a lack of studies looking at impacts of repeated and legacy effects of digestate application on soil biota. In particular the effects on soil microbial communities and functioning, have not been addressed. Similarly, the effects on soil mesofauna are underexplored, with a few studies addressing soil microarthropods and nematodes (Jothi *et al.*, 2003; Xiao *et al.*, 2007; Westphal, Kücke and Heuer, 2016; Wang *et al.*, 2019; Das *et al.*, 2022). Whilst earthworms have received more attention, the effect of repeated digestate application has not been addressed, as both Rollett *et al.*, (2020) and Moinard *et al.*, (2021) measured earthworms after two or more years following the cessation of digestate application. Within this time earthworm numbers were either no different to the no fertilised control or higher. However, it is important to determine the frequency of digestate applications in relation to life cycle duration, as Pommeresche, Løes and Torp (2017) observed that springtail abundance recovered within a season after an initial decrease due to digestate application, because of the short length of their generation time. However, earthworm generation time varies between 2 – 24 months depending on the species (Lavelle and Spain, 2001). As yet no study has been conducted to test earthworm population dynamics over several years of annually repeated digestate application. It is recommended, therefore, that long term experiments are set up to understand the impact of repeated digestate applications on soil biology, and to also include soil physical and chemical parameters as these influence biology. These studies would help provide greater confidence to end-users to utilise digestate and help to identify any issues that may arise and allow timely research interventions to mitigate them.

7.6 Conclusion

This PhD project produced a proof of concept that using soil microorganisms to temporarily immobilise anaerobic digestate supplied nitrogen for improved bioavailability to plants later in their growth cycle is possible. Furthermore, it demonstrated that it can be done using materials considered by-products: glycerol from biodiesel production, or straw from arable farms, which is an important consideration regarding the necessity of most effectively using planetary resources available for a sustainable future. The rate and magnitude of microbial immobilisation of digestate supplied nitrogen depends on the lability and rate of carbon added to the digestate, with a material ranging from labile to moderately labile applied at a rate of 24 kg-C m^3 of digestate being optimal under laboratory conditions. However, the significant immobilisation of nitrogen did not significantly influence on plant crop nitrogen uptake, yield or quality in following pot and field experiments. Two factors that may have influenced this are: i) the rate of carbon addition may still have been too low to make a significant difference and ii) that there was a mis-synchronicity between nitrogen remineralisation and crop nitrogen demand in the final development phase. However considering the relatively dry conditions of the field experiment, the benefit of early and rapid immobilisation measured under glycerol addition to digestate to reduce nitrate losses following heavy rainfall did not occur. Therefore, future works needs to focus on determining the optimal rate and type of carbon that will work under field conditions, as well as better understand the mechanisms at work to more effectively use this process to retain digestate nitrogen in the soil under field conditions. By demonstrating that adding carbon to digestate does reduce nitrogen losses with the additional benefit of building soil health, it will make digestate a more attractive option for farmers to use as a fertiliser, and therefore provide the biogas industry with a reliable end-user to utilise their digestate.

7.7 References

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Appendix A Ethical approval letter

26 November 2020

Dear Miss Van Midden ,

Reference: CURES/12456/2020

Title: Using organic fertilisers to manipulate soil microbiology for improved nutrient bioavailability

Thank you for your application to the Cranfield University Research Ethics System (CURES).

We are pleased to inform you your CURES application, reference CURES/12456/2020 has been reviewed. You may now proceed with the research activities you have sought approval for.

If you have any queries, please contact CURES Support.

We wish you every success with your project.

Regards, CURES Team

Appendix B Title change approved

07 September 2024

Dear Miss Van Midden ,

Reference: CURES/23654/2024 Project ID: 13433

Title: Using high organic carbon materials to manipulate soil microbiology for improved nitrogen bioavailability from anaerobic digestate

Thank you for your application to the Cranfield University Research Ethics System (CURES).

We are pleased to inform you your project title change request, reference CURES/23654/2024 has been approved.

If you have any queries, please contact CURES Support.

Regards, CURES Team