Exploring the environmental impacts associated with anaerobic digestate application to grassland soils

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To my family, who has nourished my passion for nature and agriculture since I was a child

“Dai diamanti non nasce niente, dal letame nascono i fior”

“Nothing grows from diamonds, from manure you can grow flowers”

-Fabrizio De Andrè-

Declaration

This thesis has not been submitted in support of an application for another degree at this or any other university. It is the result of my own work and includes nothing that is the outcome of work done in collaboration except where specifically indicated. Many of the ideas in this thesis were the product of discussion with my supervisors: Dr. Ben Surridge, Prof. Kirk Semple (Lancaster University) and Dr. Marc Stutter (The James Hutton Institute).

This thesis word length is 58951 (excluding table legends, figure captions, and reference lists) and therefore does not exceed the permitted maximum.

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Statement of Authorship

This thesis has been prepared in the alternative format, as a set of three papers presented in Chapters 3 - 5, with Chapter 3 published and Chapters 4 - 5 intended for submission to peer-reviewed journals. These chapters have co-authors in addition to my supervisory team. Please find below details of these publications with information regarding my contributions using the CRediT taxonomy, as certified by the signatures of all co-authors. Chapters 1 - 2 and 6 are introductory and discussion chapters and are not intended for submission to journals for publication.

Chapter 3 Published in *European Journal of Soil Science*

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MC was responsible for conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing-original draft; writing-review & editing.

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MC was responsible for conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing-original draft; writing-review & editing.

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Marta Cattin, Marc Stutter, Kirk T Semple and Ben W.J. Surridge. Nutrient leaching after the application of different anaerobic digestate fractions to soils with contrasting nutrient availability.

MC was responsible for conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing-original draft; writing-review & editing.

Yours sincerely,

Marta Cattin

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Abstract

Optimising the application of digestate within intensive agriculture has the potential to reduce the synthesis and use of inorganic fertilisers. Decoupling future agricultural production from a reliance on inorganic fertiliser would bring a wide range of economic, environmental and geopolitical benefits, especially since the global demand for food is predicted to rise sharply by 2050. However, there remain many uncertainties surrounding the environmental impact of digestate usage in agriculture, especially after application of different physical fractions of digestate to land (whole [WD], liquid [LD] and solid [SD]). These include effects of digestate on soil microbial communities, greenhouse gas (GHG) emissions and the leaching of nutrients to the subsurface. In this context, the thesis aimed to evaluate environmental impacts of different digestate fractions using a range of laboratory and field experimental approaches, focussing on: a) the soil microbial community composition and carbon use efficiency (CUE); b) GHG emissions and how digestate application rates can affect GHG emissions; and c) the export of multiple phosphorus (P) and nitrogen (N) fractions via leaching. All digestate fractions increased GHG emissions from grassland soils compared to control treatments, especially when application rates of LD and WD fractions were increased. However, the application of SD positively increased the soil fungal and bacterial biomass, resulting in a positive CUE when compared to WD. Surface application of WD and SD to grassland soil significantly increased the leaching of a range of P fractions, compared to both control and LD treatments, although all digestate fractions reduced the leaching of N compared to inorganic fertiliser treatments. The results

reported in this thesis highlight the need to plan the application of different fractions of digestate to land carefully, following best agricultural practices to minimise adverse environmental impacts, improve a broad range of soil health parameters and, ultimately, derive maximum agronomic benefit from the return of digestate to land.

Keywords: carbon use efficiency, digestate, grassland soil, greenhouse gases, leaching

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"The PhD is an emotional and a life trial, when you are done with it you are like a phoenix that rises from the ashes". This was one of the sentences that struck me the most when I started in 2017, something that no one can understand until you have done a PhD.

During the PhD, you push yourself to the limit, you are emotionally and intellectually invested in your research and you discover your true self. People think you must be a genius to do a PhD and of course, you have to possess some kind of intellectual capability to do one, but the real secrets are: PERSEVERANCE and PATIENCE. The PhD is the emblem of the emotional roller-coaster, a moment you laugh, the next moment you cry like a fountain and you want to quit. If I could write some rules on "how not to quit your PhD" it will probably be like this:

- Surround yourself with people that see "something" in you. In this category I will probably put my funding body, Natural Environment Research Council through the Soils Training and Research Studentships Centre for Doctoral Training (STARS CDT), which during a summer day in 2017 gave the opportunity to a scared Italian au-pair to live a dream. Especially, thank you Olivia Lawrenson for putting up with all the questions I had!
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List of Abbreviations and Acronyms

%	percentage
±	plus or minus
°C	degree Celsius (unit of temperature)
µg	micrograms
µm	micrometers
AD	anaerobic digestion
Al	aluminium
As	arsenic
B	boron
C	carbon
c.	circa
C:N	total carbon to nitrogen ratio
Ca	calcium
Cd	cadmium
CH_4	methane

Cl	chlorine
C_{micro}	microbial biomass carbon
CO_2	carbon dioxide
C_{org}	organic carbon
Cr	chrome
Cu	copper
CUE	carbon use efficiency
d	day
DM	dry matter
DRP	dissolved reactive phosphorus
DUP	dissolved unreactive phosphorus
DW	dry weight
e.g.	for example
Fe	iron
FOM	fresh organic matter
GHGs	greenhouse gases

GWP	global warming potential
h	hour
H_2S	hydrogen sulphide
ha	hectar
hPa	hectopascal
K	potassium
K_2SO_4	potassium persulfate
KCl	potassium chloride
kg	kilogram
Mg	magnesium
mg L^{-1}	milligrams per liter
Mn	Manganese
Mo	molybdenum
N	nitrogen
N:P	nitrogen to phosphorus ratio
N_2	nitrogen gas

N_2O	nitrous oxide
NH_3	ammonia
NH_4^+	ammonium
Ni	nickel
NO_2^-	nitrite
NO_3^-	nitrate
N_{org}	organic nitrogen
OM	organic matter
P	phosphorus
PE	priming effect
pH	potential for hydrogen
P_{in}	inorganic phosphorus
PLFAs	phospholipids fatty acid
PO_4^{3-}	orthophosphate
P_{org}	organic phosphorus
ppm	part(s) per million

S	sulphur
Σ	sum
SOC	soil organic carbon
SOM	soil organic matter
SOP	soluble organic phosphorus
TC	total carbon
TDP	total dissolved phosphorus
TN	total nitrogen
TOC	total organic carbon
TP	total phosphorus
TS	total solids
UK	United Kingdom
w/v	weight to volume
WHC	water holding capacity
y	year
Zn	zinc

1 Introduction and thesis objectives

Human population is predicted to rise to 12.5 billion by 2050 (UNDESA, 2019), alongside a growing demand for food production which could itself increase by 60-110% by 2050 (Alexandratos and Bruinsma, 2012; Tilman et al., 2011; Valin et al., 2014). Therefore, to meet the growing demand for food production without further degrading natural resources, farmers and other land managers are pressured to be more sustainable in agricultural practices and to improve their resource-use efficiency (German et al., 2016; Paul et al., 2019).

It has been estimated that the agricultural sector currently uses around 40% of the Earth's land surface. The increased conversion of land for agricultural production is associated with multiple impacts, including loss of biodiversity (Foley et al., 2011;Laurance, Sayer & Cassman, 2014; Machovina & Feeley, 2014), degradation of up to a quarter of the world's agricultural soils (Grunwald, Thompson & Boettinger, 2011), significant demand for water resources, pollution of two-thirds of the world's rivers to the point of saturation via leaching and runoff and inability to buffer further nutrient inputs (Liu et al., 2012). Moreover, it has been estimated that approximately 10-14% of total greenhouse gas (GHGs) emissions (as carbon dioxide [CO_2], methane [CH_4] and nitrous oxide [N_2O]) is derived from the agricultural sector (IPCC, 2014; Johnson et al., 2007).

Extensive land use change and land degradation are usually linked to intensive agricultural practices (e.g. intensive tillage, over-application of inorganic fertilisers,

conversion of natural habitat to intensively managed systems) (Dudley & Alexander, 2017). These practices may subsequently increase the mineralization rates of soil organic matter (SOM) within agricultural soils, thereby significantly decreasing soil C carbon (C), nitrogen (N) and phosphorus (P) stocks (Muhammed et al., 2018). In turn, these processes may directly impact soil biodiversity (e.g. bacterial, fungal and invertebrate populations), which under intensive agricultural management may decrease dramatically and impact the ecosystem services provided by the soil matrix (Tsiafouli et al., 2015). Intensive agricultural practices may also be directly linked to the emission of GHGs from the agricultural sector and are usually associated with use of fossil fuels during the running of machinery on farms, synthesis and import of inorganic fertilisers, particularly inorganic nitrogen and phosphorus fertilisers, intensive tillage, conversion of land and over-application of fertiliser to agricultural soils (Johnson et al. 2007). Further, the high mineralization rates of SOM during tillage and following conversion of land to agricultural production, coupled with high application rates of fertiliser, can increase the CO_2, CH_4 and N_2O emissions from agriculture (Kopittke et al., 2018).

Moreover, intensive agricultural production and fertiliser use can intrinsically compromise water quality through run off and erosion of soil particles and associated pollutants or leaching of nutrients into the subsurface (Li et al., 2020; Mander et al., 1998), in particular the nutrients nitrogen and phosphorus. When excess nutrient delivery to receiving waterbodies occurs, this can trigger a nutrient

imbalance and a range of adverse environmental impacts including eutrophication, algal blooms and hypoxia (Richardson, 1997; Paerl et al., 2002).

Therefore, in order to promote more sustainable agricultural production systems, new technologies and better practices should be devised and adopted by farmers and other land managers (IPCC, 2014). These may include: a) precision farming and associated decision support systems; b) increases in water use efficiency (e.g. improving soil structure and organic matter content to reduce water loss); c) reducing tillage; d) using more diverse crop rotation (e.g. cereal-legumes intercropping); e) implementing cover cropping (e.g. during winter to reduce runoff, soil erosion and export of excess nutrients); f) increasing nutrient use efficiency of crops (e.g. applying only the amount of fertiliser needed by crops); and g) reducing the use of inorganic fertilisers and increasing the use of new fertilisers from recycled nutrients (e.g. crop residues, compost, manure, slurry, digestate and biochar) (Paul et al., 2019; Gomiero et al., 2011). In particular, the use of organic matter-based fertiliser materials (hereby termed organic fertilisers) has many potential advantages over inorganic fertiliser use, since many organic materials (e.g. manure, slurry and digestate) are produced on-farm (e.g. animal and anaerobic digestion by-products) and are relatively cheap compared with inorganic fertilisers (RB209). Additionally, the application of organic materials to land has been shown to improve soil structure and organic matter (OM) content, increase key soil biological parameters such as microbial biomass and the size of the fungal population, can

provide available nutrients for plant uptake and can increase C sequestration into plant biomass (Dalorima et al., 2021).

However, there are still significant uncertainties and challenges surrounding the use of organic materials in agriculture, particularly because the nutrient composition of these materials can be highly variable (Lukehurst et al., 2010). In addition, the stoichiometry of these materials is often unbalanced compared to soil or crop requirements, meaning that application rates required to reach a specific N target may often lead to an excessive application of other nutrients, especially P (Fuentes et al., 2006; Sharpley and Moyer, 2000). Furthermore, farmers and other land managers still often consider these organic materials as a waste product rather than as a resource. This may result in over-application to land, particularly when application of these materials occurs in conjunction with inorganic fertilisers, and often the preferred method of application for farmers is splash-plate rather than injection, which increases the risk of water and air pollution, for example through the emission of ammonia (NH_3), CH_4 and N_2O during application (Loyon, 2018; RB209).

One potential alternative to reduce the volume of organic waste generated by farms and consequently reduce the environmental risks associated with managing these wastes, is the conversion of these wastes into material for energy production (Foster et al., 2021), such as during anaerobic digestion (AD) which is a well-established process (Anukamet al., 2019). The AD process occurs inside an anaerobic digester under anoxic conditions, leading to the production of a biogas

comprised of CH_4 (55-65%), CO_2 (35-45%), N_2O, and small amount of NH_3 and hydrogen sulfide (H_2S) (Al Seadi et al., 2008; Kaparaju et al., 2013; Insam et al., 2015). In particular, CH_4 produced during the AD process can be used as natural gas or converted into heat and electricity through a co-generator (Di Bernardo et al., 2019), whilst the H_2 produced can be used as an alternative fuel (Zappi et al., 2021). During the AD process, different types of material can be used as feedstocks to support biogas production, including waste materials from some forms of agricultural production including livestock slurry, manure, food waste and crop residues. The final residue, or digestate, from the AD process has the potential to be returned to land as a fertiliser and soil improver (Korhonen et al., 2018; Korhonen and Niutanen, 2004). Therefore, the AD process is not only a potential solution to the challenge of managing agricultural waste products and deriving value from these products via the generation of biogas, but it also supports a circular economy model if digestate is appropriately returned to agricultural soils, which can bring several advantages to modern farming systems. However, the potential environmental risks and benefits following the application of digestate to land have not been fully explored to date. This thesis focusses on investigating these potential environmental risks and potential positive benefits following digestate application to agricultural grassland soils, in the context of building an evidence base that supports optimised use of digestate in UK agriculture. In particular, the impacts of digestate application on soil microbial communities, soil C stocks, GHG emissions and leaching of nutrients remains subject to significant debate, with highly variable

results often found in past research literature (Moller, 2015), and provide key foci for the thesis.

1.1 Soil organic matter (SOM) and the soil microbial community

The application of organic materials in agriculture has the potential to improve soil structure and increase the SOM content (Dhaliwal et al., 2019). Usually, SOM is composed of 10-40% microorganisms and 40-60% stable organic matter (humus) which is considered a C sink (FAO, 2005). Together, humus and microorganisms are involved in key processes within soils, including binding soil particles into larger aggregates, improving soil structure and increasing the resistance of soil to erosion (Herrick and Wander, 1997). Applying organic materials to agricultural soil can stimulate the activity of bacteria and fungi present in the soil community, through the processes of decomposition and mineralization. For example, during decomposition microorganisms metabolise the OM supplied with the organic amendment through processes of anabolism and catabolism. During the process of anabolism new cellular material is formed by heterotrophs from simple organic compounds found in organic material (e.g. digestate), consequently increasing soil microbial biomass (Geyer et al., 2016). During the catabolic process, the organic C compounds applied with the organic material are used by bacteria for maintenance respiration (which is required to hold the size of the community constant) rather than biosynthesis, consequently increasing CO_2 effluxes (Manzoni et al., 2012).

The process of mineralisation of complex organic compounds may also return essential micro- and macro-nutrients to soil, in forms that are bioavailable for subsequent plant or microbial uptake. However, organic materials high in lignin and aromatic compounds are not completely decomposed by soil microorganisms and can be incorporated into the soil to form humus with high C stability, representing a precursor of soil formation, soil organic carbon (SOC) storage and soil stability (Rumpel et al., 2011; Stevenson & Cole, 1999). Therefore, soil formation and in particular SOM formation is important in agriculture because it is a potential sink of macro (e.g. C, N and P) and micro (e.g. zinc [Zn], copper [Cu], iron [Fe] and manganese [Mn]) nutrients in soil (Rengel et al., 1999), which are essential for crop growth (Falkowski et al., 2000).

Application of digestate in agriculture can directly supply macro- and micro-nutrients for crop growth and health, in addition to the mineralization of OM applied with digestate which can be subsequently release bioavailable forms of these nutrients (Barampouti et al., 2020). Additionally, the recalcitrant compounds present in digestate (e.g. lignin and cutin) can be used as a precursor of soil formation and increase the SOM stock. However, the OM applied during the return of digestate to land can contain simple or more complex C compounds and therefore could stimulate microbial anabolism/catabolism differently and, consequently, exert varying control on CO_2 effluxes to the atmosphere from soil bacterial respiration and SOC stocks (Reuland et al., 2021).

1.2 Greenhouse gas emissions from agricultural soils

The agricultural sector has been identified as one of the main producers of GHG effluxes into the atmosphere (Paustian et al., 2016), accounting for 10–14% of total global GHG emissions (IPCC, 2014). Of this 10-14% of total global emissions, 50–60% of the emissions are estimated to be related to N_2O and CH_4 directly linked with agricultural soils and inputs to these soils of manure/slurry and other organic amendments (Shakoor et al., 2020b). The Intergovernmental Panel on Climate Change (IPCC) provides guidelines for countries to estimate N_2O emissions along with other GHGs for reporting in their national inventories. The default emission factor for CO_2 emission after urea application is 20%, which is equivalent to the carbon content of urea on an atomic weight basis (IPCC, 2006), whilst the CO_2 emission from digestate (or livestock manure) are not included in the national inventories, since the net CO_2 emission is supposed to be zero and plants, through photosynthesis, can reduce the additional CO_2 respired (IPCC, 2006). As well as CO_2, soil CH_4 emissions are not included in the standard IPCC methodologies (IPCC, 2006) probably because in the long term, soil becomes a CH_4 sink.

Regarding N_2O emissions after organic amendment applications, the guidelines provide three “Tiers”, where Tier 1 uses global default emission factors (EFs), Tier 2 uses stratified or country-specific EFs, and Tier 3 uses measurements or complex modelling approaches requiring process-specific data at high resolutions (IPCC, 2006). An EF expresses the emission released by a specific and standard quantity of activity or input (Mathivanan et al., 2021). In the case of direct N_2O emissions from

N inputs to agricultural soils, the emission factor is the proportion of tot N input emitted as N_2O. Tier 1 (the lowest tier) uses a default emission factor derived from global N_2O measurements (IPCC, 2019, IPCC, 2006). The progression from lower to higher tiers increases the accuracy and often reduces the uncertainty in estimating N_2O emissions and is therefore recommended by the IPCC guidelines. In 2006, the IPCC published a Tier 1 EF for N_2O as 1% (as % of tot N input), whilst in the revised guidelines the EF where distinguished between "wet" and "dry" climates and within wet climates between synthetic fertilisers and other, i.e. organic or mixed N inputs (IPCC, 2019). This Tier 1 approach increases EF for synthetic fertilisers (as synthetic fertiliser, and fertiliser mixtures that include both synthetic and organic forms of N) in wet climate to 1.6% (1.3–1.9%) and decreases it for other N inputs (as organic amendments, animal manures, e.g. slurries, digested manures, N in crop residues and mineralised N from soil organic matter decomposition) to 0.6% (0.1–1.1%). However, Zhou et al. (2017) reported a much higher overall mean N_2O EF for manure application of 1.83% and noted that the EF for manure was on approximately 33% greater than that for manufactured N fertilizer. Additionally, Charles et al. (2017) identified three groups of organic materials with similar N_2O EFs: the high-risk group included animal slurries, waste waters and biosolids (mean EF 1.21%); the medium-risk group included solid manure, composts + fertilizers, and crop residues + fertilizers (mean EF 0.35%); and the low-risk group included composts, crop residues, paper mill sludge and pellets (mean EF 0.02%). The authors recommended that EFs should be site-specific and should account for organic material composition, soil characteristics, climate conditions, and whether the organic

amendment is applied alone or in combination with manufactured N fertilizers. Therefore, Thorman et al. (2020) introduced the concept that the IPCC Tier 1 values may be not appropriate for UK climatic conditions thus a Tier 2 approach, where country specific manure EFs are estimated, would be more appropriate. The authors reported that the mean direct N_2O EF for manures (e.g. pig slurry, cattle slurry, cattle farmyard manure [FYM], pig FYM, poultry layer manure, and broiler litter) applied to soils is comparable to the Tier 1 EF for wet climates (0.60%), with the variability driven by a range of factors including differences in manure composition, application method, incorporation and climatic conditions.

However, a further wide range of activity in agriculture potentially contributes to GHGs emission, although usually: i) CO_2 emissions from the agricultural sector are associated with synthesis of inorganic fertiliser and use of fossil fuels during the running of machinery on farms; ii) CH_4 emissions are linked to enteric fermentation from livestock, soil anoxic conditions (e.g. paddy rice fields), incorrect storage and land application of organic materials; and iii) N_2O emissions are associated with nitrification/denitrification processes after application of organic materials to land and incorrect manure storage (Rotz, 2018).

The use and management of digestate in agriculture can potentially influence GHG emissions into the atmosphere and consequently increase the environmental impacts associated with this sector (Rosace et al., 2020; RB209). Storage of digestate in uncovered lagoons can, in the short term at least, increase the emission of NH_3/CO_2, whilst in the medium to long term (due to crusting of the surface during

storage), anaerobic conditions can be created leading to CH_4 and N_2O emissions (Kulling et al., 2003; Amon et al., 2006). Covering the lagoons during digestate storage can reduce the emission of GHGs to the atmosphere and potentially re-introduce the CH_4 produced into the system and increase the AD plant revenue (Gioelli et al., 2011). Furthermore, land application of digestate can influence the GHG emissions due to: i) direct release of native CO_2, CH_4 and N_2O contained in digestate during spreading (Maucieri et al., 2016; Pezzolla et al., 2012); or ii) subsequent interaction between components of digestate and soil microorganisms, leading to GHG production. During decomposition of digestate by soil microorganisms the CO_2 emitted through respiration can be enhanced or, depending on the O_2 present in the soil and the level of anaerobic microsites formed, methanogens can produce CH_4, or nitrifying/denitrifying bacteria can increase N_2O production (WRAP, 2016; Dietrich et al., 2020). However, rates of GHGs emission after digestate application are highly dependent on soil structure, aeration, water infiltration, pH and the physio-chemical composition of the digestate applied, factors which can deeply influence microbial activity in soils (Firestone et al., 1989; Conrad, 2020, de la Fuente et al., 2013).

1.3 Leaching of nutrients from the agricultural sector

Whilst organic materials such as manure and slurry are now widely used in agriculture, due to their fertiliser and soil amendment properties (RB209), incorrect application of these materials may increase the risk of nutrient export through

runoff or leaching and, consequently, adverse environmental impacts (WRAP, 2016). Historically, concern has focussed on N loss to groundwaters, in particular leaching of NO_3^--N, which can threaten surface- and ground-water quality and present a high risk to human health (Lord & Athony, 2002; Schroeder et al., 2004) via associations between high concentrations of NO_3^- in drinking water and so-called blue baby syndrome in infants (McMckague et al., 2017) or livestock (Sandstedt 1990; Amdur et al. 1991). In contrast, P export to surface waters has been a focus for much research in agricultural systems, due primarily to the control exerted by P on the trophic status of surface waters and the associated risk of eutrophication (e.g. Yin et al., 2017, Awual et al., 2008, Blaney et al., 2007).

The leaching of nutrients from soil is defined as "the downward movement of dissolved nutrients in the soil profile with percolating water" (Lehmann & Schroth, 2009). Climate alongside soil physical and hydraulic properties exert important control on the risk of pollutants leaching from agricultural soil. For example, heavy rainfall events during which soil moisture levels exceed field capacity increase the risk of pollutant leaching to the subsurface (Surridge et al., 2012). However, during drought events, soil cracking, macropore formation and preferential flows may also have major impacts on the transport of nutrients to groundwater via leaching (Havlin et al., 1999). The risk of NO_3^--N leaching to groundwater from agricultural soils has been well-recognised in previous research, in particular due to the negative change of the NO_3^- molecule which results in minimal sorption to soil or the unsaturated zone matrix (e.g. Espinoza et al., 2013). In contrast, the leaching of P

and specifically the orthophosphate ion (PO_4^{3-}) is often assumed to be negligible. This may be due to the recognised strong sorption of this ion within agricultural soils (Fuentes et al., 2006). However, some recent research has begun to highlight the potential for P export via leaching, whether due to an accumulation of P within agricultural soils which exceeds the sorption capacity of these soils (Heckrath et al., 1995; Maguire & Sim, 2002; Nair, 2014), or due to geochemical conditions, such as anoxia, which can promote the mobility of orthophosphate in solution (Stutter et al., 2012; Haygarth and Turner, 2000; Surridge et al., 2007).

Digestate application to soil can supply available N and P to crops, although digestate is often applied following a N target (RB209) and due to its unbalanced total N:P, this can potentially lead to an over application of P to land (WRAP, 2016). Quantification of N and P export from land after digestate application are still relatively limited, although the present literature suggests that, based on the digestate physio-chemical composition and soil characteristics, digestate could lead to increased risk of P and N losses through run-off and leaching (Nicholson et al., 2017; García-Albacete et al., 2014, 2016; Koch et al., 2019).

1.4 UK policy on application of organic materials to land: sustainable farming incentives and farming rules for water

Political and socio-economic concerns regarding the intensification of UK agriculture and the associated environmental impact on water bodies, reduction of soil fertility and ecosystem services have led UK governance organisations to

introduce new payment schemes which will allow farmers to be paid to provide public goods (DEFRA, 2022a). The Sustainable Farming Incentive (SFI) is the first of three new environmental schemes being introduced under the Agricultural Transition Plan (DEFRA, 2020). The SFI aims to support farmers to sustainably mange agricultural land and improve food production, meanwhile reducing water pollution, increasing biodiversity, mitigating the effects of climate change and improving animal health and welfare. Moreover, the SFI strongly encourages farmers to look after soil health, by introducing two different payment schemes (introductory and intermediate levels, £22/ha and £40/ha, respectively) which reflect the level of soil management to be adopted on their land (DEFRA, 2022b). Each level consists of four actions, which aim to incentivise farmers to: a) complete a soil assessment and produce a soil management plan (e.g. assess the soil type, texture, structure and biology); b) test soil organic matter every 5 years; c) add organic matter to all land in the standard agreement at least once during the 3-year SFI standards agreement (e.g. organic manures, incorporate straw, introduce grass or herbal leys into an arable rotation); d) keep the ground covered over winter (e.g. winter cereals, cover crops, leaving weedy stubbles).

Despite the fact that farmers can be paid to maintain their soil health to a high standard, they also have to make sure their farming practices are compliant to a set of specific rules which aim to avoid pollution of water courses by the agricultural sector (Franklin et al., 2021). These rules are based on the foundation of the Water Framework Directive (WFD, DEFRA 2014) which aims to provide 'good' status for all

waters throughout Europe, by agreeing on specific management plans that are required to achieve 'good' water quality objectives for each river basin district (McDowell et al., 2016). In order to meet 'good' status and preserve the UK waterbodies from farm pollution, in April 2018 the UK government (DEFRA, 2018) introduced the 'farming rules for water' (FRfW), a list of 8 rules which in essence require farmers to: i) keep soil on the land by reducing runoff and soil erosion; ii) match nutrients to crop, and soil needs, by the correct creation of a nutrient management plan and considering the soil nutrient status and the crop need; and iii) keep livestock fertilisers and manures out of water by correct storage and correct livestock positioning in the field. The FRfW also require farmers to assess any significant risk of agricultural diffuse pollution by assessing the soil P index via soil testing from the last five years, in order to reduce P leaching and runoff, coupled with the assessment of possible nitrate leaching during application of organic materials to land, which is based on the readily available nitrogen (RAN) content of organic manures. Materials with RAN below 30% are considered low risk materials and can be applied throughout the year, whilst materials with RAN above 30% are considered high risk materials and can be spread only during a restricted period (Table 1.1)

Table 1.1 Application period allowed for organic materials with RAN above 30% (adapted from DEFRA, 2022d)

Soil type	Grassland	Tillage land
Sandy or shallow soil	1 September to the end of February	1 August to the end of February
All other soils	15 October to the end of February	1 October to the end of February

However, further restrictions are in place in areas that are classified as Nitrate Vulnerable Zones (NVZ). These NVZs are based on waters containing, or likely to contain in the future, more than 50mg/l of nitrates and almost 55% of land in England falls within an NVZ designation which is reviewed every 4 years (DEFRA, 2021). The rules that apply to an NVZ area highlight the correct use of N fertiliser and are regulated by the Environment Agency. The maximum N load from livestock manure with high RAN (including manure deposited directly by livestock and spreading) on an NVZ area is 170 kg ha^{-1} y^{-1} and can be subject to grassland derogation if the livestock manure produced on a farm is likely to exceed the amount allowed (DEFRA, 2022c); this derogation enables spreading of 250 kg ha^{-1} y^{-1} if the nitrogen comes from grazing livestock manure (e.g. sheep, cattle, deer, goats, horses). The application of organic materials to an NVZ area is subject to a restricted period (Table 1.2).

Table 1.2 Application period allowed for organic materials in NVZ areas (adapted from DEFRA, 2022d)

Soil type	On grassland	On tillage land
Sandy or shallow soils	1 Sep to 31 Dec	1 Aug to 31 Dec
All other soils	15 Oct to 31 Jan	1 Oct to 31 Jan

1.5 Thesis aim, objectives and structure

The aim of this thesis is to provide new research evidence to support improved utilisation of digestate from AD within intensive grassland production systems. More specifically, the thesis focusses on understanding how the application of digestate to agricultural soils influences: soil C stocks and the soil microbial community; the emission of key GHGs from grassland soil; and the risk of nutrient leaching from agricultural grasslands.

In order to achieve the aim of the thesis, the following five chapters and associated objectives were developed:

• **Chapter 2.** The objective of this literature review chapter was to review and provide a synthesis of: i) current knowledge relating to the anaerobic digestion process, digestate quality and utilization of digestate in agriculture; ii) the effect of the application of digestate on the soil C cycle and microbial activity; iii) the effect of the application of digestate on GHG emissions from agricultural soil; and iv) the effect of the application of digestate on nutrient leaching from agricultural soil.

• **Chapter 3.** The objectives of this experimental chapter were to determine: i) how whole and solid digestate application to grassland soils influenced soil microbial metabolism and community composition; and ii) how soil nutrient status influenced the soil C cycle, microbial activity (as carbon use efficiency) and community composition after whole and solid digestate application. To address these objectives, a 21-day microcosm experiment was conducted in the laboratory,

involving the application of two physical fractions of digestate to two grassland soils of contrasting initial nutrient status.

• **Chapter 4.** The objectives of this experimental chapter were to determine how the efflux of the key GHGs methane, carbon dioxide and nitrous oxide from grassland soils were influenced by: i) the application rate of digestate; ii) the physical fraction of digestate applied (whole, solid and liquid); and iii) initial soil nutrient status. To address these objectives, two individual, 7-day microcosm experiments were conducted using identical soils and digestate sources to those utilised in Chapter 4. These experiments included a range of GHG measurements and measurements of soil physico-chemical and geochemical properties to support interpretation of the GHG data.

• **Chapter 5.** The objectives of this final experimental chapter were to determine how: i) the application of different physical fractions of digestate (whole, solid and liquid) influenced the concentration of a range of potential pollutants (N and P) in leachate from agricultural grassland soils, and ii) the concentration of N and P found in leachate after digestate application was influenced by initial soil nutrient status. Based on a field experiment using large, intact cores of the same soil types used in Chapters 3 and 4, leachate derived from cores following multiple digestate applications and artificial rainfall events was sampled and analysed to characterise a wide range of N and P fractions. Further analyses of soil and grass samples at the end of the experiment were used to help understand the broader impacts of digestate application on the grassland system.

• **Chapter 6.** The objectives of this final chapter in the thesis were to provide a broader synthesis and discussion of the outcomes of the primary research chapters reported earlier in the thesis, to consider the potential practical implications of the research outcomes for the management of digestate in agriculture, and to examine future research needs in the broad context of this thesis.

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2 Digestate application to grassland soil: potential impacts on soil nutrient cycling and soil microbial communities

2.1 Agronomic and fertiliser properties of digestate

During the AD process, different types of material may be used as feedstocks for energy production. The range of potential feedstocks is diverse, including energy crops, agricultural waste products, animal by-products (e.g. manure and slurry), municipal bio-waste, inmdustrial and wastewater wastes (Steffen et al., 1998). Usually, different types of feedstock are co-digested together (e.g. manure and plant residues), since co-digestion can increase the CH_4 yield within biogas (Al Seadi et al., 2008). However, the quality of digestate, including the nutrient composition, physical homogeneity, viscosity and biodegradability, vary considerably among digestates derived from different types of feedstock (Barampouti et al., 2020). Feedstocks containing organic materials that are rich in sugar, starch, proteins and fat, components that are easily digested during the AD process (Al Seadi et al., 2013) and enhance the production of CH_4 within the resulting biogas, may be seen as high-quality feedstocks from the perspective of energy generation. In contrast, materials that are rich in lingo-cellulose, usually positively corrected with the age of plant residue within a feedstock, are considered more recalcitrant, because bacteria involved in the AD process digest lignin inefficiently which can limit CH_4 production within biogas (Khan & Ahring, 2019). To assess the quality of feedstock and its suitability for the AD process, basic data such as water content, organic matter or

bulky/fibrous content are used. For example, materials with a medium water content and high organic matter content are ideal for the AD process (Drosg et al., 2013). In particular, the organic matter content, pH, Tot C:N (which should be between 16:1 and 45:1 to ensure efficient performance during the AD phase) and micro/macro elements present in the feedstock play a major role in controlling the success of the initial AD process and, ultimately, digestate quality (Steffen et al., 1998).

Further important factors influencing feedstock decomposition, CH_4 production and digestate quality include temperature, the organic loading rate (OLR) and hydraulic retention time (HRT) used during an AD process (Xu et al., 2018). The organic loading is defined as the amount of organic matter (kg) entering the digester (m^3) in a day (kg day^{-1} m^3) (Labatut et al., 2018), whilst the hydraulic retention time represents the average time the feedstock remains within the reactor (Bolzonella et al., 2019). The OLR and the HRT are important for CH_4 production during the AD process, since a high OLR can increase the volatile fatty acid concentration (VFA) beyond the optimum level, which can subsequently accumulate and become toxic for methanogens, leading to a reduction of CH_4 production and, ultimately, failure of the AD plant itself (Nkuna et al., 2021). In contrast, a low HRT time can lead to an undigested feedstock and a washout of methanogens, leading to low CH_4 production and AD failure (Bolzonella et al., 2019). Therefore, a high OLR and short HRT can lead to an inefficient AD process and a digestate which contains a considerable

amount of undigested organic matter that is gradually digested during subsequent storage (Menando & Balsari, 2011).

The feedstock degradation and required retention time is also influenced by the temperature used during the AD process, which can vary between psychrophilic temperatures (below 20°C), mesophilic temperatures (between 30 to 42°C) and thermophilic temperatures (between 43 to 55°C) (Nie et al., 2021). However, the thermophilic process has shown better performances in term of pathogen degradation, shorter required retention time of material within an AD unit, improvement of digestibility and availability of feedstock, better degradation of solid feedstock and better feedstock utilisation (Kafle et al., 2014).

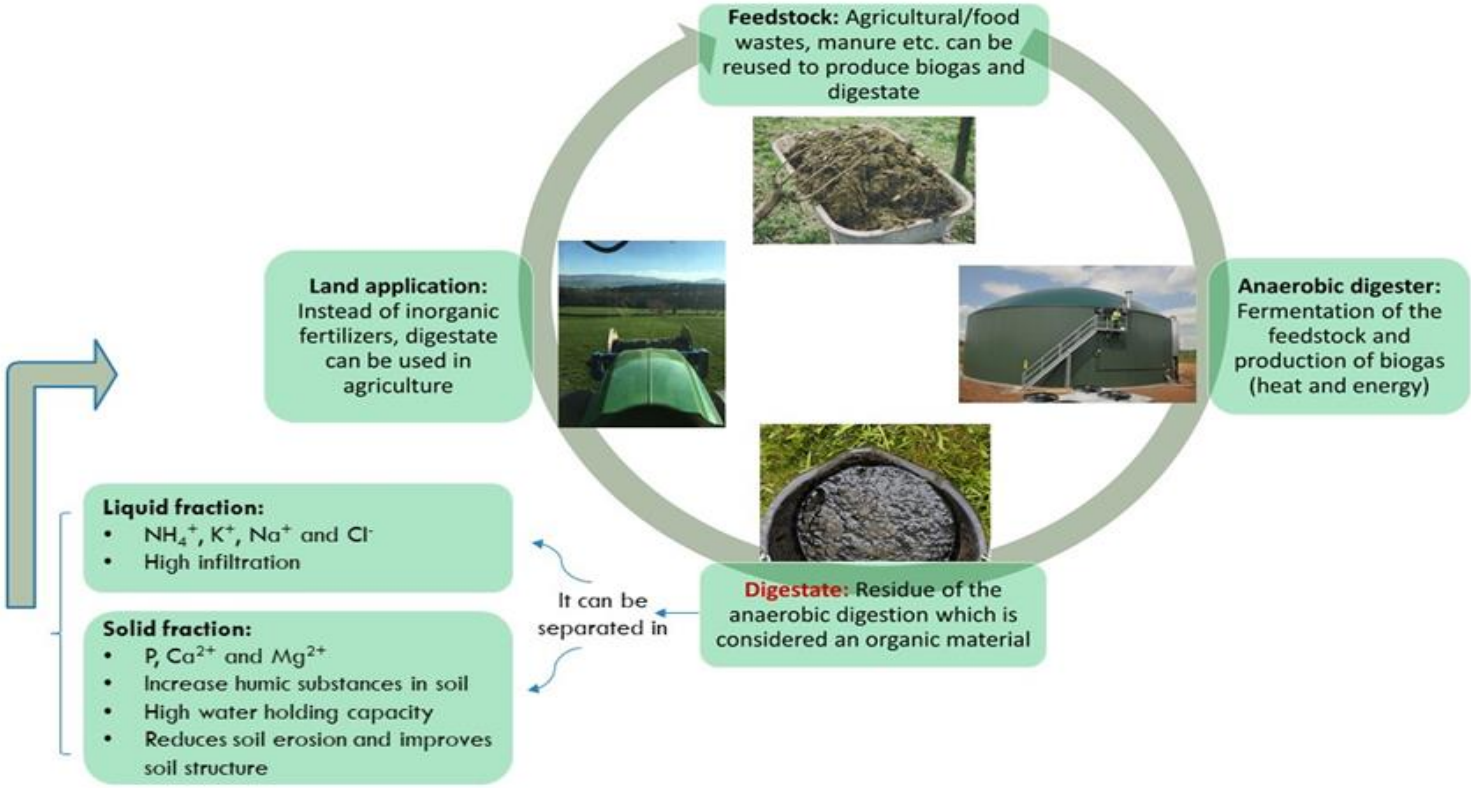

Figure 2.1 Conceptual model for increasing circularity in digestate production, starting from the feedstock/AD plant to the separation and land application of digestate

The final product of the AD process is digestate (Figure 2.1). Although the composition of digestate can be highly variable, the typical pH value is between 5.6-9, dry matter (DM) content is between 1.2-45.5%, with digestate being rich in in N, P, K and other macro- (e.g. S, Ca, Mg) and micro- (B, Cl, Cu, Fe, Mn, Mo, Ni and Zn) nutrients, often present in readily available forms for plants and crops (Tambone et al., 2010; Risberg, 2015) (Table 2.1).

Table 2.1 Typical values of whole digestate originating from known feedstock types present in the literature; the last column "digestate" represents the averaged values taken across the whole digestate from different AD plants with different feedstock types present in the literature (adapted from Barampouti et al., 2020)

	Feedstock						
Parameter	**Sewage Sludge**	**Foodwaste**	**OFMSW**	**Agricultural waste**	**Manure**	**Co-Digestion Manure + crops and/or industrial waste**	**Digestate from mixed wastes present in the literarure**
pH		7.9-8.3	8-8.3	7.5-8.4	7.3-8.6	5.6-8.3	7.5-9
DM (%)	1.9	1.4-7.88	0.72-51.2	6.41-24	2.2-9.2	1.5-24	1.5-45.7
OM (as %DM)		38-73.3	62.1-75	69-77	67.8-75	62.1-77	38-77
TN (as %DM)	0.005	0.06-1.24	0.21-7.8	0.14-2.1	0.05-0.62	0.12-5.04	0.005-5.04
Tot NH_4^+ (as %DM)		0.05-0.85	0.17-2.75	0.04-1.71	0.255-1.01	0.15-0.68	0.052-2.72
NH_4^+ (as %TN)		58-83	30-80	38-47.6	19-61	44-81	35-81
TC (as %DM)		0.44-3.56	0.20-17.72	1.92-18.48	0.59-5.07	0.41-11.6	0.41-25.2
TP (as %DM)	0.04	0.008-0.13	0.002-0.82	0.058-2.4	0.034-0.22	0.01-1	0.002-2.4
TK (as %DM)	0.00019	0.03-0.64	0.004-4	0.324-0.4	0.03-0.43	0.03-2.52	0.001-2.52
Tot Mg (as %DM)		0.0042-0.079	0.001-0.51	0.041-0.042	0.013-0.17	0.006-0.26	0.001-0.26
Mg (mg kg^{-1} DM)	<0.1						0.5-0.7
Tot Ca (as %DM)		0.014-0.41	0.036-2.56	0.077-3.1	0.044-0.85	0.01-1.56	0.01-3.1
Tot S (as %DM)		0.01-0.08	0.01-0.36	0.01-0.041	0.008-0.048	0.004-0.096	0.004-0.36
As (mg kg^{-1} DM)				28			29
Cd (mg kg^{-1} DM)	0.12	0.3-1	0.9	0.05-10	0.1-1.03	0.1-1.03	0.1-10
Cr (mg kg^{-1} DM)		6-40	6-188	0.5-55	14-364	14-364	6-364
Cu (mg kg^{-1} DM)	0.15	14-80	13-55	1-29		14-681	14-681
Mn (mg kg^{-1} DM)		0-201	<0.7		164-663	24-1100	0-1100
C:N		2.87-8.8	0.95-2.71	8.8-13.7	8.17-11.8	9.4-17.06	1.3-29.8

Dry matter (DM), Organic Fraction of Municipal Solid Waste (OFMSW), Total Nitrogen (TN), Ammonium (NH_4^+), Total ammonium (Tot NH_4^+), Total Carbon (TC), Total C-to-N ratio (C:N), Total Phosphorus (TP), Potassium (TK), Magnesium (Tot Mg), Calcium (Tot Ca) and Sulphur (Tot S), Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Manganese (Mn)

However, depending on the feedstock type (e.g. OFMSW) and type of co-digestion (e.g. co-digestated with human faeces, slaughter house residues), digestate can also contain heavy metals and pathogens (e.g. E. Coli, Salmonella spp.) that may be present at concentrations that exceed regulatory limits (BSI PAS 110, RB209). These pollutants within digestate can negatively affect soil ecosystems (Abubaker et al., 2015) and create risks for human health when digestate is used in agriculture for crop production (Nag et al., 2021), thus presenting potential limits on the use of digestate in agriculture. Whole digestate can be stored and subsequently applied to land as a form of fertiliser for crop production. However, effective partitioning of nutrients can be achieved through solid-liquid separation techniques applied to whole digestate, including sedimentation, centrifugation, drainage, or screw-press processes (Hjorth et al., 2010; Möller and Müller, 2012). After separation, individual liquid and solid fractions are produced and stored for further use in agriculture. Usually, the liquid fraction of digestate has a Tot C:N<10, is rich in K^+, Na^+, Cl^-, NH_4^+ (up to 80% of total N), contains a relatively high amount of dissolved organic C (DOC) (Marcato et al., 2008; Lukehurst et al., 2010; Bachman et al., 2016; Panuccio et al., 2016, Tambone et al., 2019) and a small amount of inorganic P (P_{in}), although these properties can vary depending on the feedstock utilised (Akhiar et al. 2017). In contrast, the solid fraction has a Tot C:N>10, is rich in recalcitrant C (e.g. lignin, cutin and humic acids) (Nkoa, 2014), it has high P_{in} (up to 90% of TP), Ca^{2+}, Mg^{2+}, S and Mn content, with only approximately 20% of the TN from whole digestate remaining in the solid fraction (Barampouti et al., 2020; Tambone et al., 2017). Thus, after solid

liquid separation, the liquid fraction is commonly used as liquid fertiliser whilst the solid fraction is often used as a soil improver (Pastorelli et al., 2021; Panuccio et al., 2019; Tambone et al., 2009) (Table 2.2).

Table 2.2 Average composition of liquid and solid fractions of digestate from different feedstocks present in the literature, reported on a wet weight basis (adapted from Barampouti et al., 2020)

	Digestate fraction	
Parameter	**Liquid**	**Solid**
pH	7.8-7.9	7.7-8.5
DM (%)	3.3-6.6	19.3-25.7
TN (as %DM)	0.32-0.51	0.33-0.65
Tot NH_4^+ (as %DM)	0.17-0.3	0.13-0.3
NH_4^+ (as %TN)	40-80	26-49.4
TC (as %DM)	2.64-3.15	9-10
TP (as %DM)	0.03-0.1	0.08-0.25
TK (as %DM)	0.29-0.52	0.25-0.48
Tot Mg (as %DM)	0.03-0.05	0.09-0.1
Tot Ca (as %DM)	0.04-0.06	0.16-0.19

Dry matter (DM), Total Nitrogen (TN), Ammonium (NH_4^+), Total ammonium (Tot NH_4^+), Total Carbon (TC), Total Phosphorus (TP), Potassium (TK), Magnesium (Tot Mg), Calcium (Tot Ca)The OM present in the solid fraction of digestate is considered biologically stable, since during AD process the easily degradable substances are used for biogas production whilst the recalcitrant compounds remain as undigested fibres (Möller, 2015). Recalcitrant compounds present in the solid fraction include lignin, cutin, humic acids, steroids and complex proteins. Cellulose/Lignin or (Cellulose+hemicellulose)/lignin ratios have been reported to indicate the degree of humification of the OM (Nkoa, 2014), since lignin and hemicellulose are possible humus precursors with high biological stability (Tambone et al., 2009). Humic substances important for soil formation are fulvic and humic acids, which contribute

to maintain soil pH buffer and cation exchange capacity. The solid fraction of digestate often has a high Tot C:N, with greater amount organic C and N present than inorganic forms of these nutrients. This high organic C content directly influences bacterial metabolic activity and slowly induces mineralization of the fresh organic matter (FOM) applied with solid fraction. During mineralization of the FOM, organic N compounds are slowly mineralized into inorganic N (N_{in}, e.g. NH_4^+ and NO_3^-) compounds, hence supplying N_{in} over the longer-term for plant uptake (Chiyoka et al., 2014). For these reasons, the solid fraction of digestate may be considered a slow release N fertiliser and a precursor for SOM formation (Crolla et al., 2013). In contrast, the liquid fraction of digestate typically has a low Tot C:N, meaning that soil microorganisms mineralize organic N faster and produce inorganic N quicker than following the application of solid digestate. However, Akhiar et al., (2017), Tambone et al., (2019) and Zheng et al. (2014) have reported that the liquid fraction of digestate still contains recalcitrant organic compounds, since this fraction of digestate has a high concentration of colloids, suspended solids and dissolved matter containing humic substances, meaning that liquid fractions of digestate can be used as a short term N- fertiliser and have the potential to be used in agriculture for increasing SOM, soil aggregate stability and microbial community size (Tambone et al., 2019). Nevertheless, this depends on the type of technique utilized during the solid-liquid separation, since different separation techniques (e.g. screw-press, centrifugation, sedimentation, pressured filtration and drainage) can result in different separation efficiencies and can alter the composition of the

resulting liquid and solid fractions, alongside the feedstock used during the AD process (Hjorth et al. 2010).

During recent years, digestate use as an organic fertiliser in agriculture has increased, since it has been estimated that using digestate to feed arable crops can save farmers up to £110/ha on bagged fertiliser costs (RB209). Whole and liquid fractions are commonly used in agriculture due to their low viscosity, whilst the solid fraction of digestate is less frequently used in agriculture due to the high DM content of solid digestate, making this fraction difficult to apply to land. Regarding whole and liquid digestate, due to their high available N content, the best application methods are shallow injection or band spreading with a trailing hose or shoe (WRAP, 2016), since during injection or band spreading less of the NH_4^+ content of digestate is lost via volatilisation as NH_3, leaving a greater proportion of the total N to be used by plants for growth or converted into NO_3^- by soil bacteria. In contrast, during splash plate application of digestate which remains a common practice in the UK, 55% of NH_4^+ is lost as NH_3 within 6h of application (RB209), resulting in both adverse environmental impacts and reductions in the agronomic benefits associated with digestate application to land.

The correct use of digestate in agriculture has been associated in past research with a wide range of potential benefits. These include increases in soil water retention, reductions in the need for herbicide/biocide use, improvements in soil structure and decreases in soil erosion (Møller et al., 2009; García-Albacete et al., 2014). For example, Garg et al. (2005) reported that, during a field experiment, after the

utilization of liquid digestate derived from agricultural waste, soil bulk density decreased from 1.47 mg cm^{-3} to 1.32 mg cm^{-3}, whilst saturated hydraulic conductivity increased from 0.52 to 0.65 cm h^{-1} and volumetric moisture content of surface soil layer at saturation increased from 42.1% to 44.1% after liquid digestate addition. Positive effects on soil properties are also confirmed by other research present in the literature (e.g. Glowacka et al., 2020; Baştabak & Koçar, 2020) where application of the whole fraction of digestate versus mineral N fertiliser increased SOM content from 5.6-15.2 to 6.4-16 g C_{org} kg^{-1} DM soil and 0.64-1.48 to 0.76-1.53 g N_{org} kg^{-1} DM soil. The fact that digestate application improved SOM is important for soil fertility and crop production, meaning that digestate can positively sustain soil functions and productivity. Further, both authors reported an increase in plant-available nutrients (e.g. P and K) after digestate application, starting from 34-81 mg kg^{-1} DM soil (P) and 51-204 mg kg^{-1} DM soil (K) and increasing up to 53-93 mg kg^{-1} DM soil (P) and 71-235 mg kg^{-1} DM soil (K). Regarding the mineral N fraction in soil (present as NH_4^++NO_3^-), digestate application increased this from 8 mg kg^{-1} DM soil to 10 mg kg^{-1} DM soil.

Moreover, it has also been observed that after the application of liquid and whole digestate fractions to land, plants are healthier (e.g. increased disease resistance), whilst the yield and quality (e.g. nutrients content) of crops can be increased (Tsachidou et al., 2018; Doyeni et al., 2021). For example, the DC-Agri project (WRAP, 2016) concluded that during spring application of whole digestate, grass yield increased by 1.1-1.5 t ha^{-1} compared with the untreated control, whilst during

autumn application of whole digestate grass yields increased by 0.4-1.5 t ha^{-1} compared with the untreated control. Additionally, this project estimated the nitrogen use efficiency (calculated based on the yield and the % of total N applied) after digestate application, which was 54% during spring and 15% during autumn. This work suggests that during spring application of digestate, the high available N content (as NH_4^+) can act as an effective replacement for inorganic fertiliser and increase crop yield and N uptake, whilst during autumn applications crop demand for N is reduced and over application can lead to N losses to the environment. In contrast, the solid fraction of digestate reaches its full potential in annual cropping systems, where it can be incorporated into a soil and contribute positively to the accumulation of OM, rather than perennial crops (e.g. grassland), since it does not infiltrate into the soil due to its mulching properties, thus it can negatively affect the crop (Ehmann et al., 2018).

It is clear that digestate application to land has the potential to substitute inorganic fertiliser application, reduce the requirements for pesticide/herbicide use by improving plant health, improve soil properties (e.g. reduce soil bulk density and increase soil aeration, increase SOM stocks) and crop yield (increase the nitrogen use efficiency) and consequently create an economic benefit for the agricultural sector. However, due to the variation in the physio-chemical properties of digestate, correct land application can be challenging, potentially leading to over application and increased soil toxicity (e.g. heavy metal accumulation and impact on soil microorganisms), pollution of groundwater of surface waters and air pollution

(Walsh et al., 2012; Moller et al., 2012). Therefore, as emphasised by Taglia et al. (2011) and Moller et al. (2015), further research should be undertaken regarding the chemical, biochemical, and biological impacts of digestate after application to land. In particular, research is required that focuses on soil C cycles and soil microbial communities, GHG emissions and risks associated with pollutant export from soils, especially after application of different fraction of digestate on agricultural land.

2.2 Bacterial stimulation and changes in the soil carbon cycle after application of different digestate fractions to land

The application of digestate to agricultural soils has the potential to deliver multiple benefits, including improving soil structure and increasing SOM content (Tambone et al., 2009). However, the application of other organic materials, including slurry or farmyard manure, to agricultural land has previously been shown to influence microbial metabolism through the degradation of freshly applied OM (FOM). The input of organic materials to soil may stimulate biosynthesis of organic C into new microbial biomass or, alternatively, may promote microbial respiration and the production of CO_2 (Figure 2.2).

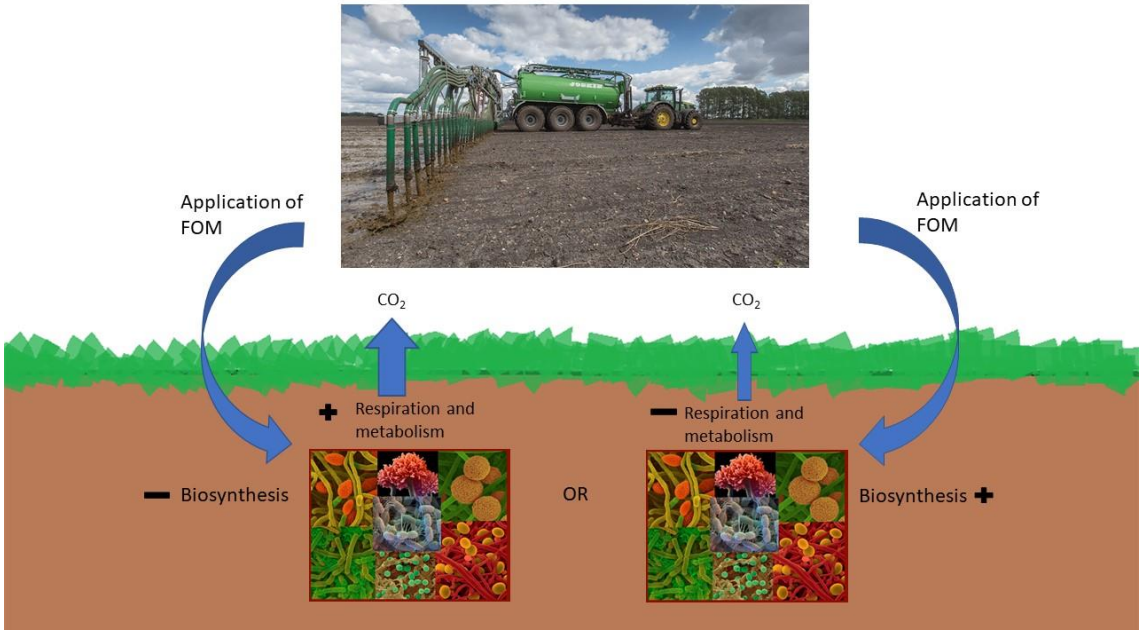

Figure 2.2 Conceptual model of microbial stimulation, CO_2 production and microbial biosynthesis after application of fresh organic matter (FOM). Increase or decrease of CO_2 production /biosynthesis is indicated with "+" or "-", arrows thickness refer to the CO_2 production into the atmosphere.

The stimulation of microbial metabolism and production of extracellular enzymes for FOM degradation, can also potentially lead to mineralization of the SOM pool (Dalenberg et al., 1989; Lipson et al., 2001), a process known as the priming effect (PE, Bingeman et al., 1953). However, the PE is strongly influenced by the C quality of the organic material applied, the soil nutrient status and the microbial biomass pool and community composition (Fontaine & Barot, 2005; Wutzler & Reichstein, 2008). For example, the application of organic materials with a high labile C fraction to a soil depleted in nutrients can stimulate microbial activity and metabolism and therefore supply enough energy to subsequently decompose the SOM and mineralise additional nutrients for use by the soil microbial community (Fontain et

al., 2003). In contrast, when FOM composed of labile C compounds is applied to a soil not depleted in nutrients, the microbial community tends to use nutrients supplied with the FOM itself, rather than mining SOM to meet the metabolic demand for nutrients. The direct determination of SOM turnover is impossible to quantify, although estimation can be achieved through the direct measurement of the C evolved from soil attributed to different microbial processes (Blagodatskaya & Kuzyakov, 2008). The estimation of C lost as CO_2 enables separation of the apparent (APE) and real (RPE) priming effect. The APE does not involve mineralisation of SOM and is mainly associated with changes in microbial biomass turnover and consequent increases of CO_2 efflux through bacterial respiration, usually lasting hours to days. The RPE involves direct decomposition of the SOM pool and consequent CO_2 release (Blagodatskaya et al., 2007; Fontain et al., 2011). Further, the PE can be positive or negative (Kuzyakov et al., 2000; Fontain et al., 2011). A positive PE occurs occur when easily degradable C compounds are added to soil and these compounds stimulate extra C decomposition in the SOM pool and N_{min} release, alongside P and other nutrients. A negative PE occurs when a reduction or immobilization into bacterial cells of the C or N added with a material occurs in the system, retarding SOM mineralization, with a consequent accumulation of C compounds in the soil.

The ability of bacteria to biosynthesize C_{org} applied with FOM, producing complex compounds that contribute to new microbial biomass, versus respiring C_{org} and releasing CO_2 may be defined as the Carbon Use Efficiency (CUE) (Manzoni et al.,

2012). A positive CUE occurs when bacteria use a C source for growth, during which the anabolic pathway is favoured and C is stored in microbial biomass (C_{micro}), with biosynthesis of C compounds into C_{micro} leading to stabilization of C in the soil and increases in the SOC and SOM pools (Wang et al., 2021; Prommer et al., 2020). In contrast, when CUE is low and/or negative, bacteria do not use a C source for anabolism and instead the catabolic pathway is favoured. This promotes high bacterial activity, which in turn can increase maintenance respiration (in which bacteria tend to maintain a given population size rather than increasing their cell growth and C assimilation), and CO_2 production, with a consequent reduction in the potential for C sequestration within soil (Geyer et al., 2016; Wang & Post, 2012; Wang et al., 2013). High bacterial activity can lead to complete utilization of the C source applied with FOM and consequent depletion of the SOM pool via mining for access to further nutrients for bacterial consumption (Dalenberg et al., 1989; Lipson et al., 2001), generating the real priming effect described above.

Multiple factors influence the CUE, for example soil temperature and moisture, quality of C source (e.g. recalcitrant C versus labile C, often described based on Tot C:N) and nutrient availability in the soil (Geyer et al., 2016). In particular, the quality of the C source present in an organic material can influence the balance between anabolic and catabolic responses within the microbial community. In the presence of recalcitrant C compounds, often defined by Tot C:N >20, bacterial catabolism increases and the production of extracellular enzymes to break down recalcitrant C compounds occurs, increasing bacterial respiration rates and the production of CO_2

(Sinsabaugh et al., 2013). In contrast, the addition of organic matter with Tot C:N<20 to soil can promote bacterial biosynthesis of C and, consequently, reduce CO_2 production (Soares & Rousk, 2019). Moreover, the CUE is directly related to whether soil is N or P limited relative to C. In a soil that is not nutrient-limited, CUE tends to increase when FOM with labile C is added, because bacteria seek to maintain a balanced intracellular composition between C and nutrients (Roller & Schmidt, 2015; Manzoni et al., 2012), reduce their respiration rates and increase their growth rates (as C_{micro}). However, when an organic material containing labile C is applied to a low-nutrient soil, for example one with high Tot C:N and, potentially, N limitation (Blagodatskaya et al., 2014; Moorhead & Sinsabaugh, 2006), bacteria tend to respire C that has been applied because maintenance respiration is increased. This is also true after application of FOM with larger or more complex molecules (e.g. recalcitrant compounds) to a low-nutrient soil, because bacteria requires the production of specific enzymes and multiple oxidation steps to degrade the recalcitrant compounds, thus bacterial catabolism increases and, consequently, so does CO_2 production (Malik et al., 2019; Sinsabaugh et al., 2009; Fierer et al., 2005, 2006). Furthermore, bacteria and fungi within the soil community have potentially different effects on CUE. For example, fungi can degrade organic material with high Tot C:N without emitting CO_2, thereby maintaining a high CUE, whilst bacteria are less efficient at degrading organic material with high Tot C:N (Blagodatskaya & Kuzyakov, 2008). Subsequently, after the Tot C:N of a material has been reduced by fungi, and in the presence of a high N source of OM in soil, bacteria may begin to act on the residues of the degraded organic material, resulting in a positive CUE, at

least within a nutrient rich soil (Geyer et al., 2016; Keiblinger et al., 2010,). Therefore, as reported by Keiblinger et al. (2010), the CUE of fungi is primarily influenced by the quantity and quality of C, whilst for bacteria CUE is influenced by the level of nutrients present in the soil, in particular N, and the quality of the C applied with FOM.

Regarding bacterial CUE, the balance between r (growth strategists; high CUE) and K (competitive strategists; low CUE) communities is a further important control (Keiblinger et al., 2010; Roller & Schmidt, 2015). During the decomposition of FOM applied to a soil that is high in available nutrients, usually the r-strategists are the first actors since they readily degrade available organic compounds present in the FOM and their reproduction rates are elevated. Subsequently, when the readily available organic compounds with the FOM have been exhausted by the r-strategists, the K-strategists degrade the remaining more recalcitrant compounds. K-strategists generally have a slower growth rate due to committing more energy to the production of extracellular enzymes and defence against predation, gradually gain competitive advantage over r-strategists and begin to degrade the remaining OM in soil via production of further extracellular enzymes (Fontain et al., 2003; Winogradzky, 1924). However, the production of these extracellular enzymes can increase the mineralization of SOM, with a consequent increase in CO_2 produced from bacterial respiration (Fontain et al., 2011, 2003; Lipson et al., 2001; Domeignoz-Horta et al., 2020; Hagerty et al., 2018). In contrast, in a soil which is relatively low in available nutrients, K-strategists dominate the soil media over r-

strategists, since r-strategists have high population turnover and require available nutrients to maintain the population size, whilst K-strategist have a lower population turnover and their optimal growth is in nutrient-poor media (Fierer et al., 2007). Therefore, an addition of FOM to a nutrient-poor soil can increase the dominance of K-strategists which out-compete r-strategists (Fontain et al., 2003).

Therefore, the addition of different fractions of digestate to soil may reasonably be expected to result in positive or negative impacts on microbial anabolism/catabolism, turnover and community composition (Mukherjee et al., 2016; Abubaker et al., 2015). A number of field and laboratory studies have reported an increase in CO_2 emission after digestate application to soil (e.g. Pezzolla et al., 2012; WRAP, 2016; Johansen et al., 2013; Maucieri et al., 2016, Table 2.3). However, in some cases the increase in CO_2 emission lasted only for a couple of hours after digestate addition to soil (e.g. Johansen et al., 2013; Maucieri et al., 2016) and was linked to bacterial respiration of the highly labile OM present in digestate. In other research, increases in CO_2 emission lasted between 60 days and 1 year after digestate application (e.g. WRAP., 2016; Pezzolla et al., 2012) suggesting that more recalcitrant C compounds present in digestate can be slowly degraded by the microbial community over significant lengths of time, or that significant quantities of CO_2 respired could be derived from bacteria utilization of native soil C.

Mukherjee et al. (2016) have introduced the concept that microbial degradation of SOM can be promoted by the addition of digestate to some soils. Interestingly, only a small number of studies have researched the effect of whole digestate application

to soils with different texture (e.g. sandy-loam and silty-loam) and OM content (Mukherjee et al., 2016; Panuccio et al., 2021). These authors report that CO_2 production was slightly higher after digestate addition to a silty-loam soil compared to the sandy-loam soil, suggesting that the beneficial effects associated with the application of digestate in agriculture (e.g. increase in C_{micro}) depend on soil characteristics rather than C loading and decomposability of the organic material applied. Furthermore, a small number of studies have investigated links between CO_2 production and changes in C_{micro} (Holatko et al., 2021; Chen et al., 2012; Cardelli et al., 2018, Table 2.3) after the application of digestate to agricultural land, compared to unamended soil or to other organic materials (e.g. farmyard manure, green manure, slurry), with these studies reporting that digestate application significantly increased C_{micro} and CO_2 emission. However, these CO_2 results are variable based on the control used, since increases in CO_2 emission after digestate application always occur when compared to an unamended soil, whilst CO_2 emissions after digestate application can be lower than green manure/slurry/farmyard manure (e.g. Chen et al., 2012; Nicholson et al., 2017) or comparable (e.g. Johansen et al., 2013).

Regarding C_{micro}, Holatko et al. (2021) and Chen et al. (2012) have reported that this parameters increased after digestate application when compared to unamended control and green manure, and the C_{micro} increase was always associated with a parallel increase in CO_2 emission. However, the increase in CO_2 emission between different digestates reported from these studies are, again, variable. For example,

the increased CO_2 emission was observed to last between 48 hours and up to several days after digestate application, primarily due to differences in digestate composition depending on the feedstock utilised during the AD process. In particular, digestate originating from food-waste had shorter-lived and more rapid increases in CO_2 emissions, because of the high labile organic compounds which are quickly utilised by bacteria for their metabolism, whilst digestate derived from more recalcitrant sources, such as agricultural wastes, contain less labile organic compounds and can sustain an increase in CO_2 production for longer periods (Panuccio et al., 2016). Changes in C_{micro} are also influenced by the degradability of a digestate, with greater increases in C_{micro} from digestate rich in easily biodegradable OM and smaller increases in C_{micro} from digestate with a more recalcitrant OM content.

Moreover, Rosace et al. (2020) suggested that the legacy of previous organic fertiliser management (e.g. soil fertilised with farmyard manure) in an agricultural soil (e.g. high C, N and P content) can adapt the microbial community to positively respond to digestate application to land. Hence, this historical context plays a major role in controlling CO_2 emissions via microbial respiration following digestate application. In this study, the CO_2 emission increased throughout 70 days of incubation after digestate application and the authors suggest that high respiration rates come from the basal respiration of an organically fertilised soil, rather than edaphic factors regarding CO_2 emissions from soil following digestate application. In soils that have a history of organic fertiliser application and with high C_{micro},

following application of FOM bacteria utilise the labile compounds for growth. After exhaustion of these labile compounds, bacteria turn into a dormancy state, without mining SOM for additional nutrients to maintain growth, yet continue to emit CO_2 through basal respiration (Joergensen et al., 2018).

Only a small number of studies have investigated how the application of different physical fractions of digestate may influence the soil microbial community and CO_2 production (e.g. Barduca et al., 2021, Grigatti et al., 2011; de la Fuente et al., 2013). Again, the results reported by these authors were found to be dependent on the feedstock utilised within the AD process, since digestate originating from highly degradable feedstock (e.g. cattle slurry co-digested with manure and maize) can still contain high amounts of readily biodegradable OM (e.g. de la Fuente et al., 2013), whilst digestate originating from agricultural waste often contains more recalcitrant organic compounds (Askri et al., 2016). However, generally the solid fraction of digestate was observed to increase by the greatest amounts the CO_2 emissions, followed by whole and liquid digestate. The CO_2 emissions after solid digestate application generally lasted longer than whole or liquid fractions (solid digestate lasted up to 56 days of incubation during the experiment conducted by de la Fuente et al., 2013), although as reported by Askari et al. (2016) this is highly dependent on the OM composition of the solid fraction of digestate. Askari et al. (2016) tested the CO_2 emissions after application of solid, whole and liquid fractions of digestate sourced from different AD plants and different feedstock type (e.g. cattle manure and slurry, biowaste of urban origins, such as paper and green waste, pig slurry and

food industry). The solid fraction of digestate increased CO_2 emissions in the long term compared to liquid and whole digestate for all the different digestates used, except for pig slurry digestate where the quantity of CO_2 produced following the application of the solid fraction of digestate was lower than for either liquid or whole fractions. Regarding liquid and whole digestate, increased CO_2 emissions occurred within the first 10 days of incubation for all the digestates studied, reaching similar levels for both fractions, except for cattle and pig slurry digestates where whole digestate produced a greater increases in CO_2 emissions than liquid digestate.

Additionally, a larger increase in C_{micro} has been observed following solid digestate application compared to whole and liquid fractions (e.g. de la Fuente et al., 2013; Barduca et al., 2021; Cattin et al., 2021). However, when de la Fuente et al. (2013) calculated the percentage of digestate C mineralized (expressed as % TOC lost as CO_2-C), they found that the highest amount of C mineralised came from the liquid fraction of digestate, followed by whole and solid fractions. These observations were also reported by Grigatti et al. (2011), where the CO_2 production from soil treated with solid and liquid fractions of digested slurry was studied, finding that lower CO_2 emissions and the greater C mineralisation originated from the liquid fraction compared to solid fraction of digestate. Grigatti et al. (2011) suggested that the highly labile OM present in the liquid fraction of digestate, compared to the more recalcitrant OM in whole and solid digestate fractions, was responsible for the high levels of mineralisation and CO_2 emissions. As reported by Barduca et al.

(2021), the solid fraction of digestate can positively influence microbial growth (which increased during their experiment) and basal respiration, but due to the stable OM, the % TC mineralised is reduced compared to more labile C compounds present in whole and liquid digestate fractions. Therefore, the solid fraction of digestate may have the potential to increase SOC stocks through an increase in C stored inside microbial cell, as evidenced by an increase in C_{micro}.

Table 2.3 Typical values of direct CO_2-C emission and C_{micro} increase after application of different fractions of digestate

Digestate fraction	Direct CO_2-C emission from digestate (kg ha^{-1})	Direct CO_2-C emission from digestate (mg kg^{-1} DW)	C_{micro} (mg kg^{-1} DW)	Material compared to	References
whole	9000			unamended soil	Pezzolla et al., 2012
				unamended soil, green manure, farm yard manure,	
	800			green manure+digestate, farm yard manure+digestate	Rosace et al., 2020
	44200			unamended soil, green/food compost , farmyard manure, livestock slurry	WRAP, 2016
		150		unamended soil, slurry, grass-clover	Johansen et al., 2013
		200	120	digestate+biochar,digestate+biochar+Humac, digestate+Humac	Holatko et al., 2021
		200	146	maize straw	Chen et al., 2012
		268	247	unamended soil, compost	Barduca et al., 2020
		1176	466	unamended soil, cattle slurry, cattle manure	de la Fuente et al., 2013
		1100		unamended soil, biochar	Mukherjee et al., 2016
				unamended soil, digested cattle slurry,	
		1200		digested cattle manure, digested pig slurry	Alburquerque et al., 2012
		1000		unamended soil, inorganic fertiliser, slurry	Cavalli et al., 2016
			1000	unamended soil, cattle slurry, inorganic fertiliser	Senbayram et al., 2014
		313		unamended soil, compost	Dietrich et al., 2020
				unamended soil, inorganic fertiliser, digested pig manure,	
			175	digested cattle manure, digested chicken manure	Doyeni et al., 2021
liquid		197	217	unamended soil, compost	Barduca et al., 2020
		936		compost, municipal solid waste	Grigatti et al., 2011
		1120	475	unamended soil, cattle slurry, cattle manure	de la Fuente et al., 2013
			175	unamended soil	Panuccio et al., 2021
		500		unamended soil, inorganic fertiliser, slurry	Cavalli et al., 2016
		379		unamended soil, compost	Dietrich et al., 2020
solid		359	231	unamended soil, compost	Barduca et al., 2021
		1100		compost, municipal solid waste	Grigatti et al., 2011
		4536	696	unamended soil, cattle slurry, cattle manure	de la Fuente et al., 2013
			215	unamended soil	Panuccio et al., 2021
		2000		unamended soil, inorganic fertiliser, slurry	Cavalli et al., 2016
		357		unamended soil, compost	Dietrich et al., 2020

Despite the research considered above, the impacts of digestate on the soil C cycle via changes in microbial CUE and in the composition of the soil bacterial and fungal communities remain poorly constrained, especially when different physical fractions of digestate with varying nutrient composition and stoichiometry are applied to soils. There has also been insufficient research focussed on the interactions between digestate application and soil nutrient status, which has been considered one of the main drivers influencing bacterial and fungal activity and, subsequently, soil C stocks and other soil health parameters (Manzoni et al., 2012; Geyer at al., 2016). These issues will be a key focus for this thesis, with objectives and experimental approaches in Chapter 3 designed to address several key research gaps in this area.

2.3 The application of digestate to agricultural soils and impacts on GHG and ammonia emissions

The application of digestate to agricultural soils has potentially significant, yet currently poorly constrained, impacts on the emission of GHGs from these soils. For example, digestate application may decrease by approximately 10% the CO_2 emissions, approximately 20% CH_4 emissions and approximately 2% N_2O emissions to the atmosphere when compared to the application of undigested feedstocks such manure or slurry (WRAP, 2016; Cavalli et al., 2017; Holly et al., 2017; Wulf et al., 2002; Moller & Stinner, 2009; Severin et al. 2015). These observations reflect the

fact that during the AD process the majority of labile C within feedstocks is converted into biogas, thereby reducing the potential for formation and release of GHGs from microbial metabolism in soil after digestate application to land (Eickenscheidt et al., 2014). However, regarding the N_2O emissions from digestate versus slurry or manure, results in the literature are variable, with digestate increasing the emission by approximately 5% or recording emissions similar to slurry or manure (Eickenscheidt et al., 2014; de la Fuente et al., 2013; Clemens et al., 2006; Johansen et al., 2013). This is because after the AD process, digestate contains more NH_4^+ than slurry or manure which, coupled with the remaining available C content of digestate, can trigger N_2O production to a greater extent than slurry or manure (Wulf et al., 2002; Senbayram et al., 2009; Sänger et al., 2010). When compared to inorganic fertilisers, the application of digestate to soils has the potential to increase GHG emissions (Zhou et al., 2017). This likely reflects the fact that, whilst the AD process reduces the lability of C within digestate compared to that within feedstocks, aerobic or anaerobic respiration and consequent production of GHGs may still be stimulated within the soil microbial community by the application of C and other nutrients within digestate.

Usually, the majority of the GHGs emitted after digestate application to land are associated with CO_2 formation, with emissions often of the order of mg CO_2-C kg^{-1} soil DM. The emission of CO_2 may be associated with the decomposition of organic compounds within the digestate itself and within native soil organic matter (see section 2.2 for CO_2 emissions from different fractions of digestate) (Drigo et al.,

2008). In contrast, CH_4 and N_2O production after digestate application to land often remain relatively low, with emissions in the range of μg kg^{-1} soil DM (Table 2.4) (Dietrich et al., 2020; Rosace et al., 2020; Czubaszek & Wysocka-Czubaszek, 2018) and often below the 1% emission threshold (of total N applied) imposed by the IPCC (2006) (Eickenscheidt et al., 2014; Pezzolla et al., 2012; WRAP, 2016). Production of CH_4 mainly occurs through anaerobic decomposition of organic materials applied to soil and native soil organic matter (Praeg et al., 2016). Anaerobic decomposition can occur in anoxic soils, for example those that are water saturated, or during the formation of anaerobic microsites during digestate injection or during high respiration rates by soil microorganism (Maucieri et al., 2016, Wulf et al., 2002). Production of N_2O occurs via denitrification or, depending on O_2 availability, during nitrification (Espinoza et al., 2013). During nitrification, in the presence of low O_2, the partial oxidation of NH_4^+ can create an accumulation of NO_2^- and promote the formation of HNO_2^- (Venterea & Rolston, 2000) which is believed to react with phenolic functional constituents of soil organic matter and consequently produce N_2O (Stevenson 1994). During denitrification, in the presence of a O_2 depleted environment, accumulated NO_3^- can be reduced to NO_2^- which is ultimately microbially transformed to N_2O (Firestone et al., 1989). In addition to these primary processes of GHG production within soil after digestate application to land, it has been reported in the literature that after the AD process, digestate may still contain CO_2, CH_4 and N_2O trapped as microbubbles and during land application these gasses are released as a short pulse of GHG emissions (Chadwick et al., 2000; Maucieri et al., 2016). However, the emissions of GHG after the application of digestate to land

are strongly dependent on multiple factors that include the initial application rate of the digestate, the application method (e.g. burial or surface dressing), the Tot C:N and the C quality (labile versus recalcitrant) of the material applied, the initial soil properties (e.g. pH, moisture content, texture) and the nutrient status of the soil (Shakoora et al., 2020a, 2021).

Increasing the application rate of digestate can increase the input of available C and N to soil and therefore potentially increase GHG emissions (Tilvikiene et al., 2020; Dietrich et al., 2020). For example, Tilvikiene et al. (2020) tested digestate added at rates of 90, 180, 270, 360, and 450 kg N ha^{-1} $year^{-1}$ and found that the greatest GHG emissions (calculated as CO_2 eq) occurred in response to the highest rate of digestate application, a pattern confirmed by Senbayram et al. (2014) where digestate was applied at 160 or 480 kg N ha^{-1} on grassland. Senbayram et al. (2014) have found that greatest N_2O emissions were also associated with the highest digestate application rate and additionally, Svoboda et al. (2015) tested digestate applied to arable soil at 120, 240, 360 kg N ha^{-1} and found that the greatest CH_4 emissions occurred at the highest digestate application rates. However, GHG emissions are also dependent on the C and N quality of the organic material applied. The application of an organic material with a low Tot C:N, such as livestock slurry, can supply labile C and available N to soil microbial communities, thereby generating increased production of GHGs. In contrast, organic materials with a high Tot C:N usually contain more recalcitrant carbon compounds that can reduce microbial respiration compared to materials with low Tot C:N and available C

compounds, therefore reducing the emission of GHGs (Askri et al., 2016; Yagi and Minami, 1990). Furthermore, the application method of the organic material can directly influence GHG emissions, since injection or incorporation of the organic material versus splash plate can create anaerobic conditions by intensifying the respiration rates from soil microorganisms, and therefore increase the CH_4 and N_2O emission to the atmosphere (Wulf et al., 2002; WRAP, 2016).

It has been reported in the literature that splash plate application versus injection of digestate can substantially increase NH_3 emissions to the atmosphere within 6 h of application, with high NH_3 emissions potentially leading to soil acidification (Nicholson et al., 2017). Emissions of NH_3 after surface application of digestate are associated with the high NH_4^+-N content and basic pH of digestate, which can promote the volatilization of NH_3 (Bicoku et al., 2018). Additionally to the application method, the N application rate, soil properties (e.g. sandy soil can increase NH_3 emission due to increased aeration), timing, temperature, wind speed, rainfall and dry matter content of the organic material can significantly influence the magnitude of NH_3 emission (Sommer et al., 2001; Bourdin et al., 2014, Nicholson et al., 2018). For example, in cattle slurry, the emission of ammonia increases with an increase in the dry matter content. However, regarding digestate, not enough research has been done on the relationship between NH_3 emission and DM content of digestate (Pedersen et al., 2021; Nicholson et al., 2017). When NH_3 is released into the atmosphere, it can produce acidic rainfall and increase the fine fraction of atmospheric particulate pollution (Pinder et al., 2008). Moreover, when NH_3 is deposited

into an aquatic ecosystem in excess, it may increase nitrogen availability and trigger eutrophication.

Svoboda et al. (2013), during a three year field experiment where digestate was surfaced applied and compared to cattle slurry, found that the NH_3 emission from digestate was 3-8% higher than cattle slurry, a pattern that was also observed in a related experiment conducted by Nyord et al. (2012). Nicholson et al. (2017), during a one year field experiment, estimated that NH_3 volatilisation losses from surface application of food-based digestate were 30–50% of total N applied and NH_3 emission was substantially greater than following application of green compost. This pattern has been confirmed by others (e.g. Riva et al., 2016; Verdi et al., 2019; Wolf et al., 2014; Matsunaka et al., 2006). Riva et al. (2016) tested surface application of digestate compared to urea that was also surface applied in a one year field experiment and concluded that ammonia emissions from digestate reached a maximum within a few hours of application and that digestate application has the potential to increase NH_3 emission by 20% when compared to urea. A similar pattern was observed by Wolf et al. (2014) and Matsunaka et al. (2006), where digestate application was compared to inorganic fertiliser during a field experiment. Wolf et al (2014) conducted a field experiment which lasted three months and reported that cumulative NH_3 emission from digestate reached 8 kg ha^{-1}, with the maximum flux within a few hours of application, whilst NH_3 emitted from mineral fertiliser was only slightly detectable. Matsunaka et al. (2006) conducted a three year field experiment and concluded that the maximum NH_3 emission from digestate

application occurred within the first few hours of application, reaching a cumulative NH_3 loss of 18.2 g m^{-2}, whilst NH_3 losses after mineral fertiliser application were not detectable.

However, only a very small number of laboratory studies have tested the effect of applying different digestate fractions to agricultural soils (e.g. Dietrich et al., 2020; Askir et al., 2016; Barduca et al., 2020) on GHG emissions. For example, Dietrich et al. (2020), tested whole, liquid and solid digestate, reporting that GHG emissions increased in the order: i) N_2O whole≈solid< liquid; ii) CH_4 whole<liquid<solid and; iii) CO_2 whole<liquid≈solid. Barduca et al. (2020) tested whole, liquid and solid digestate and found that whole and liquid digestate produced a comparable amount of N_2O emissions, whilst solid digestate produced the lowest N_2O emissions. Askri et al. (2016) tested different digestate fractions (whole, liquid and solid) from different feedstocks (e.g. cattle manure, pig manure, cattle slurry, pig slurry, biowastes) and found that the magnitude of N_2O emissions depended on the combination of the feedstock used and the digestate fraction applied. Generally, Askri et al. (2016) reported that N_2O emissions increased following the application of whole digestate across all the feedstock used, although cattle manure and pig slurry digestate produced the greatest N_2O emissions compared to the other feedstocks. The N_2O emissions from the whole fraction of digestate was followed by those from solid and liquid digestate, with greater N_2O emission from solid fraction than liquid fraction when biowaste was used as a feedstock.

Regarding the NH_3 emission from different digestate fractions, only a few field experiments (e.g. Verdi et al., 20019; Riva et al., 2016) have evaluated NH_3 losses from surface application of liquid and whole digestate after land spreading, whilst no research was conducted after land application of solid digestate, although Möller (2015) introduced the fact that solid, whole and liquid fractions of digestate can influence differently NH_3 emissions, since the NH_4^+-N redistribution is significantly different amongst fractions. Verdi et al. (2019) compared surface application of liquid digestate versus urea during a 84 days field experiment, and at the end of the experiment liquid digestate produced 66% less NH_3 volatilization than urea. Riva et al. (2016) conducted a one year field experiment where digestate, the separated liquid fraction and urea fertiliser where compared. Contrarily to Verdi et al. (2019), at the end of the experiment, whole, liquid and urea were responsible for 30%, 46% and 12% of NH_3 emission, respectively.

Additionally, GHG fluxes after the application of organic materials to land is highly dependent on soil fertility, in particular the availability of key nutrients within soil including C, mineral N, and available K (Manzoni et al., 2012). For example, soils with higher nutrient availability and labile C content have been shown to increase GHG emissions when compared to a fallow soil with lower nutrient and labile C availability (Wulf et al., 2002; Matsunaka et al., 2006). This is likely to be associated with the high SOC and nutrient composition in a well-fertilised soil, which creates a favourable environment for microbial respiration and the creation of anaerobic microsites that promote N_2O and CH_4 formation (Koops et al., 1996; Eickenscheidt

et al., 2014). In contrast, soils at lower nutrient status and labile C availability present a more adverse environment for microbial metabolism, due to relative low organic C, pH, microbial biomass, respiration and, therefore, GHG production (Russell & Cook, 1995). The input of organic material with a relatively low Tot C:N to soils with high labile C availability may lead to rapid increases in CH_4 emissions, since methanogenic bacteria are supplied with additional oxidisable organic C (Baggs et al., 2006; Topp and Pattey, 1997; Pezzolla et al., 2012). In contrast, the application of available C compounds within the organic material to soils containing lower concentrations of labile C, such as within fallow soils, may increase CH_4 emissions over the longer-term, but likely only if mining of existing SOM is triggered by the application of the organic material, since labile C is used by soil microorganism for their catabolism (and therefore CO_2 production) rather than CH_4 production (Lipson et al., 2001; Fontaine et al., 2003; Conrad, 2020). Similarly, N_2O production via either nitrification or denitrification is strongly influenced by the C quality and N availability of the organic material applied and by the C and N status of the soil (Firestone et al., 1989; Weier et al., 1993). For example, the application of a material rich in available C and N to a soil low in NO_3^- concentration can result in rapid bacterial N immobilization via biosynthesis, which in turn can lead to a decrease in NO_3^- production and the consequent decrease in N_2O emission during denitrification processes (Chadwick et al. 2000; Velthof et al., 2003). In contrast, the addition of materials high in available C and N to a soil rich in oxidised N and labile C can supply nitrifying or denitrifying bacteria with sufficient NO_3^- for the

subsequent production of high amounts of N_2O (Johansen et al., 2013; Senbayram et al., 2009).

Only rarely has past research explored the influence of soil texture (loam versus sandy soil) on GHG emissions after digestate application to land (e.g. Doyeni et al., 2021; Dietrich et al., 2020), whilst only one study has considered the influence of the SOC concentration (medium level and high level) and N contents of soil on GHG emissions (Eickenscheidt et al., 2014). During these studies, application of digestate to a sandy soil produced lower amount of CO_2, N_2O and CH_4 than application of digestate to a loamy soil, and this was associated with better aeration of the soil and a decrease in anaerobic conditions. Digestate application to a soil with higher SOC content increased N_2O emissions compared to a soil with lower SOC and this was related to the extra supply to soil microorganism of OC, which was used during denitrification processes by denitrifying bacteria for N_2O production.

Regarding ammonia emission after land application of digestate on sandy soils, which has been identified one of the main physical components to NH_3 released (Lukehurst et al., 2010), only a few studies have been reported (e.g. Wolf et al., 2014; Sänger et al., 2014; Wester-Larsen et al., 2022; Munro et al., 2017). Wolf et al. (2014) during a one year field experiment where digestate was compared to mineral fertiliser application, concluded that the greater NH_3 emission after digestate application than mineral fertiliser was due to low soil moisture and good aeration of the sandy soil used during the experiment. Munro et al. (2017) during a field experiment, compared food-waste digestate application to green compost and

livestock manure on a light sandy soil and concluded that ammonia emissions were greater from applications of food-based digestate (c.40% of total N applied) than from livestock slurry (c. 30% of total N applied). Sänger et al. (2014), tested digestate application to a sandy and silty soil and found that the cumulative NH_3 emission from the sandy soil was 2 time higher than the silty soil (10 mg N Kg^{-1} DW soil and 20 mg N Kg^{-1} DW, respectively), with the highest emissions within 24h from application. Wester-Larsen et al. (2022) during a 44 days laboratory study where different type of digestate (e.g. food waste, agro-waste and manure waste) where compared to other biowastes (compost, meat and bone meals, poultry manure, municipal solid waste) and applied to different soils (acidic sandy soil, acidic clay soil, neutral loamy soil, alkaline loamy soil, alkaline clay soil); overall, digestates produced the highest NH_3 production (64% of TN was lost as NH_3) than the other treatments applied and in the specific, the accumulated potential NH_3 volatilization was significantly higher for acidic sandy soil.

Table 2.4 Typical values of direct CO_2-C, CH_4-C and N_2O-N emissions after land application of different fractions of digestate

Digestate fraction	Direct CO2-C emission from digestate (kg ha-1)	Direct CO_2-C emission from digestate (mg kg^{-1} DW)	Direct CH_4-C emission (kg ha^{-1}) from digestate	Direct CH_4-C emission (µg kg^{-1} DW) from digestate	Direct N_2O-N emission (Kg ha^{-1}) from digestate	N_2O-N (µg kg^{-1} DW)	Material compared to	References
whole	9000		0		0.02		unamended soil	Pezzolla et al., 2012
		200					digestate+biochar,digestate+biochar+Humac, digestate+H	Holatko et al., 2021
		1176					unamended soil, cattle slurry, cattle manure	de la Fuente et al., 2013
		1100					unamended soil, biochar	Mukherjee et al., 2016
		200					maize straw	Chen et al., 2012
		1000					unamended soil, inorganic fertiliser, slurry	Cavalli et al., 2016
		1200					unamended soil, digested cattle slurry, digested cattle manure, digested pig slurry	Alburquerque et al., 2012
	44200		0.5		1.2		unamended soil, green/food compost , farmyard manure, livestock slurry	WRAP, 2016
		150				0.003	unamended soil, slurry, grass-clover	Johansen et al., 2013
	800		1		0.15		unamended soil, green manure, farm yard manure, green manure+digestate, farm yard manure+digestate	Rosace et al., 2020
		268				31.8	unamended soil, compost	Barduca et al., 2020
			0.1		0.1		unamended soil, slurry	Wulf et al., 2002
					10		unamended soil, cattle slurry, inorganic fertiliser	Senbayram et al., 2014
		313		1.6		433	unamended soil, compost	Dietrich et al., 2020
					5		unamended soil, inorganic fertiliser, digested pig manure, digested cattle manure, digested chicken manure	Doyeni et al., 2021
					1.8		unamended soil, green/food compost , farmyard manure, livestock slurry	Nicholson et al., 2017
			0		3.1		unamended soil, cattle slurry	Eickenscheidt et al., 2014
			0.1				none	Czubaszek et al., 2018
			0		0.5		unamended soil, pig slurry	Severin et al., 2015
liquid		379		18		2398	unamended soil, compost	Dietrich et al., 2020
		936					compost, municipal solid waste	Grigatti et al., 2011
		1120					unamended soil, cattle slurry, cattle manure	de la Fuente et al., 2013
							unamended soil	Panuccio et al., 2021
		500					unamended soil, inorganic fertiliser, slurry	Cavalli et al., 2016
		197				31.7	unamended soil, compost	Barduca et al., 2020
solid		359				14.6	unamended soil, compost	Barduca et al., 2020
		1100					compost, municipal solid waste	Grigatti et al., 2011
		4536					unamended soil, cattle slurry, cattle manure	de la Fuente et al., 2013
							unamended soil	Panuccio et al., 2021
		2000					unamended soil, inorganic fertiliser, slurry	Cavalli et al., 2016
		357		103		580	unamended soil, compost	Dietrich et al., 2020

Despite the research described above, the impacts of the application of digestate on GHG emissions from agricultural soils remain poorly understood, especially when different physical fractions of digestate with varying nutrient composition and stoichiometry are applied. Furthermore, insufficient research has focussed on the interactions between digestate application and soil nutrient status, which has been reported to strongly influence biogeochemical cycles and GHG emissions from soil (Conrad, 2020; Firestone et al., 1898; Shokoona et al., 2020). These variables are a further important focus for this thesis, with objectives and experimental approaches in Chapter 4 seeking to address key research gaps in this area.

2.4 Leaching and runoff of nutrients after digestate application to agricultural soils

Phosphorus (P) is one of the most important macronutrients for plant growth and development (Bolan et al., 2005). However, inorganic fertilisers are ultimately derived from phosphate rock reserves (Mackay et al., 2017). It has been estimated that rock P reserves are declining in availability, quality and sustainability (Dawson et al., 2011), thus finding alternative sources to meet agronomic demand for P and closing the P cycle is an important factor that will influence the sustainability of future agriculture.

Digestate may have potential as a substitute for inorganic P fertilisers, since it often contains a high amount of total P (TP) (between 0.4-7.9 g kg^{-1} FW), a large proportion of which may be present as inorganic orthophosphate P (P_{in}) (between

35-55% of TP) (Moller & Muller, 2012; Bachmann et al., 2016; García-Albacete et al., 2014), readily available for plant uptake. The remaining P within digestate may be present as organically-complexed P (P_{org}) (Bachmann et al., 2016; Lin et al., 2015), which may be useful for longer-term P availability because some P_{org} compounds can be mineralised by microorganisms resulting in release of P_{in} (Figure 2.3) (Espinoza et al., 2013).

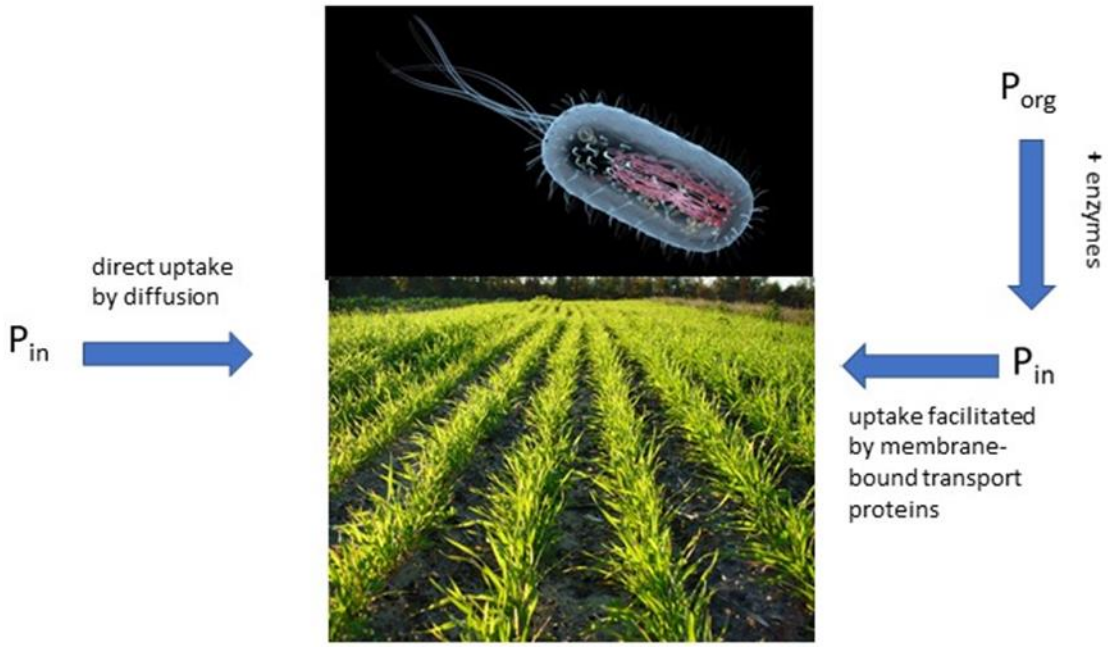

Figure 2.3 Biogeochemical cycling of P in soil, adapted from Blake et al. (2005). Orthophosphate P (P_{in}) can be taken up by diffusion, enzymes not required and incorporated into cell biomass; Organic P (from organic amendment or after cell death) is hydrolysed inro P_{in} through specific enzymes and taken up through membrane-bound transport proteins and incorporated into cell biomass

However, as reported in section 2.1, different fractions of digestate can contain different proportions of P_{in} and P_{org}, since the solid-liquid separation process results

in the partitioning of nutrients between solid and liquid digestate fractions. For example, it has been estimated that 40-50% by mass of TP is retained in the solid digestate after separation compared to the liquid fraction (Bauer et al., 2009) and that most of the TP in the solid fraction is present as P_{in} (up to 90% of TP), whilst only 5-7% of TP in the solid fraction is present as P_{org}. However, whilst the TP present in the liquid fraction is mainly present as P_{in}, the P_{org} contained in this fraction can reach substantial concentrations, for example up 13-14% of the TP retained after solid-liquid separation (Bachmann et al. 2016). However, Akhiar et al. (2017) and Lukehurst et al. (2010) reported that solid digestate contains a higher amount of P_{org} than P_{in} after separation, whilst the liquid fraction contains mostly P_{in} and only small amounts of P_{org}. Therefore, uncertainties remain surrounding the redistribution P after solid-liquid separation of digestate, especially based on the separation technique applied (Guilayn et al., 2019).

Digestate in agriculture is usually applied following specific N application rates (RB209), although these recommendations can still potentially lead to risks associated with N leaching. As reported in section 2.1, digestate usually contains high concentrations of available NH_4^+, which can be rapidly taken up by crops for their growth or can be quickly nitrified in 1-2 weeks after application (Fuchs et al. 2008; Möller and Müller 2012; Insam et al. 2015). As well as NH_4^+, plants can use NO_3^- to meet their metabolic demand for N. Whilst NH_4^+ may be sorbed and retained within the soil matrix, due to the negative charge of the NO_3^- ion, there is limited potential for NO_3^- sorption to the soil matrix (Bloom, 2010) and consequently

increases in the environmental risk of NO_3^- leaching will occur if good agricultural practices guidelines and correct N target are not followed during digestate application (Nicholson et al., 2017). Additionally, the N_{org} present in digestate can subsequently be converted into NH_4^+ and NO_3^-, which may further increase the risk of N export from agricultural land (Sharifi et al., 2019).

Leaching of NH_4^+ to water bodies can lead to dissociation into NH_3 at pH>7 and temperature>30°C, which is extremely toxic for aquatic organisms since it can pass from the bloodstream into the tissues and brain, causing damage and behavioural impairment to fish and invertebrates (Thurston et al., 1981; Thraves, 2004). At background environmental concentrations and in the presence of oxygen, NH_4^+ is converted into NO_2^- and NO_3^- during the nitrification process, although this provides an additional source of NO_3^- which can add to the risk of NO_3^- leaching following the application of fertiliser or digestate to land. The presence of NO_3^- in groundwater can alter water quality and be associated with a high risk to human and ruminant health (Lord and Athony, 2002; Schroeder et al., 2004) since high concentration of NO_3^- can cause the blue syndrome in infants (methemoglobinemia) (McMckague et al., 2017) and livestock (Sandstedt 1990, Amdur et al. 1991). Historically, N was considered the limiting nutrient for triggering eutrophication only in estuaries and the oceans. However, more recent evidence suggests that N can alter nutrient balances and ecological processes in rivers, lakes and estuaries (Smith et al., 1999) and also trigger eutrophication in these freshwater environments (Dodd & Smith, 2016).

Moreover, as detailed in section 2.1, digestate often has an unbalanced Tot N:P, in which P is relatively enriched compared to soil or crop requirements. Further, different digestate factions have different P_{in} and P_{org} contents. These characteristics mean that when digestate is applied following specific N application rates, targeting a specific P input and the input of a specific fraction of P (i.e. P_{in} or P_{org}) is challenging and often results in an over-application of P to soil (Goss et al., 2013). Further, depending on the biogeochemical properties of the soil (e.g. pH, soil microbial community, texture, phosphatase production) (Ali et al., 2019), P_{org} mineralization rates after digestate application are extremely variable and therefore it is difficult to predict the amount and rate of P_{in} released from the P_{org} pool in digestate after application to land (Westerman and Bicudo, 2005).

Over-application of P to land via digestate can occur as a result of meeting crop N targets and can lead to an accumulation of P within agricultural soils. Historically, P was perceived as relatively immobile in soil, due to significant capacity for P sorption in many soil matrices. However, immobilization of P via sorption within soils can reach a saturation point, otherwise known as a "change point". After this saturation threshold, the risk of P mobilisation and export in dissolved form increases significantly (Nair, 2014). However, the risk of P export from agricultural soils is influenced by multiple factors including: a) processes of adsorption and fixation with cations (i.e. Ca^{2+}, Mn^{2+}, Fe^{3+}, Al^{3+}) and clay particles. These cations can strongly bind to P reducing its mobility; additionally, the silicate minerals and organic matter present in clays can generate a competitive effect on P adsorption due to ligand

exchange with the surface hydroxyl groups (Fuentes et al., 2006). However, this is highly dependent on the charge density of these cations and on; b) seasonal processes, such as those associated with redox reactions, whereby in the presence of strongly reducing soil conditions, redox-sensitive cations such as Fe which normally adsorb P under oxidised conditions (Fe^{3+}), can be reduced (Fe^{2+}) and the P associated with those cations is released to solution. Leaching of P is also highly dependent on: c) soil pH, since it has been estimated that the maximum P solubility is between pH 4.5 and 6.5, which coincides with the lowest degree of P fixation by Ca, Al, and Fe minerals (Penn & Camberato, 2019); d) soil texture (sandy versus clay) and structure (e.g. cracking and preferential flow), since macropores or cracks act as preferential flow channels that can increase the risk of P export to the subsurface; and finally e) conversion into P_{in} due to soil microorganisms (e.g. through production of organic acids and hydrolytic enzymes), since the conversion can increase P mobilization into the soil-water solution (Jara et al. 2006; Hinsinger, 2001). When P is strongly immobilised within the soil matrix, export may still occur via erosion of soil particles and transport of these particles to water bodies via surface runoff, or solubilisation of P from soil and export of this P via surface runoff (Filippelli, 2002; Brandt et al., 2003; Sharpley et al., 2001). Further, when P is dissolved within soil water, export may occur through leaching into the subsurface, which is now a well-established process (e.g. Stutter et al., 2012; Haygarth and Turner, 2000). Specifically, the soil P index and associated fertilisation of soils play an important role in controlling the risk of P leaching through subsurface flows (Kleinman et al., 2015). For example, soils with a high P index are more likely to leach P than soils

with low P index, due to the relationship between agronomic P indices and the previously described change-point concentration of P in soils at which export risk increases significantly, suggested to be in the range of 60 mg Olsen-P kg^{-1} DM soil (Heckrath et al., 1995; Maguire & Sim, 2002).

A wide range of operationally-defined P fractions can reach waterbodies through export from agricultural land via leaching and surface runoff (Figure 2.4). For example, P may be exported as:

i) particulate P (PP, > 0.45 µm), which is considered a store of reactive P in the long term (due to mineralisation and solubilisation processes);

ii) total dissolved P (TDP, filtered <0.45 µm) which is the combination of all inorganic and organic forms of 'dissolved' P.

iii) dissolved reactive P (DRP, filtered <0.45 µm), comprising mainly the dissolved form of P_{in}, although this fraction can still contain colloidal or organic P compounds usually in small quantities, (e.g. Baldwin, 1998; Denison et al., 1998; Haygarth et al., 1997). DRP is the fraction assumed to be most readily available to organisms and therefore most likely to trigger eutrophication responses in receiving waters (García-Albacete et al., 2016; Turner et al., 2000; Myers and Pierzynski, 2000);

iv) dissolved unreactive P (DUP, filtered <0.45 µm comprising mainly the dissolved form of P_{org}) which is usually determined as the difference between TDP and DRP, and is normally associated with organic P compounds, inorganic polyphosphates, and mineral colloids (Vaz et al., 1992; Baldwin, 1998; Denison et al., 1998; Haygarth

et al., 1997) which can potentially become available for aquatic organisms depending on processes including solubilisation and enzyme hydrolysis (Shand & Smith, 1997; Darch et al., 2016).

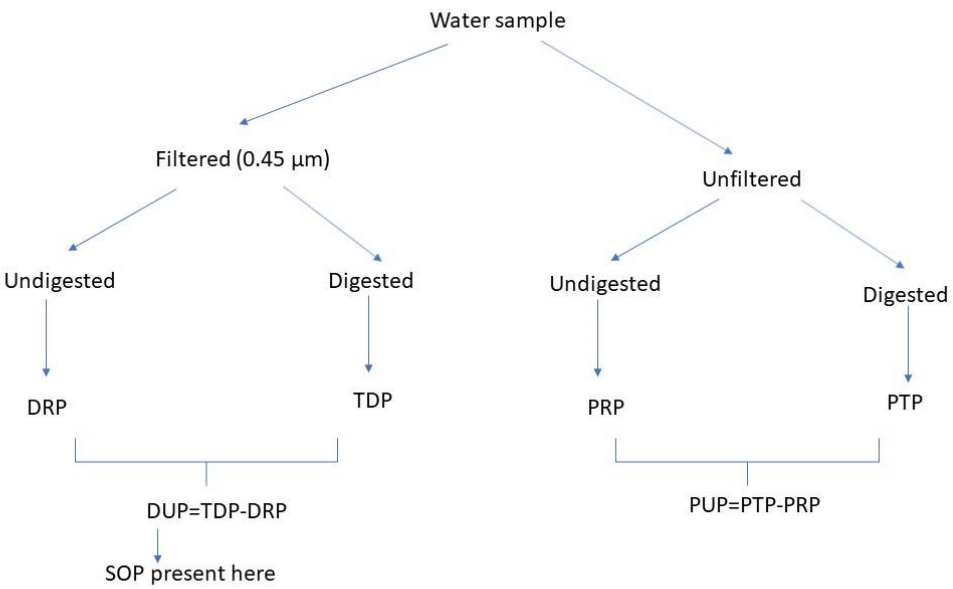

Figure 2.4 Fractionation of different P forms present in leachate or runoff, including forms estimated by difference. DRP= Dissolved reactive P; DUP= Dissolved unreactive P; SOP= Soluble organic P; TDP= Total dissolved P; PRP= particulate reactive P; PTP= Particulate total P; PUP= Particulate unreactive P

However, determination of organic P compounds by subtraction of DRP from TDP can frequently lead to bias in the estimation of soluble organic P (SOP) compounds, for example because hydrolysis of SOP during colorimetric analysis of DRP can lead to underestimates of the true organic P concentration (Hanrahan et al., 2005). These SOP compounds may potentially be extremely important in the environment, since

many organisms have the capability to produce specific enzymes that can hydrolyse SOP and release bioavailable P (P_{in}) compounds into solution (George et al., 2006). The three main groups of SOP compounds that can be hydrolysed may be defined as (Bünemann, 2008): i) hydrolysable monoester P, such as adenosine triphosphate and monophosphate (ATP, AMP), guanosine-5'-triphosphate (GTP) and compounds that build up RNA and DNA; ii) hydrolysable diester P, such as nucleic acid an phospholipids; and iii) hydrolysable inositol hexakisphosphate, which is a group of compounds where a central inositol group (in one of nine potential isometric forms) is bound between one and six phosphate groups by phosphomonoester bonds (Toor et al., 2003; Baldwin, 2013).

Regarding the P exported via leaching after digestate application to land, the literature remains extremely scarce (Table 2.5). Because high loads of P (and N) may be applied to soil during digestate application, the risks to water quality associated with digestate need to be understood more thoroughly, particularly after different digestate fractions have been applied to soil. Some past research has examined the quantity and the forms of P forms leached after digestate application (Table 2.5), focussed particularly on DRP exported after digestate application during laboratory experiments (e.g. Sogn et al., 2018; García-Albacete et al., 2014) compared to compost or unamended soil. For example, García-Albacete et al. (2014) found that whole digestate increase the risk of DRP leaching when compared to unamended soil during a column leaching experiment, but the concentration of P leached following digestate application was lower than after compost application. Sogn et

al. (2019), during a pot experiment, found that application of whole digestate increased the risk of TDP (reaching 0.04 mg P L^{-1}) leaching when compared to unamended soil, mineral fertiliser and farmyard manure. Haraldsen et al. (2011) conducted a pot experiment where liquid digestate was compared to mineral N fertiliser, cattle manure and unamended soil and found that the DRP lost from liquid digestate via leaching (reaching 0.66 mg P L^{-1}) was greater than the DRP lost from the other treatments. These authors have reported that the increase in DRP and TDP concentration in leachate after digestate application was highly correlated with the water extractable P of digestate, its wettability and the phosphorus sorption capacity of the soil (P saturation of the soil).

An increased risk of P leaching after digestate application to land has also been observed during field experiments, although only a small number of studies have been published (e.g. Vanden Nest et al., 2015; Koch et al., 2019). For example, Vanden Nest et al. (2015) tested digestate (solid digestate only) applied as a yearly dose of 37 kg P ha^{-1}, mineral fertiliser, vegetable compost, unamended soil and cattle slurry (all applied as a yearly dose of 37 kg P ha^{-1}) during a field trial. For their leaching measurement, soil samples were collected, packed in glass columns and leached in the laboratory. These authors found that solid digestate leached a TDP concentration of 0.46 mg P L^{-1}, which was higher than the other treatments (0.36 mg P L^{-1} for vegetable compost, inorganic fertiliser and cattle slurry and 0.25 mg P L^{-1} for unamended soil). During a field experiment conducted by Koch et al. (2019), intact lysimeters were used to assess the leaching of DRP and TDP before and after

whole digestate application at a rate of 48 kg P ha^{-1}. Artificial leaching events were generated, and suction caps were used to collect the DRP and TDP. The research showed that digestate application increased the DRP (0.52 mg P L^{-1}) and TDP (0.7 mg P L^{-1}) concentrations compared to unamended soil and that the DRP and TDP concentration increased steadily through each rainfall events. Further, the authors reported that most of the TDP was present as DRP and that P originated from mobilization processes within soil (rather than from digestate itself) was transported through the soil matrix and also through preferential flows.

Regarding the risk of SOP losses after digestate application, only one glasshouse study (Richards et al., 2021) has looked at the phytase-hydrolysable P present in soil amended with two digestates (food and agricultural wastes) versus unamended soil, inorganic fertiliser and wood ash. However, instead of leachate, the SOP were analysed on a soil water-extraction solution. During this pot experiment, the different amendments were applied to planted and unplanted pots and soil water extractions were conducted to estimate the DRP, TDP and phytase-hydrolysable P present in the soil matrix. At the end of the experiment, the DRP concentration found in the soil extraction after application of digestate derived from agricultural wastes was higher (0.76 mg P L^{-1}) than the DRP found in unamended soil (0.57 mg P L^{-1}) and DRP found in soil amended with food-waste digestate (0.36 mg P L^{-1}), whilst the highest DRP concentration was found in soil amended with inorganic fertiliser and ash (0.94 mg P L^{-1} and 0.96 mg P L^{-1}, respectively). Regarding the TDP concentration found by these authors in the water extracts, the highest TDP

concentration was found after ash application (1.69 mg P L^{-1}), followed by inorganic fertiliser (1.52 P mg L^{-1}), agricultural waste- digestate (1.41P mg L^{-1}) and food-waste digestate (0.23 mg P L^{-1}). Regarding the phytase-specific enzyme P solubility, this parameter was calculated as the % of the water-extracted TDP that was rendered responsive to the molybdate reagent after the enzyme treatment. After food waste digestate application, 32% of TDP was associated with phytase hydrolysable P, followed by inorganic fertiliser/agricultural waste digestate (20%) and ash/unamended soil (11%).

Further, data regarding the NH_4^+-N and NO_3^--N concentration in leachate after digestate application to land remain limited (Table 2.5), especially after application of digestate to soils at contrasting nutrient status (Nkoa, 2014). Field experiments (e.g. Tsachidou et al., 2019; Svoboda et al., 2013; WRAP, 2016) and laboratory experiments (e.g. Tshikalange et al., 2020; Sogn et al., 2019) where whole digestate was compared to unamended soil, inorganic fertiliser or other organic materials (see Table 2.5), have reported an increase in NO_3^--N concentration in leachate after digestate application (between 20 and 130 mg N L^{-1}) compared to unamended soil, associated with conversion of NH_4^+-N present in digestate to NO_3^--N within days or weeks after application to land. When digestate was compared to slurry, the NO_3^--N concentration found in leachate was variable, in some cases the NO_3^--N concentration in leachate after slurry or manure application was similar to that from digestate, whilst in other cases the concentration was lower or higher (e.g. Goberna et al., 2011; Svaboda et al., 2013; Sogn et al., 2019). These authors concluded that

the variability in leachate concentrations was related to the chemical composition of the organic material, the soil texture and microbial community composition. However, when the NO_3^--N in leachate from digestate treatments was compared to the NO_3^--N leached following inorganic fertiliser application, the concentration of NO_3^--N found in leachate from inorganic fertiliser application was higher (between 54 and 300 mg N L^{-1}) than NO_3^--N concentration found in leachate after digestate application (Svaboda et al., 2013, Sogn et al., 2019; Tshikalange et al., 2020; Tsachidou et al., 2019). This was associated with the fact that inorganic fertilisers contain already high quantities of NO_3^--N which can leach quickly after application if not properly applied.

Only a few studies have tested different digestate fractions and analysed NO_3^--N concentrations in leachate after application to land (Tsachidou et al., 2019; Haraldsen et al., 2011). For example, Tsachidou et al., (2019) and Haraldsen et al. (2011) reported that the liquid fraction of digestate produced a lower concentration of NO_3^--N in leachate (0.9 mg N L^{-1}) compared to whole digestate (20 mg L^{-1}) and mineral fertiliser (140 mg N L^{-1}), whilst when liquid digestate was compared to cattle manure or slurry the results where, again, variable. The reduction in NO_3^--N concentration in leachate after liquid digestate compared to whole digestate was related to the fact that liquid digestate has a lower viscosity than the whole fraction of digestate. Therefore, liquid infiltrated in soil to a greater extent than whole digestate, meaning that the NH_4^+-N content of the liquid fraction was effectively converted into NO_3^--N. Oher studies have reported that the NO_3^--N lost during

leaching events is highly correlated with the soil N status, fertiliser management and application rates of digestate applied (Haraldsen et al., 2011; Nicholson et al., 2017; Möller, 2015).

Regarding NH_4^+-N leaching after whole digestate application, there are only a few studies present in the literature (Tshikalange et al., 2020; Goberna et al., 2011; Matsunaka et al., 2006; Tsachidou et al., 2019) and only one study which tested the liquid fraction of digestate (Haraldsen et al., 2011). During these studies, the concertation of NH_4^+-N reported in leachate after whole digestate application was lower (between 3-20 mg N L^{-1}) than the NH_4^+-N lost from inorganic fertiliser (between 10-373 mg N L^{-1}), whilst Haraldsen et al. (2011) reported that liquid fraction had higher NH_4^+-N concentration (23 mg N L^{-1}) in leachate to inorganic fertiliser (1.4 mg N L^{-1}).

Table 2.5 Typical values of TDP, DRP, NH_4^+-N and NO_3^--N leached after application of different fraction of digestate

Digestate fraction	Direct TDP (mg P L^{-1}) from digestate	Direct DRP (mg P L^{-1}) from digestate	Direct NH_4^+-N (mg N L^{-1}) from digestate	Direct NO_3^- (mg N L^{-1}) from digestate	Direct NO_3^- (kg N ha^{-1}) from digestate	Material compared to	Reference
whole				130		unamended soil, green/food compost , farmyard manure, livestock slurry	WRAP, 2016
	0.04			17		manure, inorganic fertiliser	Sogn et al., 2018
					20	unamended soil, inorganic fertiliser	Tsachidou et al., 2019
			5	0		unamended soil, inorganic fertiliser	Tshikalange et al., 2020
					22	unamended soil, inorganic fertiliser	Matsunaka et al., 2006
	0.7	0.52				unamended soil	Koch et al., 2019
				160		unamended soil, manure	Goberna et al., 2011
					22.5	unamended soil, compost , farmyard manure, livestock slurry	Svoboda et al., 2013
	1.41-0.23	0.36-0.76				unamended soil, inorganic fertiliser, ash	Richards et al., 2021
liquid			23	0.9		unamended soil, inorganic fertiliser, compost, manure	Haraldsen et al., 2010
solid	0.46					unamended soil, inorganic fertiliser, compost, slurry	Vanden Nest et al., 2015

From this evaluation of the literature, it is clear that there remain significant uncertainties regarding the losses of P after digestate application to land, particularly following the application of different physical fractions of digestate and spanning a wider range of P forms in leachate. There has also been insufficient research focussed on the interactions between digestate application and soil P status, which has been considered one of the main drivers influencing the P leaching from soils (Heckrath et al., 1995; Maguire & Sim, 2002). Further, far more research is required, particularly under field conditions, to quantify NH_4^+-N and NO_3^--N concentrations in leachate after digestate application, including within soils of contrasting nutrient properties are following the application of different fractions of digestate (Nkoa, 2014; Al Seadi et al., 2010). These issues will be a key focus for this thesis, with objectives and experimental approaches in Chapter 5 designed to address several key research gaps in this area.

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3 Changes in microbial utilization and fate of soil carbon following the addition of different fractions of anaerobic digestate to soils

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Abstract

Applying digestate, the residue from anaerobic digestion, to soil as a replacement for inorganic fertiliser is of growing interest in agriculture. However, the impacts of different fractions of digestate on the soil carbon (C) cycle remain unclear and provide the focus for the research reported here. We examined the effects of applying whole digestate (WD) and solid digestate (SD) on carbon dioxide (CO_2-C) efflux, the concentrations of dissolved organic carbon (DOC), microbial biomass C (C_{micro}) and phospholipid fatty acids, alongside carbon use efficiency (CUE). A 21-day laboratory microcosm incubation was used to investigate the impacts of digestate

when applied to two grassland soils of high versus low initial nutrient content. Application rates for SD and WD were based on recommended nitrogen (N) inputs to grassland soils for these organic materials. Compared to control treatments, cumulative CO_2-C efflux and the concentration of DOC increased significantly after WD and SD application, although only within the low nutrient soil. Both C_{micro} and the fungal to bacterial ratio increased significantly following SD application, regardless of the initial soil nutrient content. These observations likely reflect the larger input of C, alongside the dominance of more strongly lignified compounds, associated with SD compared to WD to achieve a constant N application rate. Our results also indicate that the two digestate fractions generated significantly different CUE. The application of SD led to increases in C_{micro} and positive values of CUE, whilst decreases in C_{micro} and negative values of CUE were observed following WD application. These findings emphasise the need to carefully plan the management of digestate in agricultural production systems, to minimise negative impacts on C storage within soils whilst maximising the agronomic value derived from digestate.

3.1 Introduction

Agricultural soil is the largest active terrestrial reservoir in the global carbon (C) cycle. However, some agricultural practices, including deep tillage, over-application of inorganic fertilisers and intensification, have significantly impacted soil structural, chemical and biological conditions, increasing carbon dioxide (CO_2) emissions from soil and reducing soil organic matter (SOM) content (FAO, 2017). In contrast, soil C stocks may be increased by the promotion of agricultural practices that sequester soil organic C (FAO, 2017; Rumpel & Kögel-Knabner, 2011), through fixing atmospheric CO_2 within soil following plant photosynthesis and the transfer of CO_2 to plant biomass, or through the addition of allochthonous organic matter to soil. Additional practices may also help to reduce the environmental impacts of agricultural production, including crop rotation, improved nutrient and water application practices and the reduction of tillage intensity (IPCC, 2014). However, due to microbial metabolism, the application of organic materials to agricultural soil may also result in the release of significant quantities of CO_2, methane (CH_4) or nitrous oxide (N_2O) to the atmosphere (WRAP, 2016).

Interest in the application of digestate, the residue remaining after anaerobic digestion, to agricultural soil has grown substantially given the potential agronomic value of this material. Digestate generally has a low C-to-N ratio (C:N), is rich in NH_4^+, P, K^+, Na^+, Mg^{2+} and other macronutrients, and can improve soil structure, water infiltration rate and water-holding capacity (García-Albacete, Tarquis, & Cartagena, 2014; Möller & Müller, 2012; Tambone et al., 2010). However, there are

significant uncertainties surrounding the impact of digestate application on the C cycle within agricultural soils. This is particularly true following solid–liquid separation and the application of different fractions of digestate to soil. Separation allows for differentiation of the total nutrient content of digestate into individual phases, enhancing the potential to match digestate application to crop nutrient requirements when compared with the whole fraction of digestate without separation (Marcato, Pinelli, Pouech, Winterton, & Guiresse, 2008). Whole digestate is a mixture of fibre and liquid, with high viscosity and low infiltration potential. It is generally rich in N, P, K^+ and other macronutrient elements that are present in plant-available forms and usually has a C:N<10 (Tambone et al., 2010). In contrast, the solid fraction is rich in total P (up to 90% of total P in whole digestate may be retained in the solid fraction), much present as water extractable P, alongside Ca^{2+}, Mg^{2+}, S and Mn, usually with a C:N>10 (Bachmann, Uptmoor, & Eichler-Löbermann, 2016; Hjorth, Christensen, Christensen, & Sommer, 2010; Lukehurst, Frost, & Seadi, 2010; Marcato et al., 2008; Panuccio, Attinà, Basile, Mallamaci, & Muscolo, 2016). The forms of organic C present in the whole and solid fractions of digestate can also differ substantially. The whole fraction has been shown to be a mixture of dissolved organic carbon (DOC), which is readily available to microorganisms after application to land, and lignin compounds. In contrast, the solid fraction is dominated by recalcitrant organic C compounds, including lignin, cutin, humic acids and other complex compounds, considered as humus precursors with high biological stability (Nkoa, 2014; Tambone, Genevini, D'Imporzano, & Adani, 2009) that can promote SOM accumulation.

The application of digestate as a fertiliser in agriculture may influence C metabolism by the soil microbial community, which biosynthesizes the C into compounds for growth and/or emits CO_2 through respiration. This balance dictates the carbon use efficiency (CUE), which may be defined as the efficiency of the biosynthesis of organic C from a source material relative to its respiration (Manzoni, Taylor, Richter, Porporato, & Ågren, 2012). Usually, when CUE is positive and high the soil microbial community utilizes a C source for biosynthesis and growth, favouring the anabolic pathway, leading to C stabilization in soil. In contrast, when CUE is low and/or negative, microbial utilization of a C source for biosynthesis is less efficient, the catabolic pathway is favoured, respiration rate and CO_2 production are enhanced and C sequestration in soil is reduced (Geyer, Kyker-Snowman, Grandy, & Frey, 2016; Wang & Post, 2012; Wang, Post, & Mayes, 2013). Many factors influence the CUE, including temperature, moisture, quality of the C source (e.g., C:N) and nutrient availability in soil. For example, Sinsabaugh, Manzoni, Moorhead, and Richter (2013) reported that application of an organic material to soil that is rich in recalcitrant C (often C:N>20), such as the solid fraction of digestate, can increase bacterial catabolism in order to produce extracellular enzymes to hydrolyse C compounds and, consequently, CO_2 is produced. In contrast, the addition of organic matter with C:N<20 to soil, such as the whole fraction of digestate, can promote bacterial biosynthesis of C and, consequently, reduce CO_2 production.

Soil nutrient availability, particularly the concentrations of N and P, may also influence CUE. When soil is not N or P limited relative to C (e.g., low soil C:N), CUE

tends to increase because bacteria seek to maintain a balanced intracellular composition between C and nutrients (Manzoni et al., 2012; Roller & Schmidt, 2015) and thus microbial biomass concentration tends to increase. However, when an organic material containing labile C (e.g., the whole fraction of digestate) is applied to a low nutrient soil (high soil C:N ratio and, potentially, N limitation) (Blagodatskaya, Blagodatsky, Anderson, & Kuzyakov, 2014; Moorhead & Sinsabaugh, 2006), bacteria tend to respire C that has been applied because maintenance respiration is increased. This is also true after application of poor-quality resources (e.g., recalcitrant compounds, such the solid fraction of digestate) to a stressed environment (e.g., low nutrient availability, high temperature or low water availability), because there is an increase in the cost of producing intra/extracellular catabolism under these conditions and an increase in CO_2 production (Malik, Puissant, Goodall, Allison, & Griffiths, 2019; Sinsabaugh, Hill, & Follstad Shah, 2009). Further, bacteria and fungi within the soil microbial community have potentially different effects on CUE. For example, fungi are able to degrade organic material with high C:N without emitting CO_2-C, thereby maintaining a high CUE, whereas bacteria are less efficient at degrading organic material with high C:N (Blagodatskaya & Kuzyakov, 2008). For bacteria, CUE also differs between r (growth strategists; high CUE) and K (competitive strategists; low CUE) communities (Keiblinger et al., 2010; Roller & Schmidt, 2015).

However, the impacts of digestate on the soil C cycle via microbial effects on CUE remain poorly understood, especially when different physical fractions of digestate

with varying nutrient form and stoichiometry are applied to soils. The differing composition of whole and solid digestate may influence soil bacterial and fungal communities differently, with potential effects on C cycling and CUE. There has also been insufficient research focussed on the interactions between digestate application and soil nutrient status, which has been considered as one of the main drivers influencing bacterial and fungal activity and, subsequently, soil C stocks and other soil health parameters. In this context, the research reported here tested the following hypotheses: (a) for soil at lower initial nutrient status, the application of either WD or SD stimulates microbial respiration and reduces CUE to a greater extent than for soil at higher initial nutrient status, (b) at low or high soil nutrient status, the application of WD will stimulate microbial respiration and reduce CUE compared to SD, and (c) the application of SD increases the fungal-to-bacterial ratio in soils at both low and high initial nutrient status, when compared to WD.

3.2 Materials and methods

3.2.1 Soil sampling and initial characterization

Soils were sampled from two fields adjacent to a commercial biogas plant (Cockerham Green Energy Ltd, North- west England, UK; latitude: 53.972, longitude: −2.822) on 17 September 2018. The two fields were selected to provide contrasting initial soil nutrient properties (Table 3.1) as driven by the management history of each field. Topsoil to 15 cm depth was sampled from each field using a gouge auger and following a “W” sampling protocol (Natural England, 2008), in which samples

from 20 points along a “W” were combined into a single integrated soil sample for each field. High nutrient soil (HN) was under grass production at the time of sampling and used for grazing and silage production during previous years. This field receives liquid digestate four times per year, with the last application occurring at the end of July 2018. The low nutrient soil (LN) was fallow grassland at the time of soil sampling and had never previously received digestate. Following collection and homogenization, soils were sieved through a 2 mm mesh and stored in sealed plastic bags at 4°C until the incubations began.

Table 3.1 Initial physicochemical characteristics of soils used in the microcosm incubations (mean values reported, ±1 standard error in parentheses, n = 3). P-values represent statistical differences between High nutrient and Low nutrient soils and "n.s" indicates no statistical difference.

Soil characteristics	**High nutrient soil**	**Low nutrient soil**	**p-value**
Bulk density (g cm^{-3})	1.54 (0.14)	1.48 (0.014)	n.s
pH water (1:5 w/v)	7.31 (0.035)	5.06 (0.018)	<0.001
NO_3^- (mg kg^{-1} DW soil)	71.05 (0.51)	66.66 (0.32)	<0.01
NH_4^+ (mg kg^{-1} DW soil)	0.47 (0.044)	1.94 (0.10)	<0.01
Olsen P (mg kg^{-1} DW soil)	40.66 (1.18)	10.42 (1.10)	<0.001
P index UK (Agriculture and Horticulture Development Board, 2017)	4	1	<0.001
Water extractable Total Organic C (mg kg^{-1} DW soil)	228.61 (14.23)	61.43 (0.76)	<0.001
Soil Tot C (mg C kg^{-1} DW soil)	50298.14 (68.49)	31817.73 (39.3)	<0.001
Soil Tot N (mg N kg^{-1} DW soil)	4396.73 (160.30)	2363.93 (199.82)	<0.001
TC:TN	11.46 (0.07)	13.68 (0.50)	<0.05

DM (%)	73.06 (0.10)	75.49 (0.02)	<0.05

DM (Dry Matter); Tot C (Total Carbon, non-acidified analysis); Tot N (Total Nitrogen); Water Tot C (Water Extractable Total Organic Carbon, acidified analysis); NH_4^+ (Ammonium); NO_3^- (Nitrate); P (Phosphorus); P index (mg L^{-1} P Olsen)

3.2.2 Digestate sampling and characterization

On 24 September 2018, whole and solid fractions of anaerobic digestate were collected from Cockerham Green Energy Ltd, following sampling protocols detailed by the Agriculture and Horticulture Development Board (2017), and stored at 4°C prior to the start of the incubations. Digestate from Cockerham Green Energy Ltd is fermented in a mesophilic, single-stage digester with a retention time of 50 days. The feedstock is livestock and poultry manure, co-digested with food waste, including wheat, potatoes, tea bags and whey. Whole digestate is unpasteurised and separated into liquid and solid fractions using a screw-press. The liquid fraction is collected in covered lagoons, whereas the solid fraction is stored in an uncovered open space. Whole digestate was sampled directly from the anaerobic digester before separation, whereas the solid fraction was sampled from material that had been stored for 7 days prior to collection. The two fractions of digestate were chosen to provide contrasting properties for the experiment (Table 3.2).

Table 3.2 Physio-chemical characteristics of whole and solid digestate used in the microcosm incubations (n=1)

Parameter in fresh weight (FW)	Whole digestate (WD)	Solid digestate (SD)
DM (%)	11.6	24.3
Organic Matter (%)	8.36	84.3
pH (1:6 w/v)	8.18	8.20
TN (mg kg^{-1} FW)	8500	4836
NH_4^+-N (mg kg^{-1} FW)	4921	752.81
TP (mg kg^{-1} FW)	2869	4209
TC (mg kg^{-1} FW)	37000	109107
TC:TN	4.35	22.56

DM (Dry Matter); TP (Total Phosphorus); TC (Total Carbon); TN (Total Nitrogen); NH_4^+-N (Ammonium Nitrogen)

3.2.3 Experimental design

A microcosm incubation was carried out between 8 and 30 October 2018, involving control (Ctr), whole digestate (WD) and solid digestate (SD) treatments. Each amendment was conducted in triplicate for both HN and LN soil types, with soil × amendment combinations placed randomly in amber and Duran bottles inside a temperature-, pressure-and moisture-controlled room in the dark. The WD and SD amendments were added to soils inside separate glass containers in order to achieve the same N application rate (170 kg N (as NH_4^+-N) ha^{-1} $year^{-1}$), after the Agriculture and Horticulture Development Board (2017). This resulted in the addition of c.12,500 mg kg^{-1} dry weight (DW) soil of C for SD and 625 mg kg^{-1} DW soil of C for WD treatments to both soils. Digestate fractions were mixed thoroughly with soil and then subdivided into Duran (for respirometry) or amber bottles (destructive samples) prior to the incubation.

The moisture content of the soils was set at 50% water-holding capacity (WHC) using milliQ water (>18.2 MΩ.cm at 25°C). Control soils were left unamended without any digestate addition and only received milliQ water in order to maintain 50% WHC. Respirometry measurements were carried out using a Micro-Oxymax Respirometer (Columbus Instruments International Corp., Columbus, OH, USA), with an automated 20-channel closed circuit and with two empty bottles used as analytical blanks. For respirometry samples, the respirometer maintained a constant moisture content throughout the incubation. The concentration of CO_2 in the headspace of each Duran bottle was monitored at a partial pressure of 1,063.9125 hPa and a

temperature of 23 ± 1°C, via a specialized GL 45 three-port connection at 2 h intervals, with emission rates of CO_2-C and cumulative CO_2-C expressed as a rate (mg C h^{-1}) and as a mass (mg C), respectively. In addition, a parallel set of destructive samples was prepared using amber bottles in order to monitor changes in soil properties through time. These destructive samples were analysed at 0, 1, 2, 3, 4, 7, 14 and 21 days (for the 21 day time-point, respirometry samples were destructively sampled). The moisture content of the destructive samples was checked daily by weighing the amber bottles without lids and adding milliQ water to maintain 50% WHC. The destructive samples were placed inside the same dark controlled room as the respirometry samples.

3.2.4 Soil analyses

Destructive soil samples were analysed for microbial biomass C (C_{micro}) and dissolved organic carbon (DOC). Additional samples were taken at 0 and 21 days for analysis of phospholipid fatty acid (PLFA) content. Extraction for C_{micro} was carried out following the chloroform fumigation method (Brookes et al., 1985; Vance, Brookes, & Jenkinson, 1987). Duplicate fresh soils were extracted with and without chloroform fumigation according to Brookes et al. (1985) and Vance et al. (1987) (1:5 w/v, 0.5 M K_2SO_4, pH ~ 7, filtered Whatman No 42). The determination of TC for the two sets of extracts was carried out using a TOC-L/TN Series Analyser (Shimadzu, Kyoto, Japan) based on a combustion-reduction method. Microbial biomass C was calculated as the difference in concentration between fumigated and

unfumigated samples, with subsequent correction by K_{ec} for C evolved as CO_2 (Brookes et al., 1985; Joergensen, 1995, 1996).

Fresh soil samples were extracted in milliQ water (1:10 w/v; 15 min shaking) for DOC analyses (Jones & Willett, 2006), filtered (Whatman No 42) and the extract was analysed using a TOC-L/TN Series Analyser (Shimadzu) after sample acidification to remove inorganic C.

The PLFA extraction was carried out as described by Quideau et al. (2016), using a three-stage extraction. Frozen soil (−80oC) was freeze-dried and between 1 and 1.5 g of soil was used for the extraction. Extracted samples were analysed using a Gas Chromatograph-FID (Agilent Technology 6890N, Santa Clara, CA, USA). A C13 (methyl tridecanoate) and C19 (methyl nonadecanoate) mixed standard was used as an internal standard in order to identify the range of the retention times of the PLFAs of interest.

Soil pH was determined on fresh soil samples (1:5 w/ v; 30 min shaking) using milliQ water. Air-dried soil samples were analysed for Olsen P as described by Murphy and Rilely (1962) and Olsen, Cole, Watanabe, and Dean (1954). Samples were extracted (1:20 w/v; 30 min shaking) with a 0.5 M $NaHCO_3$ solution, with pH adjusted to 8.5, and subsequently filtered (Whatman No 42). The extracted samples were analysed using a SEAL Autoanalyzer AA3 (Seal Analytical, Fareham, UK; Method No G-103-92 Rev1; Multitest Mt7/MT8) based on the molybdenum blue colorimetric reaction. Soil dry matter (DM) and loss-on-ignition (LOI) were determined using a gravimetric

method (Allen, 1989; Gardner, 1986). Approximately 12 g of fresh soil was oven-dried at 105°C for 48 h to constant weight to determine DW. Subsequently, around 1.5 g of oven-dried soil was heated at 550°C for 6 h in a muffle furnace, left to cool over-night and subsequently weighed to determine LOI. The TC (total carbon) and TN (total nitrogen) content of soils was determined using an automated Dumas procedure on a Carbo Erba NA 1500 analyser (Erba Science, Surrey, UK), working with 30 ± 1 mg of oven-dried and ball-milled soil. Fresh soil samples were also extracted for available N using 1 M KCl (1:5 w/v, 1 h shaking) (Bremmer, 1965; McTaggart & Smith, 1993) and filtered (Whatman No 42). The filtrate was subsequently analysed for NH_4^+ and NO_3^- content using a SEAL Autoanalyzer AA3 (Seal Analytical; Method No G-102-93 Rev 2; Multitest MT7/MT8) with two different colorimetric reactions (ISO 11732, 1997 and ISO 13395, 1996, respectively).

3.2.5 Calculations for % TC respired, CUE and statistical analysis

The % TC respired from soils after the addition of digestate was calculated as:

% TC respired at each time point = (cumulative CO_2-C produced at each time point/(TC present in the soil at day 0 + TC applied in digestate amendment)) * 100

where all C terms were expressed in mg.

The CUE was estimated as described by Frey, Gupta, Elliott, and Paustian (2001) and Tiemann and Billings (2011), using the following equation:

$$CUE=dBc/(dBc+\Sigma CO_2\text{-}C)$$

where dBc is the change in C_{micro} and ΣCO_2-C is the cumulative C lost through microbial respiration during the incubation, both expressed in mg C. For both WD and SD treatments, C_{micro} and ΣCO2-C were standardised by the Ctr treatment, in order to focus on the fate of C that was added to the soil with digestate, following Tiemann & Billings (2011). The CUE of Ctr treatments was not calculated, because no C was added to soils.

Statistical analyses were performed in R version 3.6.1 (R Core Team, 2019). One-way and two-way ANOVA was employed to assess the significance of the factors

'soil' (HN, LN) and 'digestate amendment' (Ctr, WD, SD) and their interaction. Levene's tests were used to check the homogeneity of variance assumption of ANOVA, with log_{10} or square root transformations applied to data where necessary. A Tukey-test (HDS) was employed to compare individual levels where a significant factor was identified in ANOVA. For CUE, a Kruskal-Wallis test was used to assess the significance of the factors soil type and digestate amendment.

Due to the non-linear nature of many response variables across the incubations, multivariate polynomial regression was used to model time × soil type × digestate amendment interactions. Time was treated as a numerical variable and expressed from 0 to 21 days. For C_{micro} and DOC, in order to fully capture the nonlinear nature of changes through time, a cubic polynomial regression was used, whilst for cumulative CO_2-C efflux, %TC respired and fungal:bacterial linear regression models were applied. Where significant regression models were identified, T-tests were performed on cumulative CO_2-C efflux, %TC respired and fungal:bacterial data in order to determine the nature of the time × soil type × digestate amendment interaction.

In all statistical analyses, p-values < 0.05 were deemed as significant, whilst p-values between 0.05 and 0.06 were marked as borderline significant after Hofmann & Meyer-Nieberg (2018). Residual plots (S-L, Q-Q, Residual-Leverage and Cook's distance - leverage) were employed to assess the quality of the model fits and the assumption of normally distributed residuals for ANOVA, as well as the presence of leverage points or outliers. Missing observations were excluded from the analysis

and no data imputation was performed. Clear outliers, assumed to represent sample error or contamination, were removed from the datasets prior to analysis.

3.3 Results

3.3.1 Influence of treatments on CO_2-C efflux from soils

Cumulative CO_2-C efflux from HN soils was significantly greater than from LN soils across the incubations ($p<0.001$) (Table 3.3).

Table 3.3 Summary of one-way and two-way ANOVA results from microcosm incubations

	Soil	Mean	Std error	p-value	**Digestate**	Mean	Std error	p-value	**Soil × digestate interaction**	Mean	Std error	p-value
CUE	HN	-0.087	0.038	n.s.	WD	-0.22^{a}	0.17					n.s.
	LN	-0.025	0.013		SD	0.11^{b}	0.05	0.015				
Cumulative CO_2-C (mg C kg DW soil^{-1})	HN	1382.18^{a}	133.9	0.0009	Ctr	738.02^{a}	132.2		HN	Ctr: $1247.67^{a,a}$	228.53	0.00007
	LN	942.62^{b}	125.3		WD	1250.56^{b}	156.5	0.00002		WD: $1323.85^{a,a}$	239.73	
					SD	1417.88^{b}	192.5			SD: $1413.04^{a,a}$	275.16	
									LN	Ctr: $228.88^{a,b}$	45.75	
										WD: $1178.26^{b,a}$	204.43	
										SD: $1420.72^{b,a}$	275.34	
% TC respired	HN	1.44	0.25	n.s.	Ctr	1.43^{a}	0.39		HN	Ctr: $2.22^{a,a}$	0.47	0.0001
	LN	1.6	0.34		WD	2.66^{b}	0.38	0.0002		WD: $2.06^{a,a}$	0.43	
					SD	2.38^{b}	0.4			SD: $1.25^{a,a}$	0.41	
									LN	Ctr: $0.63^{a,b}$	0.14	
										WD: $3.21^{b,a}$	0.63	
										SD: $3.18^{b,a}$	0.71	
C_{micro} (mg kg DW soil^{-1})	HN	796.31^{a}	24.38		Ctr	684.60^{a}	20.14	$4.3*10^{-10}$				n.s.
	LN	698.01^{b}	21.63	0.0007	WD	663.18^{a}	22.78					
					SD	891.38^{b}	30.93					
Fungal:bacterial	HN	0.11	0.0052	n.s.	Ctr	0.11^{a}	0.003	0.005				n.s.
	LN	0.11	0.0035		WD	0.11^{a}	0.0031					
					SD	0.13^{b}	0.0067					
DOC (mg kg DW soil^{-1})	HN	166.11^{a}	9.66	0.000002	Ctr	110.45^{a}	10.68	$4*10^{-9}$	HN	Ctr: $157.12^{a,a}$	15.68	0.000002
	LN	117.03^{b}	10.15		WD	120.15^{a}	10.87			WD: $161.93^{a,a}$	15.66	
					SD	194.12^{b}	12.72			SD: $179.30^{a,a}$	19.1	
									LN	Ctr: $63.79^{a,b}$	5.69	
										WD: $78.36^{a,b}$	9.36	
										SD: $208.94^{b,a}$	16.75	

Note: Columns from left to right describe effects of initial soil nutrient status (high [HN] vs. low [LN]); effects of digestate amendment (control [Ctr], whole digestate [WD], solid digestate [SD]); and interactions between soil nutrient status and digestate amendment. "n.s" represents effects that were not statistically significant (p > 0.05). Tukey tests were employed to determine differences between individual levels of soil type and digestate amendment, with significant differences between levels denoted using superscript letters. For interactions between soil type and digestate amendment, first superscript letter represents differences between digestate amendments within each soil type, and second superscript letter represents differences between soil type within each digestate amendment.

Further, digestate amendment exerted significant control on cumulative CO_2-C efflux ($p<0.0001$), with higher cumulative CO_2-C efflux observed after the application of digestate to soils compared to control treatments, in the order Ctr<WD≈SD. However, an interaction between soil type and digestate amendment was observed ($p<0.0001$), with significant increases in cumulative CO_2-C efflux after WD and SD application only occurring within LN soils and not within HN soils.

A significant three-way interaction between time, soil type and digestate amendment was also observed for cumulative CO_2-C efflux, as shown in Figure 3.1 ($p<0.0001$). Within the LN soil, both WD and SD amendments increased cumulative CO_2-C efflux rapidly and significantly through time when compared to the control treatment, reaching +563% (SD) and +377% (WD) at 21 days compared to fluxes in the control treatment. Further, SD and WD diverged significantly from each other from 14 days onwards. Within the HN soil, only the SD amendment generated significantly higher cumulative CO_2-C efflux and only from 14 days of the incubation onwards (+20% at 21 days when compared with Ctr), whereas WD and Ctr did not differ significantly.

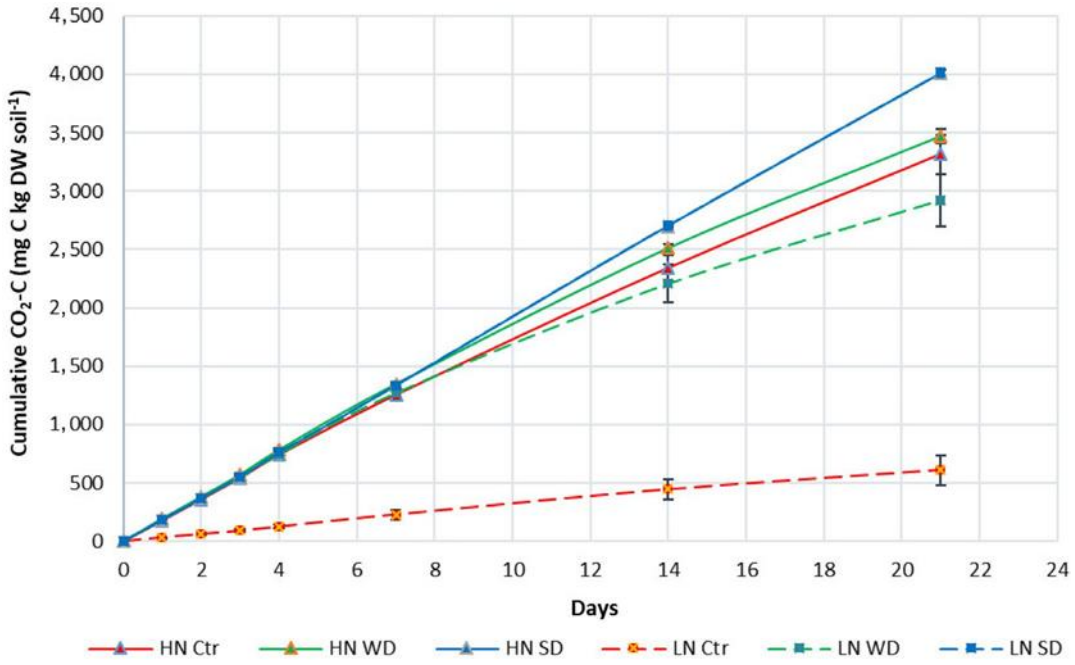

Figure 3.1 Cumulative CO_2-C produced from control (Ctr) soils or after addition of whole (WD) or solid (SD) fractions of digestate in soils with high (HN) or low (LN) initial nutrient status. HN × SD and LN × SD overlapping in the figure. Error bars ± 1 standard error.

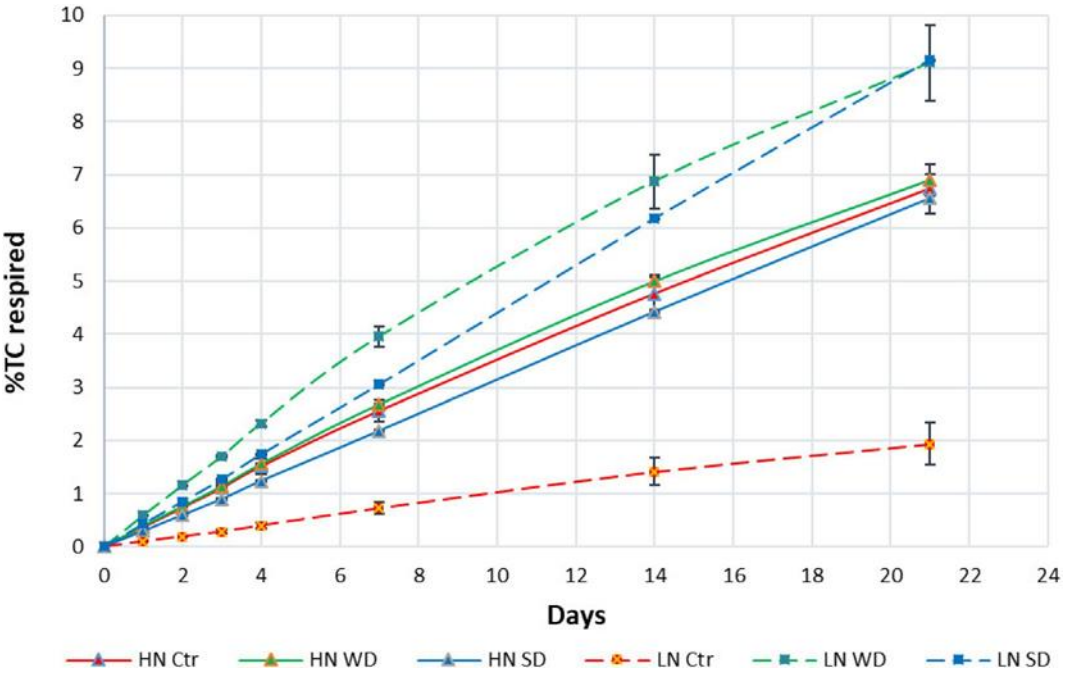

Figure 3.2 Percent total carbon (%TC) respired in control (Ctr) soils or after addition of whole (WD) or solid (SD) fractions of digestate in soils with high (HN) or low (LN) initial nutrient status. Error bars ± 1 standard error

Figure 3.2 reports the percentage of TC present in the combination of soil and digestate amendment that was respired as CO_2-C during the incubations. In contrast to cumulative CO_2-C efflux, no significant difference in %TC respired was observed between HN and LN soils. However, both WD and SD amendments resulted in significant increases in %TC respired compared to the Ctr ($p<0.001$), in the order Ctr<WD≈SD. Further, a significant interaction between soil and digestate amendment ($p<0.001$) indicated that significant increases in %TC respired following SD or WD application only occurred in the LN soil, consistent with observations related to cumulative CO_2-C efflux.

A highly significant three-way interaction between time, soil type and digestate amendment was observed ($p<0.0001$), indicating that the temporal pattern in %TC respired after the addition of digestate depended on the nature of the soil at the start of the incubation. In the HN soil, digestate amendments followed the same temporal trend as the Ctr treatment. However, in the LN soil the %TC respired increased significantly through time following both WD (+372% at 21 days) and SD (+369% at 21 days) applications compared to the control treatment, an effect that was observed from 1 day onwards in the incubations.

3.3.2 Influence of digestate amendments on the soil microbial community

Microbial biomass C was significantly higher in HN compared to LN soil ($p<0.001$). Further, C_{micro} increased significantly after the application of SD compared to either Ctr or WD treatments ($p<0.0001$), by +29% at 21 days in the HN soil and by +36% at 21 days in the LN soil compared to the Ctr treatment (Figure 3.3). No significant

interactions between soil type, digestate amendment or time were observed for C_{micro}, confirming that the significant increase following the application of SD was observed in both HN and LN soils and throughout the duration of the incubations.

Similarly to C_{micro}, the fungal-to-bacterial ratio increased significantly under the SD treatment compared to either the Ctr or WD treatments ($p<0.01$), an effect that was also consistent across both HN and LN soils.

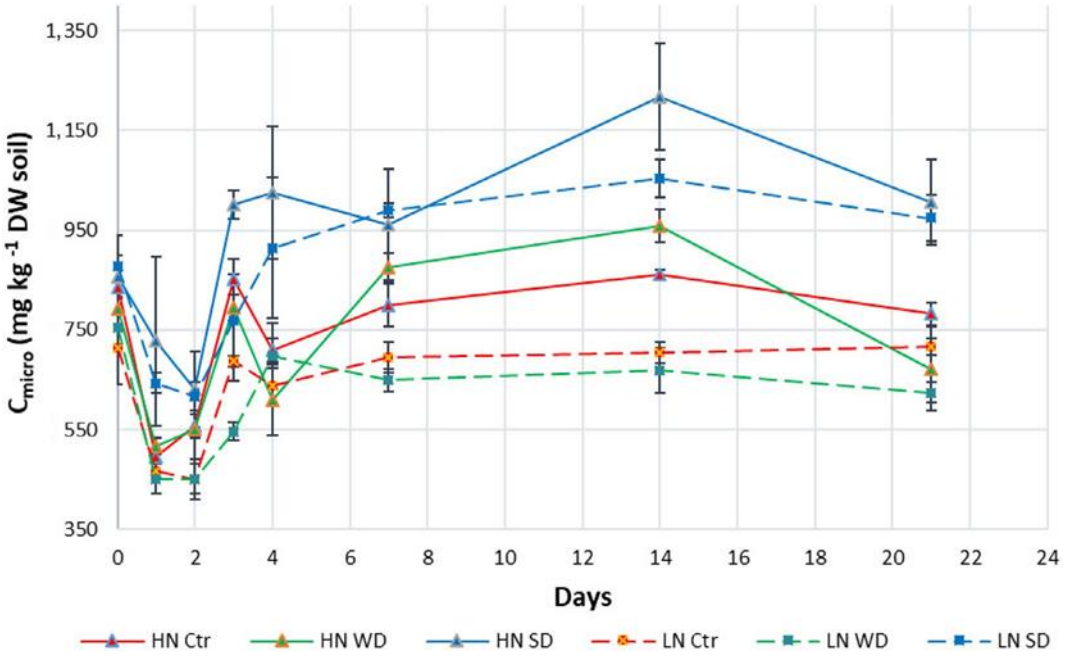

Figure 3.3 C_{micro} trends over time in control (Ctr) soils or after addition of whole (WD) or solid (SD) fractions of digestate with soils at high (HN) or low (LN) initial nutrient status. Error bars ± 1 standard error

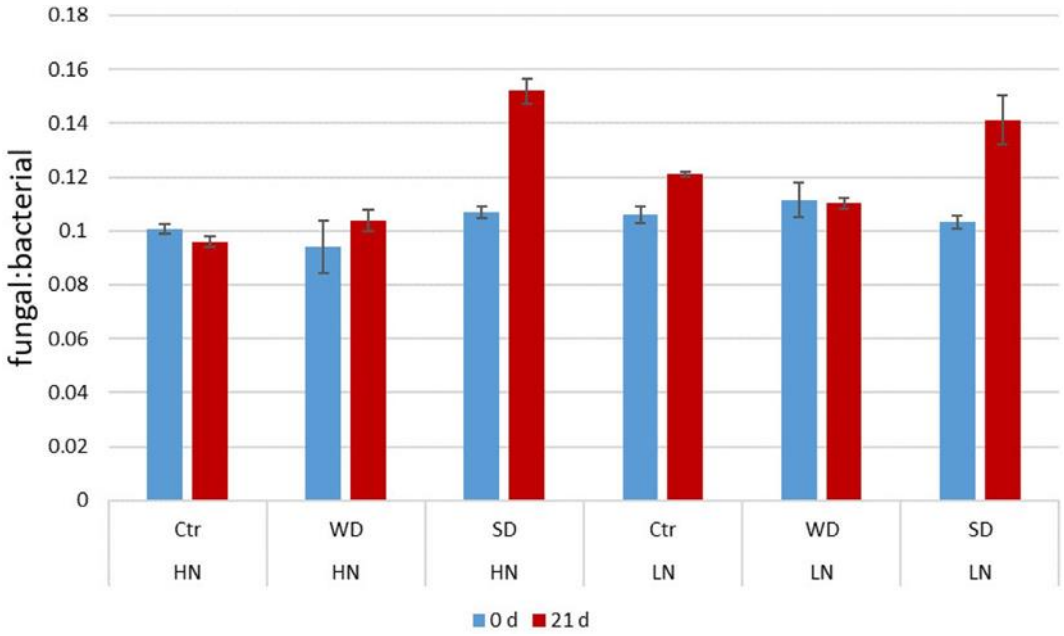

Figure 3.4 Fungal-to-bacterial ratio at 0 and 21 days in control (Ctr) soils or after addition of whole (WD) or solid (SD) fractions of digestate in soils with high (HN) or low (LN) initial nutrient status. Error bars ± 1 standard error

Further, time significantly affected the fungal-to-bacterial ratio (Figure 3.4), with a marginally significant three-way interaction observed between time, soil type and digestate amendment ($p<0.049$). The fungal-to- bacterial ratio increased significantly between 0 and 21 days following application of SD in both soils (+58% HN and +18% LN compared to Ctr), whereas the ratio decreased slightly (−8%) in the LN soil following the application of WD compared to the control ($p = 0.05$).

3.3.3 Influence of digestate amendments on dissolved organic carbon concentration

The concentration of water-extractable DOC was significantly higher in HN compared to LN soils ($p<0.0001$). Further, the application of SD to soils resulted in a significant increase in the concentration of water- extractable DOC, compared to either WD or Ctr treatments ($p<0.0001$). However, the impact of SD application

differed between soil types, with a significant increase in DOC concentration following SD application only observed in the LN soil (Figure 3.5). No interaction between time, soil type and digestate amendment was observed with respect to DOC concentration.

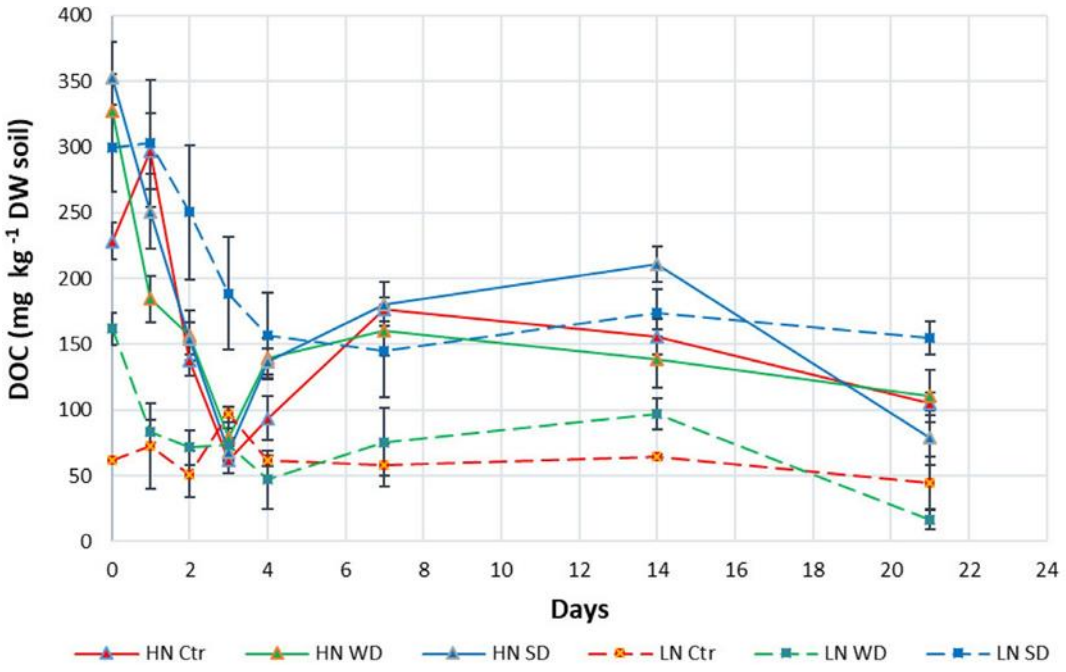

Figure 3.5 Dissolved organic carbon trends through time in control (Ctr) soils or after addition of whole (WD) or solid (SD) fractions of digestate in soils with high (HN) or low (LN) initial nutrient status. Error bars ± 1 standard error

3.3.4 Estimation of CUE after digestate amendment

Table 3.4 reports the CUE for each combination of soil type and digestate amendment used within the incubation reported here.

Table 3.4 Carbon use efficiency (CUE) following whole (WD) and solid fraction (SD) digestate amendments in high nutrient (HN) or low nutrient (LN) soils (mean values reported, ± 1 standard error in parentheses, n = 3)

Amendment	Estimation of CUE
HN × WD	-0.37 (0.33)
HN × SD	0.20 (0.050)
LN × WD	-0.07 (0.035)
LN × SD	0.02 (0.042)

No significant difference in CUE was observed between the two soil types. However, digestate amendment exerted significant control on CUE ($p<0.05$), with positive values of CUE observed following the application of SD and negative values after application of WD to soils; these effects were consistent across the two soil types used in the incubations.

3.4 Discussion

The application of digestate strongly influenced the C cycle within the soils examined during this research. This was evidenced by significant changes in the loss of C via gaseous pathways, the production of water-soluble DOC, and the biomass and composition of the soil microbial community. However, for many parameters the impact of digestate application depended on the initial soil nutrient status, on the physical fraction of digestate that was applied, and on time across the 21 days incubation. It should be noted that the history of soil management within the HN and LN soils is likely to have driven different responses between these soils to the treatments applied in the experiments reported here. For example, past digestate application to the HN soil may have been responsible for differences in microbial community composition and functional traits, compared to the LN soil. Further, our experimental system did not include the input of labile C to soil from root exudates that may alter microbial requirements for digestate-derived C. Future research will be required in order to examine the interactions within plant-microbial-soil systems, including the net impacts of these interactions on the fate of C derived from inputs of digestate to agricultural soil, and the impacts of a wider range of soil management histories.

3.4.1 The influence of digestate application on CO_2-C efflux

The efflux of CO_2-C from soil, whether expressed as an absolute flux or as a proportion of the TC within the combination of soil and digestate, increased significantly following the application of digestate. This observation is consistent with both previous laboratory and field research (e.g., Johansen, Carter, Jensen, Hauggard & Ambus, 2013; Pezzolla et al., 2012; WRAP, 2016), spanning grassland and arable soils. For example, field experiments have reported an increase in cumulative CO_2 efflux occurring across a 12-month period following four whole digestate applications (WRAP, 2016) and across a 5-month period following three applications of whole digestate (Pezzolla et al., 2012). Further, a 9-day laboratory experiment on arable soil revealed a two-fold increase in cumulative CO_2-C efflux after whole digestate addition when compared with untreated soil (Johansen et al., 2013). Although the research we report above used digestate from a single feedstock, it should also be noted that some past research has demonstrated significant effects on CO_2 efflux associated with variation in digestate feedstock and post-digestion processing (i.e. separation) techniques (e.g., Askri, Laville, & Tre, 2016). These variables were not incorporated within the experimental system used in the research reported here. The data reported above confirm that CO_2-C efflux was influenced by a significant interaction between soil type and digestate, in which increases in this gaseous flux of C following either WD or SD application only occurred in the LN soil. Increases in CO_2-C efflux following digestate application are partly consistent with de la Fuente, Alburquerque, Clemente, and Bernal (2013) and Grigatti, Di Girolamo, Chincarini, Ciavatta, and Barbanti (2011), who report

mineralization rates after the application of different fractions of digestate and their effects on CO_2-C efflux. However, de la Fuente et al. (2013) and Grigatti et al. (2011) report higher CO_2-C efflux following the application of SD compared to WD, whereas in the research reported here CO_2-C efflux did not differ significantly between the two fractions of digestate. It should be noted that the research of de la Fuente et al. (2013) involved a calcareous soil with nutrient content similar to the HN soils used in our research, whereas Grigatti et al. (2011) also used a soil more similar in nutrient content to the HN compared to LN soil used in the current research. Differences in soil type may help to explain why no significant difference in CO_2-C efflux was observed between SD and WD within the LN soil in the research reported above. However, further work would be required in order to understand why similar variation in CO_2-C fluxes after application of different fractions of digestate were not observed in the HN soils.

The efflux of CO_2-C increased rapidly from the early stages of the incubations following the application of either SD or WD to the LN soil, whether expressed as cumulative CO_2-C or as a percentage of TC present in the soil-digestate system. The effects of digestate application in the LN soil are likely to reflect the activation of dormant bacteria and stimulation of maintenance respiration after the application of either fraction of digestate (Mondini, Cayuela, Sanchez-Monedero, Roig, & Brookes, 2006). In the LN soil, rapid increases in bacterial catabolism are likely to have followed the application of WD due to the input of readily available DOC, suggesting that this C source may have been utilized quickly for enzyme production

and maintenance of respiration within a few days after application, consistent with other research (e.g., Wang et al., 2013; Wang & Post, 2012). After exhaustion of readily available C in WD, bacteria may have started to mine SOM present in the soil to meet continued demand for nutrients (Fontaine et al., 2011; Fontaine, Bardoux, Abbadie, & Mariotti, 2004), or alternatively turnover of the bacterial community may have occurred through the course of the incubation (Blagodatskaya, Blagodatsky, Anderson, & Kuzyakov, 2007), consistent with negative CUEs following the application of WD. However, the increase in CO_2-C efflux was higher and more persistent following the application of SD to the LN soil, possibly because fungal degradation of recalcitrant C compounds in SD produced C by-products that were subsequently consumed by bacterial catabolism. Alternatively, bacteria may have invested directly in enzymatic degradation of recalcitrant C such as lignin within SD, as reported by Sierra (2012). In turn, this is likely to have resulted in prolonged increases in respiration and CO_2-C efflux, consistent with Fontaine, Mariotti, and Abbadie (2003), Sinsabaugh et al. (2013) and Winogradzky (1924).

In contrast, within the HN soil, only during the later stages of the experiment and only after SD application were increases in CO_2-C efflux observed, and only when CO_2-C was expressed as a cumulative flux rather than as a percentage of TC present in the system. Following exhaustion of readily available C during the earlier stages of the incubation, by-products from fungal or bacterial degradation of recalcitrant C within SD are likely to have supported the higher efflux of CO_2-C from bacterial respiration towards the end of the incubation (Six, Frey, Thiet, & Batten, 2006). In

contrast, rapid exhaustion of readily available C, combined with the absence of an input of more recalcitrant C in WD, meant that CO_2-C efflux under this treatment did not differ significantly compared to the control within the HN soil.

Varying effects of digestate application on CO_2-C efflux between HN and LN soils are also likely to reflect differences in physicochemical conditions between the two soil types that influenced microbial metabolic responses to the input of resources within the digestate (e.g., Larsson, Vonstockar, Marison, & Gustafsson, 1995; Manzoni et al., 2012; Russell & Cook, 1995). Within the HN soil, existing neutral soil pH, higher C_{micro}, and DOC and lower C:N meant that the changes in microbial respiration following digestate input were relatively small compared to the control soil treatment. In contrast, the adverse soil conditions in the LN soil (low pH, C_{micro}, DOC and nutrient concentration) created an environment in which respiration of CO_2 from control soils was relatively low, and in which activation of dormant bacteria and subsequent increases in respiration followed the application of resources within both WD and SD (Mondini et al., 2006).

3.4.2 Changes in the soil microbial community following digestate application

Both C_{micro} and the fungal to bacterial ratio increased significantly following the application of SD, a pattern that was consistent across both HN and LN soils. Increases in C_{micro} following the application of SD were likely to be driven by higher inputs of TC compared to the WD treatment, in order to achieve a consistent N application rate across both fractions of digestate. The additional input of C resources allowed greater opportunity for biosynthesis and the accumulation of C

within new soil microbial biomass under the SD treatment. These observations related to C_{micro} are supported by other research that has examined the impact of digestate application on the soil microbial community. For example, de la Fuente et al. (2013) report increases in C_{micro} only 7 days after the application of SD, driven by the high TC applied to soil with this fraction of digestate. Further, Chen et al. (2012) carried out a 21-day incubation and report an increase in C_{micro} that was related to a shift from r-strategists to K-strategists in soil that received biogas residues.

The fungal-to-bacterial ratio of control HN and LN soils indicated a microbial community that was dominated by bacteria, consistent with other research focused on agricultural grasslands (Bardgett, Frankland, & Whittaker, 1993; Bardgett, Hobbs, & Frostegård, 1996; Bardgett & Leemans, 1995). However, this ratio increased significantly following the application of SD to both soils used in the incubations reported here, driven by an increase in fungal PLFA rather than a decrease in bacterial PLFA. This observation is likely to reflect the significant input of more recalcitrant C compounds, such as lignin, associated with SD compared to WD (Nkoa, 2014). Hydrolysis of these C compounds has been shown to rely predominantly on the action of fungi rather than bacteria (Hammel, 1997), consistent with the increase in total fungal PLFA through the incubations reported here following the application of SD and in agreement with other research (e.g., Rousk & Bååth, 2011; Walsh, Rousk, Edwards-Jones, Jones, & Williams, 2012). Fungal-produced C by-products following degradation of recalcitrant C within SD may also have sustained bacterial production (e.g., Bugg, Ahmad, Hardiman, &

Rahmanpour, 2011; Dashtban, Schraft, Syed, & Qin, 2010; Ruttimann, Vicuna, Mozuch, & Kirk, 1991), including through generating a flush of DOC, which is available for the microbial community (Möller, Miller, & Kjöller, 1999). In contrast, the limited input of recalcitrant C following WD application produced no significant change in fungal-to-bacterial ratio within the HN soil, alongside a relatively small and marginally significant decrease in this ratio within the LN soil, reflecting a decrease in total fungal PLFA within the microbial community under this treatment.

Although the concentration of DOC was significantly greater in soil following the application of SD compared to either Ctr or WD treatments, this effect was only observed within LN and not within HN soils. Within the HN soil, DOC generated following the application of SD appeared to be efficiently metabolized by the microbial community, evidenced by an increase in C_{micro} but no increase in CO_2-C efflux compared to control soils. In contrast, the application of SD to the LN soil increased DOC concentrations by the end of the incubation. This is likely to reflect unfavourable conditions for the microbial community within the LN soil, including low pH and nutrient availability, which can limit microbial metabolism of DOC, as noted in previous research (David, Vance, Rissing, & Stevenson, 1989; Guggenberger, Glaser, & Zech, 1994; Jardine, Weber, & J. F. M., 1989; Vance & David, 1989).

3.4.3 Changes in CUE following digestate application

Carbon use efficiency varied significantly between the digestate treatments used in the experiments reported here, with consistent patterns observed across both soil types. The application of WD resulted in negative values of CUE, driven by greater decreases in C_{micro} and by increased CO_2-C fluxes compared to control treatments during the incubations. Decreases in C_{micro} may reflect grazing by protozoa and/or microbial turnover (Frey et al., 2001). The input of readily degradable C substrates within WD is likely to have promoted the catabolic pathway and maintenance of respiration of bacteria to a greater extent compared to the anabolic pathway, resulting in enhanced CO_2-C effluxes and decreased biosynthesis of C within microbial cells (Geyer et al., 2016; Manzoni et al., 2012). The magnitude of the effect of WD on CUE was more pronounced in HN compared to LN soils. This observation reflects the smaller cumulative CO_2-C efflux in HN soils compared to the respective controls, generating a more negative value of CUE following the application of WD. Although C_{micro} also decreased following the application of WD to LN soils, the relatively large increase in CO_2-C efflux compared to control soils resulted in a smaller value of CUE for LN soils compared to the HN soils. These observations emphasize the potential for application of WD to result in net decreases in C_{micro}, rather than net accumulation of C within soil microbial biomass, due to the stimulation of maintenance respiration and associated utilization of C from both native soil and substrate pools (e.g., Blagodatskaya et al., 2014; Moorhead & Sinsabaugh, 2006).

In contrast to WD, positive values of CUE were observed following the application of SD to both soil types, with CUE in the range 0–0.55, as reported for soil microbial communities by Sinsabaugh et al. (2013), who accounted for substrate C:N, the assimilation efficiency of N, bacterial C:N and a CUE_{max} in their research. However, it is notable that a higher CUE was observed after application of SD to HN compared to LN soils, reflecting substantial increases in C_{micro} and relatively small increases in cumulative CO_2-C efflux in HN soils following SD application, compared to control soils. Although C_{micro} also increased in LN soils after the application of SD compared to control soils, the increases in CO_2-C efflux were far more pronounced, resulting in lower values of CUE compared to HN soils. Increase in C_{micro} following SD application to soils indicates the potential for net accumulation of C within soil microbial biomass, in particular associated with increases in soil fungal community anabolism and biomass (Keiblinger et al., 2010). However, it should also be recognized that cumulative CO_2-C fluxes following the application of SD exceeded those under all other treatments used in our experiments. Therefore, application of SD to soils can potentially generate adverse effects on absolute fluxes of CO_2 to the atmosphere, whilst at the same time contributing positively to the accumulation of C within soils.

3.5 Conclusions

The research reported here provides important new insights into how changes in the soil C cycle may follow the application of digestate to agricultural grasslands. The precise nature of these impacts is contingent on the physical fraction of digestate applied to land and on the nutrient status of the soils that receive digestate. The solid fraction of digestate drove substantial increases in CO_2-C efflux, an effect that appears to be inversely related to soil nutrient status. Microbial biomass C and the fungal-to-bacterial ratio in soil also increased following the application of the solid fraction of digestate, regardless of initial soil nutrient status. The effects of applying whole digestate to soil were more variable. Although CO_2-C efflux increased following the application of whole digestate to soil at low initial nutrient status, no significant changes in microbial biomass C or in fungal-to-bacterial ratio followed the application of whole digestate. Carbon use efficiency in soils receiving solid digestate was positive, indicating the potential for C accumulation within soil microbial biomass. However, the accumulation of C within soil was exceeded by the additional C lost from soils via CO_2-C efflux. Further, CUE was negative in both soil types following treatment with whole digestate, driven by decreases in C stored within microbial biomass and loss of C as CO_2-C.

These findings emphasize the need to carefully plan the management of digestate in agricultural production systems, in order to minimize negative impacts on C storage within soils whilst maximizing the agronomic value derived from digestate. Future research should seek to examine the impacts of a broader range of digestate

fractions (whole, liquid, solid) on the soil C cycle in long-term field experiments, including the effects of plant–soil interactions and longer-term changes in CUE and SOM. In addition, research should seek to quantify the impacts of digestate application on other environmental parameters of concern, including the emission of greenhouse gases beyond CO_2 and the potential leaching of pollutants into the subsurface.

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4 Greenhouse gas emissions following the application of anaerobic digestate fractions to agricultural grassland soils

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Abstract

Digestate application to agricultural land has the potential to increase greenhouse gas (GHG) emissions from soil (e.g. carbon dioxide [CO_2], methane [CH_4] and nitrous oxide [N_2O]). However, the impact of different digestate fractions on bacterial metabolism and subsequent GHG production remains unclear, especially during application to soils with contrasting nutrient properties. Moreover, little research has been done on the manipulation of total C:N during application of digestate to land and the consequent impacts on bacterial catabolic responses. Therefore, during the research reported here, the impacts on GHG emissions of different

digestate fractions (whole [WD], liquid [LD] and solid [SD]) applied based on recommended nitrogen (N) inputs to two grassland soils with contrasting nutrient status (high [HN] vs low [LN]) was investigated during a 7-day incubation. Compared to control treatments, GHG efflux increased following application of digestate fractions, although responses to digestate fractions differed according to the soil nutrient status. All treatments applied to the high nutrient soil similarly increased the cumulative CH_4-C and N_2O-N fluxes, whilst CO_2-C flux mainly increased after WD application. Conversely, SD application to the low nutrient soil limited the emission of CO_2-C and CH_4-C compared to WD and LD, whilst it increased the emission of N_2O-N compared to WD and LD in the low nutrient soil. Furthermore, when WD and LD were applied in an additional experiment designed to achieve a common C input (equivalent to that of SD at the previous N loading rate), GHG emissions increased substantially for both soils, especially following LD application. These findings highlight the need to plan the land application of different fractions of digestate carefully based on the soil nutrient status and to follow specific N application rates, in order to reduce the emissions of GHGs and the potential environmental impacts associated with these emissions.

4.1 Introduction

Interest in the utilisation of anaerobic digestate as an organic amendment in agriculture has grown over the past decade (Dahlin et al. 2015; Saveyn & Eder 2014), driven by a number of potential benefits associated with applying this material to land (Makádi et al., 2012). For example, the input of digestate to agricultural soil has been shown to increase soil organic matter (SOM) content, to supply macronutrients including nitrogen (N), phosphorus (P) and potassium (K) which are essential for crop yields (Tambone et al., 2010), and to improve the structure, the water infiltration and water-holding capacities of soil (García-Albacete et al., 2014). However, sub-optimum application of digestate to land can result in leaching or runoff of nutrients and may enhance the emission of the three primary greenhouse gases (GHGs), carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), from agricultural soils (WRAP, 2016; Svoboda et al., 2013; Möller, 2015). Despite this risk, the controls exerted on GHG emissions from agricultural soils by factors such as digestate application rate, digestate fraction and soil nutrient status remain poorly constrained.

Agriculture has been identified as one of the largest sectors for the emission of GHGs, responsible for approximately 10-14% of total GHG emissions into the atmosphere globally (IPCC, 2014; Johnson et al., 2007). The major practices responsible for increasing GHG emissions from agriculture are linked to intensification of fertiliser use and associated inorganic fertiliser production, enteric fermentation from ruminants, manure management, utilization of fossil fuels, field

burning and conversion of land to arable crops. These practices can be exacerbated by certain soil conditions that promote GHG emissions, including soil texture (e.g. clay soils tend to increase GHG emissions relative to sandy soils), climate zone (wet increases GHG emissions relative to dry climates and soils) and nutrient status (Shakoor et al., 2020b; Johnson et al., 2007). However, certain agricultural practices may help to reduce GHG emissions from agriculture, such as crop rotation (e.g. corn-soybean-wheat rotations), specific nutrient and water application practices (i.e. precision agriculture, slow release fertiliser application, respect of fertiliser application rates and time of application), reduction of tillage, incorporation of plant residues, or application of digestate/biochar to soil instead of inorganic fertiliser application (IPCC, 2014; Liu et al., 2019; Behnk et al., 2018).

Although some research has shown that the application of organic materials to agricultural soil compared to inorganic fertilisers may increase the emission of GHGs to the atmosphere (e.g. Zhou et al, 2017), GHG emission rates after the application of different organic materials to land are highly variable. These rates depend on factors that include the initial application rate of organic materials, the application method (e.g. burial or surface dressing), the total C:N of the material applied, the initial soil properties (e.g. pH, moisture content, texture) and the nutrient status of a soil (Shakoora et al., 2020a). Increasing the application rate of an organic material can increase the input of available C and N to soil and therefore potentially increase GHG emissions (Tilvikiene et al., 2020; Dietrich et al., 2020). However, this is also dependent on the C and N quality of the organic material applied, since organic

materials with a low total C:N (e.g. slurry) usually supply labile C and available N to soil microbial communities, whilst organic materials with a higher total C:N usually contain more recalcitrant carbon compounds that can reduce bacterial respiration and the consequent emission of GHGs (Askri et al., 2016; Yagi and Minami, 1990). Furthermore, soil organic carbon (SOC), N content and texture play major roles in controlling GHG emissions. For example, soils with high nutrient status and labile C content have been shown to increase GHG emissions when compared to a fallow soil with lower nutrient and labile C availability (Maljanen et al., 2007). This is likely to be associated with the high SOC and nutrient composition of well-fertilised soils, which creates a favourable environment for bacterial respiration and the creation of anaerobic microsites that promote N_2O-N and CH_4-C formation (Koops et al., 1996; Eickenscheidt et al., 2014). In contrast, soils with low nutrient status often create an adverse environment for bacterial metabolism, due to the low organic C, low pH, and therefore low bacterial biomass, respiration and consequently GHGs production (Russell & Cook, 1995).

With respect to the application of digestate to agricultural soils, the application of different fractions of digestate that are characterised by variable total C:N may significantly affect subsequent GHG emissions. Whole digestate (WD) generally has a low total C:N, contains fibrous material (e.g. recalcitrant lignin compounds) but also dissolved organic carbon (DOC) which is readily available to microorganisms after application to land. This fraction of digestate is also rich in NH_4^+ and other macronutrients, such as Ca^{2+}, K^+, and S (García-Albacete et al., 2014; Möller &

Müller, 2012; Tambone et al., 2010). The solid fraction of digestate (SD) usually has a total C:N >10 (Bachmann et al., 2016; Hjorth et al., 2010; Lukehurst et al., 2010; Marcato et al., 2008; Panuccio et al., 2016) and is dominated by recalcitrant organic C compounds, such as lignin, cutin, humic acids and other complex compounds, considered as humus precursors with high biological stability (Nkoa, 2014; Tambone et al., 2009) that can promote SOM accumulation. In contrast, the liquid fraction of digestate (LD) typically has a total C:N <10, is rich in NH_4^+ (up to 80% of NH_4^+ retained in the liquid fraction after solid-liquid separation) and C is mainly present as DOC (Akhiar et al., 2017).

When these different digestate fractions are applied to soil, bacterial respiration can be stimulated thereby increasing the production of GHGs (WRAP, 2016). The release of CO_2-C from soils is associated with aerobic bacterial respiration through the decomposition of organic material and native soil organic matter (Drigo et al., 2008), CH_4-C is mainly produced through anaerobic decomposition of organic materials applied to soil and native soil organic matter (Praeg et al., 2016) and N_2O-N is produced via denitrification or, depending on O_2 availability, during nitrification. However, the production of these GHGs is highly dependent on soil fertility, such as a soil low in nutrients including carbon (C), mineral N and available K versus a soil high in these nutrients, alongside the physical fraction of digestate applied to soil (Cattin et al., 2021). For example, the application of labile C within LD and WD fractions of digestate may stimulate maintenance respiration and increase CO_2-C emissions in a soil low in nutrients (Blagodatskaya et al., 2014; Moorhead &

Sinsabaugh, 2006). In contrast, the application of LD and WD fractions of digestate to a soil at higher initial nutrient status may increase biosynthesis of labile C and therefore lead to a lower CO_2-C emissions (Manzoni et al., 2012).

Few studies have focussed on the biogeochemical mechanisms of CH_4-C production after application of different physical fractions of digestate to agricultural soil. However, previous research based on manure and slurry application to such soils suggests that digestate application may increase CH_4-C production due to the creation of anaerobic microsites within soil aggregates, with these effects differing depending on soil initial C status, or through release of native CH_4-C contained in digestate (Bayer et al., 2012; Smith et al., 1985). The input of organic material with a relatively low total C:N (e.g. WD and LD) to soils with high labile C availability may lead to rapid increases in CH_4-C emissions, since methanogenic bacteria are supplied with additional oxidisable organic C (Baggs et al., 2006; Topp and Pattey, 1997; Pezzolla et al., 2012). In contrast, the application of available C compounds to soils containing lower concentrations of labile C (e.g. fallow soils), may increase CH_4-C emissions over the longer-term, but likely only if mining of existing SOM is triggered by the application of the organic material (Lipson et al., 2001; Fontaine et al., 2003; Isam et al., 2015).

Similarly, N_2O-N production during either nitrification or denitrification is strongly influenced by the C quality and N availability of the organic material applied and by the N status of the soil (Firestone et al., 1989; Weier et al., 1993). For example, application of material rich in available C and N to a soil low in NO_3^- concentration

can result in rapid bacterial N immobilization via biosynthesis, which in turn can lead to a decrease in N_2O emissions (Chadwick et al. 2000; Velthof et al., 2003). In contrast, the addition of materials high in available C and N to a soil rich in oxidised N and labile C can supply nitrifying or denitrifying bacteria with sufficient NO_3^- for the subsequent production of high amounts of N_2O-N (Johansen et al., 2013; Senbayram et al., 2009).

Regarding the application of recalcitrant C compounds with total C:N>20 (e.g. the solid fraction of digestate) to a high nutrient soil, this usually involves the production of complex enzymes for degrading the lignin compounds and consequently the stimulation of bacterial catabolism, which in turn can increase CO_2-C emissions through respiration (Cattin et al., 2021). This increase in respiration rates consequently creates anaerobic microsites within soil particles and the presence of high soil carbon content/organic material can enhance the CH_4-C emission through methanogenesis and N_2O-N emission through denitrification processes (Dietrich et al., 2012; García-Ruiz and Baggs, 2007; Wulf et al., 2002). In contrast, in a low nutrient soil the respiration rates increase because of activation of dormant bacteria and the increase in cost of producing intra/extracellular catabolism due to the addition of the C compounds to an environment which is strongly depleted in organic C and nutrients (Malik et al., 2019; Sinsabaugh et al., 2009). In addition, the presence of long-lasting C compounds to a low nutrient soil supply bacteria with C source in the long term, thereby increasing CH_4-C and N_2O-N production (Dietrich et al., 2020).

The impacts of digestate application on GHG emissions from agricultural soils remain poorly understood, especially when different physical fractions of digestate are used. There has also been insufficient research focussed on GHG emissions after digestate application to soils differing in initial nutrient status which, based on past research focussed on slurry and manure application, is likely to be a major factor influencing CO_2, CH_4 and N_2O emissions. In this context, the research reported here examined the following research questions: i) how do different digestate application rates affect GHG emissions from grassland soils?; ii) how does the application of different physical fractions of digestate influence GHG emissions from grassland soils?; and iii) how does soil nutrient status, in combination with digestate application, influence GHG emissions from grassland soils?

4.2 Materials and Methods

4.2.1 Soil sampling and initial soil characterization

Soils for the incubations reported below (section 4.2.3) were collected from two fields adjacent to a commercial biogas plant (Cockerham Green Energy Ltd, northwest England, UK; latitude: 53.972, longitude: -2.822) on 13th May 2019. The two fields were selected to provide contrasting initial soil nutrient status as driven by the management history of each field (Table 4.1, section 4.3.1). Topsoil to 15 cm depth was sampled from each field using a gouge auger and following a 'W' sampling protocol (Natural England, 2008). Within each field, gouge auger samples from 20

points along the 'W' were collected and split between two separate bags for storage and subsequent processing.

The high nutrient soil (HN) was under grass production at the time of sampling and used for grazing and silage production over multiple years. This field received liquid digestate four times per year with the last application before soil sampling at the end of March 2019. The low nutrient soil (LN) was a fallow soil at the time of sampling and had never previously received digestate. Following collection and homogenisation, soils were sieved through a 2 mm mesh and stored in sealed plastic bags at 4 °C before the incubations began. Soils were left to acclimatise to ambient (summer) field temperatures for two days at 23°C prior to the start of each incubation.

Soils were characterised before the start of each incubation for bulk density, water extractable N, dissolved organic C (DOC), pH, soil dry matter (DM), Loss-on-ignition (LOI), TC and TN. Bulk density was determined by taking an intact core of known volume from the field, which was dried at 105°C for 24h and processed as described by Rai et al. (2017). Water extractable N and DOC were determined as described by Jones & Willett (2006), using Milli-Q water (>18.2 MΩ.cm at 25°C; 1:10 w/v, 15 minutes shaking) and filtration of the resulting extract through a Whatman No. 42 filter paper. A subsample of the filtered extract was analysed for DOC using a TOC-L/TN Series Analyser (Shimadzu, Japan) after sample acidification to remove inorganic C. The remaining filtered extract was then analysed for NH_4^+ and NO_3^- using a SEAL Autoanalyzer AA3 (Seal Analytical, UK; Method No G-102-93 Rev 2;

Multitest MT7/MT8) with two different colorimetric reactions (ISO 11732, 1997 and ISO 13395, 1996 respectively).

Soil pH was determined on fresh soil samples (1:5 w/v; 30 minutes shaking) using Milli-Q water, whilst soil DM and loss on ignition (LOI) were determined gravimetrically (Allen, 1989; Gardner, 1986). Approximately 12g of fresh soil was oven-dried at 105°C for 48 h to constant weight to determine the dry matter (DM). Subsequently, around 1.5g of oven-dried soil was heated at 550°C for 6h in a muffle furnace, left to cool overnight and subsequently weighed to determine LOI.

The TC and TN content of soils was determined using an automated Dumas procedure on a Vario EL cube (Elementar, DE), working with 20±1 mg of oven-dried and ball-milled soil.

4.2.2 Digestate sampling and characterization

On 20th May 2019, whole, solid and liquid fractions of anaerobic digestate were collected from Cockerham Green Energy Ltd. Digestate from Cockerham Green Energy Ltd is fermented in a mesophilic, single stage digester with a retention time of 50 days. The feedstock is livestock and poultry manure, co-digested with food waste including cereal flour, potatoes, bird feed, wet grain, rice bran and whey. Whole digestate is separated into liquid and solid fractions using a screw-press; the liquid fraction is collected in covered lagoons, whilst the solid fraction is stored in an uncovered open-space. Digestate fractions were sampled following protocols detailed by Agriculture and Horticulture Development Board (2017). Whole

digestate was sampled directly from the anaerobic digester before separation, the solid fraction was sampled from fresh material directly after separation, whilst the liquid fraction was collected from a storage lagoon after at least 4 months of storage. Digestate fractions were stored at 4°C prior to the start of the incubation and before starting each incubation treatments were left to equilibrate at 23°C for two days, consistent with the equilibration period for soils. Physio-chemical characterisation of the three fractions of digestate was carried out by an external, ISO-accredited laboratory within five days of sample collection before the beginning of the first incubation. Recharacterization of each fraction of digestate prior the beginning of the second incubation was not performed and data presented in Table 4.2 (section 4.3.1) are assumed to accurately represent digestate characteristics at the start of the second incubation.

4.2.3 Experimental design

Two separate microcosm incubations, each of 7 days duration, were conducted between 27th May – 4th June and between 4th - 10th June 2019 respectively. Each incubation involved HN and LN soils with contrasting nutrient status (Table 4.2). Unamended soil was used as a control (Ctr) treatment, whilst whole digestate (WD), solid digestate (SD) and liquid digestate (LD) treatments were applied at the rates detailed below and mixed with each soil type (HN and LN) within separate glass bowls. Subsequently, sub-samples of the mixed soil and digestate treatments were used during each incubation.

In the first incubation, WD, SD and LD treatments were added to soils to achieve a constant N application rate of 170 kg Tot N ha^{-1} $year^{-1}$. This resulted in the addition of 104.18 mg N kg^{-1} dry matter (DM) soil for each treatment, whilst the Tot C added was 1591.2 mg kg^{-1} (DM) soil for SD, 473 mg kg^{-1} DM soil for WD and 241.06 mg kg^{-1} DM soil for LD treatments.

In the second incubation, WD and LD application rates were adjusted to achieve the same Tot C application as for the SD treatment within the first incubation. This meant that additional WD and LD was added to soils during the second incubation compared to the first, to achieve a consistent Tot C application across SD, WD and LD treatments. The SD treatment was applied at the same application rate in the second incubation as in the first incubation. This resulted in the addition of 1591.2 mg C kg^{-1} DM soil for each treatment, whilst the Tot N added was 104.18 mg kg^{-1} DM soil for SD, 389.86 mg kg^{-1} DM soil for WD and 687.77 mg kg^{-1} DM soil for LD treatments.

For both incubations, quadruplicates of each treatment within each soil type were included, with soil × treatment combinations placed using a randomised layout into two separate test set-ups: i) amber, stopper bottles with sub-seal bungs for gas-sampling of the headspace (N_2O and CH_4); and ii) two 96-deep well plates (one plate for each soil type, for CO_2 measurements). The amber, stopper bottles and the 96-deep well plates were placed inside a temperature (23 ±1°C), pressure (1063.9125 hPa) and moisture-controlled room in the dark. Soil moisture content was held at 50% water holding capacity (WHC) during the incubations using Milli-Q water. The

moisture content of the respirometry samples was checked daily (after gas sampling) by weighing each sample without lid and adding the water lost to maintain 50% WHC. Control soils were left un-amended with any digestate treatment and only received Milli-Q water to maintain 50% WHC.

4.2.4 Gas sampling and analysis

Catabolic response profiles for CO_2-C were determined using a MicroResp kit (Campbell et al., 2003; www.microresp.com) using colorimetric detection plates provided with an indicator gel. The indicator gel contains Cresol red which is pH sensitive and, based on the % CO_2-C present in the headspace of the micro-well, the colour indicator shifts from red to yellow.

A test set of detection plates were read following 6 hours of incubation to confirm the indicator gel's degree of colour saturation. From this point, plates were read after 24 hours (up to 168 hours from mixing) at 570nm using a microplate reader (FLUOstar Omega, BMG Labtech, DE). A fresh plate was used after each 24 hours having initially been read at 570 nm to establish blank values. A calibration curve was created using single-row strips provided with the MicroResp kit™ filled with indicator gel; the single-row strips were incubated for 6 hours at 23°C in 50ml falcon tubes at known %CO_2 concentrations and then read at 570nm using a microplate reader (FLUOstar Omega, BMG Labtech, DE). Subsequently, using a regression analysis, a fitted curve was used to determine the estimated values of the expression $A + B/(1 + D \times Ai)$ (www.microresp.com; A: -0.17, B: -1.244, D: -5.452 and Ai: normalised absorbance value from individual micro-well) which was

subsequently used to calculate the $\%CO_2$ present in each micro-well during the incubations. The $\%CO_2$-C produced was subsequently converted to µg CO_2-C g^{-1} DM soil emitted from each micro-well, taking into account the head-space of the micro-well, the temperature used during the incubation and the soil DW.

For NO_2-N and CH_4-C determinations, 9 ml headspace samples were taken from each sealed stopper bottle every 24 h using a 10 ml gas-tight syringe and injected into a 3 ml sealed and pre-vacuumed exetainer. Gas samples were analysed using a GC-FID (ParkinElmer instruments, AutoSystem XL Gas Chromatograph) attached to an automated sampler (HTA sampling for science, Italy). FID was used to determine CH_4-C concentration and an ECD was used to determine N_2O-N concentration, with argon used as the carrier gas. Single gas samples were taken initially from the stoppered bottle at 6h to avoid saturation of the head space and subsequent creation of anaerobic headspace conditions and then single gas samples were taken every 24h up to 168h. After each headspace sampling, the stoppered bottles were opened to check the soil moisture content and to avoid creation of anaerobic headspace conditions. Emission rates of CO_2-C, CH_4-C and NO_2-N were expressed as µg C g^{-1} DM soil h^{-1} for CO_2-C and ng C or N g^{-1} DM soil h^{-1} for CH_4 and N_2O), whilst cumulative CO_2-C, CH_4-C and NO_2-N was expressed as a mass (µg C g^{-1} DM soil for CO_2-C and ng C or N g^{-1} DM soil for CH_4-C and N_2O-N).

4.2.5 Statistical analysis

Statistical analyses were performed in R version 4.1 (R Core Team, 2020). The effects of soil type (HN, LN) and digestate amendment (Ctr, LD, WD and SD), alongside the interaction between these factors, were assessed using a one-way and two-way ANOVA on averaged cumulative GHG data in order to avoid pseudo replication, with Tukey-tests (HDS) used to compare individual levels when a significant factor was identified.

In order to assess variance homogeneity and normality distribution of the ANOVA model residuals, Levene's, Shapiro-Wilk and Kolmogorov–Smirnov tests were employed. For both incubations, the interquartile range (IQR) was computed for the cumulative CO_2-C data and any data point outside the 1.5 * IQR was identified as a statistical outlier. For cumulative CH_4-C and N_2O-N datasets, no outliers were observed in either incubation.

Square root and $\log_{10}$ transformations were applied to cumulative CO_2-C effluxes during the first and second incubation, respectively. For both incubations, a $\log_{10}$ transformation was applied to cumulative N_2O-N data, whilst cumulative CH_4-C data were analysed untransformed.

A quadratic regression was used to model the effect of soil type and digestate amendment on GHG effluxes over time. An interaction effect between time, soil type and digestate amendment was added when appropriate. Time was treated as a numerical variable and expressed from 0 to 168h. When significant regression

models were identified, T-tests were performed to determine the nature of the time × soil type × digestate amendment interaction.

In all statistical analyses, p-values < 0.05 were deemed as significant. Regression model fit was assessed by looking at residual plots such as S-L, Q-Q, Residual-Leverage and Cook's distance – leverage.

4.3 Results

4.3.1 Initial characterisation of soil and digestate properties

As illustrated in Table 4.1, HN and LN soils differed substantially in terms of their initial physio-chemical properties. In particular, the HN soil was associated with higher pH, water extractable N, water extractable organic C, TC and TN concentrations compared to the LN soil. Two batches of the HN and LN soils were used during the two incubations reported here, with different lengths of storage for each batch prior to the start of each incubation (see section 4.2.1). Although the absolute level of some soil parameters differed between the two batches, the fundamental soil physio-chemical differences marking the contrast between the HN and LN soils were maintained at the start of both incubations.

Table 4.1 Initial physio-chemical characteristics of soils used in the microcosm incubations (mean values reported, +/- 1 standard deviation in parentheses, n=4). P-values indicate statistical differences between High and Low nutrient soils and "n.s" indicates no statistical difference.

Soil characteristics	**HN soil First incubation**	**LN soil First incubation**	**p-value**	**HN soil Second incubation**	**LN soil Second incubation**	**p-value**
Bulk density (g cm^{-3})	1.23 (0.22)	1.10 (0.21)	n.s	1.23 (0.22)	1.10 (0.21)	n.s
DM (%)	67.61 (0.096)	70.40 (0.15)	<0.05	67.60 (0.30)	70.18 (0.16)	<0.05
pH water (1:5 w/v)	6.80 (0.15)	5.21 (0.05)	<0.001	7.02 (0.06)	5.05 (0.01)	<0.001
Water extractable NO_3^- (mg kg^{-1} DM soil)	105.44 (4.02)	10.12 (0.27)	<0.001	99.81 (2.83)	13.95 (0.56)	<0.001
Water extractable NH_4^+ (mg kg^{-1} DM soil)	1.02 (0.075)	0.46 (0.051)	<0.05	0.60 (0.09)	0.51 (0.071)	n.s
Water extractable organic C (mg kg^{-1} DM soil)	204.93 (17.61)	74.15 (9.68)	<0.001	386.51 (16.15)	118.55 (19.84)	<0.001
Soil Tot C (mg C kg^{-1} DM soil)	62846.47 (118.20)	30420.70 (67.45)	<0.001	65953.85 (183.53)	32698.99 (51.93)	<0.001
Soil Tot N (mg N kg^{-1} DM soil)	6313.72 (200.03)	3243.70 (254.86)	<0.001	6067.88 (159.34)	3694.66 (193.87)	<0.001
TC:TN	10.68 (0.065)	11.43 (0.060)	<0.05	10.76 (0.045)	11.68 (0.074)	<0.05

DM (Dry Matter); TC (Total Carbon, non-acidified analysis); TN (Total Nitrogen); Water Tot C (Water Extractable Total Organic Carbon, acidified analysis); NH_4^+ (Ammonium); NO_3^- (Nitrate)

Table 4.2 reports physio-chemical characteristics of the digestate fractions used in the incubations reported here. The SD fraction had a higher DM, OM, Tot C:N, TC and TN content than either WD and LD, although the NH_4^+-N content was lower for the SD compared to either the WD or LD fractions. Whilst WD and LD were broadly similar in terms of OM, TN and NH_4^+-N concentrations, the DM, TC and Tot C:N was higher in the WD fraction than within the LD fraction.

Table 4.2 Physio-chemical characteristics of whole, solid and liquid digestate used in the microcosm incubations (n=1)

Parameter in fresh weight (FW)	Whole digestate (WD)	Liquid digestate (LD)	Solid digestate (SD)
Dry Matter (%)	7.71	3.83	25.99
Organic Matter (%)	66.99	56.62	82.06
pH (1:6 w/v)	8.42	8.22	9.12
TN (mg kg^{-1} FW)	6600	5100	8007
NH_4^+-N (mg kg^{-1} FW)	3679	3162	2822.60
TC (mg kg^{-1} FW)	30100	11800	122400
Organic Carbon (by DUMAS, mg kg^{-1} FW)	23700	10300	104550
TC:TN	5	2	15

FW (Fresh Weight), DM (Dry Matter); TC (Total Carbon); TN (Total Nitrogen); NH_4^+-N (Ammonium Nitrogen)

4.3.2 Effect of digestate treatments on greenhouse gas fluxes from soil

During both incubations (Table 4.3a,b), all digestate treatments significantly increased the average cumulative CO_2-C, CH_4-C and N_2O-N effluxes compared to the Ctr treatment ($p<0.001$), regardless of the initial soil nutrient status. Digestate

treatments increased the average cumulative CO_2-C efflux in the order Ctr<SD<LD<WD and Ctr<SD<WD<LD (first and second incubation respectively), whilst the average CH_4-C efflux followed the order Ctr<SD<LD≈WD and Ctr<SD<LD<WD in the first and second incubations respectively. Similar increases in the average cumulative N_2O-N efflux were observed, in which treatments followed the order Ctr<SD<WD≈LD for the first incubation and Ctr<SD<WD<LD for the second incubation. Furthermore, the average cumulative CO_2-C, CH_4-C and N_2O-N effluxes were significantly higher for HN than for LN soils during both incubations ($p<0.001$) and significant soil × digestate treatment interactions were observed for all GHGs in both incubations ($p<0.001$).

Further analysis showed that digestate treatment and soil type affected the rate of increase in the cumulative CO_2-C and CH_4-C effluxes over time, but not the rate of change of cumulative N_2O-N effluxes. This effect on CO_2-C and CH_4-C was confirmed by a significant three-way interaction between soil × treatment × time ($p<0.01$) and is illustrated in Figure 7.1 and 7.2 (Chapter 7, Appendix). In fact, for cumulative N_2O-N efflux, trajectories over time are parallel to one another whereas for the cumulative CO_2-C and CH_4-C are not (notice the crossings in the trajectories showing the different rate of increase over time for some treatment and soil combinations).

Table 4.3 Summary of the two-way ANOVA from the a) first microcosm and b) second microcosm incubation

a)

	Soil	Mean (±s.e)	p-value	Digestate	Mean (±s.e)	p-value	Soil × digestate interaction	Mean (±s.e)	p-value
Cumulative CO_2-C (µg C g^{-1} DW soil)	HN	59.23^{a}±0.47	<0.001	Ctr	36.37^{d}±0.81	<0.001	HN	Ctr: $52.69^{c,a}$±0.83	
	LN	25.67^{b}±0.7		LD	43.94^{b}±0.82			LD: $58.42^{b,b}$±0.89	
				WD	47.23^{a}±0.9			WD: $66.79^{a,b}$±1.24	
				SD	42.06^{c}±0.93			SD: $57.79^{b,b}$±0.97	<0.001
							LN	Ctr: $21.55^{c,b}$±0.50	
								LD: $29.44^{a,c}$±0.86	
								WD: $26.83^{b,c}$±0.51	
								SD: $25.49^{b,c}$±0.74	
Cumulative CH_4-C (ng g^{-1} DW soil)	HN	9.05^{a}±0.82	<0.001	Ctr	2.82^{c}±0.52	<0.001	HN	Ctr: $3.71^{b,a}$±0.31	
	LN	3.88^{b}±0.42		LD	8.08^{a}±1.24			LD: $11.3^{a,b}$±0.17	
				WD	8.37^{a}±1.13			WD: $11.27^{a,b}$±0.51	
				SD	6.60^{b}±1.28			SD: $9.94^{a,b}$±0.63	<0.001
							LN	Ctr: $1.93^{b,a}$±0.79	
								LD: $4.89^{a,c}$±0.51	
								WD: $5.46^{a,c}$±0.36	
								SD: $3.21^{b,c}$±0.47	
Cumulative N_2O-N (ng g^{-1} DW soil)	HN	132.36^{a}±18.27	<0.001	Ctr	13.06^{c}±3.12	<0.001	HN	Ctr: $18.93^{b,a}$±4.47	
	LN	11.95^{b}±0.96		LD	79.40^{b}±26.12			LD:$148.00^{a,b}$±7.45	
				WD	109.50^{a}±37.9			WD: $205.59^{a,b}$±12.21	
				SD	87.70^{b}±27.46			SD: $157.00^{a,b}$±14.11	<0.001
							LN	Ctr: $7.27^{c,a}$±0.56	
								LD:$10.84^{b,c}$±0.72	
								WD: $13.40^{b,c}$±1.48	
								SD: $16.28^{a,c}$±1.40	

Note: Columns from left to write describe effects on initial soil nutrient status (high "HN" vs low "LN"), effects of digestate fractions (Control "Ctr", liquid "LD", whole "WD" and solid "SD") and interactions between soil and digestate amendment. Significant differences between levels were identified using superscript letters. For interaction between soil and digestate, the first superscript letter represents differences between digestate amendments within each soil type, second superscript letter represents differences between soil type within each digestate amendment.

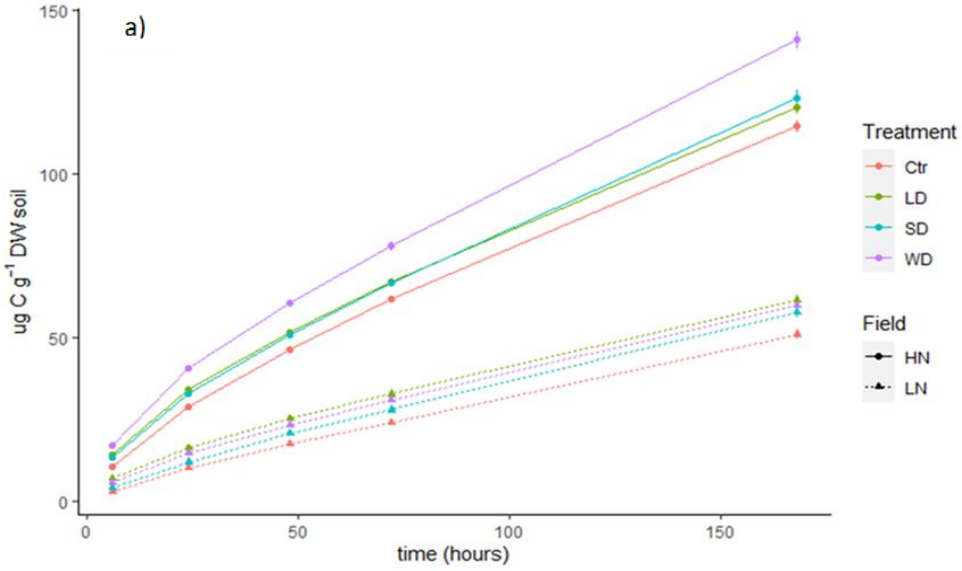
b)

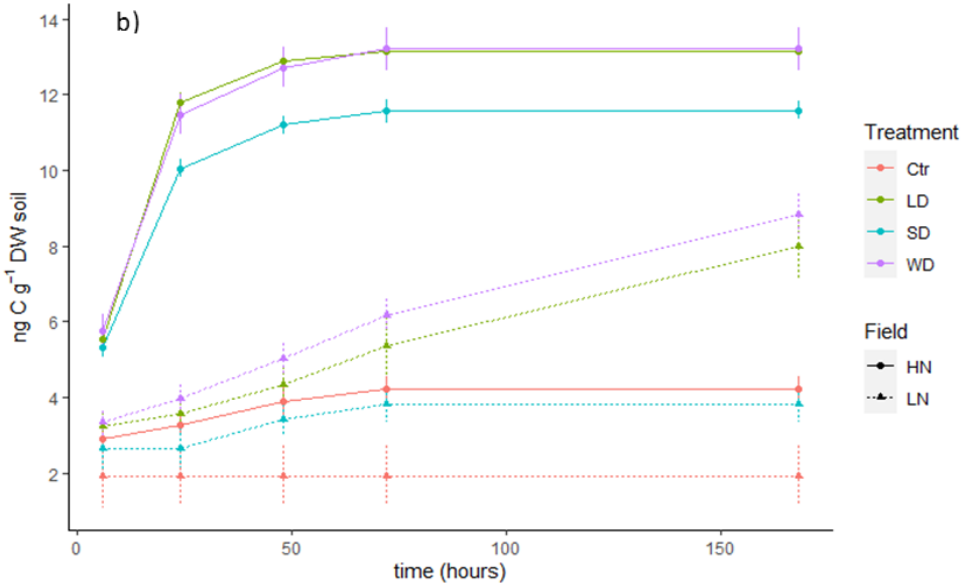
	Soil	Mean (±s.e)	p-value	Digestate	Mean (±s.e)	p-value	Soil × digestate interaction	Mean (±s.e)	p-value
Cumulative CO_2-C (μg C g^{-1} DW soil)	HN	78.17^{a}±1.87	<0.001	Ctr	42.74^{d}±2.75	<0.001	HN	Ctr: $61.40^{d,a}$±1.62	
	LN	44.05^{b}±2.20		LD	89.68^{a}±2.42			LD: $103.16^{a,a}$±2.40	
				WD	62.01^{b}±2.90			WD: $80.06^{b,a}$±1.62	
				SD	46.30^{c}±2.94			SD: $67.30^{c,a}$±1.27	<0.001
							LN	Ctr: $25.66^{c,b}$±0.38	
								LD: $76.70^{a,b}$±1.54	
								WD: $44.74^{b,b}$±1.76	
								SD: $27.85^{c,b}$±0.54	
Cumulative CH_4-C (ng g^{-1} DW soi)	HN	13.23^{a}±1.33	<0.001	Ctr	4.53^{d}±0.71	<0.001	HN	Ctr: $6.40^{d,a}$±0.19	
	LN	10.17^{b}±1.53		LD	17.47^{a}±0.25			LD: $17.30^{b,a}$±0.35	
				WD	16.53^{b}±0.95			WD: $18.85^{a,a}$±0.58	
				SD	8.43^{c}±0.65			SD: $10.03^{c,a}$±0.09	<0.001
							LN	Ctr: $2.66^{d,b}$±0.32	
								LD: $17.64^{a,a}$±0.40	
								WD: $14.23^{b,b}$±0.53	
								SD: $6.84^{c,b}$±0.53	
Cumulative N_2O-N (ng g^{-1} DW soil)	HN	228.13^{a}±51.43	<0.001	Ctr	11.73^{d}±1.58	<0.001	HN	Ctr: $15.60^{d,a}$±1.31	
	LN	28.67^{b}±5.51		LD	279.39^{a}±84.30			LD: $495.13^{a,b}$±46.82	
				WD	175.56^{b}±59.03			WD: $324.19^{b,b}$±39.15	
				SD	47.00^{c}±11.27			SD: $77.60^{c,b}$±2.76	<0.001
							LN	Ctr: $7.87^{d,b}$±0.37	
								LD: $63.65^{a,c}$±3.54	
								WD: $26.92^{b,c}$±2.21	
								SD: $16.26^{c,c}$±0.66	

Note: Columns from left to write describe effects on initial soil nutrient status (high "HN" vs low "LN"), effects of digestate fractions (Control "Ctr", liquid "LD", whole "WD" and solid "SD") and interactions between soil and digestate amendment. Significant differences between levels were identified using superscript letters. For interaction between soil and digestate, the first superscript letter represents differences between digestate amendments within each soil type, second superscript letter represents differences between soil type within each digestate amendment.

4.3.3 Effect of digestate application on GHG emissions from high nutrient soil

During both incubations, the application of digestate to the HN soil significantly increased the average cumulative efflux of all three GHGs when compared to the Ctr treatment. During the first incubation (Table 4.3a, Figure 4.1), the order of treatment effects on the average cumulative CO_2-C efflux was Ctr<SD≈LD<WD, whilst for both the average cumulative CH_4-C and N_2O-N effluxes all digestate treatments resulted in significantly higher fluxes than the control, with no significant differences between the individual digestate treatments. In the second incubation (Table 4.3b, Figure 4.2), the effects of the treatments followed the order: Ctr<SD<WD<LD (CO_2-C), Ctr<SD<LD<WD (CH_4-C) and Ctr<SD<WD<LD (N_2O-N).





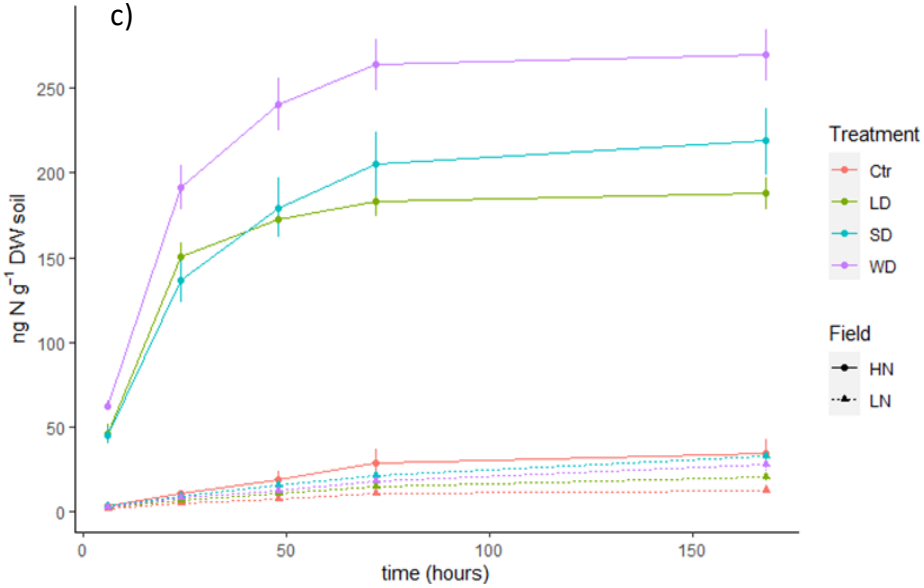


Figure 4.1 Cumulative CO_2-C (a), CH_4-C (b) and N_2O-N (c) effluxes during the first incubation in control (Ctr) soils or after addition of liquid (LD), whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) nutrient status. Error bars ± 1SE

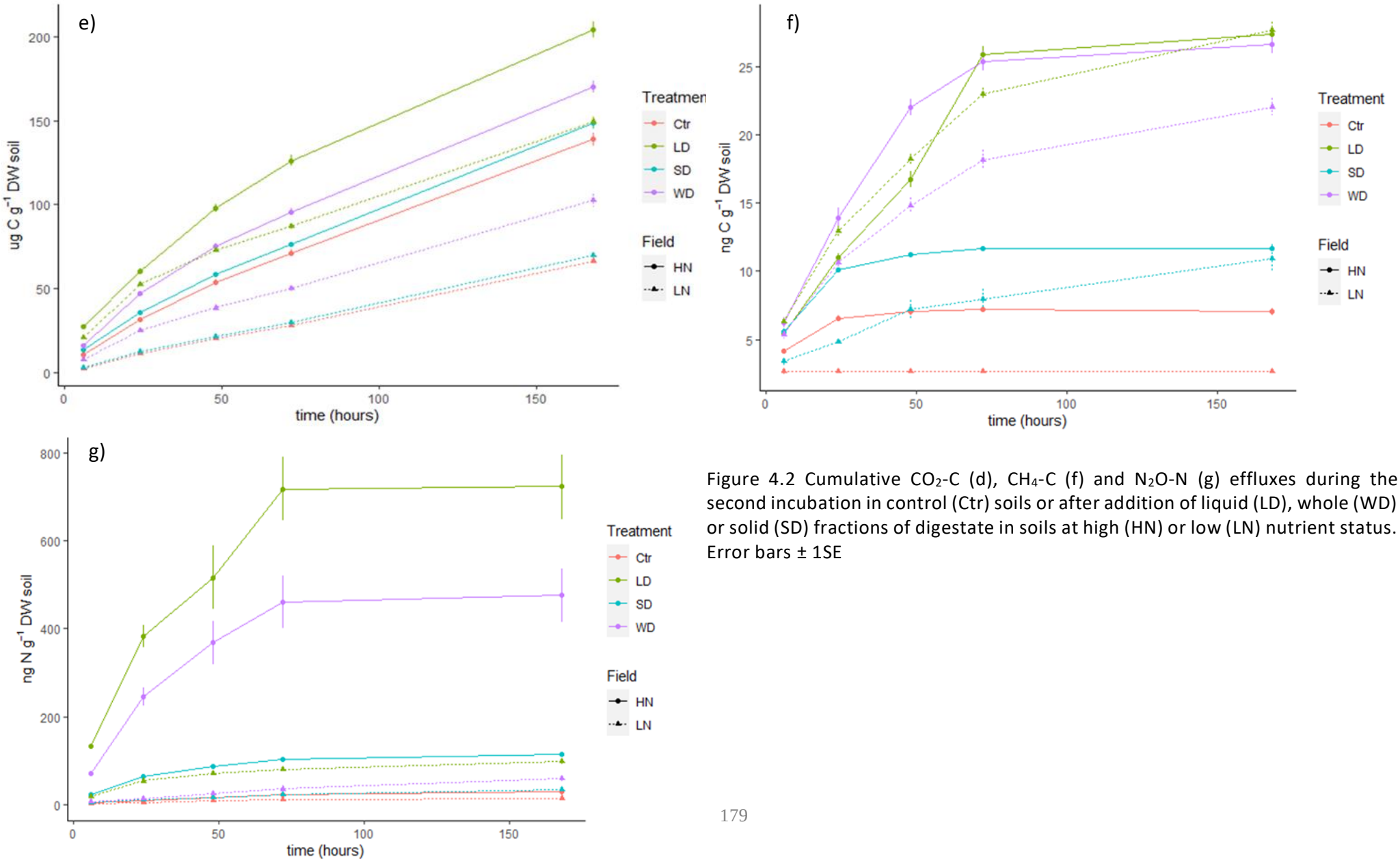


Figure 4.2 Cumulative CO_2-C (d), CH_4-C (f) and N_2O-N (g) effluxes during the second incubation in control (Ctr) soils or after addition of liquid (LD), whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) nutrient status. Error bars ± 1SE

During the first incubation, a significant three-way interaction between soil, treatment and time was observed, although only for cumulative CH_4-C efflux and not for the fluxes of either cumulative CO_2-C or N_2O-N (model estimates presented in Chapter 7, Appendix). Cumulative CH_4-C efflux increased steeply during the early stages of the incubation for SD, LD and WD treatments and subsequently flattened off, producing a total increase of +193%, +229% and +230% (SD, LD and WD respectively) at 7 days when compared with Ctr. In contrast, cumulative CO_2-C and N_2O-N increased at a similar rate to the Ctr treatment over time (parallel trajectories in Figure 7.1 in Chapter 7, Appendix) and therefore a three-way interaction was not observed

During the second incubation (Figure 4.2), cumulative CO_2-C increased rapidly only after LD addition, generating an increase in cumulative CO_2-C efflux of +41% at 7d when compared with Ctr (model estimates in Chapter 7, Appendix). Cumulative CH_4-C effluxes increased sharply and similarly during the early stages of the second incubation for LD and WD before flattening off to background levels (Figure 4.2) and generating a cumulative CH_4-C increase of +289% and + 278% (LD and WD respectively) at 7 days when compared with Ctr. In contrast, SD increased the cumulative CH_4-C effluxes only for a short time during the early stages of the incubation and then flattened off to background levels, producing a total cumulative increase of +65% at 7d of incubation when compared to Ctr (model estimates in Chapter 7, Appendix). As reported for the first incubation, the pattern of cumulative

N_2O-N efflux through time following WD, LD or SD application did not differ significantly compared to the Ctr (Figure 7.2 in in Chapter 7, Appendix).

4.3.4 Effect of digestate application on GHG emissions from low nutrient soil

During the first incubation (Table 4.3 a, Figure 4.1), all digestate treatments applied to the LN soil significantly increased the average cumulative CO_2-C efflux compared to the Ctr treatment. However, in contrast to results from the HN soil (Ctr<SD≈LD<WD), within the LN soil the order of the treatment effects on the average cumulative CO_2-C efflux was Ctr<SD≈WD<LD. Whilst all digestate treatments significantly increased the average cumulative CH_4-C efflux compared to the Ctr treatment in the HN soil, within the LN soil only the application of LD and WD significantly increased the flux of CH_4-C compared to the Ctr. Finally, the average cumulative fluxes of N_2O-N were significantly higher following the application of SD than either LD or WD to the LN soil, in contrast to results from the HN soil in which the flux of N_2O-N did not differ significantly between SD, LD and WD treatments.

During the second incubation in the LN soil (Table 4.3 b, Figure 4.2), only WD and LD treatments produced a significantly higher average CO_2-C efflux compared to the Ctr treatment, in contrast to results from the HN soil in which all three digestate treatments significantly enhanced the average CO_2-C flux compared to the Ctr. The average cumulative CH_4-C efflux was significantly higher following all three digestate treatments compared to the Ctr in the LN soil, although the flux following the LD treatment significantly exceeded that following the WD treatment, the

opposite pattern to that observed in the HN soil. With respect to the average cumulative N_2O-N flux, all three digestate treatments significantly enhanced the flux in the LN soil compared to the Ctr treatment. Whilst the order of treatment effects on N_2O-N flux was the same in the LN compared to HN soils (Ctr<SD<WD<LD), the magnitude of the increase following digestate application to the HN soil exceeded that observed within the LN soil.

Application of treatments during the first incubation in the LN soil (Figure 7.1 in Chapter 7, Appendix) did not significantly influence the trajectories for cumulative N_2O-N and CO_2-C across the 7 days of incubation. However, for cumulative CH_4-C, application of WD and LD showed a relatively constant increase throughout the 7d of incubation and produced a total increment of + 181% for WD and + 185% for LD at 7d when compared to Ctr. In contrast, the SD treatment increased the cumulative CH_4-C effluxes only for a restricted amount of time between 24h and 72h and subsequently flattened of to background levels, contributing to a total cumulative CH_4-C increase of +31 at 7d (model estimates present in Chapter 7, Appendix). In contrast, application of treatments during the second incubation in the LN soil (Figure 7.2 in Chapter 7, Appendix) significantly increased the cumulative CO_2-C, CH_4-C effluxes throughout time (model estimates present in Chapter 7, Appendix), whilst for cumulative N_2O-N in the second incubation, there were no significant differences in the estimated trajectories between Ctr and the digestate treatments.

Application of LD and WD to LN soil significantly influenced the rates of increase for the cumulative CO_2-C when compared with the Ctr; LD increased the cumulative

CO_2-C quickly during the early stages of the incubation and then it reduced the speed of increase, generating an increase in total cumulative CO_2-C at 7d of + 125% when compared with Ctr. Application of WD also increased the cumulative CO_2-C during the early stages of the incubation, but not as rapidly as LD treatments, and this rate of increase was maintained until the end of the incubation(+54% when compared to Ctr). Application of LD and WD treatments increased sharply and similarly the cumulative CH_4-C effluxes throughout time and by + 940% and+ 729% (LD and WD respectively) at 7d when compared with Ctr. The SD treatment produced a lower and sustained cumulative CH_4-C efflux, resulting in an increase of + 310% at 7d when compared with Ctr.

4.4 Discussion

The results of the incubations reported here confirm that GHG emissions from a grassland soil are significantly influenced by the fraction of digestate that is applied (WD, LD or SD) and by the application rate for each digestate fraction. The experiment also confirmed that GHG emissions after application of digestate were significantly influenced by the initial soil nutrient status, comparing a high and a low nutrient status soil (fertilised grassland versus fallow grassland), and also by time during the 7-day incubations. However, it is important to recognise that the results reported in this chapter are based on short-term laboratory incubations and that are likely to have driven different responses compared to those expected across a longer field experiment. For example, our experimental system did not include the

input of labile C to soil from root exudates that may alter GHG emissions. Moreover, GHG emissions can be determined by processes of consumption and production (Butterbach-Bahl et al., 2013; Sihi et al., 2019) which are influenced by soil structure and by environmental factors such as soil temperature and moisture (Ma et al., 2010; Wagner-Riddle et al., 2007; Jungkunst and Fiedler, 2007). Therefore, future research will be required in order to examine the interactions within plant-microbial-soil systems and the associated GHG emissions and longer (up to 12 months, IPCC, 2007) field experiments are required to investigate the behaviour of GHG emissions and soil consumption of GHGs after application of different fractions of digestate to grassland soils.

Even though two batches of HN and LN soils that had been stored for different lengths of time were used in the two experiments (firstly common N loading, secondly common C loading), the soil physico-chemical data showed that key differences between these HN and LN soils remained consistent (Table 4.1). During both incubations, cumulative GHG emissions from the HN soil significantly exceeded those from LN soil, reflecting the fact that microbial activity and associated GHG production was influenced by differences in pH, microbial biomass, DOC content and soil Tot C:N (Oertel et al., 2016; Russell & Cook, 1995). The HN soil had greater (near-neutral) pH, microbial biomass and extractable DOC relative to the LN soil (Table 4.1). These HN conditions likely favoured microbial activity and GHG emissions compared to conditions within the LN soil, also influencing the responses of the two soils to the digestate fractions. The discussion below considers the three

GHGs as affected by digestate fractions when at consistent TN addition levels, then at consistent C addition levels, addressing for each the differing responses between HN and LN soils.

4.4.1 Digestate effects on GHG emissions

The cumulative efflux of every GHG from soil increased significantly after the application of each fraction of digestate within both incubations, consistent with some past laboratory and field research across arable and grassland soils (Johansen et al., 2013; WRAP, 2016; Wulf et al., 2002). Field experiments have reported an increase in cumulative CO_2-C production over a one year period, alongside a high but short-lived increase in cumulative CH_4-C and N_2O-N effluxes which returned to background levels after a few hours or days of the start of the experiment (WRAP, 2016; Wulf et al., 2002). This increase in CO_2-C production and a short increase in CH_4-C and N_2O-N has also been reported for laboratory experiments in which incubations lasted between 9 and 80 days (Johansen et al., 2013; Senbayram et al. 2009; Alburquerque et al., 2012). During these laboratory incubations, the cumulative CO_2-C produced was greater or similar to the cumulative CO_2-C produced during both incubations reported in this chapter, whilst for cumulative N_2O-N and CH_4-C production the results were similar to the first incubation reported above. Regarding the GHG emissions generated during the second incubation reported above, the fluxes were greater than the results reported in these past studies. The long-lasting cumulative CO_2-C effluxes are likely due to the ongoing decomposition of soil organic matter (SOM) in the Ctr and the further stimulation and consequent

priming of the microbial community after application of labile C with digestate treatment (Pezzolla et al., 2012). The short-term production of cumulative CH_4-C and N_2O-N effluxes are likely due to different factors. Production of CH_4 occurs quickly and is consumed in aerated soil due to oxidation of CH_4 to CO_2 from methanotrophic bacteria, which can assimilate a large proportion of CH_4-C, often as much as half or more, into microbial biomass C (Mills et al., 2013). Labile C added with digestate may have stimulated nitrifying or denitrifying bacterial activity and the consequent creation of anaerobic microsites soon after its addition to soil (and a consequent increase of denitrification rates), which subsequently decreased due to soil aeration (Johansen et al., 2013), or the quick production of CH_4-C and N_2O-N may be related to the release of trapped CH_4-C and N_2O-N contained in digestate (Chadwick et al., 2000).

However, the data reported above also highlight that cumulative GHG effluxes are significantly influenced by the specific digestate fraction that is applied, the digestate application rate and the nutrient status of the soil to which digestate is applied.

4.4.2 Influence of individual digestate fractions on GHG effluxes following standardised total N additions

4.4.2.1 Cumulative CO_2-C efflux

Cumulative CO_2-C efflux increased after application of WD, LD and SD treatments. Higher cumulative CO_2-C production after the application of WD than LD is primarily due to the greater input of DOC to soils from WD compared to LD. In contrast, the

SD treatment supplied mainly recalcitrant C compounds (Tambone et al., 2010) which are enzymatically complex for bacteria to metabolise and therefore the cumulative CO_2-C efflux following the application of SD was the lowest of the three digestate fractions examined. These results are consistent with Cavalli et al. (2016) and Cattin et al. (2021), who observed increases in cumulative CO_2-C efflux after the addition of SD and, particularly, WD and LD treatments to soil, reflecting the high content of soluble organic matter present in LD and WD fractions compared to a more recalcitrant C present in the SD fraction. In contrast, de la Fuente et al (2013) reported an increase in cumulative CO_2-C after SD treatment compared to LD and WD, although the proportion of applied C that was mineralised was lower for the SD treatment compared to LD and WD. These authors reported that the greater increase in cumulative CO_2-C was probably due to the relatively high bioavailable OC content of the solid material, likely due to the feedstock utilised (which was 84% cattle slurry, 4% cattle manure and 11% maze-oat silage) and the retention time during the anaerobic digestion phase (Askri et al., 2016), whilst the low mineralization rate was associated with the higher proportion of lignocellulosic compounds contained in SD. In the experiments reported here, the feedstock was substantially different from the feedstock used by de la Fuente et al. (2013) and composed of more ligno-cellulose compounds which probably increased the presence of humic substances with high biological stability in the SD fraction after separation, resulting in low bacterial respiration and resulting CO_2-C efflux. These results confirm that cumulative CO_2-C effluxes can be significantly influenced by the variation in the combination of feedstock, digestate production conditions and

quality of C added to soil after digestate application. Further research is required to explore how different digestates, derived from varying feedstock compositions, influence soil bacterial activity, mineralization rates and, ultimately, GHG emissions.

A significant soil × treatment interaction was also observed (Table 4.3), meaning that cumulative CO_2-C effluxes after digestate application were dependent on the initial soil nutrient status at the start of the incubations. In the HN soil, WD application generated the highest cumulative CO_2-C efflux, followed by LD and SD which both generated a similar cumulative CO_2-C efflux. This likely reflects the fact that the amount of labile C added with WD was greater than for LD, which may have stimulated bacterial metabolism and increased the respiration rate and CO_2-C efflux in the HN soil, whilst due to the recalcitrant nature of C compounds applied with the SD treatment bacterial respiration was not stimulated to the same extent (Cattin et al., 2021). However, the HN soil was sampled from a field that has been fertilised over the long-term with liquid digestate and it is also likely that the microbial community was adapted to receiving applications of LD (Lupwayi et al., 2012; Sradnick et al., 2013; Yu et al., 2015). Conversely, after application of WD to the HN soil, the microbial community would have reacted differently (Rosace et al., 2020), increasing respiration rates and consequent CO_2-C efflux.

Application of treatments to the LN soil increased cumulative CO_2-C effluxes, although LD application to LN generated the greatest cumulative CO_2-C efflux compared to WD and SD which both behaved similarly. Whilst there was greater C input in the WD compared to the LD treatment, the microbial community in LN was

in an unfavourable environment (low pH, C_{micro}, SOC and available N), such that application of more readily-available C compounds present in the LD treatment would have triggered dormant bacteria to increase their metabolic and respiration rates (Manzoni et al., 2012; Geyer et al., 2016; Sinsabaugh et al., 2013). Further, due to the recalcitrant C compounds contained in SD, bacteria are likely to only marginally have increased metabolism and respiration rates.

4.4.2.2 Cumulative CH_4-C efflux

Cumulative CH_4-C efflux increased after WD, LD and SD addition, although it was substantially lower than cumulative CO_2-C efflux in terms of mass of C lost from soil (μg C g^{-1} DM soil for CO_2-C and ng C g^{-1} DM soil for CH_4-C), consistent with Dietrich et al. (2020). These authors reported that increases in CH_4-C efflux after digestate application were probably due to the anaerobic microbial taxa added to soils alongside the treatments, which stay active for a considerable amount of time and, when applied to a favourable environment (e.g. warm temperature, increase in soil moisture, soils rich in SOC), start producing small amounts of methane (Fetzer et al., 1993). Alternatively, increases in CH_4-C efflux may reflect immediate release of CH_4 originally present within the digestate treatments themselves (Pezzolla et al., 2012). Application of WD and LD produced similar increases in CH_4-C efflux, likely because more available C compounds were applied with these treatments compared to the SD treatment, which were subsequently used by methanogenic bacteria for their metabolism (Conrad, 2020).

Cumulative CH_4-C efflux was also highly dependent on the initial nutrient status of the soil. Application of WD, LD and SD to HN increased the cumulative CH_4-C efflux, in part because residual methane, present in a dissolved form in the treatment, was likely released after application creating a rapid spike that reached background level after 48h (Pezzolla et al., 2012; Chadwick et al., 2000; Wulf et al. 2002). In addition, due to the input of OC (e.g. DOC) present in the digestate treatments, coupled with the soil microbial community composition, high SOC and nutrient availability in the HN soil, methanogenic bacteria present either in the soil or in the digestate were supplied with oxidisable C compounds which were rapidly used for methane production (Lee at all., 2012; Severin et al. 2015, Watanabe at al., 2011). Subsequently, after these compounds has been oxidised, CH_4 production decreased to background levels (Dutaur & Verchot 2007). These results are supported by the time interaction observed within the HN soil, whereby treatments gave similar rapid increases in cumulative CH_4-C effluxes, a pattern that declined a few hours after the beginning of the incubation before reducing further to no net emission, consistent with some past research reported in the literature (Chadwick et al., 1996; Jones at al., 2005).

The application of WD and LD to the LN soil similarly increased cumulative CH_4-C efflux. However, WD and LD addition to LN followed a different temporal pattern than within the HN soil, namely a slow but sustained increased in cumulative CH_4-C efflux. This contrast may reflect a smaller population of methanogens in the LN compared to HN soil, which has a history of regular LD application (Rosace et al.,

2020). Under these conditions, the addition of OM to a soil low in C and additional nutrients may have rapidly stimulated microbial catabolism and respiration, with the small population of methanogens slowly using part of the readily available C source (DOC) for their metabolism (Conrad et al., 2020a). Subsequently, it is possible that some priming effect occurred in the LN soil, due to the low nutrient availability and the microbial activation associated with digestate input (Fontain et al., 2003; Cattin et al., 2021). Methanogenic bacteria may have started to mine the SOC (Degens et al., 1975) towards the end of the incubation for additional energy sources, maintaining increased CH_4-C emissions throughout the incubation (Conrad, 1999, 2020b). Conversely, application of SD to the LN soil did not increase overall methane efflux, although a small increase was observed only during the soil × treatment × time interaction, showing that the application of less available C compounds probably stimulated methanogens only for a short amount of time during the incubation (between 24 and 72 h) (Le Mer and Roger, 2001; Hurkuck et al., 2012), before CH_4-C efflux returned to background levels.

4.4.2.3 Cumulative N_2O-N efflux

The application of digestate significantly increased cumulative N_2O-N compared to the control treatments, consistent with other research reported in the literature (e.g. Holly et al., 2017; Dietrich et al., 2020; Rosace et al., 2020) where small increases (as ng g^{-1} DM soil) in cumulative N_2O-N were also reported after digestate application. The application of WD produced the highest cumulative N_2O-N effluxes in the research reported in this chapter, whilst LD and SD produced similar increases

in this GHG efflux. This may reflect the fact that high amounts of available C and NH_4^+ were applied with the WD treatment compared to either LD or SD treatments, triggering N_2O production during nitrification or denitrification, depending on O_2 availability (Mojeremane, 2013; Wrage et al., 2001).

However, significant differences in the impacts of digestate application on cumulative N_2O-N effluxes were observed across HN and LN soil types. The application of digestate to the HN soil increased cumulative N_2O-N efflux, due to the high amount of C and NO_3^- contained in the soil (NH_4^+ and NO_3^- soil data present in Figure 7.3 in Chapter 7, Appendix), consistent with other experiments reported in the literature (Dietrich et al., 2020; Rosace et al., 2020). This could be because during nitrification there was an accumulation of NO_2^- due to microbial oxidation of NH_4^+, promoting the formation of HNO_2^- (Venterea & Rolston, 2000) which is believed to react with phenolic functional constituents of soil organic matter and produce N_2O (Stevenson 1994). However, denitrifying bacteria are able to survive and produce N_2O over a wide range of oxygen pressures (between 0 and 20.4 kPa) (Khalil et al., 2004; Thilakarathna & Hernandez-Ramirez, 2021), thus based on our NH_4^+ and NO_3^- soil data (Figure 7.3 in Chapter 7, Appendix), denitrification processes probably occurred during the laboratory incubations reported in this chapter.

Following digestate application, bacteria were supplied with sufficient available C and N (Lupwayi et al., 2011; Sharma et al., 2005) to create an incomplete reduction of N-oxide compounds, resulting in an increase in cumulative N_2O-N emissions (Aguilera et al., 2013; Gregorich et al., 2015; Charles et al., 2017). When C and NO_3^-

availability were reduced, bacteria likely entered into a dormancy phase reducing N_2O efflux to a background level (van Gestel et al., 1993; Barton et al., 2016), a pattern that is consistent with the time interaction reported in this chapter and previous research in the literature (Thomas et al., 207; Nicholson et al., 2017).

In contrast to HN, cumulative N_2O-N efflux in LN was greatest following the SD application, although WD and LD treatments did significantly increase cumulative N_2O-N efflux compared to the control treatment. The application of WD and LD to a soil that was initially low in nutrient content may have quickly stimulated microbial N-immobilization, reducing the amount of NO_3^- produced by nitrifying bacteria and therefore reducing the available NO_3^- for denitrifying bacteria and the amount of N_2O-N produced during denitrification (Espinoza et al., 2013). Whilst the application of SD may also have induced an initial phase of N immobilization, the more recalcitrant C compounds present in the SD treatment may have stimulated bacteria to mineralise N compounds present in the SOM (Burger & Venterea, 2007; Morvan et al., 2006), thereby supplying bacteria with extra NO_3^- for N_2O-N production.

4.4.3 Influence of individual digestate fractions on GHG effluxes following standardised total C application rates

During the second incubation reported in this chapter, digestate fractions were applied to achieve a common total C addition, examining how changes in Tot C:N during digestate application can influence GHG emissions. The cumulative GHG emissions from the HN soil in this incubation were more similar to those from the LN soil compared to during the first incubation (Figure 4.3), although this was mainly

true for WD and LD treatments because the SD treatment followed the same application rate to that in the first incubation. Substantial differences in GHG emissions between the first and the second incubations reported in this chapter are largely due to the increases in DOC and NH_4^+ applied with WD and LD treatments during the second incubation, parameters which have been shown to significantly influence microbial metabolism (see section 4.1 and 4.2.3)

4.4.3.1 Cumulative CO_2-C efflux

Cumulative CO_2-C effluxes after the addition of LD and WD were nearly double those reported during the first incubation, when averaged across the two soil types. These observations reflect the increased amount of LD and WD that was applied to soil in order to reach a constant C addition across all digestate fractions, resulting in more available C and N being applied during the second compared to first incubation for these two fractions of digestate. However, LD produced a higher cumulative CO_2-C efflux than WD, reflecting the larger amount of LD applied than the amount of WD applied during the second incubation to reach the same C target. Further, the greater efflux of CO_2-C from the LD compared to WD treatment also likely reflects the more labile and readily bioavailable forms of C present within the LD compared to WD fraction.

Moreover, the cumulative CO_2-C efflux in the second incubation was highly dependent on the soil nutrient status, as shown by a significant two way interaction between soil type and digestate treatment. In HN, the LD application increased cumulative CO_2-C production to a greater extent than WD, likely due to the fact that

higher input of N and C was applied with LD than WD, which has probably increased the nutrient imbalance (more available C and N added) in the soil (Moorhead & Sinsabaugh, 2006), leading to greater mineralization of organic compounds by the soil microbial community and therefore increased CO_2-C production over time (Sinsabaugh et al., 2009; Tambone et al., 2013). Similarly to the HN soil, application of LD to the LN soil stimulated bacterial respiration to a greater extent than WD. However, in this incubation SD treatment was not significantly different from Ctr, likely because of the slightly higher TC and DOC content of the LN batch of soil used during the second incubation, which after SD application did not stimulate bacterial metabolism sufficiently to increase respiration rate and associated CO_2-C efflux (Manzoni et al., 2012). This emphasises the fact that soil nutrient status can strongly influence bacterial respiration and activity, especially after application of recalcitrant C compounds and further research is needed to investigate this pattern.

The application of WD in the second incubation generated a greater cumulative CO_2-C efflux from the LN than from the HN soil. This agrees with Cattin et al. (2021), where the authors reported that the increase of cumulative CO_2-C efflux after digestate application to LN was mainly because of the high application of DOC to a soil low in available nutrients may stimulate bacterial respiration, catabolism and enzyme production. This is also reflected in the temporal pattern of CO_2-C efflux after digestate application to the LN soil reported in the current chapter, where LD rapidly stimulated dormant bacteria (Mondini et al., 2006) and then, when bacteria began to exhaust available C compounds for enzyme production and maintenance

respiration, respiration rates reduced, consistent with other research (e.g. Wang et al. 2013; Wang and Post, 2012). In contrast, application of WD supplied bacteria with lignified substances (Tambone at al., 2010) as well as DOC, which were not easily utilised by the microbial community. Therefore, after utilization of DOC, bacteria likely started to invest in producing enzymatic compounds for lignin degradation (Sierra, 2012), leading to more prolonged increases in cumulative CO_2-C efflux (Sinsabaugh et al., 2013; Winogradzky, 1924).

4.4.3.2 Cumulative CH_4-C efflux

Consistent with the observations for CO_2-C, cumulative CH_4-C effluxes were higher during the second incubation than the first incubation, reflecting the greater input of labile C under WD and LD treatments compared to the first incubation in order to reach the same total C input as the SD treatment. Respiration of a proportion of this larger input of labile C likely contributed to increased CH_4-C effluxes, alongside possible release of higher amounts of methane contained in the digestate treatments themselves. However, these effects on CH_4-C efflux were again dependent on the soil nutrient status, as shown by a significant two-way interaction between soil type and digestate treatment. Even though a larger mass of LD was applied than WD in the second incubation, the WD treatment in the HN soil increased cumulative CH_4-C effluxes to a greater extent than the LD treatment. This likely reflects the fact that the microbial community in the HN soil, which receives regular inputs of LD, is adapted to receive LD instead of WD, meaning that WD application stimulated methanotrophic bacteria and consequently the production

of CH_4-C (Horz et al., 2002). Moreover, it is possible that application of WD also introduced more methane within the digestate fraction itself compared to LD, which was subsequent released, since LD had been stored for at least 4 months prior to the experiment and the CH_4-C content of LD may have been significantly reduced during storage (Gioelli et al., 2011). In contrast, the application of LD to the LN soil increased the cumulative CH_4-C effluxes to a greater extent than the WD treatment. This reflects the fact that the bacterial community in the LN soil is not well adapted to receive LD and the high amount of DOC added with this digestate fraction likely stimulated CH_4-C production through methanogenic metabolism. After exhaustion of available C compounds present in the digestate itself, bacteria may have started to mine SOC and maintain CH_4-C effluxes, as suggested by the temporal pattern of the treatments in LN, where a prolonged increase in CH_4-C efflux was observed.

During the second incubation, the application of SD to the LN soil significantly increased cumulative CH_4-C effluxes until the end of the incubation. It is possible that due to slight differences between soil batches used in the first and second incubations reported in this chapter, the slightly higher DOC content of LN compared to the LN batch used during the first incubation (Table 4.1) positively stimulated bacterial mining of SOC after SD application. However, little data is available on the potential correlation between physicochemical soil properties and soil CH_4 emissions (Le Mer & Roger, 2001).

4.4.3.3 Cumulative N_2O-N efflux

Cumulative N_2O-N effluxes after LD application during the second incubation were higher than after the application of WD. This likely reflects the higher amount of NH_4^+ and DOC applied to soil with LD than WD, thereby supplying nitrifying and denitrifying bacteria with sufficient C and especially N compounds to increase the cumulative N_2O-N efflux (Thomas et al., 2017). However, cumulative N_2O-N effluxes were also dependent on the soil nutrient status, showing how the nutrient composition and past management can directly influence cumulative N_2O-N efflux after digestate application (Rosace et al., 2020). Application of LD to HN increased cumulative N_2O-N effluxes to a greater extent than WD, reflecting the greater input of available C and N (NH_4^+ and NO_3^- soil data Figure 7.4, Chapter 7, Appendix) with the LD treatment compared to the WD treatment and demonstrating that, in the HN soil, cumulative N_2O-N effluxes increase proportionally to the input of C and N within the digestate applied (Millar et al., 2010). The same trend can be seen after LD and WD application to the LN soil. However, the magnitude of cumulative N_2O-N produced by LD and WD in LN is lower than observed within the HN soil, suggesting that application of readily available C and N compounds to a LN soil are quickly immobilised by the bacterial community. Nonetheless, the greater increase of cumulative N_2O-N efflux after LD application compared to WD even in the LN soil indicates that when high amounts of available N are applied to soil, nitrifying/denitrifying bacteria are quickly stimulated and this can leads to an increase in cumulative N_2O-N efflux from soil (Henault at al., 2012).

4.5 Conclusions

The research reported in this chapter explored the biogeochemical mechanisms involved in the production of GHGs from two soils at contrasting initial nutrient status, the environmental impact associated with different digestate fractions, and the implications for GHG emissions following the manipulation of total C:N during digestate application. Application of all fractions of digestate significantly increased GHG emissions when compared with control treatment receiving no digestate. However, a significantly greater emission of cumulative CO_2-C and CH_4-C was associated with WD and LD than SD, whilst greater emissions of cumulative N_2O-N were associated with WD than LD or SD. These observations suggest that the application of SD in agriculture has the potential to reduce the emission of GHGs compared to WD and LD fractions, which due to the input of highly bioavailable forms of nutrients associated with these fractions, in particular C and N, have the potential to stimulate bacterial metabolism and significantly enhance the efflux of GHGs.

Digestate application stimulated GHG production particularly strongly within the HN soil compared to the LN soil. In the latter, GHG production was stimulated over the longer-term, especially after the application of whole and liquid digestate. The results reported in this chapter suggest that the GHG content of digestate itself, coupled with application of available nutrients within digestate, past land management and soil nutrient status, are significant controls on the emission of GHGs. The application of all digestate fractions to a HN soil may increase GHG

emissions in the short term (within 48h after application) when compared to a LN soil, whilst application of SD to a LN soil has the potential to constrain GHG emissions over this short timescale. However, due to the slow mineralization of organic compounds present in the SD fraction of digestate, increased GHG emissions may be sustained over a longer period of time following the application of the SD fraction of digestate to land.

The general increase in GHG emissions during the second incubation reported in this chapter highlights that over-application of digestate can lead to significantly greater GHG emissions than when digestate is applied at the agronomically-recommended rate of 170kgN ha^{-1} y^{-1}. This observation was consistent across both soil types, with the liquid fraction of digestate producing the highest GHG emissions. This reinforces the risk that during increased application rates, the input of large quantities of available nutrients with digestate may significantly affect bacterial metabolism and, alongside the GHG content of the digestate itself, may lead to substantial increases in GHG emissions. Therefore, these findings emphasise the need to carefully plan the correct land application of different digestate fractions to different soil with contrasting nutrient status, in order to minimise GHG emissions and the associated adverse environmental impacts. However, further research is needed to examine the impact of digestate fractions on GHG emissions under long-term field conditions with different application techniques and including plant-soil interactions that were excluded from the experiments reported in this chapter. Additionally, research should seek to investigate additional environmental risks associated with digestate

application to land, such the priming effect after field application of different digestate fractions or the loss of nutrients during leaching events. These studies should be conducted at field-scale throughout a growing season, to fully characterise the potential risks associated with digestate return to land, alongside the fertiliser replacement value of these materials and the opportunity they present to close the nutrient loop associated with fertiliser use in UK agriculture.

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5 Nutrient leaching after the application of different anaerobic digestate fractions to soils with contrasting initial nutrient availability

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Abstract

Primarily due to an unbalanced nitrogen to phosphorus ratio, digestate application in agriculture has the potential to increase the risk of phosphorus (P) leaching to the subsurface. However, the concentration and speciation of P found in leachate after digestate application remain unclear, especially the potential risks associated with organic P compounds that have not been investigated in any research in this area to date. Furthermore, insufficient research has been undertaken focussing on P leaching from soils with contrasting nutrient availability, such as described by the P index, following the application of different fractions of digestate. Therefore, during a field experiment carried out between 6th April and 28th June 2021, intact soil cores were used to investigate the impacts of different digestate fractions (whole [WD], liquid [LD] and solid [SD]) versus inorganic fertiliser (control [Ctr]) on pollutant

concentrations and speciation in leachate. Treatments were applied based on recommended nitrogen (N) inputs to two grassland soils with contrasting P indices (high [HP] vs low [LP]). Four artificial leaching events were simulated and leachate analysed for a range of P forms (dissolved reactive P [DRP], dissolved unreactive P [DUP] and soluble organic P [SOP]). Compared to control treatments, the concentration of all P fractions in the leachate increased significantly following the application of WD and SD fractions, reaching 2 and 6 mg L^{-1} DRP for WD and SD respectively. However, the impacts of digestate on P in leachate varied significantly dependent on the initial nutrient status of the soil to which digestate was applied. For example, SD application to LN increased the leaching of all P parameters to a greater extent than HN, whilst application of WD to LN resulted in lower concentrations of all P parameters compared to HN. Furthermore, significant differences in leachate quality were observed after WD and SD application between individual leaching events. These findings highlight the need to carefully plan the application of different fractions of digestate during land management, based on the soil P index, in order to reduce the risk of leaching of P and consequent adverse environmental impacts. This is particularly true for the SD fraction of digestate, which may significantly increase the risk of P losses through leaching and maintain this effect throughout multiple rainfall events.

5.1 Introduction

The use of phosphorus (P) in agriculture is critical for crop development and growth (Bolan et al., 2005), meaning that P input is vital for meeting the current and likely future high demand for food production (Theregowda et al., 2019). The most common P inputs used in the agricultural sector are inorganic forms, which are present in synthetic fertilisers derived from rock phosphate (Mackay et al., 2017). However, it has been estimated that rock P reserves are declining in quantity, quality and sustainability (Dawson et al., 2011), meaning that finding alternative sources to reduce, and ultimately replace, reliance on inorganic P fertilizers through closing the P loop is an important challenge.

Inorganic P fertilisers may be substituted by organic amendments such as manure, compost or digestate, since these materials often contain high concentrations of total P (TP) (Fuentes et al., 2006) and it has been estimated that c.60% of the TP contained in organic amendments can often be present as inorganic orthophosphate (P_{in}) (Sharpley and Moyer, 2000), and therefore readily available for plant uptake. A significant proportion of the remaining P is present as organic P compounds (P_{org}) that may support longer-term P availability, because a number of P_{org} compounds can be mineralised by hydrolytic enzymes produced by microorganisms and plants, releasing P_{in} into the environment (Espinoza et al., 2013; Widdig et al., 2019). Additionally, it has been reported that symbiosis between plants and fungi *mycorrhizae* can increase the production of these hydrolytic enzymes, as well as the production of organic acids (e.g. lactic, citric, 2-

ketogluconic, malic, oxalic, malonic, tartaric, and succinic) directly from the *mycorrhizae* hyphae (Caruso et al., 2016). Production of organic acids from *mycorrhizae* hyphae can reduce soil pH and solubilise previously insoluble P compounds (e.g. P bound to Al, Fe and Ca) (Tawaraya et al., 2006). In turn, this creates an active uptake site where the diffusion of P_{in} from soil pore space to root surfaces is enhanced (Bucher, 2006) and, in exchange, plants provide carbohydrates to the fungi (Smith et al., 2003). A similar process can be observed for some soil bacteria (e.g. phosphate-solubilizing bacteria) and plants, where P_{in} within some more soluble soil minerals (e.g. apatites) can be solubilised through organic acid production and through exudate production, increasing the supply of plant-available P into the soil solution (Hinsinger, 2001; Walpola and Yoon, 2012). This occurs predominantly because the emission of CO_2 during microbial respiration and the production of organic acids via bacterial metabolism and plant exudates decrease pH (Schlesinger, 1997) around the soil minerals, allowing the release of plant-available P_{in} into the rhizosphere (Panhwar et al., 2013).

During recent years, the application of whole digestate to soils as an organic amendment in agriculture has grown considerably (Lee at al., 2021). Whole digestate is naturally rich in in N, P, K and other macro- (e.g. S, Ca, Mg) and micro- (B, Cl, Cu, Fe, Mn, Mo, Ni and Zn) nutrients, often present in readily plant-available forms (Tambone et al., 2010; Risberg, 2015). The separation of whole digestate (WD) into solid (SD) and liquid (LD) fractions is associated with redistribution of multiple nutrient elements between the individual fractions, including Total

Nitrogen (TN), ammonium (NH_4^+) and P_{in} (Panuccio et al. 2016). For example, it has been estimated that 40-50% of the TP is retained in the solid fraction after separation compared to liquid fraction (Bauer et al., 2009), with most of the TP in the solid fraction present as P_{in} (up to 90% of TP), whilst only 5-7% of TP in the solid fraction is present as P_{org} (Hjorth et al., 2010). However, it has also been estimated that TP present in the liquid fraction is mainly present as P_{in}, although the P_{org} contained in the liquid fraction can account for 13-14% of the TP retained after solid-liquid separation (Bachmann et al. 2016). However, in contrast, Akhiar et al. (2017) and Lukehurst et al. (2010) reported that solid digestate contained higher amounts of P_{org} than P_{in} after separation, whilst the liquid fractions in this past research contained mostly P_{in} and only small amounts of P_{org}. Therefore, uncertainties remain surrounding the redistribution of P after solid-liquid separation of digestate, especially based on the separation technique applied and the feedstock used during the anaerobic digestion (Guilayn et al., 2019). Moreover, it has been reported in the literature that after solid-liquid separation, the solid fraction of digestate has a lower TN and NH_4^+ content compared to liquid fraction (which retains c. 80% NH_4^+ after separation) (Tambone et al., 2017), although this is highly variable based on the feedstock utilised during the anaerobic digestion stage (Hjorth et al., 2010). For example, solid digestate originating from fibrous feedstock material, such as cattle manure and silage, may retain lower NH_4^+ and TN concentrations (c. 6.5 and 22.5 g kg^{-1} DM, respectively) than solid digestate originating from N-rich feedstock (e.g. sewage sludge and pig slurry) which contains higher NH_4^+ and TN concentrations (c. 10.4 and 47 g kg^{-1} DM, respectively). However, it is always the case that the liquid

fraction of digestate contains more NH_4^+ and TN than the solid fraction, although the absolute concentrations of NH_4^+ and TN is, again, dependent on the feedstock utilised during the AD process. For example, the liquid fraction originating from fibrous feedstock material such as cattle manure and silage can contain lower NH_4^+ and TN concentrations (c. 38.5 and 75.8 g kg^{-1} DM, respectively) than liquid digestate originating from N-rich feedstock (e.g. pig slurry and food waste) which can contain higher $NH_4^+$and TN concentrations (c. 125 and 155 g kg^{-1} DM for NH_4^+ and TN, respectively, Guilayn et al., 2019).

In agriculture, digestate is usually applied following specific N application rates (e.g. RB209), making it difficult to target a specific P application rate and a specific P form during application to land. Additionally, due to the high amount of P relative to N within digestate (Goss et al., 2013; Guilayn et al., 2019a), it is often the case that when digestate is applied to meet a recommended N rate, P is applied in excess of soil or crop requirements. Furthermore, depending on the biogeochemical characteristics of the soil and the digestate fraction applied, subsequent mineralisation rates of P_{org} applied with digestate can be extremely variable and therefore it is difficult to predict the amount and rate of P_{in} released from these compounds over various timescales (Westerman and Bicudo, 2005). Therefore, over the longer term, continued application of digestate in agriculture can contribute to P accumulation in soils, which in turn increases the risk of P export from agricultural land through leaching, overland flow and soil erosion (Brandt et al., 2003). This potentially has significant environmental consequences, including eutrophication of

receiving waters (Filippelli, 2002). This is principally due to the dissolved reactive P present in leachate or overland flow (DRP, filtered <0.45 µm; comprising mainly the dissolved form of P_{in}, see Chapter 2 section 2.3), which is the fraction most readily available to organisms and frequently linked to the triggering of algal blooms in surface waters (García-Albacete et al., 2016; Turner et al., 2000; Myers and Pierzynski, 2000). However, it has also been reported that the dissolved unreactive P fraction (DUP, filtered <0.45 µm; comprising mainly the dissolved form of P_{org}) may play an important role in the process of eutrophication in some receiving waters (Turner at al., 2000). The dissolved unreactive P fraction consists of organic P compounds, inorganic polyphosphates, and mineral colloids (Vaz et al., 1992; Baldwin, 1998; Denison et al., 1998; Haygarth et al., 1997) that can potentially be taken up by organisms within receiving waters depending on the bioavailability and solubility of these forms of P (Shand & Smith, 1997; Darch et al., 2016). This is especially the case when the availability of P_{in} in a receiving water is low, creating a situation in which it becomes an advantage to organisms to access P present within organic compounds. For example, some aquatic biota have the capability to synthesise enzymes that catalyse the hydrolysis of soluble organic P compounds (SOP) into bioavailable P (P_{in}) (George et al., 2006). Three major groups of SOP compound that can be transformed into P_{in} can be defined as (Bünemann, 2008): i) hydrolysable monoester P, such as adenosine triphosphate and monophosphate (ATP, AMP), guanosine-5'-triphosphate (GTP) and compounds that build up RNA and DNA; ii) hydrolysable diester P, such as nucleic acid and phospholipids; and iii) hydrolysable inositol hexakisphosphate, which is a group of compounds where a

central inositol group (in one of nine potential isometric forms) is bound between one and six phosphate groups by phosphomonoester bonds (Toor et al., 2003; Baldwin, 2013). Given the range of forms of P present within digestate that potentially contribute to eutrophication risk, it is important to quantify the magnitude and speciation of P_{in} and P_{org} export after digestate application to land, using this understanding to help mitigate these risks associated with digestate use in agriculture. However, previous research related to digestate application to agricultural soils has not focussed sufficiently strongly on these issues, particularly the risks associated with P export via leaching into the subsurface.

It has been commonly perceived historically that P leaching from soils was insignificant, although the potential for varied P losses into the subsurface is increasingly recognised, driven largely by soil properties (Stutter et al., 2012; Turner & Haygarth, 2000). Conditions under which leaching of P can be promoted are associated with many factors, including: a) processes of adsorption and fixation with cations (i.e. Ca^{2+}, Mn^{2+}, Fe^{3+}, Al^{3+}) and clay particles. These cations can strongly bind to P reducing its mobility, additionally, the silicate minerals and organic matter present in clays can generate a competitive effect on P adsorption due to ligand exchange with the surface hydroxyl groups (Fuentes et al., 2006). However, this is highly dependent on the charge density of these cations and on; b) seasonal processes, such as those associated with redox reactions, whereby in the presence of strongly reducing soil conditions, redox-sensitive cations such as Fe which normally adsorb P under oxidised conditions (Fe^{3+}), can be reduced (Fe^{2+}) and the P

associated with those cations is released to solution. Leaching of P is also highly dependent on: c) soil pH, since it has been estimated that the maximum P solubility is between pH 4.5 and 6.5, which coincides with the lowest degree of P fixation by Ca, Al, and Fe minerals (Penn & Camberato, 2019); d) soil texture (sandy versus clay) and structure (e.g. cracking and preferential flow), since macropores or cracks act as preferential flow channels that can increase the risk of P export to the subsurface; and finally e) conversion into P_{in} due to soil microorganisms (e.g through production of organic acids and hydrolytic enzymes), since the conversion can increase P mobilization into the soil-water solution (Hinsinger, 2001; Jara et al., 2006).

Additionally, the soil P index and fertilization processes can play important roles in controlling the risk of P leaching through the subsurface (Kleinman et al., 2015). Soils with a high P index which are constantly fertilised are more likely to reach a saturation threshold, also termed a "change point", than soils with a low P index, which in the past was indicated as 60 mg Olsen-P kg^{-1} (Heckrath et al., 1995; Maguire & Sim, 2002). When soils are at a P status below this change point threshold, P is more likely to be fixed or adsorbed to the soil matrix, since available adsorption/fixation sites are still available. In contrast, when soils contain concentrations of P above the change point, adsorption/fixation sites are saturated and the risk of P export via leaching increases. Therefore, excessive input of P_{in} to soils, for example associated with the application of digestate or manure due to the Tot N:P imbalance in these materials, can accelerate the movement of a soil towards

the “change point”, above which leaching of P is predicted to increase sharply (Nair et al., 2014).

Moreover, the fact that different digestate fractions have different NH_4^+ and N_{org} contents, means that it is difficult to predict the N_{org} mineralized through ammonification and the NH_4^+ converted into NO_3^- during the nitrification process. Additionally, estimating the amount of NH_4^+ and NO_3^- left in soil after digestate application can be difficult, since NH_4^+ and NO_3^- can be taken up by plants and leached after land application (Sharifi et al., 2019). Digestate is commonly low in NO_3^-, since due to the high pH and ionic buffering of digestate, NH_4^+ accumulates in solution but is not transformed to NO_3^- (Nutrient Value of Digestate from Farm-Based Biogas Plants in Scotland, 2016) and the available NH_4^+ content can be rapidly taken up by plants, or strongly fixed to the negative charge of many soil surfaces (WRAP, 2016; Tsachidou et al., 2019). If appropriate application of digestate is undertaken, NH_4^+ present in this material can be nitrified within soil in the first 1-2 weeks following application (Jacques et al. 2008; Möller and Müller 2012; Insam et al. 2015). However, following injudicious application of digestate, for example the application of digestate during rainfall events or soon before a rainfall event, or the over-application of digestate, NH_4^+ can be lost through leaching. Furthermore, the negative charge of NO_3^- limits the potential binding of this ion to soil particles (Bloom, 2010) and consequently increase the environmental risk of leaching if good agricultural practices, guidelines and correct N targets are not followed during digestate application (Nicholson et al., 2017).

The NH_4^+ leached to water bodies can be dissociated into NH_3 at pH>7 and temperature >30°C, which is extremely toxic for aquatic organisms since it can pass from the bloodstream into the tissues and brain, causing damage and behavioural impairment to fish and amphibians (Thurston et al., 1981; Thraves, 2004). The presence of NO_3^- in ground water can alter water quality and produce a high risk to human and ruminant health (Lord and Athony, 2002; Schroeder et al., 2004), for example because high concentrations of NO_3^- can cause blue syndrome (methemoglobinemia) in infants (McMckague et al., 2017). Historically, N was considered the limiting nutrient for triggering algal blooms only in estuaries and the oceans. However, more recent evidence suggests that N can alter nutrient balances and ecological processes in rivers, lakes and estuaries (Smith et al. 1999), potentially contributing to eutrophication (Dodd and Smith., 2016).

The leaching of P and N after digestate application to land has not been thoroughly quantified in past research. Some initial work has reported relatively low concentrations of P in leachate after digestate application, during a greenhouse pot experiment (0.04-1 mg P L^{-1}) (Song et al., 2018), when compared to compost during column laboratory experiments (García-Albacete et al., 2014), or when soil samples from pre-existing long term field experiments were re-packed into glass columns to assess P leaching (Vanden Nest et al., 2015). Only one study used a lysimeter with intact soil structure (Koch et al., 2019), which is critical for maintaining soil physical structure and hydraulic properties that will control leaching risk, although this work examined only whole digestate. At the end of the field experiment, Koch et al.

(2019) reported that digestate application increased the DRP (0.52 mg P L^{-1}) and TDP (0.7 mg P L^{-1}) concentrations compared to unamended soil. Across both laboratory and field studies, only DRP and TDP concentrations in leachate have been examined, although often only one of these parameters is reported in a given study, without consideration of the concentration of other forms of P. Further, the initial soil P status has not been one of the main controls on P leaching examined in past research and often only whole digestate has been used. The lack of knowledge surrounding different forms of P (e.g. DRP, total dissolved P [TDP], DUP and SOP) exported via leaching after the application of different fractions of digestate to land is an important research gap to address, because export of this wide range of forms of P to waterbodies may induce ecological responses and lead to significant, adverse environmental impacts.

Therefore, in this chapter, the leaching of different species of dissolved P (DRP, total dissolved P [TDP], DUP and SOP) following the surface application of multiple fractions of digestate (whole, liquid and solid) was quantified and compared to a control treatment comprising inorganic fertiliser. Experiments were undertaken using both a high P_{in} soil (>60 mg Olsen-P kg^{-1}) and a low P soil (<60 mg Olsen-P kg^{-1}), to evaluate how background nutrient status of a soil may influence the risk of nutrient export through leaching. Furthermore, due to the lack of existing data focussing specifically on the P_{org} fractions present in leachate, the amount of naturally hydrolysable P, hydrolysable monoester P, hydrolysable diester P and hydrolysable inositol hexakisphosphate contained in leachate after the application

of treatments was specifically investigated. In addition, the NH_4^+-N and NO_3^--N concentration in leachate after digestate application versus inorganic fertiliser application was investigated, since these data are poorly documented in the literature especially after application to soils with contrasting nutrient status (Nkoa, 2014). The hypotheses underpinning this experiment were that: i) soil initially at a high agronomic P index will be associated with significantly higher concentrations of dissolved P and NO_3^--N in leachate following digestate application, compared to a soil at an initially lower agronomic P index; ii) the application of SD would be associated with higher concentrations of P in leachate compared to either WD or LD, due to higher inputs of TP with the SD fraction in order to achieve recommended N application rates; iii) the application of LD would be associated with higher concentrations of NO_3^--N in leachate compared to either WD or SD, due to higher infiltration and nitrification rates in soil, since the majority of TN in LD is present as NH_4^+; iv) the concentration of P within leachate would increase significantly across individual rainfall events, due to increases in P mineralization and solubilisation within the soil profile; and v) the concentration of N (as NH_4^+-N and NO_3^--N) within leachate would decrease significantly across individual rainfall events, due to plant N uptake.

5.2 Materials and Methods

5.2.1 Experimental setup, soil-cores sampling

For the field experiment, intact soil cores (25H x 22Ø cm, metal core housings) were collected from two fields adjacent to a commercial biogas plant (Cockerham Green Energy Ltd, northwest England, UK; latitude: 53.972, longitude: -2.822) on 15th March 2021. The fields were chosen based on their contrasting P indices (Agriculture and Horticulture Development Board, 2017) and nutrient composition, as driven by the management history of each field (Table 5.1). Once collected, the soil cores were distributed randomly across individual positions within a metal holding rig and left in the field until the start of the experiment (Figure 5.1). The cores were held in place with metal wings and the gap between the core and the rig was sealed using silicone sealant.

A temperature data logger (UA-001-08, HOBO® Data Loggers) was inserted into one of the soil cores and an additional logger was inserted into the soil adjacent to the metal rigs to record temperature throughout the field experiment (Figure 5.3).

Figure 5.1 Image showing intact soil cores held within metal rigs and funnels used for leachate collection during the field experiment

The high P soil (HP) was under grass production at the time of sampling and used for grazing and silage production in previous years. This field received liquid digestate four times per year and the last application occurred on 20th February 2021. The low P soil (LP) was a fallow soil at the time of sampling and had never previously received digestate.

5.2.2 Initial soil characterisation

Separate soil samples were collected prior the beginning of the field experiment in order to characterize soil physio-chemical characteristics. On 15th March 2021, topsoil to 20 cm depth was sampled from each field using a gouge auger. Soil samples were taken from randomly selected positions in the section of a field identified for subsequent collection of intact cores and homogenised inside plastic bags. Soils were sieved through a 2 mm sieve and characterised before the start of the experiment for pH, electrical conductivity (EC), soil dry matter (DM), Loss-on-ignition (LOI), plant available N and K, Olsen P, dissolved organic C (DOC), dissolved unreactive N and P (DUN and DUP, respectively), water extractable N (as NH_4^+ and NO_3^-) and P (as PO_4^{3-}), P saturation index (PSI), oxalate extractable Al/Fe/P and total C (TC) and N (TN). Bulk density was determined by taking an intact core of soil of known volume from the field and drying at 105°C until constant weight was reached and processed as described by Rai et al. (2017).

Water extractable DOC, DUN, NH_4^+ and NO_3^- were determined as described by Jones & Willett (2006), using milliQ water (>18.2 MΩ.cm at 25°C; 1:10 w/v, 15 minutes shaking) with the extract filtered through a Whatman No 42 filter paper. A subsample of the extracted solution was analysed for DOC using a TOC-L/TN Series Analyser (Shimadzu, Japan) after sample acidification to remove inorganic C. The remaining extracted solution was then analysed for water extractable NH_4^+ and NO_3^- using a SEAL Autoanalyzer AA3 (Seal Analytical, UK; Method No G-102-93 Rev 2; Multitest MT7/MT8) with two different colorimetric reactions (ISO 11732, 1997 and

ISO 13395, 1996 respectively) and subsequently analysed for TN using a TOC-L/TN Series Analyser (Shimadzu, Japan). The difference between TN and inorganic N gave the DUN concentration. The water extractable P was determined on oven-dried soil samples (60°C for 48h, 1:10 w/v, 1 h shaking) and filtered using a 0.45 µm Acrodisc™ Syringe Filters with Supor™ Membrane. The extract was analysed as dissolved reactive P (DRP) using a SEAL Autoanalyzer AQ2 (Seal Analytical, UK; Method No EPA-118-A; Rev.3) based on an ammonium molybdate-ascorbic acid method. Subsequently, an aliquot of the sample was digested using an acidic persulfate digestion method (US EPA 365.1 method) and analysed for total P (TP) using a SEAL Autoanalyzer AQ2 (Seal Analytical, UK; Method No EPA-119-A; Rev.4). The difference between TP and DRP was interpreted as DUP.

Air-dried soil samples for oxalate extractable Al/Fe/P were extracted with a solution of oxalic acid and ammonium oxalate (pH 3) as described by Schoumans et al. (2000) (1:20 w/v, 2 h shaking wrapped in foil) and filtered (Whatman No 42). Samples were analysed for oxalate extractable Al/Fe/P using and ICP-OES (Thermo scientific, iCap 60000 series) and the concentrations were used for the calculation of the PSI as described by Schoumans et al. (2000).

Fresh soil samples for mineral N analysis were extracted with 2 M KCl (1:5 w/v, 1 h shaking) (Bremmer, 1965; McTaggart & Smith, 1993) and filtered (Whatman No 42). The solution was subsequently analysed for NH_4^+ and NO_3^- content using a SEAL Autoanalyzer AA3 (Seal Analytical, UK; Method No G-102-93 Rev 2; Multitest MT7/MT8) with two different colorimetric reactions (ISO 11732, 1997 and ISO 13395, 1996

respectively). Air dried soil samples were analysed for Olsen P as described by Murphy & Rilely (1962) and Olsen et al. (1954). Samples were extracted (1:20 w/v, 30 minutes shaking) with a 0.5 M $NaHCO_3$ solution pH adjusted to 8.5 and subsequently filtered (Whatman No 42). The extracted samples were analysed using a SEAL Autoanalyzer AA3 (Seal Analytical, UK; Method No G-103-92 Rev1; Multitest MT7/MT8) based on the molybdate blue colorimetric reaction. Air dried samples were analysed for available K as described by DEFRA (RB427, Method 63). Samples were extracted (1:5 w/v; 30 minutes shaking) using NH_4NO_3 and subsequently filtered (Whatman No 42). The extracted samples were analysed using a flame photometer (Sherwood Corning Flame Photometer 410). The TC and TN content of soils was determined using an automated Dumas procedure on a Vario EL cube (Elementar, DE), working with 20±1 mg of oven-dried and ball-milled soil. Soil pH was determined on fresh soil samples (1:5 w/v; 30 minutes shaking) using milliQ water; samples were left to settle overnight and supernatant was measured for electrical conductivity (EC). Soil DM and LOI were determined using a gravimetric method (Allen, 1989; Gardner, 1986). Approximately 12g of fresh soil was oven-dried at 105°C for 48 h to constant weight to determine the dry weight (DW). Subsequently, around 1.5g of oven-dried soil was heated at 550°C for 6h in a muffle furnace, left to cool overnight in desiccator and subsequently weighed to determine LOI.

Table 5.1 Initial physio-chemical characteristics of soils used in the field experiment (mean values reported, ±1 SE in parentheses, n=4). P-values represent statistical differences between High nutrient and Low nutrient soils and "n.s" indicates no statistical difference.

Soil characteristics	**High P soil**	**Low P soil**	**p- value**
Bulk density (g cm^{-3})	1.24 (0.027)	1.04 (0.032)	n.s
pH water (1:5 w/v)	6.65 (0.036)	5.49 (0.037)	<0.001
EC (µS cm^{-1}; 1:5 w/v)	43 (0.71)	8 (0)	<0.001
2M KCl NO_3^- (mg kg^{-1} DM soil)	24.01 (0.29)	1.79 (0.015)	<0.001
2M KCl NH_4^+ (mg kg^{-1} DM soil)	3.95 (0.61)	8.64 (0.15)	<0.001
Olsen P (mg kg^{-1} DM soil)	71.31 (0.65)	7.97 (0.64)	<0.001
Ammonium nitrate K^+ (mg kg^{-1} DM soil)	473.87 (2.71)	27.43 (0.60)	<0.001
P and K indices UK (Agriculture and Horticulture Development Board, 2017)	5 (P), 4 (K)	0 (P), 0 (K)	<0.001
Water extractable Dissolved Unreactive N (mg kg^{-1} DM soil)	13.13 (0.44)	2.10 (0.20)	<0.001
Water extractable NH_4^+ (mg kg^{-1} DM soil)	0.55 (0.084)	0.50 (0.025)	n.s
Water extractable NO_3^-(mg kg^{-1} DM soil)	31.8 (0.20)	11.75(0.17)	<0.001

Water extractable Dissolved Unreactive P (mg kg^{-1} DM soil)	4.06 (0.27)	6.49 (0.16)	<0.001
Dissolved Reactive P (mg kg^{-1} DM soil)	18.83 (0.14)	0 (0)	<0.001
Oxalate extractable Al (mg kg^{-1} DM soil)	1029.88 (37.52)	993.69 (29.42)	<0.01
Oxalate extractable Fe (mg kg^{-1} DM soil)	5343.92 (98.95)	4051.05 (128.72)	<0.01
Oxalate extractable P (mg kg^{-1} DM soil)	1274.78 (57.68)	326.25 (16.27)	<0.001
P saturation index	0.30 (0.042)	0.10 (0.014)	<0.001
Water extractable Total Organic C (mg kg^{-1} DM soil)	54.92 (1.39)	18.18 (1.94)	<0.001
TC:TN	10.70 (0.13)	10.63 (0.09)	n.s
Loss-on-ignition (LOI %)	8.36 (0.98)	7.31 (0.12)	n.s

EC (Electric Conductivity); TC (Total Carbon, Dumas); TN (Total Nitrogen, Dumas); Water Tot C (Water Extractable Total Organic Carbon, acidified analysis); NH_4^+ (Ammonium); NO_3^- (Nitrate); P (Phosphorus); K (Potassium); P and K index (mg L^{-1})

5.2.3 Digestate sampling and characterization

Whole, solid and liquid fractions of anaerobic digestate were applied on two occasions during the field experiment reported in this chapter. Therefore, in order to correctly calculate NH_4^+-N application rates for digestate fractions, digestate was collected for physio-chemical characterization on 22nd March and 23rd April 2021 from Cockerham Green Energy Ltd. Digestate from Cockerham Green Energy Ltd is fermented in a mesophilic, single stage digester with a retention time of 50 days. The feedstock is livestock and poultry manure, co-digested with food waste including potatoes, liquid sugar, silage, wet grain, tea bags and whey. Whole digestate is separated into liquid and solid fractions using a screw-press. The liquid fraction is collected in covered lagoons, whilst the solid fraction is stored in an uncovered open-space. Digestate was sampled following protocols detailed by Agriculture and Horticulture Development Board (2017). Whole digestate was sampled directly from the anaerobic digester before separation, the solid fraction was sampled from fresh material stored in an open space directly after separation, whilst the liquid fraction was collected from the lagoon after at least four months of storage. Digestate fractions were stored at 4°C prior each field application (section 5.2.4). Physio-chemical characterisation of the three fractions of digestate was performed by an externally ISO-accredited laboratory within 5 days of sample collection before each field application and these data are reported in Table 5.2.

Table 5.2 Physio-chemical characteristics of whole, liquid and solid digestate during the first and the second sampling dates (n=1)

Parameter in fresh weight (fresh matter [FW])	**Whole digestate (WD)**		**Liquid digestate (LD)**		**Solid digestate (SD)**	
	1st sampling date	2nd sampling date	1st sampling date	2nd sampling date	1st sampling date	2nd sampling date
DM (%)	8.52	8.40	2.31	2.43	23.50	28.4
Organic Matter (%)	5.08	5.53	0.90	1.10	74.27	73.9
pH (1:6 w/v)	8.51	8.38	8.15	8.10	8.99	8.94
TN (mg kg^{-1} FW)	7200	6500	2700	3200	4159.50	6361.60
NH_4^+-N (mg kg^{-1} FW)	4131	4054	1883	2256	1101.21	1601.192
TP (mg kg^{-1} FW)	1483	1691	264	231	1302.61	5573.216
WEP (mg kg^{-1} FW)	211	135	133	74.40	353.44	238.56
Tot K (mg kg^{-1} FW)	6573	6093	3201	3745	4162.55	5130.46
Tot Mg (mg kg^{-1} FW)	717	873	105	55.20	581.86	3435.55
TC (mg kg^{-1} FW)	24400	25600	7700	8090	107160	108488
TC:TN	3.39	3.94	2.85	2.53	25.76	17.05
TN:TP	4.86	3.84	10.22	13.85	3.19	1.14

DM (Dry Matter); TP (Total Phosphorus); WEP (water extractable Phosphorus); Tot K (Total Potassium); Tot Mg (Total Magnesium); TC (Total Carbon); TN (Total Nitrogen); NH_4^+-N (Ammonium Nitrogen)

5.2.4 Field experimental design

The field experiment was conducted between 6th April and 28th June 2021, involving two soils with contrasting P status ('High P' and 'Low P', Table 5.1). On 6th April 2021, the first application of inorganic fertiliser (positive Ctr), whole digestate (WD), solid digestate (SD) and liquid digestate (LD) was made to both soils. Six weeks after these first treatments, grass was harvested from each core before a second treatment application was carried out. During the first and the second treatment applications, the recommendations given in the Nutrient Management Guide (RB209, section 2, 2017) for grass silage and soil P/K indexes were followed and treatments were applied on the soil surface in order to mimic splash plate application of digestate. During the first application, cores from both soil types received ammonium nitrate (Ctr), WD, SD and LD treatments which were added to achieve a constant N application rate of 120 kg ha^{-1} (NH_4^+-N content of digestate treatments and TN content of ammonium nitrate fertiliser were used within the calculations). In addition to ammonium nitrate, the Ctr in the 'Low P' soil type received 100 Kg (Phosphate) ha^{-1} of superphosphate and 80 kg (Potash) ha^{-1} of potassium sulphate as suggested by the Nutrient Management Guide (RB209, section 3, 2017) for soils with P/K index of 0. During the second application, treatments were applied to achieve a constant N application rate of 90 kg ha^{-1} (NH_4^+-N content of digestate treatments and TN content of ammonium nitrate fertiliser was used within the calculations). Due to the large amount of SD needed during the first application, any SD material remaining on the soil surface following the first treatment was removed

prior to the second application. The Ctr in 'Low P' received 25 Kg (Phosphate) ha^{-1} of superphosphate and 120 kg (Potash) ha^{-1} of potassium sulphate in addition to ammonium nitrate, as suggested by the Nutrient Management Guide (RB209, section 2, 2017) for soils with P/K index of 0. The TN, TP, WEP, TK, TMg and TC added via WD, LD and SD during the first and second applications are reported in Table 5.3.

Table 5.3 Parameters added (mg) with the treatments during the first and second fertiliser and digestate application

Parameter (mg) added with the treatments (FW)	Inorganic fertiliser (Ctr)		Whole digestate (WD)		Liquid digestate (LD)		Solid digestate (SD)	
	1st application	2nd application	1st application	2nd application	1st application	2nd application	1st application	2nd application
NH_4^+-N (mg, application target)	456.12	342.9	456.12	342.09	456.12	342.09	456.12	342.09
TN (mg)			794.98	548.49	654.02	485.23	1722.86	1359.14
TP (mg)	380.1 (LP soil only)	95.03 (LP soil only)	163.74	142.69	63.95	35.03	539.54	1190.70
WEP (mg)			23.30	11.39	32.22	11.28	146.39	50.97
TK (mg)			725.75	514.15	775.38	567.88	1724.13	1096.11
TMg (mg)			79.17	73.67	25.43	8.37	241.01	733.99
TC (mg)			2694.10	2160.21	1865.17	1226.73	44385.56	23178.14
K (mg)	304.08 (LP soil only)	456.12(LP soil only)						

TP (Total Phosphorus); WEP (Water Extractable Phosphorus); TK (Total Potassium); TMg (Total Magnesium); TC (Total Carbon); TN (Total Nitrogen); NH_4^+-N (Ammonium Nitrogen); K (Potassium)

During both applications, treatments were applied randomly to soil cores from each soil type, as described in Table 5.4. During the application of WD and LD treatments in the field, unfortunately an error was made with the number of replicates for each treatment leading to an unbalanced experimental design. However, care was taken with the statistical treatment of the data to ensure the unbalanced design was accounted for.

Table 5.4 Replicates used during the field experiment for control (Ctr), whole (WD), liquid (LD) and solid (SD) digestate treatments

Treatments	**High P soil replicates (n=)**	**Low P soil replicates (n=)**
Ctr	4	4
WD	2	6
LD	6	2
SD	4	4

5.2.5 Artificial rainfall events and collection of leachate

A total of four artificial rainfall events were carried out, 1 week and 3 weeks after the application of treatments that occurred on the two individual dates described above. Before each rainfall event, plastic watertight pipe caps (Vital Parts, https://www.vital-parts.co.uk/) were fitted under each core (Figure 5.2a) and soil was subsequently saturated with natural rainwater, harvested from an in situ water butt, and left to equilibrate for 30 minutes. Subsequently, the pipe caps were removed and a funnel with a 2mm metal mash inserted inside was placed on the bottom of each core and directed to drain into a 1 L plastic bottle (Figure 5.2b). Natural rainwater was applied to the surface of each core until leachate was generated from the base of each core and the first 200 ml of leachate was collected for analysis. Natural rainfall was recorded using a tipping bucket rain gauge (ARG100 model) and automatically logged as 10-minute totals/averages, with the gauge located at the Hazelrigg meteorological enclosure (GR 493 578, c. 95m asl), which is situated 9 km from the location of the field experiment. Due to the unprecedented very dry and hot weather during April and June 2021, additional natural rainwater was applied to the surface of each core in order to prevent grass plants from dying and excessive drying and shrinkage of soil cores. Across April, a total of 2 x 300 ml of natural rainwater was applied to each core, whilst across June 6 x 417 ml of natural rainwater was applied to each core. During each application of rainwater, care was taken to avoid generating any additional leachate from the cores.

a)

b)

Figure 5.2 Blue pipe caps in place on base of cores during initial re-wetting prior to artificial rainfall events (a) and leachate collection during the artificial rainfall event (b)

5.2.6 Leachate sample analyses

Unfiltered water samples collected in the field were placed inside a cool box and transported to the laboratory within 30 minutes for subsequent filtration. Samples were first filtered using a Whatman GF/F glass fibre filter placed into a Nalgene™ Reusable Filter Unit and subsequently filtered using a 0.45 µm Acrodisc™ Syringe Filters with Supor™ Membrane. Filters were pre-treated with milliQ water prior filtration as reported by Karanfil et al., (2003). Filtered samples were analysed within 24 h for dissolved reactive P (DRP), soluble organic P (SOP) and dissolved inorganic N (DIN, NH_4^+-N and NO_3^--N). The DRP was analysed following the phosphomolybdenum blue methodology of Murphy and Riley (1962) and read using a microplate reader (FLUOstar Omega, BMG Labtech, DE) at 880 nm, 16 minutes after reagent addition. Subsequently, filtered samples were digested following the acidic persulphate digestion method (US EPA 365.1 method) and read using a SEAL Autoanalyzer AQ2 (Seal Analytical, UK; Method No EPA-119-A; Rev.4) for total dissolved P (TDP) determination. The difference between TDP and DRP was interpreted as dissolved unreactive P (DUP). The DIN was determined using a SEAL Autoanalyzer AA3 (Seal Analytical, UK; Method No G-102-93 Rev 2; Multitest MT7/MT8) with two different colorimetric reactions (ISO 11732, 1997 and ISO 13395, 1996 respectively) and subsequently analysed for Total N (TN) using a TOC-L/TN Series Analyser (Shimadzu, Japan). The difference between TN and DIN gave the dissolved unreactive N (DUN) concentration present in the samples.

The concentration of SOP was analysed using an enzyme addition method (Turner et al., 2002; Bunemann, 2008), in order to measure the naturally hydrolysable P (NHP, no enzyme added), monoesterase hydrolysable P (MHP; enzyme used: alkaline monoesterase from *E. Coli*. P5931, Sigma Aldrich), diesterase hydrolysable P (DHP; enzyme used: combination of alkaline monoesterase and nuclease from *Penicillium citrinium* N8630, Sigma Aldrich) and phytase hydrolysable P (PHP; enzyme used: phytase from wheat (crude preparation) P1259, Sigma Aldrich). Enzymes were dissolved in 0.5 M sodium acetate ($NaC_2H_3O_2$) buffer (pH 5) containing 2 mM of magnesium chloride ($MgCl_2$) and 2 mM zinc sulphate ($ZnSO_4$), as reported by Darch et al. (2016).

In order to store diluted nuclease from *Penicillium citrinium* (N8630, Sigma Aldrich), 50% glycerol after reconstitution in the aqueous buffer was added (https://www.sigmaaldrich.com/catalog/product/sigma/n8630?lang=en&region=GB). The phytase preparation contained phosphate, which was removed by repeated dialysis (12,000 Da membrane) in sodium acetate buffer until the DRP concentration was < 10 µg P L^{-1} (He and Honeycutt, 2001) and then centrifuge in order to remove particulate matter prior to analysis. For each enzyme determination, to 1.8 ml of sample 0.2 ml of enzyme (or buffer for NHP, or 0.1 ml of each enzyme when used in combination for DHP), was added to give a final enzyme concentration of 0.01 units ml^{-1} for alkaline monoesterase and nuclease and 0.1 units ml^{-1} of phytase. A unit of activity is defined as the quantity of enzyme that will release 1.0 µmol of inorganic P per minute at a given substrate concentration, pH and temperature (Darch et al.,

2016). Quality control standards (Glucose-6-phosphate, DNA from salmon sperm and phytic acid, all Sigma) were used during the incubation in order to assess the performance of each enzyme. Sodium azide (NaN_3) was added to the samples as a microbial inhibitor (0.1 mL, 100 mM) during the enzyme addition and samples were incubated at 37 °C for 16 h (Turner et al., 2002) before reading. Samples were read as described above for DRP. For the calibration curve, 0.2 ml buffer was added to standards for NHP, MHP and DHP determination, since enzymes do not affect the calibration curve (Turner et al., 2002), whilst for PHP determination, 0.2 ml of phytase was added to the standards since phytase affects the calibration curve.

5.2.7 Data handling and statistical analysis

Data for DHP and PHP were not considered valid and are therefore not discussed further in this chapter. The quality control standard for DHP had only a 10% recovery and this is believed to be due to the glycerol used for storing the solution after reconstitution in buffer. Despite the fact that quality control standard for PHP had a 100% recovery, the results were not used since DHP results are needed in order to calculate PHP concentration (Bunemann, 2008). Therefore, only NHP and MHP data are investigated further in this chapter, since NHP is the product of microbial hydrolysis without any enzyme addition and the quality standard for MHP had 100% recovery.

Calculations for NHP and MHP determination were carried out as followed (Darch et al., 2016):

1) (Sample + buffer concentration) – DRP concentration= NHP

2) (Sample + monoesterase concentration) – (Sample + buffer concentration) = MHP

Statistical analysis was performed using SPSS (Statistic 27, IBM). Repeated Measures ANOVA (RMA) with type III error was employed to assess the effect of soil ("HP" and "LP"), treatments (Ctr, LD, WD and SD) and the interaction with time (four individual leaching events). Levene, Shapiro-Wilk and Kolmogorov–Smirnov tests were employed in order to assess variance homogeneity, normality distribution of the residuals and probability distribution of the data. The Interquartile range (IQR) was computed for DRP, TDP, DUP, NHP, MHP, NH_4^+-N and NO_3^--N data and any data point outside 1.5 * IQR was identified as an outlier and removed from analysis. When zero values were present, they were substituted with half of the method limit of detection (Helsel et al., 1990, 2006). Square root transformation was applied to DRP and NO_3^--N data, whilst Ln transformation was applied to TDP and DUP data and no transformation was necessary for NH_4^+-N concentration data. Even though an unbalanced design existed in this experiment, after data transformation, the assumptions for the RMA test were met and therefore analysis of the datasets proceeded.

Tukey-tests (HDS) were used where significant factors were identified. With respect to the time factor, only significant Treatment × time interactions were investigated.

Despite attempts to transform the NHP and the MHP data, due to violation of Levene, Shapiro-Wilk and Kolmogorov–Smirnov tests, a Kruskal-Wallis test was used to assess the significance of the factors “soil type” and “treatment” for NHP and MHP. Further, despite attempts to transform the data, a Kruskal-Wallis test on individual soil type datasets was used to examine the effects of amendments for NHP and MHP. Due to the tests used, the effect of time was not investigated statistically for NHP and MHP and only descriptive observations of the trends over-time are reported. In all statistical analyses, p-values < 0.05 were deemed as significant.

5.3 Results

Leachate quality data from the experiments reported in this chapter are summarised in Table 5.5 below. Table columns from left to right describe effects on initial soil nutrient status (high “HP” vs low “LP”), effects of digestate fractions (Control “Ctr”, liquid “LD”, whole “WD” and solid “SD”), interactions between soil and digestate amendment and interaction between treatment and time. Significant differences between levels are identified using superscripts.

For the northwest of England, the monthly total rainfall figures for April, May and June is usually 59, 66 and 74 mm, respectively. These average rainfall totals were calculated between 1966 and 2020, with manual measurements from a 'turf wall'

gauge at Lancaster University's Hazelrigg Weather Station (Met Office Climatological station 7236). During the same months in 2021 (Figure 5.3) covering the field experiment reported here, monthly totals of 11, 107 and 29 mm (April, May and June, respectively) occurred, indicating a very dry April/June, and a very wet May. No natural rainfall occurred in April after the first digestate application on 6th April, whilst in May, between the second application of digestate (18th May) and the third rainfall event (25th May), 45 mm of rainfall was recorded. Soil temperature data showed that absolute temperature within the soil cores were sometimes higher or lower than field soil temperatures (Figure 5.4), although the temperature pattern was consistent between the cores and the field.

Table 5.5 Statistical results from the repeated measures ANOVA analyses on leachate quality after treatment applications (spans two pages)

	Soil	Mean	Std error	p-value	**Digestate**	Mean	Std error	p-value	**Soil × digestate interaction**	Mean	Std error	p-value	**Time × digestate interaction p-value**
DRP (mg P L^{-1})	HP	2.723^{a}	0.251	<0.001	Ctr	0.951^{c}	0.317	<0.001	HP	Ctr: $1.670^{b,a}$	0.449	<0.001	<0.001
	LN	2.314^{b}	0.254		LD	0.929^{c}	0.388			LD: $1.808^{b,a}$	0.449		
					WD	2.088^{b}	0.375			WD: $3.295^{a,a}$	0.634		
					SD	6.085^{a}	0.343			SD: $4.051^{a,a}$	0.449		
									LP	Ctr: $0.165^{c,b}$	0.449		
										LD: $0.091^{c,b}$	0.634		
										WD: $0.881^{b,b}$	0.401		
										SD: $8.118^{a,b}$	0.518		
TDP (mg P L^{-1})	HP	3.090^{a}	0.224	<0.001	Ctr	1.068^{c}	0.289	<0.001	HP	Ctr: $1.793^{b,a}$	0.409	<0.001	<0.001
	LP	3.070^{b}	0.25		LD	0.930^{c}	0.342			LD: $1.985^{b,a}$	0.366		
					WD	2.273^{b}	0.354			WD:$3.663^{a,a}$	0.578		
					SD	8.054^{a}	0.354			SD: $5.200^{a,a}$	0.409		
									LP	Ctr: $0.342^{c,b}$	0.409		
										LD:$0.149^{c,b}$	0.578		
										WD: $1.21^{b,b}$	0.409		
										SD: $10.180^{a,b}$	0.578		
DUP (mg P L^{-1})	HP	0.489^{a}	0.046	<0.001	Ctr	0.156^{c}	0.113	<0.001	HP	Ctr: $0.136^{c,a}$	0.159	<0.001	<0.001
	LP	0.454^{b}	0.069		LD	0.118^{c}	0.13			LD: $0.208^{c,a}$	0.13		
					WD	0.347^{b}	0.13			WD: $0.369^{b,a}$	0.225		
					SD	1.283^{a}	0.122			SD: $1.149^{a,a}$	0.159		
									LP	Ctr: $0.130^{c,b}$	0.159		
										LD: $0.058^{c,b}$	0.225		
										WD: $0.281^{b,b}$	0.13		
										SD: $1.417^{a,b}$	0.184		

NHP (mg P L^{-1})	HP	0.285	0.047	n.s.	Ctr	0.116^{c}	0.055	<0.01	HP	Ctr: 0.228^{a}	0.078	n.t	n.t
	LP	0.263	0.047		LD	0.136^{c}	0.065			LD: 0.180^{a}	0.07		
					WD	0.233^{b}	0.065			WD: 0.373^{a}	0.11		
					SD	0.410^{a}	0.078			SD: 0.307^{a}	0.11		
									LP	Ctr: 0.004^{c}	0.078		
										LD: 0.030^{c}	0.11		
										WD: 0.125^{b}	0.07		
										SD: 0.670^{a}	0.11		
MHP (mg P L^{-1})	HP	0.338	0.18	n.s.	Ctr	0.132^{c}	0.023	<0.001	HP	Ctr: 0.241^{a}	0.036	n.t	n.t
	LP	0.312	0.2		LD	0.142^{c}	0.026			LD: 0.223^{a}	0.036		
					WD	0.247^{b}	0.031			WD: 0.223^{a}	0.036		
					SD	0.717^{a}	0.026			SD: 0.368^{a}	0.036		
									LP	Ctr: 0.052^{c}	0.029		
										LD: 0.061^{c}	0.036		
										WD: 0.272^{b}	0.051		
										SD: 0.966^{a}	0.036		
NH_4^+ (mg N L^{-1})	HP	1.656b	0.388	<0.001	Ctr	5.721^{a}	0.535	<0.001	HP	Ctr: $1.557^{a,a}$	0.316	<0.001	<0.001
	LP	5.519a	0.338		LD	2.264^{c}	0.535			LD: $1.870^{a,a}$	0.316		
					WD	2.100^{c}	0.516			WD: $1.243^{a,a}$	0.447		
					SD	3.656^{b}	0.471			SD: $1.319^{a,a}$	0.365		
									LP	Ctr: $8.684^{a,b}$	0.447		
										LD: $2.729^{c,b}$	0.447		
										WD: $2.577^{c,b}$	0.258		
										SD: $5.915^{b,b}$	0.365		
NO_3^- (mg N L^{-1})	HP	12.375a	0.507	<0.001	Ctr	18.022^{a}	0.98	<0.001	HP	Ctr: $15.990^{a,a}$	1.139	<0.001	<0.001
	LP	8.349b	0.581		LD	10.408^{b}	0.735			LD: $19.032^{a,a}$	0.93		
					WD	4.799^{c}	0.625			WD:$7.575^{b,a}$	1.704		
					SD	7.620^{b}	0.697			SD: $5.757^{b,a}$	0.819		
									LP	Ctr: $20.15^{a,a}$	1.61		
										LD: $1.783^{c,b}$	1.139		
										WD: $2.024^{c,b}$	0.919		
										SD: $9.450^{b,b}$	0.819		

Note: treatments (Control "Ctr", liquid "LD", whole "WD" and solid "SD") on two soils with contrasting P index (high "HP" vs low "LP"), mean values reported, +/- 1 standard error. For interaction between soil and digestate, the first superscript letter represents differences between digestate amendments within each soil type, second superscript letter represents differences between soil type within each digestate amendment. For NHP and MHP only one superscript letter is used since the treatment effect was investigated only within soil. The acronym "n.t" refers to "not tested" (e.g. NHP and MHP), when appropriate soil × treatment and treatment × time interactions were not assessed due to the statistical test employed.

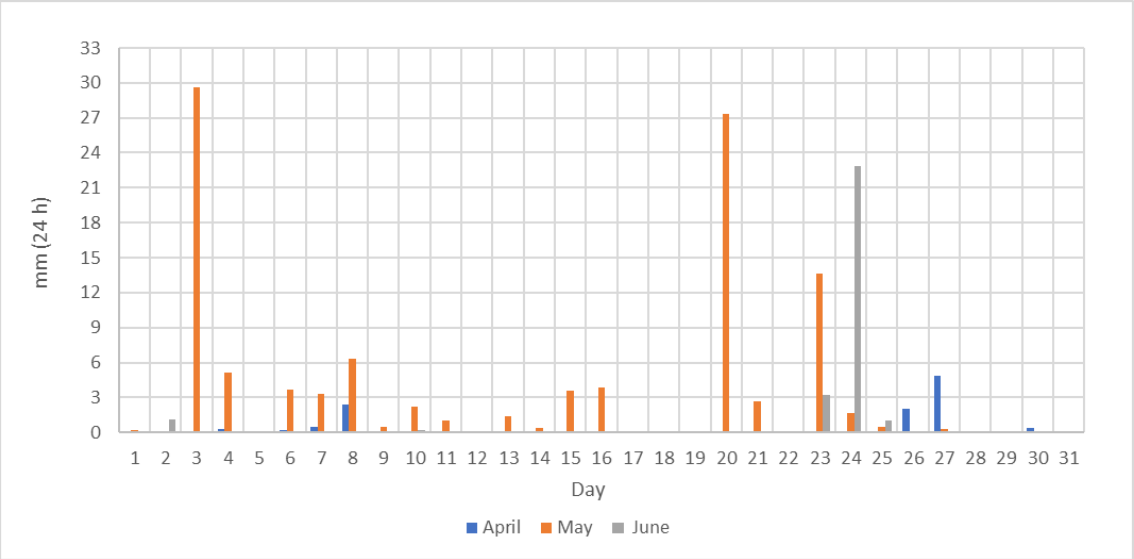


Figure 5.3 Natural rainfall data during April, May and June 2021 expressed as mm collected in 24 h.

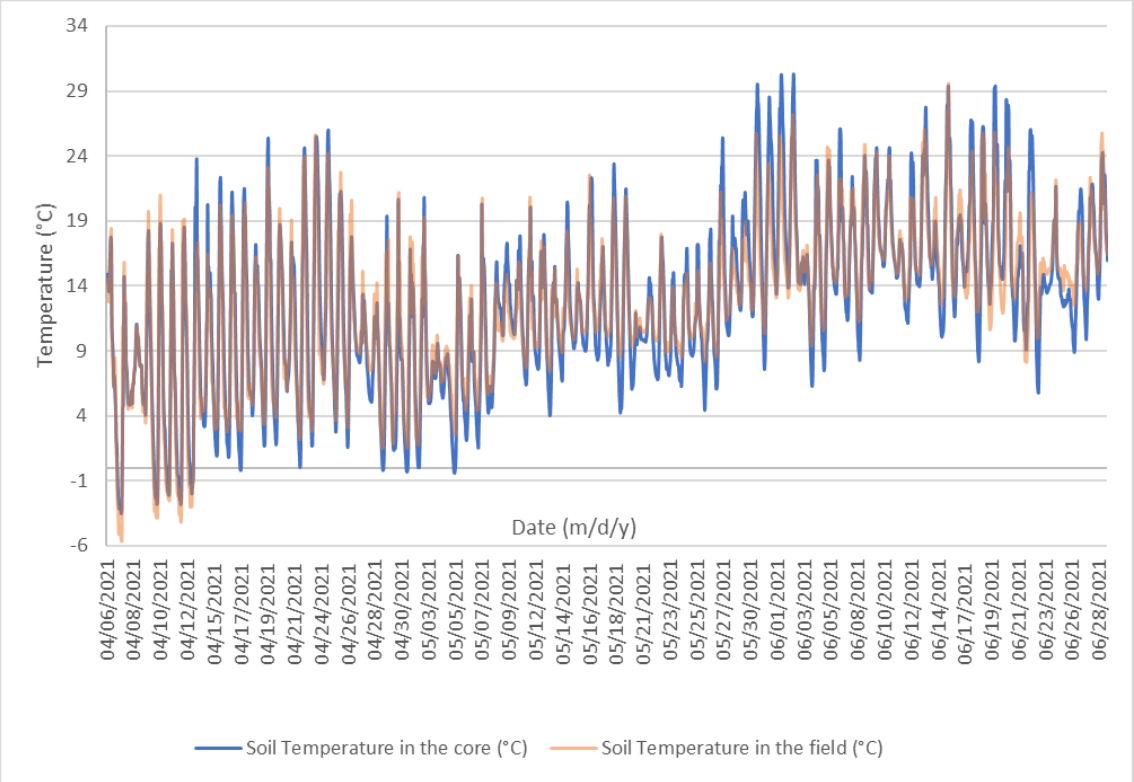


Figure 5.4 Core soil temperature data recorded every 10 minutes from the start of the field experiment (6/4/21) until the end (28/06/21). Dates are expressed as month/day/year (m/d/y)

As reported in Table 5.5, taken across the full field experiment, the concentrations of DRP, TDP, DUP and NO_3^--N were significantly higher in leachate from the HP compared to LP soil cores ($p<0.001$). In contrast, concentrations of NH_4^+-N in leachate from LP soil cores were significantly higher than leachate from HP cores ($p<0.001$). For the concentrations of both NHP and MHP in leachate, there was no significant difference between HP and LP cores.

Across the entire field experiment, the highest concentrations of P within leachate were associated with the DRP and TDP fractions (averaged across all treatments and soil types), which reached >2 mg P L^{-1}. This was strongly related to the application of both WD and SD fractions of digestate, which significantly increased the concentrations of DRP (>2 and 6 mg P L^{-1}, WD and SD, respectively) and TDP (>2 and 8 mg P L^{-1}, WD and SD, respectively) when compared to Ctr in the order Ctr≈LD<WD<SD. Furthermore, application of WD and SD significantly increased the DUP, NHP and MHP concentrations in leachate, in the order Ctr≈LD<WD<SD, although there was no significant difference in the concentration of these parameters in leachate following the application of LD compared to the control treatment. In contrast, for NH_4^+-N and NO_3^--N, the control treatment produced the highest concentrations in leachate, with the remaining treatments following the order Ctr>SD>WD≈LD for NH_4^+-N and Ctr>LD≈SD>WD for NO_3^--N.

Furthermore, a significant soil × treatment interaction was observed for all the parameters analysed in leachate ($p<0.001$), meaning that soil nutrient status significantly influenced the concentrations found in leachate following the

application of inorganic fertiliser or digestate to soil. Regarding NHP and MHP concentrations, a statistical evaluation of the soil × treatment interaction was not possible, due to the distribution of the datasets for these two parameters. Therefore, the effect of digestate and fertiliser treatment on these two parameters was only subject to statistical analysis within separate datasets for each soil type.

Within the HP soil type, the concentrations of NH_4^+-N, NHP and MHP in leachate did not differ significantly between control cores and cores receiving any of the digestate treatments. In contrast, the application of specific fractions of digestate generated significant differences in the concentrations of DRP, TDP, DUP and NO_3^--N in leachate compared to the control soil cores. For DRP and TDP, the order of leachate concentration followed Ctr≈LD<WD≈SD, with WD and SD increasing DRP concentrations by approximately 2 times and TDP concentrations by approximately 2.5 times those in the control and LD treatments, based on average concentrations across all four events. For DUP, the order of the treatments was Ctr≈LD<WD<SD, with WD and SD increasing the average concentration of this parameter by approximately 2.7 times and 8.5 times respectively compared to the control treatment. In contrast, both SD and WD treatments were associated with lower NO_3^--N concentrations in leachate compared to the control treatment (by c. 2 times), whilst there was no significant difference in this parameter between Ctr and LD treatments.

Regarding the LP soil, the concentrations of DRP, TDP, DUP, NHP and MHP in leachate were all significantly influenced by the application of digestate compared

to control soil cores, in the order Ctr≈LD<WD<SD. Compared to the control treatment, WD and SD increased the average concentrations of DRP by 5 and 50 times, TDP by 3.5 and 30 times, and DUP by 2 and 11 times, respectively. In contrast to the HP soil, the application of specific fractions of digestate also increased the concentrations of NHP and MHP in leachate compared to the control treatment. The application of WD and SD fractions significantly increased the average concentrations of NHP in leachate, by factors of approximately 31 (WD) and 168 (SD) compared to the control. For MHP concentrations, the average was increased by factors of approximately 5 and 19 following the application of WD and SD respectively, compared with the Ctr. For both NH_4^+-N and NO_3^--N, the average concentrations in leachate followed the order Ctr>SD>WD≈LD. Average concentrations of NH_4^+-N were 1.5 and 3.4 times lower in SD and WD/LD treatments respectively compared to the control, whilst average NO_3^--N concentrations were lower by factors of approximately 2 and 10 for SD and WD/LD respectively compared to the control.

The differences in leachate concentration between treatments varied significantly across the individual leaching events, as shown by a significant Treatment × time interaction for all measured parameters ($p<0.001$) (Table 5.5), apart from NHP and MHP where statistical assessment of the interaction was not possible due to the nature of these datasets (Figure 5.5d,e). For DRP and TDP (Figure 5.5a,b), concentrations associated with the LD treatment did not differ significantly from the control treatment in any of the four leaching events. In contrast, the SD treatment

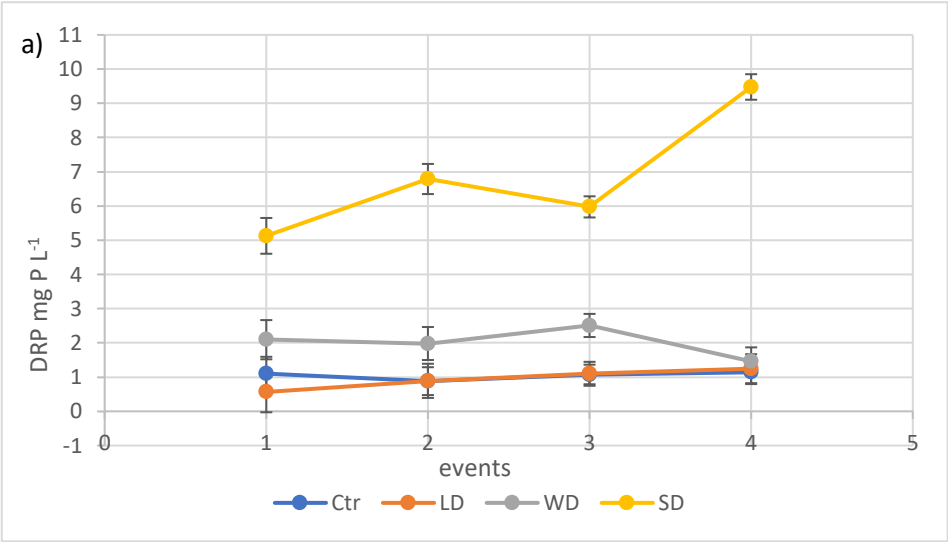
generated significantly higher concentrations of DRP and TDP than the Ctr, LD and WD treatments across all four leaching events, producing the highest DRP concentration during the fourth leaching event and the highest TDP concentration during the second and the fourth leaching events. The concentration of DRP in leachate from cores receiving the WD treatment was significantly higher than for control cores only during the second and the third leaching events, whilst it was significantly higher than for the LD treatment across the first three leaching events. For TDP, concentrations from cores receiving WD were higher than Ctr cores from the second leaching event onwards, whilst it was significantly higher than LD across all four leaching events.

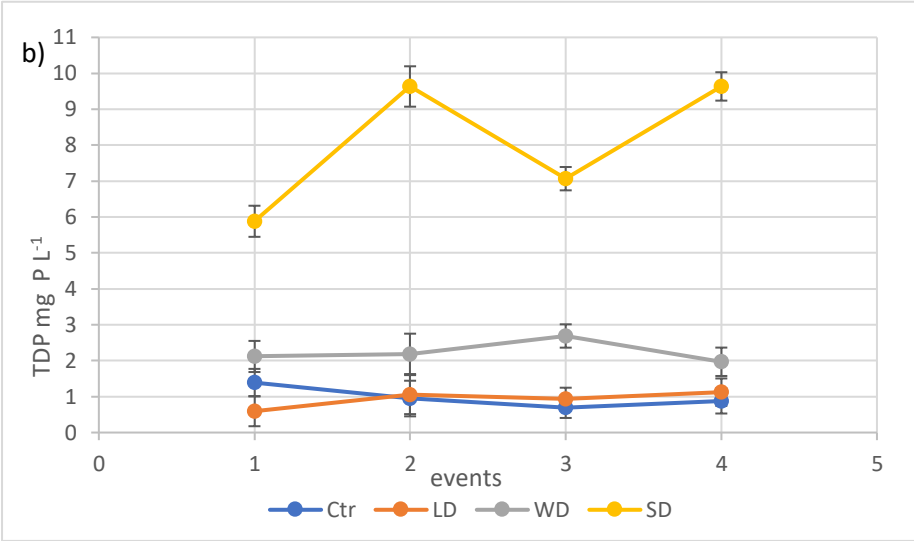
Regarding the concentration of DUP in leachate (Figure 5.5c), the LD treatment did not differ significantly from the Ctr treatment across any of the four leaching events. The application of SD generated the highest DUP concentration in leachate across all four events when compared with the Ctr and LD treatments, whilst SD treatment generated significantly higher DUP concentrations in leachate than the WD treatment during the first three leaching events. The application of WD increased the DUP concentration in leachate only during the third and the fourth leaching event when compared with the Ctr and LD treatments, with the fourth leaching event producing a DUP concentration in the WD treatment similar to SD.

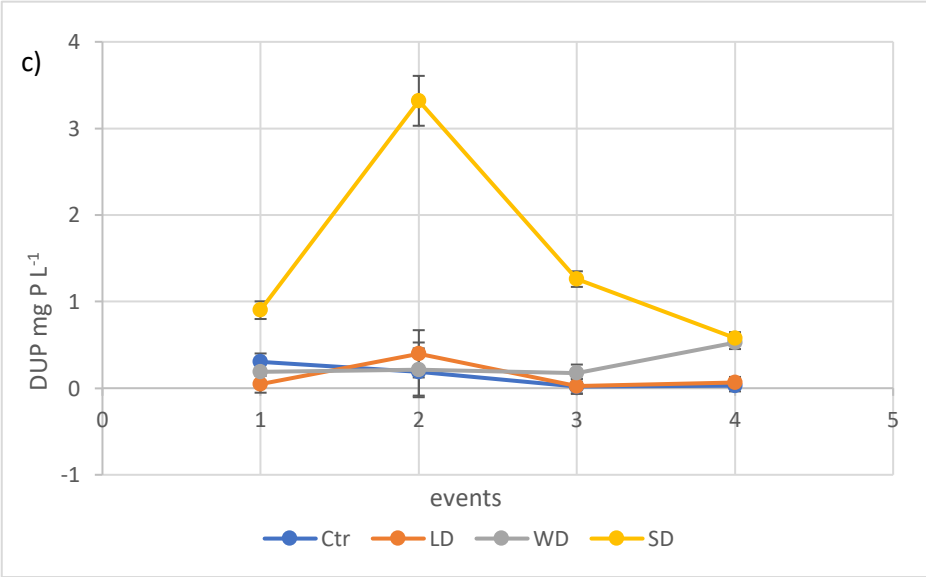
Despite the fact that a Treatment × time interaction could not be evaluated statistically for NHP and MHP, substantial differences between the concentrations of these parameters across individual leaching events were observed (Figure

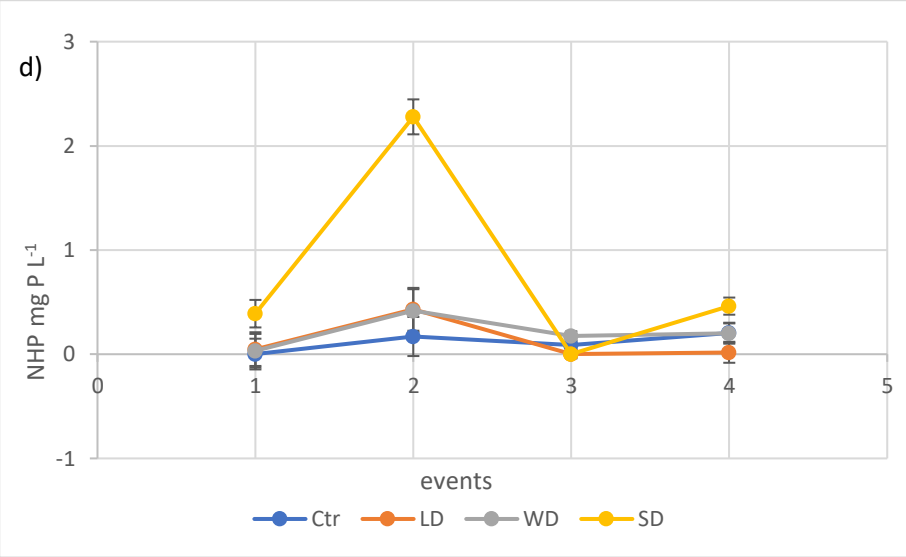
5.5d,e). These differences were more pronounced for SD than for the other treatments, particularly the increase in NHP and MHP concentrations between the first and the second leaching events, followed by substantial decreases in the concentration of both parameters during subsequent leaching events.

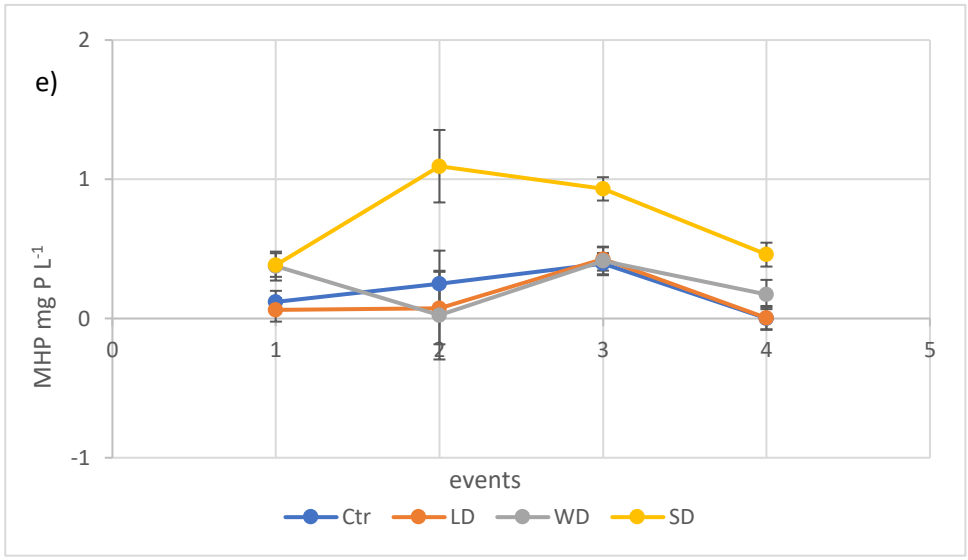
The concentrations of NH_4^+-N in leachate from LD and WD treatments were significantly lower than from the control treatments for the first and the second leaching events, whilst during the third and the fourth leaching events they were comparable to the control treatments (Figure 5.5f). The NH_4^+-N concentration in leachate after SD application was significantly lower than the Ctr only during the first leaching event. However, NH_4^+-N concentration in leachate from the SD treatment was significantly higher than from the LD and WD treatments across the first three leaching events, whilst it was similar to LD and WD during the final leaching event. Regarding NO_3^--N, all treatments generated leachate with significantly lower concentration than Ctr during the first leaching event, although WD and SD were not statistically different from each other (Figure 5.5g). During the second and third leaching events, concentrations from WD and LD treatments were significantly lower than the Ctr, whilst during the fourth leaching event these treatments generated NO_3^--N concentrations that were comparable to the Ctr. Application of SD resulted in lower NO_3^--N concentrations than the Ctr across the first three leaching events, whilst during the fourth leaching event the concentration NO_3^--N from the SD treatment was significantly higher than from Ctr, LD or WD treatments.

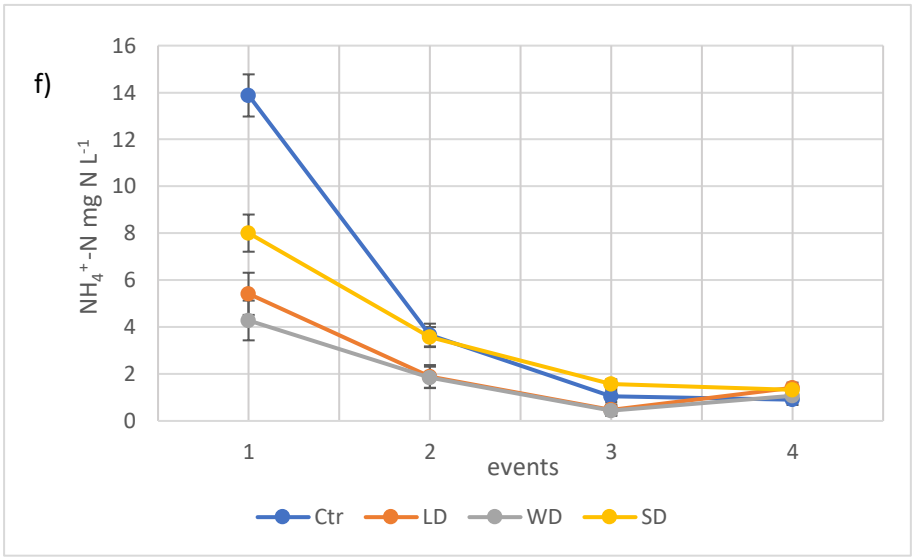

a)
DRP mg P L-1
events
Ctr
LD
WD
SD


b)
TDP mg P L-1
events
Ctr
LD
WD
SD


c)
DUP mg P L-1
events
Ctr
LD
WD
SD


d)
NHP mg P L-1
events
Ctr
LD
WD
SD





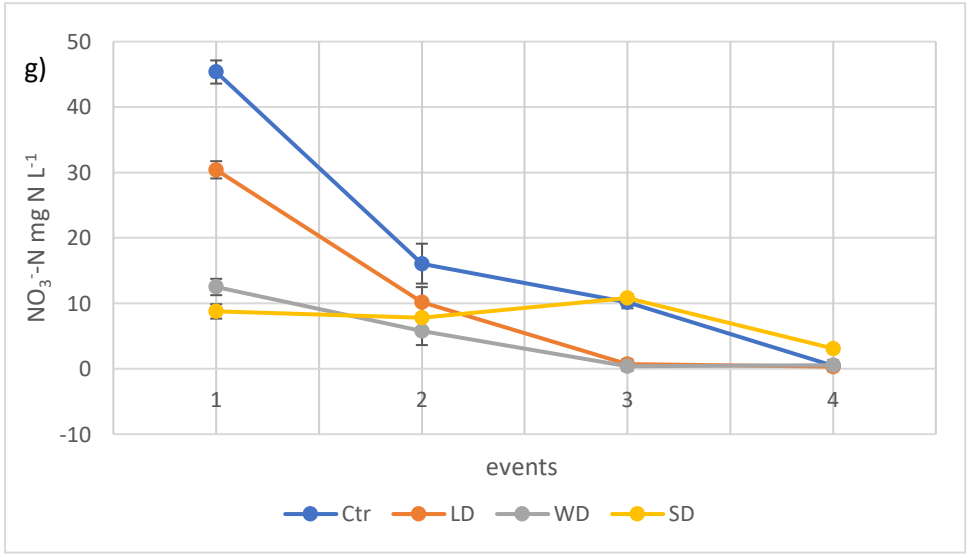


Figure 5.5 Concentration of a) DRP, b) TDP, c) DUP, d) NHP, e) MHP, f) NH_4^+-N and g) NO_3^--N in leachate during four artificial leaching events after treatment application (Control "Ctr", liquid "LD", whole "WD" and solid "SD" digestate fractions) with soil type pooled. Error bars = 1±SE

5.4 Discussion

The results reported above (Table 5.5) confirm the hypotheses that the P parameters analysed in leachate were significantly influenced by the initial soil P index, the fraction of digestate applied to grassland soil (WD, LD or SD) and the repeated rainfall events. Therefore, the effects on leachate quality of soil type, digestate treatment and the interaction between these factors is first discussed below. Subsequently, the effects of time and application of digestate treatments across the four events on leachate quality are considered.

Two soils with contrasting nutrient properties and P indices were used in the experiments reported here (Table 5.2, section 5.2.3). These soils were noticeably different in term of their physio-chemical characteristics, with these characteristics representative of an agricultural grassland soil under intensive grass silage production (HP) and under fallow conditions (LP) with no fertiliser application over the last two decades (RB209). Intact soil cores were used in the field experiment reported here, with particular care taken to ensure minimal disruption of the original soil structure during collection, in order to represent as accurately as possible the movement of water through the soil profiles and, therefore, the risks of pollutant leaching to the subsurface.

The application of the treatments followed the guidelines for grass silage given in the Nutrient Management Guide (RB209, section 2), with a target annual DM yield of +12-15 t ha^{-1}, currently the target worked towards at Cockerham Green Energy

Ltd. No inorganic P or K fertiliser was applied to the Ctr treatment in HP soil, due to the initially high P and K indices (5 and 4, respectively) of this soil. In contrast, inorganic P and K fertiliser was applied to the Ctr treatment in LP soil, due to the low value of P and K indices (0 for both) in this soil. Across the digestate treatments, SD application occurred at a higher mass compared to either WD and LD, in order to reach a consistent N target across the digestate fractions. Therefore, the quantity of TC, TN, TP and WEP applied with the SD treatment was considerably higher than with either WD or LD, as reported in Table 5.3. Furthermore, because digestate was surface-applied rather than injected, SD did not interact with the soil matrix throughout the soil profile, but instead remained largely on the soil surface. Due to the fibre content of the WD fraction, only a part of this digestate treatment infiltrated into the soil profile. In contrast, LD fully infiltrated into the soil profile after application, due to the liquid, low-fibre nature of this digestate fraction. This may have influenced the plant-soil-bacterial interaction with the different fractions of digestate and, ultimately, the concentrations of P and N found in leachate.

5.4.1 The effects of soil type on the concentration of P and N parameters in leachate

Taken across all digestate treatments and controls, DRP, TDP and DUP concentrations in leachate produced from the HP soil significantly exceeded those from the LP soil (Table 5.5). However, despite the fact that the differences in initial physio-chemical conditions of the two soils were clear, the differences in leachate quality were less pronounced (Table 5.5). This observation reflects the dominant control exerted by digestate application on leachate quality (when digestate

fractions were averaged across soils), rather than characteristics of the two soil types.

The higher overall SRP, TDP and DUP concentrations in leachate reported from the HP soil compared to the LP soil probably reflect differences in the soil P index and the degree of P saturation between the two soil types (0.3 PSI for HN and 0.1 PSI for LN) (Nair, 2014; Sims et al., 2000). The history of LD application has resulted in greater P_{in} and P_{org} pools in the HP compared to LP soil (Table 5.1) (Walsh et al., 2012) and, following further P input associated with inorganic fertiliser and digestate treatments in this experiment, contributes to significantly higher concentrations of P in leachate from the HP compared to the LP soil (Heckrath et al., 1995; Sharpley, 1995; Sims et al., 2000; Vadas et al., 2005). The LP soil had a P index of 0 and was at a lower PSI (Tab 5.1), reflecting the lack of inorganic fertiliser or digestate input in the past, and resulting in lower P concentrations within leachate during the experiment reported here. However, other research has not found a similar correlation between P index and P losses through leaching (e.g. Djodjic et al., 2004), with other soil physio-chemical properties exerting stronger control on P export via leaching, including soil texture, preferential flow, biological P release through wetting/drying cycles, high application rates of organic materials and timing of natural rainfall events (Turner & Haygarth, 2000; Djodjic & Mattsson, 2013; Allen & Mallarino, 2008). Therefore, to fully assess the potential of P leaching from soils it is necessary to take into account the soil P status, the organic

amendment used and soil physical properties including texture (Djodjic & Mattsson, 2013).

Similarly to P, concentrations of NO_3^--N in leachate were higher from the HP soil compared to the LP soil, whilst for NH_4^--N the opposite pattern was observed (Table 5.1). The higher NO_3^--N losses from HP soil than LP soil is consistent with high KCl-extractable NO_3^- within the HP compared to LP soil (Table 5.1), suggesting a larger soil NO_3^- stock in HP that is potentially at risk of leaching, compared to the LP soil. Moreover, it may be that in the HP soil nitrification processes occur more readily than in the LP soil as, due to the history of previous fertiliser and digestate applications to the HP soil, it has a higher pH, higher microbial biomass, high C content and N immobilization processes are less likely to occur, thus more NH_4^+ is converted into NO_3^- than biosynthesised in microbial cells (Möller, 2015; Firestone et al., 1989; Weier et al., 1993; Senbayram et al., 2009). In contrast, LP soil had a low pH, low microbial biomass, low C content and no significant history of fertilisation, meaning that N immobilization processes likely occur rapidly (thus more NH_4^+ is stored inside microbial cells) whilst nitrification is more limited (Rigby et al., 2013, Espinoza et al., 2013; Bárta et al., 2017). In contrast, KCl-extractable NH_4^+ was higher within the LP soil compared to the HP soil, possibly reflecting greater sorption of NH_4^+ within the LP compared to HP soil, likely due to differences in cation exchange capacity, and therefore providing a larger stock of NH_4^+ to be released to leachate.

5.4.2 Digestate effects on the concentration of P and N parameters in leachate

Taken across HP and LP soils, the application of digestate significantly increased the concentrations of DRP, TDP and DUP in leachate compared with control treatments that received inorganic fertiliser. These findings are consistent with past laboratory leaching and in-situ field lysimeter experiments (García-Albacete et al., 2014; Vanden Nest et al., 2015; Koch et al., 2019), which report increased TDP and DRP concentrations in leachate from soils following application of the whole fraction of digestate versus application of inorganic fertiliser/unamended soil. Koch et al. (2019) reported that approximately 80% of the TDP found in leachate was present as DRP, consistent with the DRP:TDP >0.8 reported in the current chapter. However, the absolute concentrations of P found in leachate within the research reported here were substantially above those reported in previous field and laboratory experiments. For example, whilst past research has reported concentrations in the range of 0.3-0.52 mg P L^{-1} for DRP and in the range of 0.04-1 mg P L^{-1} for TDP, concentrations reaching up to 6 mg P L^{-1} for DRP and 8 mg P L^{-1} for TDP were found in the experiments reported here. In particular, across DRP, TDP and DUP, the application of SD to the soil cores resulted in particularly high concentrations within leachate, reflecting the great mass of SD applied in order to reach the agronomic N target, meaning that the mass of TP, P_{org} and WSP applied was higher for SD than for all other treatments (Table 5.3). Further, the SD treatment has a higher wettability than WD and LD, due to the high fibre content of the SD fraction (García-Albacete et al., 2014). Therefore, during the artificial rainfall events, there was greater opportunity for interaction between rainwater and the SD fraction on the

soil surface compared to other fractions of digestate, and this likely resulted in greater dissolution of P (as TDP, DRP or DUP) within the subsequent leachate (García-Albacete et al. 2014).

In contrast to observations related to P, the application of digestate resulted in lower concentrations of NH_4^+-N and NO_3^--N in leachate when compared with control treatments, a pattern that has also been observed in previous laboratory and field experiments where inorganic fertiliser and digestate were applied at between 230 kg N ha^{-1} y^{-1} and 350 kg N ha^{-1} y^{-1} (Tsachidou et al., 2019; Walsh et al., 2012; Svoboda et al., 2013; Tshikalange et al., 2020). These authors tested inorganic fertiliser and digestate application on grassland and arable soils and found similar concentrations of NO_3^--N and NH_4^+-N in leachate after digestate application to those reported in the current chapter, ranging between 20 and 130 mg N L^{-1} for NO_3^--N and between 5-20 mg N L^{-1} for NH_4^+-N. However, these concentrations were below those associated with inorganic fertiliser applications in these past studies, which in these past studies reached between 54 and 300 mg N L^{-1} for NO_3^--N and between 256 and 1000 mg N L^{-1} for NH_4^+-N. This may reflect the highly soluble nature of inorganic fertiliser and the associated elevated risk of leaching compared to digestate (Han et al. 2016). Alternatively, surface application of digestate may have increased NH_3 volatilization, resulting in lower NH_4^+-N content in the digestate matrix following application to soil and consequently less NH_4^+-N being lost through leaching. Additionally, since less NH_4^+-N would have been present in the digestate matrix following application to soil after volatilisations, less N would have been nitrified,

ultimately resulting in lower NO_3^--N concentrations found in leachate (WRAP, 2016). Finally, elevated total N and C loading after digestate application vs inorganic fertiliser application (Table 5.3) may have modified the soil microbial community such that this community may have started to use the organic C and N present in digestate for cell biosynthesis, leading to less N being lost in leachate (Bárta et al., 2017).

The NH_4^+-N concentration found in leachate was greater for the SD treatment than the LD and WD treatments. The LD fraction, with low Tot C:N and high NH_4^+-N content, quickly infiltrated into soil, preventing N losses to the atmosphere via volatilisation and, consequently, increasing NH_4^+-N uptake from plants roots and conversion of NH_4^+-N to NO_3^--N through nitrification. In contrast, WD infiltrated less rapidly, due to the fibre content of this fraction, therefore increasing the opportunity for N losses into the atmosphere and reducing the risk of NH_4^+-N leaching (Delin et al., 2012). Despite the fact that SD was also applied on the soil surface and NH_4^+-N could therefore be lost via volatilisation, the greater amount of N_{org} present in SD compared to LD or WD likely led to increased mineralisation and production of NH_4^+-N by soil bacteria. This, coupled with the greater wettability of SD than WD or LD, likely increased the risk of NH_4^+-N leaching during the rainfall events (García-Albacete et al. 2014). Similar mechanisms may also explain why the NO_3^--N concentration in leachate was higher for LD and SD treatments than WD. The NH_4^+-N present in LD was quickly converted into NO_3^--N, due to rapid infiltration of this digestate fraction into soil, whilst the NH_4^+-N produced in SD during the

mineralization of N_{org} was likely quickly nitrified into NO_3^--N, increasing the risk of NO_3^--N losses during the rainfall events. In contrast, WD infiltrated into soil less readily than LD and more NH_4^+-N was lost via volatilisation. This, coupled with the small amount of N_{org} present in WD, reduced the amount of NH_4^+-N produced during mineralization and, therefore, less NH_4^+-N was converted into NO_3^--N and lost via leaching (Sørensen and Fernández, 2003; Sørensen et al., 2003).

5.4.3 Effect of digestate treatment on NHP and MHP parameters in leachate

Across the two soil types examined in this chapter, the application of WD and SD significantly increased the concentration of both NHP and MHP in leachate, compared with the control treatments. Whilst these increases in concentrations were significant, they reached much lower absolute concentrations than the DRP and TDP fractions in leachate after the application of digestate (Table 5.5). To our knowledge, no previous research has examined NHP and MHP in leachate after the application of digestate. Further, only one study has directly quantified the hydrolysable inositol hexakisphosphate (HIP) within soil extracts after digestate application to grassland soil (Richards et al., 2021), although the enzyme assay used was different to that reported in the current chapter. These authors reported that digestate contained 101–102 mg P (per pot) of monoester P, which was considered an appreciable contribution of labile P and led to the investigation of HIP. At the end of the experiment, Richards et al. (2021) found that HIP after digestate application accounted for 32% of TDP from the soil-water extraction, which was directly related to the P_{org} added with digestate. Further, Stutter (2015) analysed

the monoester content of the whole fraction of digestate using the ^{31}P NMR method and determined that 6% of the TP present in digestate was present as monoester P.

Therefore, applying digestate to soil can be associated with a substantial input of $P_{org,}$ including monoester P, suggesting a higher P_{org} load applied with SD and WD when compared with Ctr and LD (for LD, most of TP was present as WEP, Table 5.3). The increased NHP and MHP concentrations in leachate from the SD compared to the WD treatment likely reflects the higher P_{org} load applied with SD than WD and, combined with greater wettability of SD than WD, more P_{org} was lost within leachate, including NHP and MHP. This pattern is consistent with past research examining the impacts of manure application (e.g. Annaheim et al., 2015) which quantified NHP and MHP in soil extracts amended with manure and concluded that the DUP concentration was dominated by MHP, although the enzyme addition method and the enzyme used was different to that used in the research reported in the current chapter. In other field experiments conducted by Toor et al. (2003) and McDowell and Koopmans (2006), in which grassland was amended with dairy effluent, leachate was analysed following the same enzyme addition method reported here. This research found that 67% of P_{org} in leachate was present as MHP and was the most predominant form of P_{org} in the majority of soils (Magid et al., 1996). It is noticeable from Table 5.5 that the sum of NHP and MHP is higher than DUP. It has been reported in the literature that when DRP:TDP >0.8 data may be associated with high random and systematic uncertainty, increasing the risk that

during the calculations based on difference, DUP may have been underestimated (Graeber et al., 2012).

5.4.4 Soil P status and leaching of P and N after digestate application

A significant soil × treatment interaction was observed in the research reported in this chapter (Table 5.5), meaning that the effects of digestate treatment on pollutant concentrations in leachate differed depending on the initial P status and broader physicochemical properties of the two soils.

Interestingly, despite the fact Ctr, LD and WD application to the HP soil yielded higher concentrations of all P parameters in leachate than for the LP soil, likely reflecting the fact that a soil with high P index is more prone to P leaching (Nair et al., 2014), leachate from LP treated with SD showed higher concentrations of all P parameters than HP treated with SD. This was not expected because the LP soil had a lower PSI and P index than the HP soil, suggesting greater remaining capacity for P adsorption and fixation even after SD application to the LP soil (Nair et al., 2014). The observations reported in this chapter may reflect the greater capability of bacteria in the HP soil to utilise the more complex and higher quantities of C_{org} and P_{org} compounds applied with the SD treatment than the bacterial community present in the LP soil. Despite the fact that in both soils SD was applied on the surface and was not mixed with soil, thereby potentially limiting direct interaction between soil bacteria and SD, during the rainfall events in the HP soil the C and P organic compounds solubilised in water from the SD treatment may have been efficiently metabolised by soil bacteria and stored within biomass (Kehler et al.,

2021; Moritsuka et al., 2004). In contrast, within the LP soil, this flush of soluble C and P organic compounds may have increased the catabolism of bacteria and the utilisation of C compounds for maintenance respiration rather than cell growth, reducing the intracellular storage of P and increasing the loss of P via leaching. Moreover, it is possible that soil bacteria in the HP soil were stimulated after the flush of organic C compounds from SD and started to mine SOM, subsequently releasing P compounds from the soil matrix (Ehlers et al., 2010) that were subsequently leached. Furthermore, HP soil receives liquid digestate four times per year, suggesting that the native microbial community may have adapted a set of specific enzymes to transform P_{org} compounds into P_{in} compounds which can be taken up by grass or stored inside microbial cells. In contrast, the microbial community within the LP soil is unlikely to show similar adaptions, meaning that P fractions including DUP, NHP and MHP have not been utilised for microbial growth and were subsequently leached to a greater extent from the LP compared to HP soil (Turner at al., 2004).

In contrast, WD application to HP soil generated higher concentrations of the P parameters in leachate compared to LP soil treated with the same digestate fraction. This is primarily related to the soil physio-chemical composition and P index, the nutrient composition of WD and the fact that WD infiltrated more readily into soil than SD. In the HP soil, the application of available C, N and P with WD may have stimulated bacteria to increase the mineralization rates of P_{org} compounds within WD into P_{in}, therefore the increase of DRP and TDP in leachate when

compared to the Ctr. However, WD application to the HP soil still resulting in leaching of a small amount of DUP, at significantly higher concentrations than within the LP soil, meaning that not all P_{org} was converted into P_{in}. Despite the fact that HP soil has a history of being fertilised with liquid digestate, and likely has a higher microbial activity than the LP soil (Zhu et al., 2017, Richards et al., 2021), the P_{org} forms that are more complex to assimilate (e.g. inositol hexakisphosphate [McLaren et al., 2015]) may have remained in soil solution and subsequently been leached. Instead, application of WD to the LP soil likely resulted in adsorption of more P_{in} (as DRP) within the soil matrix, as may be expected within a soil at low P index and PSI. However, a small amount of DRP was lost through leaching and this was probably related to the fact that the available C, N and P applied with WD to the LP soil stimulated bacterial activity and catabolic responses (Geyer et al., 2016), which subsequently increased mineralization of P_{org} compounds from pre-existing SOM, thereby releasing extra inorganic P into the soil which was lost through leaching (Ehlers et al., 2009). Moreover, it is important to note that the LP soil has never received digestate. Therefore, the soil microbial community likely reacted differently after digestate application to the community within the HP soil (Rosace et al., 2020; Cattin et al., 2021). Therefore, after WD application to LP soil, bacteria did not have the appropriate biological enzymes to convert the P_{org} spices (e.g. NHP and MHP) into P_{in}, therefore small amounts of P_{org} compounds were found in leachate (Richards et al., 2021).

Differences in soil physio-chemical characteristics and microbial community are also noticeable in the NH_4^+-N and NO_3^--N concentrations found in leachate. Application of inorganic fertiliser (Ctr) and digestate treatments produced higher NH_4^+-N concentrations in leachate collected from the LP than HP soil. This reflects the fact that the LP soil had a higher KCl-extractable NH_4^+ concentration than the HP soil, in addition to the fact that organically amended soils such as HP tend to have a higher cation exchange capacity than unamended soils, such as LP, meaning that more NH_4^+-N was lost through leaching from the LP soil than the HP soil after the application of treatments (Miller et al., 2016). Moreover, it is possible that due to the management history of the two fields, different microbial communities reacted differently (e.g. N immobilization or mineralization) after application of inorganic fertiliser and digestate treatments (Rosace et al., 2020). The NH_4^+-N lost from the Ctr treatment in HP was comparable to the NH_4^+-N lost from digestate treatments. However, in the LP soil, concentrations of NH_4^+-N in leachate from Ctr cores were higher than in leachate following digestate treatments. In both cases, it is possible that, due to surface application of the treatments (especially for WD and SD), some NH_4^+-N was volatilised as NH_3 into the atmosphere after digestate application, thereby reducing the NH_4^+-N concentrations in leachate (WRAP, 2016). However, the remaining NH_4^+-N appeared to be utilised differently by soil bacteria and grass plants in the HP and LP soils, leading to differences in NH_4^+-N concentrations within leachate. It may be that lower microbial activity in the LP soil resulted in lower rates of nitrification and greater losses of NH_4^+-N in leachate compared to the HP soil. Alternatively, treatment addition to LP may have created an imbalanced nutrient

environment, especially for the Ctr treatment which did not contain any C compounds, therefore triggering the mineralization of SOM (Truong et al., 2018, 2021) and production of extra NH_4^+-N which was subsequently leached. Further, it may be that the application of treatments with available C and N, such as LD and WD, triggered bacterial N immobilization which reduced the amount of NO_3^--N produced by nitrifying bacteria, thereby reducing N concentrations in the leachate (Espinoza et al., 2013). The application of SD may also have induced an initial phase of N immobilization. However, the constant flushes of C compounds derived from the SD treatment during the induced rainfall events may have stimulated bacteria to mineralise N compounds present in the SOM (Burger & Venterea, 2008; Morvan et al., 2006) or within the TN present in the SD treatment, thereby supplying nitrifying bacteria with NH_4^+-N. In contrast to digestate treatments, application of Ctr to LP soil increased the NO_3^--N concentration in leachate compared to LD, WD and SD because ammonium nitrate contains available NH_4^+-N and NO_3^--N, whilst digestate treatments must go through a nitrification process before producing NO_3^--N (Tshikalange et al., 2020).

In contrast to the LP soil, in the HP soil the remaining NH_4^+-N supplied with the treatments appears to have been quickly taken up by grass plants and the soil microbial community, or nitrified into NO_3^--N, although different digestate treatments produced different concentrations of NO_3^--N. Surface application of WD and SD may have generated significant loss of NH_3 into the atmosphere via volatilisation (WRAP, 2016), meaning reduced conversion of NH_4^+-N into NO_3^--N,

whereas LD rapidly infiltrated into the soil, reducing volatilisation losses and increasing nitrification and the production of NO_3^--N. The fact that Ctr produced a similar NO_3^--N concentration to LD probably reflects the contribution of NO_3^--N from the source fertiliser, alongside nitrification of fertiliser-derived NH_4^+-N to yield NO_3^--N, followed by leaching (Tsachidou et al., 2019; Khanom et al., 2021). Digestate is considered to contain no or negligible NO_3^--N concentrations (Möller & Müller, 2012). Therefore, the reduction of NO_3^--N concentration in leachate after WD and SD application is because part of the NH_4^+-N was lost as NH_3 into the atmosphere, in addition to the fact that the TN contained in the digestate fractions has to be ammonified and then nitrified, thus the production of NO_3^--N is delayed (Espinoza et al., 2013) as is the subsequent leaching of this pollutant.

5.4.5 Effect of digestate treatments on P and N parameters across leaching events

As summarised in Figures 5.5, during four artificial rainfall events occurring between April and June 2021, the concentration of P within leachate followed a variable pattern, whilst the concentration of the N parameters in leachate generally declined consistently over time through the experiment. In general, across the four rainfall events for P fractions within leachate, there was no clear or consistent change through time, suggesting that leachate P concentrations were not simply a direct function of the P applied to soil with the treatments. However, the application of SD seemed to have generated more consistent increases through time in DRP and TDP concentration in leachate compared to the other treatments. This may reflect on-going mineralisation of P_{org} within the SD fraction of digestate and persistent

release of P to solution, in addition to the fact that the sudden freeze–thaw, followed by a drying–re-wetting cycles may have increased the bacterial lysis and consequent released of P_{org} from bacterial cells into solution (Turner et al., 2003). After the second application of the treatments, but before the third leaching event, a significant natural storm occurred, as shown by the rainfall data for the period between 18th May and 25th May in Figure 5.3. This may have led to significant leaching of pollutants through the soil cores immediately after the application of materials to the core surface. This may explain why the concentration of P fractions reported for the third leaching event were often substantially lower than after the second event (Figures 5.5), despite a fresh addition of inorganic fertiliser and digestate between these two events.

Conversely, there appeared to be a more consistent change in the concentration of NO_3^--N and NH_4^+-N across the four rainfall events, with reductions in the concentrations of NO_3^--N and NH_4^+-N across all treatments and within both soil types. This likely reflects gradual exhaustion of sources of N through a combination of previous leaching events, plant N uptake, or bacterial N immobilization, thereby reducing the mass of NO_3^--N and NH_4^+-N available for subsequent leaching. However, leaching of NO_3^--N from the SD treatment remained stable across the first three events, possibly reflecting mineralisation of organic N within this treatment and persistent supply of NO_3^--N to leachate. Whilst offering potential benefits as a slow-release fertiliser (Cavalli et al., 2017; Haraldsen et al., 2011), these data demonstrate how specific digestate fractions may continue to present risks to

leachate quality over prolonged periods of time. Despite a second N application between the second and the third leaching events, there was no significant increase in the concentration of NO_3^--N and NH_4^+-N in leachate. As described above for P, the substantial natural leaching event occurring between the second and the third artificial leaching events may have been responsible for this pattern. Further, uptake by grass plants or the soil microbial community may have contributed to reduced concentrations of N in leachate during the third and fourth leaching events.

5.5 Conclusions

The results reported in this chapter demonstrate that digestate application to grassland soil may be associated with significant increases in the risk of P export to the subsurface via leaching, compared to inorganic fertiliser application. In particular, the application of WD and SD to two, contrasting soil types has been shown to significantly increase the concentration of a range of dissolved P fractions in leachate, including both inorganic and organic P species. Given the maximum concentrations of P observed in leachate following digestate application (e.g. > 6 mg P L^{-1} for DRP), there are potentially significant environmental risks if leachate from agricultural land that has received digestate is delivered to receiving surface waters. These risks should be further explored in future research, including examining the controls on leachate quality exerted by factors such as digestate feedstock, digestate application method, agricultural production system, and a wider range of soil types. Further, this chapter reports some of the first data demonstrating that the concentration of some forms of organic P in leachate may

also be significantly increased following digestate application to land. Whilst not as directly or immediately bioavailable as DRP, the potential for these organic P compounds to be hydrolysed to meet metabolic demand for P in receiving waters emphasises that future research should consider the risks associated with organic P losses via leaching following digestate use in agriculture. In contrast to P, the concentrations of NO_3^--N and NH_4^+-N in leachate after digestate application were lower than those associated with inorganic fertiliser application, suggesting potentially positive effects of digestate use in agriculture via reducing the adverse environmental impacts associated with N exports via leaching. However, it should be recognised that, although than observed under inorganic fertiliser treatment, the absolute concentrations of N in leachate from soils that received digestate treatment could still reach relatively high levels.

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6 Synthesis of thesis outcomes and discussion of the broader environmental and economic opportunities associated with digestate application to grassland soils

6.1 Key findings of the thesis in the context of original objectives

The main aim of this thesis was to enhance understanding of significant agronomic and environmental impacts that follow the application of different fractions of anaerobic digestate to grassland soils. This research sought to contribute to the evidence-based required to develop guidelines for farmers around digestate use, incentivise better agricultural practices related to digestate, optimize the economic benefits that may be derived from digestate utilization within UK agriculture, and contribute to reductions in the reliance on inorganic fertilisers to support production in intensively manged grassland systems. More specifically, the thesis focussed on understanding changes in the biogeochemical cycles of carbon (C), nitrogen (N) and phosphorus (P) after application of different digestate fractions to grassland soils that differed substantially in initial fertility and past management history. Particular attention was paid to exploring how soil microbial communities respond to the application of different physical fractions of digestate (whole, liquid and solid) that are characterised by contrasting physico-chemical properties, including carbon quality (labile versus recalcitrant) and Tot C:N. Together, organic C (OC) input to soil and processing of allochthonous and autochthonous OC by the soil microbial community are major drivers for soil organic matter (SOM) formation,

which is critical for many soil health parameters including improving soil aeration, hydraulic conductivity, structure and resistance to erosion (Herrick and Wander, 1997). The input of fresh organic matter dictates microbial metabolic responses, which ultimately influence soil nutrient storage, respiration rates and GHG emissions. However, these soil microbial community-driven responses are also influenced by the nutrient status of the soil (high soil organic carbon [SOC], available N and P versus low SOC, available N and P) which are expected to dictate whether, after the input of fresh OM, nutrients are retained within the inorganic soil matrix or within microbial biomass, or are lost through respiration and emission of the GHGs methane, carbon dioxide or nitrous oxide. Furthermore, this thesis also investigated how the P index of a soil, alongside other soil physico-chemical properties, influenced the potential export of the nutrients N and P via leaching, a key part of evaluating the potential environmental impacts of different digestate fractions following application to land, but one that has not be subject to sufficient research to date. Export of P and N from agricultural land to receiving waters plays a potentially critical role in a range of environmental impacts that also have significant economic and social costs for human society, including triggering algal blooms as part of broader eutrophication of water bodies.

To achieve the aims of the thesis, six objectives were addressed through the research reported in the thesis. These are detailed below, before a synthesis is given regarding how the individual experimental chapters addressed each objective (see also Figure 6.1). Finally, discussion surrounding how the knowledge developed

within the thesis could potentially advance recommendations regarding optimum digestate use in agriculture, alongside consideration of priorities for future research in the field, is provided.

The research objectives addressed in this these were:

- To examine how whole and solid fractions of digestate applied to grassland soils influence soil microbial metabolism and microbial community composition;
- To determine how initial soil nutrient status influenced changes in the soil C cycle, microbial activity and microbial community composition after the application of whole and solid digestate;
- To quantify how the efflux of the key GHGs methane, carbon dioxide and nitrous oxide were influenced by application of different physical fractions of digestate to soil;
- To determine the extent to which GHG emissions were influenced by initial soil nutrient status and application rate of digestate to soil;
- To quantify how the application of different physical fractions of digestate influenced the concentration of a range of potential pollutants in leachate from agricultural grassland soils;
- To determine whether the concentration of pollutants in leachate after digestate application to land was influenced by the initial nutrient status of soil receiving digestate.

6.1.1 Fate of C compounds in whole and solid digestate fractions and soil microbial community composition

Chapter 3 examined the impact on bacterial carbon use efficiency (CUE) of applying whole and solid fractions of digestate, assessed against an unamended control soil treatment, on two soils at contrasting initial nutrient status. This experimental work was designed to address the first objective of the thesis, determining whether these two fractions of digestate exerted positive influence on the CUE and altered significantly the microbial community composition. The data reported in this chapter are among the first to evaluate the influence of digestate on CUE within soils. Chapter 3 identified contrasting impacts of digestate on the CUE depending on the fraction of digestate that was applied, with solid digestate driving an increase in the CUE whilst the application of whole digestate resulted in a negative CUE. Although the application of the solid fraction of digestate increased average cumulative CO_2-C efflux, the positive CUE associated with this digestate fraction was driven by a substantial increase in microbial biomass C (C_{micro}) following the application of solid digestate to soil. These findings highlight the potential to use solid digestate as an agriculture soil improver/conditioner, due to the potential to increase soil health parameters (e.g. bacterial and fungal biomass) and the C available for bacterial and fungal biosynthesis. However, due to the recalcitrant C compounds present in the solid fraction of digestate, coupled with low concentrations of available N (NH_4^+-N) in this digestate fraction, Chapter 3 suggested that microbial respiration of a proportion of the soil total C pool was stimulated after the application of the solid digestate fraction to soil, as evidenced

by the %TC respired (for %TC calculations, see Chapter 3 section 3.2.5) which was significantly higher after solid digestate application compared to the unamended soil. The results of this experiment suggest that important consequences for soil organic matter (SOM) stocks may follow the application of some digestate fractions to soil, because microbial stimulation coupled with limited available nutrients within the solid fraction of digestate may lead to microbial mining of the SOM to meet an increased demand for nutrients (e.g. N and P) (de la Fuente et al., 2013; Chen et al., 2012). Increases in soil microbial activity were also reported in Chapter 3 after the application of whole digestate to soil, although negative rather than positive values of CUE were reported following this digestate treatment. These observations reflect a substantial increase in cumulative CO_2-C efflux after the application of whole digestate to soil, coupled with a decrease in the C stored within microbial biomass compared to the control soil treatments. The negative values of CUE reported in Chapter 3 highlight the fact that applying the whole digestate fraction to soil may result in net decreases in microbial biomass C, due to the stimulation of maintenance respiration and associated utilisation of C from both native soil and substrate pools. These observations emphasise the potential for adverse effects on soil C storage to potentially follow the application of some digestate fractions to agricultural soils.

The second objective addressed within Chapter 3 was to determine whether the effects of applying solid and whole digestate fractions on CUE varied significantly depending on the initial nutrient status of a soil, as driven by the past management

history of agricultural land. The observations summarised above, namely positive CUE following the application of the solid fraction of digestate and negative CUE after the application of the whole fraction of digestate, were consistent across both soil types examined in this chapter. Moreover, the positive CUE following the application of the solid fraction of digestate, was associated with a greater fungal to bacterial ratio after application of solid digestate compared to control soil and soil treated with whole digestate. The increase in fungal:bacterial after application of solid digestate suggested that lignin compounds present in solid digestate were efficiently degraded by the fungal population, which in turn produced available C compounds which were utilised by bacteria for their growth. However, although not statistically significant, the magnitude of the changes in CUE following digestate application increased within the soil at high initial nutrient status compared to low initial nutrient status. For both digestate fractions, the differences in CUE between the two soil types reflect substantial variation in the extent of CO_2-C efflux between high and low nutrient soils, in particular a clear increase in CO_2-C efflux for the low nutrient soil. Therefore, data reported in Chapter 3 suggest that greenhouse gas efflux following digestate application may depend on initial soil properties, relationships that were explored in greater depth in Chapter 4.

6.1.2 Greenhouse gas emissions after whole, liquid and solid digestate application to grassland soils

Stimulation of microbial activity after the application of different fractions of digestate to soil has potentially important consequences for GHG emissions. In this context, Chapter 4 addressed the third objective of the thesis, establishing how the application of different fractions of digestate versus unamended control soils influenced the emission of CO_2, CH_4 and N_2O. Data from the experiment reported in this chapter showed that each fraction of digestate stimulated bacterial catabolic responses and significantly increased the emission of each GHG from soil, when compared to control treatments. However, the absolute change in GHG emissions varied significantly between individual fractions of digestate. Application of the solid fraction of digestate generated the lowest efflux of CO_2-C and CH_4-C of any of the digestate fractions tested in this chapter, whilst cumulative N_2O-N efflux following the application of the solid fraction of digestate was comparable to that following application of the liquid fraction and lower than following application of the whole fraction. These results support the conclusion that the solid fraction of digestate may supply soil microbial communities with substrate that stimulates anabolism and the associated biosynthesis of nutrients, consistent with observations reported in Chapter 3 related to CUE, simultaneously lowering GHG emissions compared to the other fractions of digestate tested in Chapter 4. However, it is important to note that GHG emissions following the application of the solid fraction of digestate to soil were significantly increased compared to control soils that received no treatment in the experiment reported in Chapter 4. Both liquid

and whole digestate fractions resulted in significantly higher emissions of CO_2-C and CH_4-C compared to the application of the solid fraction of digestate, suggesting enhanced stimulation of bacterial catabolism likely to due to the more labile and bioavailable C and N content of these two digestate fractions. Regarding N_2O-N emissions, when compared to solid digestate, the application of whole digestate was associated with significant increases across both experiments reported in Chapter 4, whilst liquid digestate (when compared to solid fraction) increased N_2O-N emissions only when applied at the higher rate as part of the experiments in this chapter.

The fourth objective of the thesis as addressed within Chapter 4 was to identify whether GHG emissions were influenced by the initial soil nutrient status and by the rate of digestate application to soil. During the second incubation, whole and liquid digestate were applied to reach the same total C (TC) input as solid fraction, reflects a scenario in which over-application of whole and liquid is made to land, beyond the recommendations based on target N inputs. During this second incubation, the increased application rates of whole and liquid fraction generated GHG effluxes which were greater than the GHG effluxes generated during the first incubation. Moreover, it was often the case that the GHG emissions during the second incubation were highest for the liquid fraction. This likely reflects the greater input of liquid compared to whole digestate required to achieve the constant TC input, and consequently the greater input of nutrients such as N that probably stimulated increased GHG emissions.

Application of digestate treatments to the high nutrient soil sharply increased GHG emissions when compared to the control treatment. The whole, rather than the liquid or solid, fraction of digestate was associated with a more pronounced increase in CO_2-C efflux from the high nutrient soil. This observation suggests that the nutrient composition of digestate fractions, alongside past soil management practices, may influence bacterial community responses to the input of an organic material such as digestate. For example, the high nutrient soil used in this chapter was collected from a field fertilised with liquid digestate four times per year. The soil microbial community is therefore likely to be well-adapted to the liquid fraction applied in the experiments reported in Chapter 4, potentially leading to a reduced catabolic response to the input of this fraction of digestate and lower GHG emissions. In contrast, the soil microbial community in the high nutrient soil has no history of exposure to the whole fraction of digestate, which itself contains higher quantities of nutrients in organic form compared to the liquid digestate fraction (see Table 4.2 in Chapter 4). Enhanced respiration of these organic compounds, in contrast to biosynthesis of compounds added to soil with liquid digestate, may be responsible for the greater efflux of CO_2-C observed with this digestate treatment. Regarding the solid fraction of digestate, CO_2-C effluxes are reduced compared to whole and liquid fractions, likely because the solid fraction contains a greater proportion of recalcitrant C compounds that require complex enzymatic hydrolysis pathways, often including fungal-bacterial interaction (link to Chapter 3) that limit the respiration of these C compounds and the resulting efflux of CO_2-C. In contrast to CO_2-C, each individual digestate fraction increased the efflux of CH_4-C and N_2O-N

compared to control treatments to a similar extent in the high nutrient soil. This may reflect the release of CH_4-C and N_2O-N that was native to the digestate fractions themselves, alongside a similar promotion of methanogenesis and nitrification/denitrification across all digestate fractions in the high nutrient soil that is already rich in C and N.

The application of digestate treatments to a soil at low initial nutrient status was also shown to significantly increase the emission of all three GHGs examined in Chapter 4. Increases in CO_2-C emissions were observed, particularly after the application of the liquid fraction of digestate. This likely reflects the input of a fraction of digestate that infiltrated rapidly into soil and a fraction rich in labile C. Within the unfavourable microbial environment defined by the low nutrient soil used in this experiment, the input of available C via digestate likely led to increased catabolism and therefore release of CO_2-C. The more recalcitrant C within whole and solid fractions of digestate is likely responsible for the significantly lower CO_2-C efflux from these fractions compared to the liquid fraction in the low nutrient soil, although the fluxes for these two fractions remained significantly above the flux from the control treatment. The emission of CH_4-C from the low nutrient soil was also significantly higher following whole and liquid digestate fractions. In contrast, N_2O-N emissions from the low nutrient soil were highest following the application of the solid fraction of digestate, with whole and liquid fractions producing lower fluxes but still significantly above those from the control treatments.

6.1.3 Risk of nutrient losses via leaching after the application of different digestate fractions to soils

Individual digestate fractions usually contain an imbalanced ratio of C, N and P compared to soil or crop requirements (see Chapter 5, section 5.1). When digestate application rates are based on one nutrient element, this imbalanced ratio may lead to excessive input of other nutrients to soil through digestate application. Particularly with respect to P, excessive inputs to soils can result in significant risks to the environment, primarily associated with the potential export of P from agricultural land and delivery to receiving waters where a range of adverse effects can follow, including algal blooms and broader eutrophication impacts. Therefore, Chapter 5 addressed the fifth objective of the thesis, using a field experiment with large, intact soil cores to examine the potential risks of nutrient export via leaching after digestate application, compared to a control treatment that had received inorganic fertiliser. The experiment reported in this chapter applied inorganic fertiliser and digestate as surface dressings, in order to mimic the splash plate mode of digestate application that is commonly used in UK agriculture.

The data reported in Chapter 5 demonstrate significant increases in the concentration of a number of forms of P within leachate after the application of both whole and solid fractions of digestate. This was true across a range of dissolved P fractions (TDP, DRP and DUP), spanning both inorganic and organic compounds. These significant increases in P concentration within leachate were also observed across both soil types used in the experiment reported in Chapter 5. Further, for the first time in research focussed on digestate, this chapter also examined the risk of

leaching for specific fractions of the dissolved organic P pool. Within soil at an initially low nutrient status, both whole and solid fractions of digestate significantly increased the concentrations of naturally- and monoesterase-hydrolysable P in leachate, although this significant increase was not observed for the soil at initial high nutrient status. These findings suggest that assessments of the risks associated with P export from agricultural soils after digestate application should be extended to consider organic P fractions, in addition to the more frequently-examined inorganic fractions. The experiment reported in Chapter 5 specifically addressed soluble P moving vertically through the soil profile within leachate, addressing a clear gap in existing literature with respect to P movement in the sub-surface after digestate application. However, surface runoff within the field setting may also erode and transport P-enriched soil particles following digestate application, alongside the residue of digestate if not incorporated into the soil surface after application. Therefore, future research should address both the risks of P export via surface runoff and the risk of P leaching into the subsurface, as influenced by the management of digestate in agricultural production systems. In contrast to whole and solid fractions for digestate, no significant increase in the concentration of any of the P fractions examined in leachate in Chapter 5 was observed following the application of liquid digestate to soils. This may reflect effective uptake of P within liquid digestate by the soil microbial community or by grass plants, alongside potential immobilisation of P within the soil matrix via sorption.

In contrast to P, the leaching of both NH_4^+-N and NO_3^--N following the application of liquid, whole and solid fractions of digestate was significantly reduced compared to inorganic fertiliser. This may reflect the significant quantity of organic N within both solid and whole digestate fractions, leading to slower release of NH_4^+-N and NO_3^--N via ammonification and nitrification and more efficient uptake of mineral N by the soil microbial communities and grass plants, whilst for liquid digestate the available NH_4^+-N was efficiently uptake by plants and quickly converted into NO_3^--N via nitrification processes. This ultimately leads to reduced leaching of inorganic N compared to inorganic fertiliser, in which a much greater proportion of the total N is already present as inorganic compounds that may exceed soil or crop requirements and increase the risk of N leaching. However, it may also be true that during surface application, significant quantities of N were lost from solid and whole digestate fractions as NH_3, thereby reducing the concentration of N in leachate. Therefore, the data reported in Chapter 5 emphasises the need to quantify all potential pathways and multiple elements, in order to properly constrain the risk of nutrient export following the application of digestate to agricultural soils, thereby maximising agronomic benefits and minimise the environmental costs associated with the use of organic materials in agricultural production.

Chapter 5 also addressed the sixth objective of the thesis, to examine how the risk of pollutant leaching from soil following digestate application was influenced by the initial nutrient status of soil. Whilst absolute concentrations depended on the combination of P species and digestate fraction examined, it was always the case

that across all P parameters analysed in leachate in both soils, the application of liquid digestate was not significantly different from the control treatment. However, it was always the case that across all P parameters analysed in leachate, the application of solid fraction of digestate to the low nutrient soil generated the highest concentrations in leachate. In contrast, for whole digestate, concentrations of P in leachate were usually lower in the soil at an initially low nutrient status. Lower concentrations of P in leachate following application of a material to a soil at low P index might be expected, for example due to sufficient P sorption capacity remaining within the soil to effectively retain P within the soil profile. This may explain the observations related to whole and liquid digestate, particularly as these fractions of digestate should largely infiltrate into a soil after application, offering the opportunity for sorption reactions to occur in advance of leaching events. In contrast, the predominantly fibrous nature of the solid fraction of digestate likely constrained any opportunity for sorption of P to the soil surface, meaning that P was rapidly leached once artificial leaching events were generated in the experiment. These findings emphasise that, whilst applying digestate as an improver to some soils, particularly those at low levels of fertility, may be seen positively from the perspective of parameters such as SOM, the same practice may be associated with significant environmental risk, in this case associated with P leaching into the subsurface. Further research focussed on the subsurface leaching of P after the application of digestate to land is required.

Based on the experimental work reported in Chapters 3-5 of this thesis, two conceptual models (Figure 6.1a,b) have been constructed to synthesise the potential agronomic and environmental impacts associated with applying digestate to agricultural grassland soils. These models seek to cover the role of different digestate fractions and the role of different initial soil nutrient status, two key controls examined throughout this thesis. Figure 6.1a, reflects the environmental impacts associated with different digestate fractions after application to a high nutrient soil. On the left part of the figure, whole digestate is referred as "fertiliser and soil improver", since the fibre content could positively improve the soil condition (e.g. reduction of the bulk density and increase soil aggregate stability) and the available N and P content could act as an effective fertiliser. However, whole digestate could increase the leaching of P, GHG emissions and possible mining of SOM through an increase in bacterial metabolic activity, whilst the application of this fraction of digestate could also reduce the leaching of N when compared to inorganic fertiliser. In Figure 6.1a, liquid digestate is referred to as a "fertiliser", since the available N and P content in liquid digestate can act as a quick release fertiliser after application to land. Application of liquid digestate can reduce P leaching, although it may also increase N leaching when compared to inorganic fertiliser. Furthermore, liquid digestate can increase bacterial metabolic activity, mining of SOM and GHG emissions. In contrast, solid digestate is referred to as a "soil improver" due to a high fibre content, the capability to increase overall soil health parameters and increase SOC stocks. However, solid digestate can result in an increase of GHG emissions and P leaching after application to land, whilst N

leaching is reduced after application to land. Figure 6.1b provides a summary of the same biogeochemical responses associated with digestate fractions when applied to a soil at initially low nutrient status.

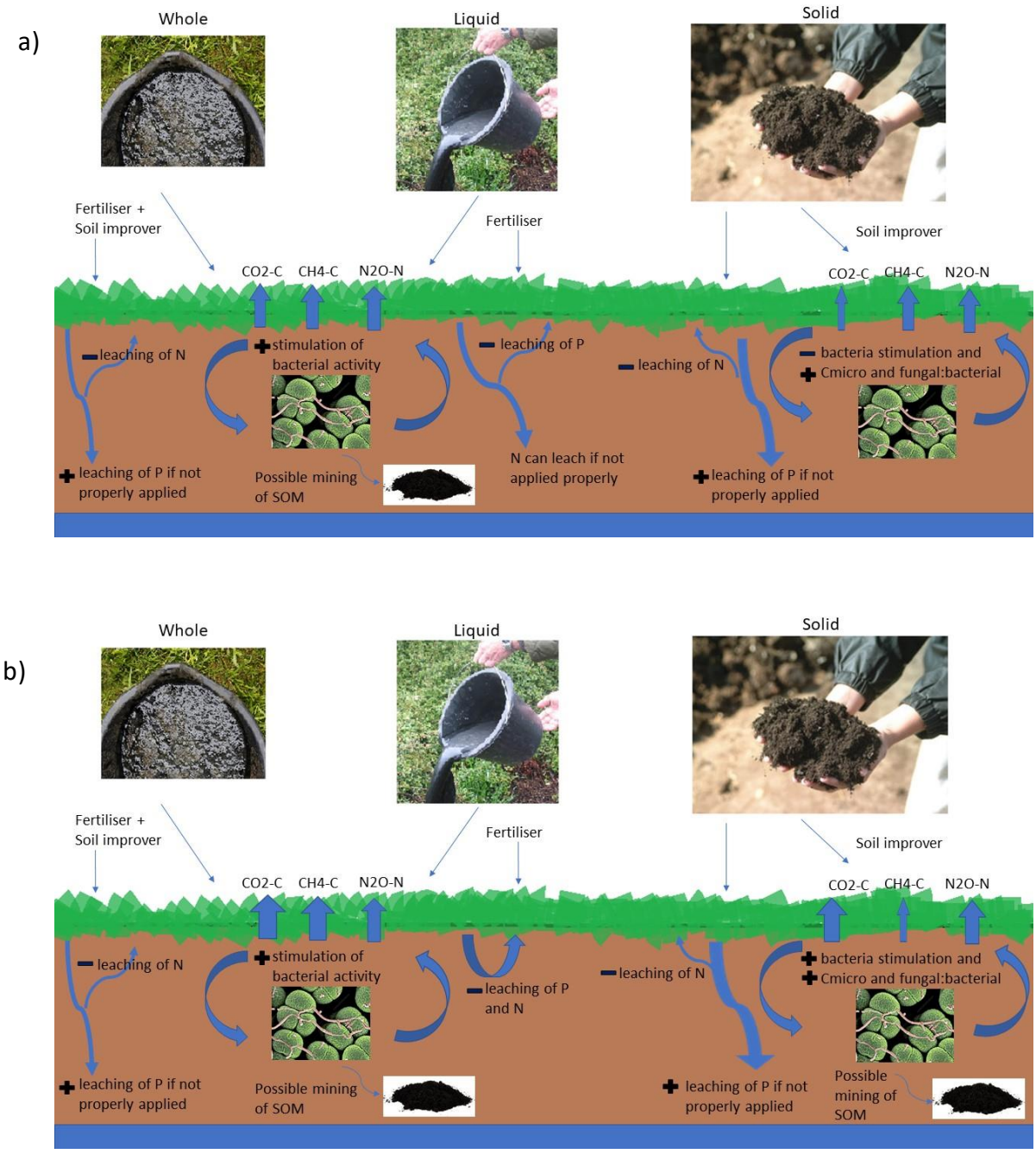

Figure 6.1 Conceptual model of application of different digestate fractions (whole, liquid and solid) in a) high nutrient soil and b) low nutrient soil and the associated environmental impact on GHGs, SOM and leaching of nutrients; the thickness of the blue arrows is directly proportional to the emission of GHGs and leaching of nutrients. Black +/- indicate "increase" or "decrease" compared to control treatments.

6.1.4 Possible agronomic and environmental benefits and disadvantages of digestate separation

Separation of digestate can have multiple agronomic and environmental benefits. As mentioned in Chapter 2 (section 2.1), separation of digestate can increase the redistribution of nutrients and enhance the fertiliser potential of the liquid fraction, compared to the whole and solid fraction. The liquid fraction of digestate could efficiently replace the inorganic fertiliser usage in agriculture and therefore provide an affordable substitution to farmers. In contrast, the solid fraction of digestate could be applied as a FYM substitution, thus improving the soil structure, increasing the SOM and reducing the bulk density. Regarding the whole fraction of digestate, this could be seen as a fertiliser and a soil improver due to its fibre content, although the higher organic N content of whole than liquid digestate could reduce its fertiliser properties; this is due to the fact that fertiliser application is usually calculated on the total N content of the material applied (RB209), hence the readily available N (RAN) applied following whole digestate application would be lower than following application of the liquid fraction. However, application of either liquid or whole digestate should respect the close period highlighted in Chapter 1 (section 1.4), since over-winter application of material with high RAN is prone to leaching and therefore could create an adverse environmental impact. However, material with a low RAN (e.g. the solid fraction of digestate) is not subjected to close period and can be applied across the entire calendar year; this is because when soil temperature is below 4° C, the N mineralization and nitrification processes are impaired, hence the risk of N leaching is significantly reduced. Moreover,

application of such material to land in autumn can increase the SOM and soil organic N reserves which can be mineralised into available N in early spring and therefore be subtracted from the spring first N application (e.g. top dressing or first fertiliser application on grassland) (RB209). Considering the digestate separation per se, the fibre removal significantly reduces the digestate volume, hence increasing the storage capacity for the liquid fraction and enabling farmers to respect the 5 months storage imposed by the Environment Agency. However, storing solid digestate on open spaces can increase the emission of NH_3, CO_2, CH_4 and N_2O (Perazzolo et al., 2015). Further, if solid digestate is stored in unconcreted surfaces (e.g. in the field) which are less than 10m from surface waters and on shallow soil, leaching/runoff of nutrients can occur and can increase the risk of adverse environmental impacts. Emission of GHGs can also occur from storage of liquid/whole digestate in uncovered lagoons, although liquid digestate is more prone to NH_3 emission than whole digestate. This is because whole digestate has a higher fibre content and lower RAN than liquid digestate, thus the formation of a crust on the surface is more likely to occur with whole digestate. During crust formation, anaerobic conditions are created and gases such as CH_4 and N_2O are more likely to be emitted (Gioelli et al., 2011), whilst liquid digestate is more prone to NH_3 and CO_2 emission than whole digestate because of the low fibre content (thus the crust formation is less likely to occur than whole fraction) and high RAN.

Therefore, digestate storage methodology and environmental impact should be considered during a nutrient management plan and coverage of lagoons and solid

fraction should be incentivised in order to reduce the GHGs emission to the atmosphere and the loss of nutrients to the water courses.

6.2 Recommendations for future research

Compared to alternative organic materials, such as livestock slurry or manure, the environmental and agronomic impacts of digestate application to land has received less focus in past research. Whilst a number of more recent studies have progressed research in this field (e.g. WRAP, 2016), there remain multiple research priorities that should be addressed in order to optimise the agronomic benefits of digestate use in agriculture whilst minimising adverse environmental impacts. A number of these key priorities are discussed below.

Although Chapter 3 examined the impact of applying solid and whole fractions of digestate on the soil C cycle and CUE, no assessment was made of the liquid fraction, perhaps the most common fraction of digestate applied to agricultural land. Future research should also consider the impacts on the soil C cycle of applying different fractions of digestate derived from a wide range of feedstocks and AD plants that employ a range of digestion processes. The combination of feedstock and AD process will create digestate with variable nutrient concentration and quality, thereby leading to potentially significant differences in impacts on the soil C cycle and CUE that should be examined. Further, the research reported in Chapter 3 did not include grass plants and future research should seek to include the full range of soil-microbial-plant interactions in terms of impact of digestate on the soil C cycle.

Further, estimates of CUE reported in Chapter 3 were based on the CO_2-C respired and C_{micro}, but this can under- or over-estimate the C respired from pre-existing SOM or from the organic material applied. In addition, the fungal-to-bacterial ratio based on PLFA analysis was used in Chapter 3 to understand microbial changes after digestate application to soil, which does not provide direct insight into the taxonomic composition of bacteria and fungi involved in the decomposition of the organic material. Therefore, future research should seek to apply ^{14}C or ^{13}C labelled digestate in order to directly track the C incorporated into C_{micro} and the C respired, using the isotopic label to discriminate between C derived from native SOM or from digestate. This research could be supported by molecular biology techniques such as qPCR to evaluate changes in bacterial and fungal species composition during the decomposition of digestate, alongside the potential to apply stable isotope probing of microbial DNA if a ^{13}C labelled digestate were applied, to directly track the microbial fate of C added to soils with digestate.

Similarly, despite the fact that Chapter 4 quantified the impact of digestate application on GHG emissions from soil, further research is required in this area. Again, the emission of GHGs after the application of digestate derived from different feedstocks and AD plants, with subsequent effects on digestate quality that will influence biodegradability and bacterial utilization, is required. Furthermore, insufficient research has focussed on CH_4-C and N_2O-N emissions, particularly following application of liquid and solid digestate to a range of soils with contrasting nutrients properties. Research in this area could employ the stable isotope labels

^{15}N and ^{13}C in order to determine whether N_2O-N is derived from nitrification or denitrification of N compounds or from SOM mining, as well as the processes responsible for the formation of CH_4-C. Further, longer-term experiments should be conducted in order to fully assess the biogeochemical mechanisms and underlying bacterial processes involved in GHG formation after digestate application. Such experiments should include vegetation that can significantly influence the availability of nutrients within soil and thereby, in combination with the soil microbial community, the production of GHGs.

Although Chapter 5 has reported some of the first data examining leachate quality after digestate application to agricultural soils, much more research is required in this area to fully constrain the risks associated with digestate. Ideally, this research would be conducted at plot to field scales, maintaining native soil structure in order to maintain soil physical and hydraulic properties that control the generation of leachate. Beyond examining leachate quality associated with a range of digestate fractions originating from different feedstock and AD plants, research should also consider the role of digestate application method in controlling leachate quality, in particular the potential contrast between surface application and injection of digestate. Additionally, at least one and preferably multiple crop growth cycles should be considered, in order to understand how the interaction between the timing of digestate application to land, crop growth, and ambient weather conditions influence leachate quality.

Future research in the area of leachate quality should also consider a range of analytical approaches to fully characterise pollutants within leachate. For example, the enzyme addition methods reported in this thesis demonstrated some limitations. Alternatively, ^{31}P NMR may be a better technique to identify and quantify different SOP compounds present in the leachate and thereby more fully characterise the environmental risks associated with digestate application to land. However, ^{31}P NMR on leachate also requires substantial method development to achieve the necessary P masses for acceptable instrumental signal to noise ratios. Whilst dissolved P fractions were quantified as part of the research reported in Chapter 5, no colloidal or particulate forms of P were analysed in leachate. Whilst the transport of particulate P through the sub-surface may be limited by size-exclusion in pore spaces, the potential transport of colloidal size fractions in leachate should be considered in future research, for example via sequential filtration approaches below the 0.45 µm pore size filter used in the research reported in Chapter 5. Furthermore, research should couple examination of leachate quality with similar approaches to overland flow generated on soils that have received digestate, in order to fully quantify the risks of pollutant export via hydrological pathways. This work should also span both dissolved and particulate P fractions, since the transport of particulate fractions to surface water may subsequently result in solubilisation/mineralisation and release of bioavailable P. Research focussed on overland flow should also examine the role of digestate application method in controlling pollutant export, given that surface application

versus injection of digestate might be expected to exert significant control on the risk of pollutant export.

The literature in the field of digestate use in agriculture indicates that both whole and liquid fractions of digestate are associated with a growing body of research. However, in comparison, much less work has focussed on the potential agronomic benefits and the environmental risks associated with applying the solid fraction of digestate to land. Future research should therefore prioritise work that is focussed on the solid fraction of digestate. Figure 6.2 summarises a number of key priorities in this area.

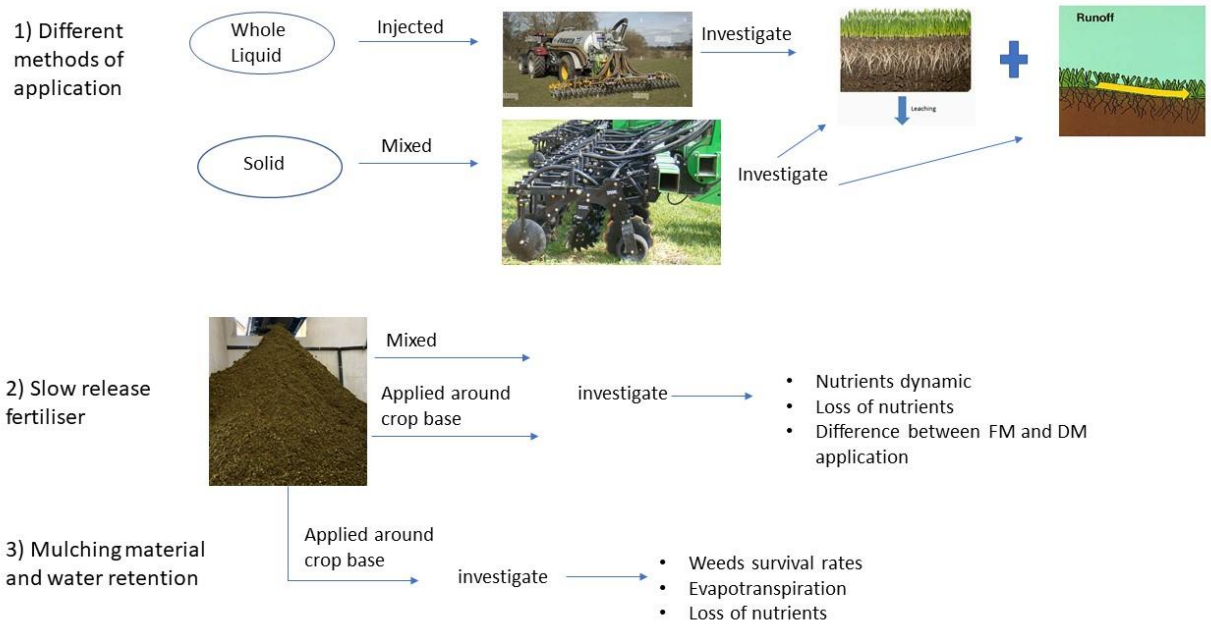

Figure 6.2 Key prioritise for future research focussed on the use of the solid fraction of digestate in agriculture.

The solid fraction of digestate has the potential to act as a slow-release fertiliser and a good mulching material that can retain soil moisture and suppress weeds,

enhancing water use efficiency and reducing reliance on herbicide use and horticultural plastic sheeting use in agriculture. However, insufficient research has been conducted to test these hypotheses, hence future field and laboratory research could focus on issues including: the slow-release fertiliser potential of solid digestate; the effects of incorporation within soil versus surface dressing immediately around crops; soil nutrients dynamics, CUE and bacterial nutrient utilization following the application of the solid fraction to land, alteration of evapotranspiration from soil and effects on plant above-ground biomass. Such research would support better understanding of the effects on soil nutrient cycles, soil organic matter and water retention/loss associated with this fraction of digestate. Further, because nutrient concentrations can vary significantly between the dry and the fresh solid fraction of digestate (observation based on analyses of the digestate used in this thesis), to reduce the environmental impact associated with over-application of this fraction, both dry and fresh material should be tested as part of future research. Further, experiments focussed on the weed-suppressing potential of solid digestate should be undertaken, for example using a thick application of solid faction around the crop base with weed survival rates being monitored.

Since solid digestate has the potential to be a slow-release fertiliser, whilst liquid digestate is a quick release fertiliser, research on the timing of the application of solid digestate, compared with application of liquid digestate, during the growth season should be undertaken. This should focus on bacterial stimulation after liquid

digestate application, decomposition of the solid fraction and the consequent release of mineral nutrients. Additionally, plant aboveground biomass should be measured and the incorporation of micro and macronutrients into the crop to give a fuller picture of the role of digestate type and timing on plant health and yield potential.

6.3 Synthesis of the thesis into a set of digestate soil application considerations and recommendations

This thesis highlights the potential to use liquid digestate as a fertiliser, whilst whole digestate can be seen as a fertiliser and a soil improver which could potentially supply available N and P to crops and improve soil aeration, decrease soil bulk density, and increase soil hydraulic conductivity due to its fibre content. Both liquid and whole digestate may increase bacterial respiration, as often occurs when materials with low Tot C:N and high C lability are applied to soil, since it has been estimated that approximately 55% of the C assimilated by microbial biomass may subsequently be lost as CO_2 when non-lignin fresh organic matter pools are decomposed (Six et al., 2006). Additionally, liquid and whole digestate can increase bacterial metabolism and subsequent production of CH_4 and N_2O. The GHG implications associated with digestate application to land need to be balanced against their potential to lead to a reduction in inorganic fertiliser use in agriculture, including accounting for the full life cycle GHG emissions associated with inorganic

fertiliser synthesis, distribution and application, alongside the economic requirements of producing and distributing inorganic fertilisers.

Unlike inorganic fertilisers, the use of solid digestate in agriculture can be considered a soil improver, offering the chance to increase C_{micro} and, consequently, improve soil aggregate stability and the formation/regulation of SOM and nutrients dynamics. Additionally, the increase in the fungal community size reported in Chapter 3 is indicative of solid digestate being a substrate with high Tot C:N that favours the soil fungal community which, together with the bacterial community, is able to effectively incorporate C within biomass (Six et al., 2006), increase C sequestration and SOC stocks, and improve soil structure and nutrient uptake. Fungi also have the potential to explore a larger volume of soil than bacteria, due to their network of hyphae and, consequently, increase potential access to nutrients for plant uptake that would otherwise be retained within SOM. Furthermore, due to a relatively high organic N and organic P content in the solid digestate fraction, microbial mineralisation can produce inorganic N and P compounds that are subsequently available for microbial or plant uptake. However, the application of the solid fraction of digestate may also increase GHG emissions in the long term, due to the presence of recalcitrant C compounds requiring multiple enzymatic steps to convert to more labile C, which ultimately lead to GHG emissions.

However, incorrect field management of digestate may lead to loss of nutrients through processes including volatilisation, leaching and runoff. Therefore, it is important that research can be synthesised and translated into recommendations

for practice. Injection (i.e. incorporation below the soil surface) of liquid and whole digestate, as opposed to surface spreading via the commonly used technique of splash plating can reduce NH_3 volatilization (Maris et al., 2021) but may also increase GHG emissions, especially N_2O-N and CH_4-N, due to the formation of anaerobic microsites and liberation of native N_2O and CH_4 contained inside the organic material (Chadwick et al., 2000). Additionally, injection of whole and liquid digestate increases the opportunity for plant-soil-microbe interaction and the uptake of nutrients by plants, if the correct application rate, timing and depth are followed, which will reduce subsequent nutrient losses via leaching and runoff (WRAP, 2016). However, injection of digestate must not be used prior to imminent rainfall events due to the fact that higher losses of nutrients may occur on freshly applied material.

The solid fraction of digestate should be mixed with soil rather than surface-dressed since this increases the uptake and the mineralization of nutrients through plant-soil-microbe interaction. Application of this fraction of digestate should be done at the end or before the start of the crop growth season, rather than in late autumn or winter. During the beginning of spring and the end of summer, the soil microbial community and plants can utilise available nutrients from the solid digestate fraction, due to warm soil temperatures. Therefore, application of solid digestate during this time will enhance decomposition and mineralisation of organic compounds by soil biota, maximising nutrient availability to crops at the same time as reducing export of excess nutrients through leaching and runoff.

In addition to the timing, the application of different fractions of digestate should be adjusted based on the soil nutrient status and management practices. For example, application of liquid and whole digestate on an established organically fertilised soil can be an efficient replacement for inorganic fertilisers and should be injected. The solid fraction of digestate may best be applied by incorporation into soil during arable tillage or grassland ploughing and reseeding, in order to increase SOC and SOM stocks and improve soil structure. This can be done especially at the end of the growth season when available soil nutrients have been depleted by exports with cropped biomass, although application of organic compounds at this time may produce a quick release of GHGs in the short term. Moreover, the solid digestate fraction can increase the C_{micro} and fungal population size, potentially linked to solubilisation of previously insoluble P stocks and making these available for microbial or plant uptake. Over the longer term, this may help to draw down existing reserves of total P in high P status soils, thereby decreasing the P index. However, since the solid fraction of digestate contains lignin compounds and organic N and P, it may also act as a sink of slow-release nutrients and may produce small emissions of GHG in the long term, especially in soils prone to flooding (Wang et al., 2016; Bhttacharyya et al., 2013).

Regarding digestate application to a low nutrient soil (e.g. a soil heavily tilled, use of inorganic fertilisers only), solid fraction of digestate should be applied first rather than whole and liquid fraction. This is because incorporation of solid digestate in a low nutrient soil can restore and build up the SOM reserves, SOC stock and improve

soil aggregate stability, water retention, increase the C_{micro} and fungal community and thus improve soil structure. Subsequently, injection of whole digestate would provide some input of more readily plant available nutrients, whilst also continuing to build up SOM with the fibre contained in the whole fraction. Ultimately, when the broader health of the soil had been improved after application of solid and whole digestate, liquid digestate can be applied as a quick release fertiliser.

6.4 Wider considerations for future usage of digestate fractions

The thesis highlighted some implications of digestate in terms of fertiliser application, soil restoration, nutrient use efficiency and a better circular economy (Figure 6.3).

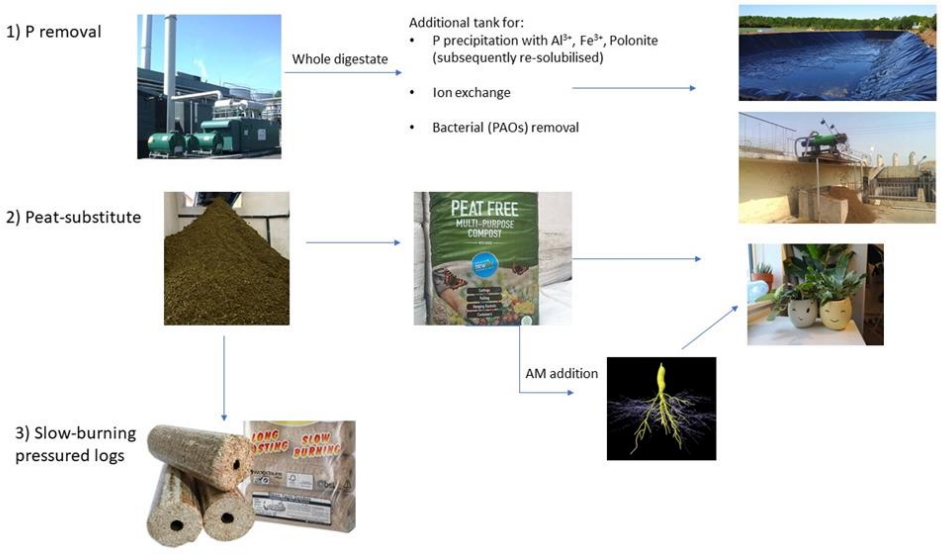

Figure 6.3 Conceptual map of additional treatment/utilization of digestate

Digestate has the potential to assist in 'closing the nutrient loop', for example by reducing reliance on imported fertiliser inputs through enhancing recycling of

nutrient resources to land. This could help to improve the resilience of multiple sectors of the UK economy that currently dependent on inorganic fertiliser supply and utilization. However, even though targeting a given N application rate during field utilization of digestate is feasible, due to the imbalanced N:P within digestate it is often the case that a desirable P application rate is not achieved and digestate application is often associated with excessive input of P to land. Therefore, new technologies should emerge to separate N and P in waste streams, including digestate, thereby allowing flexibility in attaining site-specific balanced fertiliser combinations. In this context, technological examples can be drawn from other sectors and potentially applied to the management of digestate. For example, during wastewater treatment, Al^{3+}, Fe^{3+} or Polonite (a filter media with a high and proven ability to capture phosphorus) is added to the wastewater to efficiently remove P (Bunce et al., 2018). For digestate, similar processes could be introduced via an additional treatment tank, located between the AD plant itself and a solid-liquid separator (Figure 6.3), where materials such as Al^{3+}, Fe^{3+} or Polonite are added in order to remove P. Subsequently, the precipitated P compounds (as struvite or other non-soluble compounds), can be scraped from the bottom of the treatment tank and P can be re-solubilized through the addition of acids such as sulphuric acid (Moure Abelenda et al., 2021). This approach may be a viable solution to solubilise the precipitated P into a form of P which can be subsequently used in agriculture. Such an approach could maximise the benefit derived from the P resources found in digestate, thereby reducing reliance on mining and processing of phosphorite rocks to produce inorganic fertiliser for use in agriculture. It would also help to

reduce environmental risks associated with digestate application to land, by decreasing the P concentration in digestate and the excessive input of P when digestate application rates are based on N targets. However, the financial cost of additional treatment stages at an AD plant may preclude such an approach, particularly for smaller on-farm AD units. Alternatively, ion exchange technology may be used, although this can be effective only if the PO_4^{3-} form of P is present in digestate, rather than P bound to particles or present as P_{org}. The anionic form of P can be interchanged between the liquid and the solid ion exchanger, offering simultaneous removal and recovery (Martin et al., 2009). Subsequently, P can be recovered through the addition of chemical products (e.g. acid addition), which would also regenerate the solid ion exchanger media. However, this approach is suitable for small-scale AD plants, since the chemical addition could be expensive in a full-scale AD plant (Seo et al., 2013).

Possibly the most cost-effective way to reduce the concentration of P in digestate would be through bioaugmentation products, such as phosphorus-accumulating organisms (PAOs) which increase P uptake and storage within microbial biomass. The application of PAOs in the context of digestate could be achieved by adding bacteria directly into a digestate storage tank or also by passing digestate through a filter medium impregnated with PAO bacteria. Subsequently, these PAOs can be recovered in the sludge that settles in the AD plant and returned to land. However, these processes are likely to be most effective if applied to the whole fraction of

digestate, before solid liquid separation, since it will be difficult to achieve effective P removal on the solid fraction via PAOs.

Furthermore, demand for peat-free compost or a general alternative substrate to substitute for peat materials, has grown in importance over recent years (Atzori et al., 2021). In this context, the solid fraction of digestate could potentially be used in horticulture as a potting medium. Globally, peatlands contain approximately 30% of all soil organic carbon (SOC), despite covering only 3% of the land surface (Parish et al., 2008), reflecting the fact that peat formation occurs in anoxic, low temperature environments that result in slow decay of OM (Heinemey et al., 2018). Peatlands are clearly extremely important in the global C cycle and C sequestration, therefore their continued use for potting material in agriculture ultimately releases C into the atmosphere and reduces global soil C stocks. Therefore, establishing the solid fraction of digestate as a viable substitute for peat-based products could play an important role in retaining C within peatlands and mitigating human-induced changes to the global C cycle. Furthermore, the solid fraction of digestate is rich in recalcitrant C compounds, which are precursors for SOM storage, can degrade slowly and ultimately form soil. Moreover, this fraction is rich in organic N and P, which can provide plants with a slow-release fertilization throughout the growing season and increase plant yields (Ehmann et al., 2018). Perhaps most importantly, in the case of horticultural production, using the solid fraction of digestate as alternative growing media may significantly reduce the use of pesticides, fungicides and herbicides because, during the AD process, pathogenic bacteria and seeds from

weeds are effectively destroyed. This, in turn, increases the biofortification of plants and competitiveness in modern horticulture (Rouphael et al., 2018). Therefore, the solid fraction of digestate could represent an excellent substitution for peat-based compost, with broad scope for application in horticulture and larger-scale agricultural.

As examined in Chapter 3, applying the solid fraction of digestate to soil can lead to substantial changes in soil fungal and bacterial communities, including an increase in biomass associated with both these communities and a particular increase in fungal biomass. Based on using the solid fraction of digestate as a slow-release fertiliser or as a peat-substitute, augmenting this fraction of digestate with *arbuscular mycorrhiza* (AM) could further improve crop nutrient availability within soils, alongside helping to reduce the P status of high P soils. The input of P via a range of inorganic and organic materials in agriculture can increase soil total P stocks substantially, reaching a range between 350 and 7000 kg P ha^{-1} in the first 25 cm of soil (Grant et al., 2005). However, only a small proportion of this stock of P is immediately or readily available for crop uptake, often ranging between 3-30 kg P ha^{-1} (Morel, 2002), leaving the remaining P to accumulate in soil and become a potential environmental concern. The external hyphae of AM offer the potential to explore a relatively large volume of soil and therefore extend the rhizosphere to capture areas of soil that may be rich in insoluble P, sometimes beyond 10 cm from the root surface (Jakobsen et al. 1992). Subsequently, through the action of P-solubilizing enzymes produced by AM, the bioavailability of a larger proportion of

the soil total P stock could be enhanced, thereby increasing P supply to crops and inducing a drawdown of soil total P concentrations. This has two potential benefits, firstly reducing reliance on external inputs of P to support production and secondly reducing the environmental risks that are associated with excessive soil P stocks.

Finally, the solid fraction of digestate may have potential uses beyond return to agricultural land. For example, this fraction could be used in the wood-burning industry as slow-burning pressured logs, because the solid fraction of digestate is rich in lignin compounds (Czekala, 2021). This could help to reduce pressure associated with deforestation due to the production of wood logs for fireplaces and woodstoves. However, solid digestate would have to be dried before being pressurised into logs and this can have a potential environmental impact, since high amounts of NH_3 can be emitted during the drying process (Pantelopoulos et al., 2016) with negative impacts on atmospheric pollution and respiratory diseases (Behera et al., 2013). Therefore, during the drying process, air stripping via absorption filters should be used to reduce the environmental impact of NH_3 emissions.

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7 Appendix

7.1 Supplementary information for Chapter 4

1) cumulative CH_4-C first incubation

Call:

lm(formula = CH_4N ~ timeNum * Treatment * Field + I(timeNum^2) *

Treatment * Field, data = dataCH_4_n)

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.706187	0.595676	4.543053	1.21E-05
timeNum	0.030339	0.019401	1.563776	0.120194
TreatmentLD	3.310869	0.842413	3.930221	0.000135
TreatmentSD	2.849522	0.842413	3.382571	0.000938
TreatmentWD	3.359822	0.842413	3.988331	0.000108
FieldLow	-0.7799	0.842413	-0.92579	0.356195
I(timeNum^2)	-0.00013	0.000104	-1.2188	0.22503
timeNum:TreatmentLD	0.140893	0.027438	5.135053	9.56E-07
timeNum:TreatmentSD	0.110202	0.027438	4.016479	9.72E-05

timeNum:TreatmentWD	0.136764	0.027438	4.984542	1.86E-06
timeNum:FieldLow	-0.03034	0.027438	-1.10576	0.270784
TreatmentLD:FieldLow	-2.32117	1.191352	-1.94835	0.053432
TreatmentSD:FieldLow	-2.44158	1.191352	-2.04942	0.042343
TreatmentWD:FieldLow	-2.3483	1.191352	-1.97112	0.05074
TreatmentLD:I(timeNum^2)	-0.00065	0.000146	-4.40674	2.11E-05
TreatmentSD:I(timeNum^2)	-0.0005	0.000146	-3.41678	0.000836
TreatmentWD:I(timeNum^2)	-0.00062	0.000146	-4.23444	4.19E-05
FieldLow:I(timeNum^2)	0.000126	0.000146	0.861822	0.390303
timeNum:TreatmentLD:FieldLow	-0.10814	0.038803	-2.787	0.006081
timeNum:TreatmentSD:FieldLow	-0.08311	0.038803	-2.1419	0.033983
timeNum:TreatmentWD:FieldLow	-0.08727	0.038803	-2.24904	0.026118
TreatmentLD:FieldLow:I(timeNum^2)	0.00063	0.000207	3.044542	0.002799
TreatmentSD:FieldLow:I(timeNum^2)	0.000392	0.000207	1.891864	0.060635
TreatmentWD:FieldLow:I(timeNum^2)	0.000535	0.000207	2.582414	0.010868

2) Cumulative CO_2-C second incubation

Call:

lm(formula = CO_2C ~ timeNum * Treatment * Field + I(timeNum^2) *

Treatment * Field, data = dataCO_2_c)

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.009264	0.021683	46.54604	1.3E-243
timeNum	0.017192	0.000706	24.34335	2.4E-101
TreatmentLD	0.386898	0.030329	12.7565	2.01E-34
TreatmentSD	0.105904	0.030019	3.527916	0.00044
TreatmentWD	0.189908	0.030019	6.326277	3.92E-10
FieldLN	-0.612	0.030019	-20.3872	1.79E-76
I(timeNum^2)	-6.3E-05	3.77E-06	-16.5945	2.85E-54
timeNum:TreatmentLD	-0.00295	0.000988	-2.98165	0.002943
timeNum:TreatmentSD	-0.00167	0.000978	-1.71027	0.087555
timeNum:TreatmentWD	-0.00098	0.000978	-0.99943	0.317852
timeNum:FieldLN	0.004553	0.000978	4.65653	3.69E-06
TreatmentLD:FieldLN	0.533789	0.042212	12.64551	6.72E-34
TreatmentSD:FieldLN	-0.03996	0.042212	-0.94676	0.344012
TreatmentWD:FieldLN	0.286474	0.041989	6.822551	1.62E-11
TreatmentLD:I(timeNum^2)	9.85E-06	5.27E-06	1.868685	0.061986
TreatmentSD:I(timeNum^2)	7.29E-06	5.22E-06	1.396919	0.162776

TreatmentWD:I(timeNum^2)	2.21E-06	5.22E-06	0.424419	0.67136
FieldLN:I(timeNum^2)	-1.7E-05	5.22E-06	-3.23105	0.001277
timeNum:TreatmentLD:FieldLN	-0.0058	0.001375	-4.21963	2.69E-05
timeNum:TreatmentSD:FieldLN	0.000754	0.001375	0.548477	0.583498
timeNum:TreatmentWD:FieldLN	-0.00387	0.001368	-2.82666	0.004806
TreatmentLD:FieldLN:I(timeNum^2)	2.22E-05	7.34E-06	3.023183	0.002571
TreatmentSD:FieldLN:I(timeNum^2)	-3.5E-06	7.34E-06	-0.47468	0.63513
TreatmentWD:FieldLN:I(timeNum^2)	1.63E-05	7.3E-06	2.233232	0.025774

3) Cumulative CH_4-C second incubation

Call:

lm(formula = CH_4C ~ timeNum * Treatment * Field + I(timeNum^2) *

Treatment * Field, data = dataCH_4_c)

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4.317283	0.552878	7.80875	1.39E-12
timeNum	0.068337	0.018007	3.794953	0.000222
TreatmentLD	-2.15135	0.781887	-2.75149	0.006742
TreatmentSD	1.427147	0.781887	1.82526	0.070156
TreatmentWD	-0.17941	0.781887	-0.22945	0.818861
FieldLow	-1.65352	0.781887	-2.11478	0.036273
I(timeNum^2)	-0.00031	9.61E-05	-3.24658	0.001471
timeNum:TreatmentLD	0.353695	0.025466	13.88879	7.39E-28
timeNum:TreatmentSD	0.068503	0.025466	2.689973	0.008041
timeNum:TreatmentWD	0.371075	0.025466	14.57126	1.47E-29
timeNum:FieldLow	-0.06834	0.025466	-2.68344	0.008192
TreatmentLD:FieldLow	4.151614	1.105755	3.754551	0.000256
TreatmentSD:FieldLow	-1.23819	1.105755	-1.11977	0.264786
TreatmentWD:FieldLow	1.699814	1.105755	1.537243	0.126557
TreatmentLD:I(timeNum^2)	-0.0013	0.000136	-9.56547	6.82E-17
TreatmentSD:I(timeNum^2)	-0.0003	0.000136	-2.18515	0.03059
TreatmentWD:I(timeNum^2)	-0.00151	0.000136	-11.0994	8.84E-21
FieldLow:I(timeNum^2)	0.000312	0.000136	2.295675	0.023224

timeNum:TreatmentLD:FieldLow	-0.00741	0.036015	-0.20563	0.83739
timeNum:TreatmentSD:FieldLow	0.025987	0.036015	0.721564	0.471801
timeNum:TreatmentWD:FieldLow	-0.10416	0.036015	-2.89205	0.004458
TreatmentLD:FieldLow:I(timeNum^2)	5.44E-05	0.000192	0.282861	0.777713
TreatmentSD:FieldLow:I(timeNum^2)	1.99E-05	0.000192	0.103408	0.917791
TreatmentWD:FieldLow:I(timeNum^2)	0.000552	0.000192	2.873858	0.004707

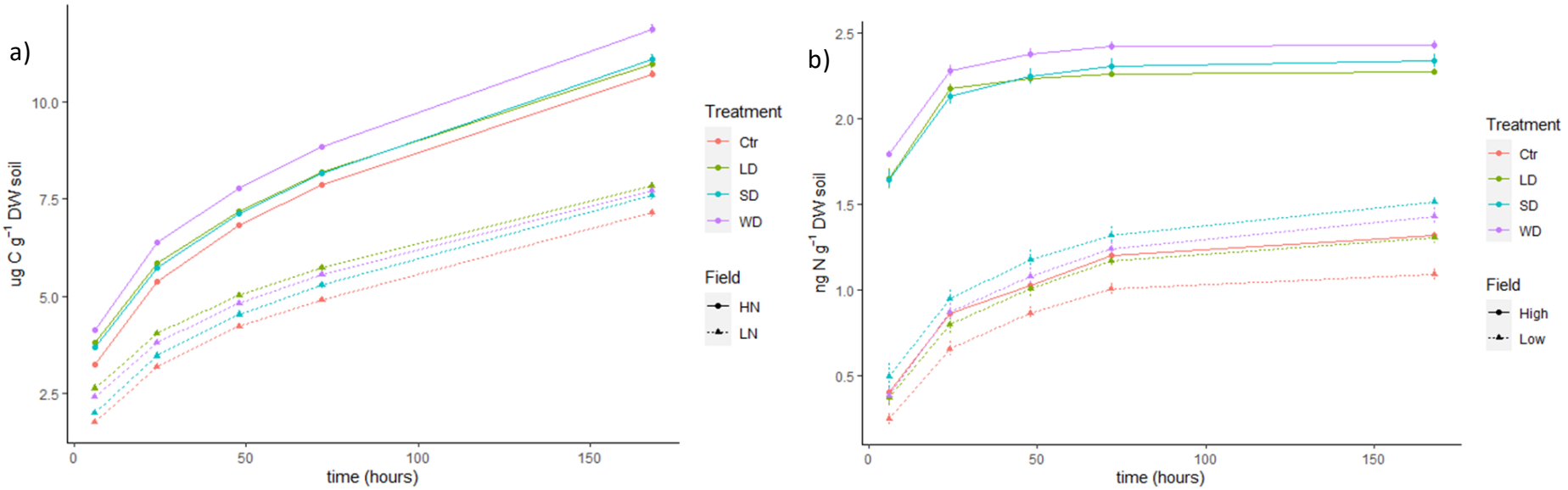

Figure 7.1 Root squared transformation of cumulative CO_2-C (a) and log_{10} transformation of cumulative N_2O-N (b) effluxes during the first incubation in control (Ctr) soils or after addition of liquid (LD), whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) nutrient status. Error bars ± 1SE

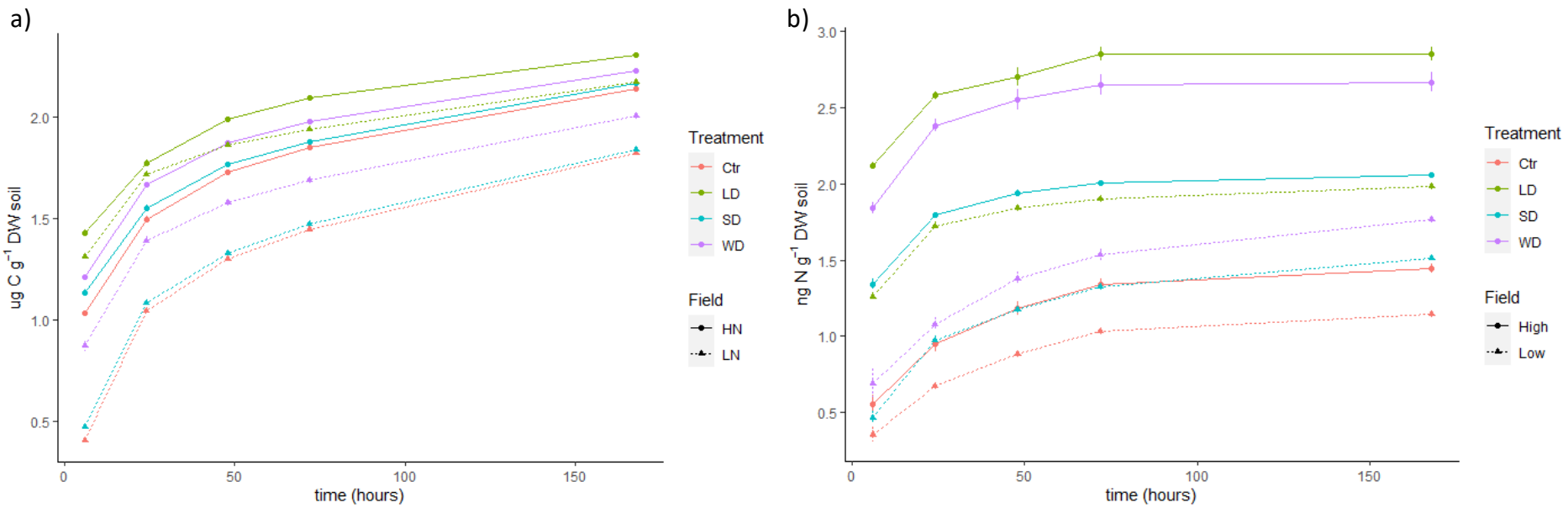

Figure 7.2 Log_{10} transformation of cumulative CO_2-C (a) and N_2O-N (b) effluxes during the second incubation in control (Ctr) soils or after addition of liquid (LD), whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) nutrient status. Error bars ± 1SE

In addition, during both incubations, a parallel set of destructive samples was prepared using amber bottles in order to monitor changes of soil water extractable NH_4^+ and NO_3^- at 0, 6, 24, 48, 72 and 168h (for the 168 h time point, respirometry samples were destructive sampled). The moisture content was checked daily in order to maintain a WHC of 50%.

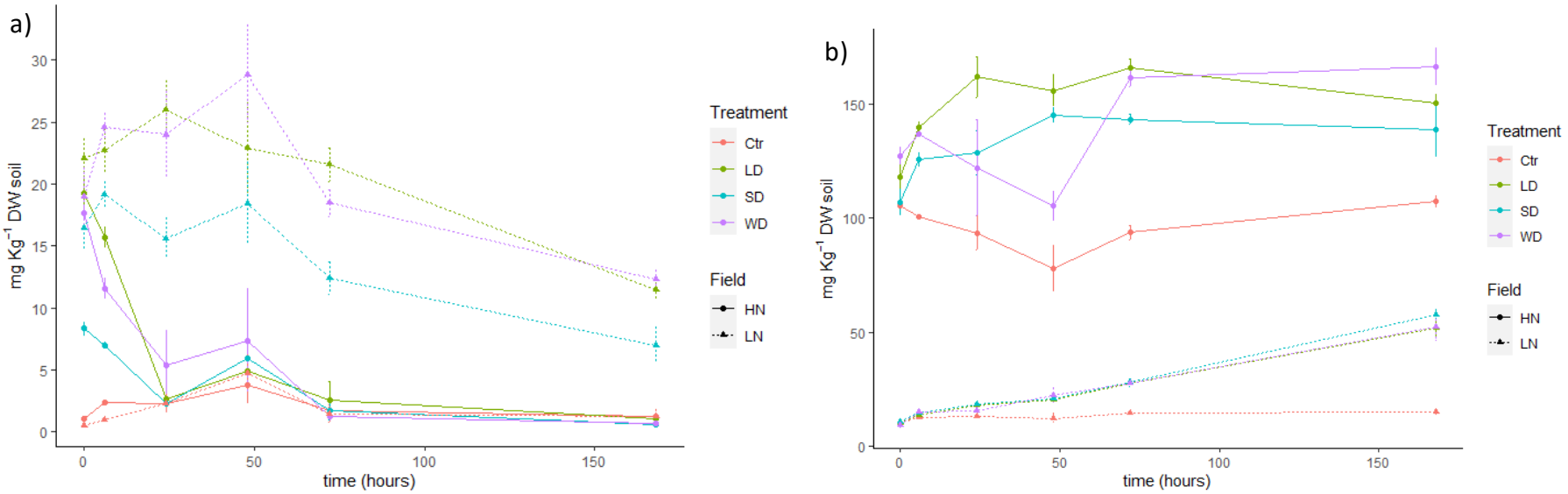

Figure 7.3 NH_4^+ (mg kg^{-1} DM soil) (a) and NO_3^- (mg kg^{-1} DM soil) (b) during the first incubation in control (Ctr) soils or after addition of liquid (LD), whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) nutrient status. Error bars ± 1SE

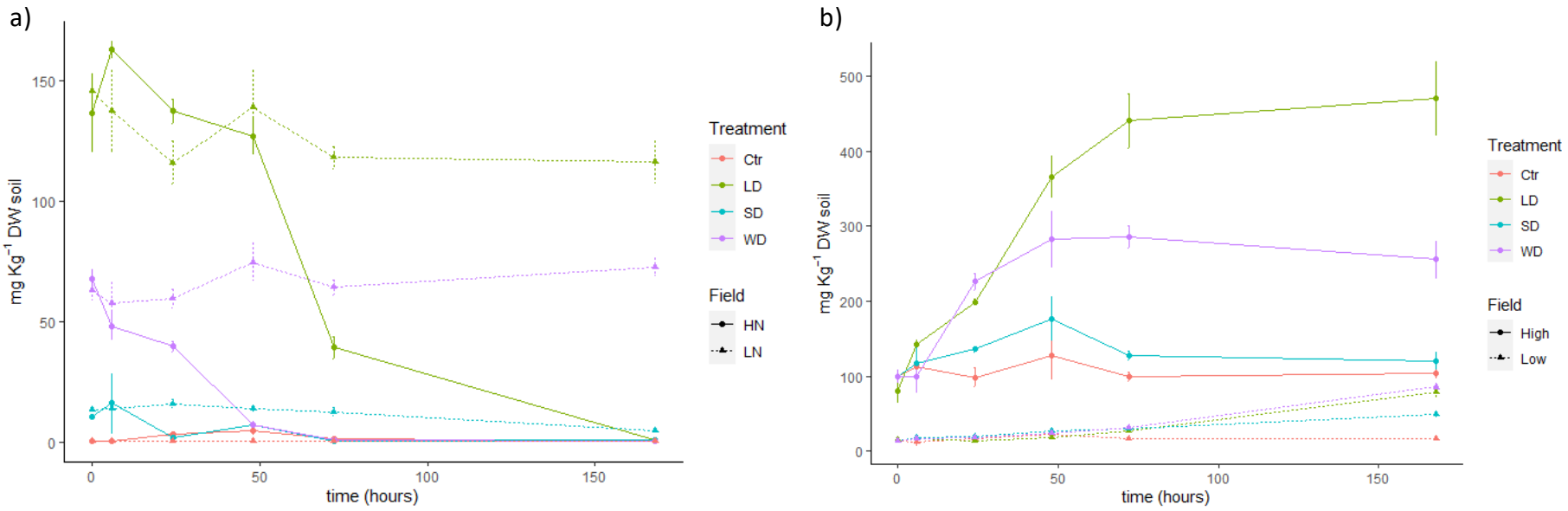

Figure 7.4 NH_4^+ (mg kg^{-1} DM soil) (a) and NO_3^- (mg kg^{-1} DM soil) (b) during the first incubation in control (Ctr) soils or after addition of liquid (LD), whole (WD) or solid (SD) fractions of digestate in soils at high (HN) or low (LN) nutrient status. Error bars ± 1SE